IMMUNOREGULATORY MECHANISMS OF INTERFERON

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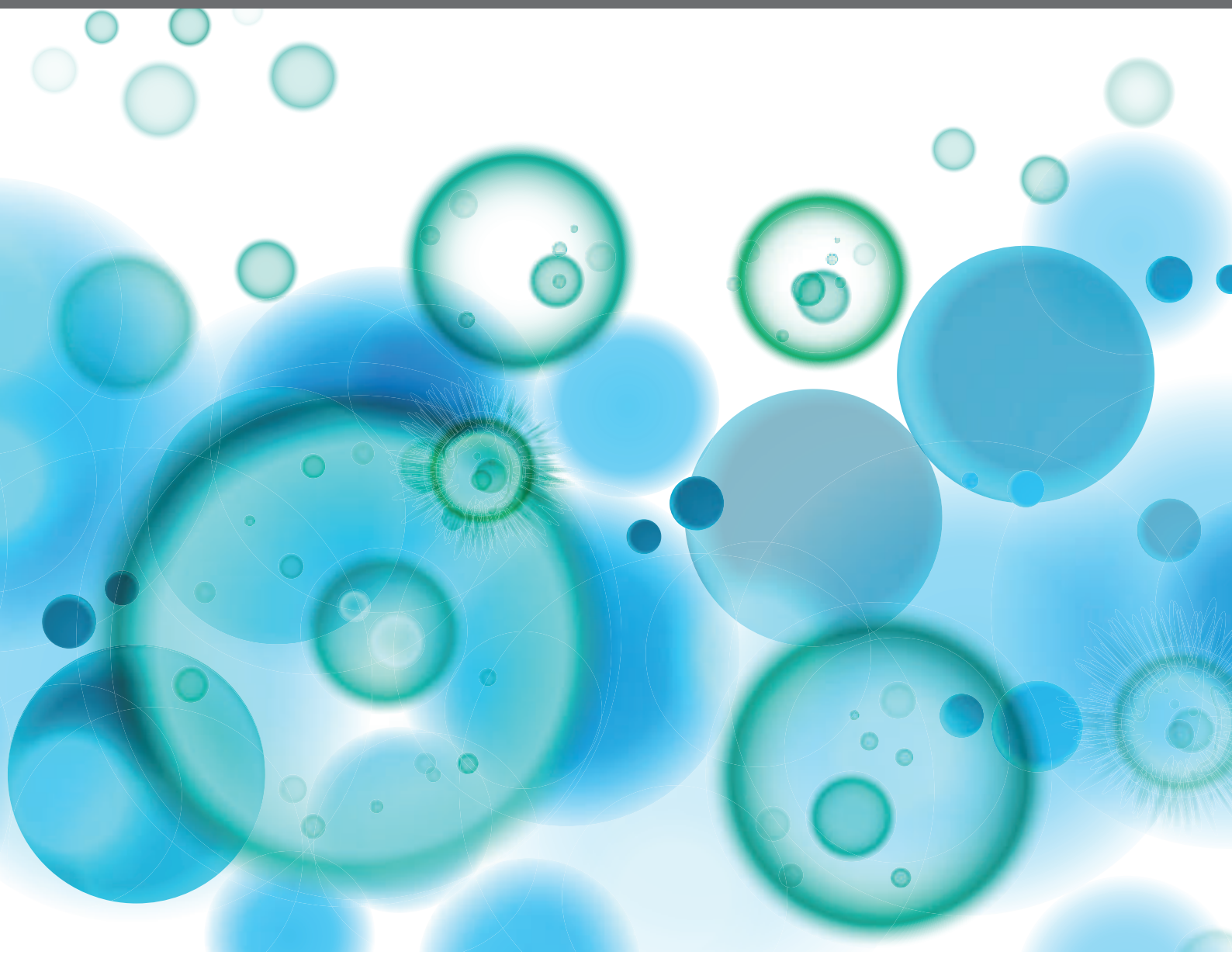

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IMMUNOREGULATORY MECHANISMS OF INTERFERON

Topic Editors:
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Interferons (IFN) belong to the family of cytokines and have been described first in the late 1950s as an inhibitory factor of viral replication. Since then, the impact of interferon has been greatly expanded and its function comprises a role not only in different types of infection, cancer and autoimmunity but importantly also in immunehomeostasis. IFN have important anti-viral effects but it is becoming more and more evident that they are true immunomodulators and have an important impact on the development and maintenance of innate and adaptive immunity.

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EDITORIAL
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Editorial: Immunoregulatory Mechanisms of Interferon

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Editorial on the Research Topic

Immunoregulatory Mechanisms of Interferon

Interferons (IFN) belong to the family of cytokines and have been described first in the late 1950s as an inhibitory factor of viral replication (1, 2). Since then, the impact of interferon has been greatly expanded and its function comprises a role not only in different types of infection, cancer and autoimmunity but importantly also in immune homeostasis. IFN have important anti-viral and anti-bacterial effects but it is becoming more and more evident that they are true immunomodulators and have a major impact on the development and maintenance of innate and adaptive immunity. IFN are classified into three groups: type I (IFN-I), type II (IFN-II, IFN-γ), and type III (IFN-III or IFN-λ). IFN can act in an autocrine and paracrine fashion upon induction by pattern recognition receptors (PRRs) sensing viral and bacterial components as well as danger associated molecular patterns (DAMPs).

This Research Topic features several **Review and Original Research articles** as well as one **Hypothesis and Theory article** on the different facets of interferon: evolution of IFN, signal transduction, role of IFN in infections, impact of IFN on metabolism and its effect on homeostasis and immune responses at barrier surfaces. Articles focus on all three types of IFN giving an important overview of current concepts of IFN functionality and emphasize the significance of IFN in immunity and beyond.

EVOLUTION OF IFN AND IFN IN FISH AND BIRDS

IFN and thereby genes for interferon are present not only in mammals but exist already in cartilaginous fish and bony fish. Secombes and Zou review the evolution of the interferon system by comparing interferon encoding genes of cartilaginous fish using mainly elephant shark and bony fish as an example with the ones in mammals. Genes for interferon and interferon receptors are present in elephant shark as well as orthologs genes of Toll like receptors (TLRs) as sensors and genes for components of IFN signal transduction emphasizing the key role and importance of interferon throughout evolution. Next to TLRs, RIG-I-like receptors comprise another important group of PRRs which are able to induce interferon expression. In the fresh water fish Grass carp, *Ctenopharyngodon Idella*, Rao et al. report that the RLR laboratory of genetics and physiology (LGP2) acts as a negative regulator of RIG-1 and MDA-5. Inhibition of RIG-1 and MDA-5 signal

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transduction is regulated by suppression of ubiquitination motifs as well as interference of LGP2 with interferon regulatory factors (IRFs). In an original research paper, Ding et al. describe two novel types of IFN-I in fish, specifically in Perciforme Fish Large Zellow Croaker *Larimichthys crocea* (*L. crocea*), namely IFN-δ and IFN-η. Sequence and phylogeny analysis of fish emphasizes the distinct IFN-I sequence motif. IFN-δ and IFN-η are induced in several different organs of *L. crocea* and are induced upon viral challenge. Moreover, fish cells respond to the novel IFN with the induction of interferon induced genes (ISGs). This work reports two novel IFN-I forms and highlights the importance of IFN for fish immunity. IFN were discovered because of their antiviral activity. These early experiments were done using influenza virus and fragments of chicken chorio-allantoic membrane (1) brought to our attention by Santhakumar et al. who provide a detailed overview of avian interferon and the induction of IFN by PRRs in birds. Interestingly, retinoic acid-inducible gene I (RIG-I), which is an important sensor in the mammalian IFN system is missing in *Galliformes* including chicken and turkeys which might influence their susceptibility to RNA viruses. Other sensors including TLRs and components involved in signal transduction are further compared and discussed and highlight thereby the similarities and differences between the mammalian and avian interferon system.

SIGNAL TRANSDUCTION OF IFN

Upon ligation of IFN receptors, signal is further transduced by a complex signaling cascade. Majoros et al. provide a conceptual framework on IFN induced signaling pathway by summarizing and discussing current knowledge of the canonical and non-canonical IFN signaling. The canonical IFN pathway involves signal transducer and activator of transcription (STAT) tyrosine phosphorylation by receptor bound activated Januse kinase (JAK) as well as STAT1 homodimers and the induction of IFN stimulated genes (ISGs) in contrast to the non-canonical IFN pathway which includes kinase dead JAKs. Recent evidence indicates that IFN is also able to influence signaling pathways not directly linked to the interferon system. Kopitar-Jerala presents a detailed overview of crosstalks between the IFN and the inflammasome system such as the regulation of expression of Caspase-11 by IFN. Bachmann et al. report a synergistic activation of inducible nitric oxid synthase (iNOS) by IFN-I in combination with IL-1β/TNF in hepatocytes. iNOS expression is also dependent in the liver on IFNAR1 in an acute liver injury model.

ROLE OF IFN IN VIRAL, BACTERIAL AND PARASITIC INFECTIONS

IFN are key modulators of immunity in infections. Schulz and Mossman discuss novel findings to counteract interferon responses upon viral infection. The steps of interferon induction and signaling are highlighted and the respective viral strategies are discussed including TLRs, RLRs, and IFNAR regulation and signaling. Recognizing viral RNA structures by the receptor RIG-1 is essential in the induction of interferon upon viral infection. Liu et al. provide a detailed overview of the signal transduction of RIG-1 as well as the importance of post-translational modification. This review further highlights recent insights into viral mechanisms of evasion to avoid host detection by interfering with the detection by RIG-1 and complements thereby the previous mentioned review. IFN-I has been identified due to its antiviral activity. Murira and Lamarre summarize in a mini review the role of IFN-I in chronic infections and discuss the role of IFN-I in immunopathology in different virally induced chronic diseases. The ubiquitous receptor expression of IFN-I is highlighted here which may be responsible for prolonged expression and sensing in chronic infections. This review article is complemented by an original research paper by Daugan et al. deciphering the importance of prolonged IFN-I sensing and its link to antigen specificity in chronic lymphocytic choriomeningitis virus (LCMV) infection. In addition to viral infections, IFN-I is key in bacterial infections and can be here beneficial or detrimental for the host. This essential role of IFN-I in bacterial infection is emphasized by Kovarik et al. Many PRRs are activated by bacterial components and can thereby also induce IFN. Deficiency of IFNAR1 influences susceptibility to bacterial infections, however, it is also important whether other IFN such as IFN-II is present or how IFNAR1 is regulated upon bacterial infection. This review highlights thereby the importance of a balance within the different groups of IFN and discusses functions of IFN by tissue tropism. Further differences and similarities of mouse and human interferon systems are discussed. The importance of IFN in infection is not limited to viral and bacterial challenge but has been also reported for protozoan infections highlighted by Silva-Barrios and Stäger. A detailed overview of current knowledge is given of the role of IFN and infection by the parasites *Plasmodium*, *Leishmania, Trypanosoma* and *Toxoplasma*. Due to complex life cycles of parasites and thereby different influences on the immune system, IFN can also play here a beneficial or detrimental role for the host. In an original research article, Sohrabi et al. investigate the expression of interferon-induced GTPases [guanylate-binding proteins (GBPs)] focusing on Gbp2b/Gbp1 and Gbp5 in different mouse strains at steady state and upon infection with *Leishmania major*. Gbp2b/Gbp1 and Gbp5 are differently expressed in the analyzed mouse strains indicating different regulation of these genes depending on genetics as well as on the level of inflammation.

ROLE OF IFN IN CANCER AND SEPSIS TREATMENT

Novel therapeutics against cancer are essential and involvement of IFN in anti-tumor immune responses are reviewed by Müller et al. This review highlights current literature of IFN-I (mainly α and β) and its immunomodulator function for different immune cells with the focus on NK cells and its importance in protective anti-tumor immune responses. The tumor environment has

come into focus in basic research of tumor immunology but also for novel therapeutic strategies. Pylaeva et al. emphasizes the importance of IFN-I in its regulation of tumor associated neutrophils. IFN restrains the subpopulation of N2 neutrophils and thereby enhances activity and function of N1 neutrophils, which are involved in tumor growth and metastasis. Additionally, IFN is discussed as a therapeutic target in infections. Rackov et al. take this further in a mini review by reconsidering IFN-I (IFN-β) in sepsis, specifically in the delayed phase of sepsis characterized by immunosuppression.

Type III IFN (IFN-λ), the most recently identified interferon group, was discovered in 2003 (3, 4). IFN-λ is further divided into IFNλ-1, λ-2, λ-3, and λ-4. Syedbasha and Egli highlight sequence similarities within this novel interferon group as well as point out differences in signal transduction between IFN-I and IFN-III. Moreover, the link between single-nucleotide polymorphisms (SNPs) in both, IFN-λ and IFNLR genes, and the clinical outcome of viral induced hepatitis are emphasized. In a complementary review, Boisvert and Shoukry highlight specifically the clinical outcome of acute and chronic hepatitis C virus infection in regard to polymorphism in the IFNλ-3 gene. Expression of IFNλ-3 influences the replication of HCV and thus is an important factor in the immune response. Moreover, this review summarizes IFNLR expression in different cells of hematopoietic origin. In contrast to the ubiquitous expression of IFNAR1/2, IFNLR is expressed mainly on epithelial cells. Lasfar et al. review the roles of IFN-III in immunity and infection at the pulmonary and vaginal mucosa and focus on IFN-III induced epithelial immunity. In an original research article, Wang et al. report that IFN-λ (λ-1, λ-2, and λ3) have an inhibitory effect on HIV infected human macrophage cultures.

ROLE OF IFN AT MUCOSAL SURFACES AND IMMUNE HOMEOSTASIS

At mucosal surfaces such as the lungs and the gut, IFN play an important role by orchestrating innate but also adaptive immunity. An appropriate immune response in response to infections is needed to ensure constant organ function. Makris et al. discuss the importance of a balanced IFN-I response in inflammation upon respiratory infections. Importantly, differences and changes of immune cells to sensitivity of IFN-I signaling in lung inflammation influences different outcomes. Moreover, the importance of IFN in pulmonary infections of viral and bacterial origin is emphasized by this review. Peteranderl and Herold highlight the interferon-TNF-related apoptosis-inducing ligand (TRAIL) signaling axis in pulmonary inflammation. Both TRAIL and IFN are induced upon viral infection, able to induce cell death and show different facets by triggering protective but also detrimental responses. The crosstalk of IFN and TRAIL is here discussed in different viral but also allergic pulmonary inflammation.

The gut harbors a plethora of different microbial species and immune responses must be well-balanced to eradicate pathogens but tolerate commensal bacteria. Kotredes et al. summarize the importance of IFN in intestinal homeostasis and inflammation. Since inflammation is also able to trigger cancer, the role of IFN in mouse models of colitis but also in human inflammatory bowel diseases are highlighted. In an original research article, Kawashima et al. identify that dsRNA of lactic acid bacteria (LAB) belonging to the small intestinal commensal flora induces IFN-I and IL-12 in human dendritic cells and thereby trigger a typical type I immune response with induction of IFN-II.

Pott and Stockinger review in detail the role of IFN-I and IFN-III (IFN-λ) in bacterial and viral infections in the gut. IFN-I is protective in enteric viral in contrast to bacterial infections. The cell tropism of the IFNLR is highlighted specifically in regard to intestinal infections in contrast to IFNAR1/2 expression, which impacts on cellular activity and infection outcome.

IFN AND METABOLISM

IFN are pleiotropic cytokines and it is becoming more and more evident that IFN are key in cellular and whole body metabolism. The review by Robertson and Ghazal commence with a retrospect reporting transient hypercholesterolemia upon interferon treatment. Further the molecular regulation of lipids including oxysterol by interferon upon infection and the impact of lipid regulation on immune cells are discussed. The link between interferon and lipid metabolism is poorly studied. In a hypothesis and theory article, Newmark et al. derive elegantly the hypothesis that the evolution of interferon driven immunity was driven by sterol metabolites. Fritsch and Weichhart review in detail the effect of IFN on cellular metabolism highlighting that an interferon induced state upon viral infection and the immune response are highly dependent on changes in metabolic pathways.

Collectively, our Research Topic highlights that IFN are pleiotropic cytokines important for the defense against infections but additionally crucial in many essential biological mechanisms and functions. Research of IFN in all fields of medical sciences is today more active and fascinating than ever.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Identification of Two Subgroups of Type I IFNs in Perciforme Fish Large Yellow Croaker *Larimichthys crocea* Provides Novel Insights into Function and Regulation of Fish Type I IFNs

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Like mammals, fish possess an interferon regulatory factor (IRF) 3 /IRF7-dependent type I IFN responses, but the exact mechanism by which IRF3/IRF7 regulate the type I IFNs remains largely unknown. In this study, we identified two type I IFNs in the Perciforme fish large yellow croaker *Larimichthys crocea*, one of which belongs to the fish IFNd subgroup and the other is assigned to a novel subgroup of group I IFNs in fish, tentatively termed IFNh. The two IFN genes are constitutively expressed in all examined tissues, but with varied expression levels. Both IFN genes can be rapidly induced in head kidney and spleen tissues by polyinosinic–polycytidylic acid. The recombinant IFNh was shown to be more potent to trigger a rapid induction of the antiviral genes MxA and protein kinase R than the IFNd, suggesting that they may play distinct roles in regulating early antiviral immunity. Strikingly, IFNd, but not IFNh, could induce the gene expression of itself and IFNh through a positive feedback loop mediated by the IFNd-dependent activation of IRF3 and IRF7. Furthermore, our data demonstrate that the induction of IFNd can be enhanced by the dimeric formation of IRF3 and IRF7, while the IFNh expression mainly involves IRF3. Taken together, our findings demonstrate that the IFN responses are diverse in fish and are likely to be regulated by distinct mechanisms.

Keywords: type I IFNs, antiviral immunity, IRF3 and IRF7 interaction, positive feedback regulation, promoter, large yellow croaker *Larimichthys crocea*

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INTRODUCTION

Teleost fish employ multiple type I IFNs to coordinate antiviral immune responses (1–3). They are classified into two major groups based on the presence of conserved cysteine residues in the mature peptide: group I IFNs containing two cysteine residues, which can be found in all teleost fish lineages, and group II IFNs containing four cysteine residues which are limited in several species, such as trout *Oncorhynchus mykiss*, salmon *Salmo salar*, and zebrafish *Danio rerio* (2, 4, 5). Phylogenetically, the two groups can be further divided into six subgroups, IFN a, b, c, d, e, and f (2). Notably, to date, the Perciformes, such as sea bass *Dicentrarchus labrax* (6), rock bream *Oplegnathus fasciatus* (7),

sevenband grouper *Epinephelus septemfasciatus* (8), and orange-spotted grouper *Epinephelus coioides* (9) have been shown to possess a single subgroup, IFNd.

The antiviral functions of fish type I IFNs have been characterized in multiple fish species. As in mammals, fish type I IFNs are able to induce expression of a variety of antiviral genes, including myxovirus resistance (Mx), protein kinase R (PKR), virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible (Viperin), and IFN-stimulated gene (ISG) 15, thus leading to an enhanced antiviral state (4, 10–12). Accumulating data suggest that fish group I and II type I IFNs may have distinct antiviral roles in different cells or at different stages of infection (3). For example, zebrafish IFNphi1 (IFNa, group I) induces a slow and powerful expression of antiviral genes, whereas zebrafish IFNphi2/3 (IFNcs, group II) trigger a rapid and transient induction of antiviral genes (5). In zebrafish larvae, IFNphi4 (IFNd, group I) exhibits poor antiviral activity (10). Consistent with these reports, salmon IFNa, but not IFNd, exerts significant antiviral effects (11). In contrast to these reports, in Perciforme species, such as rock bream, sevenband grouper, and orange-spotted grouper, IFNds (group I) are the main IFNs to mount antiviral defense to viral infection (7–9).

In general, teleost group I type I IFNs appear to be ubiquitously expressed in most cell types and tissues and are upregulated upon viral infection or viral RNA analog treatment, whereas group II type I IFNs are constitutively expressed at a very low level and induced in specific leukocyte populations, with the exception of IFNf, which can be induced in fibroblasts (2, 3, 13). Recent studies demonstrate that the six IFN subgroups in trout were differentially modulated in three trout cell types, RTG-2, RTS-11, and primary head kidney leukocytes, following stimulation with polyinosinic–polycytidylic acid [poly(I:C)]. Moreover, viral haemorrhagic septicemia virus infection of brown trout *Salmo trutta* also gave rise to differential expression kinetics in the kidney and spleen (2). Similar findings have been reported for zebrafish, salmon, and turbot *O. fasciatus* type I IFNs (10, 11, 14). These differential expression patterns of IFNs between or within group I and group II suggest that regulation of type I IFN expression in fish is very complex.

The expression of type I IFNs is controlled by two key transcription factors, the interferon regulatory factor (IRF) 3 and 7 (15). In mammals, viruses are recognized by pattern recognition receptors, including Toll-like receptors (TLRs) and retinoic acid–inducible gene I-like receptors (RLRs), which trigger distinct signaling cascades to activate IRF3 and/or IRF7, inducing expression of early phase IFNs mainly, including IFNβ. IFNβ, then, induces expression of a variety of ISGs to establish the host antiviral state through the Jak–Stat pathway and the IRF7-dependent production of the late-phase IFNs, including most of the IFNαs (16–18). Similar to mammals, fish IFN responses are also controlled by IRF3/7 and appear to be very complex. Accumulating data suggest that fish group I and group II IFN responses are governed by distinct IRFs. Group I IFN genes, including zebrafish IFNphi1, carp *Carassius auratus* IFN, and salmon IFNa1, as well as Japanese flounder *Paralichthys olivaceus* IFN (IFNd), seem to be primarily regulated by IRF3, while expression of zebrafish IFNphi3 (group II) mainly involves IRF7 (19–21). Recent studies show that zebrafish IFNphi1 and salmon IFNa1 are also activated by IRF1 and IRF7, respectively (19, 22). Furthermore, fish IFNas can significantly induce the expression of themselves and other IFN genes (5, 23), suggesting that a positive feedback regulation may exist. This observation differs from that of mammals, in which type I IFNs cannot directly induce their own expression (24, 25). Further studies demonstrate that the carp IFN facilitates phosphorylation of IRF3 that is required for activation of gene transcription, thus amplifying IFN response (20). However, the roles of the IRF3 and IRF7 in the positive feedback regulation remain largely unknown.

In this study, we report the identification of two type I IFNs from large yellow croaker (lyc) *Larimichthys crocea*. Based on the sequence and phylogenetic analyses, one IFN belonged to the IFNd subgroup while the other was assigned to a novel subgroup of group I IFNs, tentatively termed IFNh. Lyc IFNd and IFNh exhibited apparent differences in expression patterns and the ability to induce antiviral genes. IFNd, but not IFNh, was able to upregulate expression of itself and IFNh, as well as the activation of phosphorylation of IRF3 and IRF7. Furthermore, expression of the IFNd gene requires both IRF3 and IRF7, while the IFNh expression primarily involves IRF3. Collectively, the lyc IFNd may function as a key mediator for amplification of the IFN responses through IRF3 and IRF7. These findings provide new insights into the function and regulation of type I IFNs in fish.

MATERIALS AND METHODS

Ethics Statement

The studies were carried out in strict accordance with the Regulations of the Administration of Affairs Concerning Experimental Animals, under protocol license number: SYXK(MIN)2007-0004, approved by the Institutional Animal Care and Use Committee of Fujian Province. All of the surgery was performed under Tricaine-S anesthesia, and all efforts were made to minimize suffering.

Fish

Large yellow croaker *L. crocea* (lyc, weight: 103 ± 21.9 g; length: 21 ± 1.3 cm) were purchased from a mariculture farm in Lianjiang county, Fuzhou, China. Fish were maintained with a flow-through seawater supply at 25°C. After acclimating for 7 days, healthy fish were used for the challenge experiments.

Cells Lines and Virus

The lyc head kidney (LYCK) cells were isolated from the head kidney of lyc. The continuous LYCK cell lines were preserved in our laboratory and maintained at 28°C in L-15 medium (Life Technologies, Carlsbad, CA, USA), supplemented with 10% Fetal Bovine Serum (FBS, Life Technologies) according to the previous study (26). The epithelioma papulosum cyprini (EPC) cells (China Center for Type Culture Collection, Wuhan, China) were derived from fathead minnow *Pimephales promelas* and cultured at 25°C in L-15 medium supplemented with 10% FBS (27). Human embryonic kidney 293T cells (HEK293T, China Center for Type Culture Collection) were grown in DMEM (Life Technologies) containing 10% FBS, 100 U/ml penicillin, and

100 μg/ml streptomycin (Life Technologies) at 37°C in a 5% CO_2 atmosphere. Grouper spleen (GS) cells were originated from the spleen of orange-spotted grouper *E. coioides* and maintained in L-15 medium supplemented with 10% FBS at 25°C. Singapore grouper iridovirus (SGIV) was propagated in GS cells as previously described (28), and the virus stock was stored at −80°C until use. GS cells and SGIV are generous gifts from Professor Qiwei Qin in South China Sea Institute of Oceanology, Chinese Academy of Sciences.

Gene Cloning and Bioinformatics

The partial sequences of lyc IFNd and IFNh were obtained from the transcriptome library of lyc spleen tissues (29). 5′ and 3′ RACE PCR were performed to obtain the full-length cDNAs of IFNd and IFNh, as described previously (30). The cDNA for 5′ and 3′ RACE PCR was derived from the LYCK sampled at 6 h after stimulation with poly(I:C). The integrity of the cDNA sequences was confirmed by PCR with the primers covering the full-length coding sequence (Table S1 in Supplementary Material). The genomic sequence and 5′-flanking regulatory sequence of IFNd and IFNh were obtained from the lyc genome data (31) and amplified from genomic DNA of the lyc muscle with specific primers (Table S1 in Supplementary Material).

Amino acid sequence identity and similarity were calculated using the Matrix Global Alignment Tool (Matgat, version 2.0) (32). Multiple alignments were performed with CLUSTAL W2 program, and phylogenetic trees were constructed by the Neighbor-Joining and Minimum Evolution methods using the MEGA (version 6) software package. Signal peptide predictions were made using SignalP4.1 software.[1] The genomic organization of IFNd and IFNh genes was analyzed by alignment of the IFN cDNA sequences and their genomic DNA sequences using Spidey program.[2] Transcription factor binding sites were predicted using the MatInspector program.[3]

The fish IFN sequences retrieved from the databases for analysis included: *C. auratus* (*Ca*, goldfish), AAR20886; *Cirrhinus molitorella* (*Cm*, mud carp), AAY56128; *Ctenopharyngodon idella* (*Ci*, grass carp), ABC87312; *Cyprinus carpio* (*Cc*, common carp), ADI81047; *D. rerio* (*Dr*, zebrafish), AAM95448 (IFNphi1), NP_001104552 (IFNphi2), NP_001104553 (IFNphi3), NP_001155212 (IFNphi4); *D. labrax* (*Dl*, sea bass), CAQ17043 (IFN1); *E. coioides* (*Ec*, orange-spotted grouper), AGL21770 (IFN1), AGJ98284 (IFN2); *Gasterosteus aculeatus* (*Ga*, stickleback), CAM31706 (IFN1), CAM31707 (IFN2), CAM31708 (IFN3); *Haplochromis burtoni* (*Hb*, Burton's mouthbrooder), XP_005950669 (IFNal3); *Ictalurus punctatus* (*Ip*, catfish), AAV97701 (IFN), AAV97699 (IFN2); *Maylandia zebra* (*Mz*, zebra mbuna), XP_004556871 (IFNal3); *Mylopharyngodon piceus* (*Mp*, black carp), AKM15287; *O. mykiss* (*Om*, trout), CAM28541 (IFNa1), NP_001153977 (IFNa2), CCV17397 (IFNa3), CCV17398 (IFNa4), NP_001153974 (IFNb1), NP_001158515 (IFNb2), CCV17399 (IFNb3), CCV17400 (IFNb4), CCV17401 (IFNb5), CCV17402 (IFNc1), CCV17403 (IFNc2), CCV17404 (IFNc3), CCV17405 (IFNc4), CAV07949 (IFNd1), CCV17406 (IFNe1), CCV17407 (IFNe2), CCV17408 (IFNe3), CCV17409 (IFNe4), CCV17410 (IFNe5), CCV17411 (IFNe6), CCV17412 (IFNe7), CCV17413 (IFNf1), CCV17414 (IFNf2); *O. fasciatus* (*Of*, turbot), AFP94213 (IFN1), AFP94213 (IFN2); *Oreochromis niloticus* (*On*, tilapia), XP_005950669 (IFNω1), XP_005469255 (IFNω3), XP_003453450 (IFNal3); *Oryzias latipes* (*Ol*, medaka), BAU25609 (IFN1); *P. olivaceus* (*Po*, Japanese flounder), BAA02372; *Pundamilia nyererei* (*Pn*, cichlid), XP_013771349 (IFNal3); *S. salar* (*Ss*, salmon), ABD39320 (IFNa1), ABD39321 (IFNa2), ACE75687 (IFNa3), ACE75691 (IFNb1), ACE75693 (IFNb2), ACE75689 (IFNb3), ACE75692 (IFNc1), XP_014048249 (IFNc2), ACE75688 (IFNc3), DAA64377 (IFNd); *Sparus aurata* (*Sa*, gilthead seabream), CAT03221 (IFN1), CAT03222 (IFN2), CAT03223 (IFN3), CAT03224 (IFN4); *Takifugu rubripes* (*Tr*, Fugu), CAM82750 (IFN1), CAM82751 (IFN2); *Tetraodon nigroviridis* (*Tn*, spotted green pufferfish), CAD67779.

Production of Recombinant lyc IFN Proteins

To obtain the recombinant IFN (rIFN) proteins, the coding sequences of IFNd and IFNh, with the signal peptide deleted, were inserted into the pCMV-Flag 2C vector (Stratagene, La Jolla, CA, USA) using gene-specific primer sets (Table S1 in Supplementary Material) and expressed as a fusion protein with the FLAG tag in HEK293T cells. 3×10^6 HEK293T cells were plated in 9-cm tissue culture dishes (Biofil, Guangzhou, China) and transfected with 18 μg of rIFN plasmid using 36 μl of Fugene® HD transfection reagent (Promega, Madison, WI, USA). At 48 h after transfection, cells were harvested for analysis of the expression of rIFN proteins. The recombinant proteins were then purified using ANTI-FLAG® M2 affinity gel (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturers' instructions. Briefly, the harvested cells were lysed with the lysis buffer [TBS (50 mM Tris–HCl, pH 7.4, 150 mM NaCl), 1 mM EDTA, and 1% Trition X-100] and incubated with ANTI-FLAG® M2 affinity gel for 1 h at 4°C. Then the beads were washed with TBS, and the recombinant proteins eluted with TBS containing 3 × FLAG peptides (200 ng/μl, Sigma-Aldrich). After dialyzed against phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH7.4), the purified proteins were concentrated using an ultrafiltration centrifuge tube (Millipore, Bedford, MA, USA) and stored at −70°C after filtration with a 0.45-μM filter. The purified rIFN proteins were quantitated using Bradford protein quantitation assay by Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

Antiviral Activity Assays in Grouper Spleen Cells

The GS cells were seeded onto the 6-well plates (Thermo Fisher Scientific) for 18 h. The cells were pretreated with rIFNd or rIFNh at a final concentration of 50 ng/ml or PBS (as a control) for 2 h; then, the cells were infected with SGIV at a multiplicity of infection of 2. At 24 h postinfection, the cells were observed microscopically for cytopathic effect (CPE) (Leica Microsystems, Wetzlar, Germany).

[1] http://www.cbs.dtu.dk/services/SignalP
[2] http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey
[3] http://www.genomatix.de/

The expression of two SGIV envelope protein genes, ORF049 and ORF072, was detected by real-time PCR. Briefly, infected cells were harvested at 24 and 48 h postinfection. Total RNA was extracted using the SV total RNA Isolation System (Promega) according to the manufacturer's instructions, and reverse-transcribed into first-strand cDNA using an Oligo dT-Adaptor primer (TaKaRa, Dalian, China). Real-time PCR was performed with gene-specific primer sets (Table S1 in Supplementary Material). *E. coioides* β-actin (*Ec*β-actin) was amplified as an internal control with the *Ec*actin-F/*Ec*actin-R primers (Table S1 in Supplementary Material). Real-time PCR was performed on the Mastercycler ep gradient realplex4 system (Eppendorf, Germany) using SYBR® Premix ExTaq™ (TaKaRa). Cycling conditions were 3 min at 94°C, then 40 cycles at 94°C for 5 s, 60°C for 10 s, and 72°C for 10 s. The fluorescence output for each cycle was analyzed upon the completion of the entire run. The expression levels of SGIV genes, ORF049 and ORF072, were normalized by *Ec*β-actin using the $2^{-\Delta\Delta CT}$ method (33). Each experiment was repeated three times.

Expression Analysis of lyc IFN Genes

To determine the tissue expression profiles of IFN genes, tissues including brain, gills, heart, head kidney, intestine, liver, skin, spleen, and stomach were collected from five healthy lyc fish. Total RNA was isolated using the Trizol reagent (Life Technologies) and treated with RNase-free DNase I (TaKaRa). After reverse transcription, real-time PCR was carried out using gene-specific primer sets (Table S1 in Supplementary Material) and the cycling conditions were 30 s at 95°C, followed by 40 cycles at 95°C for 5 s, 58°C for 15 s, and 72°C for 20 s. The expression levels of IFN genes were normalized by β-actin using the $2^{-\Delta\Delta CT}$ method as above and expressed as the ratio of the IFNd expression levels in the spleen.

To understand the modulation of IFN gene expression upon poly(I:C) challenge, one group of 25 fish was intraperitoneally injected with poly(I:C) (Sigma-Aldrich, St. Louis, MO, USA; 1 mg/ml in PBS) at a dose of 0.2 mg/100 g fish. Another group of 25 fish was injected with sterile PBS at a dose of 0.2 ml/100 g fish as a control. The head kidney and spleen were collected from five fish in each group at 4, 8, 12, 24, and 48 h postinjection, frozen immediately in liquid nitrogen, and stored at −80°C until RNA extraction. Total RNA was extracted from head kidneys and spleens collected at the described time points postinjection. Real-time PCR was then performed using the conditions described above to detect the expression levels of two IFN genes at different time points postinjection. The relative expression levels of IFN genes were normalized by the reference gene β-actin. Fold change of gene expression level was obtained by comparing the normalized gene expression level of poly(I:C)-injected fish with that of the PBS-injected fish (defined as 1) at the same time point.

Treatment of LYCK Cells with rIFN Proteins

To determine the bioactivities of IFNd and IFNh, the LYCK cells were plated in 6-well plates with a density of 1×10^6 cells/well and treated with rIFNs at a final concentration of 50 ng/ml or PBS (as a control). Three replicate wells were used for each treatment. The LYCK cells were harvested at 0, 2, 4, 8, and 20 h posttreatment, and total RNA was extracted as described above. Expression levels of lyc IFNd, IFNh, MxA, PKR, IRF3, and IRF7 genes were determined using real-time PCR as described previously. Fold change of gene expression level was calculated by comparing the normalized gene expression level in rIFN-treated cells with that in PBS-treated cells (defined as 1) at the same time point. Each experiment was repeated three times.

Luciferase Activity Assay

For luciferase assays, the recombinant plasmids were constructed by inserting the promoter regions of two IFN genes and a series of their respective deleted fragments into the dual luciferase reporter plasmid pGL3-Basic (pGL3-IFNPs, primers in Table S1 in Supplementary Material; Promega). The EPC cells (5×10^4/well) were seeded in 96-well plates (Thermo Fisher Scientific) overnight and cotransfected with 100 ng of pGL3-IFNP plasmid or pGL3-Basic plasmid (control) and 2 ng pRL-TK plasmid using the Fugene® HD transfection reagent. After 48 h, the luciferase activity of total cell lysates was measured on a GloMax 20/20 luminometer (Promega) according to the Dual-Luciferase® Repoter Assay System (Promega). The firefly luciferase activity was normalized to the Renilla luciferase activity (pRL-TK, Promega), and the IFNP relative luciferase activity (IFNP Rel. Luci. Act.) was expressed as the ratio of normalized luciferase activity in cells transfected with pGL3-IFNPs versus that in control cells transfected with the pGL3-Basic plasmid. To further study the effect of IRF3 and IRF7 on IFN promoter activity, the complete ORFs of lyc IRF3 and IRF7 were cloned into the pCMV-HA vector (pCMV-HA-IRFs, primers in Table S1 in Supplementary Material). The resulting plasmids [pGL3-IFNPs (50 ng), pCMV-HA-IRF (50 ng)/pCMV-HA (50 ng), and pRL-TK (1 ng)] were cotransfected into the EPC cells. The IFNP relative luciferase activity was expressed as the ratio of normalized luciferase activity in cells cotransfected with pGL3-IFNPs and pCMV-HA-IRF3/7 versus that in control cells cotransfected with pGL3-Basic and pCMV-HA plasmids. All data were obtained from three independent experiments with each performed in triplicate.

Preparation of IRF3 and IRF7 Polyclonal Abs

To produce the polyclonal anti-IRF3 and anti-IRF7 antibodies (Abs), the DNA binding domains (DBD; $IRF3^{1\text{-}113aa}$, *KKF34018*; $IRF7^{1\text{-}110aa}$, *KKF30244*) were amplified using gene-specific primers (Table S1 in Supplementary Material) and inserted into the pET-32a vector. The recombinant proteins were expressed in *E. coli* BL21 (Novagen, Madison, WI, Germany) as a fusion protein and purified as described previously (30). The purified proteins were injected into the white New Zealand rabbits to raise polyclonal Abs using the standard method (34). The polyclonal Abs were pre-adsorbed using *E. coli* lysate supernatants to remove the irrelevant Abs and purified using the HiTrap™ Protein A HP system on AKTAprime™ Plus (GE Healthcare, Piscataway, NJ, USA).

Western Blotting

To determine the specificity of the rabbit anti-IRF3 and anti-IRF7 Abs prepared above, HEK293T cells were transfected with pCMV-HA-IRF3, pCMV-HA-IRF7, and pCMV-HA (Clontech, as control) for 48 h. The lysates of the transfected cells were separated by 12% SDS-PAGE and electrophoretically transferred to a PVDF membrane (Millipore). The membrane was blocked in 5% (w/v) non-fat milk in TBST buffer (25 mM Tris–HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20) at 25°C for 1 h, incubated with primary Abs [rabbit anti-IRF3 or anti-IRF7 Abs (1:1000); or rat anti-HA Ab (1:8000, Sigma-Aldrich)] at 4°C overnight, then incubated with HRP-conjugated secondary Abs (goat anti-rabbit Ab, 1:3000, Sigma-Aldrich; or goat anti-rat Ab, 1:5000, Solarbio, Beijing, China) for 1 h at 25°C. All washing operations were performed on the SNAP i.d. system (Millipore) using three TBST buffer washes. The membrane was stained using the ECL system.

To detect the endogenous IRF3 and IRF7, the LYCK cells were cultured in a 6-cm plate (2.5×10^6) and treated with poly(I:C) and rIFNd or rIFNh at a range of doses for 12 h. Total protein was incubated with or without 20 U of calf intestinal alkaline phosphatase (CIAP) at 37°C for 30 min and, then, separated by 12% SDS-PAGE and transferred to a PVDF membrane using PierceG2 Fast Blotter equipment (25V for 10 min; Pierce, Rockford, IL, USA). The primary Abs (rabbit anti-IRF3 and anti-IRF7 Abs, 1:1000) were incubated using 1% (w/v) non-fat milk in TBST buffer (0.1% Tween 20). The secondary Ab was HRP-conjugated goat anti-rabbit Ab (1:3000). Immunoreactive proteins were detected using an ECL system.

Co-immunoprecipitations

To detect the interaction of lyc IRF3 and IRF7, complete ORF of IRF3 was cloned into the pCMV-Flag 2C vector (pCMV-Flag-IRF3, primers in Table S1 in Supplementary Material). The immunoprecipitation method for analysis of IRF3 and IRF7 was performed using ANTI-FLAG® M2 affinity gel (agarose beads conjugated with murine anti-Flag monoclonal Ab) according to the manufacturers' instructions. In brief, 3×10^6 HEK293T cells were seeded in 9-cm tissue culture dishes overnight and then cotransfected with 1.8 mg of pCMV-Flag-IRF3 and pCMV-HA-IRF7 (at a ratio of 1:1) using 36 μl of Fugene® HD transfection reagent. Cells transfected with pCMV-Flag-IRF3/pCMV-HA, pCMV-Flag/pCMV-HA-IRF7 or empty vectors were used as controls. At 48 h after transfection, cells were harvested and lysed with cell lysis buffer (Beyotime, Nantong, China). The IRF3-Flag immune complexes were then immune-precipitated from supernatants of cell lysates using ANTI-FLAG® M2 affinity gel for 1 h at 4°C. The beads were washed with cell lysis buffer for five times and eluted by boiling beads in 5 volumes of SDS-PAGE loading buffer. Finally, the samples, including controls, were used for SDS-PAGE and Western blotting analyses using anti-Flag Ab or anti-HA Ab (Sigma-Aldrich) against the fusion protein.

EMSA

EMSA was performed as previously described (35). Briefly, the lysates of HEK293T cells transfected with pCMV-HA-IRF3 or pCMV-HA-IRF7 were prepared for DNA–protein binding reactions. The wild-type and mutated oligonucleotides (Table S1 in Supplementary Material) for EMSA probes were biotin-labeled using an EMSA Probe Biotin Labeling Kit (Beyotime) according to the manufacturer's instructions. DNA–protein binding reactions were carried out using an EMSA/Gel-Shift Kit (Beyotime) at 25°C according to the manufacturer's instructions. In parallel, to determine the specificity of the DNA–protein binding reactions, competition experiments were performed with 100 × excessive unlabeled wild-type or mutated probes. After a 20 min incubation, the completed reactions were separated by non-denaturing 4% PAGE, and the gel was subjected to autoradiography using a LightShift® Chemiluminescent EMSA Kit (Pierce).

RESULTS

Gene Cloning and Sequence Analysis of Two lyc IFNs

The lyc IFNd (*KU144879*) and IFNh (*KU144880*) genes were identified and their coding sequences determined. The complete IFNd cDNA is 934 bp in length, with an open reading frame (ORF) translating into a protein of 185 aa, where a signal peptide of 22 aa can be predicted. The deduced IFNd protein contains two cysteine residues (C1: C^{23} and C3: C^{125}) conserved in the mature peptides of fish group I type I IFNs (**Figure 1A**, Figure S1A in Supplementary Material). The full-length cDNA of IFNh is 822 bp, containing an ORF encoding a protein of 190 aa, with a predicted signal peptide of 21 aa. Although the deduced IFNh protein contains six cysteine residues, only two are aligned with the conserved cysteine residues (C1: C^{22} and C3: C^{108}) in the fish group I type I IFNs (**Figure 1A**, Figure S1B in Supplementary Material). Both lyc IFNs possess some typical features of type I IFNs in teleost fish, including a distinctive family signature motif ([FYH]-[FY]-X-[GNRCDS]-[LIVM]-X^2-[FYL]-L-X^7-[CY]-[AT]-W) at the C-terminus and a gene organization of five exons and four introns (Figures S1A,B in Supplementary Material).

The phylogenetic relationships of the lyc IFNs with other fish IFN homologs were studied. The phylogenetic tree with the Neighbor-Joining method shows that lyc IFNd falls into a major clade with the fish IFNd subgroup (**Figure 1B**). To our surprise, the lyc IFNh does not cluster with any known group I type I IFNs (IFNa, IFNd, and IFNe), but forms a separate clade with IFNs from zebra mbuna *M. zebra*, Burton's mouthbrooder *H. burtoni*, tilapia *O. niloticus*, and Nyerere's Victoria cichlid *P. nyererei* (**Figure 1A**, Table S2 in Supplementary Material), which is likely to represent a novel subgroup of group I IFNs. Additionally, the phylogenetic tree constructed by the minimum evolution method gives a similar tree topology (Figure S2 in Supplementary Mateiral).

Multiple sequence alignment revealed that all of the IFNh members share a well-conserved signature motif, which has some amino acid variation compared with other group I IFN members (**Figure 1A**). Homology comparison showed that lyc IFNd exhibits the highest sequence identity of 82.8% with sea bass IFNd, followed by 82.3–66% identity with IFNd members from other Perciforme species, whereas a low sequence identity of 17.1–31.7% with members of other subgroups (Table S2 in Supplementary Material). Lyc IFNh shares 48.4–55.2% sequence identity to its homologs in other fish species, but

only 17.2–35.1% to those of other subgroups (Table S2 in Supplementary Material). These results further support the classification of IFNh as a novel subgroup, which is distinct from the six subgroups of type I IFNs already known.

Expression Analysis of Iyc IFN Genes

The Iyc IFNd and IFNh were constitutively expressed in all tissues analyzed, with varied expression levels detected. For example, the IFNd and IFNh were most highly expressed in the head kidney and liver, respectively (**Figures 2A,B**). Administration of poly(I:C) by intraperitoneal injection resulted in significant induction of IFNd and IFNh expression in head kidney and spleen, with the highest increases at 4 h for both genes (**Figures 2C,D**). Notably, the IFNh was more responsive than IFNd, showing remarkable increases of 1185- and 695-fold in head kidney and spleen, respectively (**Figures 2C,D**).

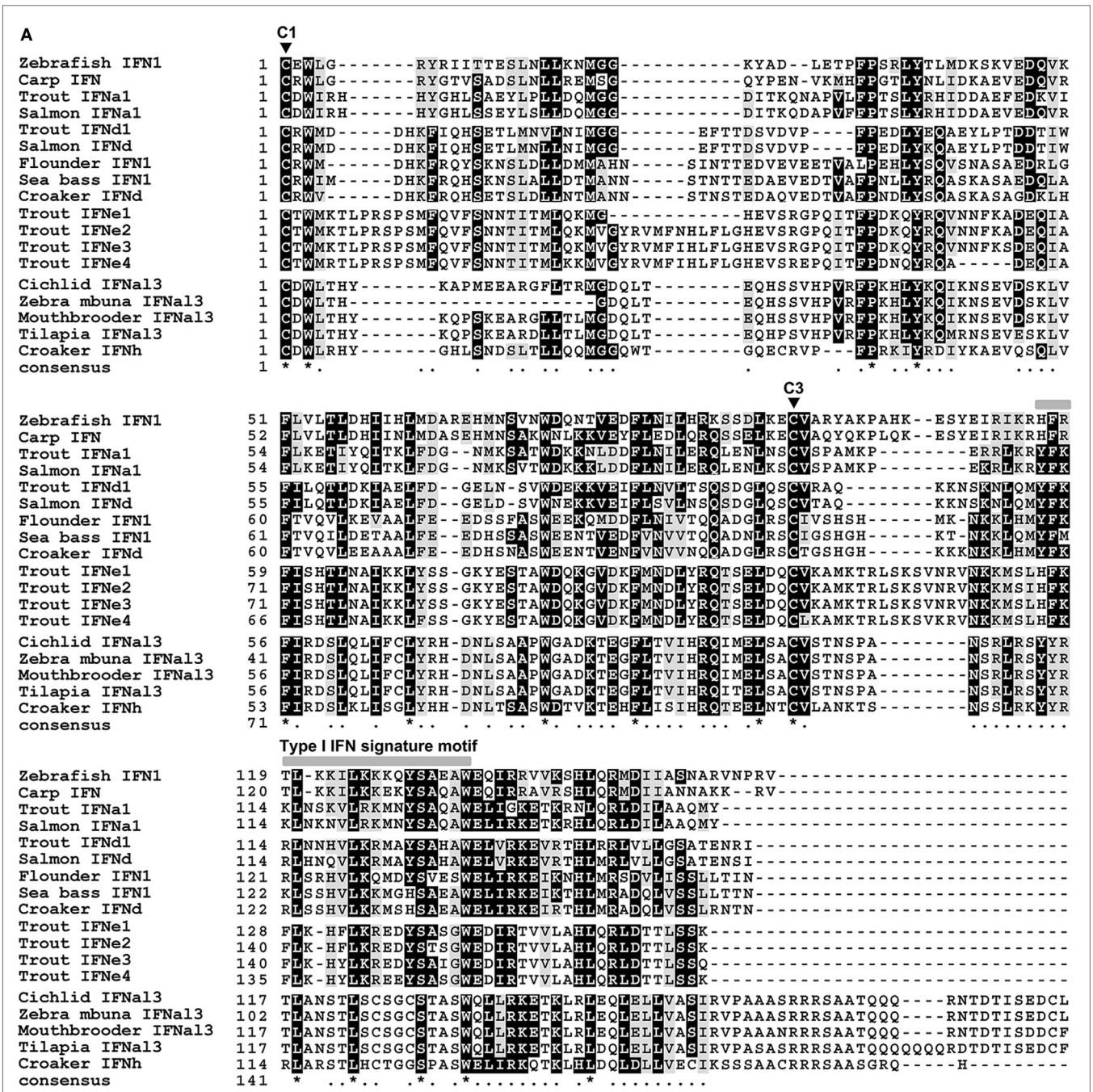

FIGURE 1 | Sequence and phylogeny analysis of fish IFNs.

(Continued)

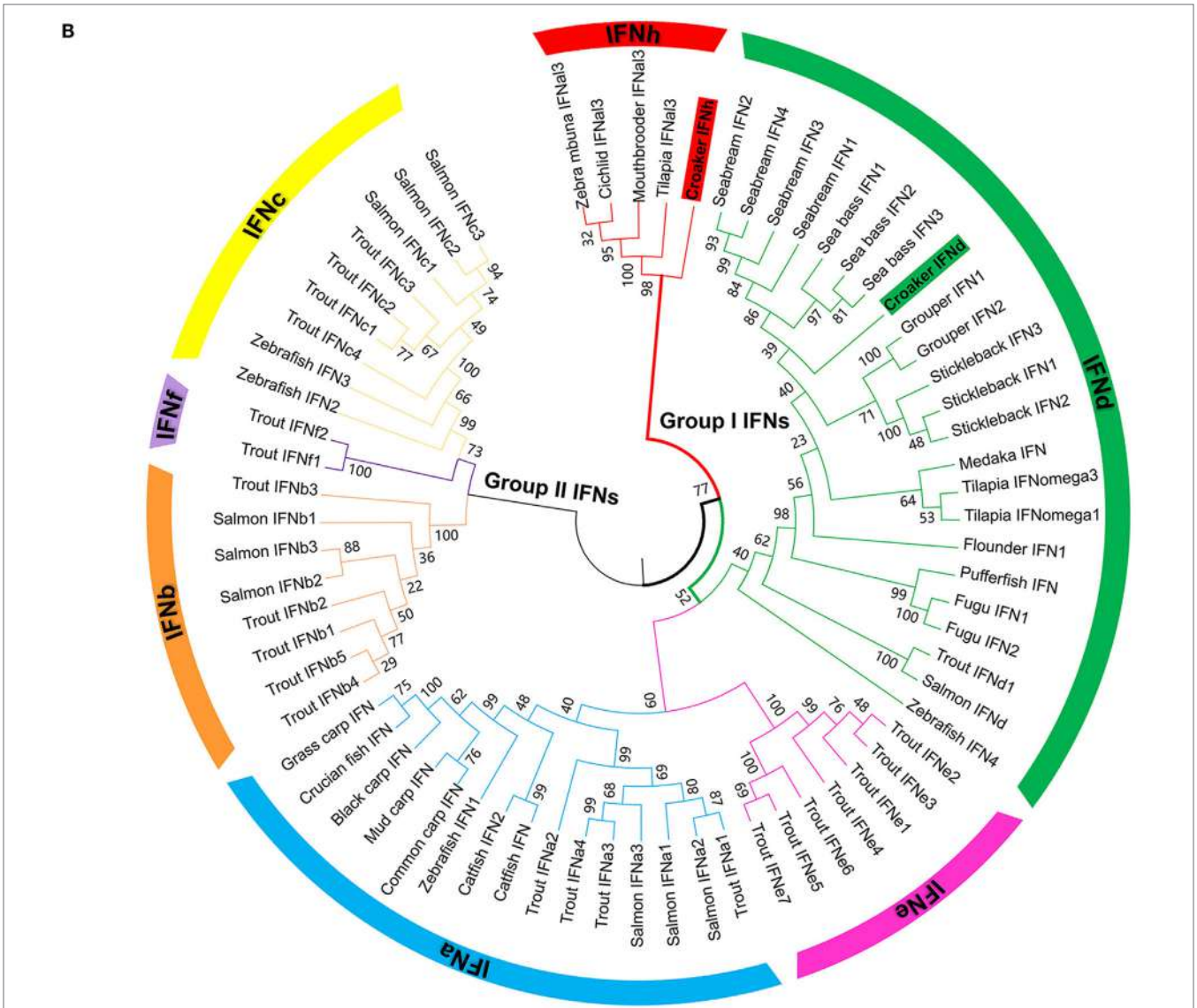

FIGURE 1 | Continued

(A) Multiple alignment of mature peptide sequences of group I type I IFNs (IFNa, d, e, and h) from large yellow croaker and other teleosts. Sequence alignments were obtained using CLUSTAL W2 program, and the conserved residues are shaded using BOXSHADE (v3.21). The two highly conserved cysteine residues of group I type I IFNs (C1 and C3) are indicated by triangles. The signature motif of type I IFNs are marked above the alignment. Identical residues are indicated by stars, while similar residues are indicated by single dots. **(B)** Phylogenetic tree of fish type I IFN family members based on the genetic distances of deduced amino acid sequences. Deduced amino acid sequences of type I IFN family members were aligned, and the tree was constructed with the Neighbor-Joining method using the MEGA (version 6) software package. The tree is bootstrapped 10,000 times, and the bootstrap values of the major branches are shown as percentages.

Antiviral Activity of lyc IFNs

To investigate whether lyc IFNs were able to induce the expression of antiviral genes, the LYCK cells were treated with rIFNd or rIFNh produced in HEK293T cells. Unsurprisingly, the two major antiviral genes, MxA and PKR, were significantly increased by the treatment of rIFNs. Interestingly, rIFNh gave rise to a more rapid activation of MxA and PKR, than the rIFNd (**Figures 3A,B**).

The antiviral activity of recombinant lyc IFNd and IFNh was examined using a cell line (GS) derived from orange-spotted grouper, where an infection model was already established (28, 33, 36). When stimulated with rIFNd or rIFNh for 24 h, the GS cells exhibited significant induction of MxA and PKR expression, confirming the cross-activity in the GS cells (**Figures 3C,D**). Subsequently, the GS cells were used for assessing the antiviral activity of recombinant lyc IFNd and IFNh. Pre-treatment with rIFNd and rIFNh 2 h prior to SGIV infection resulted in significant inhibition of CPE compared with the control cells (**Figure 3E**), indicating that lyc IFNd and IFNh were able to provide enhanced protection of GS cells against SGIV infection. This is supported by the obviously reduced expression of viral genes in the rIFN-treated GS cells (**Figures 3F,G**). These results,

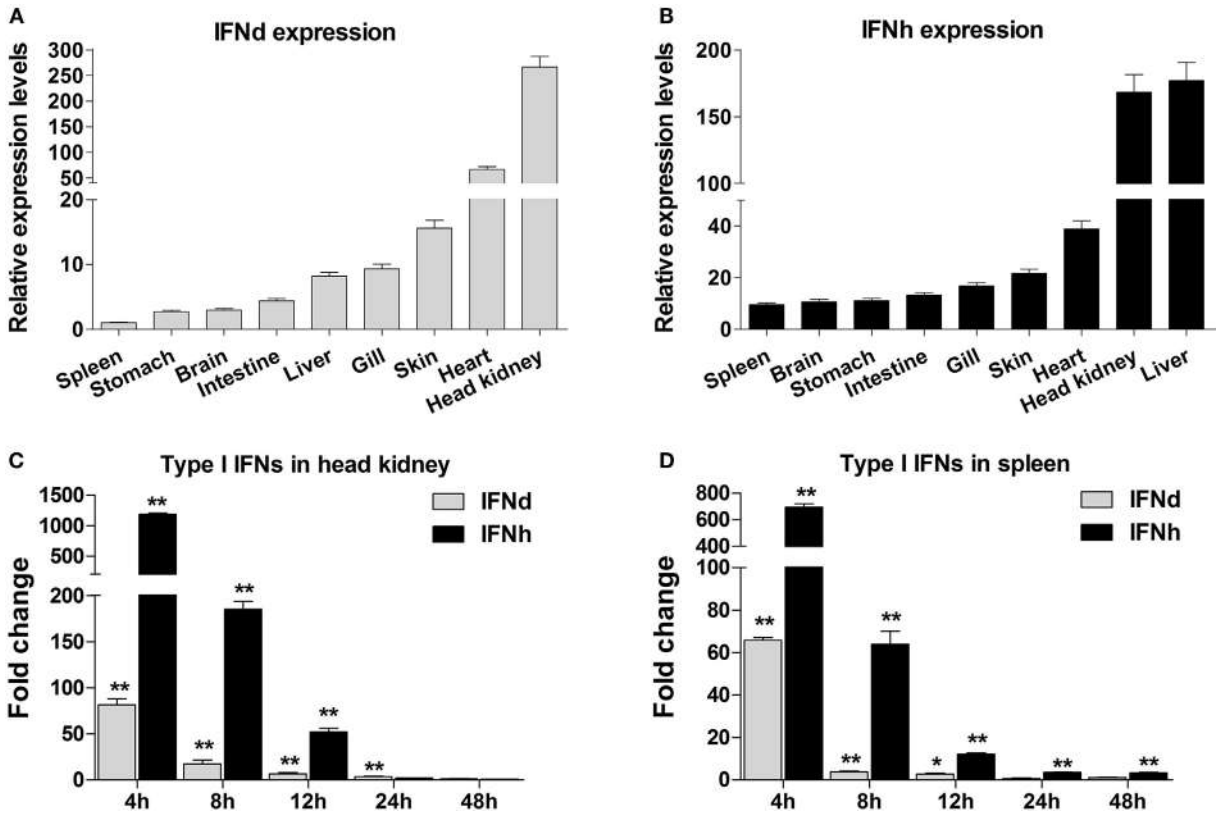

FIGURE 2 | Expression analysis of large yellow croaker IFNd and IFNh. **(A,B)** Tissue expression profile of IFNd **(A)** and IFNh **(B)** genes. Total RNA was extracted from various tissues of five healthy fish and used for real-time PCR analysis. The expression levels of IFN genes were normalized by β-actin using the $2^{-\Delta\Delta CT}$ method and expressed as the ratio of the IFNd expression levels in the spleen. The tissues were ordered according to the relative expression levels from the lowest to the highest. **(C,D)** Expression modulation of IFNd and IFNh genes in the head kidney **(C)** and spleen **(D)** after poly(I:C) induction. Each fish was intraperitoneally injected with 0.2 mg poly(I:C)/100 g fish or PBS (as a control), and head kidney and spleen tissues were collected from five fish in both groups at different time points postinjection for real-time PCR analysis. The expression levels of IFN genes were normalized by β-actin and the normalized expression levels compared between the poly(I:C)-injected fish and the PBS-injected fish (defined as 1) to obtain the relative fold changes at different time points. Error bars represent the standard error of the mean (± SEM) of three repeated experiments. $^{*}p < 0.05$; $^{**}p < 0.01$. The data were analyzed by two-tailed Student's *t*-test.

thus, indicated that both lyc IFNd and IFNh exhibited antiviral activity against SGIV in GS cells.

Activation of the IFN Response by lyc IFNd and IFNh

To investigate whether lyc IFNd and IFNh were able to activate the IFN responses, the LYCK cells were stimulated with the recombinant lyc IFNs, and expression levels of lyc IFNd and IFNh were analyzed at 2, 4, 8, and 20 h postinduction. Surprisingly, the lyc IFNd significantly upregulated expression of both lyc IFNd and IFNh genes (**Figures 4A,B**). Furthermore, the transcript levels of lyc IRF3 and IRF7 were significantly increased by lyc IFNd treatment, with a greater increase of IRF3 transcripts than that of IRF7 transcripts (**Figures 4C,D**). In contrast, the lyc IFNh had no effect on the expression of lyc IFNd, IFNh, IRF3, and IRF7 (**Figure 4**).

Next, we examined the phosphorylation of lyc IRF3 and IRF7 in the LYCK cells, following treatment with poly(I:C) and rIFNs. For this, polyclonal anti-IRF3 and anti-IRF7 Abs were generated and verified to specifically recognize their corresponding proteins expressed in HEK293T cells by Western blotting, thus excluding the possibility of cross-recognition between these two Abs (Figure S3C in Supplementary Material). In the LYCK cells treated with poly(I:C), both unphosphorylated and phosphorylated lyc IRF3 and IRF7 proteins were increased (**Figures 5A,B**). Similar effects were observed in the cells stimulated with rIFNd (**Figures 5C,D**). However, the rIFNh did not alter the levels of unphosphorylated and phosphorylated IRF3 and IRF7 (**Figures 5E,F**). These results suggest that the lyc IFNd, but not IFNh, was involved in the activation of IRF3 and IRF7, leading to induced IFN expression.

Promoter Analysis of IFNd and IFNh Regulation

To understand why the two IFNs elicited distinct activities in induction of the IFN responses, the 1.2 kb 5′-flanking regions of lyc IFNd and IFNh gene promoter were analyzed to search for putative binding sites of transcription factors, including IRF3 and

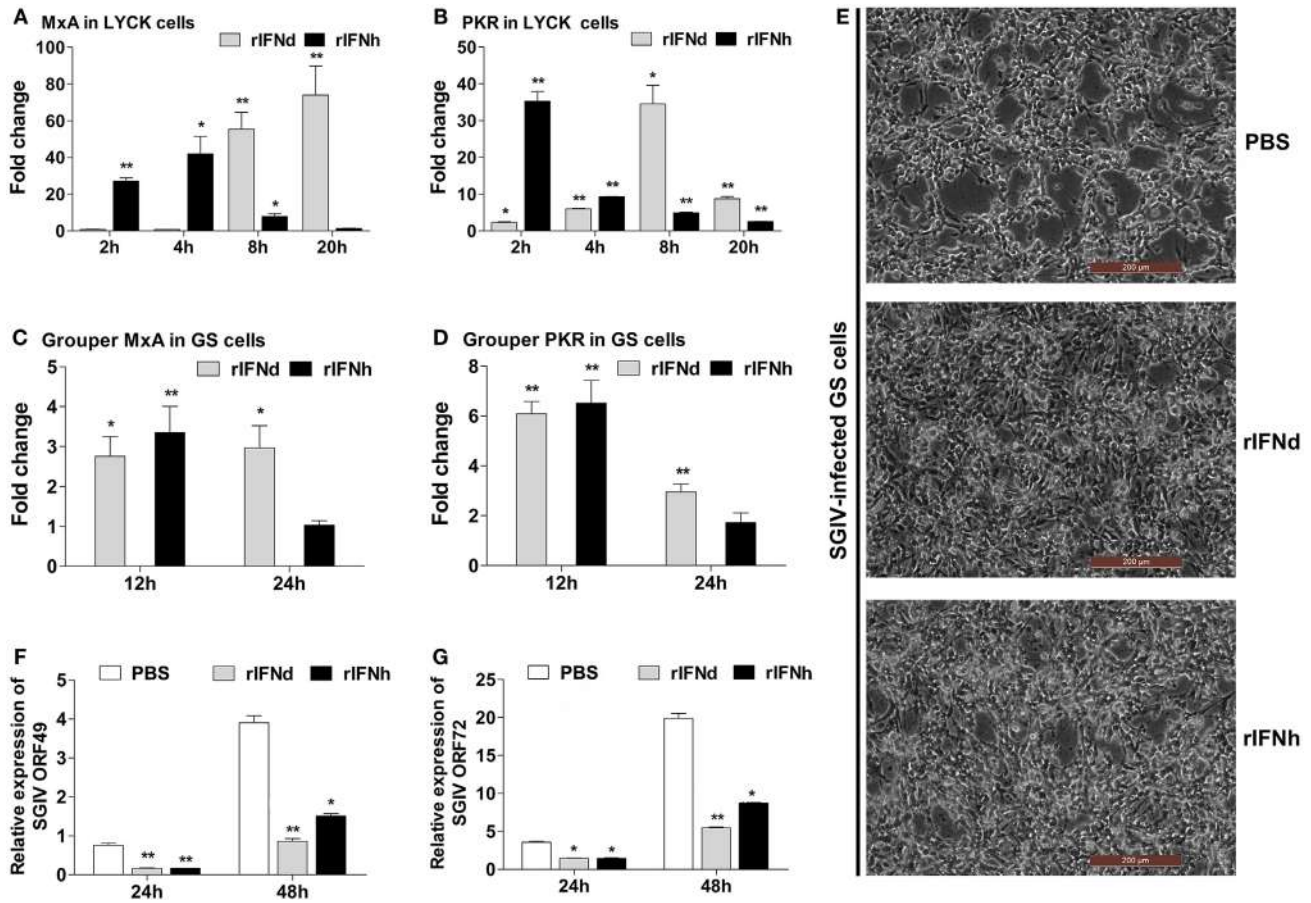

FIGURE 3 | Antiviral activities of recombinant large yellow croaker IFNd and IFNh. (A,B) Induction of large yellow croaker MxA and PKR gene expression by rIFNs in LYCK cells. LYCK cells were plated in 6-well plates (1×10^6 cells/well) and then treated with rIFNs at a final concentration of 50 ng/ml or PBS (control). LYCK cells were sampled at the indicated time points and used for real-time PCR analysis of MxA and PKR gene expression. The relative expression was normalized to the expression of β-actin, and fold induction was calculated by comparing the relative gene expression in rIFN-treated cells with that in PBS-treated cells (defined as 1) at the same time point. **(C,D)** Induction of grouper MxA and PKR gene expression by rIFNs in GS cells. The experiments were performed as described above. **(E)** GS cells were pretreated with rIFNs at a final concentration of 50 ng/ml or PBS (control) for 2 h; then, the cells were infected with SGIV at MOI 2. At 24 h postinfection, GS cells were observed for CPE using microscopy. **(F,G)** At 24 h and 48 h postinfection, the expression levels of SGIV ORF049 **(F)** and ORF072 **(G)** genes were detected by real-time PCR, and normalized to that of *Ec*β-actin. All data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (±SEM) of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$. The data were analyzed by two-tailed Student's *t*-test.

IRF7. As shown in **Figure 6**, the predicted binding sites for transcription factors were mainly located within 796 bp upstream of the transcription start site of IFNd promoter and 655 bp upstream of that of IFNh promoter. The lyc IFNd promoter contained one predicted IRF3 binding site and two IRF7 binding sites, while the IFNh promoter only had two predicted IRF3 binding sites. Binding sites for other transcription factors, such as the NF-κB, ATF-2, and PAX5 and NFAT families, were also predicted (**Figures 6A,D**). Luciferase assays further showed that both full-length IFNd and IFNh promoters (IFNdP1 and IFNhP1) had the ability to initiate the transcription of the luciferase reporter gene (**Figures 6B,E**) and that their expression could be enhanced by poly(I:C) treatment (**Figures 6C,F**).

To determine the active IRF binding sites, several different truncated mutants of IFNd and IFNh promoters were constructed. The IFNdP2 construct, containing two IRF7 binding sites (IRF7 site 1 at −306 bp and IRF7 site 2 at −147 bp) and an IRF3 binding site at −76 bp, was found to have the maximal transcriptional activity. However, after deleting the IRF7 site 1 (−306 bp) on IFNdP3, the promoter activity of this construct was largely reduced (**Figure 6B**), suggesting that the IRF7 site 1 was important for initiating the IFNd expression. IFNdP6 only containing the IRF3 binding site (−76 bp) exhibited no activity as well (**Figure 6B**). Similarly, the IFNhP2 construct, containing a distant IRF3 binding site (IRF3 site 1, −171 bp) and a proximal site (IRF3 site 2, −55 bp), showed the maximal transcriptional activity relative to the other constructs, and the deletion of the IRF3 site 1 (−171 bp) resulted in a significantly reduced promoter activity, suggesting that the IRF3 site 1 was essential for the IFNh promoter activity (**Figure 6E**). In contrast, the constructs only

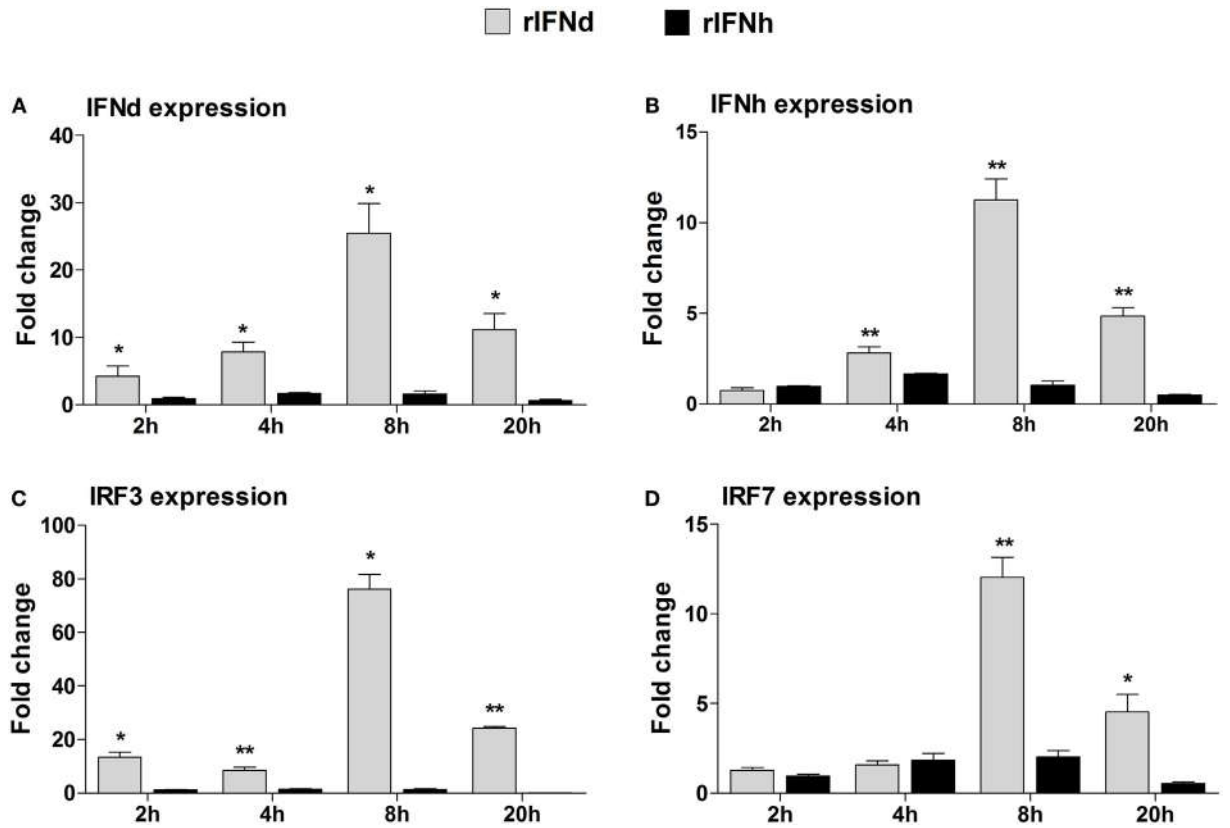

FIGURE 4 | Modulation of IFN-responsive genes by recombinant large yellow croaker IFNs in LYCK cells. LYCKs were plated in 6-well plates (1×10^6 cells/well) and then treated with rIFNd or rIFNh at a final concentration of 50 ng/ml or PBS. LYCK cells were sampled at the indicated time points and used for real-time PCR analysis of large yellow croaker IFNd **(A)**, IFNh **(B)**, IRF3 **(C)**, and IRF7 **(D)** gene expression. The relative expression levels of these genes were normalized by β-actin. Fold change of gene expression level was obtained by comparing the normalized gene expression level in rIFN-treated cells with that in PBS-treated cells (defined as 1) at the same time point. All data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (±SEM) of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$. The data were analyzed by two-tailed Student's *t*-test.

containing the IRF3 site 2 (−55 bp) did not induce luciferase activity, suggesting that this IRF3 site was not required for triggering the IFNh expression (**Figure 6E**).

Further transfection experiments were performed to gain insights into the regulatory roles of IRF3 and IRF7 on IFNd and IFNh expression. First, we confirmed that the exogenous IRF3 and IRF7 were produced in the EPC cells when transfected with expression constructs of lyc IRF3 or IRF7 (Figure S3D in Supplementary Material). The full-length IFNd promoter (IFNdP1) could be activated by overexpression of IRF7 (3.4-fold, **Figure 7A**). The truncated constructs IFNdP2 and P3, which contained the IRF7 site 1 (−306 bp), also showed induced luciferase activity in IRF7-overexpressed cells, whereas the IFNdP4 construct without the IRF7 site 1 did not show any altered luciferase activity (**Figure 7B**), indicating that the IRF7 site 1, but not the IRF7 site 2, was important for the IFNd expression. However, overexpression of IRF3 did not affect the luciferase activity driven by all the IFNd promoter constructs (**Figure 7B**).

In contrast, overexpression of IRF3 significantly enhanced the luciferase activity yielded by the full-length IFNh promoter (IFNhP1) and IFNhP2 constructs, but not the IFNhP3 construct where the IRF3 site 1 (−171 bp) was absent (**Figures 7C,D**), confirming that the IRF3 site 1 was essential for the activation of IFNh. The luciferase activity of the IFNh promoter was also increased by overexpression of IRF7 (2.6-fold), but considerably lower than that by overexpression of IRF3 (66.9-fold, **Figure 7C**). These results indicate that IRF3 and IRF7 have specific roles in regulating IFNd and IFNh, respectively.

Interestingly, cotransfection of IRF3 and IRF7 plasmids yielded much higher luciferase activity of the IFNd promoter (19.1-fold) than that of IRF7 alone (6.1-fold, **Figure 7E**). Again, the synergistic effect of IRF3 and IRF7 appeared to involve the IRF7 site 1, since overexpression of IRF3 and IRF7 did not stimulate the luciferase activity of the IFNdP4, P5, and P6 constructs lacking the IRF7 site 1 (**Figure 7F**). However, overexpression of IRF3 and IRF7 did not increase IFNh promoter activity to a greater extent than IRF3 alone (**Figure 7G**). Co-immunoprecipitation of IRF3 and IRF7 in the transfected cells revealed that lyc IRF3 could interact with lyc IRF7, which facilitated the formation of the IRF3 and IRF7 heterodimer and was important to the enhanced

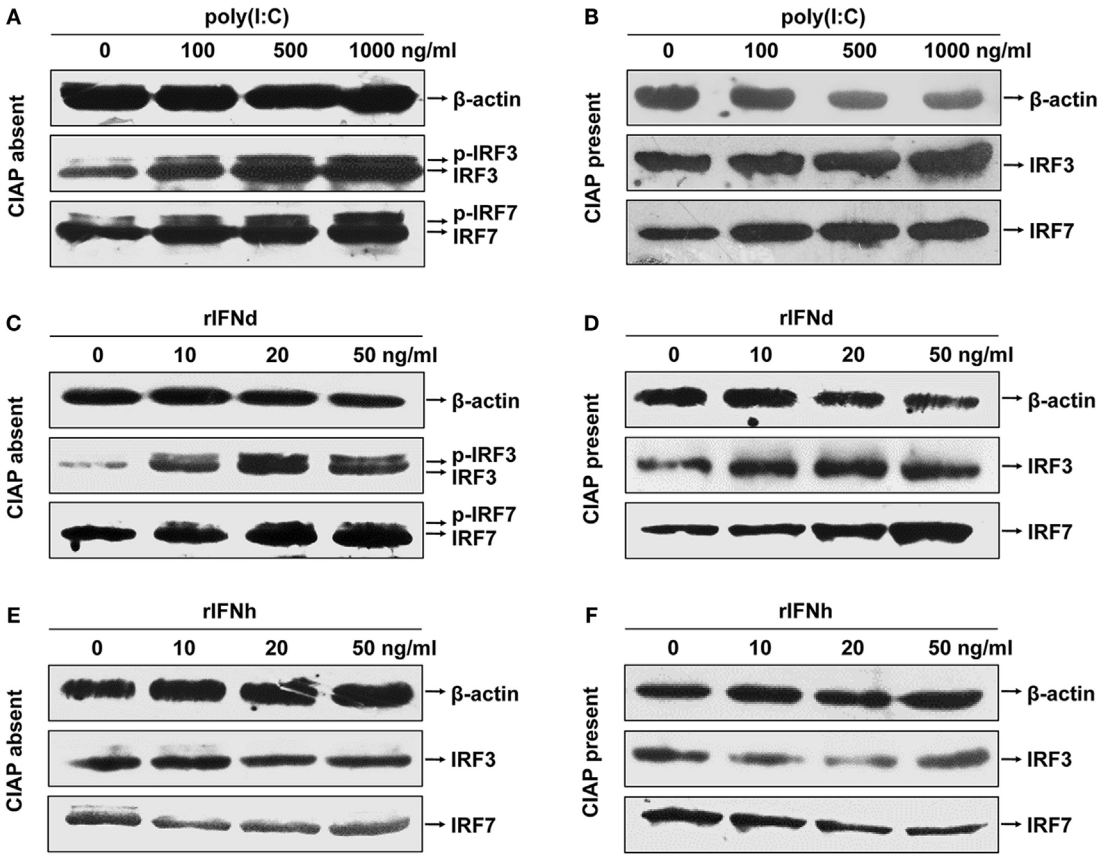

FIGURE 5 | Activation of large yellow croaker IRF3 and IRF7 in response to poly(I:C) and rIFNs. LYCK cells were in 6-cm culture dishes (2.5×10^6 cells/dish) overnight and then treated with poly(I:C) **(A,B)**, rIFNd **(C,D)**, and rIFNh **(E,F)** at a range of doses as indicated for 12 h. LYCK cell extracts were incubated with or without 20 U of calf intestinal alkaline phosphatase (CIAP) each sample for 30 min and then used to detect the induction and phosphorylation of IRF3 and IRF7 proteins by Western blotting analysis.

effect (**Figure 7H**). These results reveal that the IRF3 and IRF7 have distinct roles in regulating fish IFN expression, controlling the expression of IFNd and IFNh, respectively, and interaction of IRF3 and IRF7 could further enhance the IFNd, but not IFNh expression.

Binding of IRF3/IRF7 to IFN Promoters

The EMSA assay was performed to verify the IRF3/7 binding motifs in the IFN promoters characterized. The oligonucleotide probes were synthesized for the predicted IRF3 and IRF7 binding sites and incubated with cell lysates containing recombinant IRF3 or IRF7 *in vitro*. It is apparent that the rIRF3 was able to bind to the oligo probes of the predicted IRF3 binding sites in the IFNd promoter (−76 bp) and IFNh promoter (−171 bp). The formation of DNA–rIRF3 complex was specific since it could be blocked by excessive unlabeled control probes (100×) (**Figures 8A,B**). Furthermore, retardation of the IRF3 probes was not observed in the presence of rIRF7 protein, indicating that the IRF7 could not bind to the two IRF3 binding sites (**Figures 8A,B**). Subsequent mutations of the nucleotides in the IRF3 binding site resulted in dissociation of the DNAc–rIRF3 complex (**Figures 8D,E**). Similarly, the specific binding of rIRF7 and IRF7 binding site 1 (−306 bp) in the IFNd promoter was also confirmed (**Figures 8C,F**).

DISCUSSION

Teleost type I IFNs are classified into two groups based on the cysteine patterns in their mature peptides, with group I and II containing either two or four cysteines, respectively (3). Recent studies have shown that they can be further divided into six phylogenetic subgroups, including IFNa, b, c, d, e, and f (2). In the present study, we have identified two type I IFNs, IFNd and IFNh, in large yellow croaker, of which IFNh belongs to a novel subgroup (termed IFNh subgroup) of group I IFNs, based on the cysteine pattern and phylogenetic analyses (**Figure 1**, Figure S2 in Supplementary Material). The IFNh homologs were also discovered in zebra mbuna, Burton's mouthbrooder, tilapia, and cichlid (Table S2 in Supplementary Material), suggesting that an IFN subgroup is commonly present in the Perciforme lineage in addition to the IFNd subgroup found previously (6–9). Thus, the Perciforme fishes possess at least two subgroups of group I IFNs, IFNd and IFNh. This is the first report that two subgroups of type

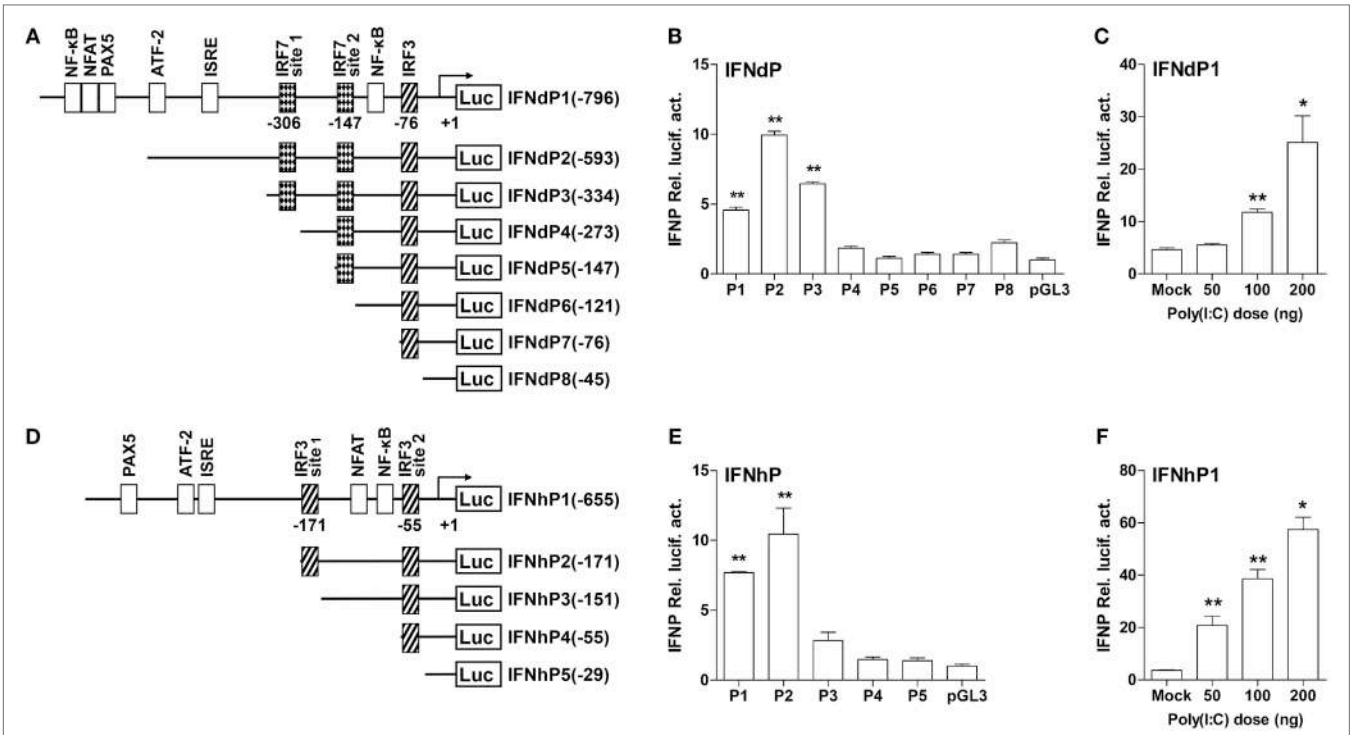

FIGURE 6 | The structure and transcriptional activity of large yellow croaker IFN promoters. **(A,D)** Schematic representation of IFNd **(A)** and IFNh **(D)** promoters and a series of deletion constructs. **(B,E)** Transcriptional activity of IFN promoters. EPC cells (5×10^4/well) were seeded in 96-well plates overnight and cotransfected with 100 ng of pGL3-IFNdP plasmid **(B)** or pGL3-IFNhP plasmid **(E)** and 2 ng of pRL-TK using the Fugene® HD transfection reagent. Transcript levels were determined by real-time PCR. **(C,F)** Induction of large yellow croaker IFN promoter activity by poly(I:C). EPC cells (5×10^4/well) were seeded in 96-well plates overnight and cotransfected with 50 ng of pGL3-IFNdP plasmid **(C)** or pGL3-IFNhP plasmid **(F)**, poly(I:C) (as indicated doses), and 1 ng of pRL-TK using the Fugene® HD transfection reagent. After 48 h of transfection, the cells were harvested for detection of luciferase activity. All data were obtained from three independent experiments with three replicates in each experiment. Error bars represent ±SEM of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$. The data were analyzed by two-tailed Student's *t*-test.

I IFNs exist in Perciforme fishes, making the number of fish IFN subgroups to seven, the IFNa, d, e, and h belonging to the group I IFNs, while the IFNb, c, and f to the group II IFNs.

It is well known that teleosts have experienced a third round (3R) of whole-genome duplication (WGD) during their early evolution, and this WGD event is believed to contribute to the diversification of fish type I IFN family (37, 38). Salmonids are thought to have undergone an additional WGD event compared with other teleost species and possess six IFN subgroups, IFNa, d, and e subgroups of group I IFNs and IFNb, c, and f of group II IFNs (2, 39). Some, if not all of these, subgroups exist in primitive teleosts and may have been lost in certain teleost lineages during evolution. For instance, only three of the subgroups, IFNa, c, and d are present in cyprinids, such as zebrafish and carps (11, 40–42). However, the IFNh subgroup identified here cannot be assigned to any subgroups found in salmonids and cyprinids and, hence, represents a novel phylogenetic group. Considering that salmonids and cyprinids are relatively primitive teleosts, we suggest that the IFNh subgroup found in the Perciformes might have diverged more recently in evolution.

The expression patterns of type I IFNs in fish have been relatively well studied. In general, group I IFNs (IFNa, d, and e) appear to be constitutively expressed in most cell types and fish tissues and are inducible by viral RNA analogs or viral infection. In this study, the IFNd and IFNh (group I IFNs) were also shown to be constitutively expressed in all examined tissues, but with different levels of expression in tissues (**Figures 2A,B**). Upon poly(I:C) stimulation, the increase of IFNh transcripts was greatly higher than that of IFNd (1185-fold increases versus 82-fold increases in head kidney, and 695-fold increases versus 66-fold increases in spleen) (**Figures 2C,D**), indicating that the IFNd and IFNh are differentially modulated by poly(I:C) in head kidney and spleen. The results are in line with those observed in other fish species, such as trout, zebrafish, salmon, and medaka *O. latipes* (2, 10, 11, 43). Subsequent functional analyses demonstrate that the IFNh was more potent in triggering a rapid induction of the antiviral genes MxA and PKR than the IFNd (**Figures 3A,B**), suggesting that these two IFNs may play distinct roles in regulating early antiviral immunity.

Interferon regulatory factor 3 and IRF7 are master transcriptional factors that regulate type I IFN gene expression (44, 45). Mammalian IRF3 is constitutively expressed in most cell types and cannot be induced by IFN or viral analogs at the transcriptional level, whereas fish IRF3, on the contrary, has been confirmed to be a typical ISG (20, 46–49). The likely cause of this discrepancy may be explained by the presence of

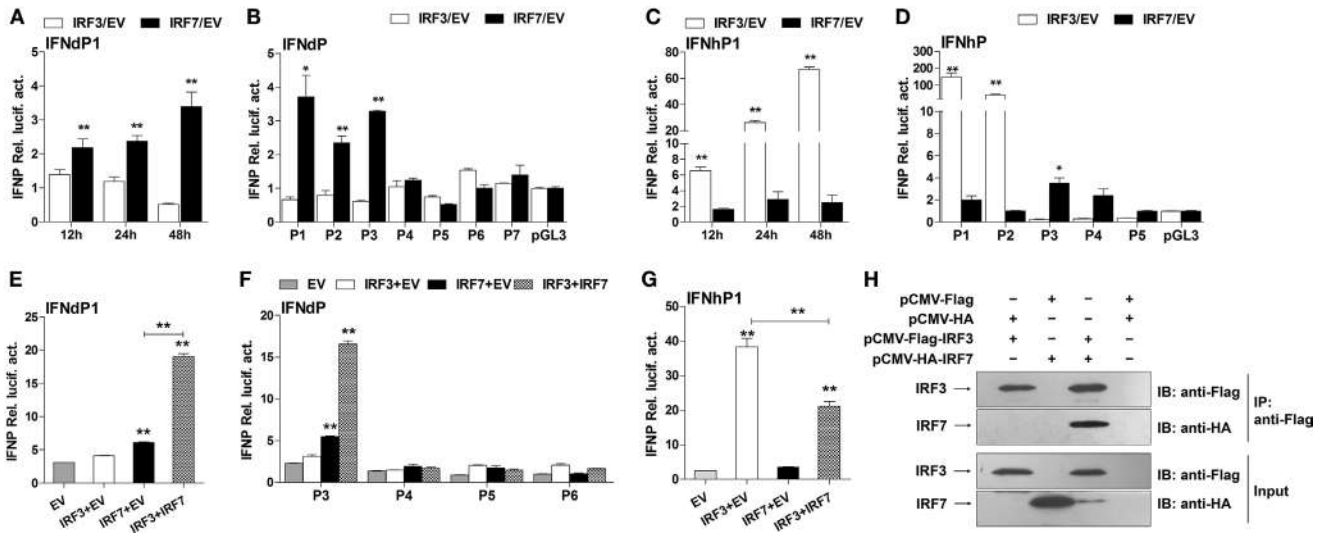

FIGURE 7 | **Effects of IRF3 and IRF7 on induction of IFN promoter activity**. **(A)** Full-length IFNd promoter (IFNdP1), **(B)** the IFNdP deletion constructs, **(C)** full-length IFNh promoter (IFNhP1), **(D)** the IFNhP deletion constructs. EPC cells (5×10^4/well) were seeded in 96-well plates overnight and cotransfected with 50 ng of pGL3-IFNP plasmids, 50 ng of pCMV-HA-IRF3 or -IRF7, and 1 ng of pRL-TK. **(E–G)** Cooperative effect of IRF3 and IRF7 on IFN promoter. EPC cells were seeded in 96-well plates overnight and cotransfected with 50 ng of IFNdP1 **(E)**, the IFNdP deletion constructs **(F)** or IFNhP1 **(G)**, the indicated expression constructs (100 ng total at a ratio of 1:1), and 1 ng of pRL-TK. After 48 h of transfection, the cells were harvested for detection of luciferase activity. The empty vectors (EV) were used as controls. All data were obtained from three independent experiments with three replicates in each experiment. Error bars represent ±SEM. $^*p < 0.05$; $^{**}p < 0.01$. The data were analyzed by two-tailed Student's *t*-test. **(H)** Interaction of large yellow croaker IRF3 and IRF7. Protein extracts from HEK293T cells cotransfected with plasmids expressing Flag-tagged IRF3 and HA-tagged IRF7 were immunoprecipitated with murine anti-Flag monoclonal antibody. Immunoprecipitated complexes (IP) and whole cell lysates (Input) were analyzed by immunoblot (IB) for IRF3 and IRF7 using antibodies against Flag and HA.

the IFN-stimulated response element (ISRE) motifs in the fish IRF3 promoters, including large yellow croaker (Figure S1C in Supplementary Material) (20, 46). In contrast to the IRF3, both mammalian and fish IRF7 can be induced by type I IFNs (48–51). Interestingly, the activation of both IRF3 and IRF7 in mammals requires virus-induced phosphorylation (17, 52), coordinating the appropriate initiation of IFN responses during virus infection. In the present study, not only were lyc IRF3 and IRF7 induced at the transcriptional level but also were phosphorylated by the treatment of IFNd or poly(I:C) (**Figures 4C,D** and **5C,D**), suggesting distinct mechanisms for the activation of IRF3 and IRF7 between fish and mammals (17, 46, 52). The IFN-induced IRF3/IRF7 activation may be IFN type-specific, as the lyc IFNh failed to induce the expression and activation of IRF3 and IRF7 (**Figures 5E,F**).

In mammals, IRF3 functions mainly for the initiation of IFNβ gene expression, while IRF7 is more critical at the late stage for IFNα gene induction (16, 17). It is found that IRF7 governs the overall IFN responses and synergistically promotes the expression of type I IFNs with IRF3 (15, 52, 53). In fish, the IRF3 and IRF7 also display distinct roles in regulating type I IFN expression (19–22, 54). For example, the IRF7 binding sites are present in the promoter of IFNd, but not IFNh (**Figure 6**). The IRF7 could trigger IFNd expression by itself and induce much higher IFNd expression together with IRF3 (**Figures 7A,E**), in agreement with the previous observations in carp and zebrafish, where cooperation of IRF3 and IRF7 led to higher induction of IFN expression than IRF3 or IRF7 alone (54). In fact, lyc IRF3 and IRF7 could form heterodimer (**Figure 7H**), which may be transported into the nucleus and bind to the IFN promoter more effectively than the IRF3 or IRF7 homodimer alone. The EMSA assays further showed that both lyc IRF3 and IRF7 specifically bound to the IFNd promoter at the sites of −76 and −306, respectively (**Figures 8A,C**). Curiously, the lyc IFNh expression involves IRF3. Overexpression of IRF3 greatly enhanced the transcriptional activity of the IFNh promoter (**Figure 7C**), likely through binding to the IRF3 site 1 (−171) in the IFNh promoter (**Figure 7D**), as shown by the EMSA that IRF3, but not IRF7, specifically was involved in interaction with this motif (**Figure 8B**).

In summary, we have identified two type I IFNs in large yellow croaker, one of which was assigned to a novel subgroup of fish group I IFNs (IFNh). The two IFNs (lyc IFNd and IFNh) showed apparent differences in expression patterns and ability to induce antiviral genes. Only IFNd, but not IFNh, was able to activate phosphorylation of IRF3 and IRF7 and trigger the expression of itself and IFNh. Furthermore, the expression of IFNd can be enhanced by the synergistic effect of IRF3 and IRF7, and the IFNh expression mainly involves IRF3. Thus, a positive feedback regulation, which was mediated by IFNd-induced IRF3 and IRF7 activation, was proposed in lyc (**Figure 9**). This IFN-induced IRF3 and IRF7 activation may represent a unique mechanism regulating fish IFN responses (**Figure 9**), which differs from that in mammals (17, 46, 52). The results provide new insights into the

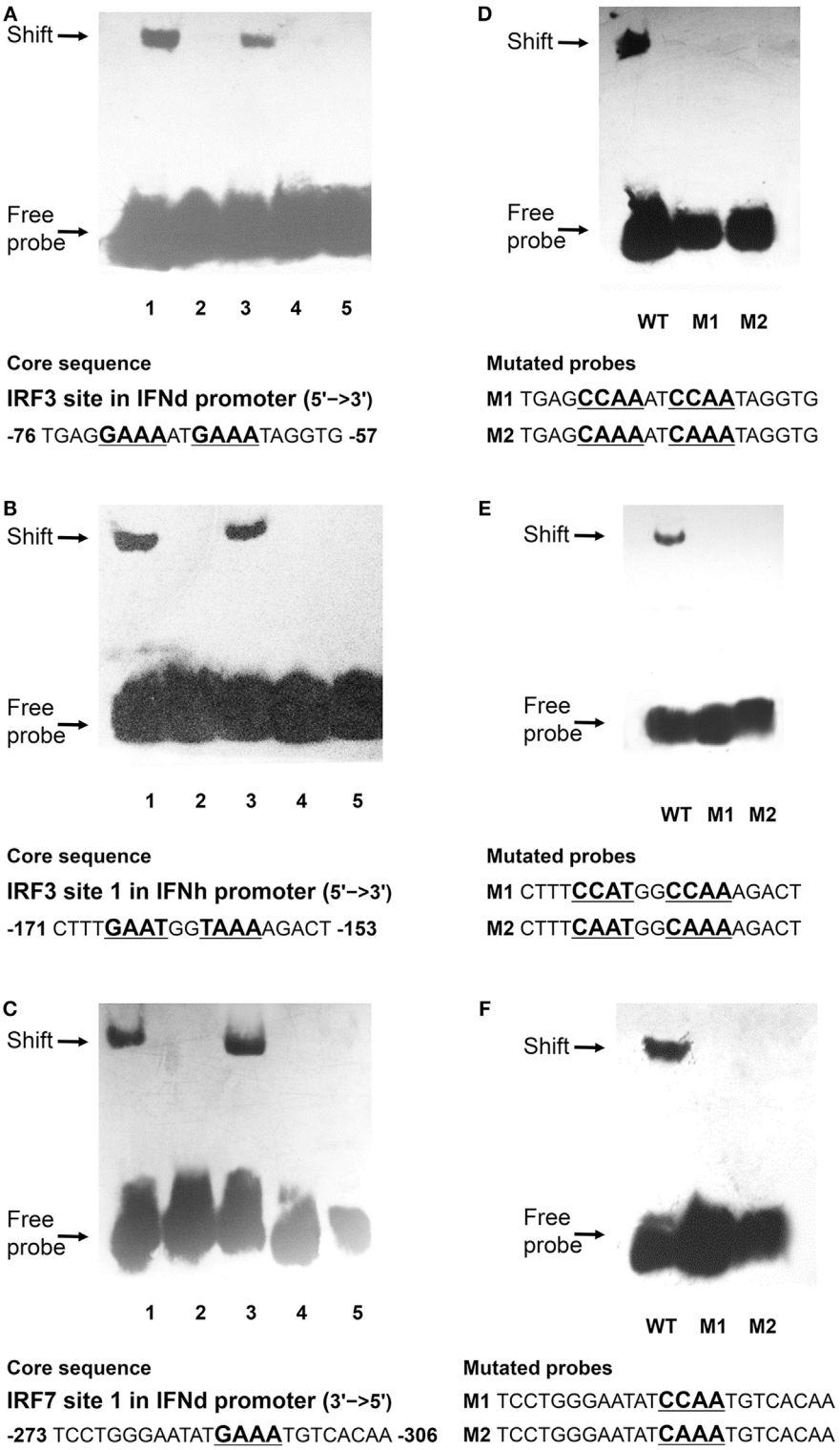

FIGURE 8 | **Binding reactions of large yellow croaker IRFs and IFN promoters**. Biotin-labeled EMSA probes were incubated with lysates of HEK293T cells containing rIRF3 or rIRF7 proteins. **(A,B)** 1. IRF3 probes of IFNd **(A)** and IFNh **(B)** plus IRF3; 2. IRF3 probes only; 3. 100× unlabeled mutated probes plus IRF3; 4. 100× unlabeled wild-type probes plus IRF3; 5. IRF3 probes plus IRF7. **(C)** 1. IRF7 probes of IFNd plus IRF7; 2. IRF7 probes only; 3. 100× unlabeled mutated probes plus IRF7; 4. 100× unlabeled wild-type probes plus IRF7; 5. IRF7 probes plus IRF3. **(D)** Mutated IRF3 probes of IFNd and **(E)** mutated IRF3 probes of IFNh plus IRF3. **(F)** Mutated IRF7 probes of IFNd plus IRF7. After a 20 min incubation, the completed reactions were separated by electrophoresis on a 4% non-denaturing polyacrylamide gel for EMSA. WT, wild-type probes; M1 and M2: mutated probes.

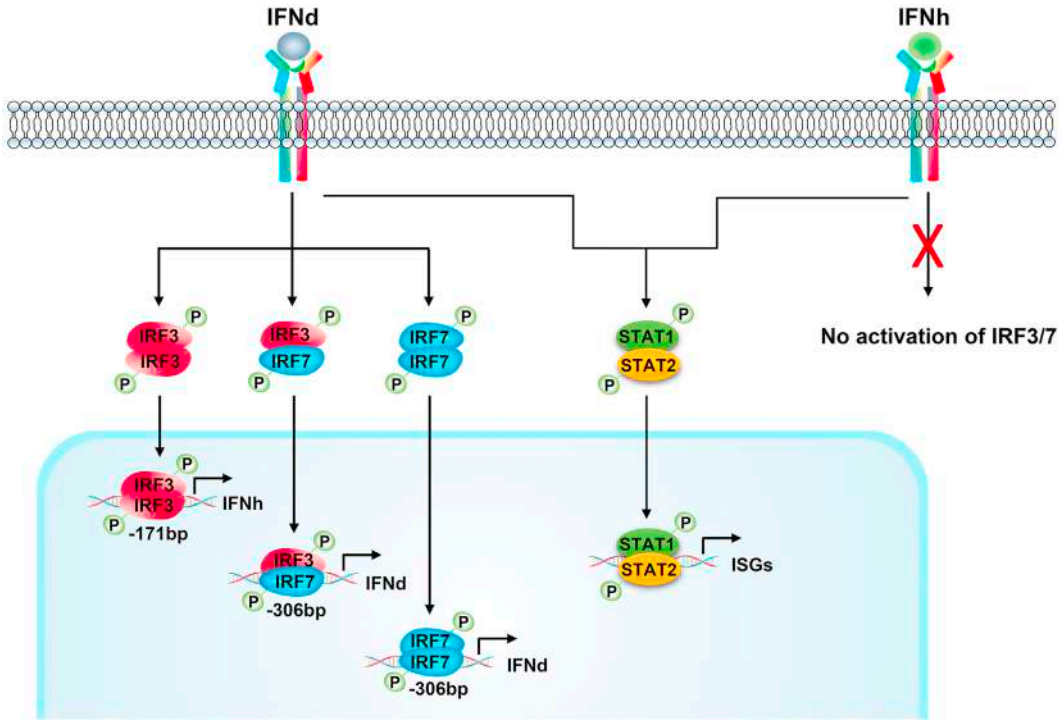

FIGURE 9 | Positive feedback regulation of type I IFN response mediated by IFNd-induced IRF3 and IRF7 activation in large yellow croaker. The large yellow croaker IFNd induced the expression of itself and IFNh by activating IRF3 and IRF7. The dimeric form of IRF3 and IRF7 or IRF7 may be transported into the nucleus and bind to the corresponding sites in the IFNd promoter, thus upregulating the expression of IFNd. The activated IRF3 binds to the IRF3 site 1 (−171bp) present in the IFNh promoter and induces the expression of IFNh. In contrast, the large yellow croaker IFNh has no effect on the expression of itself and IFNd and expression and phosphorylation of IRF3 and IRF7. Both, IFNd and IFNh, are able to induce the expression of ISGs, including MxA and PKR, possibly through the Jak–Stat pathway.

regulation and function of fish type I IFNs and further reveal the complexity of the regulatory mechanisms.

AUTHOR CONTRIBUTIONS

YD performed most of the experiments, analyzed the data, and wrote the manuscript. XH prepared the GS cells and SGIV and performed the experiments for antiviral activities. JA helped with the first draft and performed the statistical analysis. XC designed the research, mentored all other authors, and wrote the final version of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00343

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REVIEW
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Viral Evasion Strategies in Type I IFN Signaling – A Summary of Recent Developments

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The immune system protects the organism against infections and the damage associated with them. The first line of defense against pathogens is the innate immune response. In the case of a viral infection, it induces the interferon (IFN) signaling cascade and eventually the expression of type I IFN, which then causes an antiviral state in the cells. However, many viruses have developed strategies to counteract this mechanism and prevent the production of IFN. In order to modulate or inhibit the IFN signaling cascade in their favor, viruses have found ways to interfere at every single step of the cascade, for example, by inducing protein degradation or cleavage, or by mediate protein polyubiquitination. In this article, we will review examples of viruses that modulate the IFN response and describe the mechanisms they use.

Keywords: virus, type I interferon, evasion, innate immune signaling, NFκB

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INTRODUCTION

The mammalian immune system evolved to detect and fight viral infections effectively. The induction of type I interferon (IFN), predominantly IFN-α and IFN-β, forms the first line of defense. The type I IFN response consists of two parts. First, the cell produces type I IFN, when triggered by a viral stimulus. The IFN is then secreted and, in the second part of the response, it is sensed by the producing, as well as neighboring cells, resulting in the production of IFN-stimulated genes (ISGs) [reviewed in Ref. (1)].

Viruses, which have coevolved with their host, develop strategies to counteract the signaling cascades of the innate immune system and ensure their replication. Recently, several reviews were published, describing the innate immune evasion strategies of individual viruses or virus families, such as influenza virus (2, 3), Phleboviruses (4), Herpes viruses (5–7), Coronaviruses severe acute respiratory syndrome (SARS) and middle east respiratory syndrome (MERS) (8), human immunodeficiency virus (HIV) (9, 10), as well as multiple RNA viruses (11, 12). Moreover, there are recent articles that review how viruses prevent detection by pathogen recognition receptors (PRRs) (13, 14) and how viruses modulate innate immune signaling by use of viral deubiquitinases (15).

In this review, we will compare the different strategies viruses have developed to suppress innate immune signaling of individual components of the innate immune signaling cascade. Due to the tremendous amount of data in this field, we will focus on recent discoveries. Older studies were summarized in Ref. (16, 17).

VIRUS RECOGNITION

Invading viruses are recognized by PRRs [reviewed in Ref. (14)]. The most important viral markers for the innate immune system are viral nucleic acids. The detection of viral DNA through the cGAS-Sting pathway and the counter measurements taken by viruses have been reviewed recently (18) and are not part of this review.

Viral RNAs, which are mostly double-stranded (ds-)RNA, are recognized by three PRRs: the endosomal toll-like receptor 3 (TLR3), the cytoplasmic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and the nucleotide-oligomerization domain (NOD)-like receptors (NLRs) (19). TLR3 and the RLRs are important for inducing the type I IFN response, whereas NLRs have been shown to regulate interleukin-1β (IL-1β) maturation through activation of caspase-1 (20). The group of RLRs consists of RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). The three receptors have a similar structure, all containing a caboxy-terminal domain, which functions as a repressor domain (RD) in RIG-I and LGP2 (21) and a central helicase domain, but LGP2 lacks the caspase activation and recruitment domains (CARDs) that function in signaling [reviewed in Ref. (19, 22)]. Both the helicase and the carboxy-terminal domain are required for RNA binding. RIG-1 and MDA-5 detect specific viral RNA PAMPs, while LGP2 negatively regulates RIG-I signaling and promotes RNA binding to MDA5 [reviewed in detail in Ref. (14)].

In unstimulated cells, RIG-I and MDA-5 are kept in a repressed state due to phosphorylations on serine and threonine residues in the CARDs and carboxy-terminal domains (23, 24). Upon binding of RNA, both RIG-I and MDA-5 undergo conformational changes, resulting in release of their CARDs (25, 26). Recruited phosphatases remove the phosphate residues, and E3 ubiquitin ligases attach Lys63-linked ubiquitin polymers onto the CARDs and C-terminal domain of RIG-I, which are important for RIG-I tetramerization (27–31).

RNA-bound RIG-1 then interacts with 14-3-3ε, a mitochondrial trafficking protein, and the TRIM25 ubiquitin ligase, which together transport RIG-I to the mitochondria (32). There the CARDs of RIG-I or MDA-5 interact with the CARD of the mitochondrial activator of virus signaling (MAVS, also known as IPS-1, VISA, and Cardif), which is an essential signaling adaptor protein. The activation of MAVS has recently been reviewed in detail in Ref. (33).

TLR3 interacts with TRIF, which serves as a molecular platform and forms physical interactions with several adaptor molecules (34). By interacting with upstream adaptors, TRIF undergoes conformational changes and recruits the downstream TNF receptor-associated factor (TRAF)3 and TRAF6 [reviewed in Ref. (35)]. The kinase receptor-interacting protein-1 (RIP-1) is part of both the signaling pathways downstream of TLR3 and RIG-I. It can interact with TRIF to induce NFκB activation (36). Moreover, the dsRNA-activated TLR3 can recruit TRIF, RIP-1, and Caspase-8 and induce apoptosis (37). Also, RIP-1 and its adaptor protein Fas-associated protein with death domain (FADD) are part of the signaling cascade downstream of RIG-I and MDA-5 and involved in the activation of the transcription factors interferon regulatory factor (IRF)3 and IRF7 (38). TRAF3 serves as a linker between the upstream adaptor proteins (TRIF or MyD88 for TLRs and MAVS for RLRs) and the downstream signaling kinases TBK1/IKKε or IRAK1/IKKα. The recruitment of TRAF3 to the TLR or RLR signaling complexes activates the E3 ligase activity of TRAF3, which then catalyzes its own K63-linked ubiquitinylation. Subsequent TRAF3 activates TBK1/IKKε or IRAK/IKKα [reviewed in Ref. (39)] (**Figure 1**).

Viruses target RIG-I directly or indirectly to block the type I IFN response. The phlebovirus Toscana Virus expresses a non-structural protein, which directly interacts with RIG-I and induces its proteasomal degradation (40, 41). Foot-and-mouth disease virus (FMDV) proteins L^{pro}, $3C^{pro}$, and 2B increase the RIG-I mRNA expression but decrease the protein expression of RIG-I. L^{pro} and $3C^{pro}$ both induce RIG-I degradation, whereas the mechanism of how 2B reduces RIG-I protein levels has not been solved yet (42). Other viruses target RIG-I indirectly. Hepatitis B virus (HBV) induces miR146a, which then posttranscriptionally inhibits the expression of RIG-I and suppresses the production of type I IFN (43).

The dengue virus NS3 protein binds to 14-3-3ε and prevents the translocation of RIG-I to MAVS. The binding site on NS3 is a highly conserved phosphomimetic motif, which was verified by generation of a virus containing a mutation in this motif (44).

It has been proposed that in certain cell types RIG-I requires sentinels, such as the protein DDX60, which associates with RIG-I and promotes the RIG-I RNA-binding activity (45, 46). Other studies question DDX60 acting as a broadly active enhancer of antiviral responses (47, 48) and instead suggest that DDX60 only functions in the antiviral response to specific viruses, such as hepatitis C virus (47). However, there are data indicating that influenza A virus and hepatitis C virus attenuate IFNβ-promoter activation by targeting the sentinel DDX60. Both viruses activate the epidermal growth factor (EGF) receptor, which in turn phosphorylates DDX60 on Tyr-793 and Tyr-796. This results in the attenuation of DDX60-dependent RIG-I activation. In addition, independent of its role as sentinel for RIG-I viral RNA recognition, DDX60 plays a role in viral RNA degradation (46) (**Figure 1**).

Mitochondrial activator of virus signaling is blocked by different viruses in various ways. The dengue virus protein NS4A targets MAVS, and the interaction prevents the binding of MAVS to RIG-I (49). The porcine reproductive and respiratory syndrome virus (PRRSV) 3C-like protease (3CLSP), by contrast, cleaves MAVS in a proteasome- and caspase-independent manner at Glu268 (E268/G269). Both cleavage products fail to activate the type I IFN response (50). Likewise, the hepatitis C virus protein NS3-4A (51, 52), as well as the highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) protein nsp4 (53) have been shown to cleave MAVS and block RLR signaling. The porcine epidemic diarrhea virus (PEDV) also targets MAVS in small intestinal epithelial cells (IECs). However, the exact mechanism has not been solved yet (54) (**Figure 1**).

The SARS coronavirus protein ORF9b not only influences antiviral signaling but also alters host cell mitochondria morphology by inducing degradation of the dynamin-like protein (DRP1). MAVS becomes concentrated into small puncta in the presence

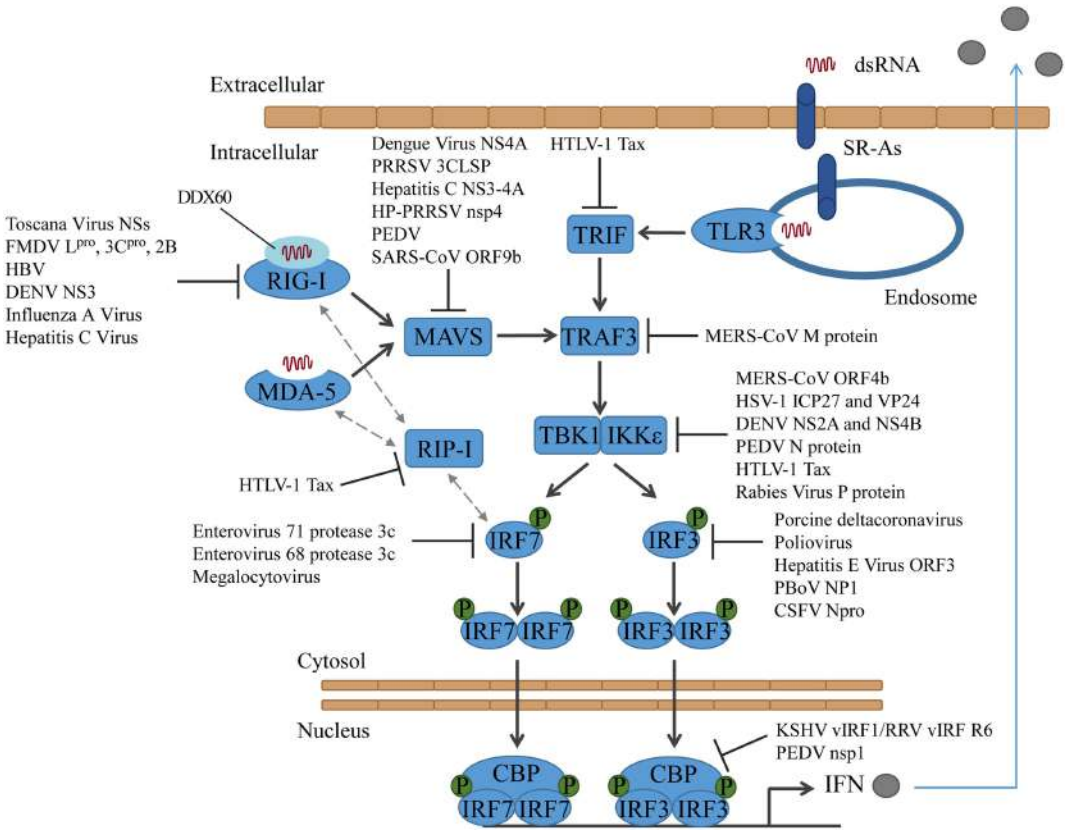

FIGURE 1 | Activation of interferon regulatory factors and the counteractions taken by viruses. DsRNA is sensed by PRRs, which results in the activation of different adaptor proteins and the recruitment of TRAF3. TBK1 and/or IKKε are activated and phosphorylate IRF3 and/or IRF7, which then translocate into the nucleus to induce type I IFN expression. CSFV, classical swine fever virus; DENV, dengue virus; FMDV, foot-and-mouth disease virus; HBV, hepatitis B virus; HP-PRRSV, highly pathogenic porcine reproductive and respiratory syndrome virus; HSV-1, herpes simplex virus type 1; HTLV-1, human T-cell lymphotropic virus type I; KSHV, Kaposi's sarcoma-associated herpesvirus; MERS-CoV, middle east respiratory syndrome coronavirus; PBoV, porcine bocavirus; PEDV, porcine epidemic diarrhea virus; PRRSV, porcine reproductive and respiratory syndrome virus; RRV, rhesus macaque rhadinovirus.

of ORF9b (55). In addition, ORF9b triggers K48-linked ubiquitinylation of MAVS, by targeting the poly(C)-binding protein 2 (PCBP2) and the HECT domain E3 ligase AIP4. Under normal conditions, PCBP2 controls MAVS levels by linking the AID4 E3 ubiquitin ligase with MAVS (56). In addition to MAVS, also the levels of TRAF3 and TRAF6 are reduced by ORF9b. However, it is unlikely that TRAF3 and TRAF6 are targeted directly. More likely, they are degraded due to their interaction with MAVS (55) (**Figure 1**).

Human T-cell lymphotropic virus type I (HTLV-1) protein Tax disrupts innate immune signaling in multiple ways: it binds to the RIP homotypic interaction motif (RHIM) domains of RIP-1 and disrupts the interaction between RIP-1 and RIG-I or MDA-5 and the activation of the type I IFN promoter. Tax also binds to TRIF and thereby interrupts the TLR3 signaling cascade. Finally, Tax blocks the association between RIP-1 and IRF7, which resulted in repression of the IRF7 activity (57) (**Figure 1**).

Middle East respiratory syndrome coronavirus M protein interacts with TRAF3 and disrupts the interaction between TRAF3 and TBK1, which ultimately leads to a reduced IRF3 activation. For the interaction with TRAF3, the N-terminal transmembrane domain of the MERS-CoV M protein is sufficient (58), similar to what has been shown for SARS-CoV before (59) (**Figure 1**).

ACTIVATION OF TRANSCRIPTION FACTORS AND IFN TRANSCRIPTION

Triggering of the TLR3- and RLR-signaling cascade results in the activation of the transcription factors NFκB and IRF3/IRF7. In its inactive state, the transcription factor NFκB is complexed with its inhibitor IκB (60). Upon stimulation, IκB is phosphorylated by the IκB kinase (IKK) complex, which is composed of two catalytic subunits, such as IKKα and IKKβ, and a regulatory subunit, such as NFκB essential modulator (NEMO) (61). The phosphorylation of IκBα induces its polyubiquitination through the E3 ubiquitin ligase β-transducin repeat-containing protein (β-TrCP) and subsequent proteasomal degradation (62), allowing NFκB to translocate into the nucleus and induce the expression of target genes (63) (**Figure 2**).

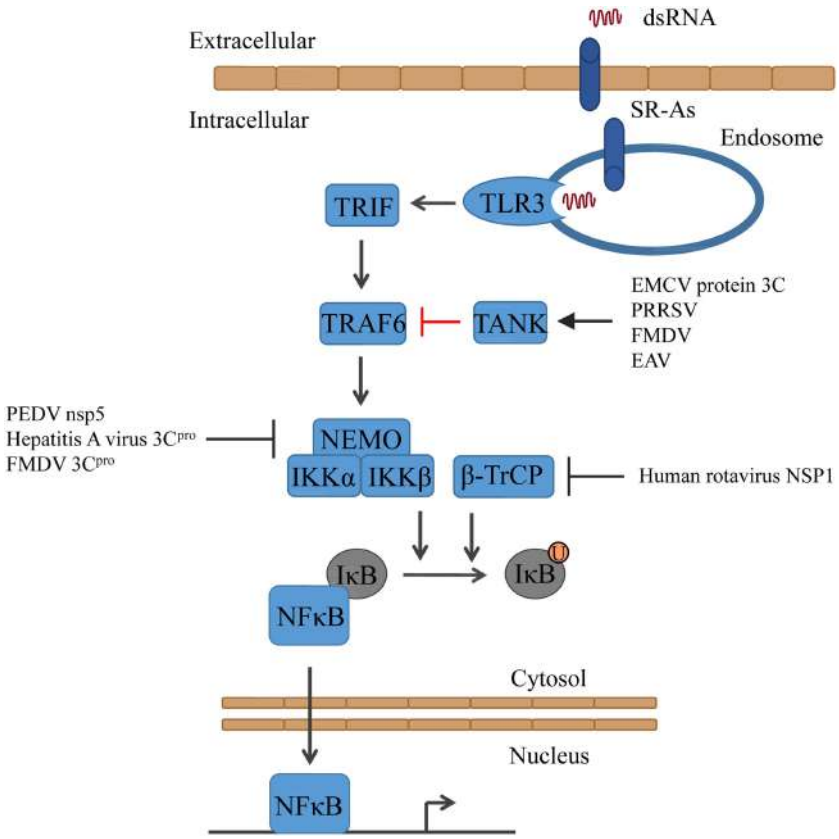

FIGURE 2 | Activation of NFκB signaling and the counteractions taken by viruses. Triggering of TLR3 results in the activation of first TRAF6 and subsequently of IKK (consisting of NEMO, IKKα, and IKKβ). Together with β-TrCP, IKK mediates the ubiquitinylation of IκB, resulting in the release of NFκB. EAV, equine arteritis virus; EMCV, encephalomyocarditis virus; FMDV, foot-and-mouth diseases virus; PEDV, porcine epidemic diarrhea virus; PRRSV, porcine reproductive and respiratory syndrome virus.

Encephalomyocarditis virus (EMCV) protein 3C cleaves TRAF family member-associated NFκB activator (TANK), which inhibits TRAF6-mediated NFκB activation, on Gln291. As a result, NFκB is activated and the unstable C-terminal fragment of TANK is subjected to proteasomal degradation (64). Also, other viruses express proteases that cleave TANK, although on other residues, such as porcine reproductive and respiratory syndrome virus (PRRSV) (TANK is cleaved by Nsp4), FMDV (protease 3C cleaves TANK), and equine arteritis virus (EAV) (TANK is cleaved by Nsp4). Thus, TANK seems to be a common target of several positive RNA viral proteases (64) (**Figure 2**).

Several viruses have been shown to disrupt IFN signaling by cleaving NEMO. PEDV 3C-like protease, nsp5, cleaves NEMO at Gln231 (65), whereas the hepatitis A virus 3C protease ($3C^{pro}$) cleaves NEMO at Gln304 (66) and the picornavirus FMDV protease $3C^{pro}$ at Gln383, removing the C-terminal zinc finger domain from the protein (67). The human rotavirus has developed another way. Its non-structural protein 1 (NSP1) has been shown to inhibit the NFκB pathway by degrading β-TrCP and consequently stabilizing IκB (68) (**Figure 2**).

TANK-binding kinase 1 (TBK1) and inhibitor of κB kinase ε (IKKε) are classified as non-canonical serine/threonine kinases and are both able to induce IRF3 and IRF7 phosphorylation and subsequent dimerization (69–72). However, while TBK1 is constitutively expressed in most cell types, the expression of IKKε is more restricted (73). Upon stimulation, TBK1 and IKKε are recruited by adaptor proteins to signaling complexes to be activated by phosphorylation on Ser172 and both have been shown to be subjected to K63-linked polyubiquitination [reviewed in Ref. (73, 74)]. For TBK1, K63-linked polyubiquitination seems to be important for TLR- and RLR-induced IFN production, as ubiquitin chains might serve as a platform for the assembly of TBK1 signaling complexes. Moreover, deubiquitinases are able to terminate the TBK1-mediated pathway by cleaving the K63-linked ubiquitin chains [reviewed in Ref. (74, 75)]. Activated TBK1/IKKε phosphorylates IRF3 and/or IRF7 in the cytosol at specific serine residues. This phosphorylation results in homo- or heterodimerization of IRF3 and IRF7 and nuclear translocation (76, 77). Interestingly, while IRF3 is constitutively expressed, IRF7 is expressed at low levels in most cell types and expression is induced upon IFN signaling. Therefore, in most cells, IRF7 strongly enhances the production of IFN [reviewed in Ref. (78)]. Once phosphorylated IRF3 and/or IRF7 dimers have translocated into the nucleus, they bind to the transcription coactivator

CREB-binding protein (CPB)/p300 (79, 80). Together with other factors, such as NFκB, they form the enhanceosome on the IFNβ promoter and induce the expression of type I IFN [reviewed in Ref. (76)].

The viral proteins that target TBK1 act by either blocking activation of TBK1 by MAVS or by inhibiting activation of IRF3 by TBK1. The MERS-CoV protein ORF4b blocks IFNβ production by binding to TBK1 and IKKε and suppressing the formation of a MAVS/IKKε complex (81). In addition to inhibiting TBK1/IKKε activation, ORF4b can also inhibit the production of IFNβ in the nucleus; however, the mechanism has not been solved yet (81). Recently, two herpes simplex virus proteins have been shown to target TBK1/IKKε and inhibit the phosphorylation of IRF3: ICP27 (82) and VP24 (83). Also, dengue virus serotype 4 non-structural proteins NS2A and NS4B, as well as the NS2A and NS4B proteins of other Dengue viruses, inhibit the phosphorylation of TBK1 (84) and PEDV N protein has been shown to interact with TBK1, hampering the association of TBK1 with IRF3 and preventing the activation of IRF3 activation (85). The human T-cell leukemia virus type 1 oncoprotein Tax has been shown to also interact with TBK1. However, studies came to contradicting results on how that influences the production of IFNβ. While one group showed that Tax activates TBK1 and the production of IFNβ (86), another group showed that Tax suppresses the IFN production by interaction with TBK1 (87). Interestingly, when a recent study tested how the rabies virus P protein of street strains behaves compared to laboratory-adapted strains with regard to the induction of type I IFN, they found that both street strains and laboratory strains inhibit TBK1-mediated signaling, but only the P protein of street strains also interacts with and inhibits IKKε-inducible IRF3-dependent IFNβ expression (88) (**Figure 1**).

Interferon regulatory factor 3 is targeted by many viruses to impair innate immune signaling. Most viruses inhibit the phosphorylation and thereby also the dimerization and translocation of IRF3, such as the porcine deltacoronavirus (89) or poliovirus (90). Hepatitis E virus protein ORF3 also suppresses IRF3 phosphorylation, but in an indirect way. It activates the signal regulator protein α (SIRP-α), which negatively regulates type I IFN induction (91). In contrast, porcine bocavirus (PBoV) NP1 protein does not affect IRF3 expression, phosphorylation, or nuclear translocation. Instead, it interacts with the DNA-binding domain of IRF3 and inhibits the DNA-binding activity (92). A very interesting way of how to circumvent the host innate immune response was found when studying gammaherpesviruses Kaposi's sarcoma-associated herpesvirus (KSHV) and rhesus macaque rhadinovirus (RRV). They express several viral homologs to the IRFs, called viral IRFs (vIRFs). These vIRFs have found multiple ways to suppress type I IFN production. For KSHV, different strategies have been reviewed in Ref. (6). Recently, the RRV vIRF R6 has been shown to interact with the transcriptional coactivator CBP in the nucleus, similar to the KSHV vIRF1. As a result, CBP cannot form a complex with the phosphorylated IRF3, and the IFN expression is not induced (93–95). Interestingly, RRV R6 is the first vIRF for which an association with the viron could be shown. Therefore, vIRF V6 can shut down the type I IFN response shortly after the cell was infected, rendering the cell more susceptible to infection (95). The PEDV protein nsp1 also targets CBP. Nsp1 induces CBP degradation in a proteasome-dependent manner and thereby interrupts enhanceosome assembly and the production of type I IFN (96) (**Figure 1**).

For most of these interactions, the molecular mechanisms have not been unraveled yet. A protein that has been shown to interact with and induce proteasomal degradation of IRF3 some time ago is classical swine fever virus (CSFV) Npro (97, 98). Recently, the molecular mechanism has been published. IRF3 and Npro interact direct and form a soluble 1:1 complex. Moreover, it was shown that Npro interacts with the full-length IRF3, not with individual domains, and that Npro binds the constitutively active form of IRF3 in the presence of CPB. Thus, Npro interacts with both the monomer and the active IRF3 dimer and likely targets both species for ubiquitinylation and proteasomal degradation (99).

Interferon regulatory factor 7 is targeted by two human enteroviruses, such as enterovirus 71 and enterovirus 68. They downregulate IRF7 by cleaving it with their protease 3c, leaving the cleavage products unable to induce IFN expression. While enterovirus 71 cleaves IRF7 once at Gln189–Ser190 (100), Enterovirus 68 cleaves it twice, the cleavage sites being Gln167 and Gln189 (101). Moreover, megalocytivirus, a DNA virus that infects marine and freshwater fish, induces the expression of the host microRNA pol-miR-731, which then specifically suppresses the expression of IRF7 (102) (**Figure 1**).

TYPE I IFN SIGNALING

The type I IFNs act in an autocrine, paracrine, or systemic manner to stimulate antiviral responses. They are recognized by the IFNα/β receptor (IFNAR), which consists of the subunits IFNAR1 and IFNAR2 expressed on virtually all cell types (103). The interaction of type I IFN with the receptor results in the phosphorylation and activation of the IFNAR1- and IFNAR2-associated tyrosine kinases tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1), which then phosphorylate IFNAR tyrosine residues, resulting in the recruitment and activation of signaling molecules, such as the signal transducer and activator of transcription (STAT) family of transcription factors (104, 105). Upon activation, STAT1 and STAT2, together with IRF9, form the IFN-stimulated gene factor 3 (ISGF3), which then translocates into the nucleus to induce transcription of ISGs [reviewed in detail in Ref. (106–108)].

Several viruses target IFNAR to prohibit IFN binding and signaling. Influenza virus induces the degradation of IFNAR1. Hemagglutinin (HA) triggers the phosphorylation and ubiquitinylation of IFNAR1, thus promoting protein degradation (109). Encephalitic Flaviviruses, such as tick-borne encephalitis virus or West Nile virus, inhibit IFNAR1 surface expression. Their protein NS5 binds the cellular dipeptidase prolidase (PEPD), which is involved in IFNAR1 maturation and accumulation, activation of IFNβ-stimulated gene induction, and IFN-dependent viral control. This interaction inhibits IFNAR1 intracellular trafficking and glycosylation but does not promote IFNAR1 degradation (110) (**Figure 3**).

Both STAT1 and STAT2 are targeted by many viruses to suppress ISG induction. PEDV induces Stat1 ubiquitinylation and targets it for degradation in the proteasomes (111).

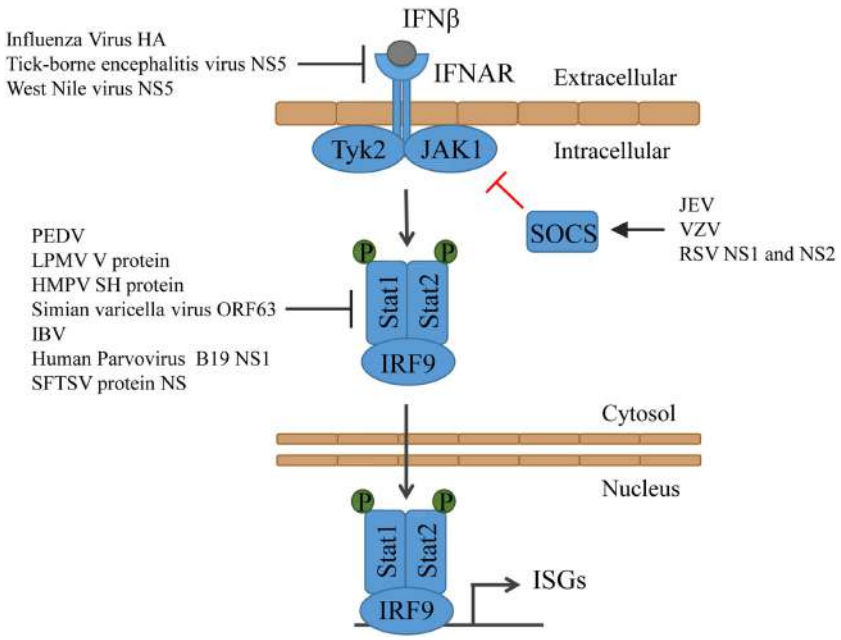

FIGURE 3 | Type I IFN signaling and the counteractions taken by viruses. IFN binds to its receptor and thereby activates Tyk2 and Jak1, which then phosphorylate Stat1 and Stat2. Together with IRF9, Stat1 and Stat2 form the ISGF3, which translocates into the nucleus and induces the expression of ISGs. HMPV, human metapneumovirus; IBV, infectious bronchitis virus; JEV, Japanese encephalitic virus; LPMV, La Piedad Michoacán Mexico Virus; PEDV, porcine epidemic diarrhea virus; RSV, respiratory syncytial virus; SFTSV, severe fever with thrombocytopenia syndrome virus; VZV, varicella-zoster virus.

Some viruses evolved to prevent the phosphorylation of Stat1 or Stat2. The paramyxovirus La Piedad Michoacán Mexico Virus (LPMV) V protein binds to Stat2 and prevents the type I IFN-dependent phosphorylation and nuclear translocation of Stat1 and Stat2 (112). Similarly, human metapneumovirus (HMPV) protein SH impairs Stat1 expression, phosphorylation, and activation (113). Simian varicella virus not only inhibits Stat2 phosphorylation but also promotes degradation of IRF9 in a proteasome-dependent manner through its protein ORF63 (114). Also, infectious bronchitis virus (IBV) inhibits phosphorylation and nuclear translocation of Stat1. However, despite detailed analyses, it is unclear which viral protein is responsible. It was, however, shown that the accessory protein 3a contributes to IBV resistance to type I IFN, although the target is unknown as well (115). In case of the human Parvovirus B19, it becomes evidently clear that both the virus and the immune system constantly evolve to prevail. While its protein NS1 suppresses Stat phosphorylation, the immune system senses the protein and triggers the production of type I IFN (116). SFTSV, an emerging tick-borne pathogen, developed multiple ways to prevent ISG induction. The viral non-structural protein NS impairs Stat1 expression, phosphorylation, and activation (117) and interacts with STAT2 and sequesters STAT1 and STAT2 into viral inclusion bodies, where they are trapped (118) (**Figure 3**).

The JAK-STAT signal transduction pathway is negatively regulated by the suppressor of cytokine signaling (SOCS) family of proteins in form of a classical feedback loop (119, 120). Some viruses induce the expression of SOCS to take advantage of this mechanism to minimize the induction of ISGs. Japanese encephalitic virus (JEV) downregulates the expression of microRNA miR-432, which then results in upregulated SOCS5 levels (121). Varicella-zoster virus (VZV) infection induces the expression of SOCS3 (122) and respiratory syncytial virus (RSV) nonstructural proteins NS1 and NS2 induce upregulation of SOCS1 and SOCS3, which also inhibited the induction of chemokines (123) (**Figure 3**).

HOST SHUT OFF

Viruses fully depend on the translation machinery of the host cell for replication. Accordingly, they have evolved multiple ways to hamper host protein synthesis [reviewed in Ref. (124)]. One way is to shut off host protein synthesis. For some time, it was thought that Gamma- and Deltacoronaviruses do not induce host shutoff, such as Alpha- and Betacoronaviruses do. However, a recent study showed that the infectious bronchitis Gammacoronavirus induces host shutoff using its protein 5b. It seems like 5b is a functional equivalent of nsp1, the host shutoff protein of Alpha- and Betacoronaviruses (125).

CONCLUSION

Viruses evolved to have various strategies to circumvent the innate immune response by blocking the production of type I IFN or the expression of ISGs. While these diverse strategies may appear contradictory between viruses, several factors require consideration. For example, the use of clinical isolates versus

laboratory-passaged strains could yield different results, particularly with RNA viruses that rapidly accumulate mutations due to error-prone RNA-dependent RNA polymerases. Moreover, the choice of cell line can greatly influence experimental outcomes, as many immortalized or transformed continual cell lines harbor mutations in critical innate immune signaling (126). Likewise, the use of genetic knockout versus knockdown cell lines or organisms can influence experimental outcomes, as can the experimental procedures themselves, particularly when endogenous interactions are disrupted with the use of overexpression approaches.

Studying the mechanisms used by viruses to prevent an immune response is of great importance for the development of new strategies to limit the sequelae of viral infections. Identification of key immune evasion proteins allows development of antivirals to target these proteins. Alternatively, identification of key cellular antiviral pathways allows development of strategies to enhance these pathways to overwhelm incoming viruses. Information on key immune evasion factors further facilitates the engineering of safe and effective vaccine strains and designing strategies to target new emerging viruses from the same or closely related family.

AUTHOR CONTRIBUTIONS

KS and KM conceptualized the scope of the review article. KS wrote the review with input from KM.

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Type I Interferon Impairs Specific Antibody Responses Early during Establishment of LCMV Infection

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Elicitation of type I interferon (IFN-I) has been shown to both enhance and impair cell-mediated immune responses in acute and persistent viral infections, respectively. Here, we show that, in addition to its effect on T cells, IFN-I drives impairment of specific antibody responses through interaction with B cells in the acute phase of lymphocytic choriomeningitis virus (LCMV) infection. This impairment was limited to the T cell-dependent B cell response and was associated with disruption of B cell follicles, development of hypergammaglobulinemia (HGG), and expansion of the T follicular helper cell population. Antigen-specific antibody responses were restored by ablation of IFN-I signaling through antibody-mediated IFN-I receptor blockade and B cell-specific IFN-I receptor knockout. Importantly, IFN-I receptor deficiency in B cells also accelerated the development of LCMV neutralizing antibodies and alleviated HGG. These results provide a potential therapeutic target toward efficient treatment measures that limit immunopathology in persistent viral infections.

Keywords: LCMV, interferon type I, antibody formation, immunopathology, neutralizing antibodies

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INTRODUCTION

The humoral immune response plays a central effector role against viral infection whereby induction of effective antibody (Ab) responses serves as an important correlate toward pathogen clearance. However, during persistent viral infections, e.g., with human immunodeficiency virus (HIV), hepatitis C virus (HCV), or the murine infection model lymphocytic choriomeningitis virus (LCMV), emergence of neutralizing Abs (nAbs) against these highly mutable viruses is delayed, initially narrow in specificity and ineffective against the established infection; as such, the Ab response bears negligible impact on the progression of the disease (1). Accompanying the delayed induction of nAbs, infected hosts also exhibit an altered immunological milieu that features aberrancies to the humoral response such as: (i) dysregulation of B cell subpopulations (2, 3); (ii) hypergammaglobulinemia (HGG) (4, 5); (iii) increase of polyreactive Abs (6, 7); and (iv) impaired response to vaccines (4, 8). Altogether, these perturbations result in a diminished antigen-specific Ab response and an enhanced non-specific polyclonal response. Notably, these immunomodulatory effects are driven directly by viral pathogenic mechanisms and indirectly through immunopathogenesis triggered by host antiviral responses (9). Presently, it is yet to be determined whether this immunological disruption occurs as a function of chronicity or due to mechanisms initiated during the acute stage of the viral infection.

Concomitant with dysregulation of the humoral immune response, an increase in T follicular helper (T_{FH}) cells has also been observed during persistent HIV (10) and HCV (11) infections as well as the chronic phase of LCMV infection (12). In the LCMV model, expansion of T_{FH} cells has been attributed to polarization of the CD4 T cell compartment toward T_{FH} responses, which suggests a role of cytokines such as type I interferon (IFN-I) that skew differentiation and maturation toward T_{FH} and away from T helper type 1 (T_H1) cells (13). The role of IFN-I signaling with respect to T cells is well characterized and increasing evidence shows that this antiviral cytokine has both enhancing and immunosuppressive effects on the T cell response upon viral infection (14, 15). Two recent studies clearly outlined the bipolar effect that IFN-I renders on T cell-mediated immune responses by comparing the expression profile of IFN-I and IFN-stimulated genes (ISGs) in LCMV Armstrong (acute) versus LCMV Clone 13 (Cl13; persistent) infection (16, 17). Collectively, this research revealed that although protective upon transient elicitation such as in acute infections, prolonged elevation of IFN-I levels postinfection led to immunosuppression of T cell responses. In these studies, sustained expression of IFN-I was shown to drive upregulation of immunosuppressive molecules such as PD-1 and IL10 as well as disruption of splenic architecture and dampened effector CD8+ T cell (CTL) responses (16–21). Altogether, this contributes to the failure of viral clearance and eventual persistent infection.

Similarly, IFN-I production has also been shown to enhance the development of the Ab response against acute viral infections or vaccine antigens (22–27). Akin to T cells, the effect of IFN-I on B cell responses has been shown to drive increased cellular activation and class switching recombination (CSR) in the T-cell-dependent arm of the humoral immune response (23, 25, 27–29). The upregulation of ISGs in B cells from HIV-viremic patients (30) is also indicative of a role played by IFN-I during chronic infections.

However, unlike the deleterious role played by the cytokine against T-cell responses during persistent infection, the effect of IFN-I on B cell responses in this context is yet to be fully elucidated. In this report, we use the LCMV mouse model to further characterize the molecular mechanisms that drive the modulation and resulting humoral immune dysregulation during persistent virus infection.

RESULTS

LCMV Infection Impairs the Humoral Response to T-Dependent Antigens

Although the influence of escape mutations within the glycoprotein envelope of LCMV as well as dysregulated T cell responses have been implicated in the late appearance of nAbs (31–33), it is unclear whether broader modulation of the immune response also contributes to the disrupted Ab response. To directly evaluate this, we analyzed the Ab response against the model T-dependent (TD) antigen, nitrophenyl (NP) coupled to chicken gamma globulin (CGG) in the context of CTL-controlled LCMV (34, 35), and Ab-controlled vesicular stomatitis virus (VSV) infection (36). The focal point of these experiments was based on the NP response rather than comparing the antiviral response to clearly distinguish and determine modulation to the global immune response independent of LCMV versus VSV whose pathogenic determinants drive distinctive responses. C57BL/6 (B6) mice were infected with either LCMV Cl13 or LCMV WE (acute); VSV Indiana or mock infected with culture media only. All groups were contemporaneously immunized with NP-CGG, which predominantly elicits an IgG1 response (37). At various time points after immunization, the NP-specific IgG1 serum response was monitored by ELISA, which revealed that Ab titers were drastically reduced in LCMV Cl13-infected mice, compared to VSV-infected or mock-infected control mice (**Figure 1A**). Furthermore, this impairment was also present albeit to a lesser extent in mice infected with LCMV WE (**Figures 1A,B**, left panel). Although LCMV-Cl13-associated impairment of NP-specific responses declined after day (d)12, the increase in the IgG1 responses thereafter did not attain the levels observed in VSV-infected or mock-infected groups for the duration of the experiment (30 days) (**Figure 1A**). The kinetics of the disrupted NP-specific response and the impact by both the acute and persistent strains of LCMV suggest that the immunological process that drives this phenotype occurs early after infection whereas the Cl13 strain featured more adverse impairment due to viral persistence. Upon infection with LCMV Cl13 or VSV as above and simultaneous immunization with a T-independent Type 2 (TI-2) antigen, NP-FICOLL, NP-specific IgG3 (**Figure 1C**), and IgM (**Figure 1D**) responses were similar in all groups although a trend toward weaker IgM responses in the LCMV-infected group was observed at latter time points. These results demonstrate that LCMV predominantly impairs the TD response. Similar results were also observed in LCMV WE-infected mice (data not shown). Despite the impairment of the TD response, however, the affinity maturation process was unaltered by LCMV Cl13 infection. As illustrated in **Figure 1E**, the ratio of high affinity anti-NP IgG1 Abs binding to NP_4-BSA versus the total anti-NP IgG1 response, measured using NP_{26}-BSA, reflected a similar increase in Ab affinity at various time points in all three experimental groups. Again, similar results were observed for LCMV WE (data not shown). Thus, although diminished in serum concentration, the quality of the NP-specific response was not affected by LCMV infection. Importantly, the reduction in the NP-specific IgG1 response occurs in the context of increased total IgG serum levels that is evident by d12 in LCMV Cl13- and d8 in LCMV WE-infected mice compared to VSV- or mock-infected animals (**Figure 1F**). These results are in agreement with the emergence of polyclonal non-specific B cell activation and resultant HGG that is observed during LCMV infection (38) and other persistent viral infections such as HIV (4, 6) or HCV (39).

To further characterize the duration of LCMV-associated effects on the NP-specific response, we immunized mice 4 days before (d-4) and on d4, d8, d12, d20, or d30 after LCMV WE or VSV infections (Figure S1 in Supplementary Material). In this particular experiment, the kinetics of viral clearance within a limited window are important to facilitate accurate assessment of the effect of Ab-response impairment by LCMV. As such,

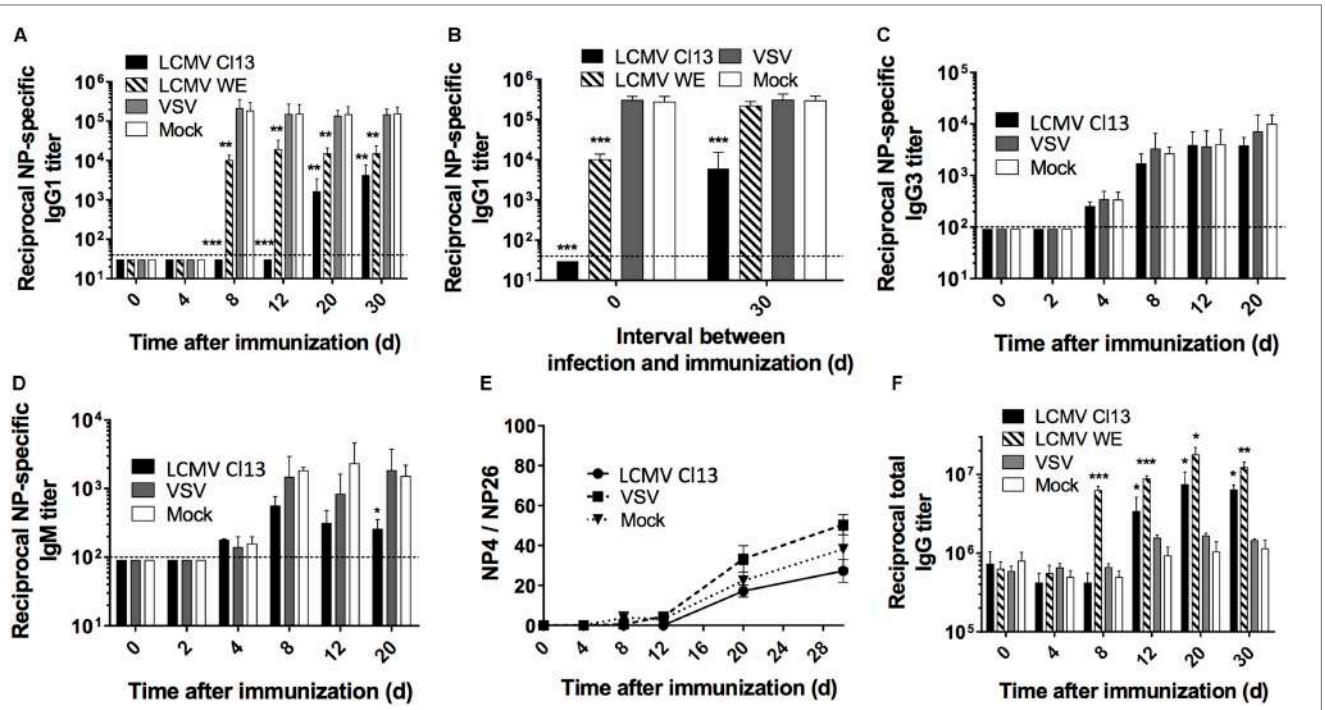

FIGURE 1 | **LCMV infection impairs the NP-specific Ab response in a T cell-dependent manner**. B6 mice (four per group) were infected with LCMV Cl13 (black), LCMV WE (hatched), VSV (gray), or mock infected (white). Mice were immunized the same day (except when indicated otherwise) with an i.p. injection of NP_{53}-CGG in alum **(A,B,E,F)** or NP_{40}-FICOLL in PBS **(C,D)**. **(A)** NP-specific IgG1 response monitored by ELISA following NP_{53}-CGG immunization. **(B)** Mice were infected as above and immunized with NP_{53}-CGG the same day (d0) or 30 days after infection (d30), and IgG1 NP-specific responses were monitored by ELISA on d8 postimmunization. NP-specific IgG3 **(C)** or IgM **(D)** responses monitored by ELISA following NP_{40}-FICOLL immunization. **(E)** ELISA plates were coated with NP_4-BSA or NP_{26}-BSA and high affinity Ab responses were measured as a ratio of Abs binding to NP_4-BSA versus the total anti-NP IgG1 response binding to NP_{26}-BSA. **(F)** Total serum IgG responses following concomitant infection and NP_{53}-CGG immunization. Statistical analysis was performed by individual *T*-tests between experimental groups and the mock-infected group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. The dotted line represents detection threshold. **(A–E)** Representative of two independent experiments. **(F)** Representative of four independent experiments.

the acute strain, LCMV WE, was used given that this variant is cleared from lymphoid organs within about a week (35). As shown in Figure S1A in Supplementary Material, the Ab response in the VSV-infected group was unaffected irrespective of the time interval between infection and immunization. On the other hand, NP-specific IgG1 responses in LCMV WE-infected mice revealed similar levels of impairment upon immunization on d4, d8, and d12 postimmunization. However, the impact on the NP-specific response upon immunization on d4 before or d20 after infection was less severe relative to the other time points. This indicates that B cell responses: (i) were less susceptible to LCMV impairment if established prior to infection; (ii) start to recover by d20 following LCMV WE infection before returning to normal levels by d30 postinfection; and (iii) remain impaired past d30 following infection with LCMV Cl13 (**Figure 1B**, right panel). Finally, changes in affinity maturation did not attain statistical significance irrespective of the time interval between LCMV infection and immunization (Figure S1B in Supplementary Material). Taken together, these results indicate that LCMV impairs the development of specific Ab responses early following establishment of infection, which is sustained in the context of persistent infection.

LCMV Infection Modifies the Lymphoid Microenvironment and B Cell Function

To gain further insight into the immunological milieu within which the impairment of TD NP-specific Ab responses occurred, we evaluated B cell populations and the splenic microenvironment in the three infection groups on d8 postinfection/immunization. Similar to a previous report (40), we observed perturbations in the splenic architecture whereby immunohistochemistry revealed a dramatic disruption of B cell follicles in LCMV-infected mice relative to VSV- and mock-infected controls with B cells being mostly found outside of the follicles (**Figure 2A**). Counterintuitive to this disruption, while total numbers and proportions of splenic B cells were contracted in the LCMV Cl13-infected group (**Figure 2B**), the number and proportion of GC B cells were significantly increased (**Figure 2C**). Expansion of the GC B cell population suggested enhanced activation of B cells in the LCMV group. Given that this phenomenon could lead to increased differentiation into effector B cell subsets [e.g., plasma cells (PCs)], we evaluated the splenic Ab-secreting cell (ASC) population on d8 after infection and immunization with NP-CGG using ELISPOT (**Figure 2D**). In agreement with the expansion of GC B cells and the presence

of HGG, the total number of IgG-secreting cells in the LCMV Cl13-infected group was elevated. Conversely, the quantity of NP-specific IgG-secreting cells was significantly decreased in the LCMV Cl13 group, correlating with the depressed antigen-specific Ab response. Similar results were also observed in the bone marrow compartment to which the PCs migrate after

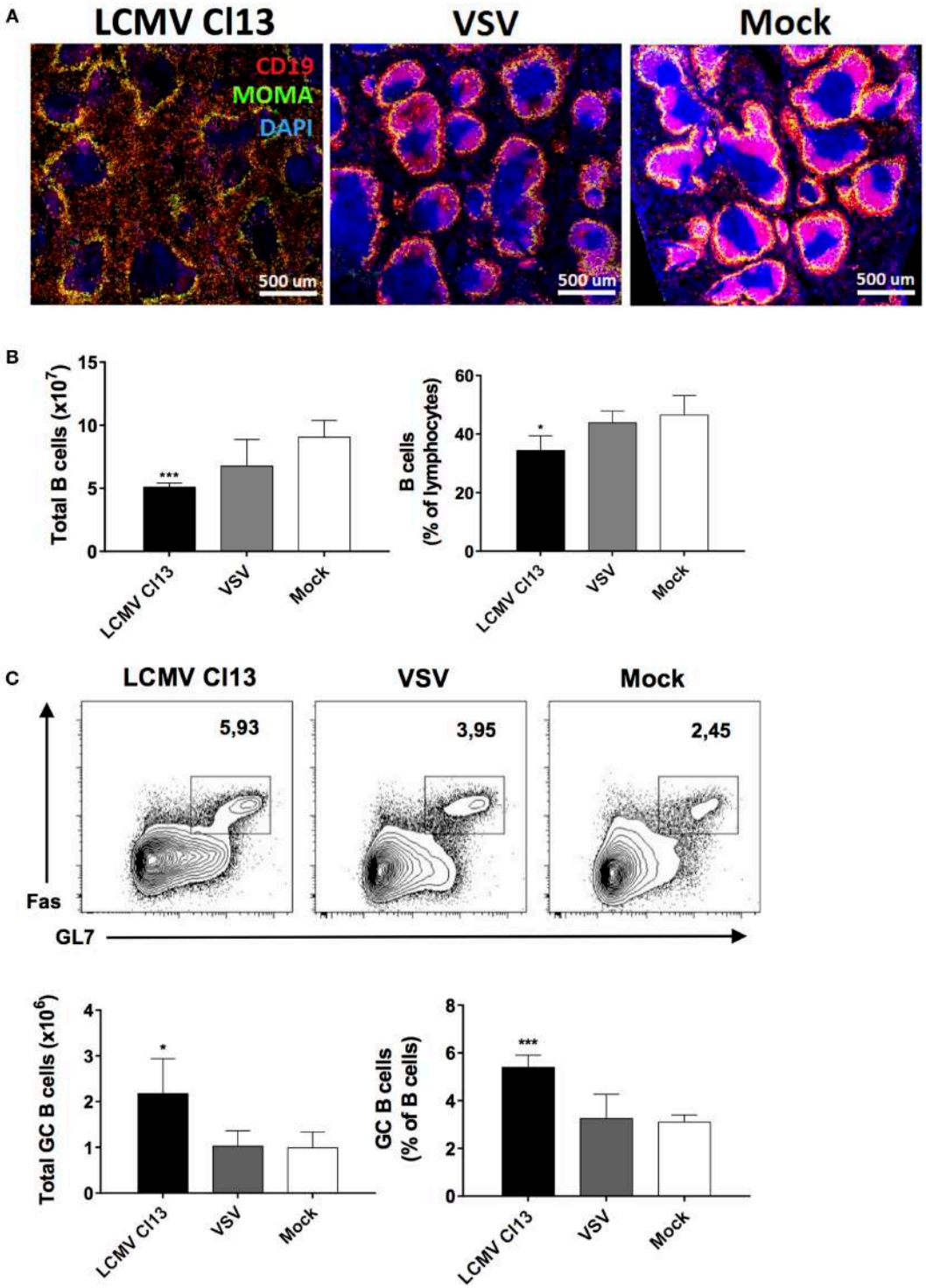

FIGURE 2 | LCMV infection disrupts the splenic follicular architecture while increasing GC B cell numbers and non-specific Ab responses.

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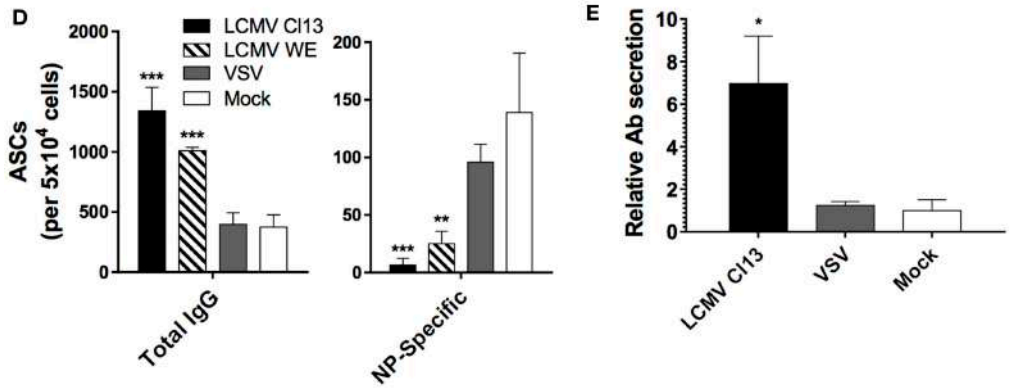

FIGURE 2 | **Continued**

B6 mice (four per group) were infected with LCMV Cl13 (black), LCMV WE (hatched), VSV (gray), or mock infected (white). Mice were immunized the same day with an i.p. injection of NP_{53}-CGG in alum and TD B cell responses were analyzed on d8 after infection. **(A)** Immunofluorescence showing CD19 (red), MOMA-1 (green), and DAPI (blue) expression on spleen sections. **(B)** Total B cell numbers and proportions were enumerated by flow cytometry. **(C)** Number and proportion of splenic GC B cells. **(D)** Number of total and NP-specific IgG-secreting cells detected by ELISPOT. **(E)** Relative Ab secretion of ASCs calculated by *ex vivo* measurement of secreted Abs produced by 10^5 splenocytes. Statistical analysis was performed by individual *T*-tests between experimental groups and the mock-infected group. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **(A)** Representative of two independent experiments. **(B–E)** Representative of four independent experiments.

differentiation in the secondary lymphoid organs (data not shown) and these features were observed in LCMV WE-infected animals as well (**Figure 2D**). Last, consistent with the increase in the number of IgG-secreting ASCs due to polyclonal B cell activation during LCMV infection (38), ASCs from this group also displayed an increased secretory capacity (**Figure 2E**). Expressed here as a ratio, the *ex vivo* Ab concentration from splenic ASCs was quantified from culture supernatants, which revealed that following LCMV Cl13 infection, ASCs secreted a significantly higher quantity of Ab relative to VSV- or mock-infected mice.

LCMV Infection Triggers the Expansion of T_{FH} and an Increase in Their Effector Function

Upon phenotypic characterization of the CD4 T cell compartment, we observed that there was a contraction in the absolute number of CD4 T cells (**Figure 3A**, top panel), which was more evident in the proportion of CD4 T cells relative to total lymphocytes in the LCMV-infected groups (**Figure 3A**, bottom panel). This coincided with a significant increase in the T_{FH} compartment ($CD4^+CD62L^-CD44^+CXCR5^+Bcl\text{-}6^+$) as shown in **Figure 3B**. These results are supported by previous work, which demonstrated that LCMV increased differentiation of CD4 T cells into T_{FH}, and this redirected differentiation program was sustained in LCMV Cl13 due to viral persistence (12). Based on these changes in the T_{FH} population and their potential influence on the humoral immune response, we sought to determine whether the essential costimulatory and signaling molecules that comprise interaction between T_{FH} and GC B cells were similarly modified during LCMV infection. Using phenotypic analysis by flow cytometry, we analyzed the expression of PD-1, which has been shown to be an ideal marker to distinguish GC from non-GC T_{FH} (41). Here, we observed a significantly higher proportion of T_{FH} cells expressing increased levels of PD-1 in LCMV Cl13-infected mice (**Figure 3C**) indicating a higher number of GC T_{FH} relative to the other cohorts. Likewise, proportions of T_{FH} expressing high levels of ICOS were also significantly elevated relative to mock-infected controls, which was also the case for VSV-infected mice albeit to a lesser extent. Surprisingly, while proportions of B cells expressing PD-L1 similarly increased (**Figure 3D**), those expressing ICOSL were reduced whereas the ligand pair CD40:CD40L remained unchanged across the three infection groups (**Figures 3C,D**). Serum levels of BAFF were also elevated in LCMV Cl13-infected mice along with BAFF, IL-21, and IL-4 mRNA and protein expression in CD4 T cells (**Figures 3E,F**). As previously mentioned, predilection toward T_{FH} differentiation in the context of persistent infection can occur as a result of prolonged expression of IFN-I. However, whether the effects on the humoral response are solely due to a modulated T_{FH} response shaping the B cell response or more direct impact of IFN-I on the B cells is unknown. To determine the potential role of T_{FH} immunomodulation on perturbation of the humoral response, we administered blocking Abs against PD-1, which comprises a key molecular interaction between GC B cells and T_{FH} (42). The LCMV WE strain was used here given that interfering with the PD-1 pathway during the early phase of systemic LCMV Cl13 infection has been shown to induce lethal CD8 T cell-mediated immunopathology (18, 43). As illustrated in **Figure 3G**, blockade of PD-1 prior to infection and immunization with NP did not alter the NP-specific and total IgG titers relative to the untreated control group. Similar results were obtained following PD-L1 blockade (data not shown). Therefore, in our model, humoral disruption was immutable to blockade of the PD-1 pathway suggesting a more direct role of IFN-I on B cell function.

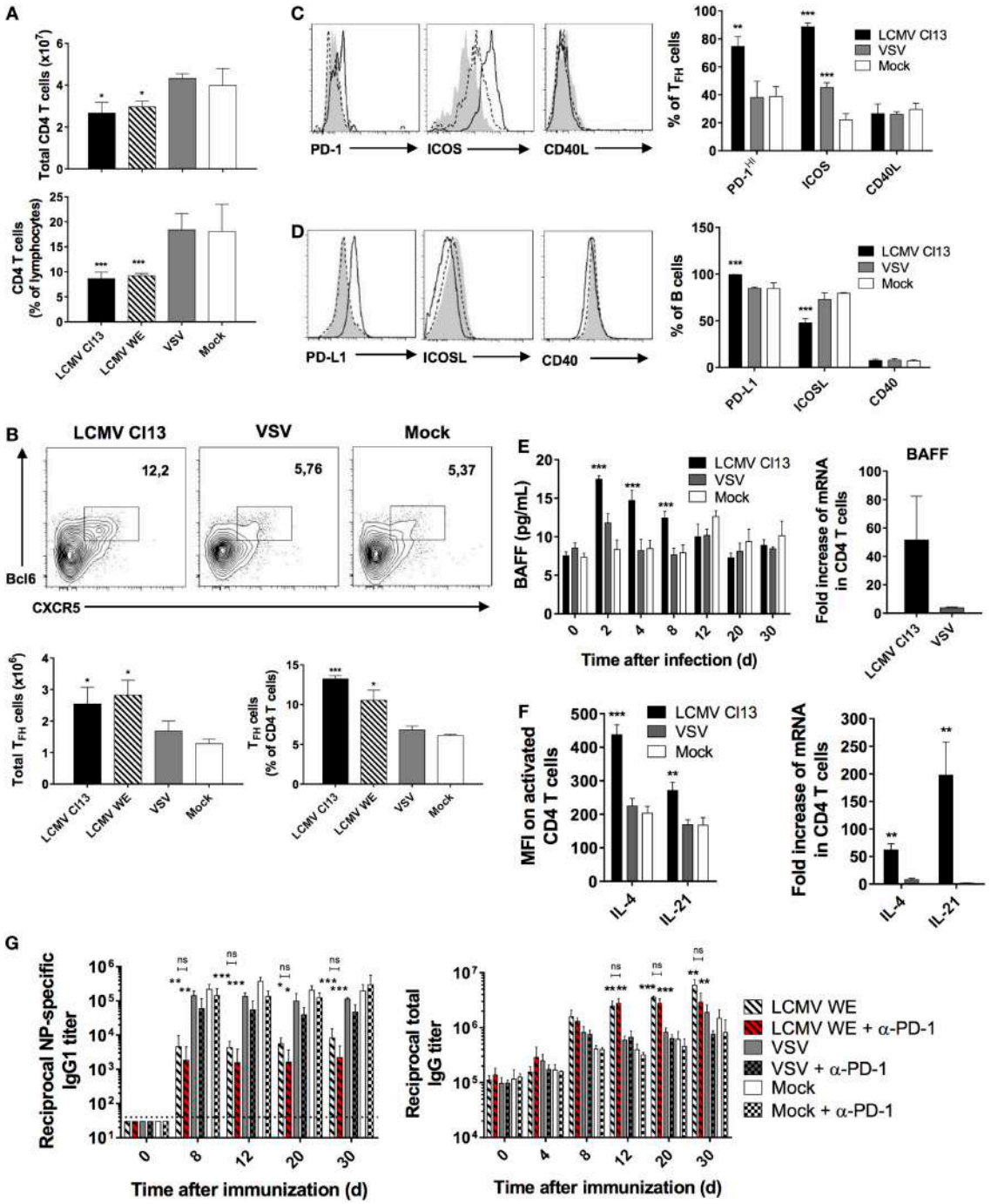

FIGURE 3 | **LCMV infection triggers the expansion of T_{FH} cells and induces their expression of B cell-activating cytokines**. B6 mice (four per group) were infected with LCMV Cl13 (black bars or lines), LCMV WE (hatched), VSV (gray bars or dotted lines), or mock infected (white bars or shaded area). Mice were immunized the same day with an i.p. injection of NP_{53}-CGG in alum and $CD4^+$ T cells were analyzed on d8 after infection. **(A)** Total $CD4^+$ T cell numbers (top panel) and proportions (bottom panel) determined by flow cytometry. **(B)** T_{FH} cell numbers and proportions determined by flow cytometry. **(C)** Proportion of T_{FH} cells expressing PD-1, ICOS, and CD40L and **(D)** B cells expressing PD-L1, ICOSL, and CD40 determined by flow cytometry. **(E)** Serum BAFF concentration (left panel) and BAFF mRNA expression in total splenic CD4 T cells (right panel) measured using ELISA and qRT-PCR, respectively. **(F)** Intracellular cytokine levels (left panel) and mRNA expression (right panel) of IL-4 and IL-21 in CD4 T cells measured using flow cytometry and qRT-PCR, respectively. **(G)** NP-specific IgG1 response (left panel) and total IgG response (right panel) monitored upon infection with LCMV WE (hatched bars), VSV (gray), or mock-infected (white) infection along with injection of PD1-blocking Ab (red hatched or checkered bars). Statistical analysis was performed by individual *T*-tests between experimental groups and the mock-infected group and between non-treated and anti-PD1 Ab-treated LCMV-infected groups when indicated with brackets. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. **(A,B)** Representative of four independent experiments. **(C–E,G)** Representative of two independent experiments. **(F)** Representative of three independent experiments.

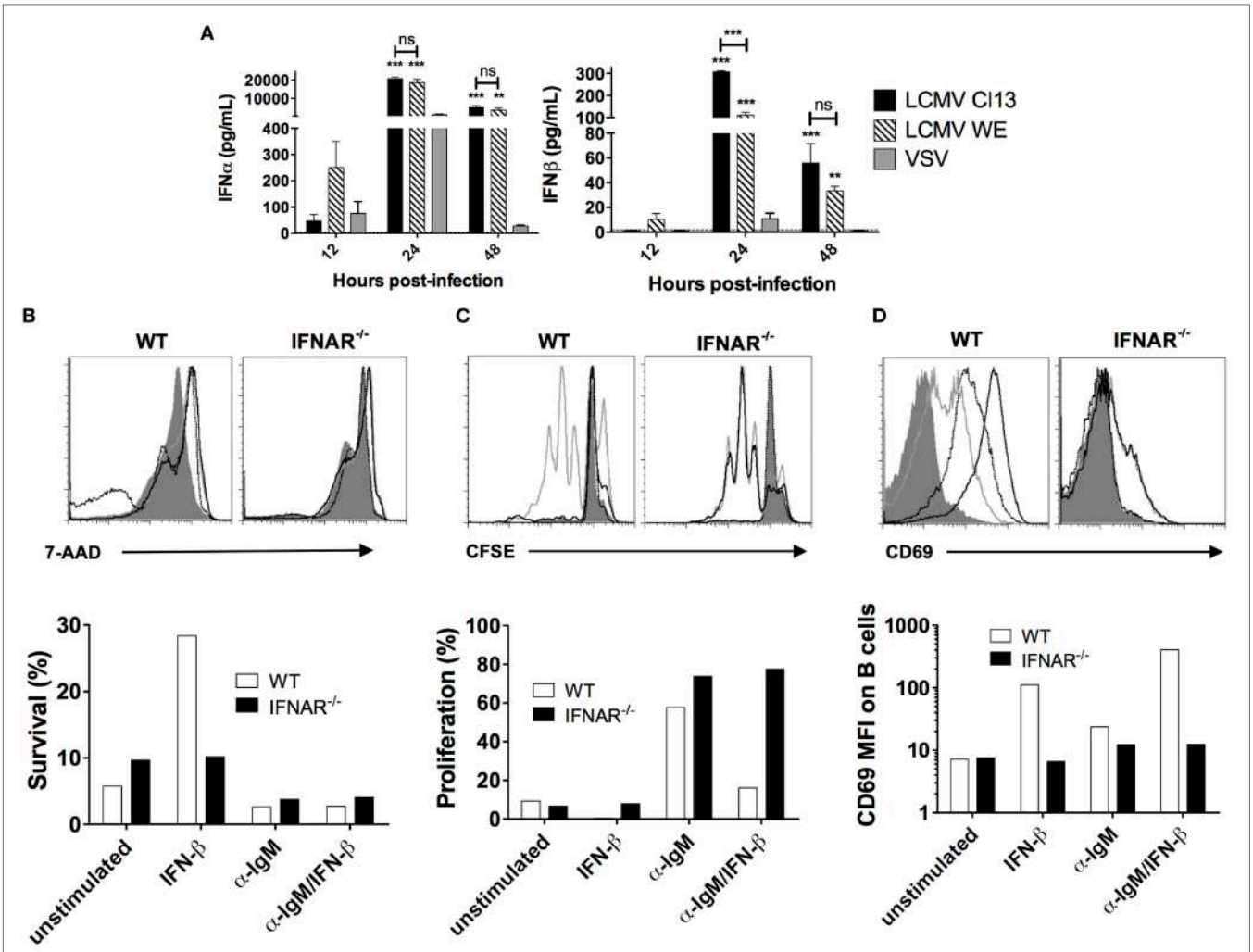

FIGURE 4 | IFN-I acts in competition with BCR signaling to induce B cell survival and proliferation. **(A)** Serum level of IFN-I in B6 mice ($n = 4$) infected with LCMV Cl13 (black), LCMV WE (hatched), or VSV (gray) measured using ELISA. Statistical analysis was performed by *T*-test between LCMV and VSV-infected groups. ***$p < 0.001$. **(B–D)** 2×10^6 B cells from WT B6 (white bars) or IFNAR $^{-/-}$ (black bars) mice were cultured for 7.5 h (for proliferation and CD69 expression) or 4 days (for survival) without *in vitro* stimulation (solid dark gray), or with α-IgM (light gray line), IFN-β (black dotted line), or both (black line). **(B)** 7-AAD exclusion, **(C)** CFSE dilution, and **(D)** CD69 expression were measured by flow cytometry. Representative of two independent experiments.

IFNAR Signaling Is Essential for LCMV-Mediated Humoral Immune Response Disruption

Previous studies have demonstrated a rapid and robust increase in LCMV-induced IFN-I levels in the serum (16, 17), which, as illustrated in **Figure 4A**, was also observed in our LCMV cohorts as well as in VSV-infected mice albeit to a much lesser extent and for a shorter duration. Next, we performed *in vitro* stimulation of B cells with IFN-β in the presence or absence of BCR signaling to evaluate any modifications in survival and proliferation. Here, B cell samples were harvested from both wild-type B6 and IFNAR$^{-/-}$ mice to determine specific action by IFN-I. As shown in **Figure 4B** and consistent with a previous study (24), addition of IFN-β in the WT B cell culture sustained B cell survival by fivefold after 4 days, whereas B cells from IFNAR$^{-/-}$ mice did not respond to IFN-β, as expected. Of note, IFN-I stimulation in this setting is likely equivalent to acute infection and our results are in agreement with the impact of IFN-I on B cells as shown in previous reports (23, 25, 27–29). Interestingly, upon stimulation through the BCR, survival of cultured B cells was diminished regardless of addition of IFN-β. Furthermore, while BCR stimulation increased B cell proliferation, the addition of IFN-β completely abrogated the BCR-dependent increase in proliferation (**Figure 4C**). We also measured B cell activation by evaluating expression of the activation marker CD69 upon which we found that IFN-β increases B cell activation independently of BCR stimulation (**Figure 4D**). Given the enhancement of survival independent and antagonistically to BCR signaling, these results suggest that IFN-I signaling could potentiate the increase of non-specific B cells while impairing the development of antigen-specific B cell responses.

To directly evaluate the effect of IFN-I signaling on the humoral response in our *in vivo* model, we performed LCMV Cl13 infection and co-immunization with NP in mice that were treated with either IFNAR blocking or isotype control Abs. Although IFNAR blockade prior to LCMV Cl13 infection has been shown to enhance viral clearance in a CD4 T cell-dependent manner (16, 17), its impact on the Ab response during the progression of a chronic infection has not been thoroughly assessed. As depicted in **Figure 5A**, LCMV-specific-binding Ab responses were not significantly affected by IFNAR blockade as observed in previous reports (16, 17). Remarkably, however, NP-specific serum IgG1 titers were restored to levels present in VSV- or mock-infected animals upon IFNAR blockade (**Figure 5B**). In addition, restoration of the anti-NP IgG1 response was observed following either: a single anti-IFNAR administration conducted on d-1 prior to the infection/immunization (**Figure 5C**) or a series of 11 anti-IFNAR treatments conducted every third day until d30 postinfection/immunization (**Figure 5D**). Despite this result, it is important to note that the effect of the short-term Ab treatment regimens seemingly waned over time (**Figures 5B,C**). IFN-I has been shown to induce CSR primarily toward an IgG2a/c subtype (27). To ascertain that the LCMV-associated depletion of NP-IgG1 responses was not solely due to the skew toward NP-specific IgG2c responses, we assessed whether the recovery of NP-specific IgG1, upon IFNAR blockade, was inversely related to IgG2c titers in our experimental cohorts. As shown in **Figure 5E**, although low-level NP-specific IgG2c titers were detected starting on d12 following immunization in LCMV-infected mice compared to the mock-infected group, similar levels were also observed in the VSV-infected group indicating a general effect driven by viral infection. Expectedly, IFNAR blockade reduced NP-specific IgG2c titers in LCMV-infected mice denoting a role for IFN-I in the observed CSR to IgG2c. These results indicate that, the IFN-I response generally elicited during all viral infection induces CSR to IgG2c of some antigen-specific B cells, and this effect is unlikely limited to LCMV infection or more broadly, persistent infections; thus, neither the diminished NP-specific IgG1 Ab response observed during LCMV infection nor its recovery upon IFNAR blockade is accounted for by a skewing toward or away from IgG2c responses. Altogether, these results affirm that the suppressive effect observed on the NP-specific IgG1 response during LCMV infection is dependent on IFN-I signaling and independent of CSR to IgG2c. Finally, consistent with a previous report that showed the induction of HGG in IFNAR$^{-/-}$ mice following LCMV infection (38), HGG was unchanged by any of the IFNAR blockade regimen (**Figures 5F–H**).

We next evaluated the impact of IFNAR blockade on the TD B cell response during LCMV infection. Consistent with the normalized IgG1 response against NP, the number of NP-specific IgG-secreting cells returned to that found in VSV- or mock-infected animals (**Figure 6A**, right panel) upon IFNAR blockade while total IgG-secreting cells remained elevated (**Figure 6A**, left panel), in agreement with sustained HGG. Surprisingly, although IFNAR blockade led to the restoration of total splenic CD4+ T cell proportions (**Figure 6B**), the increase in T_{FH} observed following LCMV infection remained unchanged (**Figure 6C**). Moreover, the treatment bore limited impact on IL-4 and IL-21 expression in CD4 T cells (**Figure 6D**), GC B cell proportions (**Figure 6E**), and relative Ab secretion by ASCs (**Figure 6F**). However, relative to the isotype control, anti-IFNAR treatment resulted in moderate modulation in the expression levels of PD-L1, ICOSL, and ICOS (**Figures 6G,H**). As observed, T_{FH} and GC B cell populations remained elevated upon IFNAR blockade despite the recovery of NP-specific Abs. Next, we sought to determine whether the structure of B cell follicles was restored upon IFNAR blockade similar to the rescue of lymphoid architecture as described in previous reports (16, 17, 20). Here, we observed that only incomplete recovery of the B cell follicle structure occurred suggesting that LCMV infection induces disruption of B cell localization in a partially IFNAR-independent manner (**Figure 6I**). Based on the role of CXCR4 in the trafficking of B cells in lymphoid follicles and resultantly their structure (44), we examined its expression level upon IFNAR blockade. As shown in **Figure 6J**, left panel, we observed a remarkable elevation in CXCR4 expression in total B cells, which was restored to base levels upon IFNAR blockade. Surprisingly however, this increase in CXCR4 expression levels was not observed in the GC compartment in the LCMV-infected group compared to VSV- or mock-infected animals (**Figure 6J**, right panel). Nonetheless, IFNAR blockade also significantly reduced CXCR4 expression in GC B cells. These results illustrate a significant role played by IFN-I produced during LCMV infection in altering CXCR4 expression and consequently the trafficking and localization of B cells outside of follicular structures. This interplay suggests a mechanistic outline by which IFN-I modulates the humoral immune response.

B Cell-Intrinsic IFN-I Signaling Directly Disrupts the Antigen-Specific Humoral Response

Our data so far have suggested an effect of IFN-I on B cells. Therefore, to determine the role of B cell-intrinsic IFN-I signaling on the impaired humoral response observed during LCMV infection, we developed a chimeric model by reconstituting irradiated B6 mice with a mix of bone marrow cells from B cell-deficient (J_HT) mice (45) and IFNAR$^{-/-}$ mice. As a result, we obtained chimeras in which only the B cells are deficient in IFN-I signaling. Upon LCMV Cl13 infection and NP-CGG immunization of J_HT/IFNAR$^{-/-}$ chimeras, we observed restoration of the NP-specific response to levels observed in the VSV- or mock-infected B6 mice (**Figure 7A**), which was consistent with the IFNAR blockade experiment. Interestingly, whereas IFNAR blockade did not ameliorate HGG, the levels of total serum IgG in LCMV Cl13-infected J_HT/IFNAR$^{-/-}$ chimeras were significantly lower than those in WT animals and normal relative to VSV and mock-infected mice (**Figure 7B**). Yet, despite the normalization of humoral immune responses, J_HT/IFNAR$^{-/-}$ mice still exhibited only partial rescue of the splenic marginal-zone and B cell follicle structures (**Figure 7C**). Perhaps the most significant impact observed by the absence of B cell-specific IFNAR signaling was the accelerated emergence of nAbs in the chimeras compared to both J_HT/B6 chimeras,

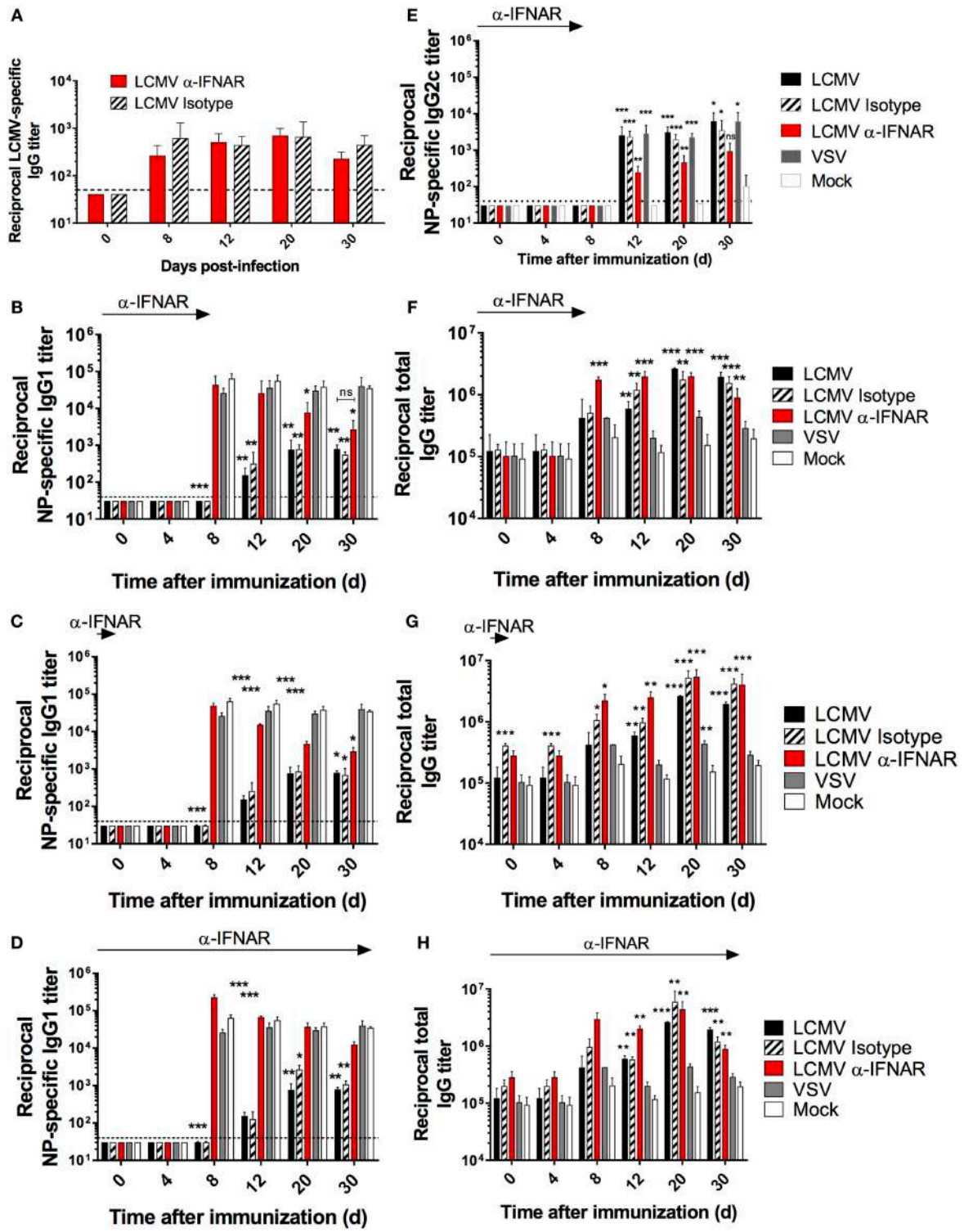

FIGURE 5 | **IFNAR blockade restores NP-specific IgG1 responses during LCMV infection**. B6 mice (four per group) were treated with α-IFNAR Ab (red), isotype control Ab (hatched), or left untreated (black) and were infected the next day with LCMV Cl13, VSV (gray), or mock infected (white). Mice were immunized the same day with an i.p. injection of NP_{53}-CGG in alum. **(A,B,E,F)** IFNAR blockade was conducted every second day until d8. In another series of experiments, anti-IFNAR treatment was stopped after either one injection (d-1) **(C,G)** or 11 injections every third day (d30) **(D,H)**. **(A)** LCMV nucleoprotein-specific IgG titers, **(B–D)** NP-specific IgG1, **(E)** NP-specific IgG2c, **(F–H)** total IgG titers monitored by ELISA. Statistical analysis was performed by individual *T*-tests between experimental groups and the mock-infected group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. The dotted line represents detection threshold. Representative of two independent experiments.

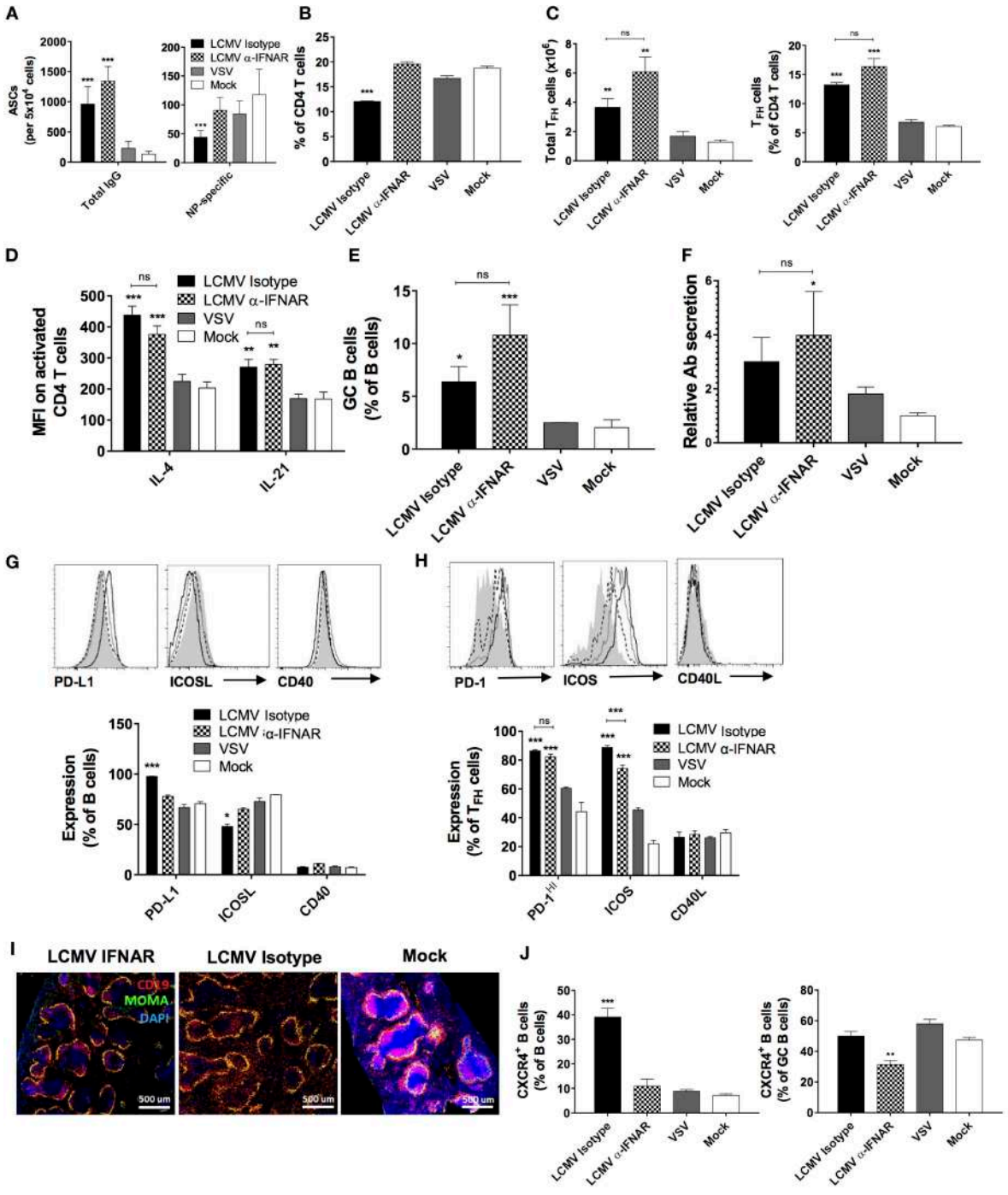

FIGURE 6 | IFNAR blockade partially restores TD B cell responses and splenic follicular structures. B6 mice (four per group) were treated with α-IFNAR Ab (checkered) or isotype control Ab (black) and were infected the next day with LCMV Cl13, VSV (gray), or mock infected (white). Mice were immunized the day of the infection with an i.p. injection of NP_{53}-CGG in alum. IFNAR blockade was conducted every second day until analysis on d8. **(A)** Number of total (left panel) and NP-specific IgG-secreting (right panel) cells detected by ELISPOT. **(B)** Proportion of CD4 T cells among total lymphocytes and **(C)** total numbers (left panel) and proportions (right panel) of T_{FH} cells among total CD4 T cells as determined by flow cytometry. **(D)** Intracellular IL-4 and IL-21 levels in T_{FH} cells and **(E)** proportion of splenic GC B cells determined by flow cytometry. **(F)** Secretory capacity of ASCs as determined by measuring the quantity of secreted Abs produced by 10^5 splenocytes. **(G)** Proportion of B cells expressing PD-L1, ICOSL, and CD40. **(H)** Proportion of T_{FH} cells expressing PD-1, ICOS, and CD40L. **(I)** Splenic follicular structures visualized using immunofluorescent staining of CD19 (red), MOMA-1 (green), and DAPI (blue) in tissue sections. **(J)** Proportion of $CXCR4^+$ B cells among total (left panel) and GC B cells (right panel) determined by flow cytometry. Statistical analysis was performed by individual *T*-tests between experimental groups and the mock-infected group and between isotype and IFNAR blocking Ab-treated groups when indicated with brackets. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. **(A,J)** Representative of three independent experiments. **(B–I)** Representative of two independent experiments.

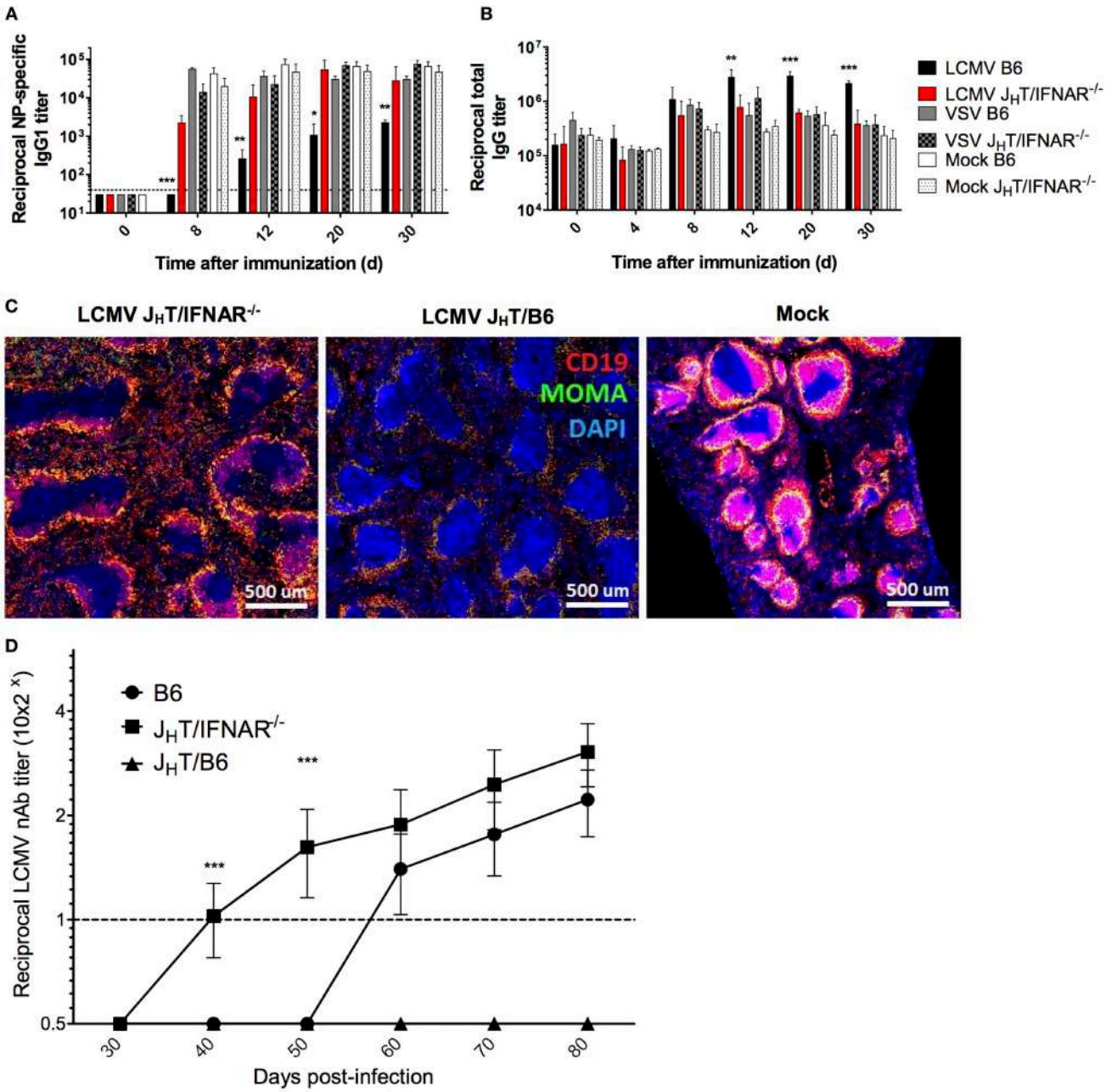

FIGURE 7 | B cell-specific disruption of IFNAR restores NP-specific Ab responses and accelerates the development of LCMV nAbs. **(A–C)** B6, J_HT/IFNAR$^{-/-}$, or J_HT/B6 mice (four per group) were infected with LCMV Cl13 (black for B6; red for J_HT/IFNAR$^{-/-}$), VSV (gray), or were mock infected (white). Plain bars represent B6 mice and checkered bars J_HT/IFNAR$^{-/-}$ B cell bone marrow chimeric mice. **(A)** NP-specific IgG1 and **(B)** total IgG responses monitored using ELISA on d8 following infection/NP_{53}-CGG immunization. **(C)** Splenic follicular structures visualized using immunofluorescent staining of CD19 (red), MOMA-1 (green), and DAPI (blue) on spleen sections obtained on d8 postinfection. **(D)** Neutralization assay showing accelerated nAb responses in J_HT/IFNAR$^{-/-}$ chimeric mice upon infection with LCMV Cl13. **(A–C)** Representative of two independent experiments. **(D)** Compilation of three independent experiments (B6 mice, $n = 21$; J_HT/IFNAR$^{-/-}$ mice, $n = 25$; J_HT/B6 mice, $n = 8$). Statistical analysis was performed by individual *T*-tests between experimental groups and the mock-infected group for **(A–C)** and one-way ANOVA for **(D)**. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. The dotted line represents detection threshold.

used as controls to account for any changes driven by irradiation and reconstitution and more importantly, WT B6 mice (**Figure 7D**). The fact that J_HT/B6 chimeras did not produce any detectable LCMV nAbs suggests that only partial reconstitution of the humoral response was achieved in the chimeric system. This result further underscores the significance of the accelerated nAb response observed in the J_HT/IFNAR$^{-/-}$ chimeras. Collectively, these data illustrate a potent effect borne by IFN-I

signaling on B cells, which upon negation results in a normal NP-specific humoral response as well as enhanced induction of LCMV nAbs.

DISCUSSION

The immune response in the LCMV infection model has been classically defined as cell mediated (46) whereas the role of the humoral immune response has only been considered relevant in the context of reinfection (47). Although the absence of an initial robust nAb response has been primarily attributed to antiviral escape mechanisms (32, 48), the immunological processes that drive the disruption of humoral immunity during persistent infection are yet to be elucidated.

In this report, we reveal that emergence of humoral dysfunction during LCMV infection occurs in an IFN-I-dependent manner in which antigen specificity in the TD immune response was impaired; this effect was notably more evident in LCMV Cl13- relative to WE-infected animals. To gain deeper insight into the nature of immunological impairment, we tracked the immune response against NP rather than the actual viral antigens in different experiments to distinguish viral specific effects from global immune responses. Collectively, the presence of humoral disruption observed with both LCMV strains illustrates that pathogenic mechanisms present in the acute phase of infection as well as sustenance of viral burden in the face of prolonged LCMV Cl13 infection both play a role in the observed perturbations. However, this impairment is not a universal consequence of any viral infection or of IFN-I production *per se* as infection with VSV, which also promotes IFN-I production, albeit to a lesser extent than LCMV, does not lead to a disrupted humoral response; as such, LCMV-specific factors also likely play a role in the muted Ab response. Whereas blockade of IFN-I signaling using anti-IFNAR Abs reestablished the anti-NP response, HGG remained present consistent with a previous report in IFNAR$^{-/-}$ (38) and the expansion of the T_{FH} and total IgG-secreting cell populations was still observed. Furthermore, the expression of costimulatory molecules between B and T cells was partially restored to levels intermediate between isotype treated and VSV-infected controls. In addition, treatment with anti-PD-1/PD-L1, which was conducted prior to infection and co-immunization, bore no impact on LCMV-mediated dysregulation. These findings along with the observed effect of IFN-I on B cell survival and proliferation *in vitro* suggested that LCMV-induced IFN-I acted directly on B cells. The significance of this interaction was demonstrated using J_HT/IFNAR$^{-/-}$ chimeric mice in which only B cells carried the receptor knockout. Remarkably, the absence of IFN signaling on B cells restored normal humoral function with NP-specific Ab titers similar to those observed in VSV- or mock-infected mice. Moreover, B cell-specific IFNAR ablation normalized HGG consistent with a recent report in the Leishmania infection model (49). In light of the incapacity of IFNAR blockade to limit HGG, this suggests that IFN-I signaling on cells other than B cells also contributes to regulating HGG development although the exact nature of this contribution remains to be defined. Altogether, these results revealed the role of IFN-I signaling on B cells in impairing Ag-specific responses albeit possibly indirectly through the abnormal expansion of non-specific B cells.

Along with the recovery of the antigen-specific humoral response, we observed a reduction in the extent of destruction to splenic follicular architecture upon both the blockade and B cell-specific knockout of IFNAR. Similar to the recovery of the general splenic tissue organization observed in previous studies (16, 17), we surmise that this occurs in part due to preservation of cellular trafficking and localization in the splenic microstructure. Shuttling back and forth between the light zone (LZ) and dark zone (DZ) of the GC is actuated by differential expression of chemokine receptors such as CXCR4 and CXCR5 (50, 51). Upon analysis of CXCR4 expression on B cells, we observed that the LCMV-associated increase in total B cells was completely reversed by anti-IFNAR Ab administration. However, since CXCR4 expression on GC B cells was unchanged upon LCMV infection, we surmise that the overall increased expression of the chemokine receptor upon infection most likely occurred in extrafollicular B cells. It is important to note that this change to cellular trafficking and localization likely represents only a fraction of similar such changes that alter splenic structure during LCMV infection.

In addition, these changes to B cell trafficking also suggest altered positive selection thresholds of effector cells. This possibility is further advanced by the increase in GC B cell numbers as well as the expansion of the non-specific ASC population. Along with the observation of T_{FH} increase in LCMV-infected animals, we deduce that the positive selection process in which antigen-specific B cell clones compete for survival signals from T_{FH} is dysregulated. In this setting, the threshold for positive selection is lowered based on the aberrant expansion of T_{FH} cells. As a result, B cells exhibiting lower antigen specificity or non-specificity, which in the normal functional setting would not be selected, receive survival signals. Consequently, this expansion of non-specific B cells diminishes the likelihood of cognate T_{FH}–B cell interactions taking place in a productive manner, which leads to an impairment of specific Ab responses (see **Figure 8** for proposed model). However, the affinity maturation process is still intact despite this disruption based on the stability of the high affinity NP_4-BSA Ab titers in all the infection groups. Thus, the positive selection mechanism is functional yet stunned by the influx of non-specific B cell clones. Most significantly, the accelerated LCMV nAb response observed in the J_HT/IFNAR$^{-/-}$ chimeras relative to WT B6 and J_HT/B6 can also be accounted for by such a mechanism. In this setting, the WT response features increased activation of non-specific B cell clones. Consequently, the response to neutralizing epitopes, which are immunorecessive, is further diminished by the effects of IFN-I signaling. This is a significant characterization given that the elicitation of nAbs and moreover, broadly nAbs, is also delayed in chronic infection settings such as HIV and HCV whose immunological profiles closely mirror those observed in LCMV.

Another important aspect of human persistent virus infection is poor vaccination responses in infected patients. Using NP co-immunization, our model recapitulates the diminished response against a secondary antigen and elucidates the role played by IFN-I-mediated signaling in the chronic infection setting. Other

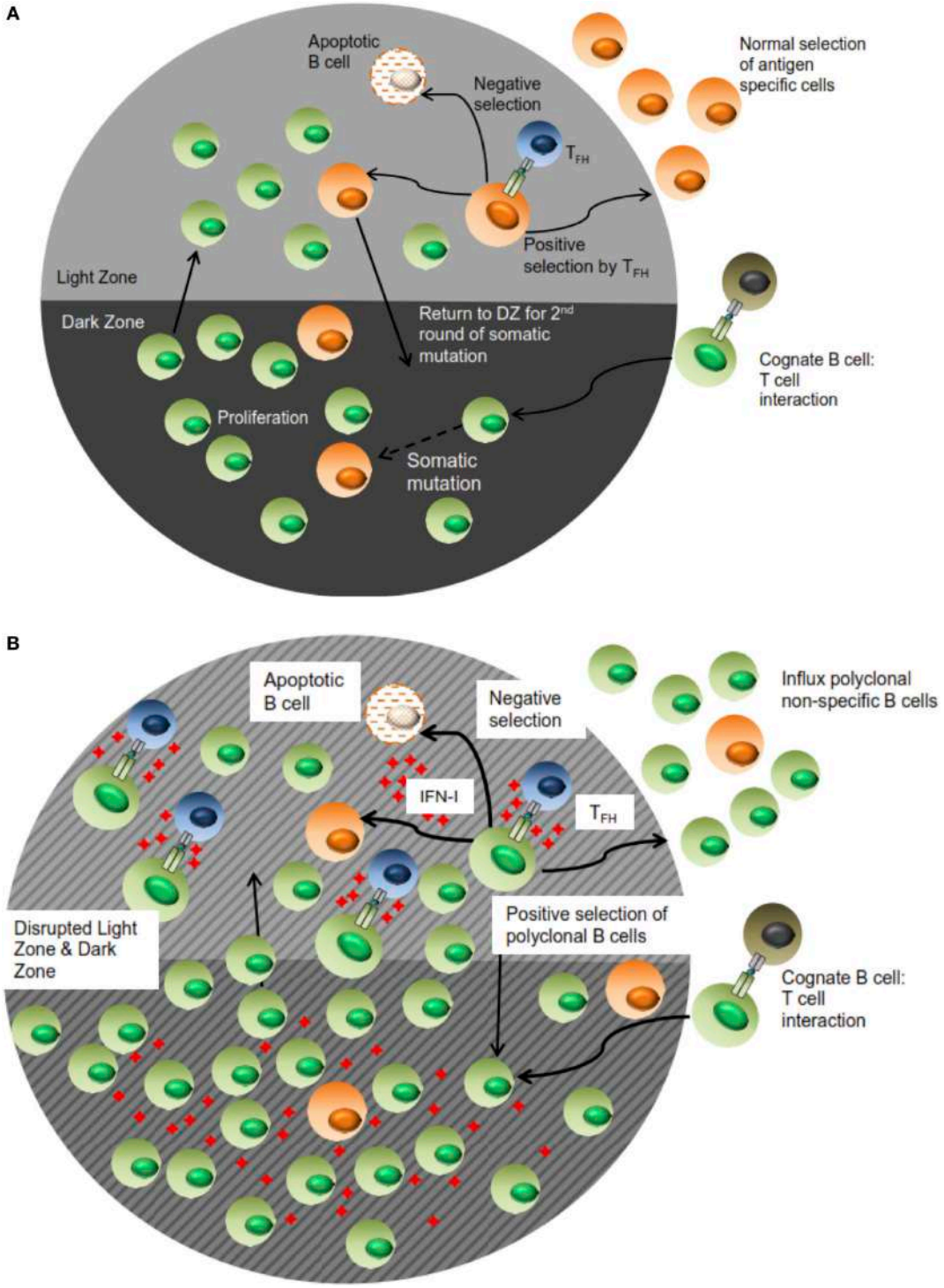

FIGURE 8 | **Proposed schematic of B cell selection during a normal versus impaired humoral immune response during LCMV infection**. In **(A)**, antigen specificity of the resultant Ab response is facilitated by effective selection of B lymphocytes in the LZ of the GC response. Some of these cells migrate to the periphery as effector cells while others cycle back into the DZ for proliferation in an iterative process. During LCMV and more generally, other chronic infections **(B)**, the high concentration of IFN-I along with sustained elicitation and T_{FH} expansion triggers selection of non-specific B cells in the context of a disrupted follicular architecture; as such, antigen-specific effector cells and consequently, Ab responses, are "drowned" out by the influx of non-specific cells. This figure only represents a snapshot of the process in which the immune response is further honed in **(A)**, whereas the dilution and loss of specificity in **(B)** is exacerbated in subsequent rounds.

studies have also shown that poor responses against vaccines are correlated with an increased PD-1:PD-L1 profile in HIV and HCV infected patients (10, 52).

In conclusion, the lack of an effective humoral immune response during LCMV infection is driven by general modulation to the humoral immune microenvironment by IFN-I. This modulation is limited to the TD response and is established early following infection; as well, blockade of IFN-I-mediated effects allows the immune response to proceed normally. Altogether, there are many aspects of the immune response to consider in their entirety, but the significance of IFN-I signaling offers an ideal anchor for future analysis and provides a therapeutic target against persistent viral infections to potentially boost Ab responses as well as limit immunopathogenesis.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were reviewed and approved by the Institut national de la recherche scientifique (INRS) animal care committee under protocol 1302-05 and in accordance with AAALAC and Canadian Council on Animal Care (CCAC) guidelines.

Mice, Infections, and Immunizations

Six- to eight-week-old B6 female mice were purchased from Charles River Laboratories (St-Constant, QC, Canada). J_HT mice were obtained from Rolf M. Zinkernagel, Zurich, Switzerland. IFNAR$^{-/-}$ mice on a B6 background were obtained from Ulrich Kalinke, Hannover, Germany. All mice were maintained at the INRS animal facility until use.

To generate mixed bone marrow chimeric mice, bone marrow cells from 8- to 12-week-old B6, J_HT, and IFNAR$^{-/-}$ mice were mixed at a ratio of 4:1 (J_HT/IFNAR$^{-/-}$ or J_HT/B6) and injected intravenously (i.v.) into lethally irradiated (2 × 600 rads) B6 mice. Mice were left untouched for ≥5 weeks to allow for immune reconstitution as determined by flow cytometry.

All viral strains used in this paper were obtained from Rolf M. Zinkernagel, Zurich, Switzerland. Infections with LCMV Cl13, LCMV WE, or VSV Indiana were carried out by i.v. injection with 2×10^6 pfu of virus or with culture media alone (mock).

For T cell-dependent Ab response characterization, mice were immunized intraperitoneally (i.p.) with 50 μg alum-precipitated 4-Hydroxy-3-nitrophenylacetic hapten conjugated to CGG (NP-CGG; Biosearch Technologies, Petaluma, CA, USA), at a ratio of 53:1, or with precipitated alum alone as a control. For T cell-independent Ab response characterization, mice were immunized i.p. with 5 μg 4-hydroxy-3-nitrophenylacetic hapten conjugated to AminoEthylCarboxyMethyl-FICOLL (NP-FICOLL; Biosearch Technologies, Petaluma, CA, USA) in PBS, at a ratio of 40:1, or with PBS alone as a control. All mice were maintained under specific pathogen-free conditions and immunizations or adoptive transfers were carried out at 6–10 weeks of age.

Blocking Abs were used to block PD-1 and IFNAR signaling. For PD-1 blockade, 250 μg of blocking Ab or isotype control (BioXcell, West Lebanon, NH, USA) were injected i.p. every 3 days starting 1 day before infection and continuing throughout the whole experiment. For IFNAR blockade, 500 μg of blocking Ab or isotype control (Leinco Technologies) were injected i.v. every 2–3 days starting 1 day before infection and stopping at various time points as indicated.

B Cell *In Vitro* Stimulation and CFSE Staining

Splenic B cells were isolated using the EasySepTM Mouse B Cell Enrichment Kit (Stemcell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. B cells were resuspended to 2×10^7 cells/mL in PBS containing 2% FBS and CFSE (Fisher, Waltham, MA, USA) was added to a final concentration of 1 μM. Samples were incubated for 5 min at room temperature and staining was quenched by addition of an equal amount of FBS. Cells were washed with PBS/2% FBS and resuspended in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 55 μM 2-mercaptoethanol. The 3×10^6 cells/well were seeded in 24-well plates and stimulated with either 10 μg/mL goat anti-mouse IgM (Jackson Immunoresearch, West Grove, PA, USA) and 500 U/mL mouse IFN-β (Quansys Biosciences, Logan, UT, USA) or respective combinations and cultured (37°C, 5% CO2) for either 7.5 h or 4 days.

Antibodies and Detection Reagents

The following staining reagents were used: anti-CD3 (145-2C11), anti-CD4 (H129.19), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD45R (RA3-6B2), anti-PD-1 (29F.1A12), anti-PD-L1 (10F.9G2), anti-ICOSL (HK5.3), anti-CD40 (HM40-3), anti-CD40L (MR1), anti-CXCR4 (L276F12), and anti-IL-4 (11b11) were from Biolegend (San Diego, CA, USA); anti-CD19 (eBio1D3), anti-ICOS (7E.17G9), anti-IL-21 (mhalx21), anti-CD69 (H1.2F3), and anti-GL7 (GL-7) were from eBiosciences (San Diego, CA, USA); anti-Bcl-6 (K112-91), anti-CXCR5 (2G8), and anti-Fas (Jo2) were from BD Biosciences (Franklin Lakes, NJ, USA). PNA (B-1075; Vector Laboratories, Burlington, ON, Canada) was used for staining germinal center B cells and MOMA-1 (Abcam, Cambridge, UK) for staining MZ macrophages.

Flow Cytometry

Single-cell suspensions of splenocytes were stained on ice with the appropriate Abs in PBS containing 1% (v/v) bovine serum albumin (BSA, Fisher, Waltham, MA, USA) and 0.1% (w/v) sodium azide (GE Healthcare, Mississauga, ON, Canada). Non-specific staining was blocked by 2.4G2 Ab against CD16/32 [Biolegend; prepared as previously described (53)]. Samples were fixed with 1% (v/v) paraformaldehyde and analyzed on a BD LSR II Fortessa (BD Bioscience). For intracellular cytokine staining, splenocytes were stimulated *ex vivo* for 5 h with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Saint-Louis, MI, USA) (100 ng/mL), ionomycin (Sigma-Aldrich) (1 μM), and Brefeldin A (Sigma-Aldrich) (10 μg/mL). Samples were fixed and permeabilized prior to incubation with Ab in permeabilization buffer (eBiosciences). Dead cells were excluded based on positive staining with 7-Amino-Actinomycin D (7-AAD)

(BD Pharmingen), and doublets gated out using FSA-A/SSC-A. Data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Quantitative RT-PCR

$CD4^+$ T cells were magnetically selected using the EasySep system (StemCell, Vancouver, BC, Canada), frozen, and conserved at −80°C. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) and quantified by Nanodrop ND-1000 (Fisher, Waltham, MA, USA). cDNA was synthesized from total RNA using the Superscript III First-Strand Synthesis SuperMix (Applied Biosystems) and frozen at −80°C until use. Gene expression was determined by quantitative PCR using TaqMan Gene expression System (Life Technologies, Burlington, ON, Canada) on a Rotor-gene 6000 system (Corbett, Concorde, NSW, Australia). Standard commercial TaqMan probes were used for IL-4, IL-21 and BAFF (Life Technologies). Samples were normalized to GAPDH and represented as fold change over mock-infected mice using the ΔΔCT method (54).

ELISA and ELISPOT Assay

For NP-specific serum Ig detection, 96-well plates (Mabtech, Cincinnati, OH, USA) were coated overnight at 4°C with 0.1 µg of NP-BSA conjugated at a ratio of NP to BSA ranging from 4:1 to 26:1. For total serum IgG detection, microtiter plates were coated overnight at 4°C with unlabeled anti-IgG (Jackson ImmunoResearch, West Grove, PA, USA) at 2 µg/mL. Non-specific binding was blocked with 10% (v/v) fetal bovine serum and 0.2% (v/v) Tween 20 in PBS. Horseradish peroxidase (HRP)-conjugated secondary Abs: anti-IgM, anti-IgG1, anti-IgG2c, anti-IgG3, and anti-IgG (total IgG) (Jackson ImmunoResearch, West Grove, PA, USA) were detected with 0.04% (w/v) o-phenylenediamine and 0.8% H_2O_2 (v/v) in citrate buffer. Serum BAFF detection was done by coating 96-well plates overnight at 4°C with 2 µg of anti-BAFF Ab (R&D Systems, Minneapolis, MN, USA) in carbonate buffer. Non-specific binding was blocked with 1% (v/v) BSA in PBS. HRP-conjugated anti-BAFF secondary Ab (R&D Systems) was detected with 0.04% (w/v) o-phenylenediamine and 0.8% H_2O_2 (v/v) in citrate buffer. Serum IFN-α and -β detection was done using Verikine kits (PBL Assay Science, Piscataway Township, NJ, USA) in conditions recommended by the company. LCMV nucleoprotein-specific IgG serum Abs were determined by ELISA using plates coated with purified recombinant LCMV nucleoprotein-GST as previously described (55).

For ASC determination by ELISPOT, a mouse IgG ELISPOT^{Plus} kit (Mabtech, Nacka Strand, Sweden) was used. Briefly, 96-well nitrocellulose plates (Millipore, Etobicoke, ON, Canada) were coated overnight at 4°C with anti-IgG antibody. Non-specific binding was blocked with 5% (v/v) FBS in PBS. Cell suspensions obtained from spleens were added to wells (10^4 cells for detection of IgG and 10^5 for NP) in duplicate and incubated overnight at 37°C in a 5% CO_2 atmosphere. B cell spots were developed by sequential washes with PBS, addition of biotinylated anti-IgG or NP-BSA-Biotin, washes with PBS, addition of streptavidin-alkaline phosphatase, washes with PBS and addition of 5-Bromo-4-chloro-3-indolyl phosphate substrate for 15 min. Spots were counted using an AID Elispot Reader (Autoimmun Diagnostika GmbH, Strasberg, Germany).

Immunochemistry

Freshly harvested spleens were flash frozen in OCT (Electron Microsopy Sciences, Hartfield, PA, USA) in liquid nitrogen, stored at −80°C and processed for sets of 10-µm section sizes with a cryostat (Microm HM 525; GMI, Ramsey, MN, USA). Tissue sections were fixed on slides in 75% acetone and 25% ethanol (v/v) for 5 min and incubated with primary reagent in PBS for 1 h at room temperature. The following primary reagents were used: anti-mouse CD19-PE (RA3-6B2, eBiosciences, 1:10000) and anti-mouse MOMA-1-FITC (Abcam, Toronto, ON, Canada, 1:200). Tissue sections were then washed in PBS and incubated with a secondary Alexa 488 anti-FITC (Life Technologies) to amplify FITC signal or strep-A488 if needed. Tissue sections were then mounted with Prolong (Life Technologies), dried overnight, and observed using a LSM780 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Ab Secretion Quantification

Freshly prepared splenocyte suspensions were plated in triplicates in 96-well flat bottom plates at 10^5 cells per well in culture medium containing DMEM, 10% FBS, 1% streptavidin/penicillin, 1% L-glutamine, 1% sodium pyruvate, and β-mercaptoethanol. Cells were incubated for 48 h at 37°C and culture media was harvested to quantify Ab by ELISA. This quantitation was compared to the numeration of ASCs obtained using ELISPOT to define an individual secretion per ASC cell.

LCMV nAb Quantification

Lymphocytic choriomeningitis virus nAbs were quantified as described previously (56). In brief, serial 2-fold dilutions of 10-fold prediluted sera were incubated with LCMV for 90 min at 37°C in 96-well plates. MC57G mouse fibroblasts were added and incubated for 1 h to allow cells to settle and be infected by non-neutralized virus; cells were then overlaid with 1% methyl-cellulose in MEM. After 48 h, cell monolayers were fixed with 4% formalin and infectious foci were detected by intracellular LCMV staining of infected cells with rat anti-LCMV mAb VL-4.

Statistical Analysis

Data were analyzed using Prism 6 (GraphPad Software, Inc.). Statistical significance was assessed as indicated using unpaired two-sided *T*-test, or a one-way ANOVA with Tukey's multiple comparisons test. p Value <0.05 was considered significant. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Data are represented as means ± SD.

AUTHOR CONTRIBUTIONS

MD and AM designed and performed experiments, analyzed data, and wrote the paper; BM designed and performed experiments and analyzed data; AG and ET performed experiments; PL helped to analyze data; JF designed parts of the study and analyzed data; AL directed the study, analyzed data and organized, designed, and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00564/full#supplementary-material.

FIGURE S1 | Kinetics of the LCMV WE-dependent impairment of the NP-specific Ab response. B6 mice (four per group) were infected with LCMV WE (black), VSV Ind (gray), or mock infected (white). Mice were then immunized with an i.p. injection of NP_{53}-CGG in alum following a varying immunization schedule (from d-4 to d30). **(A)** NP-specific IgG1 response in each differentially timed experiment monitored by ELISA. **(B)** ELISA plates were coated with NP_4-BSA or NP_{26}-BSA and high affinity Ab responses were measured as a ratio of Abs binding to NP_4-BSA versus the total anti-NP IgG1 response binding to NP_{26}-BSA in each differentially timed experiment. Statistical analysis was performed by individual *T*-tests between experimental groups and the mock-infected group. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

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REVIEW
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IFN-λ: A New Inducer of Local Immunity against Cancer and Infections

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IFN-λ is the newly established type III IFN with unique immunomodulatory functions. In contrast to the IFN-α/β family and to some extent IFN-γ, IFN-λ is apparently acting in specific areas of the body to activate resident immune cells and induces a local immunity, instrumental in preventing particular infections and also keeping transformed cells under control. Mucosal areas of lung and gastrointestinal tracts are now under scrutiny to elucidate the immune mechanisms triggered by IFN-λ and leading to viral protection. New evidence also indicates the crucial role of IFN-λ in promoting innate immunity in solid cancer models. Based on its unique biological activities among the IFN system, new immunotherapeutic approaches are now emerging for the treatment of cancer, infection, and autoimmune diseases. In the present review, we highlight the recent advances of IFN-λ immunomodulatory functions. We also discuss the perspectives of IFN-λ as a therapeutic agent.

Keywords: IFN-λ, mucosal immunity, viral infections, immunotherapy of cancer, inflammation, NK cells

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INTRODUCTION

Human IFN-λs are represented by four functional and highly homologous subtypes IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4 (1, 2). In contrast to the other IFN-λ subtypes, IFN-λ4 is selectively expressed in the human population and weakly released by IFN-producing cells (3). However, all the four IFN-λ protein subtypes are clustered on chromosome 19 and are grouped in a new IFN family, called type III IFN, distinct from type I and type II IFNs, respectively, representing the classical IFN-α/β family and IFN-γ. In mice, only two functional genes located on chromosome 7 and encoding IFN-λ2 and IFN-λ3 have been characterized (4). In contrast to its human counterpart, the murine IFN-λ1 gene ortholog is a pseudogene as reported in several mice strains. However, we did not find yet a corresponding IFN-λ4 in mice. Type III IFNs use a unique receptor, the IFN-λ receptor, and induces similar JAK–STAT signaling pathway as type I IFNs (5, 6). Although it has been well established that in addition to JAK1, TYK2 is crucial in mediating the activity of both type I and type III IFN, new evidence in patients with a defect in TYK2 shows an impaired response for type I IFN only (7), suggesting a Tyk2-independent signaling for type III IFN. Upstream cell signaling is quite distinct between type I and type III IFN. Type I IFN interacts with a receptor formed by IFNAR1 and IFNAR2 (8). However, type III IFNs bind to the specific receptor chain IFN-λR1, and IL-10R2, a receptor subunit shared by IL-10 cytokine

family members IL-10, IL-22, and IL-26 (6). In contrast to type I and type II IFN receptors, the unique type III IFN receptor for IFN-λ, IFN-λR1 is not ubiquitously expressed (5, 9), suggesting that IFN-λ may eradicate specific viral infections and also elicit a more local immunity against pathogens and cancers. This has important consequences for therapeutic targeting (10, 11).

In addition to its restricted interaction mainly with epithelial cells (EC), IFN-λ may also induce cell signaling that differs to some extend from IFN-α/β signaling. Currently, it has been established that the antiviral patterns of IFN-λ are quite distinct from those of IFN-α (10). In oncology and autoimmune diseases, the role of IFN-λ seems also to differ in many aspects from IFN-α (11).

Furthermore, in contrast with type I, type III IFN is prone to a particular genetic reactivation or deactivation in the human population as illustrated with IFN-λ4 and related genetic polymorphism (3). Tremendous research efforts are still ongoing to understand the impact of this genetic aspect of type III IFN on the prevalence of diseases, particularly hepatitis. IFN-λ4 has been linked with the failure to clear hepatitis C virus (HCV) infection and decreased response of HCV patients to IFN-α therapy (12, 13). IFN-λ4 can be produced only by people who carry the IFN-λ4–ΔG allele (rs368234815), known for predicting HCV clearance (3, 14). The inherited IFN-λ4–ΔG allele is the main variant in Africans, while the minor variant is found in Asian people (15). Therefore, a negative genetic selection for IFN-λ4–ΔG allele could be driven by infectious agents, including HCV (16).

As highlighted in many reviews, the activity of IFN-λ is highly prominent in EC in comparison with other cell types (1, 3, 5, 9–11, 17–24). However, the significance of the restricted action of IFN-λ remains elusive. We still have to understand the role of this specific interaction of IFN-λ on the protection of epithelial surfaces from exposure to pathogenic microbes and the development of carcinomas. The goal of this review is not an exhaustive description of the IFN-λ biology, which has been abundantly reported in many important reviews (5, 10, 20, 22–25). We have been focusing our review on the potential links between the prominent activity of IFN-λ on EC and its associated immunity against viral infections and cancer.

IFN-λ AND THE EPITHELIUM TRACT DEFENSE AGAINST VIRAL INFECTIONS

Accumulating evidence strongly suggests that IFN-λ plays a major role in providing the frontline defense for the epithelium against viruses. The epithelium is formed by closely packed EC with practically no intercellular spaces. However, EC are not isolated from immune cells. Respiratory, urogenital, and gastrointestinal (GI) tracts forming the major mucosal areas in the body show complex association between the epithelium and the immune cells in variable proportions, endowing mucosal surfaces with a particular immunity against the harmful environment (26). By lining mucosal surfaces, EC are under continuous attack by viruses. The first response of cells infected with virus is the release of IFN. The released IFN provides neighboring healthy cells an antiviral state, allowing them to stop viral spreading (**Figure 1**). However, clearance of infected cells requires immune cells intervention. Both type I and type III IFNs are expressed by host cells in response to viral infection. However, depending on the site of virus attack, host cells exhibit differential expression of type I and type III IFNs (5, 25, 27). The mechanisms leading to the induction of IFN expression, the establishment of antiviral state, and the clearance of infected cells are well documented particularly for type I IFN (10). In contrast, we are still striving to understand the role of IFN-λ-regulated antiviral mechanisms in mucosal surfaces on which increasing reports indicate a critical role of type III IFN.

ROLE OF IFN-λ IN THE CONTROL OF VIRAL INFECTIONS OF THE RESPIRATORY TRACT

Respiratory tract infections by viruses are common and mainly concern the sinus, the throat, and the lungs. In comparison with IFN-α, IFN-λ is predominantly induced by respiratory viruses (28–31). Currently, IFN-λ is designated as a therapeutic candidate against the influenza A virus (IAV) infection (32, 33). In infants hospitalized for respiratory syncytial virus (RSV)- or human rhinovirus (HRV)-associated bronchiolitis, RSV elicited higher levels of IFN-λ subtypes when compared with HRV (34).

It has been postulated that in order to increase infection, respiratory viruses can also suppress IFN-λ antiviral response. Influenza virus- and RV-induced epidermal growth factor receptor activation has been found to suppress IFN regulatory factor (IRF)-1-induced IFN-λ production and increased viral infection (35). NS1 and NS2 proteins of the human RSV also inhibit IFN-λ production, occurring *via* IFN regulatory factor (IRF)-3, NF-κB, and proinflammatory cytokines suppression (36, 37). More recently, it has been reported that excessive expression of IFN-λ in the lung during IAV infection is associated with a suppression of IFN-λ signaling by SOCS-1 (31). The authors suggested that the suppression of cytokine signaling by virus-induced SOCS-1 leads to an adaptive increase in IFN-λ production by the host to protect cells against viral infection. This increase of IFN-λ production further induces the expression of SOCS-1 at late stage of infection, which in turn, inhibits the activation of JAK–STAT signaling. Finally, this vicious cycle results in excessive production of IFN-λ and impaired antiviral activity.

One of the main concerns about viral lung infections such as the one caused by IAV is the subsequent inflammation. Although IFN-α is highly efficient in suppressing IAV, in contrast with IFN-λ, it exacerbates the inflammation by overstimulating the immune system and driving immunopathology (32). Therefore, in agreement with its weak targeted actions on immune cells surrounding infected EC, IFN-λ may constitute the treatment of choice in viral infections associated with inflammation (11). In favor of IFN-λ as therapeutic option for viral infection associated with inflammation, we can mention early studies on asthma, showing a deficiency in IFN-λ (38) and the role of IFN-λ treatment in suppressing respiratory viral infections and allergic airway inflammation (39). However, other immune mechanisms could also occur after IFN-λ treatment. It has been strongly

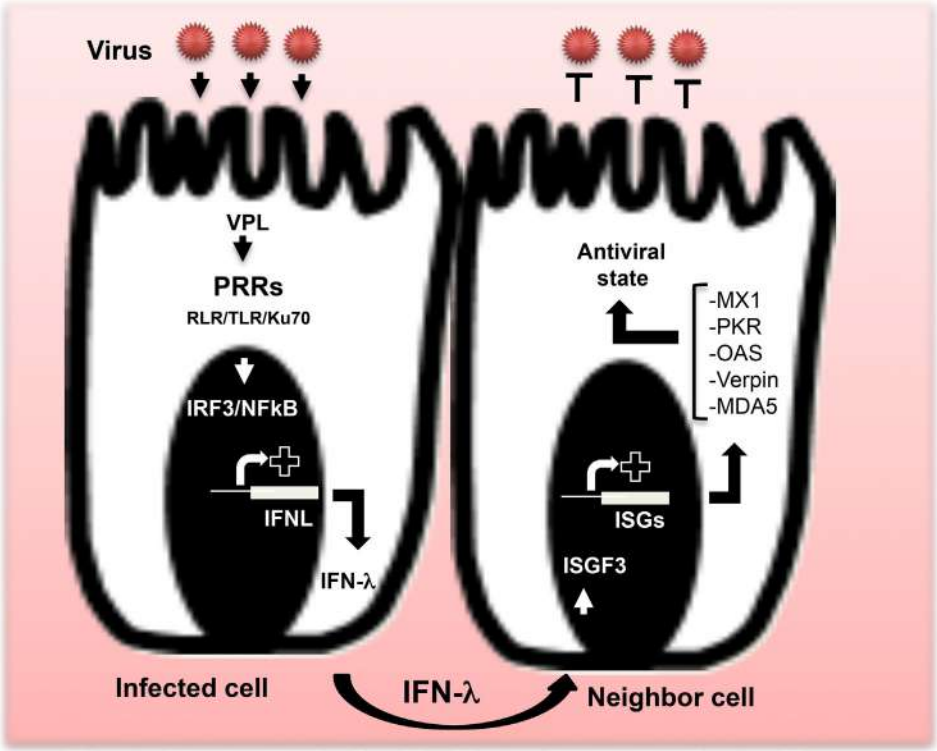

FIGURE 1 | **Production of IFN-λ and establishment of the antiviral state**. After virus attack, IFN-λ genes are induced. When viral particle ligands (VPL) are sensed by pattern recognition receptors (PRRs), particular transcription factors, mainly IRF-3 and NF-κB, are induced to allow IFN-λ gene expression and subsequent release of IFN-λ proteins outside the infected cell. Dependent on the type of virus, various PRRs are involved, including members of the RIG-I-like receptor and toll-like receptor families, as well as the DNA sensor Ku70. Released IFN-λ induces an antiviral state in neighboring cells *via* the induction of interferon-stimulated gene factor leading to the expression of specific antiviral interferon-stimulated genes (ISGs), including myxovirus resistance 1, protein kinase R, melanoma differentiation-associated protein 5, verpin, and 2′-5′-oligoadenylate synthetase. Products of those antiviral ISGs inhibit virus replication and provide an antiviral state to cells.

suggested that by upregulating indoleamine 2,3-dioxygenase during influenza virus infection, IFN-λ may induce an immune suppression (40).

ROLE OF IFN-λ IN THE CONTROL OF VIRAL INFECTIONS OF THE GASTROINTESTINAL TRACT

Currently, several studies indicate that IFN-λ plays a predominant role in controlling viral infections of the GI tract (5, 10, 41, 42). In response to viral infections, IFN-λ is highly produced by intestinal EC and induces a strong antiviral response (27). However, recent studies show that this strong effect of IFN-λ resulted from a synergistic effect with IL-22 (43). ZEB1 has been shown to play a role in the activation of IFN-λ gene expression at the transcriptional level, in addition to IRF-3 and NF-κB (10, 44). Interestingly, the role of IFN-λ in controlling viral infections of the GI tract cannot be compensated by IFN-α/β in suckling mice (25).

In contrast to type I IFN, type III IFN was not involved in controlling viral infection of lamina propria in agreement with the lack of response of effector immune cells to IFN-λ but not to IFN-α (**Figure 2**). Therefore, type I and type III IFNs are not redundant cytokines at least in the GI tract (25, 45).

Regarding the role of IFN-λ during rotavirus infection, divergent results between virus strains and research groups have been reported. Early studies showed that IFN-λ is highly effective in controlling the murine rotavirus infection in suckling mice (27, 43). However, Lin et al. demonstrated that the effect of IFN-λ is dependent on the rotavirus strain used (46, 47). IFN-λ was able to control rotavirus infection when suckling mice received the heterologous but not the homologous rotavirus. Although the homologous rotaviruses used by those different groups are quite similar, the IFN-λR1 knock-out mice used in the more recent study were generated differently. In this study, only exon 3 of *IFN-λR1* gene is lacking, while in the transgenic mice used by the other groups, the entire *IFN-λR1* gene is missing. Although the first generated IFN-λR1-deficient mice have been extensively studied by many groups for almost a decade (48), comparison between the two IFN-λR1-deficient mice is warranted. Important standardizations of virus dosage, virus strain, host strain, and diet are also required for minimizing the variance in the experimentation.

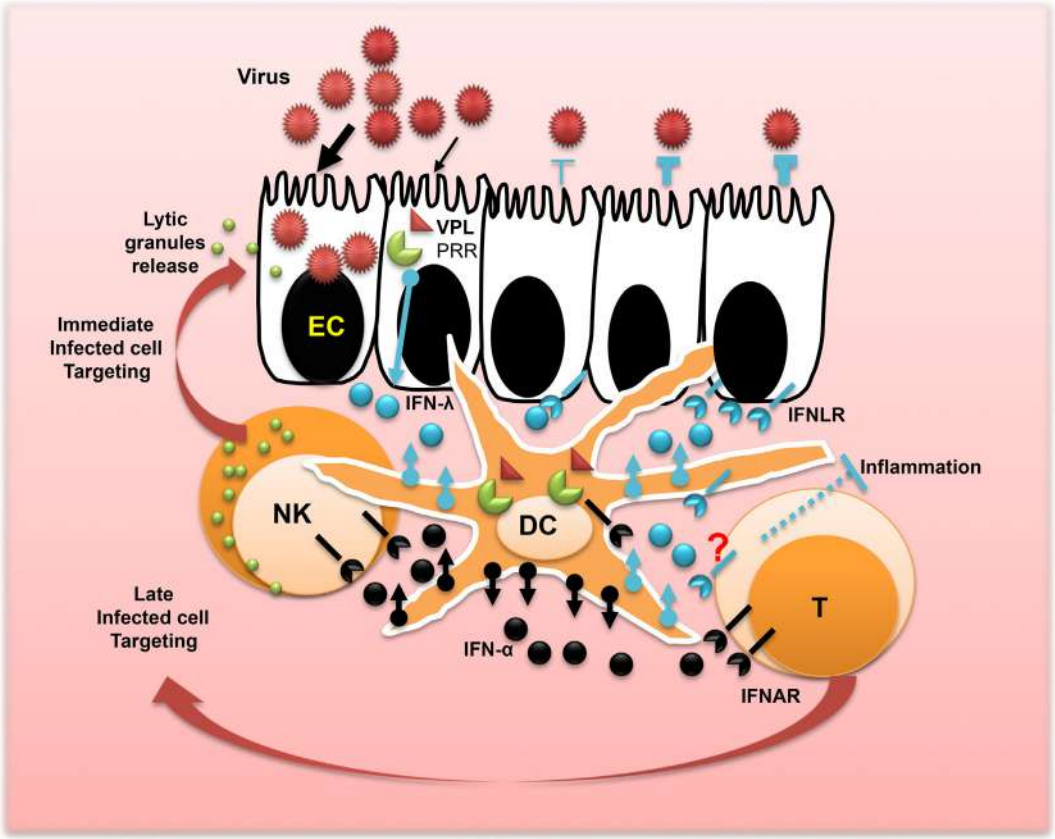

FIGURE 2 | **Concerted action of IFN-λ and IFN-α promotes mucosal immunity and viral protection**. After the interaction of the epithelium surface of the mucosa with viruses, epithelial cells release IFN-λ. The process leading to IFN-λ production is triggered by the interaction of a viral particle ligand (VPL) such as DNA or RNA with the cellular pattern recognition receptors. Dendritic cells (DCs) are the main immune cells of the mucosa involved in sensing viral infections and producing high amounts of IFN-λ and IFN-α. This produced IFN-λ fuels the antiviral protection of the epithelium and in concert with IFN-α may shape local immunity and control inflammation. Released IFN-α by DCs also plays a central role in controlling viral dissemination in the lamina propria and the promotion of T cell immunity and natural killer cell activation for immediate targeting and clearance of infected cells toward stopping viral spread.

ROLE OF IFN-λ IN THE CONTROL OF VAGINAL MUCOSA IMMUNITY

Herpes simplex virus particularly 2 (HSV-2) is the prevalent cause of genital ulceration in humans worldwide with lifelong latent infection of female genital mucosa (49). Early studies in mice using a model of localized HSV-2 infection demonstrate that IFN-λ completely prevents virus replication in the vagina. IFN-λ has been shown to induce significant virus suppression associated with a complete remission from the genital viral disease. Antiviral effects of IFN-λ were superior in comparison with IFN-α (48, 50, 51). In a recent study using BAC transgenic mice, expressing firefly luciferase under transcriptional control of the Mx2 gene promoter, it has been also demonstrated that IFN-λ reactivity was most prominent in mucosal surfaces, including the genital area; however, IFN-α response was strong in the liver, spleen, and kidney (52). Furthermore, the vaginal mucosa expresses high levels of IFN-λ. In contrast to IFN-α, NF-κB plays a major role in promoting IFN-λ expression (53). The primary source of IFN-λ was attributed to dendritic cells (DCs), indicating that IFN-λ response plays a crucial role in promoting vaginal mucosa immunity (**Figure 2**). Ongoing investigations, using IFN-λ transgenic models, will likely determine the effector cells responsible for the control of vaginal infection by IFN-λ.

PERSPECTIVES AND CHALLENGES OF IFN-λ AS A NEW PLAYER IN MUCOSAL SURFACES

The role of IFN-λ in controlling viral infection of mucosal surfaces is increasingly studied (5, 10). The interaction between IFN-λ and the EC of the mucosa is a crucial step in establishing this antiviral protection. In comparison with IFN-α, the antiviral effect of IFN-λ on the lining epithelium appears significantly superior. However, IFN-λ fails to control viral infection of immune areas underlying the epithelium. Conversely, IFN-α appears more efficacious than IFN-λ in protecting immune tissues and lamina propria from viral infection. By playing complementary roles, both IFN-λ and IFN-α seem instrumental in protecting mucosal

surfaces from viruses. However, the role of immune cells that contributes to the antiviral activity of IFN-λ and IFN-α remains poorly understood. Besides DCs, we still poorly understand the contribution of other immune system components in IFN-λ functions within the mucosa. It has been clearly demonstrated that the mean source of IFN-λ in the mucosa is coming from the DCs. This released IFN may fuel the antiviral protection of EC, and probably in concertation with IFN-α, modulates mucosal immunity and inflammation (**Figure 2**). However, studies in this field concern mostly IFN-α. The role of IFN-α in activating immune cells during viral infection of the mucosa and related inflammation has been relatively well studied (10). The question yet to be answered is, in the context of IFN-α and viral infections, what is the role of IFN-λ in innate immunity and inflammation, particularly in neonates and infants?

Due to their innate immune deficiency, neonates and infants are highly sensitive to respiratory and GI virus infections leading to high risk of mortality (54, 55). Immaturity of natural killer (NK) cells has been demonstrated as the primary factor for increased susceptibility to viral infections in early life for both human and mice (56–59). NK cell responses' impairments are associated with a significant deficiency in the production and the release of lytic granules (60), in agreement with early studies demonstrating that the transfer of adult NK cells to suckling mice induces a protection against viral infection (61). This conclusion has been corroborated by a recent report demonstrating that NK cell deficit can be reversed in suckling mice (62). In parallel, as reported earlier, it has been shown that IFN-λ plays a crucial role in viral infections of suckling mice (27, 43, 46, 47), suggesting that IFN-λ may induce antiviral functions at least partially *via* NK cells, and those functions of IFN-λ are missing in neonates and infants due to a potential deficiency of IFN-λ production or response. The aptitude of NK cells to respond rapidly without prior sensitization makes them at the front line of defense against infection (63, 64). NK cells are well armed for sensing and killing virus-infected cells (**Figure 2**). *In vivo* activation of NK cells by IFN-λ has been well documented in cancer models (6, 65). Significant NK cell impairment of NK cell tumoricidal activity has been reported in IFN-λR−/− mice (66). More recently, we have demonstrated that a cooperation between IFN-λ and IFN-α promoted local NK cell antitumor actions (67). We believe that within mucosal surfaces, IFN-λ in combination with IFN-α may play an important role in recruiting and activating NK cells to clear viral infections. Privileged interaction of IFN-λ with EC may not only induce the antiviral state but also contribute to the attraction of immune cells *via* the release of potential chemokines.

In addition to its role in mucosal immunity and viral infections, IFN-λ has been recently proposed as the treatment of choice for IAV infection because its antiviral activity was not associated with an exacerbation of inflammation in contrast to IFN-α (32). However, the mechanisms leading to the potential anti-inflammatory role of IFN-λ remain elusive. In the case of IAV infection, it has been indicated that IFN-λ was simply acting on EC without overstimulating the immune system and driving immunopathology like IFN-α. However in collagen-induced arthritis, the anti-inflammatory role of IFN-λ has been demonstrated (68). Apparently, IFN-λ decreased significantly neutrophil population in the joints of diseased mice. This occurred in association with a reduction of interleukin-1β level, which is thought to play a crucial role in inflammation.

CONCLUSION

We currently see a clearer picture about the role of IFN-λ and its possible therapeutic uses. All studies highlight the crucial role of IFN-λ on EC, which are the first line of attack by pathogens, toxins, and other damaging agents. The majority of infections and cancers concern epithelial cell types. This strongly suggests that beyond its well-described antiviral and antitumoral roles, IFN-λ may have immunomodulatory roles for indirectly protecting EC from different damages. New ideas have already emerged about the role of IFN-λ on effector cells orchestrating inflammation and autoimmunity. However, for building successful strategies against cancer and infection diseases, the interaction between IFN-λ and IFN-α should be taken into consideration. Based on new evidence from viral infections and cancer studies a concerted action of IFN-α and IFN-λ seems crucial in the complexity of interactions between diseased cells and surrounding immune cells.

AUTHOR CONTRIBUTIONS

AL: designed the plan of the manuscript and the figures and wrote the manuscript. AZ, AT, and KC-S: discussion on the plan and the references used in the manuscript and contribution in writing the manuscript.

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Type-I Interferon Responses: From Friend to Foe in the Battle against Chronic Viral Infection

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Type I interferons (IFN-I) have long been heralded as key contributors to effective antiviral responses. More widely understood in the context of acute viral infection, the role of this pleiotropic cytokine has been characterized as triggering antiviral states in cells and potentiating adaptive immune responses. Upon induction in the innate immune response, IFN-I triggers the expression of interferon-stimulated genes (ISGs), which upregulate the effector function of immune cells (e.g., dendritic cells, B cells, and T cells) toward successful resolution of infections. However, emerging lines of evidence reveal that viral persistence in the course of chronic infections could be driven by deleterious immunomodulatory effects upon sustained IFN-I expression. In this setting, elevation of IFN-I and ISGs is directly correlated to viral persistence and elevated viral loads. It is important to note that the correlation among IFN-I expression, ISGs, and viral persistence may be a cause or effect of chronic infection and this is an important distinction to make toward establishing the dichotomous nature of IFN-I responses. The aim of this mini review is to (i) summarize the interaction between IFN-I and downstream effector responses and therefore (ii) delineate the function of this cytokine on positive and negative immunoregulation in chronic infection. This is a significant consideration given the current therapeutic administration of IFN-I in chronic viral infections whose therapeutic significance is projected to continue despite emergence of increasingly efficacious antiviral regimens. Furthermore, elucidation of the interplay between virus and the antiviral response in the context of IFN-I will elucidate avenues toward more effective therapeutic and prophylactic measures against chronic viral infections.

Keywords: type-I interferon, chronic viral infection, immunopathology, IFNAR, immunoregulation

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INTRODUCTION

Upon viral infection, the immune response comprises a multi-layered coordination of effector functions broadly characterized as a progression from innate to adaptive immunity. Within the immunological milieu, Type I interferons (IFN-I) play a central role in driving an antiviral state in non-immune cells as well as orchestrating antiviral immune responses through: (i) inhibiting viral replication in infected cells in the innate stage of the immune response; (ii) activating and enhancing antigen presentation in the "early induced" immune response, and (iii) triggering the adaptive immune response through direct and indirect action on T and B cells that make up the memory

response [reviewed in Ref. (1)]. Therefore, this cytokine acts as a master regulator whose induction in the early stages of viral infection modulates downstream signaling cascades that promote both pro-inflammatory and anti-inflammatory responses depending on the context of activation as discussed below. Whereas the protective role of IFNs has been widely characterized, emerging lines of evidence illustrate a deleterious effect borne by IFN-associated immunopathology (2, 3). These characterizations bear particular importance given the historic use and ongoing studies on IFN therapy in the treatment of chronic viral infections [e.g., HCV (4) and HIV (5–8)], autoimmune diseases [e.g., systemic lupus erythematosus (9)], and cancer (10–13). Whereas the advent of new therapies has spurred a trend toward IFN-free treatments in HCV, HIV, and oncology, IFN therapy is still considered to be a significant therapeutic agent due to its efficacy against HCV-associated complications [e.g., hepatocellular carcinoma (4)] and combinatorial effect in cancer therapy (14). In addition, cost restriction due to the price of emergent therapies also sustains the use of IFN-based therapies (15).

Described here in the context of viral infections, this review focuses on the course of IFN-I upon (i) elicitation; (ii) downstream signaling in various cell types, and (iii) the consequent binary effect on immunity. Collectively, we discuss the development of IFN-driven antiviral responses and key features that highlight potential targets toward effective treatment measures against chronic viral infections.

DIVERSITY IN IFN-ASSOCIATED IMMUNE RESPONSES

Type I interferons can be broadly characterized into three groups: IFN-I, Type II (IFN-II), and Type III (IFN-III) with subcategories therein based on gene loci of the IFN transcribing genes as well as difference in their cognate receptors. IFN-I is the largest and most well-characterized group with seven classes: IFNα, IFNβ, IFNδ, IFNϵ, IFNκ, IFNω, and IFNτ whereas IFN-II comprises IFNγ. IFN-I and IFN-II signal through IFNαR1/R2 (IFNAR) and IFNγR1/R2 (IFNGR), respectively. The last class IFN-III, otherwise classified as "IFN-like cytokines," consists of interleukin (IL)-28A (IFNλ2), IL28B (IFNλ3), and IL29 (IFNλ1) and signals through IL-28RI/IL10R2 receptor chains [reviewed in Ref. (16)].

Upon pathogen-encounter, a plethora of cells are induced into IFN-I expression through recognition of pathogen-associated molecular patterns by putative pattern recognition receptors (PRRs), such as (i) toll-like receptors (TLRs) (17–24), (ii) retinoic-acid inducible gene I (RIG-I) (20, 25), (iii) melanoma differentiation-associated gene 5 (26), and (iv) nucleotide-binding oligomerization domain-containing protein (27). Consequent to PRR activation, signal transduction occurs through downstream transcription regulators called IFN regulatory factors (IRFs). This family of nine members, IRF1–IRF9 [see Table 1 in Ref. (28) for summary] offers yet another layer of diversity in the IFN response; convergence to and transcription by different sets of IRFs is determined by the nature of the sensing PRR, which resultantly determines the nature of the subsequent IFN responses.

The third layer of diversity entails the initiation of transcription by IRFs, which is facilitated by the variety of signal transduction pathways triggered upon elicitation of IFNs. Upon ligation of IFNAR, signal transducer and activator of transcription 1 (STAT1) and STAT2 are induced through phosphorylation by the tyrosine kinase 2 (TYK2) and Janus kinase (JAK1). Thereafter, STAT1 and STAT2 form a trimeric transcription factor, IFN-stimulated gene factor 3, by assembling with IRF9 that subsequently migrates into the nucleus to initiate transcription of IFN-stimulated genes (ISGs) by binding to the promoter regions known as IFN-stimulated response elements (ISRE) (29). Within this signal cascade lies combinatorial differences through which IFNs foster both proinflammatory and anti-inflammatory responses. For example, while signaling by IFN α/β through IFNAR typically leads to heterodimerization of STAT1 and STAT2, homodimerization between STAT1 and STAT3 may occur concurrently or alternatively upon IFNAR signaling. This different pairing of downstream STAT dimers therefore results in (i) the aforementioned engagement of ISRE toward antiviral responses (STAT1/3 heterodimers), (ii) the induction of pro-inflammatory responses by binding to IFNγ response elements (GAS) (STAT1 homodimers), or (iii) binding of STAT3-binding elements (SBE) to trigger an anti-inflammatory response (STAT3 homodimers) [reviewed in Ref. (30)].

Importantly, whereas STAT1 drives a pro-inflammatory, pro-apoptotic response, STAT3 dimerization favors an anti-inflammatory response that negatively regulates the action of STAT1 (31); we surmise that this is likely a homeostatic mechanism to counter the immunopathological effects of sustained IFN-associated pro-inflammatory responses. However, in the context of IL-6 cytokine signaling, the anti-inflammatory effect of STAT3 upon IFNAR signaling can also be counteracted through a negative feedback loop as well; this further underscores the multiplicity of interactions that govern IFN-I-associated signaling and its downstream effects (31). Lastly, in addition to the plethora of molecular interactions, the presence of IFN-I receptors on various cell types [e.g., hematopoietic stem cells (32, 33), macrophages (34–36), dendritic cells (DCs) (37–43), and natural killer (NK) cells (35, 44–47)] further enhances the impact of IFN-I upon induction.

IFN-I RESPONSES IN CHRONIC INFECTION

It is important to consider that the antiviral effects of IFN-I have been primarily made in the framework of an acute infection in which the intricate interplay of well-timed and tightly regulated IFN responses functions optimally toward resolution of an infection. What are the effects of prolonged IFN-I production such as in the case of chronic infections? This is an open question that is gaining increasing traction based on emerging data on the deleterious effects of IFN-I in the chronic setting. Importantly, various combinations of IFN-I are used as therapeutic measures particularly in chronic infections. Given the historical and continued use in clinical applications, this is a crucial factor to consider given the multifaceted ways in which IFN elicitation and response are regulated in a fine balance whose perturbation bears

impact ranging from hematopoiesis to mature differentiated adaptive immune responses.

IFN-I RESPONSES IN LYMPHOCYTIC CHORIOMENINGITIS VIRUS (LCMV) INFECTION

The deleterious effect of IFN-I responses has been brought into sharper focus more recently by two independent studies using a chronic (LCMV-Clone 13) versus acute (LCMV-Armstrong) infection model, which revealed that viral persistence was diminished by *in vivo* IFNAR blockade (2, 3). In their analyses, Teijaro et al. illustrated that IFNAR blockade led to the rescue of IFNγ$^+$ CD4 T cells, which as discussed comprise the T helper 1 (T_H1) cellular subsets that potentiate cytotoxic T lymphocyte (CTL) responses. Strikingly, this study revealed that the size of the CTL subpopulation was not changed despite the enhanced viral clearance observed; thus, functional quiescence (similar to exhaustion) in the face of sustained IFN-I signaling partially facilitates impairment of viral clearance by CTLs. A significant finding in these studies was that in addition to the net detrimental effects of sustained IFN-I, elicitation of high concentration of the cytokine early in the course of infection correlated with viral persistence.

As outlined, IFN-related mechanisms are governed by feedback loops to ascertain homeostasis and prevent immunopathology. An example of these coordinate measures is observed in the switch from T_H1 responses toward T follicular helper (T_{FH}) cells. Fahey et al. originally depicted this transition using LCMV. By comparing LCMV-Armstrong versus LCMV-Clone 13, they observed that while mice infected with an acute strain of the virus did not bear any aberrant elevation of T_{FH} markers, the chronic phase of LCMV-Clone 13 infection exhibited increased proportions of T_{FH} cells depicted by putative markers such as (i) CXCR5; a B cell homing chemokine receptor; (ii) ICOS; an inducible T cell costimulatory molecule; and (iii) inducible T cell costimulatory OX40, also known as TNFRSF4. A significant distinction to make here is that T_{FH} cells were also present in the acute infection but these abated upon resolution of the infection (48). In follow-up analyses, Osokine and colleagues revealed that this switch occurred in an IFN-I-dependent manner wherein the absence of IFN signaling, T_H1 responses were maintained; in the presence of IFN-I, the cytokine actively suppressed the emergence of *de novo* T_H1 cells in a pre-programed function that occurred early in the priming stages of the infection (49). The underlying principle behind this transition is to curb the T_H1 response, which triggers IFNγ expression that in turn activates CTLs and NK cells. From a homeostatic point of view, prolonged effector function of these cells may lead to excessive cytotoxicity and other detrimental effects resulting in host tissue damage.

However, in the event of viral persistence, this skew toward T_{FH} responses results in a number of aberrant responses that hinder viral clearance. Decades-long characterization of CTL exhaustion has been at the forefront of chronic-infection immune response perturbations [(50), reviewed in Ref. (51)]. Initially characterized in LCMV infection as well, exhausted CTLs were observed to be refractory to activation signals, prone to apoptosis, and feature an upregulation of inhibitory markers (52–56). Notably, the aforementioned switch to T_{FH} from T_H1 results in diminished activation of CTLs based on the resultant reduction of the second activation signal required to fully activate naïve CTLs. As shown by Fuller et al., the absence of T_H1 licensing (57) along with the reduction of IFNγ due to contraction of T_H1 cell populations as infection progresses toward chronicity leaves CTLs in a pseudo-activated state characterized as exhaustion.

That the T_{FH} subpopulation is atypically expanded in chronic infections (48, 49) also imposes dysregulation on their close immunological counterparts, the B cells. In the context of a chronic infection, perturbations such as atypical B-cell subpopulations, hypergammaglobulinemia (HGG), and polyspecificity are well characterized (58–66). Along with others, we observed the extensive impact of IFN-mediated responses on humoral immunity both directly and indirectly in the context of viral persistence. In our study, we found that in addition to the indirect T_{FH}-associated humoral response perturbation, there was a direct IFN-I-mediated effect on B cells (67). Comparing LCMV-Clone 13 versus LCMV-WE (acute), we observed sustained ablation of antigen specificity against a secondary immunogen, nitrophenylacetyl-chicken gamma globulin (NP-CGG), in the former whereas the latter only showed transient impact on antigen specificity. Furthermore, we also evaluated antigen specificity of NP-CGG in the context of vesicular stomatitis virus (an acute infection), which remained unchanged. Remarkably, we observed the rescue of antigen specificity upon IFNAR blockade in addition to a recovery of lymphoid architecture similar to previous studies (2, 3, 67, 68). Most importantly, we also assessed the humoral response using a chimeric mouse model comprising reconstitution of irradiated B6 mice with a mix of bone marrow cells from J_HT (B-cell deficient) (69) and IFNAR$^{-/-}$ mice. Here, we observed that in the absence of IFNAR signaling in B cells, neutralizing antibodies (nAbs) against LCMV were elicited more robustly and earlier than in wildtype mice and control J_HT/B6 chimeras. These results are in agreement with previous findings by Price et al. who also showed that in the absence of IFN-I signaling, nAb responses against influenza virus developed more efficiently (70). Recently, the direct effect of IFN signaling on B cells has also been illustrated using *Leishmania donovani*, which is the etiological agent of the chronic disease, visceral leishmaniasis. In this study, Silva-Barrios et al. illustrated that B-cell activation occurred in an IFN-associated, TLR-dependent manner that culminated in disruption of the humoral immune response that typifies other chronic infections. Similar to our findings, they also observed the reduction of HGG upon B-cell-specific IFNAR knockout in mice (71), which further supports the role played by IFN signaling toward this phenomenon.

IFN-I RESPONSES IN HCV INFECTION

In the perspective of human infection, the role of IFN responses is particularly important based on the widespread use of IFN therapy against chronic viral diseases such as HCV (4), HIV (5–8, 72), and more broadly in clinical setting such as systemic lupus erythematosus (9), melanoma, and other neoplastic indications

[(11–13), reviewed in Ref. (10)]. It is important to state that the standard of care in HCV is slowly moving away from IFN-based therapy, whereas HIV anti-retroviral therapy is almost entirely IFN-free except in impoverished regions. Of note, although some of these conditions are non-viral infections, they all feature antigenic persistence and therefore resemble chronic viral infections despite different etiologies. Given the pervasive influence of IFN-I responses and data revealing both positive as well as negative effects of the cytokine, it is also imperative to critically delineate the effect of IFN-I in chronic disease settings.

Generally, the immunopathology associated with IFN-I, e.g., aberrant cellular populations, inadequate immune responses, and disrupted cytokine environments are also observed in HCV. On a molecular level, most characterizations of IFN cellular responses have been made using *in vitro* models, e.g., HCV pseudoparticles (73) and HCV cell culture (74, 75) systems whereby the impact of IFN is observed in the context of both endogenous expression in cell culture and exogenous supplementation akin to administration of therapy. Detection of viral RNA occurs through typical PRR-recognition pathways [(76, 77), reviewed in Ref. (78)], upon which upregulation of ISGs occurs (79). Interestingly, researchers observed a coincidence between low response rates to IFN treatment in patients with high baseline levels of IFN in their plasma (80). In this study, Sarasin-Filipowicz and colleagues revealed that hepatocytes obtained from chronically infected, non-responder patients bore non-responsive signaling to IFN treatment *ex vivo*. Similarly, evidence of attenuation in IFN responses in the chronic phase of HCV is also suggested by the prevalence of ineffective CTL responses upon delayed induction of IFNα-therapy, whereas functional effector activity was maintained or restored in spontaneous resolvers or responders, respectively (81). At the transcriptional level, clues toward IFN-resistance are posited by the discovery of proviral ISGs whereby recent work has shown that some ISGs work to promote the HCV resistance in cell culture. For example, overexpression of ubiquitin-specific protease 18 (USP-18), which functions as a negative regulator of IFN signaling drives, a proviral response highlighted by evidence of up regulation in HCV patients who do not respond to IFN treatment (82). Conversely, USP18$^{-/-}$ mice are resistant to viral infection (83). Here, USP18 works in concert with ISG-15, therefore inhibiting effective JAK/STAT signaling; based on the significance of this signaling pathway toward effective IFN signaling, the expression of these ISGs results in diminished IFN responses and counterintuitively facilitate HCV replication (84, 85). Important to note here is that transcription of both antiviral and proviral ISGs are driven by ligation of IFN receptors. Similarly, the presence of "negative regulators" such as these is therefore likely a negative feedback mechanism, which when functioning optimally reverts the host immunological milieu to "steady-state". However, against chronic infection, the presence of such processes also contributes to desensitization to therapeutic IFN-administration in HCV patients with high levels of IFN expression (86). In this setting, the consequent evocation of ISGs such as USP-18 and ISG-15 renders the patients non-responsive to therapy (87, 88). This feature also underscores the possibility that efficacious virologic responses against persistent infection are blunted over time due to the presence of proviral ISGs. Along with the IFN-led dysregulation described in the LCMV model, the presence of dysregulation at the ISG level further renders the immune response in a state of flux and incapable of clearing the infection.

IFN RESPONSES IN HIV INFECTION

The progression of the HIV-associated IFN-I response closely mirrors that observed upon HCV infection. This evolution has been elegantly laid out using a simian immunodeficiency virus (SIV) model in rhesus macaques. In this study, Sandler et al. observed that IFN blockade *in vivo* accelerated advancement to AIDS with unchecked SIV replication whereas IFNα administration conferred resistance to the host upon challenge (89). However, in line with the observation of desensitization discussed in HCV, they also observed that sustained IFN administration led to a reversal of host resistance to infection and conversely, resembled the IFN blockade scenario in which the SIV reservoir was enlarged along with CD4$^+$ T-cell depletion and AIDS. Notably, CD4$^+$ T-cell depletion in this setting could be a function of the cellular tropism of the virus rather than solely the direct effect of IFN-mediated effects.

Furthermore, a wealth of research has also underscored the elevated IFN signature observed in the chronic stage of HIV infection, which correlates with high levels of viral load and thus, failed viremia control. Following transcriptome analyses on CD4$^+$ T cells, Rotger et al. found that ISGs were upregulated in untreated patients relative to patients on therapy and healthy controls. In addition, upon induction of antiretroviral therapy and reduction of viremia, the ISG profiles in patient T cells reverted to those observed in the cohorts of HIV-infected individuals who maintain a CD4 T cell count of ≥500 (elite controllers) whose IFN level, and resultantly ISG expression is at a lower baseline (90). These findings were supported by previous findings of ISG upregulation *in vitro* and *in vivo* in CD4$^+$ T cells from chronically infected HIV$^+$ patients relative to healthy controls (91). Furthermore, despite similarity in expression levels in the acute phase of infection, the absence of hyperactivated IFN expression is a distinctive factor between pathogenic and non-pathogenic forms of SIV; while pathogenic SIV_{mac} in rhesus macaques features an elevated IFN signature and resultant disease and the non-pathogenic SIV_{agm} and SIV_{smm} in African green monkeys and Sootey mangabeys, respectively, neither exhibit aberrant IFN upregulation nor immune activation (92–94).

Lastly, the differences between pathogenic and non-pathogenic forms of SIV are partially driven by distinct signaling potentials through PRRs in pDCs (94); strong signaling through TLRs is observed in pathogenic SIV, which results in a surge of IFN that further propagates an immunopathogenic response as outlined in the various scenarios described above.

CLOSING REMARKS AND OUTLOOK

It is important to note that causality between prolonged IFN expression and viral persistence is yet to be fully determined: does prolonged IFN diminish the immune response leading to viral persistence or does persistent infection lead to prolonged

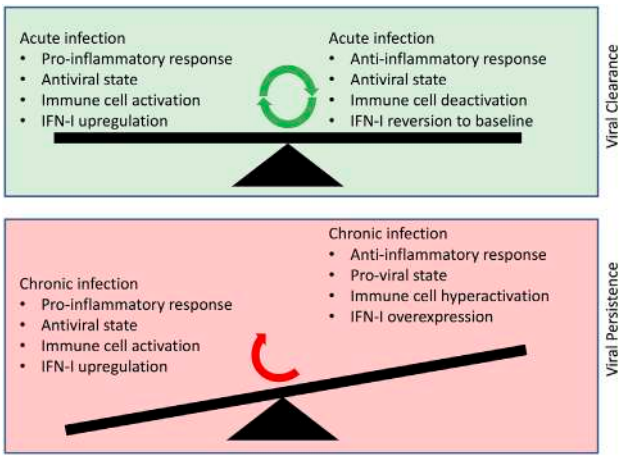

FIGURE 1 | Depiction of changes in Type I interferon (IFN-I) roles in viral clearance vs. viral persistence. (Upper panel) Balance of functional IFN-I signaling, which results in viral clearance within the context of an acute infection. (Lower panel) Dysfunctional IFN-I signaling in the face of a chronic infection resulting in aberrant immune cell activation and viral persistence.

IFN expression whose dysregulation of immune responses is misconstrued as cause rather than effect? Nevertheless, the dizzying network of IFN-activating and IFN-inhibiting responses highlights the complexity in elucidating the exact nature of the IFN-related immunopathology in chronic infection (summarized in **Figure 1**). Intuitively, disruption of the delicate balance using exogenous IFN may result in less efficacious responses and adverse event profiles in therapeutic administration of IFN (95, 96). On the contrary, the multiplicity of pathways and molecules offers avenues that can be useful toward more effective therapeutic approaches by specific targeting of the deleterious moieties. For example, targeting proviral ISGs may offer an incisive approach toward triggering effective IFN responses and through their rescue, obviate exogenous IFN administration. From a prophylactic perspective, induction of nAbs in the absence of IFN signaling in B cells offers insight into the mechanisms that drive the delayed effective humoral response in diseases such as HIV and HCV. Given that the emergence of broadly nAbs against these chronic infections is delayed and in a highly altered immunological milieu, delineating the role of IFN-I facilitates a more comprehensive understanding of the conditions present during elicitation of broadly nAbs. In this regard, it is tempting to speculate that perhaps modulation of the IFN response along with the appropriate immunogen may advance vaccine work in these chronic infections along with other prophylactic measures as well. Altogether, these emergent insights bear significant impact on our understanding of the role of IFN-I in the immune response and importantly, its use in therapeutic settings. Guided by these findings, future work will more clearly determine the delicate balance that tips IFN responses from friend to foe.

AUTHOR CONTRIBUTIONS

AM contributed to the conceptualization of the subject, literature search, and writing the manuscript. AL contributed to the conceptualization of the subject, critical review of compiled literature, and writing the manuscript.

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REVIEW
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Effects of Interferons and Viruses on Metabolism

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Interferons (IFNs) are potent pleiotropic cytokines that broadly alter cellular functions in response to viral and other infections. These alterations include changes in protein synthesis, proliferation, membrane composition, and the nutritional microenvironment. Recent evidence suggests that antiviral responses are supported by an IFN-induced rewiring of the cellular metabolism. In this review, we discuss the roles of type I and type II IFNs in regulating the cellular metabolism and biosynthetic reactions. Furthermore, we give an overview of how viruses themselves affect these metabolic activities to promote their replication. In addition, we focus on the lipid as well as amino acid metabolisms, through which IFNs exert potent antiviral and immunomodulatory activities. Conversely, the expression of IFNs is controlled by the nutrient sensor mammalian target of rapamycin or by direct reprograming of lipid metabolic pathways. These findings establish a mutual relationship between IFN production and metabolic core processes.

Keywords: glycolysis, oxidative phosphorylation, fatty acid synthesis, fatty acid oxidation, cholesterol synthesis, mTOR, immunometabolism

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INTRODUCTION

Type I and II interferons (IFNs) are important cytokines that are induced upon viral infections (1). They promote a so-called "antiviral state" that limits viral replication in infected cells and viral spreading in non-infected cells. Additionally, IFNs are expressed during bacterial infections or autoimmune diseases and exert potent immunomodulatory functions. The human type I IFN family

Abbreviations: 2-DG, 2-deoxyglucose; 25-HC, 25-hydroxylcholesterol; ACC, acetyl coenzyme A carboxylase; Acetyl-CoA, acetyl coenzyme A; ACLY, ATP citrate lyase; ADP, adenosine diphosphate; ADV, adenovirus; αKG, α-ketoglutarate; AMPK, 5-adenosine monophosphate-activated protein kinase; ATP, adenosine triphosphate; cGAS, cyclic guanosine monophosphate-adenosine monophosphate synthase; ER, endoplasmic reticulum; F6P, fructose-6-phosphate; FA, fatty acid; $FADH_2$, flavin adenine dinucleotide; FAO, fatty acid oxidation; FAS, fatty acid synthesis; G6P, glucose-6-phosphate; Glut4, glucose transporter 4; HBV, hepatitis B virus; HCMV, human cytomegalovirus; HCV, hepatitis C virus; Hif1α, hypoxia-inducible factor 1α; HIV, human immunodeficiency virus; HSV1, herpes simplex virus; H_2O_2, hydrogen peroxide; IDO1, indoleamine-2,3-dioxygenase 1; IFN, interferon; IFNAR1/2, interferon alpha and beta receptor subunit 1/2; IFNR, interferon receptor; IL-1, interleukin-1; IRG1, immune-responsive gene 1; NOS2, nitric oxide synthase 2; IRF, interferon-regulatory factor; IRG, immune-responsive gene; ISG, interferon stimulated gene; Kyn, kynurenine; LCMV, lymphocytic choriomeningitis virus; LPS, lipopolysaccharide; MDA5, melanoma differentiation antigen 5; MHV-68, murine gammaherpesvirus-68; MNK, mitogen-activated protein kinase (MAPK)-interacting kinase; mTOR, mammalian target of rapamycin; mTORC1/2, mTOR complex 1/2; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; OAA, oxaloacetate; OAS, 2′,5′-oligoadenylate synthetases; $ONOO^-$, peroxynitrite; OXPHOS, oxidative phosphorylation or electrone transport chain; PAs, polyamines; pDC, plasmacytoid dendritic cell; PKR, double-stranded RNA-activated protein kinase; PRR, pattern recognition receptor; ROS, reactive oxygen species; SAT1, spermidine-spermine acetyltransferase; SFV, semliki forest virus; SREBP 1/2, sterol regulatory binding protein 1/2; STAT, signal transducer and activator of transcription; STING, stimulator of interferon gene; SOD1, superoxide dismutase 1; TCA, tricarboxylic acid; TLR, Toll-like receptors; Treg, regulatory T cell; Trp, tryptophan; TYK2, tyrosine kinase 2; UDP-GlcNAc, uridine diphosphate-*N*-acetylglucosamine; VSV, vesicular stomatitis virus; VV, vaccinia virus; WNV, West Nile virus.

consists of 13 IFNα subtypes (14 in mice), one single IFNβ gene, and some further poorly analyzed genes (2). The sole representative of class II IFN is IFNγ, which is mainly produced by T cells and NK cells (2). IFNγ generally activates innate responses by augmenting inflammatory cytokine and chemokine production, microbial killing, and antigen presentation of macrophages and dendritic cells (3). Upon stimulation of extra- and intracellular pattern recognition receptors (PRR), including Toll-like receptors (TLR), nucleotide-binding oligomerization domain-like receptors, and retinoic acid-inducible gene I-like receptors, many immune cells, but also non-hematopoietic cells, are capable of inducing type I IFNs by a concerted activation of transcription factors called IFN-regulatory factors (IRFs) (4). Expression of IFNs is also dependent on the sensing of the extra- and intracellular microenvironment by the mammalian target of rapamycin (mTOR) network (5). mTOR complex 1 (mTORC1) integrates the main classes of nutrients and energy sources [amino acids, glucose, lipids, and adenosine triphosphate (ATP)] to couple the environmental status with cellular activation and translation (5). Activation of mTORC1 is required to induce the translation as well as the activation of IRFs, including IRF5 and IRF7, to maximize type I IFN production (6–9). IFNα and IFNβ bind a heterodimeric membrane receptor consisting of the interferon alpha and beta receptor subunit 1 (IFNAR1) and IFNAR2 (10). Receptor engagement activates the receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which phosphorylate and activate the transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT2 (10). In contrast, the dimeric IFNγ receptor consists of the interferon gamma receptor 1 (IFNGR1) and IFNGR2 and activates the receptor-associated tyrosine kinases, JAK1 and JAK2, which solely activate STAT1 (11).

Type I IFNs and IFNγ induce the transcriptional upregulation of several hundred interferon stimulated genes (ISGs) (1, 4). Three families of ISGs have been extensively studied with respect to their antiviral activities. These genes encode the double-stranded RNA-activated protein kinase (PKR), the 2′,5′-oligoadenylate synthetases (OAS), and the Mx protein(s) (1, 12). They actively participate in inhibiting viral replication by different mechanisms. PKR is an IFN-inducible and RNA-dependent kinase that phosphorylates the translation initiation factor 2α (eIF2α), which inhibits cellular and viral translation (13). Activation of OAS by binding of dsRNA stimulates RNase L activity, which cleaves cellular and viral ssRNA to inhibit protein expression (13). Mx proteins are GTPases that often associate with nucleocapsid-like viral structures to trap and inhibit viral replication (14).

This review focuses on additional roles of IFNs involving the regulation of the cellular metabolism. The following sections discuss recent evidence and older observations of how type I and II IFNs modulate metabolic pathways to generate an antiviral state and influence subsequent immune responses.

CELLULAR METABOLISM

The principal purpose of metabolism is the conversion of nutrients to energy to maintain all cellular processes and the delivery of building blocks for the biosynthesis of proteins, lipids, nucleic acids, and some carbohydrates. Viruses are incapable of metabolizing on their own and are, therefore, completely dependent upon host metabolism. Their life cycle requires an energy-demanding synthesis of high levels of proteins, glycoproteins, nucleic acids, and sometimes lipids. Therefore, there is a mutual relationship between viral replication, metabolism, and host defense. First, we will discuss the basic principles of metabolism. Afterward, we will continue to elaborate on specific pathways of metabolism that are affected by IFNs or viral infection.

Energy Metabolism

The central nutrients, used by eukaryotic cells to generate energy in the form of ATP, are carbohydrates, amino acids, and fatty acids (FAs) (15). In the presence of oxygen, non-proliferating cells take up the carbohydrate glucose and metabolize it in the cytoplasm to pyruvate through a process called glycolysis (**Figure 1**). This results in a net production of two ATPs and the reduction of two nicotinamide adenine dinucleotide (NAD) molecules to NADH. Pyruvate can be transported into the mitochondria, where it is oxidized into acetyl coenzyme A (acetyl-CoA) with the production of one molecule of carbon dioxide and one more NADH. Acetyl-CoA acts as fuel for the tricarboxylic acid (TCA) cycle (also known as citric acid or Krebs cycle), through which it is completely oxidized to carbon dioxide with the net production of three molecules of NADH, one molecule of ATP (or guanosine triphosphate GTP), and one molecule of the reduced form of flavin adenine dinucleotide ($FADH_2$) (15). The molecules of NADH and $FADH_2$, generated until this point, are the inputs for the electron transport chain. They are used to establish a proton gradient at the inner mitochondrial membrane, which finally generates ATP from adenosine diphosphate in a process called oxidative phosphorylation (16) (**Figure 1**). In summary, from one molecule of glucose, theoretically, 36 equivalents of ATP can be generated in eukaryotes, although due to proton leakage and inefficiencies of the ATPase, the observed yield is about 30 ATPs (17). Importantly, glucose is not the only energy source, which can be used by eukaryotic cells. The amino acid glutamine is a second carbon source that can be converted to α-ketoglutarate (αKG) as oxidative substrate to fuel the TCA cycle (16, 18). Moreover, fatty acid oxidation (FAO) in the mitochondria generates acetyl-CoA, NADH, and $FADH_2$, which are further used to generate ATP (19). FAs are the most energetic nutrients, yielding the highest levels of ATP on an energy per gram basis. Hence, glycolysis and the TCA cycle are the central cellular respiratory systems of eukaryotic cells (15).

Anabolic Metabolism

When cells start to proliferate, there is increasing demand of nutrients for energy production as well as biosynthesis of novel molecules (20). Therefore, proliferating cells increase glucose uptake and glycolysis, but do not oxidize all of the additional glucose-derived pyruvate in the TCA cycle. Instead, the pyruvate is reduced to lactate despite the presence of oxygen, which is therefore called aerobic glycolysis (21) (**Figure 1**). This effect was first described in tumor cells by Otto Warburg and is now called

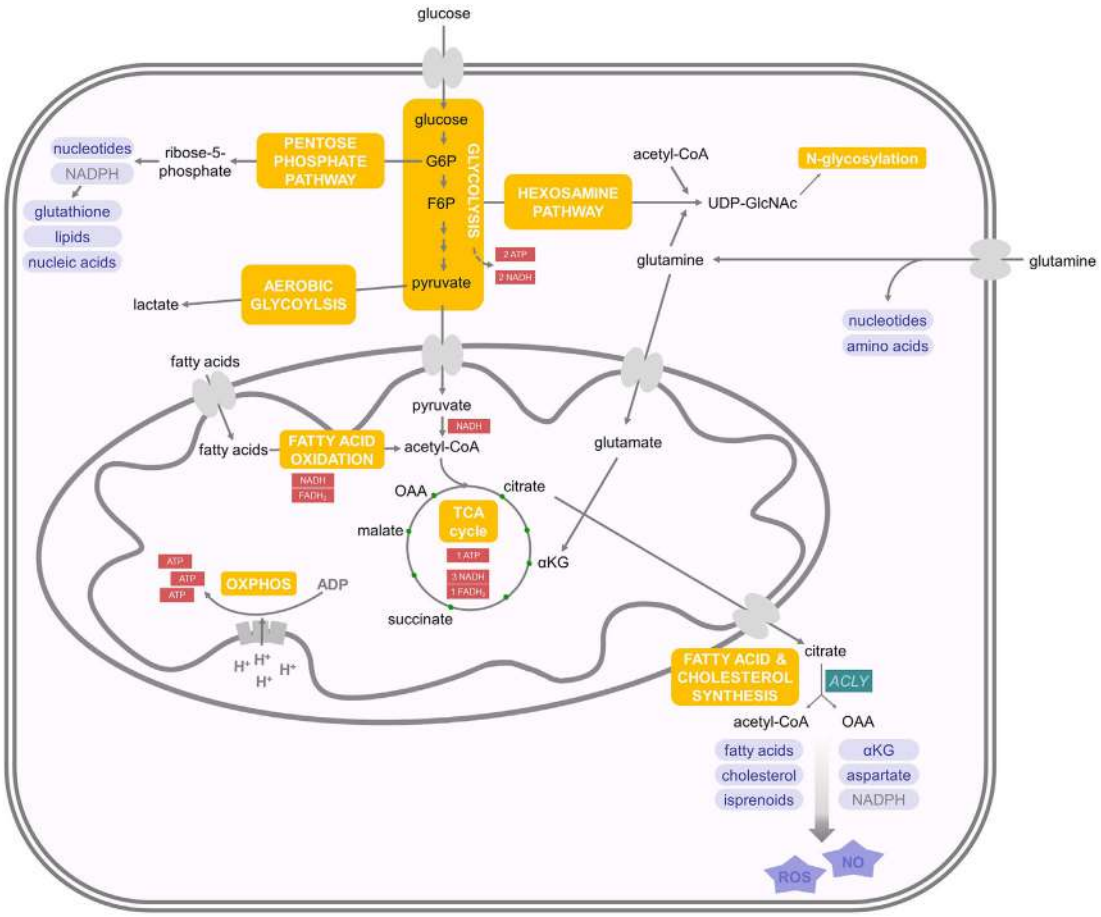

FIGURE 1 | **Energy and biosynthetic metabolism**. Glucose is taken up and metabolized in the cytoplasm to pyruvate in a process called glycolysis. Pyruvate is then transported into the mitochondria and oxidized into acetyl coenzyme A (acetyl-CoA), which enters the tricarboxylic acid (TCA) cycle. The molecules NADH and $FADH_2$ produced until this point are the inputs for the electron transport chain. Another important energy source are fatty acids, whose oxidation delivers acetyl-coA. Aerobic glycolysis takes place in proliferating (and cancer) cells and describes the phenomenon of increased glucose uptake and glycolysis with the subsequent production of lactate. Glutamine is another carbon source that can be transformed to αKG and, therefore, enters the TCA cycle. Glutamine can also be used as nitrogen donor in the hexosamine pathway, which requires F6P and is important for N-glycosylation of proteins. G6P can feed into the pentose phosphate pathway, which is important for the production of nucleotides and NADPH. Mitochondrial citrate can enter the cytoplasm and feeds into *de novo* fatty acid synthesis. For further details consult the text.

the Warburg effect (22). It is important to note that TCA flux is reduced but maintained during aerobic glycolysis in proliferating cells. Although aerobic glycolysis generates only two molecules of ATP, it is thought to generate cellular building blocks for rapidly proliferating cells (15). However, also amino acids are important contributors to increased cell mass in proliferating cells (23). Many glycolytic intermediates provide backbone carbons for multiple non-essential amino acids or function as substrates for the biosynthesis of phospholipids and triacylglycerols. In addition, the glycolytic intermediate glucose-6-phosphate (G6P) can feed into the pentose phosphate pathway to generate ribose-5-phosphate, which is important for nucleotide biosynthesis as well as the conversion of nicotinamide adenine dinucleotide phosphate to its reduced form NADPH (**Figure 1**). NADPH is used as reducing agent in lipid or nucleic acid synthesis and protects against cellular oxidative stress by generating reduced glutathione that inactivates reactive oxygen species (ROS) (e.g., H_2O_2) and free radicals (16). αKG, derived from glutamine, can be metabolized to malate and then to pyruvate to support NADPH generation in a process called glutaminolysis (16). Furthermore, glutamine is used as nitrogen donor for the biosynthesis of nucleotides, non-essential amino acids, and hexosamines. The hexosamine pathway requires fructose-6-phosphate (F6P) from glycolysis and acetyl-CoA, in addition to glutamine to produce uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc), which is important for N-glycosylation of proteins (24). Therefore, N-glycosylation represents a nutrient-sensitive protein modification, which regulates the glycosylation of IFNs and viral glycoproteins (25). This modification is involved in protein trafficking, in viral entry, and in evading the immune system's detection by some viruses (26).

As described above, during aerobic glycolysis, the TCA cycle is sustained in proliferating cells by glucose-derived pyruvate, as well as by replenishing depleted intermediates in the form of, e.g., glutamine in a process called anaplerosis (27, 28). The TCA cycle

contributes many intermediates that act as biosynthetic substrates. For example, mitochondrial citrate can feed into *de novo* FA and cholesterol synthesis upon its export to the cytoplasm, where it is converted to acetyl-CoA and oxaloacetate by ATP citrate lyase (ACLY). Cytoplasmic acetyl-CoA is then the substrate for FAs, cholesterol, and isoprenoid synthesis (**Figure 1**). Phospholipids are generated from FAs and, together with cholesterol, form the majority of the lipid bilayers of the cellular membranes. Oxaloacetate is further metabolized to yield αKG and NADPH (29). Alternatively, oxaloacetate can be transaminated to aspartate, which acts as a carbon source in nucleotide biosynthesis. In addition, ACLY-derived acetyl-CoA and oxaloacetate can serve as precursors for nitric oxide (NO) and ROS production (30, 31).

EFFECTS OF IFNs ON ENERGY METABOLISM

While it has been known for a long time that viral infections and IFNs interfere with lipid metabolism including FA and cholesterol synthesis (described below), recent studies have shown a more general influence of IFNs on the energy metabolism of cells.

Generally, a theme emerges that type I IFNs promote glycolysis (**Figure 2**). For example, IFNβ stimulates a PI3K/AKT-dependent glucose uptake in mouse embryonic fibroblasts that may enhance ATP production (32). Inhibition of IFNβ-induced glycolysis with 2-deoxyglucose (2-DG), a competitive inhibitor of hexokinase, the first enzyme in the glycolysis cascade, enhances replication of coxsackievirus B3 *in vitro* (32). This suggests that enhanced glycolysis may support the establishment of an antiviral state. Similarly, injection of the synthetic dsRNA poly(I:C), a TLR3 and melanoma differentiation antigen 5 agonist, into mice induces an increase in glycolysis in splenic $CD11c^+$ $MHCII^+$ DCs *ex vivo* (33). This increase is dependent on IFNAR1 and thus mediated by type I IFNs. Increased glycolysis often is accompanied by a decreased oxidative consumption, and this Warburg effect depends on expression of hypoxia-inducible factor 1α (Hif1α) and is required to efficiently prime $CD8^+$ and $CD4^+$ T cells *in vivo* (33). In macrophages, TYK2 and IFNAR1 are also required for an increase in glycolysis-mediated lactate production (34). IRF5 increases glycolysis in macrophages through a glycolytic gene expression induced by activation of AKT2 (35). In a human squamous carcinoma cell line, expression of type I IFN-regulated STAT1 promotes aerobic glycolysis and decreases oxidative phosphorylation,

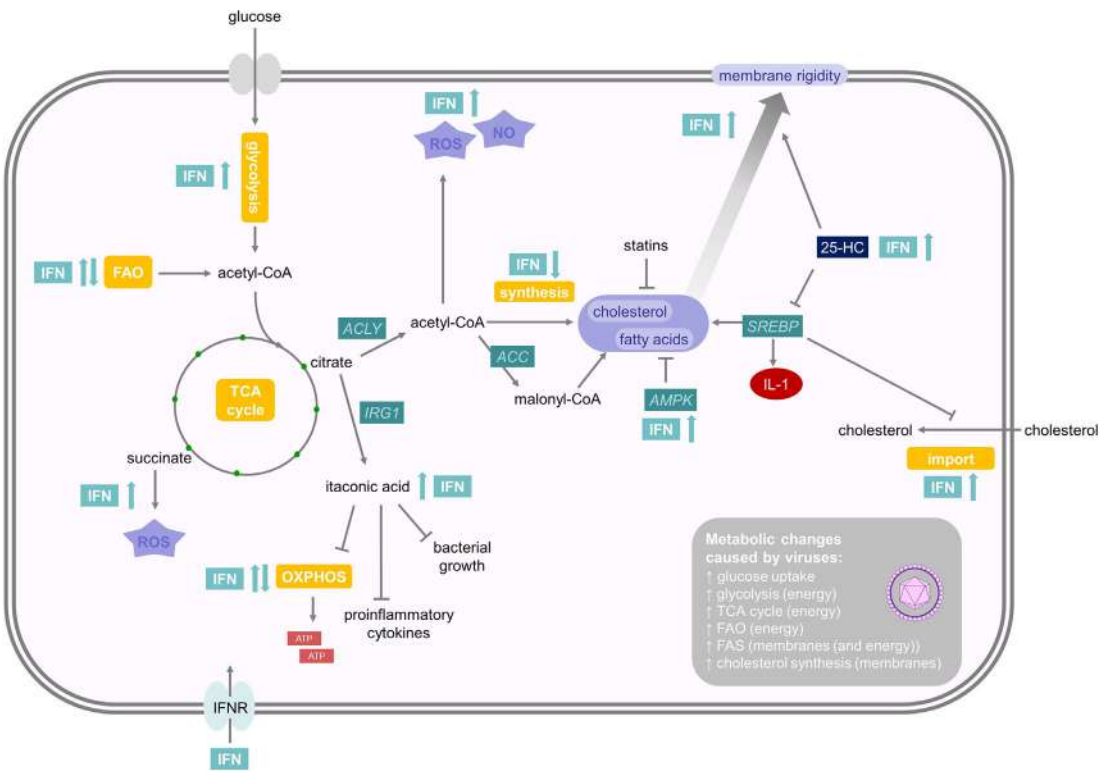

FIGURE 2 | Effects of interferons (IFNs) on energy and lipid metabolism. Type I IFNs promote glycolysis while mitochondrial respiration is regulated cell-type specifically. Citrate can induce the formation of itaconic acid or acetyl coenzyme A (acetyl-CoA). Itaconic acid is a bactericidal metabolite, which inhibits proinflammatory cytokine expression and mitochondrial respiration. Acetyl-CoA can either promote NO and reactive oxygen species (ROS) production or initiate fatty acid (FA) and cholesterol synthesis. As viral replication is an energy-demanding process, which depends on protein and nucleotide synthesis, most viral infections enhance FA and cholesterol synthesis, which, on the other hand, can be reduced by adenosine monophosphate-activated protein kinase and statins. 25-HC is a soluble antiviral factor that broadly inhibits growth of many enveloped viruses by inhibiting sterol regulatory binding protein and enhances membrane rigidity. IFNs also promote subsequent NO and ROS production. For further details consult the text.

which contributes to tumor growth in a xenograft model (36). Other studies also showed that mitochondrial respiration and ATP production are diminished upon type I IFN treatment in mouse L929 or human Daudi cells (37). In humans, IFNβ-treated multiple sclerosis patients exhibited a dose-dependent reduction of ATP levels in isolated $CD4^+$ T cells (38).

However, there are also important examples, where type I IFN stimulates oxygen consumption. Plasmacytoid dendritic cells (pDCs) are specialized immune cells devoted to the production of large amounts of type I IFNs after viral recognition (39). Mouse pDCs upregulate oxidative phosphorylation and ATP production 24 h after stimulation with poly(I:C) or directly after type I IFN treatment through an autocrine loop (40). This boost in energy production is required for full immune effector functions *in vitro* and for fighting lymphocytic choriomeningitis virus (LCMV) infection *in vivo*. This increase in oxidative phosphorylation and mitochondrial respiration is fueled by FAO (40). Interestingly, the FAs required for FAO seem to be a result of *de novo* fatty acid synthesis (FAS) from glycolysis-derived pyruvate. The stimulating effect of type I IFN on increased oxygen consumption was also observed on conventional DCs, keratinocytes, or memory T cells, but not on effector T cells (40). This increase in ATP and mitochondrial fitness may support the energetic demands of high cytokine production in pDCs and in non-hematopoietic cells to support survival during viral infection. In contrast, stimulation of human pDCs with influenza virus induced a Warburg-like remodeling of the energy metabolism, including enhanced glycolytic flux and decreased mitochondrial respiration (41). These studies in total suggest that type I IFN, by canonical pathway activation through IFNAR1, Tyk2, and STAT1, mediates an induction of glycolysis, whereas mitochondrial respiration seems to be regulated cell-type specifically (**Figure 2**). Interestingly, type I IFN and IFNγ induce lipolysis in cultured adipocytes and in mice *in vivo* and may thus supply cells with FAs (42). However, this function of IFN has not been thoroughly investigated.

A decrease in oxidative phosphorylation reduces mitochondrial ATP production, which may still be compensated by ATP produced through aerobic glycolysis, whose flux can be dramatically increased when glucose is not limited (43). Reduced mitochondrial respiration frees TCA intermediates, which can be used in subsequent biosynthetic reactions. For example, activation of macrophages with IFNγ and lipopolysaccharide (LPS) induces high levels of glycolysis and a break of TCA flux. This leads to the accumulation of succinate and citrate in conjunction with induction of FAS (44). Succinate can drive mitochondrial ROS production (45), which is a conserved response against many pathogens (46) but may also cause tissue pathology (as discussed below) (47). Naujoks et al. showed that type I and II IFNs control *Legionella pneumophila* infection in alveolar macrophages by induction of a bactericidal molecule (48). In fact, *Legionella*-infected macrophages induce IFN-dependent expression of immune-responsive gene (IRG) 1 that mediates production of itaconic acid (also known as methylenesuccinic acid). This molecule is bactericidal against a number of extracellular multidrug-resistant, Gram-positive, and Gram-negative bacteria (48). Itaconic acid is produced by IRG1 through decarboxylation of cis-aconitate, a TCA intermediate that is formed from citrate (49, 50) (**Figure 2**). Except of being a bactericidal metabolite, itaconic acid also inhibits proinflammatory cytokine expression (51) and mitochondrial respiration (51, 52). Stimulation of macrophages with poly(I:C), IFNγ, or LPS can also increase the expression and activation of ACLY (31, 34). This, in turn, enhances the conversion of citrate into acetyl-CoA and oxaloacetate, which promotes subsequent NO and ROS production.

MODULATION OF LIPID SYNTHESIS BY VIRUSES AND IFNs

Viral replication depends on a massive induction of protein and nucleotide synthesis. Therefore, most viruses themselves upregulate carbon fluxes and promote efflux to nucleotide and amino acid biosynthesis (53). Additionally, virus entry, replication, and assembly rely on membranous networks, surrounding and residing within the host cells. These include the plasma, the endolysosomal, and the endoplasmic reticulum (ER) membranes (54–56), which all function as scaffolds to recruit and concentrate viral and host components, necessary for viral replication and assembly (57). Many viruses induce changes in membrane fluidity and a massive proliferation of membranes such as the ER, which is the place for translation of secretory and membrane proteins and for N-glycosylation (54, 55). Obviously, enveloped viruses need not only to induce membrane generation but also alter the composition of the cell membrane to meet their needs for effective infectious progeny particles (58). However, viral replication is also a highly energy-demanding process; therefore, utilizing all available energy to produce ATP is rate-limiting for some viruses (58).

FA Synthesis and IFNs

In light of these functional prerequisites, it comes as no surprise that most viral infections enhance FA and cholesterol synthesis to support generation of membranes and ATP production (53) (**Figure 2**). For example, human cytomegalovirus (HCMV) upregulates most metabolic pathways in infected fibroblasts and drives flux from glycolysis through the TCA cycle to FAS (59). Inhibition of FAS suppresses replication of HCMV (59). Mechanisms of HCMV-induced metabolic reprograming include the activation of the glucose transporter Glut4 and inductions of ACLY and acetyl coenzyme A carboxylase (ACC) (53, 60). After ACLY-dependent generation of acetyl-CoA in the cytoplasm, ACC carboxylates acetyl-CoA to malonyl-CoA, which is a critical rate-limiting step in FAS (19). ACLY and ACC are currently evaluated as therapeutic targets for cancer, obesity, diabetes, and viral infections (61, 62). Similarly, influenza A, flaviviridae family members including hepatitis C virus (HCV) and West Nile virus (WNV), enteroviruses including poliovirus and coxsackievirus B3, rotavirus, rift valley fever virus, and respiratory syncytial virus depend on FAS for viral replication, making its modulation an attractive therapeutic target (63–65). 5′-adenosine monophosphate-activated protein kinase gets activated after certain virus infections, such as rift valley fever virus or coxsackievirus B3, and potently inhibits FAS (66, 67). Some viruses, such as influenza A, use FAS to induce the production of

prostaglandin E2, which inhibits IFN expression and promotes apoptosis in macrophages (68). Viral replication is a highly energy-demanding process. Therefore, utilizing all available energy is critical and rate-limiting for some viral infections. Hence, some viruses depend on FAS and their degradation by FAO to produce ATP. In this respect, Vaccinia virion assembly is dependent on ATP synthesis fueled by FAS and FAO (69). Dengue virus, on the other hand, induces FAO by an autophagy-dependent processing of lipid droplets and triglycerides to generate ATP for efficient replication (70). Nevertheless, dengue virus also induces FAS to support virus replication (71). Hence, channeling the FAs from biosynthesis to catabolism by the induction of FAO, as seen in pDCs (40), could represent a novel powerful antiviral mechanism of IFN. However, further work is required to elucidate whether this represents a general antiviral mechanism.

Cholesterol Homeostasis and IFN Responses

Many viruses do not only modulate FAs, but also cholesterol homeostasis to enhance their replication efficiency. For example, WNV upregulates biosynthesis of cholesterol, redistributing it to viral membranes in the phase of replication (72). Moreover, HCV, hepatitis B virus (HBV), measles, human immunodeficiency virus (HIV), and dengue virus also change cholesterol pathway gene expression in a variety of cellular systems (73–76). Pharmacological disruption of cholesterol synthesis, e.g., by statins, often results in the inhibition of viral replication (77–82). Recent evidence has shown that an important antiviral mechanism of type I IFN seems to be inhibition of cholesterol and fatty acid biosynthesis derived from glucose (83, 84). Type I IFN reduces cholesterol synthesis upon CMV, herpes simplex (HSV1), semliki forest virus, vaccinia virus (VV), and adenovirus (ADV) infection in bone marrow-derived macrophages, which is dependent on IFNAR1 and TYK2 (83). Similarly, infection with murine gammaherpesvirus-68 (MHV-68) reduces cholesterol and long chain FAS in macrophages (84). It is important to note that total cholesterol levels are not strongly affected upon inhibition of cholesterol synthesis by IFN due to an enhancement of cholesterol import (84) (**Figure 2**). Similarly, WNV infection enhances cholesterol synthesis, but total cholesterol levels do not change (72). Inhibition of cholesterol biosynthesis has a direct antiviral effect. The sterol regulatory binding protein 2 (SREBP2), together with SREBP1, are the main transcription factors involved in coordinating the regulation of the sterol biosynthesis pathway (85). IFNs potently inhibit the transcription and expression of SREBP2 *via* IFNAR1 (83). On the other hand, WNV-induced redistribution of cellular cholesterol downregulates the IFN-stimulated JAK–STAT antiviral signaling response to infection potentially by influencing lipid raft signaling (72).

Fascinatingly, limiting cholesterol synthesis alone induces spontaneous type I IFN production and enhances antiviral immunity (84). Deletion of SREBP2 or the ER chaperone SCAP, which regulates SREBP2, reduces synthesis but enhances the uptake of cholesterol. This shift induces spontaneous IFN signaling that is strongly enhanced upon viral infection in bone marrow-derived macrophages or mouse embryonic fibroblasts. The IFN response in these cells is dependent on the cGAS–STING–TBK1–IRF3 pathway (84). The stimulator of interferon gene (STING) protein is anchored on the ER and Golgi apparatus, suggesting that cholesterol levels and potentially lipid rafts in these membranes may modulate STING signaling. Moreover, it is interesting to note that cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS), which senses DNA from viral infections, is evolutionarily conserved with OAS, another important antiviral protein that is induced by IFNs (86). In summary, many viruses increase cholesterol and FAS. This is counteracted by type I IFN signaling, which limits FA and cholesterol synthesis. Reducing cholesterol synthesis alone induces IFN production establishing an inflammatory circuit, which links the regulation of the sterol pathway with the antiviral IFN defense responses. However, one report noted enhanced cholesterol synthesis derived from acetate in IFNβ-treated HeLa cells (87).

Regulation of Membrane Function by 25-Hydroxycholesterol

Cholesterol-derived metabolites such as oxysterols are important systemic mediators that regulate many immunological functions (88). IFNs or viral infections lead to the induction and secretion of one oxysterol in macrophages: 25-hydroxycholesterol (25-HC) (89) (**Figure 2**). 25-HC is a soluble antiviral factor, generated from cholesterol by IFN-dependent activation of cholesterol-25-hydroxylase *via* STAT1 (89). 25-HC broadly inhibits growth of many enveloped viruses, such as vesicular stomatitis virus, HSV, HIV, MHV68, and Ebola virus, by suppressing membrane fusion between the virus and the host cell (90, 91). Mechanistically, 25-HC seems to incorporate into the membrane and/or modify the membrane composition (91). Indeed, an IFN-dependent increase in plasma membrane rigidity has long been observed in several previous studies (92–94). IFNβ augments membrane rigidity already after 30 min, and this is maintained for 2 days making it a powerful antiviral mechanism to prevent viral infection and spreading (93, 95). Nevertheless, type I IFNs decrease membrane contents of saturated FAs and increase unsaturated FAs (92). In patients with chronic hepatitis C infection, treatment with IFNα2 reduces the deformability and membrane fluidity of red blood cells, which may result in hemolytic anemia, a frequent side effect of IFN therapy (96). Another downside of IFN-induced 25-HC expression is its capacity to inhibit SREBP1, which not only drives FAS but also stimulates transcription of interleukin-1, a secreted inflammatory protein with wide-ranging antibacterial functions (97). This may explain why IFNs, produced during viral infections, enhance the subsequent susceptibility to bacterial or fungal infections (98–100). Generally, there is increasing awareness of a close relationship between membrane lipid dynamics and innate immune responses (101).

AMINO ACID METABOLISM AND IFNs

IFNs Deplete Polyamines to Restrict Virus Replication

Polyamines are a family of small polycationic molecules, derived from decarboxylation of the amino acid ornithine, that classically comprise three molecules: putrescine, spermidine, and spermine

(102). Spermine is generated from spermidine, which itself is produced from putrescine (**Figure 3**). Ornithine, which generates putrescine, is produced from L-arginine by arginase (103). Polyamines bind DNA, RNA, and proteins and are implicated in supporting transcription, translation, and deacetylation to influence a plethora of different cellular functions, including proliferation, apoptosis, autophagy, and gene regulation (103). Spermidine–spermine acetyltransferase (SAT1 or SSAT) acetylates spermidine and spermine, which promotes either their conversion back to putrescine or their export from the cell (102, 103). Interestingly, type I IFNs induce the expression of SAT1 and, therefore, deplete spermidine and spermine levels (104). The depletion of these two polyamines has a strong antiviral effect and inhibits replication of the RNA viruses, Zika virus and Chikungunya virus (104). Mechanistically, polyamines seem to be important for transcription and translation of viral RNA and proteins. Limiting polyamine synthesis, therefore, emerges as a novel antiviral strategy and SAT1 constitutes an important ISG.

IFNs Stimulate Arginine-Dependent NO Production

NO is a gaseous and inorganic free radical best known for its vasodilatory and microbicidal effects (105). However, NO is also an important mediator in intracellular inhibition of viral replication, which results in lower viral yields and more efficient host clearance of the infection (106). NO is produced by the enzymatic modification of L-arginine to L-citrulline by NO synthases (NOS) (**Figure 3**). NOS type 2 (NOS2, iNOS) is an IFNγ-inducible protein in macrophages and requires IRF1 as a transcription factor, which itself is regulated by STAT1 (106, 107). Molecularly, the antiviral activities of NO are poorly described, but one demonstrated mechanism is nitrosylation of viral molecules (108). For example, NO S-nitrosylates the cysteine residue in the active site of Coxsackievirus protease 3C, thus inhibiting protease activity and interrupting the viral life cycle (109). In addition, the generation of NO by NOS2 depletes the common substrate L-arginine and, subsequently reduces polyamine levels, as described above. Moreover, this relieves a feedback inhibition mechanism, because polyamines can directly inhibit NOS2 (110). Hence, IFN-induced NOS2 and SAT1 induction have antiviral effects due to a coordinated shift from polyamine synthesis to NO production.

Type I IFNs Promote Oxidative Stress and Tissue Damage

Viral or bacterial infections often cause immunopathology and tissue damage, not only because of the pathogens destroying the tissue but because of an overactivation of the immune system, which promotes tissue destruction. For example, excessive type I and II IFN production can drive tissue damage by proinflammatory actions on innate and adaptive immune cells, as well as the induction of apoptosis (111–113). IFNγ can cause the production of ROS, which induces apoptosis (114). NO contributes to tissue damage, especially if substantial numbers of IFN-activated macrophages produce large micromolar quantities of NO (115).

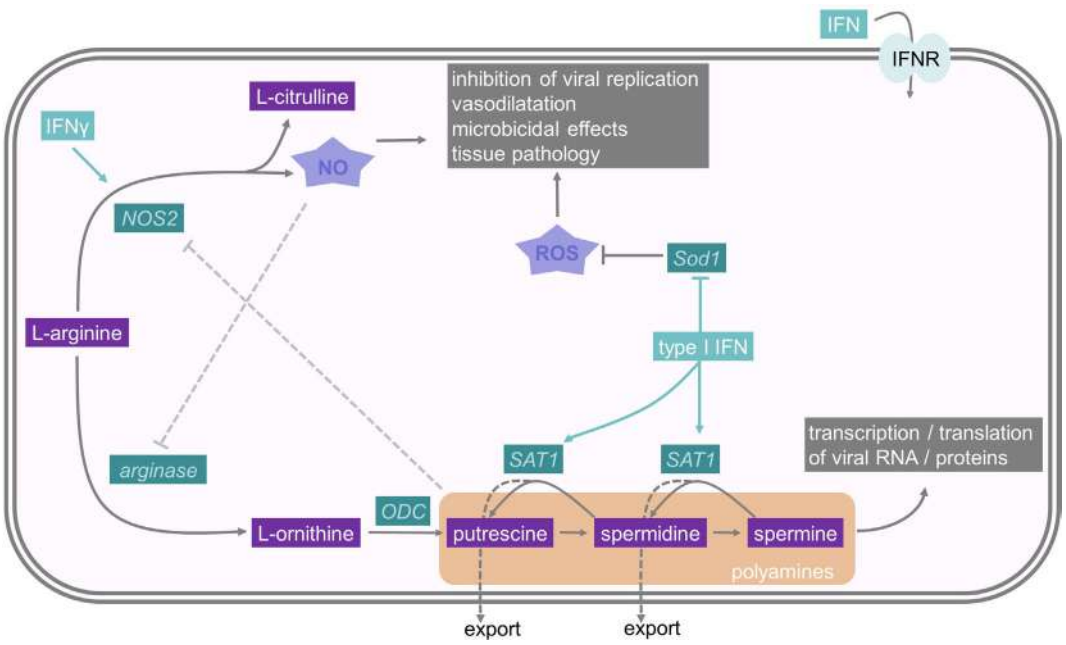

FIGURE 3 | Interferons and their influence on nitric oxide and polyamine metabolism. The polyamines putrescine, spermidine, and spermine derive from the amino acid L-arginine. One rate-limiting enzyme in polyamine synthesis is ODC, while Spermidine–spermine acetyltransferase (SAT1) is an important enzyme in polyamine catabolism. As polyamines are important for viral replication, SAT1 constitutes an important interferon stimulated gene. NO also derives from L-arginine, and therefore, depletes the substrate for PA synthesis. It has microbicidal effects and reduces viral replication. Sod1 is an antioxidative molecule which resolves oxidative stress. For further details consult the text.

First, NO can have proinflammatory effects on other cells of the immune system causing hyperactivation and immunopathology (105). Moreover, NO can rapidly react with hydrogen peroxide (H_2O_2) to form peroxynitrite ($ONOO^-$), which nitrates proteins and is highly toxic, leading to the accumulation of injurious intracellular oxidants and to DNA damage (116). This NO-induced oxidative stress causes cytotoxicity, which promotes cellular and organ dysfunction (115). Currently, there is no clear-cut way of predicting whether NO has a more important role in viral clearance or in tissue pathology for a particular viral pathogen.

There are additional metabolic mechanisms explaining how IFN signaling can promote immunopathology. Infection of mice with LCMV causes a dysregulation of the redox system in the liver. In this infection model, the early production of type I IFN causes tissue pathology due to the downregulation of superoxide dismutase 1 (Sod1) in the liver (117). Sod1 is a ubiquitously expressed antioxidative molecule, which can protect cells from oxidative stress by scavenging O_2^- radicals (118) (**Figure 3**). Hence, type I IFN-mediated oxidative stress may be a key mediator of virus-induced liver damage, and this suggests that early antioxidant treatment may be therapeutically helpful in ameliorating tissue damage. On the other hand, this oxidative stress, induced by the downregulation of Sod1, may also be part of an immediate antioxidant host defense system against pathogens (117).

Depletion of Tryptophan as an Immunomodulatory Mechanism of IFN

Indoleamine-2,3-dioxygenase 1 (IDO1) is an intracellular, non-secreted enzyme, which catalyzes the production of kynurenine (Kyn) derivatives from the essential amino acid tryptophan (Trp) (119). The IDO1 promoter region contains two IFN-stimulated response elements and three IFNγ-activated sites. Hence, IFNγ is the most potent inducer of IDO1 expression in many cells, including macrophages, fibroblasts, and pDCs (120, 121) (**Figure 4**). Although type I IFNs are able to directly induce IDO1, the maximum IDO1 expression requires co-stimulation with TNF-α or LPS (122, 123). Strong activation of IDO1 by IFNγ decreases serum levels of Trp. Since many microbial organisms rely on Trp, its degradation by IDO1-expressing cells of the innate immune system seems to be a major immune mechanism against bacterial infections (124). In fact, IFNγ-induced IDO1 expression seems to be protective in *Toxoplasma gondii*, *Salmonella enterica* serovar Typhi (*S.* Typhi), or *Chlamydia pneumoniae* infections (124, 125). For example, IFNγ-primed macrophages effectively contain intracellular replication of *S.* Typhi depending on the activation of IDO1 (125).

IDO1 expression also plays an important role during viral infections, such as HIV, influenza, Epstein–Barr, HBV, and HCV (124, 126). However, in viral infections, the induction of IDO1 by IFN seems to be generally harmful by the establishment of an immunotolerogenic microenvironment (124). Trp is important for activation and proliferation of T cells. Hence, Trp depletion inhibits T cell immunity and, moreover, the oxidation of Trp by IDO1 generates Kyn derivatives, which promote T cell tolerance by induction of regulatory T cells (121) (**Figure 4**). Therefore, mice lacking IDO1 exhibit significantly lower morbidity after sublethal influenza A infection by generating a stronger influenza-specific effector CD8 T cell response, though viral clearance rates are unaffected by IDO1 ablation (127). Similarly, genetic ablation of IDO1 or chemical inhibition with 1-methyl-D-L-tryptophan suppresses viral replication of murine leukemia virus *in vivo* and upregulates type I IFN production (128). In conclusion, in various chronic infections, autoimmune diseases, and cancer, an increased expression of IDO1, besides its antiviral effects, may promote an immunosuppressive environment, which potentially contributes to disease (119, 121, 123, 125).

Tryptophan Depletion Suppresses mTOR-Mediated Translation and Modulates IFNγ-Dependent Metabolic Processes

The importance of translational control of many cellular responses, including metabolism, is increasingly appreciated (129, 130). On the one hand, type I IFNs cause massive translational inhibition as antiviral strategy (131), but on the other hand, they do promote the translation of ISGs, including PKR (10). Molecularly, AKT-mTORC1, mTORC2, and MAP kinase-interacting kinases (MNK), as well as eukaryotic initiation factor 4E (eIF4E) signaling are transiently activated by type I IFNs. An increase in ISG mRNA translation follows, which represents the early phase of IFN response (132–135). However, in primary human macrophages, stimulated by TLR2 ligands, IFNγ induces a strikingly different response. It reprograms metabolic pathways toward enhanced mitochondrial pathways and oxidative

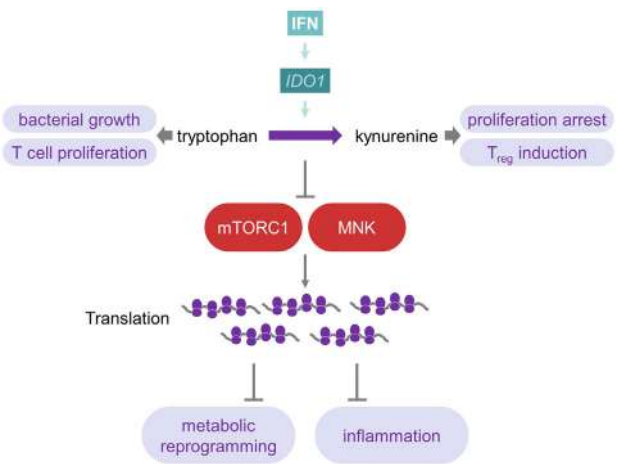

FIGURE 4 | Interferon influences tryptophan metabolism to reprogram metabolism and inflammation. IDO1, which is induced by IFNs, catalyzes the production of Kyn from Trp. As many microorganisms rely on this amino acid, this represents a mechanism against bacterial infections. Furthermore, Trp is important for T-cells, and its depletion, therefore, inhibits T-cell effector immunity, while Kyn promotes T-cell tolerance by inducing T_{regs}. Depletion of Trp causes inhibition of mTOR complex 1 as well as MAP kinase-interacting kinases and, therefore, induces changes at the translational level of metabolism and inflammation. For further details consult the text.

phosphorylation by inhibition of mTORC1 as well as MNK (136) (**Figure 4**). IFNγ treatment of patients with sepsis also enhances mitochondrial oxidative phosphorylation in peripheral blood mononuclear cells (137). mTORC1 and mTORC2 are well-known to control a wide array of metabolic pathways, including glycolysis, oxidative phosphorylation, and lipid metabolism (5, 138). Moreover, IFNγ suppresses the translation of repressors of inflammation, including HES1, HEY1, and IκBα, *via* mTORC1 in human macrophages (136, 139). The translational inhibition of these molecules promotes an inflammatory response and may contribute to the potent proinflammatory effects of this cytokine (140). Similarly, diminishing translation by blocking mTORC1 with rapamycin favors the translation of the more abundant pro-inflammatory cytokines such as IL-12 and blocks the translation of low abundant mRNAs such as IL-10 (5, 141). Mechanistically, IFNγ induces IDO1 expression (as explained above) by depleting intracellular tryptophan levels, and this suppresses mTORC1 (136). The amino acids leucine, arginine, as well as tryptophan are sensed by mTORC1 at the level of the lysosome. Only if sufficient amino acids are present, full mTORC1 activation by growth factors or PRR ligands occurs (142, 143). Additionally, IFNγ inhibits expression of the macrophage colony-stimulating factor receptor and interferes with the expression of SIRT1, a major deacetylase that influences energy metabolism and longevity (136, 144). Together, these data indicate that both the control of the cellular metabolism and mTORC1 activation by IFNγ may be central mediators of this pleiotropic proinflammatory molecule.

CONCLUDING REMARKS

Based on recent and older studies, a theme is emerging, which shows that IFNs are potent modulators of basic cellular processes (**Table 1**). Viruses rewire the metabolism of the host cell to efficiently replicate and produce infectious particles. Therefore, interfering with distinct metabolic pathways seems to constitute one of the core antiviral properties of IFNs. In this context, we described that inhibition of FA and cholesterol synthesis as well as induction of NO are to date the best studied metabolic actions of IFNs. Future studies should focus on expanding the investigation of the influence of IFNs on the cellular energy metabolism including FAO. Moreover, IFN-mediated metabolic effects may be mediated by metabolic-derived protein and epigenetic modifications such as N-glycosylation, methylation, or acetylation (44, 145, 146).

However, some considerations need to be taken into account when studying metabolic processes. First, the metabolism of immortalized cell lines may be notably different from that of primary cells. Proliferating cell lines harbor mutations in pathways that regulate metabolic processes such as the mTOR pathway and already show a Warburg effect to allow infinite proliferation. Moreover, the composition of the cell culture medium profoundly affects the cellular metabolism. Culture media often contain nutrients that far exceeds the amounts observed in tissues and thus may mask the importance of individual metabolic pathways for specific immunologic functions. Finally, pharmacological inhibitors are instrumental in metabolic studies (147), but they may show off-target effects and should, therefore, be complemented with genetic studies to elucidate whether an observed metabolic shift is the cause or the consequence of a change in the cellular phenotype (148).

The expansion of our knowledge on immunometabolism and the role of IFNs suggest novel avenues for metabolic therapies. In this regard, it might be possible in the future to target specific

TABLE 1 | Metabolic changes caused by interferons (IFNs).

Effects of IFNs	Cell type	Reference
Generally, IFNs cause a translational inhibition, but promote the transcription of IFN-stimulated genes		(1, 4, 10, 12, 130, 131–134)
↑Glucose uptake	mouse embryonic fibroblasts, human plasmacytoid dendritic cells (pDCs)	(31, 40)
↑Glycolysis	Splenic CD11c$^+$ MHCII$^+$ DCs *ex vivo*, macrophages, human squamous carcinoma cell line	(32, 34, 35, 43)
↑Aerobic glycolysis	Macrophages	(33)
↓Oxidative phosphorylation and adenosine triphosphate (ATP) production	Human squamous carcinoma cell line, mouse L929 or human Daudi cells, human CD4$^+$ T cells *ex vivo*, pDCs	(35–37, 40)
↑Oxidative phosphorylation and ATP production	Primary human macrophages, peripheral blood mononuclear cells, pDCs, conventional DCs, keratinocytes, or memory T cells	(39, 135, 136)
↑Lipolysis	Adipocytes of mice *in vivo*	(41)
↑Itaconic acid	Alveolar macrophages	(47)
↑NO, reactive oxygen species	Macrophages, primary hepatocytes, macrophages	(30, 33, 113, 114)
↓Fatty acid and cholesterol synthesis	HeLa cells	(86)
↑25-hydroxycholesterol	Macrophages	(88)
↑Membrane rigidity	Daudi cells, L9292 cells, human monocytes, RSa, RSb, IF r and RD-114 cells, red blood cells of patients with hepatitis C infection	(91–95)
↓polyamine synthesis	Huh7 cells, BHK-21 cells	(103)
↑IDO1 expression, ↓of tryptophan	Macrophages, fibroblasts, pDCs, *Toxoplasma gondii*, *Salmonella enterica* serovar Typhi (*S.* Typhi), or *Chlamydia pneumoniae* infections	(119, 120, 123, 124)
↑AKT–mTOR complex 1, mTORC2, MAP kinase-interacting kinases, eukaryotic initiation factor 4E	Huh-7, Huh-7.5, 293T, MT-4, *STAT1*$^{-/-}$ Fib, reviewed in, MEFs	(131–134)
↓Translation of repressors of inflammation	Human macrophages (135, 138)	(135, 138)

pathways that are critical for viral replication, such as FAS or cholesterol synthesis. More generally, distinct immune cells may be more dependent on specific metabolic processes than others, and hence, more vulnerable to allow specific immunometabolic targeting of cells *in vivo* (149, 150). Moreover, the support of antiviral actions of IFNs by providing metabolites may also be possible. In conclusion, the reciprocal regulation of IFNs and metabolic processes advances our understanding of immunometabolism and may hold future surprises for our understanding of immunity in health and disease.

AUTHOR CONTRIBUTIONS

SF and TW conceived and wrote the manuscript.

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REVIEW
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The Essential Role of Type I Interferons in Differentiation and Activation of Tumor-Associated Neutrophils

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Type I interferons (IFNs) were first characterized in the process of viral interference. However, since then, IFNs are found to be involved in a wide range of biological processes. In the mouse, type I IFNs comprise a large family of cytokines. At least 12 IFN-α and one IFN-β can be found and they all signal through the same receptor (IFNAR). A hierarchy of expression has been established for type I IFNs, where IFN-β is induced first and it activates in a paracrine and autocrine fashion a cascade of other type I IFNs. Besides its importance in the induction of the IFN cascade, IFN-β is also constitutively expressed in low amounts under normal non-inflammatory conditions, thus facilitating "primed" state of the immune system. In the context of cancer, type I IFNs show strong antitumor function as they play a key role in mounting antitumor immune responses through the modulation of neutrophil differentiation, activation, and migration. Owing to their plasticity, neutrophils play diverse roles during cancer development and metastasis since they possess both tumor-promoting (N2) and tumor-limiting (N1) properties. Notably, the differentiation into antitumor phenotype is strongly supported by type I IFNs. It could also be shown that these cytokines are critical for the suppression of neutrophil migration into tumor and metastasis site by regulating chemokine receptors, e.g., CXCR2 on these cells and by influencing their longevity. Type I IFNs limit the life span of neutrophils by influencing both, the extrinsic as well as the intrinsic apoptosis pathways. Such antitumor neutrophils efficiently suppress the pro-angiogenic factors expression, e.g., vascular endothelial growth factor and matrix metallopeptidase 9. This in turn restricts tumor vascularization and growth. Thus, type I IFNs appear to be the part of the natural tumor surveillance mechanism. Here we provide an up to date review of how type I IFNs influence the pro- and antitumor properties of neutrophils. Understanding these mechanisms is particularly important from a therapeutic point of view.

Keywords: type I interferons, neutrophils, tumor, inflammation, neutrophil polarization

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INTRODUCTION

The significance of type I interferons (IFNs) in cancer immune surveillance is well established by now. These cytokines were first characterized late in the 1950s as cytokines with antiviral activity (1). In the mouse, type I IFNs comprise a large family of cytokines with at least 12 IFN-α, IFN-β, IFN-ε and IFN-κ (2, 3). All of them signal *via* a common receptor IFNAR, and they induce the expression

of several 100 IFN-inducible genes and have a broad range of biological functions (2). Within the type I IFNs, IFN-α and IFN-β are best characterized. Importantly, a hierarchy of expression has been shown to exist for these cytokines (4, 5), where IFN-β is induced first. When it binds to IFNAR, IFN-β in a paracrine and autocrine fashion triggers a cascade of type I IFNs, including IFN-α and IFN-β. The only exception to this rule are plasmacytoid dendritic cells (pDCs), which can start immediately with the secretion of IFN-α (6). Besides, its importance for the induction of the IFN cascade, IFN-β is also constitutively expressed in low amounts under normal non-inflammatory conditions (7). This was clearly demonstrated by non-invasive imaging using the new luciferase reporter mouse, but also by *ex vivo* determination of the enzymatic activity of luciferase in various tissues (4). The reason for such constitutive expression of IFN-β might be the priming of the immune system to persist in a pre-activated state that guarantees a faster and stronger type I IFNs response when necessary. Notably, using luciferase reporter mouse, it could be demonstrated that growing tumors induce type I IFNs expression mainly in tumor-infiltrating dendritic cells (DCs) (8).

Besides their role in antiviral and antimicrobial responses, type I IFNs shape innate and adaptive immunity (9), influence the maintenance of cellular homeostasis (10), hematopoiesis (11), and lymphocyte development (12). In addition, type I IFNs show strong antitumor activity (13) and are involved in cancer immunoediting (14). The mechanisms of how type I IFNs contribute to the immune surveillance against tumors are not fully understood, notwithstanding their beneficial effects in the cancer therapy (13). In the context of cancer, type I IFNs were found to play a key role in supporting host immune responses through the activation of multiple immune cells, e.g., T-cells, natural killer (NK) cells, DCs, and macrophages. In recent years, it has become apparent that type I IFNs affect also neutrophil activation and promote antitumor functions of these cells.

The inflammation has been recently associated with increased susceptibility for cancer (15). Consequently, neutrophils as a central component of this process play an essential role in inflammation-driven tumorigenesis. Moreover, neutrophils represent an independent prognostic marker in a broad variety of neoplasias. In the past, these cells were viewed as solely dedicated to phagocytosis and the production of reactive oxygen species (ROS). Now, they are recognized for an extreme versatility with regard to function (16, 17) and play manifold roles during tumor development (8, 18). Neutrophils affect primary tumor growth by influencing its angiogenesis (18), but also by direct killing of tumor cells (8). Moreover, neutrophils can facilitate the spread of tumor cells to distant organs in a process called metastasis (19, 20). Neutrophils are apparently controlled by factors produced by the primary tumor and are responsible for the preference of metastasizing tumor cells to certain organs. Type I IFNs have a substantial influence on this process (20).

The tumor microenvironment represents a special niche that is extremely influencing infiltrating immune cells. The concept of immune cell polarization was described initially for macrophages (antitumor M1/pro-tumor M2), but recently polarization of neutrophils is getting growing attention. Neutrophils appear to have contradicting phenotypes in the tumor microenvironment, i.e., tumor promoting (N2) or inhibiting (N1) (16), depending on the cytokine milieu in the tumor. Cytokines that are known to control neutrophil polarization are type I IFNs, driving neutrophil differentiation into N1 antitumor state (8). Of note, strict classification into N1 or N2 phenotypes is certainly an oversimplification. More likely, these two immune phenotypes spot the end points of a continuum of functional states exhibited by neutrophils in tumor milieu, which can be regulated by the environmental cues. Here, we provide an up to date review of how type I IFNs influence the pro- and antitumor properties of neutrophils.

TUMOR MICROENVIRONMENT AND THE PHENOTYPE OF NEUTROPHILS

Tumor cells, as well as infiltrating immune cells, produce wide range of cytokines, chemokines, and growth factors. This leads to the activation and recruitment of other immune cells, such as neutrophils, monocytes, and lymphocytes. Moreover, the tumor microenvironment plays a significant role in the differentiation and functional properties of such cells. In the early steps of tumorigenesis, the infiltration of immune cells into the tumor tissue serves as one of the tumor killing mechanisms and provides protection against tumor progression. When this line of defense is insufficient and tumor escapes the immune response, the balance shifts to suppressive anti-inflammatory microenvironment. This effect was initially described and widely studied for macrophages and their polarization into antitumor M1 and pro-tumor M2 (21–26).

Neutrophils were previously thought to be terminally differentiated, short lived myeloid cells. Recent studies, however, confirmed that tumor-associated neutrophils (TANs) can also exhibit antitumor N1 or pro-tumor N2 properties, similarly to macrophages (16). The different role of such neutrophil phenotypes in tumor progression and their influence on the prognosis of the disease were assessed. Some studies revealed strong antitumor properties of neutrophils (27), including antibody-dependent or direct cytotoxicity (28) mediated by ROS release (29) and production of neutrophil extracellular traps (NETs) (8). Moreover, neutrophils potentiate antitumor immune responses by the recruitment of other immune cells to the tumor site (30). Recently, the role of neutrophils as possible antigen-presenting cells (APCs) was suggested. These cells were shown to modulate activation of $CD4^+$ and $CD8^+$ T cells *via* expression of costimulatory molecules like CD86, ICAM-1, OX40L, and 4-1BBL (31–33).

At the same time, tumor-supporting activities of neutrophils were revealed, demonstrating the role of these cells as efficient inhibitors of host immunosurveillance. Moreover, neutrophils were shown to stimulate tumor angiogenesis *via* secretion of vascular endothelial growth factor (VEGF) and matrix metallopeptidase 9 (MMP9) (18). This leads to the better vascularization of the primary tumor and its growth. Of note, not only primary tumor growth is maintained by neutrophils, but also the formation of metastases can be enhanced by these cells. Pro-tumor neutrophils upregulate the expression of pro-metastatic proteins, e.g., Bv8, S100A8, and S100A9, but also VEGF and MMP9 in pre-metastatic lungs of IFN-deficient mice. This phenomenon,

together with the enhanced infiltration of lungs by these cells, leads to improved metastatic load in IFN-deficient mice (20). N2 neutrophils are characterized with immature nucleus shape and reduced tumor cell killing capacity (34); they were also shown to recruit regulatory T cells in tumors by expression of CCL17 (35). Accordingly, in clinical studies, the percentage of neutrophils in blood and neutrophil/lymphocyte ratio was shown to be negative predictors of patient outcome in different types of cancer (36, 37).

Although two functionally different types of neutrophils were described, clear markers allowing distinguishing pro- and antitumor neutrophils are still not available. Nevertheless, factors that determine the phenotype shift are in general similar to those for macrophages (16). Type I IFNs are considered as N1-promoting cytokines and transforming growth factor beta (TGF-β) has been suggested to be N2 inducer (38). TGF-β is a well-known immunosuppressive cytokine, expressed also in tumors, which circulating form was shown to correlate with tumor progression (39). The functional antagonism between type I IFNs and TGF-β is observed not only for neutrophils but also was shown for human peripheral blood mononuclear cells (40) and it could be due to the antagonisms between signaling pathways of these cytokines. Smad2 and Smad3, the downstream molecules of the TGF-β signal transduction pathway, were shown to negatively regulate LPS-induced macrophage activation by suppressing multiple steps in the IFN-β/STAT1 pathway, including the inhibition STAT1 transcriptional activity (41). Similar results were shown for myeloid cells of the central nervous system (CNS), where TGF-β impaired the ability of such cells to acquire a resolving anti-inflammatory phenotype *via* downregulation of IFN regulatory factor 7 (IRF7) (42). Thus, TGF-β may potentially influence neutrophil type I IFN-dependent functions and polarization by modulation of STAT1 pathway. Nevertheless, the regulation of type I IFN and TGF-β pathways seems to be more complicated since a positive crosstalk between IFN-α and TGF-β signaling was observed in preneoplastic rat liver, resulting in activation of both; STAT1 and Smad2/3 pathways (43).

Deficiency in endogenous type I IFN signaling seems to play a significant role in the switch of immune response from antitumor to pro-tumor one. Moreover, there is an evidence of changing phenotype of TANs with a shift to pro-tumorigenic properties during tumor progression (44), which can be explained with continuous fluctuation of cytokines and chemokines.

Of note, an alternative concept of anti-inflammatory low-density neutrophils (LDNs) and pro-inflammatory high-density neutrophils (HDNs) in tumor situation emerged recently (45). Due to the literature, HDNs represent a homogenous population of mature segmented neutrophils, while LDN population consists of both immature (banded/MDSC) and mature neutrophils. HDNs are characterized as cells with high cytotoxicity against tumors while LDNs have no cytotoxicity, representing the pro-tumor neutrophils subset (45). Yet, there is no comprehensive data showing the influence of type I IFNs on the LDL/HDL balance in the tumor-bearing hosts. The current knowledge on the heterogeneous populations of mature and immature neutrophils, including LDNs, low-density granulocytes (LDGs), granulocytic myeloid-derived suppressor cells (G-MDSCs), and immunosuppressive neutrophils is summarized recently by Scapini et al. (46).

ENDOGENOUS TYPE I IFN SIGNALING IN NEUTROPHILS

The receptor of type I IFNs (IFNAR) belongs to the family of type II cytokine receptors that trigger the activation of the JAK-STAT pathway. Ligand that binds to the receptor activates the cascade of phosphorylation of JAK molecules, which in turn phosphorylate tyrosine residues on the receptor chains leading to the subsequent STAT protein phosphorylation and activation. IFNAR primarily utilizes JAK1 with some accessory role for JAK2 and TYK2, and activates STAT1, STAT2, or STAT3 pathways. Phosphorylated STAT proteins undergo dimerization and shuttle to the nucleus where they bind promoter regions and regulate gene transcription (47). Type II cytokine receptors are involved in a number of neutrophil function including regulation of survival, differentiation, and activation (48–50). Different expression of mediating proteins and the regulation of intracellular signal transduction pathways determine the final effect of type I IFNs under certain conditions (51). STAT1/STAT3 functional imbalance with the shift to STAT3 activation and following antiapoptotic protein expression is known to be crucial for tumorigenesis (52). In this case, IFN-β was shown to suppress cancer growth and metastasis rate through inhibition of STAT3 signaling in tumor cells (53).

In the context of cancer, type I IFNs show strong antitumor properties. IFN gene therapy was associated with sustained local production of IFN-β that efficiently suppressed tumor growth in prostate and bladder cancer as well as melanoma, renal cell carcinoma, and colon carcinoma (54–56). This effect was ascribed to the induction of tumor cell apoptosis (57) and the inhibition of tumor angiogenesis due to decreased VEGF expression in different tumors (58, 59).

A new line of studies devoted to the effects of IFNs in tumor conditions was induced by the growing evidence that the immune system plays a significant role in the regulation of oncogenesis. Type I IFNs were shown to stimulate antitumor immune responses *via* several mechanisms reviewed by Parker et al., including enhancement of immune recognition of tumor cells by upregulation of major histocompatibility complex (MHC) class I and tumor antigen expression on tumor cell surface, increasing NK cytotoxicity, and switching macrophage phenotype from M2 to M1 (60). Moreover, antigen-presenting properties of DCs were shown to be improved, effector T cell proliferation enhanced, and suppressive activity of regulatory T cells reduced by these cytokines.

Neutrophils lately are being recognized as key players that regulate tumorigenesis and metastatic processes, modulation of their differentiation and activation by type I IFNs becomes an important area of research. The absence of endogenous type I IFN signaling results in shift of neutrophil phenotype to tumor-supporting one. Several factors can be responsible for this phenomenon, including genetic peculiarities of molecular signaling pathways (61, 62), maturation state of the neutrophils, and exogenous influence. While the genes for intracellular proteases and other cytotoxic proteins were shown to be expressed at earlier stages of maturation, the genes for proteins responsible for signal transduction from IFNAR, and, therefore, mediating the release of abovementioned cytotoxic factors, are preferentially induced

during terminal differentiation (63). Immature state of circulating human neutrophils exposed to granulocyte/macrophage colony-stimulating factor (GM-CSF) *in vitro* was characterized with downregulation of IFN signaling pathway, including IFNAR, IFN-γ receptor as well as JAK1, JAK2, STAT1, and STAT2 (63). Colony-stimulating factors are usually overexpressed in tumor environment. Considering the fact that these factors induce the release of immature neutrophils from the bone marrow (BM) as well as support the immunosuppressive state in circulation (64–66), one can expect a downregulation of IFN-mediated signal transduction pathways and decreased efficiency of antitumor immune responses in the presence of such growth factors. Similar feedback loop was described for VEGF, one of the factors supporting tumor angiogenesis, which is capable to downregulate IFNAR expression (67).

Although type I and type II IFN signaling pathways share common intracellular mediators, e.g., STAT1, STAT2, and STAT3, they are shown to exhibit different regulatory role in tumorigenesis. While IFN-γ is known mainly as an agent regulating tumor cell survival, type I IFNs primarily modulate host immune responses against tumors (68). The exclusive effect of type I IFNs on the host immune system was also confirmed by studies of Wu et al. that could demonstrate enhanced tumor growth in animals lacking IFNAR but able to produce endogenous type I IFNs (20). In this case, tumor growth was similarly enhanced as in IFN-β-deficient animals. The constitutive lack of endogenous IFN-β (*Ifnb1*$^{-/-}$ mice) as well as the lack of type I IFN signaling (*Ifnar1*$^{-/-}$ mice) leads to increased growth of different types of tumors (B16F10 melanoma, 4T1 mammary carcinoma, LLC carcinoma, and MCA205 fibrosarcoma) (8, 18, 20, 34, 69) and enhanced metastatic processes (20). The strong pro-tumor phenotype of *Ifnb1*$^{-/-}$ mice confirms the hypothesis that the expression of all alpha IFNs strongly depends on the previous IFN-β expression. Even if pDCS are indeed able to express alpha IFNs without previous stimulation, the comparable elevated tumor growth in *Ifnb1*$^{-/-}$ and *Ifnar1*$^{-/-}$ mice demonstrates its irrelevance. Enhanced tumorigenesis in type I IFN-deficient mice is accompanied by strong accumulation of neutrophils in primary tumors. These neutrophils show reduced cytotoxicity, increased pro-angiogenic properties, and are resistant to apoptosis.

TYPE I IFNs INFLUENCE THE TURNOVER AND THE LIFESPAN OF NEUTROPHILS IN TUMOR ENVIRONMENT

Neutrophils for long time were believed to be short-living cells. This was most probably due to *ex vivo* manipulation techniques that limited the lifespan of these cells. Recently, the perspective is changing and there are data demonstrating much longer neutrophil lifespan that can reach up to approximately 10–20 days (70). Neutrophil homeostasis in the organism is maintained through a balance of neutrophil production, release from the BM, and clearance from the circulation (71). The BM serves as a reservoir for neutrophils that can be rapidly mobilized in response to inflammatory stimuli. However, at steady state, only a small fraction of the total BM neutrophil pool is released into circulation (72). In the absence of pro-inflammatory stimuli, neutrophils undergo spontaneous apoptosis and are phagocyted by tissue macrophages (73). Several stimuli can prolong neutrophil survival, including infectious factors associated with bacterial infections (LPS) as well as colony-stimulating factors, e.g., granulocyte colony-stimulating factor, G-CSF (74).

Tumors are known to produce the whole spectrum of colony-stimulating factors (IL-3, G-CSF, and GM-CSF) (64–66, 75) that potentially influence proliferation of progenitor cells, neutrophil release from BM, and prolongation of their lifespan in tissues. Other sources of G-CSF are endothelial cells (76) and neutrophils themselves (34). Type I IFNs were shown to downregulate G-CSF expression on gene and protein level (20, 34). G-CSF is known to be a major regulator of neutrophil development, mobilization, and differentiation. It has been shown to mobilize neutrophils from BM to the blood *via* STAT3 activation and regulating CXCL2/CXCR2 axis (20, 77) as well as to suppress neutrophil apoptosis (78). Furthermore, recently Casbon et al., using a multistage mouse model of breast cancer, could show that tumor-derived G-CSF was responsible for both the development and activity of immunosuppressive neutrophils in cancer (79). Similarly, Spiegel et al. could recently show that G-CSF-induced neutrophils act to promote metastasis in 4T1 lung metastasis model *via* inhibition of NK cell-mediated clearance of intraluminal tumor cells. Moreover, such neutrophils facilitate extravasation of tumor cells into lungs *via* secretion of IL1β and matrix metalloproteinases (80).

Since G-CSF is upregulated in type I IFNs-deficient tumor-bearing mice (34), this phenomenon could be responsible for the observed neutrophil expansion in bloodstream and tumors of such mice (18). Generally, TANs are characterized with prolonged lifespan comparing to other tissue neutrophils (81). Andzinski et al. demonstrated that endogenous type I IFNs influence neutrophil survival and lifespan. In type I IFNs-deficient mice bearing B16F10 melanoma, the neutrophil life span was prolonged due to apoptosis suppression. Neutrophilic granulocytes from such *Ifnb1*$^{-/-}$ tumor-bearing mice expressed higher amounts of BCL-xL and showed decreased effector caspase 3 activity as well as inhibited expression of death receptor Fas (34). Fas expression on neutrophils has been shown to be involved in spontaneous extrinsic cell death signaling (82). Even though Fas ligand-induced apoptosis is considered not to be a major mechanism in steady state (83), it has been demonstrated to be important under inflammatory conditions, for example, in cancer (84). An additional factor that has been revealed to induce neutrophil apoptosis is TNFα (85). Notably, type I IFN signaling has been shown to increase expression of TNFα by neutrophils (8). Decreased neutrophil apoptosis in the absence of endogenous type I IFNs could be also due to the decreased production of cytotoxic ROS by TANs (34).

TYPE I IFNs INFLUENCE NEUTROPHIL MIGRATION IN TUMOR-BEARING MICE

The process of neutrophil migration to the site of inflammation depends on the several ligand–receptor interactions, including

chemokine sensing and sensing of activated endothelium in inflammatory site. Retention of immature neutrophils in BM is due to high expression of CXCR4 on the cell surface and its interaction with CXCL12 secreted by stromal cells. Attenuation of CXCR4 signaling and upregulation of CXCR2 on neutrophils is an important mechanism by which these cells are mobilized into the circulation under inflammatory conditions (71). Subsequent neutrophil migration to tissues is determined by interacting of surface chemokine receptors and chemokines forming concentration gradient. Mature neutrophils are characterized by the high expression of CXCR2 (86, 87). The ligands of this receptor (CXCL8 in humans and CXCL1, CXCL2 in mice) are responsible for homing of mature neutrophils into tissues. Lungs, liver, and spleen are the major producers of CXCR2 ligands under normal conditions and conclude considerable neutrophil marginated pool in microvascular bed (88). It is suggested that the high expression of CXCR2, CXCR4, and CCR7 ligands in lungs, liver, BM, and lymph nodes is one of the reasons responsible for metastases homing toward these organs in certain types of cancer (89, 90).

Tumor tissue seems to be a significant source of chemokine ligands of CXCR2 (91–93) and forms chemokine gradient attracting neutrophils. Low CXCL1 or CXCL2 level in BM and high level of these chemokines in the tumor form gradient in tumor-bearing mice, thus attracting neutrophils into tumor site. Correspondingly, the expression of CXCR2 is the highest on neutrophils from BM and blood, and is downregulated after reaching the tumor (69). Of note, the migration of neutrophils is downregulated by endogenous type I IFNs *via* suppression of chemokines. Expression of CXCL1 and CXCL2 in blood and tumor was significantly higher in *Ifnb1*$^{-/-}$ mice as compared to wild-type (WT) controls. On the other hand, the expression of CXCL5, which is known to compete with CXCL1 and CXCL2 for CXCR2-binding site, was upregulated in blood of WT mice. This could be responsible for the inhibited migration of neutrophils into tumor tissue in WT animals, since they are trapped in the blood due to high concentration of CXCL5. Treatment of tumor-bearing IFN-deficient mice with recombinant murine IFN-β downregulated CXCL1 and CXCL2 expression in blood and tumor to the levels observed in control mice (69).

An additional chemokine/receptor axis involved in migration of neutrophils into tumor site is CXCL12/CXCR4 axis. This axis has also been shown to be downregulated by type I IFNs. Endogenous type I IFNs inhibit CXCR4 expression on neutrophils and block CXCL12 expression in tumors leading to suppressed migration of neutrophils toward the tumor (69).

Rolling, adhesion, and migration of neutrophils to the site of inflammation are mediated by the interaction of endothelial adhesion molecules and their ligands on leukocytes (94). Mature and activated neutrophils are characterized with decreased surface expression of L-selectin CD62L (95). Importantly, in the absence of endogenous type I IFNs tumor-bearing mice show significantly increased percentage of CD62L^{+} circulating neutrophils (8), which could result in increased migration to tumor site. All described mechanisms explain the increased migration of neutrophils into tumors leading to the enhanced tumor growth in IFN-deficient mice.

REGULATION OF OXIDATIVE BURST BY TYPE I IFNs

Type I IFNs were shown to regulate the most prominent anti-tumor feature of neutrophils, i.e., their ability to directly kill tumor cells (8). Neutrophil cytotoxicity includes both direct and antibody-dependent cell-mediated cytotoxicity (*via* recognition of opsonized cells). Functional activity of neutrophils is determined by large spectrum of secretory granules and vesicles rather than production of proteins *de novo*. The primary azurophilic granules containing myeloperoxidase and other acid hydrolases, as well as neutral proteases (cathepsin G, elastases, and collagenases), are responsible for pathogen degradation. Secondary (specific) granules are large stores for soluble mediators as well as for NADPH oxidase that supports oxidative burst. The tertiary granules and secretory vesicles support migration and interaction of neutrophils with the environment (96).

Cytotoxicity depends on developmental stage of the cell (63) but also on the microenvironment. In animal models, TANs show reduction of cytotoxicity in comparison to blood-derived neutrophils, indicating further influence of the tumor milieu on neutrophil activation and function (8). Type I IFN signaling is essential for neutrophils to facilitate some of their functions (63). Accordingly, decreased spontaneous production of cytotoxic ROS by tumor-infiltrating neutrophils was demonstrated in mouse models deficient in endogenous IFNs (34), which was linked to significantly reduced cytotoxicity of tumor neutrophils in such mice, compared to IFN-sufficient animals. Treatment of *Ifnb1*$^{-/-}$ mice with recombinant IFN-β-restored neutrophil cytotoxicity (8).

DECREASED NEUTROPHIL EXTRACELLULAR TRAP FORMATION BY NEUTROPHILS DEFICIENT IN ENDOGENOUS TYPE I IFNs

Neutrophil extracellular traps consist of nuclear or mitochondrial-derived web-like DNA strands released from neutrophils that are equipped with histones and bactericidal proteins. The process of NETs release is called NETosis and it is an unique form of cell death. NETosis is a mechanism of distinct killing of extracellular pathogens with high local concentration of effector components (97). The intracellular components shifted extracellularly become a target for macrophages, which destroy attached pathogen as well (98). During this process, neutrophils kill extracellular pathogens while minimizing damage to the host cells. Tumor environment obviously and strongly activates neutrophils and initiates NETs release (8). There are conflicting data about the role of NETs formation during tumorigenesis. On the one hand, it is postulated that released NETs improve efficient tumor cell killing by neutrophils (99). On the other hand, there are studies suggesting NETs as a mechanism supporting metastasis formation (100). One could speculate that the subsequent fate of trapped tumor cells depends on the activation of the neutrophils and their ROS release.

Interferons seem to influence the process of NETosis. Priming with IFN-α or IFN-γ with subsequent C5a activation triggers release of NETs in mature human neutrophils. Notably, immature neutrophils are not able to release NETs in this condition, probably due to the lack of IFN signaling pathway mediators (63). Another animal model study revealed that blood neutrophils isolated from type I IFN-deficient tumor-bearing mice display significantly lower NETs formation capacity compared to WT controls. This is accompanied by less efficient tumor cell killing by these cells and was in agreement with observed enhanced tumor growth in such mice (8).

SUPPRESSION OF PRO-ANGIOGENIC PROPERTIES OF NEUTROPHILS BY TYPE I IFNs

Effective angiogenesis is essential for successful tumor growth. One of the developmental hallmarks of a tumor is the induction of angiogenesis, i.e., the formation of new blood vessels. Small tumors up to a size of 1–2 mm^3 can be supplied with oxygen and nutrients by the surrounding tissue. For larger tumors, this is no longer sufficient. The tumor has to alter its angiogenic phenotype and the so-called angiogenic switch – the induction and assembly of tumor vasculature – has to take place (101, 102). Interestingly, myeloid cells like neutrophils are known to take part in tumor vascularization since they are known to be the source of the variety of pro-angiogenic factors, including VEGF and angiogenic chemokines (103). MMPs and other enzymes released by neutrophils provide degradation of extracellular matrix and facilitate vessel growth (104).

The role of type I IFNs in inhibition of tumor angiogenesis has been suggested before (105, 106). Recent data show also the significant impact of TANs on angiogenic processes in tumor and the important role of type I IFNs in the modulation of these processes (18). In *Ifnb1*$^{-/-}$ mice, considerably higher tumor growth was observed, accompanied by boosted angiogenic processes. Enhanced content of fully developed functioning vessels, completely covered by pericytes, was found. Moreover, the number, area and the perimeter of vessels in tumors of such mice were significantly higher than in WT mice. Notably, these tumors were strongly infiltrated by neutrophils that were found in close vicinity of vessels. Neutrophils isolated from *Ifnb1*$^{-/-}$ mice show significantly higher expression of VEGF and MMP9 (18). Moreover, CXCR4 was upregulated on these cells. CXCR4, is known to be overexpressed in highly vascularized tumors (107, 108), and its ligand CXCL12 is apparently induced under hypoxic conditions in accordance with the triggering of the angiogenic switch. Depletion of neutrophils in this model led to reduction of number of developed vessels and subsequent retardation of tumor growth as compared to untreated animals (18). *In vitro* treatment of TANs isolated from *Ifnb1*$^{-/-}$ mice with low levels of IFN-β restored expression of pro-angiogenic factors to control levels (18).

Certain chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8) are known to mediate angiogenic processes through direct activation of endothelial cells *via* CXCR2 receptor or recruit pro-angiogenic immune cells and endothelial progenitors to the neovascular niche (109). Type I IFNs were shown to decrease production of some of these chemokines, which could serve as an additional antiangiogenic mechanism during tumorigenesis (69).

Reactive oxygen species are also considered to be regulators of endothelial cell functions. While high amounts of ROS are toxic for endothelial cells and reveal antiangiogenic properties, low concentrations of NO and H_2O_2 can serve as intracellular mediators of signal transduction to stimulate vascular smooth muscle cells to support angiogenesis [reviewed by Irani (110)]. Thereby, increased IFN-dependent ROS production by tumor-infiltrating neutrophils (34) can additionally exert an antiangiogenic effect.

THE REGULATION OF ADAPTIVE IMMUNE RESPONSES BY NEUTROPHILS STIMULATED WITH TYPE I IFNs

Accumulating data suggest that neutrophils may influence adaptive immunity by acting either indirectly (*via* APCs) or directly on T cells. T cells are considered to be key players involved in antitumor immunity; yet, many other components of the immune system take part in this process. Antigen presentation is an important link between innate and adaptive immune responses. Two main mechanisms are involved in this process. Fragments of intracellular pathogens are presented on MHC class I complex to $CD8^+$ cytotoxic T cells. Extracellular pathogens, after phagocytosis and procession, are bound in phagolysosome with MHC class II molecules and are presented to $CD4^+$ T-cells (111). The third mechanism of antigen presentation, called "cross-presentation," shares features of previous two. In this process, APCs translocate extracellular antigen from the endocytic vesicle to the cytosol and present it on MHC class I to $CD8^+$ T cells (cross-priming) (112). In the last two cases, appropriate activation of APCs is necessary to induce effective immune response. In the absence of activating stimuli or in anti-inflammatory environment, APCs stimulate abortive T-cell responses, which lead to tolerance (113).

All neutrophils constitutively express MHC class I. Murine neutrophils, both circulating and resident, are known to express MHC class II and can potentially play a role in antigen presentation together with macrophages and DCs. To the contrary, human circulating neutrophils do not express MHC class II under normal conditions, but there is an evidence of antigen-presenting function of these cells in certain inflammatory conditions, including autoimmune diseases (114, 115) as well as after treatment with GM-CSF (116) or IFN-γ (117). In patients with cancer, no MHC class II expression on circulating neutrophils was observed (33), arising a question about TANs participating in antigen presentation. Immature neutrophils that are released from BM as a result of tumor-driven emergency myelopoiesis were shown to become activated with cytokines released in tumor microenvironment (GM-CSF, IL-4, TNF-α) and acquire molecular features characteristic for DCs. Such activated DC-like cells express DC-associated surface molecules cluster of differentiation CD1a, CD1b, CD1c, MHC class II, and costimulatory molecules CD80, CD86, CD40, as well as ICAM-1

and CD5. At the same time, these cells downregulate CD15 and CD65 expression. Altogether, this leads to effective presentation of antigen to $CD4^+$ T cells, thus activating antitumor immune responses (118). Recently, Eruslanov et al. demonstrated that neutrophils augment T cell proliferation in a positive-feedback loop *via* upregulation of ICAM-1 and costimulatory molecules like CD86, OX40L, and 4-1BBL on the neutrophil surface (32). ICAM-1 was also shown to act as a costimulatory molecule taking part in antigen presentation to T cells and is crucial for T cell activation under conditions where costimulation by CD80 and CD86 is low (119). High ICAM-1 expression can, therefore, induce the activation of cytotoxic $CD8^+$ T cells (120) as well as repress the secretion of immunosuppressive IL-10 by $CD4^+$ T cells (121). Mature neutrophils upregulate ICAM-1 (122) and, therefore, can participate in antigen presentation. Importantly, it was recently demonstrated that type I IFNs strongly upregulate ICAM-1 expression on neutrophils (8).

Type I IFNs support systemic immunity against tumor targets by upregulation of MHC class I expression as well as enhancement of cytotoxic T cell responses and activation of NK cells (60). They are also known to induce MHC class II expression on monocytes (123) as well as to induce cross-priming of $CD8^+$ cells against exogenous antigens (112).

Thus, neutrophils are able to influence adaptive immune responses, either by directly presenting peptide–MHC class I complexes, MHC class II complexes, or by delivering peptides to other APCs for presentation. Cross-presentation by these cells occurs actually earlier than in professional APCs (31). Possibly, neutrophils may directly present peptide to effector T cells *in vivo*, inducing cytokine production, whereas DCs after receiving neutrophil-derived antigenic peptides may migrate to lymphoid organs to initiate T cell responses (124).

Apart from activating T cells *via* antigen presentation, neutrophils could attract T cells to the sites of inflammation, e.g., growing tumors. $CD8^+$ T cells are attracted to the inflamed tissue *via* CXCL12 (125). Notably, this chemokine was shown to be produced by TANs and downregulated by type I IFNs (18). Moreover, neutrophils produce cytokines-stimulating T cell differentiation and activation, e.g., IFN-γ or TNF-α. Leschner et al. could show that expression of TNF-α is strongly enhanced in blood and tumors of tumor-bearing mice (126). Importantly, type I IFNs upregulate TNF-α expression in TANs (8), thus regulating lymphocyte antitumor responses. On the other hand, peripheral blood neutrophils, under specific conditions, e.g., late stage of tumor, can also suppress antigen non-specific T cell proliferation through the release of arginase-1, TGF-β, and the production of ROS (127–129). Expression of ROS was also shown to be stimulated by type I IFNs (8, 34), once again demonstrating the strong involvement of this cytokine family in the activation of adaptive immune responses leading to the restriction of tumor growth.

THE EFFICIENCY OF METASTATIC SPREAD DEPENDS ON NEUTROPHILS AND IS INHIBITED BY TYPE I IFNs

Metastases are associated with unfavorable prognosis in cancer (130). Metastatic spread is a complex process that includes cells shedding from a primary tumor, their migration in circulation, extravasation, and initiation of secondary tumor growth. Recently, it was postulated that metastases from primary tumors do not migrate and home undirected into sites of secondary growth, but are guided by cells that form the so-called pre-metastatic niche (19, 29, 131). Major component of the pre-metastatic niche are neutrophils. They are apparently controlled by factors produced by the primary tumor and are responsible for the preference of metastasizing tumor cells to certain organs. Different neutrophil-mediated mechanisms of metastatic spread are described, including promotion of tumor cell extravasation by binding ICAM-1 on tumor cells (132) or by catching tumor cells *via* NETs (100).

Endogenous type I IFNs play essential role in modulating neutrophil functions in context of metastatic processes. In mice lacking endogenous type I IFNs, higher metastatic load was observed in the lung as compared to WT animal, which was accompanied by strong neutrophil accumulation in this organ (18, 20). One of the reasons attributed to this phenomenon was the elevated plasma level of G-CSF and increased expression of CXCR2 on neutrophils (20). In the absence of endogenous type I IFN signaling, neutrophils express more CXCR2 and are capable to extravasate more actively to the organs producing high levels of CXCL1 or CXCL2. Such organs that are predisposed to metastasis formation are lungs, liver, and spleen, as mentioned previously. Neutrophils accumulating in pre-metastatic lungs support tumor cell extravasation and proliferation by release of pro-metastatic proteins, e.g., Bv8, MMP9, S100A8, and S100A9. S100A8 and S100A9 are known to influence tumor cell proliferation, survival, and migration (133) as well as to stimulate migration and proliferation of neutrophils themselves. Bv8 induces tumor cell extravasation (134) and increases neutrophil accumulation within pre-metastatic tissue. MMP9 is responsible for formation of leaky vasculature in the pre-metastatic lung (131) and supports tumor cells survival in this organ. The expression of all above factors is significantly enhanced in type I IFN-deficient mice and is suppressed by the recombinant IFN treatment (18, 20). Notably, G-CSF, that is also downregulated by type I IFNs, is known to enhance expression of Bv8, S100A8, S100A9, and MMP9 in neutrophils and thus might also be directly involved in regulating pre-metastatic niche formation (134). Neutrophils from IFN-deficient mice show also reduced cytotoxicity against tumor cells leading to enhanced metastasis in such mice (20). Moreover, Bidwell et al. demonstrated that, in a mouse model, early initiated administration of recombinant type I IFNs leads to reduced bone metastases and prolonged survival of the host (135). This indicates that endogenous type I IFNs effectively suppress the formation of pre-metastatic niche on multiple levels.

CLINICAL ASPECTS OF TYPE I IFN-MEDIATED POLARIZATION OF NEUTROPHILS

The efficacy of type I IFN therapy for various malignancies has been investigated for many years. IFN therapy has been clinically evaluated as the treatment of melanoma (136, 137), renal cell carcinoma (138, 139), myeloproliferative disorders (140, 141), lymphomas (142), neuroendocrine tumors (143) as well

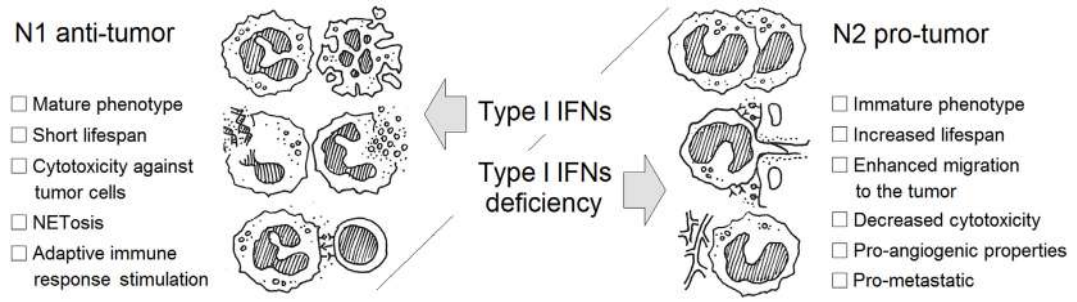

FIGURE 1 | Type I interferon (IFN)-dependent regulation of neutrophil polarization in cancer. Neutrophils may be divided into N1 antitumor and N2 pro-tumor cells in tumor situation. The activation and differentiation of these cells during tumorigenesis is determined by the growth factor and cytokine milieu in the tumor. Type I IFNs are potent drivers of the transition into antitumor N1 phenotype.

as vascular neoplasias including pulmonary hemangiomatosis (144), infantile hemangiomas (145), Kaposi's sarcoma (146), and malignant hemangiopericytomas (147).

Lately, the role of type I IFNs in modulation of immune cell activation in tumor context is getting attention. It is generally accepted that immune cells play important role in the regulation of tumor growth. Neutrophils, both circulating and tumor associated, represent an independent prognostic marker in a broad variety of neoplasias (148, 149); therefore, several studies aimed to modulate the immune system in order to suppress pro-tumoral components and enhance antitumoral immune responses. This has determined the increasing interest in type I IFN treatment. The evidence that IFNs play a role in neutrophil polarization was supported with clinical observations. Recently, the increase of ICAM-1 expression on the neutrophils isolated from melanoma patients undergoing adjuvant type I IFN therapy was shown (8). Notably, the treatment was associated with reduced migratory capacity of neutrophils in such patients. Blood-derived neutrophils from melanoma patients upon adjuvant type I IFN treatment significantly downregulate their IL-8 receptor (CXCR1 and CXCR2) expression. It affects neutrophil migration from the BM and is of high clinical importance due to poor prognosis for tumor patients with elevated neutrophil numbers in blood and tumor. Notably, neutrophil amounts in type I IFN treated patients were lower, compared to untreated patients (8).

Immunotherapy with alpha IFNs is used for patients with different types of malignancies. Nevertheless, its efficacy is limited and only a small proportion of patients benefit from such treatment. Notably, the level of responsiveness to IFN treatment varies among individuals. This might be due to genetic polymorphism in type I IFN-related genes that have been shown to exert a significant impact on survival and therapy outcome in melanoma patients (61). Importantly, humans with impaired type I IFN signaling, due to STAT2 deficiency, have been identified (62). Another reason for impaired therapy response could be a suppression of pathways involved in IFN signal transduction in different microenvironment conditions, e.g., in GM-CSF presence (63). One of the factors reflecting the sensitivity of neutrophils to IFN-α therapy is a study by Azuma et al. showing a favorable survival predictive response correlated with a decrease

TABLE 1 | Type I IFN-dependent regulation of neutrophil polarization in cancer.

	Sufficient type I IFN signaling	Impaired type I IFN signaling
Polarization of neutrophils	**N1 anti-tumor**	**N2 pro-tumor**
THE TURNOVER AND THE LIFESPAN OF NEUTROPHILS		
Neutrophil expansion in bloodstream and tumor	↓	↑
Expression of G-CSF by neutrophils	↑	↓
Expression of pro-apoptotic factors by neutrophils (caspase 3, TNFα, Fas, ROS production)	↑	↓
Expression of antiapoptotic factors by neutrophils (Bcl-XL)	↓	↑
MIGRATION OF NEUTROPHILS TO THE TUMOR SITE		
Neutrophil expansion in bloodstream and tumor	↓	↑
CXCR2 – CXCL1, CXCL2 axis activation	↓	↑
CXCR4 – CXCL12 axis activation	↓	↑
CD62L expression on circulating neutrophils	↓	↑
KILLING OF TUMOR CELLS		
Neutrophil cytotoxicity against tumor cells	↑	↓
ROS production by TAN	↑	↓
Neutrophil extracellular traps formation	↑	↓
REGULATION OF ADAPTIVE IMMUNE RESPONSE		
Expression of co-stimulatory molecules (ICAM-1)	↑	↓
Expression of cytokines (TNFα)	↑	↓
ANGIOGENESIS AT THE TUMOR SITE		
The number of fully developed vessels in the tumor	↓	↑
Expression of VEGF, MMP9 by TAN	↓	↑
CXCR2 – CXCL1, CXCL2 axis activation	↓	↑
CXCR4 – CXCL12 axis activation	↓	↑
ROS production by TAN	↑	↓
FORMATION OF THE PRE-METASTATIC NICHE		
Metastatic load in organs	↓	↑
Accumulation of neutrophils in metastatic organs	↓	↑
Expression of pro-metastatic proteins (Bv8, S100, and MMP9)	↓	↑
CXCR2 – CXCL1, CXCL2 axis activation	↓	↑

Neutrophils may be biased into N1 antitumor and N2 pro-tumor cells in tumor situation. The activation and differentiation of these cells during tumorigenesis is determined by the growth factor and cytokine milieu in the tumor. Type I interferons are potent drivers of the transition into antitumor N1 phenotype.
GCSF, granulocyte colony-stimulating factor; TNFα, tumor necrosis factor alpha; ROS, reactive oxygen species; TAN, tumor-associated neutrophils; VEGF, vascular endothelial growth factor; MMP9, matrix metallopeptidase 9.

in the number of circulating neutrophils after IFN-α treatment in patients with metastatic renal cell carcinoma (150).

CONCLUDING REMARKS

Type I IFNs are one of few cytokines known to alter polarization of neutrophils in tumor-bearing hosts. IFNs drive neutrophils to an antitumor and antimetastatic phenotype in numerous ways: due to restriction of neutrophil survival and migration to tumor site, *via* enhancement of neutrophil cytotoxicity and NETs formation, suppression of pro-angiogenic properties of neutrophils, and inhibition of the pre-metastatic niche formation by these cells (**Figure 1**; **Table 1**). Importantly, IFNs need initial trigger, such as inflammation accompanying tumor growth, to exert their neutrophil polarizing effect. In healthy, tumor-free mice, no alteration in neutrophil activation and polarization due to IFNs could be observed. Inflammation and cytokine milieu in tumor, together with functional type I IFN signaling, are responsible for subsequent alteration of neutrophil activation leading to modifications of their phenotype into tumor inhibiting. In the situation when IFN signaling is disturbed, neutrophils are polarized into pro-tumor phenotype and effectively support tumor growth. Thus, the vicious circle enhancing tumor progression and metastasis is formed. In this situation, restoring the pool of type I IFNs by using exogenous treatment should modulate neutrophilic phenotype providing therapeutic option to overcome neutrophil-mediated immunosuppression thus leading to the restriction of tumor growth.

AUTHOR CONTRIBUTIONS

EP: drafting of the manuscript, writing of the manuscript, final approval of the submitted version, agreed to be accountable for all aspects of the work. SL: writing of the manuscript, final approval of the submitted version, agreed to be accountable for all aspects of the work. JJ: concept and design of the manuscript, drafting and writing of the manuscript, final approval of the submitted version, agreed to be accountable for all aspects of the work, submission of the manuscript, and corresponding author.

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REVIEW
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Interferon Control of the Sterol Metabolic Network: Bidirectional Molecular Circuitry-Mediating Host Protection

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The sterol metabolic network is emerging center stage in inflammation and immunity. Historically, observational clinical studies show that hypocholesterolemia is a common side effect of interferon (IFN) treatment. More recently, comprehensive systems-wide investigations of the macrophage IFN response reveal a direct molecular link between cholesterol metabolism and infection. Upon infection, flux through the sterol metabolic network is acutely moderated by the IFN response at multiple regulatory levels. The precise mechanisms by which IFN regulates the mevalonate-sterol pathway—the spine of the network—are beginning to be unraveled. In this review, we discuss our current understanding of the multifactorial mechanisms by which IFN regulates the sterol pathway. We also consider bidirectional communications resulting in sterol metabolism regulation of immunity. Finally, we deliberate on how this fundamental interaction functions as an integral element of host protective responses to infection and harmful inflammation.

Keywords: cholesterol, sterol, interferon, metabolism, miRNA, oxysterol, 25-hydroxycholesterol, miR-342-5p

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INTRODUCTION

Immunity depends on and employs metabolic pathways for its function. Our knowledge of the molecular and functional mechanisms for this coupling has grown dramatically in recent years and it is now accepted that a remodeling of glycololytic, lipid biosynthetic, and associated homeostatic molecular "circuitry" is an integral component of innate and adaptive immune responses (1–3). In particular, multiple immune-mediated mechanisms for the transcriptional, posttranscriptional, translational, and posttranslational regulation of lipid biosynthesis, storage, influx, and efflux in immune cells have been described (4–7). Broadly, with some notable exceptions, these mechanisms have been defined *in vitro* in specific cell types (e.g., macrophages) and their general significance and relative importance *in vivo* have yet to be fully characterized.

Abbreviations: 7α,25-HC, 7α,25-dihydroxycholesterol; 25-HC, 25-hydroxycholesterol; CH25H, cholesterol 25-hydroxylase; EBI2, EBV-induced G-protein coupled receptor 2 (also known as GPR183); HCV, hepatitis C virus; HDL, high-density lipoprotein; HIV-1, human immunodeficiency virus 1; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; HSV1, herpes simplex virus type 1; IAV, influenza A virus; IFN, interferon; IRF, interferon regulatory factor; LDL, low-density lipoprotein; LXR, liver X receptor; MCMV, murine cytomegalovirus; MHV-68, murine gammaherpesvirus 68; SREBP2, sterol regulatory-binding protein 2; SREBF2, sterol regulatory-binding transcription factor 2; TLR, toll-like receptor; VSV, vesicular stomatitis virus; VZV, varicella zoster virus; WNV, West Nile virus.

Immediately after infection, the ligation of cellular pattern-recognition receptors by, for example, dsRNA leads to an induction of NFkB, ATF2/c-jun, and interferon regulatory factor 3 (IRF3), a rapid upregulation of IFNα/β gene expression and secretion of type I IFNs by cells. The autocrine/paracrine binding of IFNα/β or IFN-γ (from activated NK and T cells) to type I or type II IFN receptors, respectively, leads to the activation of JAK/STAT signaling pathways and rapid alterations in the abundance of hundreds of transcripts in the cell. These IFN-stimulated changes reflect an acute re-programing of the cell to resist infection and limit cellular damage. **Figure 1** shows a high-resolution temporal (every 30 min for the first 12 h) analysis of genome-wide alterations in gene expression upon IFN-γ activation of bone marrow-derived macrophages. Importantly, alongside many IFN-stimulated genes, this data reveal an equivalent number of transcripts are significantly suppressed by IFN.

While interferon (IFN)-stimulated genes (ISG) such as *NOS2*, *OAS2*, *MX2*, and *IFITM3* have intensively investigated antiviral or antibacterial effects, IFN downregulated transcripts have received relatively little attention (8–11). Notably, a statistical over-representation analysis of the IFN suppressed genes presented in **Figure 1** identified the sterol metabolic network as a significantly over-represented component of this dataset. Importantly, consequent mechanistic studies demonstrated that a suppression of sterol biosynthesis is an integral component of the innate immune response to infection (4). This work raised several significant questions about the coupling of sterol metabolism to immunity. In particular, what are the molecular mechanisms by which IFN mediates a downregulation of the sterol biosynthesis pathway and how does the suppression of sterol biosynthesis benefit the infected host? Recent studies are beginning to answer some of these questions.

Here, we first discuss early clinical work showing iatrogenic effects of IFN on sterol metabolism. Next, with an emphasis on molecular oxysterol and miRNA-mediated mechanisms, we consider what is known about how IFN regulates sterol metabolism. Overall, we advance the notion that the mevalonate–sterol pathway is an effector arm of immunity and highlight how this response helps the host limit excessive inflammation and resist infection.

HYPOCHOLESTEROLEMIC EFFECTS OF IFN TREATMENT IN HUMANS

Although interest in IFN-mediated regulation of the sterol pathway has increased dramatically in recent years, IFN-induced alterations in cholesterol in humans have been reported for several decades (**Table 1**). In 1979, Baillie and Orr reported that acute viral infections are regularly associated with reductions in

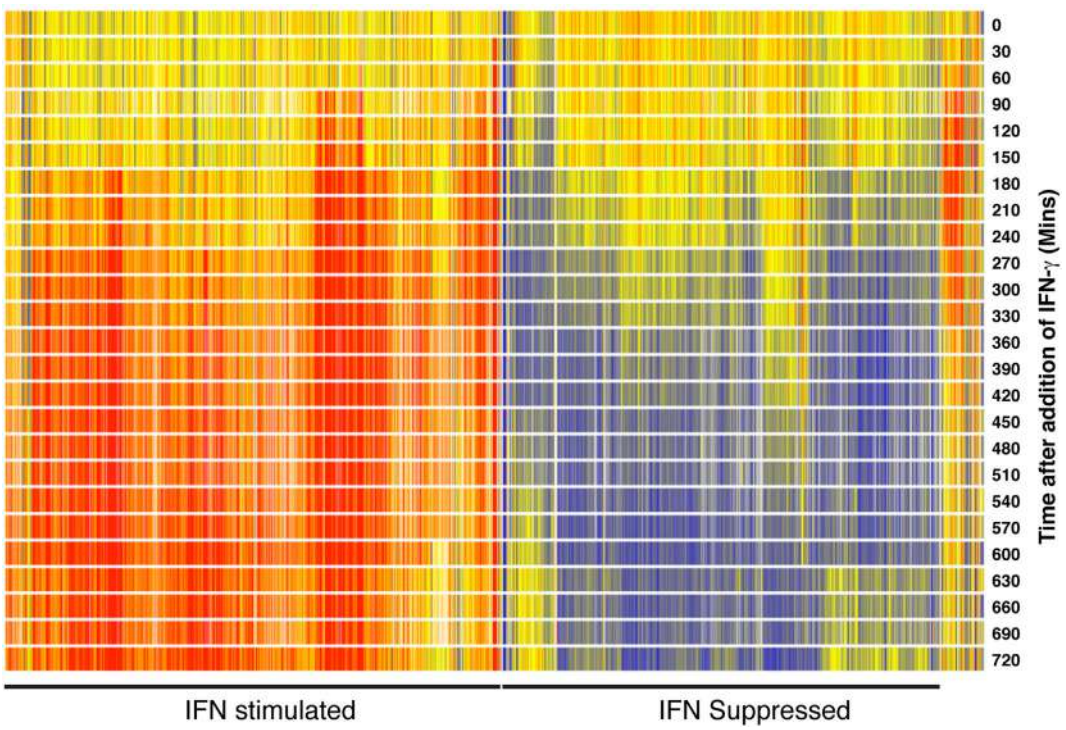

FIGURE 1 | Heat map showing 1,048 genes significantly increased or decreased in expression after interferon (IFN) simulation of macrophages. Bone marrow-derived macrophages were mock treated or treated with 10 U/ml IFN and then sampled at 30-min intervals for a period of 12 h. Total RNA was then labeled and hybridized to Mouse Agilent V2 (G4121A) microarrays. Gene expression is shown as a pseudo-color—blue, decrease; red, increase. Explorative and statistical analyses were undertaken as previously described (4). Data are available for download from the NCBI gene expression omnibus (https://www.ncbi.nlm.nih.gov/geo/) (series GSE42504).

TABLE 1 | Representative clinical studies reporting decreases in cholesterol following treatment with type 1 or 2 IFN.

IFN type	Year	Treatment	Disease context	Observation	Reference
Partly purified human leukocyte IFN	1980	1× healthy male volunteer: 10× daily SC injections of 3×10^6 IU. Two further volunteers: 1× SC of 3×10^6 IU, then $3 \times 1.5 \times 10^6$ IU on consecutive days	Healthy volunteer	Drop in high-density lipoprotein (HDL) cholesterol in all volunteers 7 days after treatment	(13)
Human leukocyte IFN	1981	3×10^6 IU IFN IM daily for 1 week. 6 × healthy male	Healthy volunteer	Total and HDL plasma cholesterol decreased in all 6 subjects	(17)
Human IFN-α prepared from buffy coat leukocytes rIFN-αA (Hoffman–LaRoche Inc., Nutly, NJ, USA)	1984	Daily IM injection of 3×10^6–9×10^6 U of (A) over 28–57 days Daily IM injection of 3×10^6–5.4×10^7 U of (B) for 15 days	Cancer	Significant decrease in HDL and total cholesterol	(18)
rIFN-β_{ser} (modified rIFN-β: Ser_{17} substituted for cysteine)	1985	Escalating dose regime: IM and IV injection from 1×10^6 to 4×10^8 U, twice weekly	Cancer	Decrease in serum cholesterol	(19)
rIFN-α2	1986	3×10^7 U/m^2 IV for 5 days consecutively every 3 weeks	Cancer	Significant decrease in plasma cholesterol. Effect specific to low-density lipoprotein (LDL) and HDL. VLDL or triglycerides unchanged	(20)
rIFN-β_{ser}	1987	Patients randomly assigned to 1 of 2 dose regimens. 4.5×10^6 U (3 males and 7 females) or 9×10^7 U (8 males and 3 females) of IFN-β_{ser} IV daily in a double blind manner for 10 days followed by 11 days off	Cancer	Significant dose-dependent decrease in mean plasma total cholesterol and LDL concentrations (24–36 h after initiation of treatment). Approx. 25% reduction in plasma cholesterol concentration after 10 days of treatment	(14)
IFN-α-n1 (Wellferon—highly purified combination of natural human IFNα from lymphoblastoid cells)	1988	9× men received IM treatment	Refractory condylomata acuminata	All patients had significant decrease in HDL cholesterol levels. Total cholesterol decreased—change not significant	(21)
rIFN-β_{ser}	1990	Randomized, double-blind trial of two doses of IFN-β_{ser} (4.5×10^6 and 9×10^7 U). IV injections daily for 10 days with 11 days rest before treatment reinitiated	Cancer	Statistically significant change in cholesterol	(15)
rIFN-γ	1990	29 patients treated IV at doses escalating from 2×10^5 to 10^8 IU/m^2 in 9 successive steps (at least 3 patients/step). Injections of rIFN gamma were repeated every 72 h for 15 days	Cancer	Hypocholesterolemia observed in 18 patients	(22)
rIFN-β_{ser}	1992	4.5×10^6 U daily IV for 5 weeks to normal and hypercholesteremic patients	Hypercholesteremia	Significant 15% reduction of total cholesterol in normal and hypercholesterolemic subjects. IFN induced significant reductions in LDL cholesterol of 25% in normal subjects and of 40% in hypercholesterolemic subjects. Significant decreases in LDL apoB observed only in the normal group	(16)
rIFN-α2b	1995	44 patients were treated with human recombinant interferon (IFN)-alpha 2b (3×10^6 U 3× per week for up to 12 months). 8 control patients	Hepatitis C virus (HCV)	Blood lipids evaluated after 3, 30, and 90 days of treatment. HDL, cholesterol, apolipoprotein A-I, and HDL3 decreased within 4 weeks of starting IFN treatment	(23)
rIFN-α2a	1997	39 patients: recombinant IFN alpha-2a (9×10^6 U/day) administered IM for 2 weeks, and then 3× a week for 6 months	HCV	Serum cholesterol concentration significantly decreased 1 week after start of IFN administration. 67% of reduction attributable to HDL-cholesterol	(24)

(*Continued*)

TABLE 1 | **Continued**

IFN type	Year	Treatment	Disease context	Observation	Reference
rIFN-α2b (Intron A, Schering–Plough, Kenilworth, NJ, USA)	1998	36 patients received therapy with recombinant IFN-α2b for 6 months; 34 patients received 5×10^6 U and 2 patients 6×10^6 U, 3× a week	HCV	Reduction in HDL-cholesterol and apoA1 levels. Total, LDL, and lipoprotein(a) levels unchanged during treatment	(25)
rIFN-β (Frone, Serono, Madrid, Spain)	2000	IFN-β SC (6×10^6 U) 3× a week for 6 months	HCV	Cholesterol concentration decreased slightly in HDL subfractions	(26)
rIFNβ-1a (Avonex; Biogen Idec, Inc., Cambridge, MA, USA) rIFNβ1b (Betaferon—cys_{17} replaced by ser_{17}, lacks met_1 and carbohydrate moieties—Schering, Berlin, Germany) rIFNβ1a (Rebif, Ares-Serono, Geneva Switzerland)	2004	95 patients: 6×10^6 U/week IM and SC IFNβ1a (Avonex) 92 patients: 8×10^6 U IFNβ1b every other day SC 41 patients: 22 μg 3× SC/week. IFNβ1a (Rebif) 25 patients: 3× SC/week 4 μg IFNβ1a (Rebif)	MS	Highly significant sustained decrease (−8%) in mean cholesterol level in plasma of IFN-treated MS patients	(27)
rIFNβ-1a (Avonex; Biogen Idec, Inc., Cambridge, MA, USA)	2006	255 patients were included in the study	MS	Decrease in blood cholesterol	(28)
Peg-rIFN	2016	520 patients treated with pegIFN or combination of IFN-free direct acting antivirals (DAA)	HCV	IFN-based therapy decreased total circulating cholesterol, while IFN-free DAA increased cholesterol levels	(29)

systemic cholesterol in patients (12). Subsequently, Cantell et al. (13) showed that the administration of partly purified human leukocyte IFN to volunteers led to a 20% drop in high-density lipoprotein (HDL), a transient declining trend in total cholesterol and put forward the first proposal that viral infections elicit a drop in cholesterol *via* the induction of IFN (13). **Table 1** presents a chronological summary of wide-ranging studies in which natural and recombinant type I and type II IFNs have been administered to volunteers or patients with cancer, multiple sclerosis, human papilloma, or hepatitis C virus (HCV) infections. In all studies, despite differences in the preparation of IFN used, a drop in circulating total cholesterol and/or HDL was observed. Arguably, the strongest clinical evidence comes from prospective double blind studies such as those reported by Rosenzweig et al. (14) and Borden et al. (15). The former utilized a double blind analysis to demonstrate a dose-dependent effect of IFN administration on plasma cholesterol (14). The latter employed a prospective double blind placebo-controlled analysis of IFN treatment in renal carcinoma patients, demonstrating a significant decrease in mean plasma total cholesterol (15). It is worth noting that Rosenzweig et al. (14) also showed that the effects of IFN were not permanent and that after cessation of treatment circulating cholesterol levels returned to normal in patients (14). In subsequent metabolic tracer experiments, the primary effect of IFN was shown to occur *via* a modulation of cholesterol synthesis (16).

In summary, the induction of hypocholesteremia by IFN has been recorded clinically for many years *via* an analysis of total cholesterol, HDL, or LDL in the circulation. Despite this recognition, physiological roles related to human health and underpinning this observation have not been further investigated.

THE MEVALONATE-STEROL PATHWAY IS AN INTRINSIC COMPONENT OF THE IFN RESPONSE TO INFECTION

Alongside, clinical studies demonstrating exogenously administered IFN can regulate sterol metabolism, a number of groups have also associated cholesterol regulation with IFN responses in experimental animal studies. In 1984, Kuo et al. showed that IFN-inducing agents significantly reduced cholesterol deposits in the aortas of rabbits fed a pro-atherogenic diet (30). Further, in 1987, Pereira et al. showed that a hypercholesteremic diet resulted in an increased susceptibility to murine hepatitis virus in A/J mice—a result in part due to a decreased response to IFN and reduced antiviral state (31). A key question in this context is: *what benefit to the host is conferred by the IFN regulation of sterol metabolism?* While studies prior to 2011 showed that toll-like receptor 3 (TLR3) or TLR4 ligation results in an IFN-independent inhibition of cholesterol efflux from the cell, little was known at this point about how IFN signaling directly influences cholesterol homeostasis and the physiological purpose this could serve (32). In 2011, Blanc et al. demonstrated that viral infection or treatment of macrophages with type I or II IFN results in a coordinate, negative regulation of the entire sterol biosynthesis pathway and that inflammatory cytokines such as TNF, IL-6, and IL1β are incapable of eliciting a similar effect. This study further showed that the regulation of the sterol pathway by IFN is, at least partly, due to a reduction in SREBF2 transcription and SREBP2 abundance and that this event is an integral component of the cell-intrinsic antiviral response (4). Notably, a recent study highlighted the

interdependent reciprocal nature of the molecular circuitry coupling IFN and sterol metabolism. In 2015, York et al. described a STING-dependent recognition of decreased flux through the sterol biosynthetic pathway leading to positive feedback that enhances the type I IFN response and antiviral gene expression in the context of gammaherpesvirus infection (33). The implications of this data are discussed later in this review.

FUNCTIONAL ROLES FOR THE MEVALONATE-STEROL PATHWAY IN GOVERNING ADAPTIVE IMMUNE RESPONSES

Beyond the intracellular and/or cell-intrinsic environment, IFN-mediated regulation of sterol metabolism has the potential to influence many aspects of immunity. The functions that the sterol metabolic network plays in a wide range of adaptive immune responses have recently been reviewed (3). These include: an absolute requirement for SREBP2 functionality during activated T lymphocyte clonal expansion, a requirement for flux through the sterol biosynthesis pathway during the activation of T regulatory cell function, the observation that a hypercholesteremia can alter the balance of the Treg and T effector cells, and the induction of lymphocyte hyper-proliferation due to impaired cholesterol efflux (34–38). Cholesterol is also indispensable in the formation of lipid raft microdomains—crucial to the assembly of cell surface signaling molecules such as the T and B cell receptors—and has recently been identified as a critical allosteric regulator of TCR priming (39, 40). Notably, cholesterol is not the only output of the sterol biosynthesis pathway on which cells depend. For example, prenylation of the Ras family of GTPases by the side branch of the mevalonate pathway is integral to the control of T cell differentiation, proliferation, and cytokine production [reviewed in Ref. (41)]. Further, sterol pathway intermediates have also been identified as endogenous ligands for the transcription factor RORγt. RORγt is required for the differentiation of naïve CD4+ T lymphocytes into T_H17 cells, a subset of lymphocytes associated with a range of autoimmune diseases and mediating protective immune responses to pathogens such as *Klebsiella pneumoniae*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, and *Candida albicans* (42–45). Santori and colleagues identified sterol pathway intermediates generated downstream of lanosterol and above zymosterol as natural ligands of RORγt, while Hu et al. suggested that desmosterol (downstream of zymosterol and recently shown to be negatively regulated by IFN) is a potential endogenous ligand for this transcription factor (7, 46). Through the utilization of chemical library screening studies, the oxysterol 7β,26-dihydroxycholesterol (synthesized from 7β-hydroxycholesterol, a metabolite immediately downstream of cholesterol) has also been identified as a potent ligand of RORγt (47). In summary, the sterol metabolic network is increasingly viewed as integral to the activation and differentiation of T lymphocytes. More work, however, is required to better understand the precise mechanisms by which the network and/or specific metabolites function in these processes.

In B lymphocytes, IFN regulation of sterol metabolism may lead to alterations in lipid raft cholesterol composition and, in doing so, affect antigen processing/presentation (48) and B-cell receptor signaling (48–50). Notably, significant roles for the inflammatory sterol pathway product 25-hydroxycholesterol and 7α,25-dihydroxycholesterol in class-switching and the chemoattraction of B lymphocytes to germinal centers have recently been described and these will be discussed in detail later.

BENEFITS OF IFN-MEDIATED STEROL REGULATION DURING INFECTION

An IFN-mediated suppression of sterol metabolism has the potential to directly curtail the replication of microorganisms in the host. Pathogens with a dependency on the host sterol metabolic network include HCV, human immunodeficiency virus (HIV), Ebola, the Herpesvirus family [HCMV, murine cytomegalovirus (MCMV), herpes simplex virus type (HSV1) MHV-68, and varicella zoster virus (VZV)], Influenza A virus (IAV), *Listeria monocytogenes*, and *M. tuberculosis* (51–57). Importantly, the requirements of these organisms on the system vary dramatically. For example, lipid rafts play an integral role in the entry, assembly, and release of a wide range of unrelated viruses (enveloped and non-enveloped) such as HIV1, Ebola, Influenza A, and Echovirus 1 [reviewed here in Ref. (58)]. In contrast, HCMV uses cholesterol for envelopment and limiting the availability of intracellular cholesterol levels has been shown to restrict infectivity of this virus (59). The replication of several viruses also requires prenylation of host and/or virus proteins. For example, hepatitis D virus requires prenylation of its large delta antigen for optimal virion morphogenesis, HCV requires the geranylgeranylated host protein FBL2 for replication, and respiratory syncytial virus (RSV) F glycoprotein binds to the prenylated host protein RHOA enabling membrane fusion (60–62). In the context of bacterial infection, Listeriolysin O, the major virulence factor of the intracellular bacteria *L. monocytogenes*, is a cholesterol-dependent cytolysin (CDC) responsible for a wide array of functions including disruption of the internalization vacuole (54). Further, *M. tuberculosis* has cholesterol uptake machinery, an enzyme system capable of catabolizing sterols for growth and potentially utilizes sterols as a carbon and energy source (55, 63).

It is perhaps unsurprising, given the essential role sterol metabolism plays in their replication, that examples are appearing of pathogens subverting or co-opting the regulation of this system for their own benefit. In 2007, Mackenzie et al. showed that a West Nile virus (WNV) infection of Vero cells induced an upregulation of cholesterol biosynthesis and redistribution of cholesterol resulting in defective IFN-stimulated JAK/STAT signaling. This result was attributed to a disrupted recruitment and activation of the type 1 IFN receptor and IFN signaling proteins and emphasizes the tight integration of IFN and cholesterol regulation in the cell (64).

In summary, evidence increasingly reveals an intimate molecular coupling between IFN signaling and the sterol metabolic network. This underscores the importance of immune-mediated

regulation of sterol metabolism as an integral component of the host response to infection.

CELLULAR MECHANISMS FOR IFN REGULATION OF THE STEROL METABOLIC NETWORK

A prototypic outcome of IFN signaling in the cell is the transcriptional activation or suppression of hundreds of genes. Over the past 5 years, significant progress in characterizing which of these genes contribute to regulation of cholesterol in the cell has been made. In this regard, oxysterol and miRNA-mediated mechanisms have risen to prominence and, in the following sections, we will review what is known about the functions of IFN-elicited CH25H/25-HC and miRNA-mediated sterol regulation. We will then conclude by considering mechanisms of cholesterol regulation by the "conventional" ISG Viperin and the IFITM protein family.

IFN-INDUCED 25-HYDROXYCHOLESTEROL IN INFECTION AND IMMUNITY

While cholesterol is a critical component of cell membranes and a precursor of bile acids and steroid hormones, at high concentrations, it may be toxic to the cell. Intracellular cholesterol homeostasis is, therefore, stringently controlled by tightly coupled regulatory mechanisms including influx and efflux, esterification, and storage and biosynthesis (65). Oxysterols are oxygenated forms of cholesterol formed directly from cholesterol (or oxysterols derived from cholesterol) by enzymatic and non-enzymatic mechanisms (55). Functionally, oxysterols such as 22(R)-hydroxycholesterol and 24(S)-hydroxycholesterol potently bind ligand-activated transcription factors liver X receptor (LXR)-α and/or LXR-β and induce the upregulation of cholesterol homeostasis-related proteins such as ABCA1—responsible for cholesterol efflux from the cell (66, 67). Notably, however, despite its identification over 50 years ago and an early demonstration of potent sterol biosynthesis regulatory feedback functionality, until recently, physiological roles for 25-hydroxycholesterol (25-HC) have proven elusive (68, 69).

25-HC binds the INSIG protein in the ER and, in doing so, prevents SREBP2 transport to the golgi/nucleus and cholesterol biosynthesis [reviewed in Ref. (70)]. It is not, however, a strong activator of the LXRs nor does it play a significant role in systemic cholesterol homeostasis *in vivo* (71). In 2009, independent studies showed that CH25H, the enzyme responsible for 25-HC synthesis, is transcriptionally upregulated in macrophages following treatment with a TLR agonist (72, 73). Park and Scott (74) then showed that type I IFNs are also capable of upregulating CH25H (74). While this evidence supported the notion that 25-HC may play a role in immunity, in fact, studies had been emerging for decades implicating 25-HC in the immune response to infection. In 1986, Kournikakis et al. demonstrated that 25-HC can suppress antibody-dependent cell cytotoxicity, and in 1998, Moog et al. showed 25-HC (but not cholesterol) can inhibit HIV *in vitro* (75, 76). Over the next decade, several groups independently investigated the effects of 25-HC on HCV subgenomic replicon replication and found the oxysterol inhibited this process (77–80). Notably, the effects of 25-HC are not restricted to viruses, and in 2006, Howe and Heinzen described a partial inhibition of the bacteria *Coxiella burnetii* following treatment of Vero cells with this oxysterol (81).

BROAD ANTIVIRAL FUNCTIONALITY OF 25-HC

Interest in the regulation and functions of 25-HC (and its derivatives) has increased dramatically in recent years [reviewed in Ref. (82)]. In 2012, Gold et al. demonstrated that ATF3 directly suppresses the transcription of *CH25H* and the production of 25-HC. They further showed that a deletion of ATF3 in APOE$^{-/-}$ mice results in enhanced aortic 25-HC expression and foam cell development (83). In 2013, Blanc et al. showed that 25-HC is the only oxysterol synthesized (and secreted) in significant quantities by murine macrophages after IFN activation and demonstrated that the transcription of *CH25H* is directly regulated by IFN through the binding of STAT1 to its promoter. These studies also showed that physiological levels of 25-HC have a broad antiviral functionality mediated, in the case of cytomegalovirus (CMV), post-entry *via* regulation of the sterol biosynthesis pathway. Data presented by Blanc et al. supported an important role for the prenylation side-branch of the sterol biosynthesis pathway, rather than cholesterol, in mediating antiviral effects against CMV (52). At the same time, Liu et al. (84), using a molecular screening approach, also identified CH25H as an important IFN-stimulated gene and demonstrated a broad antiviral functionality for 25-HC (84). Contrary to the CMV-related work of Blanc et al., however, Liu et al. found this effect was mediated *via* an inhibition of pathogen [vesicular stomatitis virus (VSV) and (HIV)] entry to the cell. The distinct modes of 25-HC action described likely reflect differences between the cell/virus systems examined. Liu et al. further showed that CH25H$^{-/-}$ mice are more susceptible to MHV-68 infection and the therapeutic administration of 25-HC to humanized mice suppressed HIV-induced T cell depletion (84). Together, these studies identified a significant new role for 25-HC as an effector in the immune response to infection and, since 2013, several independent studies have described further roles for 25-HC in this context. In 2014, Roulin et al. showed that 25-HC suppresses picornavirus infections by displacing cholesterol binding to the oxysterol sterol-binding protein (OSBP1). In doing so, 25-HC disrupts a cholesterol-phosphatidylinositol 4-phosphate counter-current essential for formation of the replication organelle at ER–Golgi membrane contact sites (85). Building on early studies investigating sterol pathway regulation, in 2015, Lu et al. showed that, alongside its ability to inhibit SREBP2 migration to the nucleus, IFN-elicited 25-HC induces a rapid proteosomal degradation of HMGCR in macrophages (6, 86). Work has also shown that 25-HC can inhibit a wide range of unrelated enveloped and non-enveloped viruses including

poliovirus, Hepatitis B and C viruses, human papillomavirus (HPV-16), human rotavirus, encephalomyocarditis virus, and SFTS virus (87–92). Recent studies have also revealed more detail regarding the regulation of CH25H. Mboko et al. (93) showed that CH25H expression in mice is, at least partly, dependent on IRF1 and Xiang et al. (94) demonstrated an IFN-independent induction of CH25H in hepatocytes in response to viral infection (93, 94). In this regard, evidence to-date suggests that CH25H gene expression is regulated in a cell-specific manner and it cannot be considered a prototypic ISG. While the majority of studies have broadly focused on the ability of 25-HC to suppress infection *via* regulation of lipid metabolism, data from Shibata et al. (5) suggest that it may also achieve this *via* a specific activation of the GCN2/eIF2α/ATF4 branch of the integrated stress response (ISR) (5).

Notably, recent studies describe direct interactions of both CH25H and 25-HC with gene products of the microorganism. Chen et al. (95) describe a direct interaction between CH25H and NS5A of HCV leading to an inhibition of NS5A dimerization and inhibition of HCV replication (95). More recently, Ren et al. (96) describe an INSIG homolog with predicted 25-HC-binding capacity in the bacterium *Mycobacterium vanbaalenii* (96). What physiological role this would play, however, remains unclear.

In summary, it is now accepted that 25-HC is an important component of the IFN-induced response to infection and a range of studies have identified divergent mechanisms for the inhibition of entry, replication, and exit from the cell.

25-HC AS AN INFLAMMATORY MEDIATOR

Recent evidence has emerged supporting a role for 25-HC as a pro- and/or anti-inflammatory mediator. In 2010, it was demonstrated that 25-HC has the capacity to suppress CCR7 expression and thus impair DC migration (97). Wang et al. subsequently described a 25-HC-elicited RIG-I-dependent induction of IL-8 and Raccosta et al. showed that 25-HC can bind CXCR2 (98, 99). In 2014, Data from Reboldi et al. showed that in macrophages, through its ability to antagonize SREBP, 25-HC can reduce IL-1β expression and inflammasome activation. They further demonstrated that CH25H$^{-/-}$ mice are more sensitive to septic shock and have an enhanced ability to suppress *L. monocytogenes* infection (100). In contrast, Gold et al. (101) describe 25-HC as an amplifier of inflammation, showing a reduction in pro-inflammatory gene expression in poly I:C treated CH25H$^{-/-}$ macrophages and decreased inflammatory pathology in the lungs of Influenza virus-infected mice (101). Further evidence of a pro-inflammatory role for 25-HC has very recently emerged from Jang et al. (102) who describe a role for the oxysterol as an endogenous signal for NLRP3/inflammasome activation during cerebral inflammation (102). At present, therefore, evidence would appear to support multiple roles for 25-HC in the regulation of inflammation.

An important consideration in the analysis and interpretation of 25-HC-related data is the concentration of exogenous oxysterol utilized *in vitro*. Others, and ourselves, have demonstrated that nanomolar concentrations of 25-HC elicit profound effects in primary macrophages, e.g., Ref. (52, 100). In the literature, however, functional roles for 25-HC have been defined after treatment of cells with considerably higher concentrations (e.g., 10–100μM)—for instance (99, 102). Caution should be exercised when interpreting data from experiments utilizing arguably supraphysiological concentrations of the oxysterol. In this regard, more work—in particular *in vivo*—is required to characterize the specific concentrations, circumstances, locations, and times at which pro- or anti-inflammatory effects are observed during infection.

CH25H AND ACQUIRED IMMUNE RESPONSES

In 2009, Bauman et al. described a role for 25-HC in the direct repression of B cell proliferation and immunoglobulin class switching (72). Oxysterols are often subject to consecutive modifications in order that a functional effector molecule can be synthesized, and in 2011, two groups identified 7α,25-HC—generated *via* the hydroxylation of 25-HC by CYP7B1—as a ligand for the receptor EBI2 (71, 82, 103, 104). While studies of 25-HC have broadly focused on its production and function in macrophages, 7α,25-HC is primarily synthesized in radiation resistant stromal cells (105). The 7α,25-HC receptor EBI2 is expressed throughout the immune system and, to-date, has been shown to play a critical role in B lymphocyte and dendritic cell biology. In B lymphocytes, EBI2 binding of 7α,25-HC, induces a series of temporally regulated migratory events. B cells first move to the outer follicles of lymphoid tissues, then migrate to the T cell margin, and finally move to interfollicular regions before EBI2 is downregulated and germinal centers form (82, 106). Ultimately, binding of 7α,25-HC to EBI2 and subsequent B cell repositioning events are crucial to antibody responses and CH25H$^{-/-}$ mice have reduced IgG and IgM responses to T cell-dependent antigens (107, 108). In dendritic cells, EBI2 and 7α,25-HC are also crucial and determine cellular migration/location and ability of these cells to support CD4 and B cells responses to blood borne antigens (82, 109).

Importantly, roles for 25-HC and downstream metabolites in the regulation of T lymphocyte responses are emerging. In 2014, data from Chalmin et al. suggested that 7α,25-HC may direct the migration of activated T cells into the CNS in a model of autoimmune encephalomyelitis. Further, a very recent study from Li et al. (110) has described a role for 25-HC in T helper cell development. Specifically, through an interaction with T lymphocyte EBI2, 7α,25-HC functions to orientate T cells at the interface of the follicle and the T cell zone. In doing so, it promotes T_{FH} cell differentiation by facilitating interactions first between the lymphocytes and ICOSL^{HI} CD25^{+} dendritic cells and subsequently between lymphocytes and B cells (110).

Evidence is rapidly accumulating that multiple complementary mechanisms are responsible for the molecular coupling of IFN to sterol metabolism. In this context, Singaravelu et al. (111) recently described an ability of 25-HC to induce the expression miR-185 and, in doing so, regulate host lipid metabolism pathways critical to HCV replication (111). This finding will be discussed in more detail later.

HOW DOES 25-HC SUPPRESS INFECTION?

For almost four decades, a physiological role for 25-HC remained elusive, however, multiple lines of evidence now show that the direct induction of *CH25H* expression and 25-HC synthesis by IFN is a fundamentally important feature of immune responses to infection. A key unanswered question is: *what are the mechanisms by which 25-HC can suppress infection?*

Studies to-date have primarily utilized 25-HC as a research tool to study the functional role of cholesterol homeostasis and its effects on membrane composition, vesicular trafficking, and isoprenylation. The addition of exogenous side-chain oxysterols such as 25-HC to cells is known to elicit trafficking of cholesterol from the membrane to the ER—an event that may perturb membrane architecture and the orientation and composition of, for example, lipid rafts (112). *Via* an interaction with OSBP1, 25-HC is also known to disrupt a cholesterol-phosphatidylinositol 4-phosphate "counter-current" required for ER to golgi cholesterol transport and Rhinovirus replication (85). In 2013, Liu et al. found 25-HC-inhibited membrane fusion and cellular infections by HIV, Ebola, and HCV (84). In contrast, Blanc et al. (52) found that 25-HC had a minimal effect on MCMV entry and, by utilizing a mathematical model, estimated that entry-related effects of this oxysterol account for only 10% of its overall antiviral activity for this virus. Data from Blanc et al. (52) support the view that 25-HC primarily exerts its effects by limiting mevalonate–sterol biosynthesis pathway flux. In particular, flux associated with the isoprenoid branch is responsible for protein prenylation (52). Prenylation refers to the posttranslational modification of proteins by the covalent addition of farnesyl (C_{15}) or geranylgeranyl (C_{20}) to conserved amino acid motifs and is key to protein–membrane interactions/intracellular localization of, for example, the Rab proteins. The Rab GTPase superfamily has more than 20 members playing essential roles in vesicle trafficking and protein localization in the cell. Prenylation is the key to this function as it allows attachment of the protein to the lipid bilayer. An ability to inhibit the prenylation and, therefore, the function of Rab GTPases may allow 25-HC to influence a wide range of pathogens that depend on or utilize this family of proteins. For example, Rab11 is key to the recycling endosome pathway in cells and plays a critical role in the assembly of multiple negative strand RNA viruses such as respiratory syncitial virus, Influenza A, and Sendai virus (113–115). Notably, the direct prenylation of pathogen proteins is also an important event in some bacterial infections. For example, the PelH and AnkB proteins of *Legionella pneumophila* are known to require farnesylation, while SifA of *Salmonella typhimurium* requires geranylgeranylation for membrane association (116–118). The effects of 25-HC on these bacteria have yet to be characterized. Given the complexity of the Rab superfamily and the differential dependency of a range of pathogens on its functions, more work is required to determine whether 25-HC effects are mediated *via* this route.

An intriguing possibility is that microorganisms may exploit the disruption of Rab prenylation by 25-HC. Rab5a contributes to lysosomal degradation of *L. monocytogenes* in macrophages and a disruption of its geranylgeranylation in this context may prove advantageous to the bacterium. In this regard, data show that the growth of *L. monocytogenes* is greater in WT macrophages when compared to their $CH25H^{-/-}$ counterparts (100).

While several studies have identified an important role for 25-HC *in vivo,* questions remain regarding: where and when 25-HC is synthesized after infection, how 25-HC synthesis is regulated in different anatomical locations, functional *in vivo* intra- and extracellular concentrations, half-life in the tissues and circulation and therapeutic potential (84, 108). In 2014, Ikegami et al. analyzed oxysterol concentrations in serum of patients with chronic HCV infection and found 25-HC levels 44% greater than those in the controls. Notably, 25-HC levels *decreased* significantly in these HCV-infected patients after they had received PEGylated IFN and Ribavirin therapy for a period of 3 months (119).

The presence of INSIG homologs with potentially conserved 25-HC-binding capacity in bacteria and yeast raises the intriguing possibility that the oxysterol can directly influence these organisms. An incomplete understanding of sterol metabolism in these organisms and, in some cases, an absence of SREBP, SCAP, or HMGCR homologs makes progress in this field challenging at present (96).

IFN-INDUCED miRNA REGULATION OF STEROL METABOLISM IN INFECTION AND IMMUNITY

miRNA are small (20–25 nt) RNA encoded in introns, exons, and intergenic regions of the mammalian genome and are typically co-expressed with a protein-coding or non-coding primary transcript. miRNA function to regulate gene expression *via* imperfect base-pairing to the 3′UTR of mRNA which results in the targeting of the transcript for degradation and/or an inhibition of translation. A key functional characteristic of miRNA is that they can target and regulate the expression of multiple transcripts in the cell. Since the discovery that miRNA, in particular miR-33, function to regulate cholesterol homeostasis, interest in this area has grown dramatically and more than 20 miRNAs are now known to directly target the sterol metabolic network [reviewed in Ref. (120)]. Notably, a small number of sterol-associated miRNA have been shown to be IFN regulated and a subset of these also contribute to the immune response to infection. Here, we will review what is known about these IFN-regulated sterol regulatory miRNA and discuss the mechanisms employed to inhibit pathogens.

STEROL PATHWAY TARGETING BY miR-342-5P GENERATES BROAD ANTIVIRAL IMMUNITY

miR-342 is encoded in an intron of the Ena-vasodilator-stimulated phosphoprotein gene (*EVL*) in the mouse or Ena-Vasp-Like (*EVL*) gene in the human and co-transcribed with this transcript. Transcription of miR-342 can be induced by all-trans retinoic acid or IFN and suppressed by CpG island methylation upstream of *EVL*. Processing of the primary transcript results in the production of a pre-miRNA hairpin encoding two functional

miRNA—miR-342-3p and miR-342-5p (7, 121–124). *In vivo*, the *EVL* transcript is primarily expressed in cells of the immune and nervous systems, however, a systematic tissue and cell-type analysis of miR-342 expression has yet to be undertaken (125). In macrophages, miR-342 has been identified as a PU.1-regulated miRNA contributing to myeloid differentiation and miR-342-5p shown to be a pro-inflammatory mediator capable of enhancing miR-155 expression (123, 124). miR-342-5p has recently been implicated in the regulation of SREBP1 and SREBP2 in a cancer cell line; however, biological roles and precise mechanisms for the miRNA in relation to sterol biosynthesis and the immune response were not addressed (126). In this regard, we recently demonstrated that, in BMDM, miR-342-5p is directly regulated by IFN *via* IRF1 (7). We further showed that miR-342-5p directly targets the master transcriptional regulator of the pathway SREBP2, multiple members of the sterol biosynthesis pathway including (*IDI1* and *SC4MOL*) and can reduce miR-33 abundance in the cell (7). In doing so, miR-342-5p contributes to IFN-induced suppression of the sterol metabolic network—reducing the abundance of both sterol pathway metabolic intermediates and total cholesterol in macrophages. Notably, IFN-induced miR-342-5p suppression of the sterol metabolic network enables this miRNA to inhibit the replication of unrelated viruses including Influenza A and HSV1 (7). This study, in conjunction with our previous analysis of the antiviral effects 25-HC, highlights the complex, temporally coordinated, and redundant molecular circuitry utilized by the cell to regulate the sterol metabolic network during infection.

A summary of the molecular circuitry underlying the regulation of sterol metabolism by IFN is presented in **Figure 2**. In murine BMDM, *CH25H* mRNA expression is regulated by STAT1 and increases dramatically in the first half hour after activation of cells by IFN (52). In contrast, miR-342-5p expression increases from 2 to 3 h after IFN-γ activation of BMDM and is regulated by IRF1. Data from others and ourselves suggest, therefore, that a sequential IFN-elicited regulation of the sterol metabolic network exists in which 25-HC provides a rapid mechanism for decreasing sterol biosynthesis. It does this *via* an immediate proteosomal degradation of HMGCR and subsequent inhibition of SREBP2 nuclear translocation. This leads to a suppression of viral entry and/or replication *via* an inhibition of sterol pathway flux—an outcome that will also activate STING to further stimulate type I IFN production. Since IFN-stimulated ATF3 swiftly inhibits CH25H transcription and 25-HC is rapidly catabolized, the CH25H response is primarily effective in limiting sterol synthesis for the first few hours of IFN induction. Importantly, however, miR-342-5p then further promotes a longer, sustained fine-tuning of sterol metabolism, and antiviral effects in the cell by targeting *SREBF2* RNA and transcripts encoding select enzymes of the sterol biosynthesis pathway (e.g., *IDI1* and *SC4MOL*). In this role, miR-342-5p complements and reinforces the antiviral functions of the rapidly induced oxysterol 25-HC on sterol biosynthesis.

While *in vitro* data show that an inhibition of endogenous miR-342-5p can reduce the antiviral effects of exogenous IFN by 40–50%, the relative importance of this miRNA—and also 25-HC—*in vivo* are not known. A critical next step, therefore, will be the production of single miR-342-5p and combined miR-342-5p/CH25H KO murine models in which the individual and combined functions of the miRNA and oxysterol can be tested in the context of infection.

miR-122 POSITIVELY REGULATES CHOLESTEROL, FACILITATES HCV REPLICATION, AND IS DOWNREGULATED BY IFN

Arguably, the first miRNA associated with IFN responses to infection and the regulation of cholesterol metabolism was miR-122. miR-122 is a tissue-specific miRNA highly expressed in hepatocytes in which it constitutes around 70% of all miRNA present in the cell. In agreement with this strict tissue-specific expression, we failed to detect miR-122 in resting or IFN-activated bone marrow-derived macrophages (7). In 2005, Jopling et al. (127) demonstrated a direct interaction between miR-122 and the 5′ region of the Hepatitis C genome and showed that this interaction results in the facilitation of viral replication (127). Subsequently, a role for miR-122 in the regulation of lipid metabolism was revealed when the administration of an antisense oligonucleotide ("antagomir") to mice resulted in a significant reduction in circulating cholesterol levels (128, 129). Further analyses of miR-122 KO animals confirmed an absence of miR-122 results in reduced plasma cholesterol levels; however, it remains unclear what the specific sterol-related targets of miR-122 are and how this miRNA acts to regulate systemic cholesterol levels (130, 131). Notably, in 2007, miR-122 was identified as an IFN-regulated miRNA whose abundance decreased by around 80% in Huh cells treated with IFNβ (132). Pedersen et al. further showed that the transfection of a miR-122 inhibitor into cells could suppress HCV replication with a similar magnitude of regulation to that induced by IFNβ alone (132). This and other findings have led to the development and *in vivo* testing of therapeutic miR-122 inhibitors that show promise for the treatment of chronic HCV infection (133, 134).

In summary, while miR-122 couples IFN to the regulation of sterol metabolism and, by direct interaction with the viral genome, plays a fundamental role in the replication of HCV, it is currently unknown whether the IFN suppression of miR-122 directly influences circulating cholesterol levels and whether this plays a role in modulating host responses to infection.

miR-185 IS REGULATED BY 25-HC AND INHIBITS VIRUS REPLICATION BY TARGETING LIPID METABOLISM

Recent evidence suggests that miR-185 functions to regulate sterol metabolism in the liver during an immune response to infection. In the absence of an infection or IFN treatment, data from hepatocytes show that miR-185 expression is downregulated when cholesterol is depleted *in vitro* and that expression of this miRNA is directly regulated by SREBP1c *via* a single sterol response element in its promoter (135). Multiple studies have further shown that this miRNA can regulate *SR-BI*, *SREBP1c*, *SREBP2*, *HMGCR*, *and LDLR* transcript abundance (126, 136, 137). Notably, in 2015, Li et al. showed that HCV can

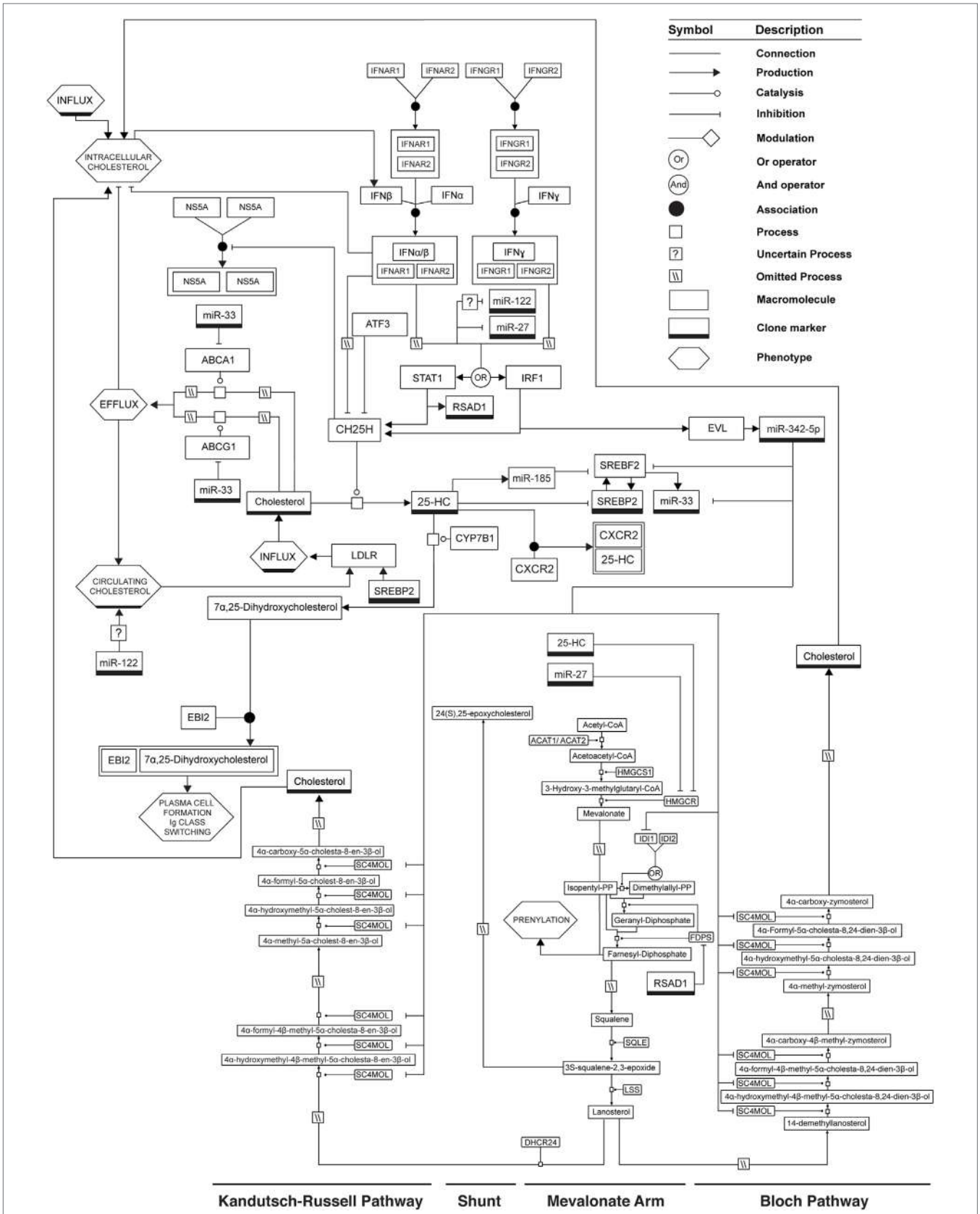

FIGURE 2 | **Mechanisms by which IFN can regulate the sterol metabolic network**. See legend for glyph notation.

upregulate SREBP2 *via* a core protein-mediated suppression of miR-185 (138) while Singaravelu et al. demonstrated that miR-185 expression in hepatocytes is upregulated by 25-HC and restricts HCV replication *via* a repression of cellular lipid uptake and biosynthesis (111). Data suggest, therefore, that miR-185 is antiviral, indirectly upregulated by IFN through 25-HC and exerts its effects (at least in hepatocytes) *via* a suppression of the sterol metabolic network. This mode of regulation was not detected in activated or infected murine macrophages and the general antiviral significance of these observations in alternative cell types has yet to be tested (7). Importantly, however, these data strongly support previous findings demonstrating a membrane-independent antiviral mechanism for 25-HC.

miR-27 INTEGRATION OF IMMUNITY AND LIPID METABOLISM

Over the past decade, functional roles for miR-27 have been investigated in the context of several viral infections. In this regard, significant attention has focused on the ability of Herpesvirus saimiri and murine CMV to induce a reduction in miR-27 abundance and the virus-related mechanisms mediating this reduction are now relatively well characterized (139–142). Notably, the functional consequences and benefit to the Herpesviruses of this reduction are incompletely understood with studies focusing on a suppression of miR-27-inducing constitutive T cell activation (Herpesvirus saimiri) or the suggestion that this event enhances IL-10 production during MCMV infection (142). Since 2013, several studies have described a miR-27 regulation of lipid (including sterol) metabolism. In 2013, Vickers et al. described a miR-27-mediated reduction in *HMGCR* abundance and a sensitivity of miR-27 to lipid levels *in vivo* (143). Also at this time, Shirasaki et al. described a HCV (but not IFN) induction of miR-27a in hepatocytes and a repression of *ABCA1*, *SREBP1*, and *SREBP2* by the miRNA. They further showed that an inhibition of miR-27 increased cellular lipids/viral replication and an over-expression of the miRNA resulted in a reduction in viral infectivity and enhanced IFN signaling (144). In 2014, Singaravelu et al. showed HCV induction of miR-27 is accompanied by the formation of large, abundant lipid droplets in hepatocytes. Zhang et al. further demonstrated this miRNA directly targets *ABCA1*, *LPL*, and *ACAT1*, and, in doing so, reduces cholesterol efflux/uptake and regulates the balance of free versus esterified cholesterol in THP1 cells (145). Notably, recent work from Zheng et al. (146) describes a type 1 IFN downregulation of miR-27 in macrophages resulting in enhanced SIGLEC1/TRIM27 expression. As a consequence, IFN signaling was suppressed and VSV replication enhanced (146). Taken together, the above studies suggest that miR-27 couples infection-induced IFN responses to the regulation of sterol metabolism. Importantly, however, the significance of miR-27 and the relative importance of its sterol-regulatory effects in the context of specific infections and cell types are, incompletely understood. In this regard, we and others have demonstrated that MCMV is dependent on the sterol metabolic network for its replication. It may be hypothesized, therefore, that a suppression of miR-27 functions to upregulate the sterol metabolic network and, in doing so, enhances viral replication.

Alongside the examples discussed above, several other miRNAs hold promise as IFN-regulated modulators of the sterol metabolic network. Others, and ourselves, have demonstrated an IFN-elicited downregulation of miR-33 (7, 147). Recent work from Lai and colleagues demonstrates that miR-33 promotes pro-inflammatory signaling *via* an ABCA1/ABCG1 augmentation of lipid raft microdomains (147). IFN-mediated downregulation of this miRNA, therefore, may serve to suppress the ongoing inflammatory response. Both viral infection and IFNγ can induce the expression of miR-19b—known to target *ABCA1* (148, 149). Further, type 1 IFN (and hepatitis B virus) suppress the expression of miR-145—a miRNA known to target *ABCA1* and HPV and play a role in the progression of atherosclerosis (150–154).

In summary, through their ability to simultaneously regulate multiple genes and propensity for fine-tuning rather than the induction of dramatic alterations in RNA expression, miRNA are ideally suited to the task of coordinating protective functions of the sterol metabolic network (on which the cell depends). Notably, work to-date supports cell- or tissue-specific expression of IFN-regulated miRNAs. In this regard, a great deal is still unknown about where and when IFN-regulated miRNA are expressed *in vivo*, how they are regulated by IFN and what their targets are in particular cell types in distinct species. In this regard, knockout miRNA models remain relatively scarce. There is a pressing need, therefore, for the development of new models to enhance our understanding of sterol regulatory miRNA and the roles they play in host protection against infection.

ISG REGULATION OF THE STEROL METABOLIC NETWORK DURING INFECTION

While this review has focused on IFN-elicited oxysterol and miRNA-related mechanisms, several "conventional" ISG, integral to the cellular response to infection, also elicit their effects *via* the sterol metabolic network.

VIPERIN

Work characterizing the IFN-regulated gene Viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible, or *RSAD2*) has demonstrated that this protein can inhibit both RNA and DNA viruses (155–158). Viperin can decrease HCMV late gene expression, block the release of Influenza A and HIV-1 particles from the cell, and limit the replication of HCV, dengue virus, and WNV [reviewed in Ref. (159)]. Importantly, *in vitro* data show that Viperin inhibits Influenza A budding by disrupting cell membrane lipid raft integrity and increasing membrane fluidity. A key feature of this mechanism is the binding of Viperin to the sterol pathway enzyme farnesyl diphosphate synthase (FDPS)—an enzyme integral to sterol biosynthesis and the processes of farnesylation and geranylgeranylation (156, 158). Together, these observations suggest Viperin functions to inhibit Influenza A release *via* regulation of the sterol metabolic

network; however, a precise mechanism has yet to be determined. Unlike Influenza A and other viruses such as HIV-1 and Ebola, HCV does not bud from lipid rafts. HCV does, however, have an intimate relationship with cellular lipid metabolism—in particular, a dependency on lipid droplets. It has been suggested, therefore, that Viperin may inhibit HCV replication by altering the lipid composition of these droplets *via* its interaction with FDPS. This has not, however, been confirmed (160). Very recently, a TLR4/IRF3-dependent Viperin-induced reduction in membrane cholesterol and sphingomyelin was found to be key to the inhibition of Rabies virus replication in RAW264.7 cells (161). Taken together, the above studies highlight the importance of Viperin as a very early IFN-induced antiviral protein. While our mechanistic understanding is incomplete, it is notable that Viperin exerts at least some of its effects *via* the specific targeting of a key enzyme in the sterol metabolic network and a profound alteration of cellular membrane composition. Further work is required to confirm a conclusive link between these two observations and investigate mechanisms by which pathogens can exploit this protein for their own benefit (162).

IFITM3

While the IFN-inducible transmembrane (IFITM) proteins were first described some 20 years ago, their antiviral properties remained unknown until 2009 when Brass et al. demonstrated a functional role in cellular resistance to Influenza A, WNV, and Dengue virus (163). Since 2009, a plethora of studies have demonstrated the importance of IFITM proteins in suppressing virus-related morbidity and mortality and have characterized roles for the IFITM proteins in responses to a range of enveloped and non-enveloped viruses [reviewed in Ref. (164)]. Much of this work has focused on the ability of IFITM proteins to inhibit viral entry and/or the very early stages of viral replication. In this regard, in 2013, Amini-Bavil-Olyaee et al. demonstrated that IFITM1, 2, and 3 interact with vesicle-associated membrane protein A (VAPA) (165). VAPA is a highly conserved protein, generally found in the ER and, importantly, known to play a role in cholesterol homeostasis *via* its interaction with OSBP. Under normal circumstances, OSBP is found in the cytoplasm where it serves as a cholesterol sensor and, together with VAPA, functions to redistribute cholesterol from the ER to other organelles in the cell. Amini-Bavil-Olyaee et al. (165) found that an IFITM-mediated disruption of the VAPA–OSBP interaction results in cholesterol levels increasing dramatically in late-endosomal compartments. They attributed a block in VSV release into the cytosol to this alteration in membrane composition (165). Notably, subsequent studies suggest an IFITM-mediated regulation of SNAREs and/or regulation of protein lateral mobility may explain the ability of these proteins to inhibit IAV entry to the cell (166). Interestingly, Munoz-Moreno et al., very recently, described a role for IFITM2 in protecting Vero cells against African Swine Fever Infection—a DNA virus (167). In agreement with previous work, they also described an IFITM-associated accumulation of cholesterol in cells, however, it remains unclear whether an IFN-induced IFITM-mediated regulation of the sterol metabolic network plays a direct role in the antiviral functions of this family of proteins.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Three decades after Cantell and colleagues speculated that IFN regulates sterol metabolism, the first evidence for a molecular coupling of IFN to the sterol metabolic network was provided by a systems biology investigation of macrophage responses to infection (4, 13). Given the wealth of clinical and molecular data now available, it is clear that an IFN-mediated reshaping of the sterol metabolic network is an integral, core component of the immune response to infection. The functional outcomes of this event are, however, only beginning to emerge for the wide array of immune-related cells and tissues in the body and are likely to be complex and context dependent. For example, in secondary lymphoid organs, 25-HC is indispensible as an intermediate metabolite crucial to B cell, T cell, and DC migration and antibody class switching. At a cell-intrinsic level, however, this oxysterol can also inhibit viral entry and replication. The latter occurs *via* the regulation of sterol biosynthesis through SREBP2 and HMGCR in macrophages. A key question arising from work to-date is: *how does the sterol metabolic network influence immunity?* Studies now show that the molecular coupling between IFN and sterol metabolism is bidirectional. In this regard, the recent work of York et al. (33) is fascinating as it demonstrates the influence of sterol metabolic flux on antiviral IFN signaling (33). Similarly, Reboldi et al. recently showed that the transcription factor SREBP2, whose function is tightly coupled to cholesterol homeostasis, functions to regulate inflammatory responses to infection and it has been demonstrated that a cholesterol loading of macrophages leads to a reduction in miR-342-5p abundance (33, 100, 168). In this context, relatively little is known about how the microbiome and diet, in particular, cholesterol intake, affect IFN responses to infection and this is an important question for the future.

Work to-date emphasizes the complexity of the molecular circuitry governing the regulation of sterol metabolism by IFN and vice versa. Given IFN directly or indirectly regulates the expression of several hundred genes and the inherent complexity of the sterol metabolic network; it is likely that new bidirectional regulatory mechanisms will continue to appear. For example, we have shown that alongside the posttranscriptional and post-translational effects of miR-342-5p and 25-HC, IFN can also repress SREBF2 transcription. The mechanism for this is unclear, however, epigenetic modifications may play a critical role and this will be important area to pursue in the future.

Given the many recent advances in our understanding of the role sterol metabolism plays in immunity to infection, how can we translate our new knowledge to clinical applications? While changes in systemic cholesterol levels may be of diagnostic value, the therapeutic targeting of host metabolic pathways for anti-infective treatment represents the most exciting application of our knowledge to-date. While statins are a widely utilized, clinically approved, therapy for regulating sterol metabolism and can inhibit a range of pathogens *in vitro*, *in vivo* utility in the context of infectious diseases remains inconclusive. The emergence of new pathogens and threat of antibiotic resistance means it is imperative that we develop new methods for treating

infectious diseases. While studies have explored oxysterol and miRNA inhibitor regulation of sterol metabolism in a preclinical and clinical context, legitimate concerns have been raised about the pharmacokinetics and potential side-effects of both. For example, the miR-122 inhibitor Miraversin can be effectively delivered *in vivo* and substantially reduces HCV replication in a Chimpanzee model. Importantly, however, Miraversin administration is typically accompanied by an increase in circulating cholesterol leading to concerns that the cardiovascular health of recipients may be affected. Further, while miR-342-5p regulates sterol biosynthesis and, in doing so, can suppress viral replication it also targets AKT1 and, as a result, can promote inflammation (124). An important objective, therefore, will be to identify the specific mechanisms by which IFN-induced regulators of the sterol metabolic network function to suppress pathogen replication and specifically target these molecules. In doing so, undesired off-target effects will be reduced. In this context, several groups have already explored prenylation as a viable therapeutic target. Prenylation inhibitors are available as an oral medication and show promise in the treatment of, for example, HDV (169).

In conclusion, the sterol metabolic network has now moved center-stage in the context of IFN responses to infection and is increasingly recognized as a fundamentally important immune-metabolomic system holding great promise in the next decades as target for diagnostic and therapeutic intervention.

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Type III Interferons in Hepatitis C Virus Infection

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The interferon (IFN)-λ family of type III cytokines includes the closely related interleukin (IL)-28A (IFN-λ2), IL-28B (IFN-λ3), and IL-29 (IFN-λ1). They signal through the Janus kinases (JAK)-signal transducers and activators of transcription pathway and promote an antiviral state by the induction of expression of several interferon-stimulated genes (ISGs). Contrary to type I IFNs, the effect of IFN-λ cytokines is largely limited to epithelial cells due to the restricted pattern of expression of their specific receptor. Several genome-wide association studies have established a strong correlation between polymorphism in the region of IL-28B gene (encoding for IFN-λ3) and both spontaneous and therapeutic IFN-mediated clearance of hepatitis C virus (HCV) infection, but the mechanism(s) underlying this enhanced viral clearance are not fully understood. IFN-λ3 directly inhibits HCV replication, and *in vitro* studies suggest that polymorphism in the IFN-λ3 and its recently identified overlapping IFN-λ4 govern the pattern of ISGs induced upon HCV infection of hepatocytes. IFN-λ can also be produced by dendritic cells, and apart from its antiviral action on hepatocytes, it can regulate the inflammatory response of monocytes/macrophages, thus acting at the interface between innate and adaptive immunity. Here, we review the current state of knowledge about the role of IFN-λ cytokines in mediating and regulating the immune response during acute and chronic HCV infections.

Keywords: hepatitis C, IFN-λ3, IFN-λ4, liver, SNP, HCV clearance, SVR

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INTRODUCTION

The interferon (IFN)-λ family of cytokines was first described in 2003 by two independent groups (1, 2). By using computational analysis of unknown genes potentially corresponding to cytokines that were related to interleukin (IL)-10 and type I IFNs, Sheppard et al. identified three new cytokines, IL-28A, IL-28B, and IL-29 (2). Expression of these three cytokines could be induced in peripheral blood mononuclear cells (PBMCs) and other cell types upon poly I:C stimulation or viral infection. Furthermore, these cytokines demonstrated antiviral activity and were shown to bind to a new receptor, IL-28Rα, that forms a heterodimer with IL-10R2. Around the same time, Kotenko et al. also identified three new genes related to the IFN type I and IL-10 families (1). The new cytokines were named IFN-λ1, IFN-λ2, and IFN-λ3 (equivalent to IL-29, IL-28A, and IL-28B, respectively). The newly described cytokines were shown to bind to a new receptor complex composed of IFN-λR1 (equivalent to IL-28Rα) and the IL-10R2, signal through the Janus kinases-signal transducers and activators of transcription (Jak-STAT) pathway, and exhibit antiviral activities *via* the induction of interferon-stimulated genes (ISGs) and upregulation of major histocompatibility complex (MHC) class I. In 2013, a dinucleotide frameshift variant rs368234815 (previously termed ss469415590)

(TT or ΔG) was identified in the IFN-λ region. This frameshift variant was shown to create a novel gene, IFNL4, encoding the IFN-λ4 protein (3). This new protein was related to IFN-λ3 (29.1% identity and 40.8% similarity between both proteins). Expression of IFN-λ4 activated the Jak-STAT pathway and resulted in the expression of ISGs (3). In this article, we will use the nomenclature of IFN-λ genes, protein, and polymorphisms according to the Human Genome Organization Gene Nomenclature Committee. Alternative names for IFN-λ genes and proteins (including IFN-λ specific receptor) are listed in **Table 1**.

TISSUE TROPISM OF TYPE I VERSUS TYPE III IFNs

Type I and type III IFNs are related and may act in parallel *via* the same pathways. Type I IFNs (IFN α/β) can act on multiple cell types and tissues because their specific receptors (IFNAR1 and IFNAR2) are ubiquitously expressed. In contrast, IFN-λR1 expression is rather restricted and as such it affects a much more limited set of cells and exhibits reduced side effects (4). IFN-λR1 is mostly expressed by cells of epithelial origin including hepatocytes (5, 6). However, its expression on hematopoietic cells remains controversial. This issue is discussed in more detail below, but it is generally believed that the main immune cells expressing IFN-λR1 are dendritic cells (DCs) (4, 7, 8). Most studies assessed the expression of IFN-λR1 by polymerase chain reaction (PCR), evaluating the mRNA level, which might not accurately reflect expression of the protein on cell surface. It was demonstrated that immune cells [B cells, T cells, and natural killer (NK) cells] express mostly a shorter splice variant of IFN-λR1 that can be secreted (9). This secreted form could bind IFN-λ with moderate affinity and inhibit its effects. This could explain at least in part why immune cells express IFN-λR1 mRNA but lack responsiveness to IFN-λ treatment.

ASSOCIATION OF TYPE III IFN POLYMORPHISMS WITH HCV SPONTANEOUS CLEARANCE AND RESPONSE TO IFN THERAPY

Hepatitis C virus (HCV) infection is a global health problem. Only 25% of individuals acutely infected with HCV are able to eliminate the virus spontaneously, while the majority (~75%) develops persistent infection and chronic liver disease including fibrosis, cirrhosis, and liver cancer (10). Until 2011, the only available treatment for HCV was a combination of ribavirin and pegylated IFN-α (11). This non-specific treatment was modestly effective, especially in individuals infected with genotype 1, resulting in ~50% sustained virological response (SVR) rate defined as undetectable viral load 24 weeks following the end of treatment (12). Furthermore, the course of treatment was long (48 weeks) and associated with multiple side effects, thus significantly impacting the quality of life of the patients (13). Factors associated with higher odds of spontaneous resolution or response to IFN therapy include virus genotype, gender, and ethnicity, suggesting that genetic factors are key determinants of viral clearance (14, 15). Individuals of European ethnicities were more likely to achieve SVR compared to individuals of African ancestry (14, 16). These differences accompanied by the difficulties and side effects associated with IFN treatment prompted research into genetic factors that can predict SVR. Several genome-wide association studies demonstrated a link between single-nucleotide polymorphisms (SNP) near the *IFNL3* gene encoding IFN-λ3 and HCV infection outcome and response to treatment. These major polymorphisms are listed in **Table 2**. Ge et al. demonstrated that the IFN-λ3 rs12979860 SNP predicted the response to IFN treatment in an American cohort composed of multiple ethnicities infected with HCV genotype 1 (17). The favorable allele (CC genotype) was not only overrepresented in the treatment responder group but was also more prevalent in the European population compared to the African population where the unfavorable TT genotype was more prevalent. Moreover, the IFN-λ3 rs12979860 genotype was a better predictor of treatment outcome than ethnicity, since African Americans with the CC genotype were more likely to achieve SVR than the European American bearing the TT genotype (17). That study also demonstrated that the CC genotype was associated with higher baseline viral loads in all groups tested. Two other studies confirmed the same association with polymorphism in the IFN-λ3 region in Australian (18) and Japanese cohorts (19) and identified an additional SNP (rs8099917). This SNP was associated with HCV genotype 1 treatment response in the Australian cohort and confirmed with other cohorts (18). This study also used quantitative reverse transcription PCR to demonstrate that healthy individuals carrying the favorable allele (TT) expressed higher levels of IFN-λ2 and IFN-λ3 transcripts in peripheral blood. In the Japanese cohort, both SNPs (rs12979860 and rs8099917) were associated with treatment response (19).

The favorable rs12979860 CC genotype was also associated with spontaneous clearance in untreated individuals from six different cohorts (20). In this study, Thomas et al. also observed

TABLE 1 | Type III IFN genes and proteins.

	Gene	Alternate gene names	Protein	Alternate protein names
Receptor	*IFNLR1*	*IL-28RA, IL-28R1, IFNLR*	IFN-λR1	IL-28RA, IL-28Rα, IL-28R1
Cytokines	*IFNL1*	*IL-29*	IFN-λ1	IL-29
	IFNL2	*IL-28A*	IFN-λ2	IL-28A
	IFNL3	*IL-28B*	IFN-λ3	IL-28B
	IFNL4	–	IFN-λ4	–

TABLE 2 | Type III IFN gene polymorphisms.

SNP	Common name	Alternative names	Favorable allele	Unfavorable allele
rs12979860	IFN-λ3	IL-28B IFNL4 rs12979860	CC	TT
rs8099917	IFN-λ3	IL-28B	TT	GG
rs368234815	IFN-λ4	ss469415590	TT	ΔG
rs117648444	IFN-λ4-P70S		AA (IFN-λ4-S70)	GG (IFN-λ4-P70)

that the C allele was more represented in Europeans compared to African individuals. More importantly, they demonstrated that the C allele was associated with spontaneous resolution of HCV infection in both ethnic groups. Moreover, the protective effect appeared to be recessive, since there was no difference between heterozygous individuals bearing the CT genotype and homozygous individuals bearing the TT genotype. This study also genotyped >2,000 individuals worldwide and demonstrated that the C allele was most prevalent in East Asia, whereas the T allele was most prevalent in Africa and an intermediate pattern with both alleles was observed in Europe. Similar results were obtained by Rauch et al. who sequenced the IFN-λ3 rs8099917 SNP and showed association of the unfavorable allele with establishment of a chronic infection and treatment failure in HCV monoinfected and HCV/HIV coinfected individuals (21). Finally, the IFN-λ3 rs12979860 SNP was also associated with spontaneous clearance and jaundice in a single-source cohort (22). The German anti-D cohort consists of 2,867 women who were exposed to HCV genotype 1b after treatment with anti-D immunoglobulin. Fifty-two percent of infected women achieved spontaneous clearance. This cohort enabled the evaluation of the role of IFN-λ3 polymorphism in spontaneous clearance without the confounding effect of virus genetics. In this cohort, it was possible to analyze genetic factors associated with spontaneous clearance in 190 women. Results demonstrated that spontaneous clearance was strongly associated with the IL-28B/IFN-λ3 genotype (22). The highest rate of clearance was observed in women homozygous for the favorable C allele (CC, 64.2% clearance), the lowest rate of clearance was observed in women homozygous for the unfavorable T allele (TT, 6.1% clearance), and intermediate levels of clearance were observed in heterozygous women (CT, 24.4% clearance) (22). IFN-λ3 favorable genotype was also associated with clearance upon reinfection in high-risk people who inject drugs (23).

In 2013, a new dinucleotide polymorphism rs368234815 (previously termed ss469415590) located near the *IFNL3* gene was identified, and the variants TT or ΔG were associated with a frame shift resulting in either production of a new protein, IFN-λ4, (ΔG) or absence of the protein due to the introduction of a frameshift creating an early stop codon (TT) (3, 24). This new polymorphism was in high linkage disequilibrium with the IFN-λ3 rs12979860 polymorphism and was found to be a stronger predictor of HCV spontaneous resolution and treatment outcome of chronic HCV (3, 25, 26). Another group reported association of the TT/ΔG polymorphism with HCV treatment outcome in a large European cohort (27). Given that the IFN-λ3 rs12979860 was located within the newly discovered IFN-λ4 region, it was suggested to change its nomenclature to IFN-λ4 rs12979860 (24).

MECHANISMS UNDERLYING THE ROLE OF IFN-λ POLYMORPHISMS IN HCV CLEARANCE

The exact mechanisms underlying the role of IFN-λ polymorphisms in HCV clearance are not well understood. It was proposed that such polymorphisms may influence the expression of IFN-λ cytokines during HCV infection and their downstream effects on expression of ISGs and innate and adaptive immune cells. Although it was demonstrated early on that IFN-λ SNPs may influence expression of the IFN-λ transcripts in PBMCs (18), data evaluating the circulating levels of IFN-λ cytokines during acute and chronic HCV were inconclusive. Data from the chimp model of HCV infection demonstrated that type III IFNs were strongly induced upon HCV infection at the gene and protein level and correlated with ISG expression and viral load (28). In humans, while some studies associated the favorable IFN-λ3 CC allele with higher serum levels of IFN-λ (29), others demonstrated the reverse correlation (7). One report also found no difference in serum levels of IFN-λ between HCV treatment responders and non-responders (30). Our group has demonstrated that serum levels of IFN-λ3 were highly variable, but were lower in individuals bearing the favorable IFN-λ3 CC allele (31). Altogether, type III IFN genotyping has been, so far, a more accurate predictor for HCV infection or treatment outcome compared to the circulating levels of the cytokines.

How the expression of IFN-λ4 would interfere with HCV clearance or treatment response is not fully understood. It was shown that the protein is only poorly secreted (3, 32). Nevertheless, the protein could interact with the same receptor as IFN-λ3 (IFN-λR1 and IL-10R2) and displayed similar levels of activation of ISGs and antiviral activity (32). It remains possible that IFN-λ4 has other functions apart from activation of ISGs, perhaps through the interaction with an intracellular receptor. Both IFN-λ3 and IFN-λ4 polymorphisms were associated with the level of expression of the type I IFN receptor *IFNAR1* in PMBCs (33). Individuals carrying both favorable alleles expressed the highest level of *IFNAR1*, while individuals bearing both unfavorable alleles exhibited lower levels. Treatment of PBMC with IFN-α confirmed that individuals with both favorable alleles and the highest *IFNAR1* expression also exhibited the highest ISG induction.

Several early studies demonstrated a link between the liver expression levels of ISGs before IFN treatment initiation and treatment outcome (34–37). A higher level of expression of a set of ISGs and genes involved in IFN regulatory pathways (ISG15 and USP18) was observed in non-responder patients before treatment and predicted treatment outcome (34). Furthermore, the level of expression of several ISGs was shown to correlate with IFN-λ genotypes, with the unfavorable alleles associated with higher hepatic levels of ISGs (37, 38). Unphosphorylated IFN-stimulated gene factor 3 (ISGF3) is induced by type III IFNs and sustains expression of USP18, a negative regulator of IFN signaling, resulting in unresponsiveness to IFN-α treatment (39). Comparison of levels of ISGs before and after treatment further demonstrated that the high basal expression levels in non-responders did not increase above pretreatment level, whereas there was a strong ISGs induction in the SVR group (40). This suggests that the baseline high ISG levels in non-responders render them unresponsive to further IFN stimulation upon therapy. Extending this hypothesis to explain why the expression of IFN-λ4 would be detrimental for HCV infection and treatment outcome, patients expressing a less active variant of the IFN-λ4 protein had better odds of achieving spontaneous clearance or SVR (41). This study demonstrated that

the IFN-λ4-S70 protein (SNP rs117648444) exhibited reduced ISG activation and antiviral activity *in vitro*. When comparing infection and treatment outcome in a large cohort, individuals bearing genetic variants resulting in no IFN-λ4 production had the highest odds of clearance/SVR, followed by those expressing the IFN-λ4-S70 impaired protein and finally those expressing the IFN-λ4-P70 fully active protein had the lowest odds of achieving clearance/SVR.

On the basis of current knowledge, we can elaborate a model where during acute HCV infection, innate immune responses are induced in hepatocytes that trigger the production of type III cytokines that stimulate a variety of antiviral ISGs. In individuals carrying the favorable IFN-λ3 rs12979860 CC and IFN-λ4 rs368234815 TT alleles, production of IFN-λ is controlled, and the induction of ISGs is more focused leading to an effective antiviral state and increased rate of spontaneous resolution of infection. In contrast, carriers of the unfavorable alleles exhibit stronger IFN-λ production and a more diverse array of ISGs. This will also induce the expression of USP18, an inhibitor of the IFN signaling pathway, leading to an impaired antiviral state and to an increased propensity to develop chronic infection. This effect is not absolute, and some individuals carrying the favorable alleles will develop chronic infection. In chronically infected individuals carrying the favorable allele, basal levels of IFN-λ and ISGs will be relatively low and treatment with IFN-α can induce a potent antiviral state leading to viral clearance or SVR. In individuals carrying the unfavorable allele, a high basal level of IFN-λ, ISGs, and USP18 will lead to a refractory state and unresponsiveness to the IFN-α treatment, and failure to respond to treatment (**Figure 1**).

TYPE III IFNs AND INNATE IMMUNITY IN THE LIVER

HCV is a hepatotropic infection and investigation of the early steps of viral replication, and the innate immune response is hindered by the difficulty to access the infected tissue, i.e., the liver. *In vitro* systems usually show low level of viral replication, and animal models to study the immune response are limited to chimpanzees that are no longer used in research. Thus, limited information is available about the activation of the innate immune response

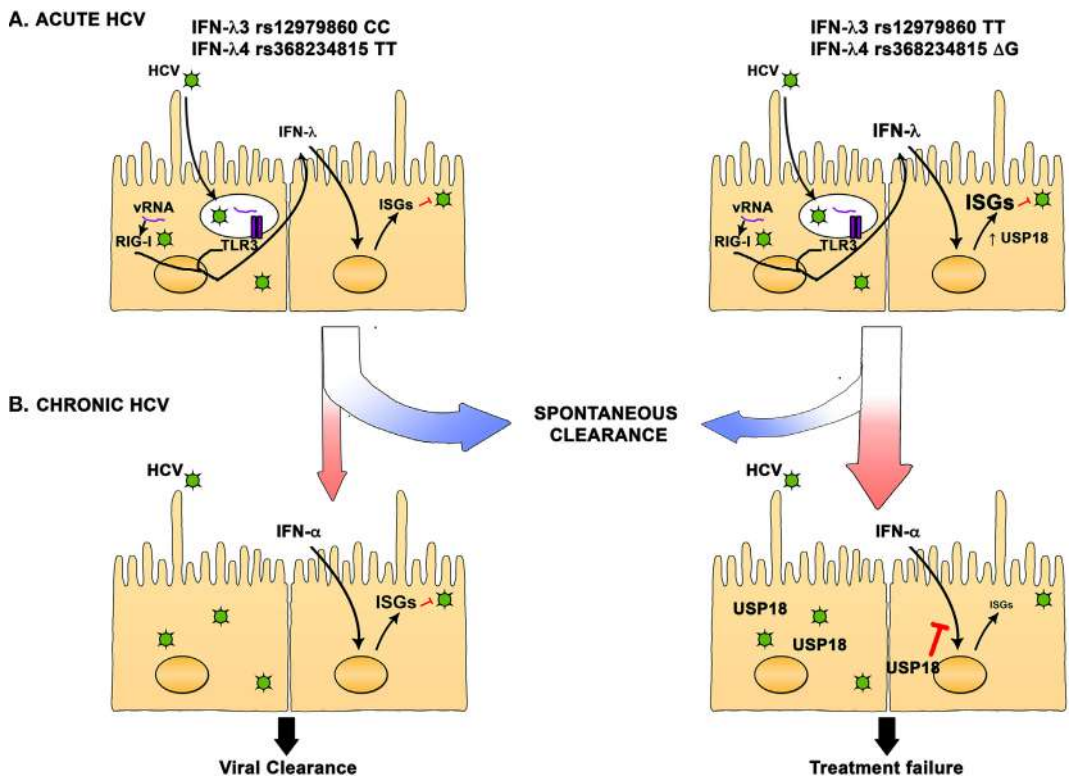

FIGURE 1 | **Interferon (IFN)-λ polymorphisms are associated with hepatitis C virus (HCV) spontaneous resolution and response to treatment**. **(A)** In HCV acutely infected individuals, IFN-λ3 rs12979860 CC and IFN-λ4 rs368234815 TT favorable alleles are strongly associated with spontaneous resolution. Hepatocyte infection triggers the production of IFN-λ that then induce the expression of hundreds of interferon-stimulated genes (ISGs). In individuals carrying the favorable alleles, production of IFN-λ is moderate, and the ISGs induction is more focused, leading to an effective antiviral state and increased rate of spontaneous resolution of infection. On the other hand, carriers of the unfavorable alleles exhibit stronger IFN-λ production and a more diverse array of ISGs can be detected. This will also induce the expression of USP18, an inhibitor of the IFN signaling pathway, leading to an impaired antiviral state and an increased rate of chronic HCV infection. **(B)** In chronically infected individuals carrying the favorable allele, basal level of IFN-λ and ISGs is relatively low and treatment with IFN-α can induce a potent antiviral state leading to viral clearance or SVR. In individuals carrying the unfavorable allele, a high basal level of IFN-λ, ISGs, and USP18 will lead to a refractory state and unresponsiveness to the IFN-α treatment and failure to clear the infection.

in the liver of HCV-infected individuals during acute infection. Nevertheless, chimpanzee data have demonstrated strong induction of ISGs in the liver early after infection irrespective of the outcome toward resolution or chronicity (42, 43). Examining the kinetics of type I versus type III IFNs demonstrated that HCV-infected chimpanzees exhibited rapid induction of type III IFNs in the liver. This was associated with upregulation of ISGs but minimal induction of type I IFNs (44). Similarly, infection of primary human hepatocytes (PHHs) induced production of type III IFNs that were associated with induction of a distinct set of ISGs compared to type I IFNs (44, 45). Sheahan et al. used PHHs and laser capture microdissection to compare the transcriptional profile of HCV-infected hepatocytes to adjacent uninfected cells (46). They demonstrated that infected cells had a transcriptional profile dominated by innate immunity genes, including induction of IFN-λ genes only in infected cells. Interestingly, when comparing gene expression from donors of different IFN-λ genotypes, they demonstrated that even if a greater number of genes were induced in the unfavorable (TT) allele group, the response in the favorable allele group (CC) was more focused toward antiviral and cell death responses, and unsurprisingly, viral replication was more limited in donors bearing the favorable allele (46). Onabajo et al. also used an *in vitro* system of PHHs and hepatic cells and demonstrated that IFN-λ4, while highly retained inside cells, is also secreted and induces strong ISGs response in surrounding cells, including the expression of IP-10 (47). However, IFN-λ4 expression was associated with reduced proliferation and increased cell death (47). Ferraris et al. used PHHs of different IFN-λ3/4 genotypes to investigate the mechanisms associated with HCV clearance (48). Treatment of HCV-infected cells with either IFN-α or IFN-λ1 decreased viral load only in cultures carrying the favorable IFN-λ3/4 alleles (48). They also showed that, in both PHHs and liver biopsies of HCV-infected subjects, IFN-λ1, IFN-λ3, and ISGs production were higher in carriers of the unfavorable allele. Silencing of IFN-λ1 in unfavorable allele context restored IFN-α antiviral activity, suggesting that the high basal IFN-λ and ISG expression blocked further activation by IFN-α treatment (48). The unresponsiveness observed in the context of the unfavorable IFN-λ3/4 alleles was shown to be driven by upregulation of USP18 (39). In liver biopsies from individuals with chronic HCV, it was also shown that the favorable IFN-λ4 rs368234815 TT genotype was associated with increased degranulation capacity (CD107a+) from T, NK, and NKT cells, which correlated with serum ALT and AST levels (49). This suggests increased innate immune activation in the livers of these individuals.

Hepatocytes are not the only source of type III IFNs in the liver. Hepatic stellate cells (HSC), normally in a quiescent state, become activated following liver damage induced by HCV infection and may modulate intrahepatic immune responses. HSCs activated with the TLR-3 ligand poly I:C exhibit an antiviral effect when co-cultured with HCV-infected hepatocytes (50). Supernatants of activated HSCs demonstrated an antiviral effect that could be blocked by antibodies specific to the IL-10R2. These *in vitro* results strongly suggest that HSCs can participate in the innate immune response in the liver *via* the production of IFN-λ. Finally, DCs can also act as a key source and regulator of type III IFNs in the liver and the peripheral blood.

IFN-λ INTERACTION WITH HEMATOPOIETIC CELLS

The interaction of type III IFNs with hematopoietic cells is not fully understood. In contrast to type I IFNs whose receptors are ubiquitously expressed, type III IFNs have a limited number of target cells, because their receptor (IFN-λR1 and IL-10R2 heterodimer) expression is highly restricted to the cells of epithelial origin including hepatocytes and few hematopoietic cells (4–7). Although, IFN-λR1 transcripts could be detected in several hematopoietic cells, it has been problematic to detect its expression on cell surface. It has also been reported that hematopoietic cells may express a soluble splice variant that may influence their capacity to respond to type III IFNs (9, 51). In the following sections, we will discuss in details the effect of type III IFNs on different types of hematopoietic cells.

Monocytes and Macrophages

It was shown that IFN-λR1 was expressed on monocyte-derived macrophages, but not monocytes (52). Monocyte-derived macrophages responded to IFN-λ1 treatment by phosphorylation of STAT-1 and increased production of cytokines such as tumor necrosis factor (TNF), IL-10, and IL-12p40 following TLR stimulation. Similar effects were observed after treatment with IFN-λ2 or IFN-λ3. Furthermore, contrary to IFN-α, IFN-λ1 enhanced cell surface expression of *IFNGR1* on monocyte-derived macrophages, thus enhancing IL-12p40 and TNF production after stimulation with IFN-γ (52). Polymorphism in the IFN-λ3 SNP rs12979860 also impacted the activation of monocytes where individuals of the TT unfavorable genotype produced lower levels of IL-12 upon activation of their monocytes with the TLR ligand R848 (53). Thus, a better IFN-λ response could potentiate the antiviral and inflammatory response of monocytes and may indirectly mediate viral clearance by boosting the induction and priming of the adaptive immune response.

NK Cells

Cytotoxic and antiviral functions of NK cells depend on a tightly regulated balance between activation and inhibitory signals. The main inhibitory mechanism is *via* binding of the killer cell-Ig-like receptors (KIR) with MHC class I molecules (54). The polymorphism within the KIR and MHC class I genes results in interactions of different strengths and degrees of activation of NK cells that correlate with HCV infectious outcome (55). IL-28B/IFN-λ3, HLA-C, and KIR variants could additively predict response to IFN therapy in chronic HCV, suggesting a collaborative effort between type III IFNs and NK cells during viral clearance (56). Activation of NK cells, associated with the success of IFN-based treatment, was studied in relation to IFN-λ3 polymorphism, and patients carrying the unfavorable IFN-λ3 allele expressed higher levels of expression of the inhibitory receptor NKG2A on NK cells and were more likely not to respond to treatment (57).

These observations further underscored the potential effect of type III IFNs on NK cells.

In the context of acute infection, *KIR2DS3* and the IFN-λ3 SNP rs12979860 unfavorable T allele synergized to increase the risk of chronic infection (58). This study also suggested a direct link between IFN-λ and NK cells, showing reduced IFN-γ production by NK cells upon IFN-λ treatment (58). However, these data were difficult to reproduce in other cohorts. The IFN-λ3 rs12979860 CC genotype was associated with decreased levels of the inhibitory receptor NKG2A after infection resolution (31). Individuals bearing the CC genotype also displayed increased NK cell function measured by IFN-γ production after stimulation irrespective of infectious outcome suggesting that IFN-λ genotype influenced NK cell function but that this was not sufficient to achieve spontaneous HCV clearance (31). Although *IFNLR1* mRNA expression could be detected in NK cells (59, 60), they express very low levels of the specific type III IFN receptor (IFN-λR1) on cell surface, even after IFN-α stimulation (31, 61, 62). Treatment of purified NK cells with IFN-λ had no effect on neither NK cytotoxicity nor cytokine production (31, 60, 62, 63). On the other hand, it was reported that the level of expression of IFN-λR1 could be upregulated by IFN-λ treatment (59) and studies in IFN-λR1$^{-/-}$ mice have demonstrated that this receptor is required for optimal antitumoral *in vivo* activity of NK cells (64), suggesting that in some activation context, NK cells could become sensitive to type III IFNs.

Given the lack of activation of NK cells by IFN-λ (31, 60, 62, 63), indirect mechanism were investigated. IFN-λ1 affected NK cells indirectly *via* the activation of monocyte derived macrophages. Macrophages activated by IFN-λ1 produced cytokines of the IL-12 family (IL-12p40) that could then activate NK cells leading to increased IFN-γ production. This activation was determined by polymorphisms in the IFN-λ3 gene, and the presence of monocytes was essential (53). This suggests that HCV-infected individuals bearing the unfavorable IFN-λ3 allele have an impaired monocyte function. Monocytes can activate NK cells through the production of IL-12 or IL-18. Stimulated monocytes from CC genotype background produced significantly more IL-12p40 and IL-12p70 compared to monocytes of the CT or TT genotype. Blocking IL-12 and not IL-18 abolished the IFN-λ association with the level of NK cell activation by monocytes, suggesting that IL-12 is a major player in the interplay between monocyte and NK cells that is associated with IFN-λ3 genotype in HCV-infected subjects (53).

Analysis of NK cell phenotype and function in chronic HCV infection demonstrated that CD56^{bright} NK cell subsets are significantly more cytotoxic than in healthy donors based on TRAIL and CD107a expression (65). This effect was independent of the IFN-λ3 rs12979860 genotype, but subjects carrying the TT genotype exhibited the highest levels of TRAIL+ and CD107a+IFN-γ+NK cells. In the same study, CD56^{dim} NK cells of TT genotype individuals produced more TNF-α. Accordingly, individuals with the TT genotype also had a higher proportion of polyfunctional NK cells (65).

In conclusion, whether by a direct or indirect mechanism, it appears that type III IFNs can modulate NK cells activation and functions but further investigation is required to identify the exact mechanism.

Dendritic Cells

Dendritic cells are important antigen-presenting cells and have a central role in mediating the link between the innate and adaptive immune response. DCs also are a major source of type III IFNs (8, 60, 66). Stimulation of DCs (*in vitro* and *ex vivo*) with HCV RNA induced the production of both type I and type III IFNs and the levels were associated with IFN-λ3 rs12979860 genotype, with the favorable CC allele leading to the highest IFN type III production (66). Type I and type III IFNs produced by DCs could control HCV replication *in vitro*, suggesting again an important role for type III IFN in HCV infection (66). During chronic HCV infection, serum levels of IFN-λ1 were lower compared to HCV resolvers and healthy controls (29). In acute HCV infection, IFN-λ1 serum levels were variable as described earlier (29, 31). Interestingly, HCV proteins E2 and NS3 inhibited IFN-λ1 production by stimulated DCs, suggesting that IFN-λ1 is an important immune mediator in HCV infection (29). On the other hand, treatment of DCs with IFN-λ altered their function toward a dysfunctional help to T cells (7). IFN-λ-treated DCs exhibited decreased T cell stimulation capacity by upregulating PDL1 expression. In addition, IFN-λ-treated DCs promoted the expansion of regulatory T cells (Tregs), further impeding with the immune response. Further research will be needed to clarify the role of IFN-λ and DCs during a viral infection, such as HCV.

CD4 and CD8 T Cells

The link between IFN-λ and T cells is less studied compared to the link with cells of the innate immune response. A study by Bes et al. showed that CD4 T cell responses to HCV, assessed by IFN-γ enzyme-linked immunospot (ELISpot) assay, were of a higher frequency in the unfavorable allele (non CC) group (67). This was unexpected since a stronger immune response is normally associated with HCV spontaneous clearance. However, they tested a limited number of patient (n = 69; with 38 samples with positive ELISpot response), and there was a lot of variability between samples. In a more recent study, Scheurich et al. tested the breadth and frequency of CD4 responses to HCV and stratified their results according to the IFN-λ3 rs12979860 genotype (68). However, they did not find any difference between the various IFN-λ groups. Genetics studies showed that polymorphisms of MHC class I and MHC class II are associated with spontaneous clearance of HCV infection, independently of the IFN-λ3 polymorphism (69, 70). Protective alleles were shown to have additive effect, suggesting that innate and adaptive immunity contribute independently to the prediction of a favorable outcome following acute HCV infection. However, these independent associations do not rule out the possibility that type III IFN could modulate CD4 T cells responses. Indeed, data from other models suggest that IFN-λ can indeed impact CD4 T cells (71–73). It was reported that naive and memory CD4 T cells express IFN-λR1 mRNA and that T cell stimulation in presence of IFN-λ inhibited IL-4, IL-5, and IL-13 production, thus impeding the development of Th2 helper response without affecting cell proliferation (72, 73). IFN-λ also

inhibited the upregulation of IL-4Rα on the surface of stimulated naive CD4 T cells, thus limiting the Th2 polarizing effect of IL-4 on these cells. IFN-λ-treated cells expressed significantly less GATA3, the transcription factor that is master regulator of Th2 differentiation, further supporting the hypothesis that IFN-λ has inhibitory effect on the development of a Th2 response (73). Whether such mechanisms are implicated during HCV infection remains to be seen. It is also tempting to speculate that the effect of IFN-λ polymorphism on production of IL-12 by monocytes may indirectly influence priming of the HCV-specific CD4 and CD8 T cells and the generation of antiviral Th1 responses.

B Cells

The role of the B cells and neutralizing antibody responses during acute HCV infection remains unclear. Some studies showed that early anti-HCV antibody response is associated with higher rate of spontaneous clearance of the virus during primary infection and reinfection (74–78), but other studies showed no association (79–82). To our knowledge, no data are currently available on the role of IFN-λ polymorphisms in the B cell response against HCV but it can be inferred from other models. Generally, the role of IFN-λ on B cells was only modestly studied. One recent article by de Groen et al. demonstrated that both naive and memory B cells express IFNLR1 mRNA and that IFN-λ1 can activate B cells (83). Treatment of B cells with IFN-λ1 led to increased expression of ISGs (Mx1 and OAS1) as well as increased expression of TLR7. IFN-λ1 also enhanced IgM and IgG production from TLR7/8-stimulated B cells. Finally, IFN-λ1 stimulation increased the proliferation of B cells stimulated with TLR7/8 agonist. However, after TLR2 or TLR9 stimulation, IFN-λ1 had no effect on the antibody production or proliferation (83). Globally, this study suggests that IFN-λ1 can enhance B cell response, but only under certain stimulation conditions.

In another context, Egli et al. showed that the IFN-λ3 rs8099917 GG genotype was associated with a higher rate of seroconversion after influenza vaccination in a cohort of immune-suppressed transplant patients (84). In addition, GG genotype carriers showed lower Th1 responses after PBMC stimulation with influenza antigens, suggesting that the IFN-λ3 rs8099917 genotype can affect the Th1/Th2 balance. Accordingly, adding IFN-λ3 in the stimulation medium increased the Th1 cytokine production and reduced Th2 cytokine production (84). These observations were confirmed in a cohort of healthy volunteers where IFN-λ3 treatment enhanced Th1 cytokine profile and reduced production of Th2 cytokines after influenza stimulation (84). Also, IFN-λ3 decreased B cell proliferation and antibody production. Interestingly, it was also demonstrated that adding peptides that block the effect of IFN-λ3 during the stimulation led to an increased antibody production, suggesting that blocking IFN-λ3 during influenza vaccination could improve the seroconversion rate and thus have a better protective effect (84).

These two aforementioned studies used different type III IFNs to stimulate B cells (IFN-λ1 or IFN-λ3) as well as different antigenic stimulation. Hence, there is no clear conclusion on whether type III IFNs exert a beneficial or detrimental effect on B cell function and antibody production, and additional studies with standardized stimuli are warranted.

SUMMARY OF THE ROLE OF TYPE III IFNs ON HEMATOPOIETIC CELLS

Within the hematopoietic compartment, DCs are the main producers of IFN-λ, and they can travel between the liver and peripheral blood. The production of IFN-λ by DCs can inhibit HCV RNA replication in hepatocytes. However, HCV proteins E2 and NS3 can also inhibit IFN-λ production by DCs. Hematopoietic cells express variable levels and splice variants of IFN-λR1, and conflicting results were obtained about the effect of IFN-λ treatment on these cells. HCV exposed DCs or DCs treated with IFN-λ display reduced stimulation of T cells by upregulating expression of PDL1 and enhanced proliferation of Tregs. Monocytes are responsive to IFN-λ treatment resulting in IL-12 and IL-18 production. In turn, these cytokines can influence NK cell functions, and thus, IFN-λ is an important component of the innate immune response to HCV. The role of IFN-λ on CD4 and CD8 T cells as well as on B cells in the context of HCV remains understudied, but studies suggest that IFN-λ could modulate the CD4 Th1/Th2 balance and can also have a positive or negative impact on IgG production by B cells (**Figure 2**).

TYPE III IFNs AND HCV-SPECIFIC IMMUNITY DURING PREGNANCY

In HCV-infected women, a sharp decrease in HCV viral load is sometimes observed after childbirth, suggestive of a boost in the immune response following delivery (85, 86). It was recently demonstrated that beside a stronger T cell response, the presence of the favorable CC IFN-λ3 rs12979860 genotype was significantly associated with this high decrease in viral load (87). Considering the high linkage disequilibrium between IFN-λ3 rs12979860 genotype and IFN-λ4 rs368234815 genotype, the IFN-λ4 was also associated with the decrease in viral load postpartum (87). It is well known that women's immune system is altered through pregnancy to avoid a reaction against the fetus (88). It is postulated that the innate immune system will play a significant role against pathogens, while the adaptive immune responses are dampened by increased Tregs activity (89, 90). Another recent study showed that the expression of innate immunity genes is enriched in postpartum women compared to control (91). Interestingly, ISGs level in women of the CT or TT IFN-λ3 rs12979860 genotype remained elevated as late as 24 weeks after childbirth, while women with the CC IFN-λ3 genotype were comparable to non-pregnant controls (91). This reflects what was observed in the context of acute HCV where individuals with non-favorable CT or TT IFN-λ3 genotype had a higher baseline ISG expression in the liver.

IFN-λ DURING TREATMENT WITH DIRECT-ACTING ANTIVIRALS (DAA) AND HCV-RELATED LIVER DISEASE

With the development of novel DAAs that are highly effective (~100%) against most genotypes, treatment has switched to IFN-free regimens. Limited studies have indicated that polymorphism

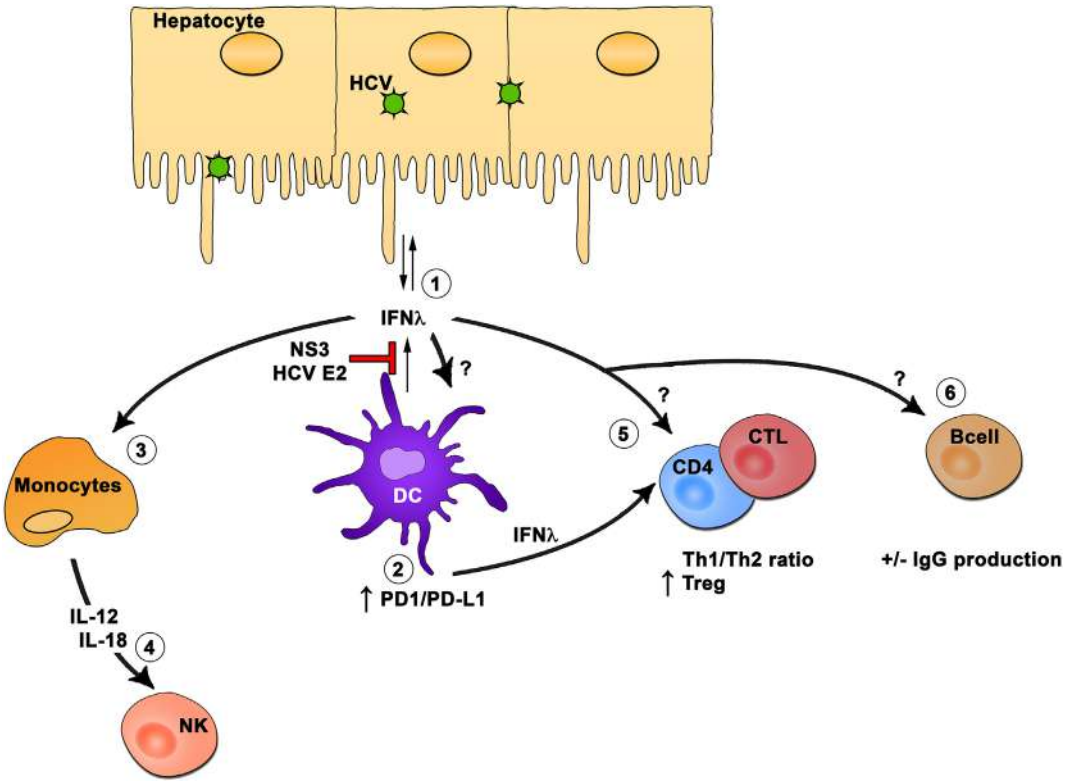

FIGURE 2 | **Interferon (IFN)-λ modulation of hematopoietic cells**. In the hematopoietic compartment, dendritic cells (DCs) are the main producers of IFN-λ (1). The production of IFN-λ by DCs can inhibit hepatitis C virus (HCV) RNA replication in hepatocytes. However, HCV proteins E2 and NS3 can inhibit IFN-λ production by DCs. Hematopoietic cells express variable levels and splice variants of IFN-λR1, and conflicting results were obtained about the effect of IFN-λ treatment on these cells. HCV-exposed DCs or DC treated with IFN-λ display reduced stimulation of T cells through upregulation of PDL1 and enhanced proliferation of regulatory T cells (2). Monocytes are responsive to IFN-λ treatment resulting in interleukin (IL)-12 and IL-18 production (3). In turn, these cytokines will influence natural killer cell function (4), and thus, IFN-λ is an important component of the innate immune response to HCV. The role of IFN-λ on CD4 and CD8 T cells (5) as well as on B cells (6) in the context of HCV remains understudied, but studies suggest that IFN-λ could modulate the CD4 Th1/Th2 ratio and can also have a positive or negative impact on IgG production by B cells.

in the IFN-λ region may influence the response to DAAs, especially if IFN is still used in the combination (92, 93). However, given the high rate of response to DAAs and the availability of multiple products on the market, IFN-λ has lost its predictive value, and testing for it before treatment is no longer recommended except in specific situations where a DAA and IFN combination may still be warranted (94).

Chronic HCV infection is associated with an increased risk of liver-related illness, such as fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). The link between IFN-λ polymorphisms and HCV-related liver disease is not completely clear and was reviewed in detail elsewhere (95). One study showed that the favorable CC IFN-λ3 rs12979860 allele, associated with better chance of HCV clearance, is also associated with higher blood ALT levels, indicative of increased liver inflammation. However, IFN-λ rs12979860 polymorphism was not associated with fibrosis progression in the same cohort (96). Another study did not find any association of the IFN-λ polymorphism with any of the observed associated liver illness (decompensated cirrhosis, HCC, liver-related death, and all-cause mortality) (97). On the other hand, Bochud et al. demonstrated that particularly in non-genotype 1 HCV-infected individuals, the favorable IFN-λ alleles were associated with increased inflammation and higher fibrosis scores (98). In agreement with this, Eslam et al. observed a significant association between IFN-λ3 rs12979860 polymorphism and liver necroinflammatory activity, serum level of AST and ALT, as well as fibrosis score and progression (99). Once again the association was stronger in individuals infected with HCV genotype 3 than those infected with HCV genotype 1. Also, in patients carrying the IFN-λ4 rs368234815 unfavorable allele (ΔG), there was a correlation between the frequency of CD107a expressing cells and the serum ALT levels, suggestive of increased liver damage (49). In the context of HCC, two studies associated IFN-λ3 rs12979860 unfavorable CT or TT alleles with liver cirrhosis and the development of HCC in patients chronically infected with HCV (100, 101). However, this was not confirmed in two other independent studies in Japanese (102) or Italian cohorts (97). Finally, HCV-related liver disease is a multifactorial problem, and the independent association of genetic factors may not be a clear cut. Fortunately, with the development of highly effective DAAs, it is expected that these complications will be less frequent as SVR will be achievable in most patients.

CONCLUDING REMARKS

Type III IFNs exhibit strong antiviral activity, and yet, the expression of a functional IFN-λ4 protein was strongly associated with failure to clear HCV infection either spontaneously or after IFN-based treatment. A recent study suggested that humans suppress IFN-λ4 expression through various mechanisms and hence immune functions may be dependent on other type III IFNs (103). Data accumulated so far suggest that a higher baseline ISG expression level is associated with induction of a refractory state, where further IFN treatment has no beneficial effect. With the new era of anti-HCV IFN-free DAA therapies, the role of type III IFNs during therapy has become somewhat irrelevant but its role in mediating spontaneous clearance during acute HCV infection and modulating the cross-talk between innate and adaptive immunity remains highly pertinent. This is applicable not only for HCV infection but also for other viral infections and response to vaccines. Furthermore, the recently described role of type III IFN polymorphisms in driving immunity postpartum is just the tip of the iceberg as it will become increasingly relevant to mother–infant health and vertical transmission of various pathogens.

AUTHOR CONTRIBUTIONS

Both the authors reviewed the literature and wrote this manuscript.

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REVIEW
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Type I Interferons in Bacterial Infections: A Balancing Act

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Defense against bacterial infections requires activation of the immune response as well as timely reestablishment of tissue and immune homeostasis. Instauration of homeostasis is critical for tissue regeneration, wound healing, and host recovery. Recent studies revealed that severe infectious diseases frequently result from failures in homeostatic processes rather than from inefficient pathogen eradication. Type I interferons (IFN) appear to play a key role in such processes. Remarkably, the involvement of type I IFNs in the regulation of immune and tissue homeostasis upon bacterial insult may have beneficial or detrimental consequences for the host. The reasons for such ambivalent function of type I IFNs are not understood. The disparate effects of type I IFNs on bacterial infections are in marked contrast to their well-established protective roles in most viral infections. In this review, we will focus on type I IFN effector mechanisms which balance processes involved in immune and tissue homeostasis during specific bacterial infections and highlight the most important missing links in our understanding of type I IFN functions.

Keywords: type I interferon, bacterial infection, cytokines, chemokines, innate immunity, resilience to infections, immunomodulation, immunosuppression

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INTRODUCTION

Successful defense against pathogens requires both, the eradication of the infectious agent by the immune system as well as tissue protection against the damaging effects of the immune response. Increasing evidence indicates that many if not most infectious diseases result from insufficient resilience, i.e., from a failure of the infected host to repair and regenerate destroyed tissues, rather than from inefficient pathogen clearance (1–4). Mechanisms which preserve the integrity of host tissues during the intensive inflammatory response against the pathogen remain incompletely understood. Recent studies established that successful tissue protection during infection requires systems which balance the immune response as well as mechanisms which restore tissue homeostasis. These mechanisms are often interdependent and result from messengers like growth factors, cytokines, or lipids produced by immune cells (1–4). Examples include the anti-inflammatory cytokine IL-10, the tissue regeneration promoting IL-22 and amphiregulin, the tissue remodeler TGF-β, or the pro-resolving lipid lipoxin (5–8). Remarkably, several recent studies demonstrated that type I interferons (IFNs) can also act as critical resilience-promoting cytokines during infections with several streptococcal species (9–11). Such protective functions are in marked contrast to detrimental effects of type I IFN during infections with many other bacterial species (12, 13). The reasons for the ambivalent roles of type I IFNs in bacterial infections remain poorly understood. However, it appears that the ability of type I IFNs to both suppress and stimulate immune responses is of critical importance

(**Table 1**; **Figure 1**). This review focuses on the role of type I IFNs in balancing pro- and anti-inflammatory processes as well as cell survival and cell death programs during antibacterial defense and discusses how these effects determine the outcome of an infection.

TYPE I IFN INDUCTION BY BACTERIA

Type I IFNs were described more than a half century ago as products which are secreted by virus-infected cells and interfere

TABLE 1 | Effects of type I interferons (IFN) signaling in bacterial infections.

Pathogen	Type of bacteria	Route of infection	Model of infection	Effect of type I IFN signaling	Mechanism	Reference
Streptococcus pneumoniae	Gram+, extracell	Intranasal; intratracheal	Model of lung infection	**Protective**	Protection against epithelial barrier damage	(10, 11, 14)
Streptococcus pyogenes	Gram+, extracell	Subcutaneous	Model of invasive cellulitis	**Protective**	Prevention of IL-1β-driven systemic hyperinflammation	(9, 15)
Group B streptococcus	Gram+, extracell	Intraperitoneal (adults); subcutaneous (neonates)	Model of systemic infection/sepsis	**Protective**	Protection against bacteremia	(16, 17)
Legionella pneumophilia	Gram−, intracell	Intranasal	Model of lung infection	**Protective**	Inhibition of intracellular replication of the pathogen and protection against bacteremia	(18, 19)
Helicobacter pylori	Gram−, extracell	Oral	Stomach infection/ gastric mucosa infection	**Protective**	Induction of CXCL10 and reduction of bacterial burden in gastric mucosa	(20)
Staphylococcus aureus	Gram+, extracell	Intranasal	Model of lung infection	**Detrimental**	Exacerbated inflammatory cytokine production and leukocyte recruitment	(14, 21)
Mycobacterium tuberculosis	Intracell	Aerogenic	Model of lung infection	**Detrimental**	Immunosuppression (inhibition of IL-1 production and Th1 responses)	(22–24)
Listeria monocytogenes	Gram+, intracell	Tail vein injection	Model of systemic infection	**Detrimental**	Induction of apoptosis	(25, 26)
		Intraperitoneal	Model of systemic infection	**Detrimental**	Induction of apoptosis in the spleen and supression of IFN-γ production	(27, 28)
		Tail vein injection	Model of systemic infection	**Detrimental**	Suppression of IFNGR expression	(29)
		Intragastric	Model of gastrointestinal infection	**Protective**	Upregulation of protective cytokines limits hepatic inflammation	(28)
		Through food	Model of gastrointestinal infection	**No effect**		(30)
Francisella tularensis subspecies *tularensis*	Gram−, intracell	Intranasal	Model of tularemia	**Detrimental**	Inhibition of IL-17A	(31)
Francisella tularensis subspecies *novicida*	Gram−, intracell	Intradermal	Model of intradermal infection	**Detrimental**	Induction of macrophage death, inhibition of IL-17A and increased bacterial loads	(31, 32)
Salmonela enterica serovar Typhimurium	Gram−, intracell	Tail vein injection; intraperitoneal	Model of systemic infection	**Detrimental**	Enhancement of macrophage necroptosis and failure to control baterial burden	(33)
		Oral	Model of gastrointestinal infection	**Detrimental**	Immunosppression (inhibition of IL-1β, CXCl1 and CXCL2)	(34)
Coxiella burnetii	Gram−, intracell	Intratracheal	Model of lung infection	**Detrimental**	Promotion of dissemination	(35)
		Intratracheal infection with intraperitoneal rIFNα administration	Model of lung infection	**Detrimental**	Inhibition of inflammatory response in lungs	
		Intratracheal infection with intratracheal rIFNα administration	Model of lung infection	**Protective**	Reduction in bacterial dissemination	
Postinfluenzal bacterial pneumonia		Intratracheal; oropharyngeal aspiration	Model of lung infection	**Detrimental**	Attenuation of inflammatory response and leukocyte recruitment	(36–39)

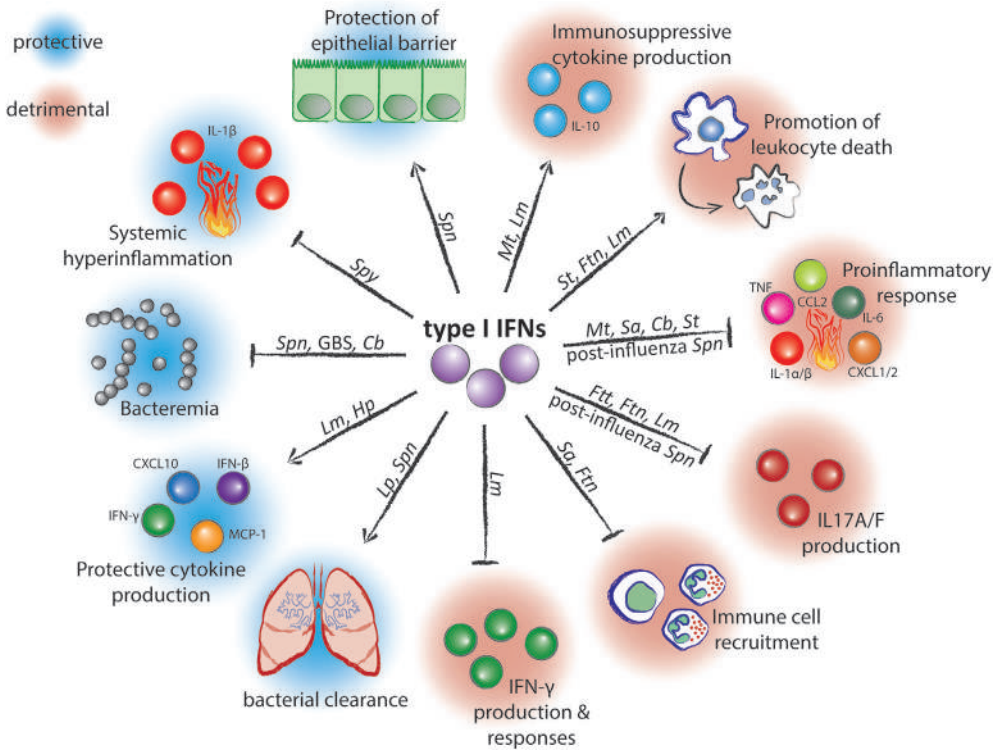

FIGURE 1 | **Mechanisms of action and effects of type I interferons (IFNs) during infection with bacterial pathogens**. Arrow-headed lines represent stimulation and bar-headed lines represent inhibition by type I IFNs. Pathogen abbreviations: *Spn*, *Streptococcus pneumoniae*; *Spy*, *Streptococcus pyogenes*; GBS, Group B Streptococcus; *Cb*, *Coxiella burnetii*; *Lm*, *Listeria monocytogenes*; *Hp*, *Helicobacter pylori*; *Lp*, *Legionella pneumophilia*; *Sa*, *Staphylococcus aureus*; *Ftn*, *Francisella tularensis* subspecies novicida; *Ftt*, *Francisella tularensis* subspecies tularensis; *Mt*, *Mycobacterium tuberculosis*; *St*, *Salmonella enterica* serovar Typhimurium.

with virus replication in autocrine and paracrine ways (40). It is now known that type I IFNs are cytokines produced in response to viral, bacterial, and fungal pathogens, as well as parasites. The effector mechanisms of type I IFNs mainly derive from products of genes which are transcriptionally regulated by type I IFN signaling. Type I IFNs induce hundreds of interferon-stimulated genes (ISGs) through activation of the homodimeric STAT1 and the heterotrimeric STAT1–STAT2–IRF9 (i.e., ISGF3) transcription factors (41). Bacteria trigger type I IFN production mostly following the recognition of bacterial nucleic acids or the Gram-negative cell wall component lipopolysaccharide (LPS) by innate immune receptors (12, 42). The induction mechanisms have been best studied for IFN-β which belongs together with IFNα4 to the first type I IFNs produced during infection and is the driver of other type I IFN genes (43, 44). The induction of IFN-β by bacterial DNA is complex and involves different pathways. The most common mode of IFN-β induction by bacterial DNA is through the cytosolic DNA sensor cyclic GMP-AMP synthase, as described for *Francisella novicida*, group B streptococcus (GBS) (*Streptococcus agalactiae*), *Legionella pneumophila*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Neisseria gonorrhoeae* (45–51). The cytosolic DNA sensor Ifi204 (IFI16 in humans) contributes to IFN-β induction in the course of *F. novicida* and *L. monocytogenes* (48, 49). The signaling events downstream of these DNA sensors involve the STING–TBK1–IRF3 pathway driving the IFN-β gene transcription. *L. monocytogenes* can activate this pathway also independently of DNA sensing. This alternative mechanism is driven by binding of the bacterial product c-di-AMP to STING (51, 52). The recognition of bacterial DNA through unmethylated CpG motif-containing DNA by the endosomal Toll-like receptor 9 can also contribute to IFN-β induction, although the importance and relevance of this pathway in the context of the overall IFN-β production and host response have not been entirely clarified (42). Bacterial RNA has been recently established as another key IFN-β inducer. A short and highly conserved sequence found in the bacterial 23S rRNA is recognized by the mouse TLR13 leading to Myd88- and IRF5-dependent IFN-β induction (9, 53–56). Human cells employ TLR8 as IFN-β-inducing RNA sensor rather than TLR13 which is missing in humans (9, 53, 57–59). The precise nature of bacterial RNA triggering the human TLR8 remains to be identified.

IFN-β induction by LPS occurs after binding of this ligand to the TLR4 and the following internalization into the endosome (60). Subsequently, a TRIF-dependent activation of the kinase TBK1 causes phosphorylation of the transcription factor IRF3 to stimulate IFN-β gene transcription. Endosomal signaling has been also implicated in IFN-β induction by TLR2 in response to

Gram-positive bacteria, although this mechanism appears to be restricted to specific immune cells and/or pathogens (61–63). The cytosolic receptors NOD1 and NOD2 were reported to trigger IFN-β production following infection with *Helicobacter pylori* and *M. tuberculosis*, respectively (20, 64). NOD2 engagement can induce IFN-β also in responses to *Staphylococcus aureus* (14).

While the pathways causing IFN-β induction by bacteria are relatively well understood, more studies are needed to assess the importance of individual pathways for the overall IFN-β production in whole organism rather than cells. One of the rare studies on this topic revealed that the dominant IFN-β-inducing pathway during infection with *Streptococcus pyogenes* (group A streptococcus) is the TLR13-mediated RNA recognition pathway (9).

Additional work is also needed to clarify the key IFN-β producing cells, as investigated during, e.g., *L. monocytogenes* infections (65–67) and the reported cell type-specific features of IFN-β induction (12, 15, 16).

TIPPING THE BALANCE I: BENEFITS OF IMMUNOMODULATORY EFFECTS OF TYPE I IFN SIGNALING DURING BACTERIAL INFECTIONS

Type I IFNs' ability to stimulate immune responses against viruses has been established very early after their discovery but it soon became clear that these cytokines exhibit also immunosuppressive activities. The first evidence for such immunosuppressive activities was provided in a study showing that type I IFNs were able to reduce carrageenin-induced footpad swelling (68). Thus, type I IFNs are now regarded as immunomodulatory cytokines capable of enhancing or dampening the immune response, depending on the context. This ambiguousness contributes to the disparate and still incompletely understood roles of type I IFNs during bacterial infections. Importantly, no unifying principles have been found to date: neither the beneficial nor detrimental effects of type I IFN signaling correlate with the broad pathogen classification into Gram-positive and -negative, extra- and intracellular pathogens, or the route of infection (**Table 1**; **Figure 1**).

Immunosuppressive effects of type I IFN signaling are beneficial during infection with the Gram-positive largely extracellular human pathogen *S. pyogenes* (9). *S. pyogenes* is the causative agent of mild (e.g., pharyngitis and scarlet fever) but also invasive and life-threatening infections (e.g., cellulitis, necrotizing fasciitis, and streptococcal toxic shock syndrome). Mice deficient in the type I IFN receptor IFNAR1 are more susceptible to subcutaneous *S. pyogenes* infection, which is a relevant model of severe invasive infection of the soft tissue (15). Type I IFN signaling promotes resistance against *S. pyogenes* by suppressing the transcription of the *Il1b* gene (9). The absence of type I IFN signaling results in an unrestricted production of IL-1β thereby causing a lethal hyperinflammation and organ damage. Importantly, type I IFN signaling balances rather than prevents *Il1b* transcription so that a controlled and life-saving IL-1β production is achieved (9). The key IFN-β producer and effector cells in this infection model are both LysM+ and CD11c+ myeloid cells (9).

Immunomodulation by type I IFN signaling is protective during infection with the human Gram-positive extracellular pathogen GBS (16, 17). GBS is regarded as commensal microbe asymptomatically colonizing the skin and mucosal tissues of 30% people, yet it is the leading cause of severe neonatal pneumonia and meningitis in developed countries. The absence of IFNAR1 results in increased bacterial loads during both subcutaneous GBS infection of neonate mice and intravenous infection of adult animals (16, 17). Similarly, type I IFN signaling is protective against uncontrolled bacteremia during infection with the Gram-negative intracellular bacterium *L. pneumophila*, which is a frequent cause of the severe pneumonia, Legionnaire's disease (18). Type I IFN signaling inhibits in a cell-autonomous way replication of *L. pneumophila* inside the infected cell (18, 19). The organismal physiology of the protective effects of type I IFN signaling during infection with GBS and *L. pneumophila* remains to be elucidated so that it is presently unclear whether immunosuppressive or immunostimulatory effects of type I IFNs drive the resistance against these two pathogens.

Stimulation of the immune response by type I IFN signaling is advantageous in defense against the Gram-negative pathogen *H. pylori* (20). *H. pylori* is a frequent cause of chronic gastritis and is associated with increased risk of gastric ulcers and stomach cancer. Deficiency in type I IFN signaling causes increased *H. pylori* loads in the stomach of orally infected mice. The lack of type I IFN responses is associated with decreased levels of the chemokine CXCL10 suggesting that type I IFNs promote defense against *H. pylori* by stimulating CXCL10-driven inflammation (20). Immunostimulatory effects of type I IFNs are beneficial also during gastric infection with the food-borne Gram-positive intracellular pathogen *L. monocytogenes* (28). *L. monocytogenes* infects the gastrointestinal tract, where it traverses the epithelial barrier and spreads into distant organs. Deficiency in type I IFN signaling results in an increased bacterial dissemination and is accompanied by diminished production of several pro-inflammatory cytokines, including TNF and IL-6 upon gastric infection using oral gavage (28). Interestingly, type I IFN signaling plays no role in an infection model using food contaminated with *L. monocytogenes* (30).

TIPPING THE BALANCE II: DISADVANTAGES OF IMMUNOMODULATORY EFFECTS OF TYPE I IFN SIGNALING DURING BACTERIAL INFECTIONS

Immunosuppression by type I IFN signaling is detrimental during infection with the intracellular pathogen and causative agent of tuberculosis, *M. tuberculosis* (22–24). Type I IFN signaling-mediated inhibition of IL-1 cytokines during *M. tuberculosis* lung infection blunts the antimicrobial defense and results in increased local as well as systemic bacterial loads (24). The key type I IFN effector cells are transplantable inflammatory monocyte-macrophage cells and DCs (24). The precise mechanism of

IL-1 inhibition by type I IFN signaling in this infection model is not resolved but includes both direct as well as indirect mechanisms (24). The indirect IL-1 inhibition appears to be mediated by the anti-inflammatory cytokine IL-10 which is known to be upregulated by type I IFNs (69). The importance of type I IFN signaling in *M. tuberculosis* infections is underlined by type I IFN signaling-associated gene expression pattern found in blood cells of patients with active tuberculosis (70). The detrimental function of type I IFN signaling during *M. tuberculosis* lung infection is converted into a protective one if IFN-γ signaling is missing (71). Under such conditions, type I IFNs inhibit the polarization of macrophages into infection-permissive alternatively activated macrophages.

Inhibition of immune response by type I IFNs is deleterious during infection with the facultative intracellular Gram-negative bacterium *F. novicida* (31). *F. novicida* is a subspecies of *Francisella tularensis* which infects humans through the skin or aerosol droplets and causes ulceroglandular or pneumonic tularemia, respectively. IFNAR1-deficient mice infected intradermally with *F. novicida* respond by an increased IL-17 production compared to WT animals and, correspondingly, are more resistant against infection (31). Similar increase in resistance is also observed during lung infection with *F. tularensis* (31). The key IL-17 producers during *F. novicida* infection are IL-17A+ γδ T cells which show enhanced expansion in the absence of type I IFN signaling.

Interferon-β exacerbates infection with *S. typhimurium* by reducing the ability of the host to launch a complete immune response (34). *S. typhimurium* is Gram-negative intracellular pathogen associated with gastroenteritis in humans and a severe disease resembling typhoid fever in mice. Mice deficient in IFN-β are more resistant against oral infection with *S. typhimurium* and display enhanced expression of IL-1β and the neutrophil chemoattractants, CXCL1 and CXCL2 (34). These changes are attributable to IFN-β-mediated inhibition of these genes in macrophages and are independent of *S. typhimurium*-induced macrophage death.

Listeria monocytogenes-induced type I IFN signaling downregulates the expression of both type II IFN receptor subunits, IFNGR1 and IFNGR2, thereby decreasing the responsiveness of macrophages and DCs to IFN-γ (29). The suppression of the IFN-γ signaling results in an increased susceptibility to *L. monocytogenes* infection. Increased susceptibility to *L. monocytogenes* infection is also caused by type I IFN-mediated induction of anti-inflammatory IL-10 (72).

Immunosuppressive effects of type I IFNs are harmful during postinfluenzal bacterial pneumonia (36–39). The immune response during a secondary postinfluenzal infection with the Gram-positive extracellular pathogen *S. pneumoniae*, a key causative agent of pneumonia, is impaired. This is caused by the ability of type I IFNs to suppress production of the neutrophil chemoattractants CXCL1 and CXCL2, the macrophage chemoattractant CCL2, and the inflammation-promoting cytokine IL-17 (36–38). The resulting reduction of leukocyte infiltration in the lung diminishes the capability of the host to control bacterial growth. Similar alterations in the immune response appear to be responsible for the increased susceptibility of mice to postinfluenzal infection with *S. aureus* and *Pseudomonas aeruginosa* (39). The mechanisms of the immunosuppressive effects of type I IFN signaling during postinfluenzal bacterial infection are not well understood but they might act downstream of the type I IFN-mediated inhibition of IL-1 cytokines.

Enhancement of the inflammatory response is associated with detrimental effects of type I IFN signaling during lung infection with the Gram-positive extracellular pathogen *S. aureus* (21). IFNAR1-deficient mice exhibit a lower TNF and IL-6 production and decreased leukocyte infiltration in the lung compared to WT animals suggesting that type I IFN signaling causes an exacerbated tissue damage (21). The pathogenicity of *S. aureus* strains correlates with the levels of type I IFNs induced during infection with differently virulent strains (14).

TIPPING THE BALANCE III: REGULATION OF TISSUE AND CELL INTEGRITY BY TYPE I IFNs DURING BACTERIAL INFECTIONS

Type I IFN signaling plays an indispensable role in the preservation of the epithelial barrier and epithelial integrity during lung infection with *S. pneumoniae* (10, 11). Type I IFN signaling promotes the maintenance of lung epithelial tight junctions during *S. pneumoniae* infection thereby reducing the passage of the pathogen from alveoli into the lung parenchyma (10). IFNAR1-deficient mice display increased permeability of the lung epithelium and enhanced invasiveness of *S. pneumoniae* infection associated with higher bacterial burden in distant organs. Type I IFN signaling protects the barrier function of the lung during *S. pneumoniae* infection also by promoting survival of the alveolar epithelial type II cells as revealed by IFNAR1 deletion specifically in this subtype of the barrier epithelium (11).

A common detrimental effect of type I IFN signaling during bacterial infections is the induction of various types of leukocyte cell death. Type I IFN-facilitated apoptosis of macrophages and lymphocytes appears to contribute to the increased susceptibility of WT mice compared to IFNAR1-deficient animals to intravenous and intraperitoneal infection with *L. monocytogenes* (25–27, 73). Type I IFN-facilitated death of macrophages is associated also with harmful effects of type I IFNs during infection with *F. novicida* and *S. typhimurium* (32, 33). *F. novicida* promotes macrophage death by type I IFN-mediated inflammasome-activation whereas *S. typhimurium* employs type I IFN induction to stimulate RIP-dependent macrophage necroptosis.

CONCLUSION AND FUTURE DIRECTIONS

Ample evidence exists for the pivotal role of type I IFNs in regulation of defense against bacterial pathogens. The complex and often disparate effects of type I IFNs on the outcome of different bacterial infections provide chances to exploit type I IFNs and their inducers as well as effectors for adjuvant therapies tailored to specific infectious diseases. A prerequisite for the development of such therapies is a detailed understanding of the molecular, cellular, and organismal physiology of type IFNs in the course

of bacterial infections. The following topics appear particularly important since they represent rather underexplored yet critically important areas.

Pathogen Species

The inconsistent roles of type I IFNs during infections with different bacteria remain a significant and challenging topic in the current research. Only few common principles of type I IFN action have been found to date. They include the cell death-promoting effects of type I IFNs which contribute mostly to detrimental functions of type I IFN signaling. Another frequent observation is the suppression of IL-1β and neutrophil chemoattractants—these effects are, however, associated with both beneficial and harmful consequences for the infection outcome. Future studies employing pathogens which have not yet been analyzed in detail, such as *Klebsiella pneumoniae*, uropathogenic *E. coli*, or *Clostridium difficile*, might reveal novel common principles.

Infection Route and Tissue-Specific Features of Type I IFN Signaling

The complexity of type I IFN function in bacterial infections is further increased by the distinct effects of type I IFNs in response to the same but differently administered pathogen. Type I IFNs are harmful followed intraperitoneal or intravenous infection with *L. monocytogenes* but protective in a physiologically more relevant intragastric infection (25–28). In contrast, type I IFN signaling has no impact on the overall outcome of *L. monocytogenes* infection after ingesting pathogen-contaminated food (30). These observations suggest that type I IFN signaling has, with regard to bacterial infections, distinct functions in different tissues/organs. This implication is supported by a recent study showing that exogenous type I IFN has, depending on the site of administration, disparate effects on the course of lung infection with *Coxiella burnetii* (35). Infections with *C. burnetii*, a Gram-negative intracellular bacterium, in humans occur after inhalation of bacteria and result in Q fever which can develop into an atypical pneumonia. Lung infection with *C. burnetii* in mice has a more severe course in WT compared to IFNAR1-deficient animals indicating that type I IFN signaling is disadvantageous in this infection model (35). Consistently, intraperitoneally administered type I IFN exacerbates *C. burnetii* infection. However, intratracheal delivery of type I IFN ameliorates the course of *C. burnetii* infection. The mechanisms of these distinct effects of type I IFN signaling in different tissues remain to be elucidated. Future studies should investigate other pathogens known of using various routes of infection and focus on physiologically most relevant routes.

Most Significant Type I IFN Inducers and Effectors

Modulation of type I IFN production during bacterial infection might represent a powerful approach in therapy of infectious diseases. Therapeutic targeting of type I IFN production requires the knowledge of the most important type I IFN-inducing pathway in a given infection. As most bacterial pathogens employ more than one pathway to stimulate type I IFN production, future efforts should focus on the identification of the most crucial bacterial and cellular components involved in type I IFN induction. These studies will need to use a combination of bacterial and animal host genetics for functional assessment and suitable reporter as well as imaging systems for type I IFN detection *in vivo*. On the effector side, recent studies provided a number of novel type I IFN-induced factors which interfere with pathogen replication and survival. Notably, various type I IFN-inducible small GTP-binding proteins have recently been showed to significantly contribute to the effects of type I IFNs [e.g., Ref. (74–77)]. These and yet to be discovered effectors represent potential targets for therapeutic intervention.

Human versus Mouse Systems

While many host defense mechanisms are well conserved among mice and men, important differences exist. For example, the mouse type I IFN inducer TLR13 is not expressed in humans (9, 53). Conversely, the human but not mouse TLR8 appears to be involved in type I IFN induction by bacterial RNA (59). Some antimicrobial functions of human neutrophils are enhanced by type I IFNs (78) whereas such stimulatory effects have so far not been described in mouse neutrophils. Future work should put more emphasis on studies of common and distinct features of type I IFN functions in bacterial infections.

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All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Host and Viral Modulation of RIG-I-Mediated Antiviral Immunity

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Innate immunity is the first line of defense against invading pathogens. Rapid and efficient detection of pathogen-associated molecular patterns *via* pattern-recognition receptors is essential for the host to mount defensive and protective responses. Retinoic acid-inducible gene-I (RIG-I) is critical in triggering antiviral and inflammatory responses for the control of viral replication in response to cytoplasmic virus-specific RNA structures. Upon viral RNA recognition, RIG-I recruits the mitochondrial adaptor protein mitochondrial antiviral signaling protein, which leads to a signaling cascade that coordinates the induction of type I interferons (IFNs), as well as a large variety of antiviral interferon-stimulated genes. The RIG-I activation is tightly regulated *via* various posttranslational modifications for the prevention of aberrant innate immune signaling. By contrast, viruses have evolved mechanisms of evasion, such as sequestrating viral structures from RIG-I detections and targeting receptor or signaling molecules for degradation. These virus–host interactions have broadened our understanding of viral pathogenesis and provided insights into the function of the RIG-I pathway. In this review, we summarize the recent advances regarding RIG-I pathogen recognition and signaling transduction, cell-intrinsic control of RIG-I activation, and the viral antagonism of RIG-I signaling.

Keywords: innate immunity, antiviral, infection, RIG-I, type I IFNs, virus–host interaction

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INTRODUCTION

Eukaryotic organisms rely on the host innate immune system to defend against viruses or other pathogenic microbes in early phases of infection. The innate antiviral immune response starts with the detection of evolutionarily conserved structures, termed pathogen-associated molecular patterns (PAMPs), by a set of germline-encoded pattern-recognition receptors (PRRs). With respect to their cellular localization, ligand specificity, and functions, PRRs are categorized into distinct families including the toll-like receptors, nucleotide-binding oligomerization domain-like receptors, C-type lectin receptors, retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) (1–5), as well as cytosolic viral DNA sensors such as cyclic GMP-AMP synthase (6, 7). Following the detection of specific viral PAMPs, PRRs trigger the activation of intracellular signaling cascades, ultimately leading to the production of type I interferons (IFNs), as well as pro-inflammatory cytokines. Secreted IFNs are crucial for the induction of numerous interferon-stimulated genes (ISGs); the products of which are major forces in controlling and restricting viral infections, thereby establishing a cellular antiviral state as well as helping to shape the adaptive immune response (8). Recent studies showed that viruses have evolved complex strategies to affect multiple stages of the host antiviral defense, from inhibiting the viral detection to manipulating components of the signaling pathways (9, 10).

To ensure successful antiviral defenses and to avoid aberrant or dysregulation of host immune signaling, antiviral pathways need to be tightly regulated at each level. In this review, we will summarize the cell-intrinsic regulation of RIG-I receptor activity, as well as the viral strategies to subvert the RIG-I signaling machinery.

RIG-I STRUCTURE AND LIGAND INTERACTIONS

The three members of the RLR family: RIG-I, MDA5 (melanoma differentiation factor 5), and LGP2 (laboratory of genetics and physiology 2) are expressed in most cell and tissue types. They function as cytoplasmic sensors for the recognition of a variety of RNA viruses and subsequent activation of downstream signaling to drive type I IFN production and antiviral gene expressions. These three RLR proteins are RNA-dependent ATPases belonging to the DExD/H-box family of helicases (11). Structurally, RLRs have a similar central helicase core that is comprised of two helicase domains, Hel1 and Hel2 with an insertion termed Hel2i. In addition, they all have a C-terminal domain (CTD). However, only RIG-I and MDA5 contain two N-terminal caspase activation and recruitment domains (CARDs) (3) (**Figure 1A**). Among these three, RIG-I is the founding member and hence the most intensively studied member of this family. Each domain of RIG-I plays unique roles during RIG-I autorepression and activation. In brief, the CTD and helicase domain are involved in RNA ligand binding and ATP hydrolysis-involved conformational changes (12–14), whereas the RIG-I CARDs facilitate interaction with other downstream CARD containing molecules (15).

Retinoic acid-inducible gene-I has been shown to be involved in the recognition of a variety of RNA viruses in the cytoplasm, such as the Sendai virus, influenza A and B viruses (IAV, IBV), vesicular stomatitis virus, measles virus (MV), Newcastle disease virus, Ebola virus (EBOV), dengue virus (DENV), and hepatitis C virus (HCV) (16–19). The short double-stranded (ds) RNA with a triphosphate (ppp) motif at the 5′-end, as found in these viral genomes, were shown to be a key signature recognized by RIG-I (20, 21). The 5′ppp dsRNA of viral nucleocapsids has also been characterized as stimulating RIG-I (22). 5′-Diphosphate-bearing RNA (5′ppRNA), either naturally contained in viruses, produced by *in vitro* transcription, or *via* chemical synthesis, were all shown to bind to RIG-I and were sufficient to activate RIG-I (20, 23). Physiologically, the control of *in vitro* and *in vivo* infections of reoviruses, which bear the 5′ppRNA genome, relies on RIG-I functionality (24). It is worth noting that the *in vitro*-synthesized 5′pppRNA sequences also trigger RIG-I activation (25). These agonists have demonstrated their therapeutic potential as broad-spectrum antiviral agents and could be optimized as vaccine adjuvant candidates (26–30). Furthermore, the recognition of several DNA viruses, including herpes simplex virus type 1 (HSV-1), Epstein–Barr virus (EBV), vaccinia virus (VACV), and adenovirus, *via* the RNA polymerase III were found to be RIG-I-dependent (31, 32). Interestingly, the RIG-I-mediated upregulation of STING is required for protection against the HSV-1 by the RIG-I agonist, offering new evidence of the overlapping between RIG-I signaling and the host response to DNA viral infection (33).

Notably, viral RNA triggered RIG-I signaling also mediates the inflammatory response *via* distinct pathways. The first involves the formation of the RIG-I inflammasome through interactions between RIG-I, ASC, and caspase-1 and the stimulation of IL-1β release. The second involves the adaptor proteins CARD9, Bcl-10, mitochondrial antiviral signaling protein (MAVS), and the activation of nuclear factor-κB (NF-κB) (34, 35). Upon RNA ligand binding, RIG-I undergoes a series of conformational changes and posttranslational modifications (PTMs) to achieve full activation (further detail below).

RIG-I SIGNALING TRANSDUCTION

Activated RIG-I recruits its downstream adaptor molecule MAVS (also known as IPS-1, CARDIF, and VISA) through CARD–CARD-mediated interactions (36, 37). The oligomeric RIG-I CARD assembly and the polymeric formation of MAVS, together serve as a signaling platform for protein complexes that mediate the bifurcation of signaling into two branches. One branch recruits tumor necrosis factor receptor-associated factors (TRAF)-2/6 and the receptor-interacting protein 1 to subsequently activate the IKK complex, resulting in NF-κB activation (38). The other branch signals through TRAF3 and activates the TANK/IKKγ/IKKϵ/TBK1 complex, leading to the phosphorylation and dimerization of interferon regulator factors (IRF)-3 and -7 (39, 40). Activated IRF3/7 and NF-κB then translocate to the nucleus, together with ATF2, c-Jun, and the transcription coactivator CREB-binding protein/p300, to coordinate the IFN and pro-inflammatory gene expressions (41). Once secreted, IFNs bind to specific cell surface receptors and activate the JAK–STAT pathway. The activated transcription factors STAT1, STAT2, and IRF9 form the interferon-stimulated gene factors (ISGF3) complex. ISGF3 then translocates to the nucleus and coordinates the transcription of hundreds of ISGs including RIG-I, thus generating an amplifying loop leading to the accumulation of RIG-I during several types of infections (8) (**Figure 1B**).

MECHANISMS OF RIG-I ACTIVATION

RIG-I Autorepression

Structural and biochemical studies have demonstrated that the activation of RIG-I is a multi-step process and is primarily regulated by conformational changes and PTMs. When initially identified as a dsRNA sensor, it was hypothesized that RIG-I was under negative regulation in physiological conditions. The over expression of the CARD domain of RIG-I alone demonstrated superior signaling activity than full length RIG-I in absence of viral PAMPs (2). Studies by Saito et al. showed that the deletion of CARD was dominant-negative for RIG-I signaling. By contrast, the deletion of repressor domain (RD) resulted in constitutive signaling, whereas RD expression alone ablated RIG-I signaling actions. Together, these findings provided the model of RIG-I autoregulation in which the RD is predicted to mask CARDs for signaling transduction in uninfected cells (42). The crystal structural analysis further delineated the models of autorepressed and ligand activated states of RIG-I, respectively. In a

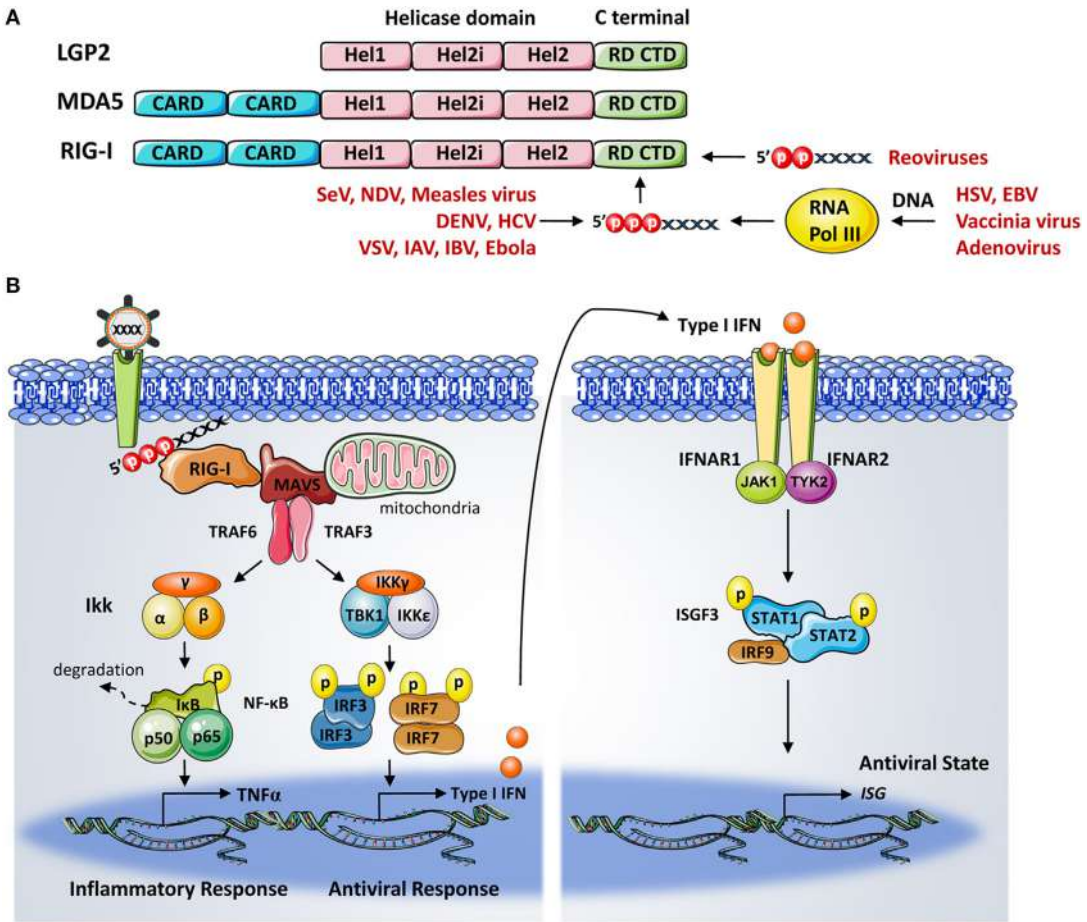

FIGURE 1 | **(A)** Domain structure of retinoic acid-inducible gene-I (RIG-I). RIG-I belongs to the DExD/H-box family of helicases and is comprised of helicase domains 1 (Hel1) and 2 (Hel2) with a Hel2i insertion, N-terminal caspase activation and recruitment domains, and a C-terminal domain (CTD) or repressor domain. RIG-I CTD is responsible for recognizing a plethora of RNA viruses with short 5′ triphosphate (5′ppp) RNA and 5′-diphosphate-bearing RNA structures. RIG-I also detects 5′ppp RNA species synthesized through the transcription of viral DNA by RNA polymerase III. **(B)** The cytoplasmic pattern-recognition receptor RIG-I is essential for recognizing RNA viruses with a 5′ppp signature. Upon viral RNA recognition, RIG-I recruits the adaptor protein mitochondrial antiviral signaling protein to activate the TBK1–IKKε complex and IKKα–IKKβ complex, which are responsible for the activation of transcription factors interferon regulator factor (IRF) 3, IRF7, and nuclear factor-κB. These transcription factors then translocate to the nucleus and coordinate the induction of type I interferons (IFNs). This is followed by the binding of the IFNs α and β to their cognate receptor, which will lead to the transcriptional activation of interferon-stimulated genes (ISGs) by the JAK/STAT signaling pathway. The products of ISGs are key factors in limiting pathogen spreading.

ligand-free state, CARDs and Hel2i interactions hinder dsRNA binding and inactivate RIG-I (14). The binding of 5′ppp dsRNA to RD leads to a conformational switch of RIG-I, which releases the autorepressed CARDs and exposes the helicase domain for ATP binding (14, 43). ATP hydrolysis is essential for RIG-I signaling. It enables RIG-I to translocate along the dsRNA, and further promotes the oligomerization of RIG-I CARDs. These processes assemble RIG-I into a filamentous architecture which facilitates the CARD–CARD interactions with the mitochondrial MAVS, leading to the subsequent signaling transduction for IFN production (44, 45). Importantly, RIG-I ATPase activity also plays a role in distinguishing self-RNA from non-self-RNA (46). It was reported that RIG-I ATP hydrolysis increases the binding affinity of RIG-I and dsRNA ligands; whereas the RIG-I mutants deficient in ATP hydrolysis promotes the interaction of RIG-I and self-dsRNA and results in unintentional immune signaling (47).

Posttranslational Control of RIG-I

Ubiquitination

One of the first PTMs of RIG-I following the initial ligand recognition is performed by the robust ubiquitination machinery (**Figure 2**). Mass spectrometry analysis revealed that TRIM25, a member of the tripartite motif (TRIM) protein family possessing E3 ligase activity, induces the covalent Lys63-linked ubiquitination of RIG-I. Mechanistically, the C-terminal SPRY domain of TRIM25 interacts with CARD1 and facilitates the ubiquitination of CARD2 at K172 (48). The RIG-I–TRIM25 ubiquitination complex, associates with the adaptor protein 14-3-3ε and translocates to mitochondria for MAVS binding (49). Mutation

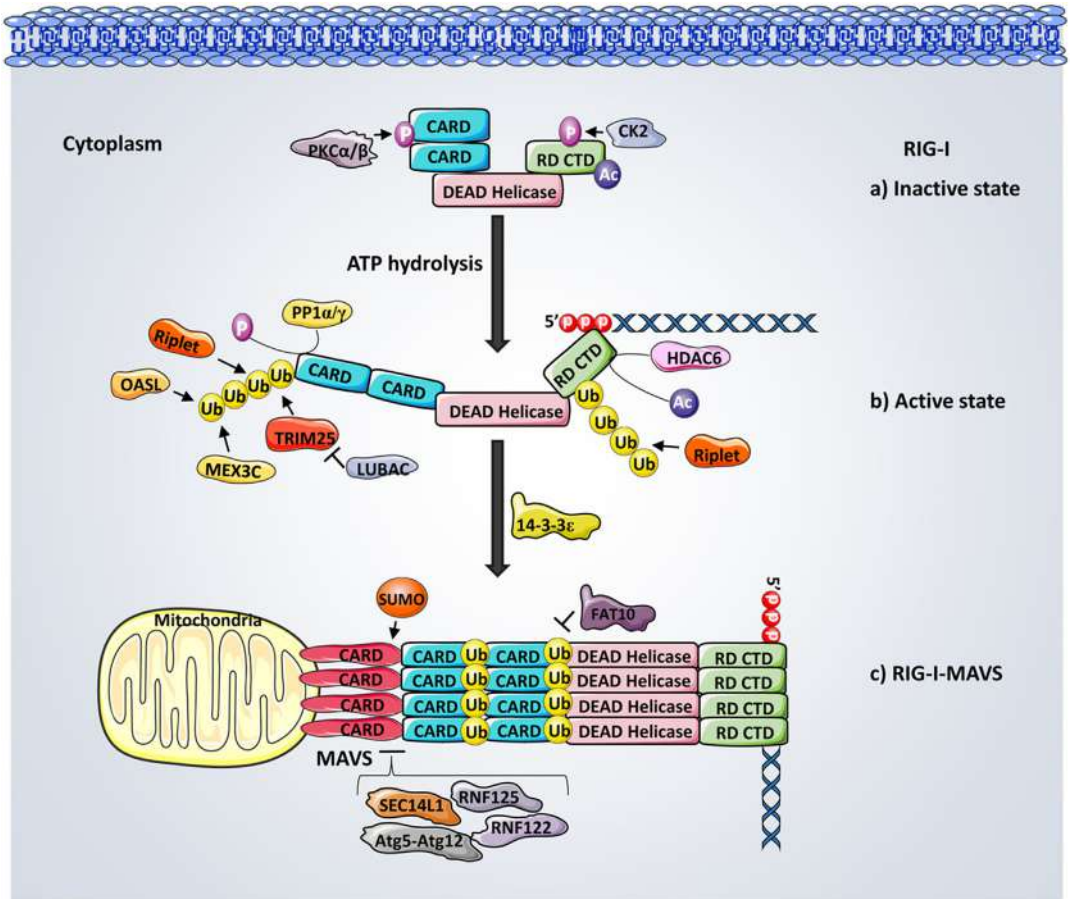

FIGURE 2 | **Regulation of retinoic acid-inducible gene-I (RIG-I) activation**. (a) In resting cells, RIG-I is kept inactivated through the phosphorylation of caspase activation and recruitment domains (CARDs) and C-terminal domain (CTD) mediated by casein kinase II and protein kinase C-α/β, respectively. (b) Following the binding of 5′ triphosphate (5′ppp) RNA and ATP hydrolysis, RIG-I is dephosphorylated by phosphoprotein phosphatase 1-α/γ and results in a conformational change that opens CARDs. HDAC6-mediated deacetylation of RIG-I CTD is critical for RIG-I and 5′pppRNA binding. The Lys63-linked ubiquitination of RIG-I mediated by TRIM25, Riplet, oligoadenylate synthetases-like protein, and MEX3C at both CARDs and CTD further activate RIG-I and facilitate its tetramerization. (c) Interactions between RIG-I–TRIM25 complex and 14-3-3ε promote RIG-I translocation to mitochondrial mitochondrial antiviral signaling protein (MAVS) for downstream signaling, leading to interferon production. Interactions between TRIM25, RIG-I, and MAVS are further negatively regulated by the Lys48-linked ubiquitination, which is meditated by LUBAC, RNF125, and RNF122. SEC14L1 and Atg5–Atg12 both inhibit the signaling by interrupting RIG-I–MAVS interactions, whereas SUMOylation promotes RIG-I–MAVS binding.

of K172 disrupts the interaction between RIG-I and MAVS thus abrogating downstream signaling and IFNs production (50). Furthermore, a RIG-I splice variant which lacks the TRIM25 interaction domain acts as a feedback inhibitor of RIG-I signaling transduction upon viral infections (48). In addition, Riplet (RING-finger protein leading to RIG-I activation, also named RNF135 or REUL), another E3 ubiquitin ligase, also promotes RIG-I ubiquitination. Multiple sites within the CARDs, as well as within the CTD of RIG-I, were identified as the crucial ubiquitin anchoring residues (51–53). Among which, K63-linked polyubiquitination (pUb) at Lys788, is demonstrated as being critical for RIG-I activation. However, unlike TRIM25-induced ubiquitination, Riplet induced RIG-I pUb is dispensable for RIG-I-RNA binding but is essential for releasing CARD from its autorepressed state. This enhances TRIM25 functionality as well as promoting the oligomerization of RIG-I and the activation of MAVS (54). MEX3C (Mex-3 RNA binding family member C), another recently identified E3 ligase, also mediates Lys63-Ub at K99 and K169 of CARD, playing a critical role in RIG-I activation (55). In addition, the oligoadenylate synthetases-like (OASL) protein, although not an E3 ubiquitin ligase itself, contains a dsRNA-binding groove and enhances RIG-I activation by mimicking the K63-linked pUb through its ubiquitin-like (UBL) domain (56, 57). Non-covalent binding of K63-ubiquitin chains to CARDs also potently activates RIG-I (58). Recent structural analysis suggests that covalent and non-covalent binding of ubiquitin synergistically stabilize RIG-I tetramerization and enhance polymerization of MAVS CARDs (59).

On the other hand, several deubiquitinating enzymes (DUBs) were identified to remove K63-linked pUb chains from RIG-I, thus dampening RIG-I signaling. The tumor suppressor protein cylindromatosis (CYLD) removes K63-linked pUb chains from

RIG-I as well as TBK1 and IKKε to inhibit the IRF3 response, serving as a pathway negative regulator (60). Syndecan-4, a newly identified negative regulator of RIG-I, functions through attracting CYLD to RIG-I complex, thus potentiating the K63-mediated deubiquitination of RIG-I (61). In addition, the ubiquitin-specific protease (USP) family members, such as USP3 and USP21, were also identified as inhibitors of RIG-I activation by deubiqutinating RIG-I (62, 63).

In contrast to K63-linked ubiquitination, which promotes protein activation, K48-linked ubiquitination triggers proteasomal degradation of its target. For instance, the RING-finger protein 125 (RNF125), together with the ubiquitin E2 ligase UbcH5, conjugate K48-linked ubiquitin to RIG-I and MAVS, targeting them for proteasomal degradation and thereby inhibiting downstream signaling (64). Similarly, RNF122 was recently demonstrated to mediate the proteasomal degradation of RIG-I by delivering the K48-linked ubiquitin to RIG-I CARDs (65). The linear ubiquitin assembly complex (LUBAC) has been shown to promote K48 pUb of TRIM25, leading to its degradation (66). Conversely, the deubiquitinase USP15 antagonizes LUBAC by removing K48-linked ubiquitin from TRIM25, leading to its stabilization and thereby promoting RIG-I-mediated antiviral signaling (67).

Phosphorylation

In parallel with ubiquitination, phosphorylation has emerged in the past several years as a critical regulator of the RIG-I signaling transduction (**Figure 2**). Protein purification and mass spectrometry analysis identified that phosphorylation of Thr170 in the CARDs antagonizes RIG-I signaling by inhibiting TRIM25-mediated Lys172 ubiquitination and MAVS binding (68). Ser8 phosphorylation of CARDs also serves as a negative regulator of RIG-I (69). In addition, the CTD of RIG-I is constitutively phosphorylated at Thr770 and Ser854/855 by casein kinase II to promote intermolecular interactions between CTD and CARDs, thereby maintaining RIG-I at an autorepressive state to prevent premature downstream signaling (70). A recent mass spectrometry analysis revealed that IKK phosphorylates RIG-I at Ser855, thereby providing a negative feedback regulation of RIG-I (71). Furthermore, conventional protein kinase C-α (PKC-α) and PKC-β have also been shown to phosphorylate CARDs, thus suppressing RIG-I–TRIM interaction and subsequent antiviral responses (72). In fact, RIG-I signaling activity is controlled by a dynamic balance between phosphorylation and dephosphorylation. Dephosphorylation of RIG-I occurs rapidly with the presence of viral RNA. A functional siRNA screen identified phosphoprotein phosphatase 1-α (PP1α) and PP1γ as essential phosphatases responsible for CARDs dephosphorylation at Ser8 and Thr170, leading to RIG-I signal activation and viral inhibition (73).

Acetylation

In addition to the ubiquitination and phosphorylation described above, acetylation modulation has recently started to gain more acknowledgment for controlling RIG-I activity (**Figure 2**). Mass spectrometry has identified the acetylation of two lysine residues (K858 and K909) in the CTD of RIG-I at its inactivate state and are deacetylated during viral infection (74). The mutation of these two sites restricts RIG-I from undergoing the virus-induced interaction with MAVS. K858 and K909 acetylation of RIG-I has also been shown to control the PAMP RNA-induced RIG-I oligomerization (75). The cytoplasmic deacetylase HDAC6-mediated removal of K909 acetylation has been shown as critical for RIG-I binding to dsRNA during viral infections (76). Furthermore, HDAC6-dependent RIG-I deacetylation also regulates RIG-I oligomerization upon ligand binding, thus facilitating RIG-I activation (75).

Other Regulatory Mechanisms

RIG-I signal transduction is further regulated by additional PTMs, regulatory proteins, and other cellular processes (**Figure 2**). It is worth noting that a number of UBL proteins including SUMO, ISG15, FAT10, and Atg8–Atg12 are involved in these positive or negative regulatory mechanisms (77). SUMOylation serves as a positive regulator of RIG-I by enhancing the RIG-I and MAVS binding (78). On the contrary, the HLA-F adjacent transcription 10 (FAT10), an UBL modifier protein, was shown to negatively regulate RIG-I by modulating RIG-I solubility through a non-covalent association with CARDs (79). In addition, IFN-induced ISG15 negatively regulates the RIG-I mediated signaling in a feedback-loop control manner (80). SEC14L1 has been observed competing with MAVS for RIG-I CARD binding (81). Furthermore, autophagy has been reported to be involved in RIG-I modulation through its key regulator, the Atg5–Atg12 conjugate. Atg5–Atg12 has been found to suppress RIG-I–MAVS interaction, thereby inhibiting downstream signaling (82). Recently, deamidation of CTD has been described as a distinct means to induce RIG-I activation. For examples, vGAT (glutamine amidotransferase), from KSHV (kaposi's sarcoma-associated herpesvirus) and γHV68 (murine gamma herpesvirus 68), recruits cellular phosphoribosylformyglycinamide synthase to deamindate and activate RIG-I (83, 84).

VIRAL ANTAGONISM OF RIG-I SIGNALING

In order to establish infections, viruses have developed sophisticated mechanisms to counteract host immune responses. With regard to RIG-I signaling, these include mechanisms such as altering viral genomes and their intermediate transcripts to avoid detection, manipulating the activation and degradation of RIG-I and MAVS, as well as modulating downstream signaling cascades. Studying these antagonistic viral strategies has greatly broadened our understanding of RIG-I activation and regulation.

Sequestration of Viral RNAs

Since 5′ triphosphate (5′ppp) is an important feature recognized by RIG-I, modification of this motif has long been described as one of the major mechanisms for viruses to antagonize RIG-I signaling. Crimean–Congo hemorrhagic fever virus, Borna disease virus (BDV), and hantavirus (HTNV) remove the 5′ppp group on their genome posttranscriptionally, make RIG-I unable to bind to viral RNA, and therefore incapable of triggering RIG-I activation (85). Mechanistically, HTNV uses the "prime and realign" strategy to generate a 5′-terminal monophosphorylate

(86, 87). BDV on the other hand, employs genome trimming to form a 3′-terminal overhang as well as convert 5′ppp to 5′p to avoid detection by RIG-I (88). The arenavirus presents an unpaired 5′ppp-nucleotide overhang to evade recognition by RIG-I (89). The 5′-end of viral RNA can also be modified through RNA-capping pathways. For example, the genomic RNA of polioviruses linked to Vpg (viral protein genome-linked) to cap the 5′-end from exposure to RIG-I (90). The 5′-end capping with 7-methyl guanosine and methylation of 5′ppp dsRNA at the 2′-*O* position makes viral RNA non-distinguishable from the host mRNAs, and therefore does not stimulate RIG-I (91, 92).

By contrast, some viruses encode viral proteins to prevent RNA recognition. The EBOV utilizes its VP35 protein to sequester viral RNA (18). The crystal structural analysis indicates that the VP35 interferon inhibitory domain competes with RIG-I for dsRNA binding by forming an "end-cap" complex with dsRNA, resulting in substantially diminished activation of RIG-I (93). Similarly, the marburg virus VP35 spirals around the dsRNA backbone and end-caps the dsRNA to escape from RIG-I detection (94, 95). The IAV non-structural protein 1 (NS1) possesses dsRNA-binding properties to shield viral RNA from RIG-I (96). IAV has also been shown to antagonize RIG-I activation *via* its viral polymerase subunit PB2. PB2 position 627K in the mammalian strain increases PB2-nucleocapids binding affinity, thus inhibiting RIG-I interaction with the nucleoprotein-encapsidated 5′ppp RNA (22, 97).

In addition to altering and concealing their genome to prevent RNA binding, viruses also re-localize viral RNA to specific cellular compartments, such as mitochondria, endoplasmic reticulum (ER), and Golgi, to avoid cytosolic surveillance by RIG-I. For instance, the DENV conceals dsRNA in the intracellular membrane as an escape strategy (98). ER is an important organelle for viral entry, replication, and assembly. The severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) has been shown to induce a modified ER to hide its replicating RNA from detection (99). These viral antagonism strategies highlight the importance of cellular organelle localization in viral–host interactions during innate antiviral responses.

Manipulation of RIG-I–MAVS Signaling

Modulation of the PTMs

As reviewed above, ubiquitination represents one critical PTM mechanism of RIG-I activation and, not surprisingly, is an attractive target for viral manipulation (**Figure 3A**). Viruses have evolved ways to inhibit K63-linked ubiquitination of RIG-I by interacting with the E3 ligases TRIM25 and Riplet. For instance, IAV NS1 from various strains has been shown to suppress TRIM25-mediated RIG-I CARDs ubiquitination. Among all the TRIM25 binding amino acids identified in NS1, R38/K41 and E96/E97 were described as critical in interfering with the coil-coiled domain of TRIM25. These interactions resulted in an inhibition of TRIM25 multimerization and therefore blocked the RIG-I CARDs ubiquitination (100). Intriguingly, NS1-TRIM25 binding is found to be preserved in human and avian, but lost in mouse, indicating a species-specific manner of inhibition. This study further demonstrates that the NS1 suppression of RIG-I ubiquitination in mouse is Riplet-dependent (101). Conversely, phosphorylation of NS1 at Thr49 was recently identified as impairing the NS1–TRIM25 interaction, thereby suppressing its antagonistic activity of RIG-I signaling (102). Phosphorylation of another site on NS1, Thr80, has also been reported to disrupt NS1 binding affinity with RIG-I (103). Similar to IAV, the IBV non-structural NS protein (NS1-B) has recently been described as inhibiting RIG-I ubiquitination, which involves TRIM25-NS1 C-terminal effector domain interaction and the RIG-I/TRIM25/NS1-B complex formation (104). By contrast, the protease NS3-4A of HCV functions differently, rather than inhibiting TRIM25, it is thought to target the E3 ligase Riplet. NS3-4A directly disrupts Riplet, abolishes Riplet-mediated RIG-I ubiquitination, and further reduces the interaction between TRIM25 and RIG-I (54).

On the other hand, some viruses encode enzymes that directly deubiquitinate RIG-I. For instance, KSHV encoded deubiquitinase ORF64 cleaves Lys63-ubiquination chains on CARDs, blocks CARDs interaction between RIG-I and MAVS, thereby downregulating RIG-I signaling (105). Other viruses including arterivirus, nairovirus, SARS-CoV, and foot-and-mouth disease virus (FMDV) have also been reported to downregulate RIG-I ubiquitination through their viral encoded DUBs (106, 107).

Few viruses have been shown to manipulate RIG-I regulation with regards to targeting the phosphorylation or dephosphorylation process of RIG-I. Nevertheless, it was reported that MV efficiently escapes antiviral response *via* suppressing RIG-I dephosphorylation in dendritic cells (DCs). In this study, the growth arrest and DNA damage protein (GADD34) was shown to form complexes with PP1 to facilitate RIG-I activation. The MV infection induced DC-SIGN signaling results in an inhibition of GADD34-PP1 phosphatases activity and thereby impairs RIG-I activation (108).

Degradation of RIG-I and MAVS

Another distinct strategy used by viruses to antagonize RIG-I signaling is the direct cleavage or degradation of the receptor and multiple members of the signaling cascade (**Figure 3A**). RIG-I has been reported in some studies to be cleaved by the proteinase $3C^{pro}$ during infections with picornavirus, coxsackievirus B3 (CVB3), and enterovirus 71 (EV71) (109, 110). The encephalomyocarditis virus directs both caspase- and proteasome-dependent degradation of RIG-I (111). Intriguingly, the NS1–NS2 degradasome of the respiratory syncytial virus (RSV) has been shown to mediate the proteasomal degradation of RIG-I (112).

Mitochondrial antiviral signaling protein is also a well-studied molecule which is often targeted by many types of viral-induced cleavage. For example, the hepatitis A virus (HAV) cleaves MAVS for proteolysis by its protease $3C^{pro}$ (113). Both CVB3 proteinase $2A^{pro}$ and $3C^{pro}$ trigger MAVS cleavage at different sites during infection, and the cleavage of MAVS by EV71 is accomplished *via* its $2A^{pro}$ activity (110, 114). In addition, serine protease NS3-4A of HCV cleaves MAVS, removing it from the mitochondria, thereby inhibiting downstream signaling (36, 115). In a parallel fashion, many viruses mediate cellular proteolytic degradation of MAVS to attenuate RIG-I antiviral responses. Hepatitis B virus viral protein HBx triggers the proteasome-mediated degradation of MAVS through Lys136 ubiquitination (116). Another study

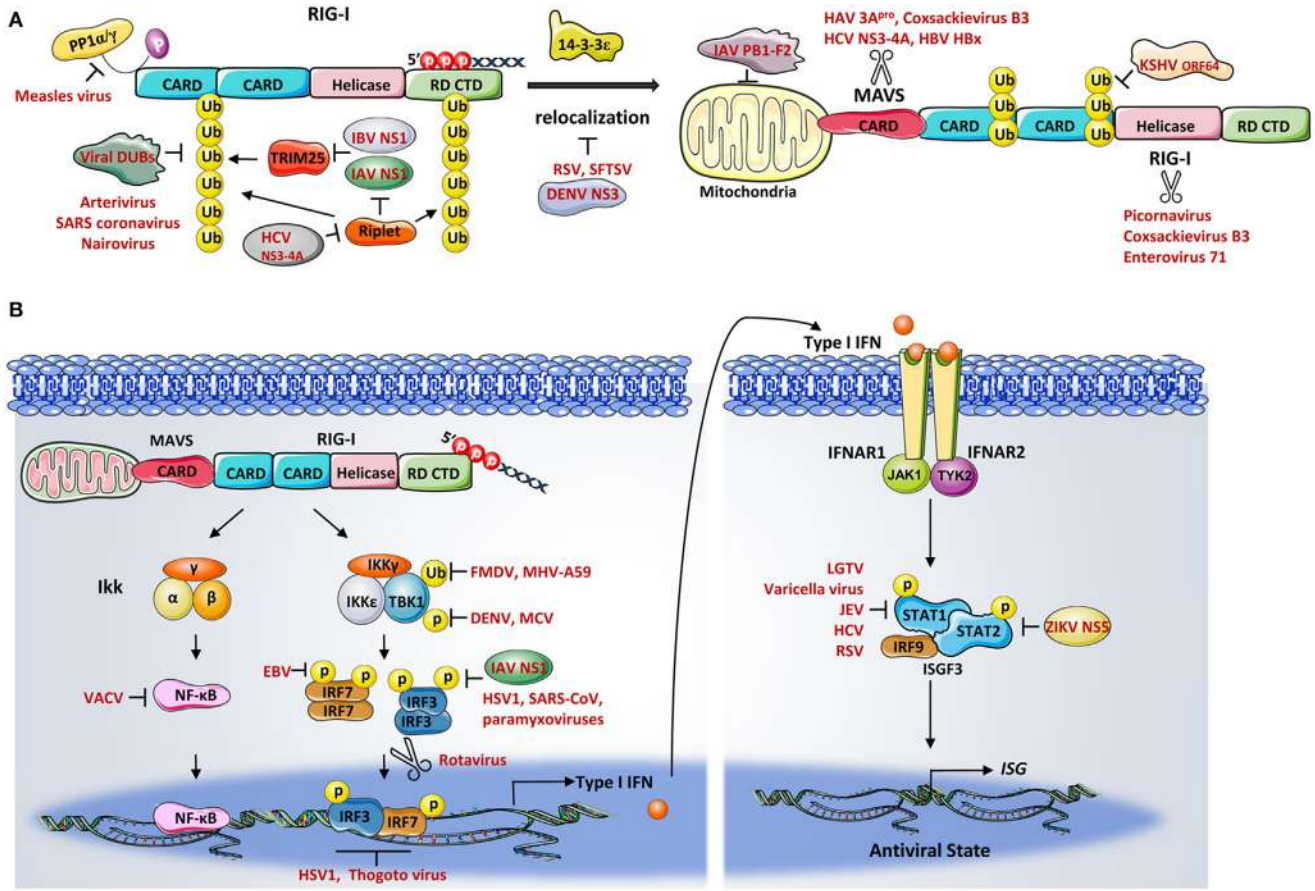

FIGURE 3 | **(A)** Viral inhibition of retinoic acid-inducible gene-I (RIG-I)– mitochondrial antiviral signaling protein (MAVS) signaling. Numerous viral proteins have been described as RIG-I signaling antagonists which inhibit the activation of RIG-I and its adaptor protein MAVS. Influenza A virus (IAV) NS1 and influenza B virus NS1 were both shown to suppress the TRIM25-mediated ubiquitination of RIG-I caspase activation and recruitment domains. The protease NS3-4A of hepatitis C virus functions by targeting Riplet. A number of virus-encoded deubiquitination enzymes from various viruses remove Lys63-linked ubiquitin from RIG-I, resulting in signaling suppression. The measles virus efficiently blocks RIG-I dephosphorylation to prevent RIG-I activation. Dengue virus (DENV), respiratory syncytial virus, and thrombocytopenia syndrome virus (SFTSV) were involved in disrupting RIG-I translocation to the mitochondria. Numerous virus-encoded proteases were shown to directly cleave or degrade RIG-I and MAVS. Additionally, the PB1-F2 of IAV decreases the mitochondrial membrane potential thereby inhibiting RIG-I–MAVS interaction. **(B)** Viral modulation of downstream signaling components. Ubiquitination- and phosphorylation-mediated TBK1 activation were shown be antagonized by a number of viruses, including foot-and-mouth disease virus, mouse hepatitis virus A59, molluscum contagiosum virus, and DENV. IAV NS1, herpes simplex virus type 1, severe acute respiratory syndrome coronavirus (SARS-CoV), and several paramyxoviruses have been shown to interfere with interferon regulator factor (IRF) 3 phosphorylation. Epstein–Barr virus suppresses IRF7 transcriptional activity. The rotavirus targets both IRF3 and IRF7 for degradation. The binding of transcription factors to the IFNB promoter is also challenged by herpes simplex virus and the thogoto virus. Other viruses subvert the JAK–STAT signaling to inhibit the production of interferon-stimulated genes. STAT1 and STAT2 are therefore often targets by a number of viruses.

reported that the HAV cysteine protease ABC targets MAVS for proteolysis at mitochondrial membrane (113). Additionally, viral modulation of cellular organelles such as mitochondria also affects RIG-I–MAVS signaling. The PB1-F2 of IAV, for instance, has been described as decreasing the mitochondrial membrane potential, resulting in the acceleration of mitochondrial fragmentation, thereby inhibiting RIG-I–MAVS signaling (117–119).

It is important to note that the proper localization of RIG-I and MAVS is a prerequisite for effective signaling transduction. MAVS resides on the mitochondrial membrane, peroxisomes, and mitochondria-associated membranes for antiviral signaling. In fact, a RIG-I translocon has been identified to direct RIG-I redistribution from cytosol to membranes during viral infection (49). Studies have shown that several viruses encode proteins to disrupt the proper localization of RIG-I or MAVS as a novel mechanism of regulation, such as NS3 of DENV (113), nucleoprotein of RSV (120), and non-structural proteins of thrombocytopenia syndrome virus (SFTSV) (121).

Modulation of Downstream Signaling Components

To ensure successful RIG-I signaling transduction, the kinase activities of TBK1 and IKKε are tightly controlled *via* various regulatory mechanisms and are common targets of viruses

(**Figure 3B**). For example, both the leader proteinase (L^{pro}) of FMDV (122) and the non-structural protein 3 (ns3) of the mouse hepatitis virus A59 (123) inhibit ubiquitination of TBK1. Dengue virus serotype4 non-structural proteins, NS2A and NS4B, as well as the FLIPs proteins encoded by the molluscum contagiosum virus (MCV), all reduce TBK1 phosphorylation, thereby preventing its activation (124, 125). Several viruses have been shown to prevent the formation of functional TBK1-containing complexes. The K7 protein of the VACV prevents TBK1/IKKε complex-induced IRF activation by targeting host DEAD box protein 3 (DDX3) (126). Two other viruses, the NY-1 HTNV and SARS-CoV, disrupt the TBK1–TRAF3 and TANK–TBK1/IKKε complex, respectively (127, 128). Moreover, SFTSV has been shown to irreversibly re-localize TBK1 and IKK from mitochondria and sequester the TBK1/IKKε/IRF3 complex *via* the formation of inclusion bodies, causing signaling cascade termination (129).

Viral regulation of the transcription factors, IRFs and NF-κB, further serve as points of control in RIG-I signaling (**Figure 3B**). One of the best studied examples is the inhibition of IRF3 activity by the IAV NS1 protein (130). Besides this, the HSV-1, rabies virus, SARS-CoV, as well as several paramyxoviruses have been demonstrated to interfere with the phosphorylation state of IRF3, thereby blocking IFN induction (131–134). The EBV conjugates SUMO to IRF7 at lysine 452 to decrease IRF7 transcriptional activity (135). The rotavirus NS1, targets both IRF3 and IRF7 for degradation to prevent IRFs from undergoing dimerization (136). Viruses have also developed various means to suppress the IRF3 DNA binding ability. Herpes simplex virus, thogoto virus, and KSHV, all developed strategies to downregulate IRF3 transcriptional activity by either disrupting IRF3 binding complex formations or competing binding regions on the IFNB promoter (137–139). Viral strategies in inhibiting cytoplasmic or transcriptional activities of NF-κB have been extensively studied during the VACV infection. Studies reported that multiple proteins encoded by VACV and HSV-1 suppress NF-κB activation (140–143).

Viruses have also developed multiple inhibitory mechanisms to counteract the IFN stimulation of ISGs by targeting STAT1 and/or STAT2 (**Figure 3B**). For example, the langat virus was shown to inhibit the phosphorylation of both STAT1 and STAT2 (144). Varicella viruses and the Japanese encephalitis virus, both block the JAK/STAT1 pathway through multiple mechanisms including inhibiting STAT proteins phosphorylation and nucleotranslocation (145, 146). The non-structural protein NS5 of several flaviviruses, have been shown to target STAT proteins *via* distinct mechanisms. For example, MNV NS5 inhibits STAT1 phosphorylation, whereas DENV NS5 interacts with UBR4 to promote STAT2 degradation (147, 148). By contrast, the Zika virus NS5 induced proteasomal degradation of STAT2 was recently identified as UBR4 independent (149). Furthermore, other viruses, such as HCV (150), RSV (151), and paramyxovirus (152), also demonstrate negative regulation of the JAK–STAT pathway.

CONCLUDING REMARKS

Studies from the past decade have well established RIG-I as one of the principal PRRs for the recognition of cytoplasmic viral RNA, as well as defining its critical role in the induction of IFNs during viral infections. Our understanding of the RIG-I-mediated antiviral response has been greatly expanded with the key discoveries made regarding the molecular mechanism of RIG-I regulation, such as ubiquitination, phosphorylation, and acetylation. Meanwhile, investigating viral strategies to manipulate RIG-I responses not only allow us to understand the viral pathogenesis, but also significantly contributed to our knowledge of how RIG-I is activated and regulated. These new insights into the viral-mediated RIG-I regulations are important for vaccine and drug development aiming to suppress infectious diseases and enhance immune responses.

AUTHOR CONTRIBUTIONS

YL wrote the manuscript. RL and DO revised and approved the manuscript.

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REVIEW
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Protozoan Parasites and Type I IFNs

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For many years, the role of interferon (IFN)-I has been characterized primarily in the context of viral infections. However, regulatory functions mediated by IFN-I have also been described against bacterial infections and in tumor immunology. Only recently, the interest in understanding the immune functions mediated by IFN-I has dramatically increased in the field of protozoan infections. In this review, we discuss the discrete role of IFN-I in the immune response against major protozoan infections: *Plasmodium*, *Leishmania*, *Trypanosoma*, and *Toxoplasma*.

Keywords: protozoan infections, IFN-I, *Leishmania*, *Toxoplasma*, *Plasmodium*, *Trypanosoma*

INTRODUCTION

Innate and adaptive immune responses are key factors in the control of infectious and chronic diseases; the balance between these two systems is mainly orchestrated by cytokines. Interferons (IFNs) are a large family of cytokines that were first discovered in 1957 in the context of viral infections. The name IFN is due to the capacity of these antiviral factors to interfere with viral replication in mammalian cells (1). Numerous studies have been carried out since their discovery, which allowed the identification of several related molecules. Based on their structural characteristics and the restricted affinity by the receptor molecule with which they directly interact, IFNs are classified into three main groups: type I (IFN-I), type II (IFN-II), and the recently identified type III (IFN-III) (2).

The IFN-I family includes two main classes of related cytokines: IFN-α, which comprises 13 different subtypes encoded by 13/14 different genes; and IFN-β, a product encoded by a single gene and a group of other less studied IFNs (IFN-ϵ, IFNδ, IFNκ, IFNτ, IFNω) (2). The ability to produce and respond to IFN-I is distributed in a wide variety of cells. This confers several autocrine and paracrine effects that have been extensively characterized mainly in viral infections. IFN-I signaling is mediated through a common cell surface receptor, the IFN-I receptor (IFNAR) (3, 4).

The IFN-II family is represented by a single gene product, IFN-γ, and is mainly produced by T lymphocytes and natural killer (NK) cells. IFN-II responses are mediated by the binding of IFN-γ to a heterodimeric molecule, the IFN-γ receptor (IFNGR), ubiquitously expressed in a wide range of cells. IFNGR is involved in the modulation of different cell functions and is a key factor for host defence to intracellular pathogens in various infection models (5).

Finally, the IFN-III family, also known as IFN-λ, comprises four different subtypes: IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4. The members of this novel IFN family interact through a unique receptor, the IFN-λ receptor (IFN-λR). In contrast to IFNAR and IFNGR, the expression of IFN-λR is mainly restricted to cells of epithelial origins. The role of IFN-III has yet to be better characterized; however, they appear to induce similar responses to IFN-I (6).

The crosstalk between IFNs and their specific receptors elicits an intracellular signaling cascade that mainly enhances inflammatory responses. The well-characterized signaling cascades of IFN-I and IFN-II are fairly similar. In both cases, Janus kinase 1 (JAK1) and tyrosine kinase 2, associated with IFNAR and IFNGR, are activated. This results in activation and following formation of a heterodimer complex comprised by the cytoplasmic transcription factor signal transducer and activator

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of transcription 1 and 2 (STAT1/STAT2). STAT1/STAT2 dimers can be translocated to the nucleus and interact with the IFN regulatory factor 9 to form the IFN-stimulated gene factor 3 complex, leading to the transcription of IFN-stimulated genes (ISGs). By contrast, IFN-II signaling through IFNGR activates the JAK/STAT pathway leading to the transcription of pro-inflammatory targets downstream of γ-activated sequences (2, 7).

Interferon (IFN)-I production is mainly induced in response to the activation of receptors on the membrane and/or cytosol, such as pattern recognition receptors (PRRs). PRRs can be activated by conserved pathogens component and endogenous molecules. In most of the cases, the production of IFN-I is related to the activation of PRRs that recognize xenogeneic or autologous nucleic acid, such as toll-like receptors (TLRs) (8).

Interferon (IFN)-I is historically best known for their capacity to elicit antiviral responses; however, they also play a role in bacterial infections and autoimmune diseases (4). The role of IFN-I in regulating the immune response against pathogens is fairly complicated. IFN-I can have enhancing or suppressive effects depending on the disease, the stage of infection, and the amount produced. For instance, IFN-I enhances the antigen-presenting capacity of DCs (9–11), favors the development of T cell responses (12–14), and promotes antibody responses (15, 16) during acute viral infections. By contrast, type I IFNs play an immunosuppressive role during chronic viral infections (17–19), reduce IFN-γ responsiveness in macrophages (20, 21), block B cell functions at high concentrations (22, 23), and can promote the expression of immunosuppressive factors such as IL-10 and PDL-1 (24–27). This duality is also observed in the context of autoimmune diseases, where IFN-I plays a pathogenic role in systemic lupus erythematosus and Sjogren's syndrome (28, 29), whereas it has therapeutic effects in multiple sclerosis (30).

While IFN-γ has been widely characterized in the modulation of the immune response against protozoan infections, the contribution of IFN-I to host defence against parasites is less clear. In the past few years, a growing body of literature suggests an important role for IFN-I during protozoan infections, particularly in the innate immune response.

In this review, we provide a brief overview of IFN-I mediated effects on the host response in various protozoan infection models and the possible mechanisms involved.

PROTOZOAN PARASITES AND IFN-I

Interferon (IFN)-I is involved in the modulation of innate immune responses promoting antigen presentation and NK cell functions. They are also known to play a role in the regulation of the adaptive immune system, promoting the development of antigen-specific T and B lymphocytes against numerous pathogens and inducing immunological memory (7). In most of the cases, these key features are important factors that limit pathogen proliferation; however, IFN-I may also lead to disease exacerbation. Protozoan parasites such as *Plasmodium*, *Leishmania*, *Trypanosoma*, and *Toxoplasma* are causing diseases that are among the most lethal and widespread around the world, primarily affecting populations of developing countries. The contribution of IFN-I in the host immune response to these pathogens will be discussed below.

Plasmodium

Plasmodium parasites are the causative agents of malaria, one of the most widespread diseases in the world. The infection presents itself in a wide range of pathologies that can degenerate into severe anemia and the high-risk cerebral malaria (CM). Members of the *Plasmodium* genus have a complex life cycle between an invertebrate (female mosquitoes of the *Anopheles* genus), in which the sexual cycle occurs, and a mammalian host. During the mosquito blood meal, sporozoites are inoculated into the dermis of the mammalian host. In the initial phase of infection, circulating sporozoites can reach lymph nodes, where the priming of B and T cells occurs, or migrate to the liver (31, 32). Within the liver, sporozoites transform first into schizonts within hepatocytes and then into merozoites. This phase is asymptomatic and is known as the pre-erytocytic stage (33). Merozoites are then released into the blood stream. Once they reach the blood, merozoites invade red blood cells, where they undergo cyclic asexual replication initiating the typical symptomatic manifestations of blood-stage malaria, which are caused by the exponential growth of the parasite and massive destruction of erythroid cells (34).

Most of the current knowledge about the immune response to *Plasmodium* parasites has been derived from a combination of *in vitro* and *in vivo* observations in human patients (e.g., *P. falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi*, and *P. ovale*) and murine models of infections (e.g., *P. berghei*, *P. yoelii*, *P. chabaudi*, and *P. vinckei*) (34).

During the pre-erythrocytic stage, sporozoite invasion of hepatocytes and subsequent development into merozoites can be blocked by sporozoite-specific antibodies generated by previous exposure to malaria or by immunization; however, this stage is not completely efficient because sporozoites remain in circulation for a short period of time. When T cell priming takes place, infected hepatocytes can be eliminated by cytotoxic CD8 T cells. CD8 T cells, IFNγ, and TNF are critical components required for elimination of infected hepatocytes in humans and the mouse model (35). However, the immune response at this stage is insufficient and released merozoites can reach erythrocytes giving rise to blood-stage malaria (35).

In the erythrocytic stage, early interaction between merozoites and innate immune cells such as dendritic cells, monocytes, macrophages, NK cells, NKT cells, and γδT cells is important for the control of parasite replication and the resolution of infection (33). This phase is characterized by a strong pro-inflammatory response, mediated by the activation of NK, NKT, CD8, and CD4 T cells that produce large amounts of IFNγ and other pro-inflammatory cytokines. IFNγ activates phagocytic cells, such as macrophages, enhancing the secretion of pro-inflammatory cytokines and promoting phagocytosis of circulating parasites and infected red blood cells, which results in the control of parasitemia (36). Polyreactive and specific antibodies against blood-stage malaria can limit parasite propagation between erythrocytes by opsonization and agglutination of parasites and infected erythrocytes; however, humoral responses during the infection are dependent on the presence of circulating merozoites (37). Infected erythrocytes on the surface express parasitic protein which allows them to bind to vascular endothelial cells and avoid clearance. This event induces obstructions in the blood

flow and is associated with a strong inflammatory response and the development of CM (33).

Although IFN-γ is the most extensively studied IFN in malaria infection, part of the attention has now been diverted to type I IFNs. IFN-I can have a host-protective or detrimental effect, depending on the stage of the infection or the species of *Plasmodium* involved.

One of the first reports involving type I IFNs demonstrated that administration of mouse serum containing high levels of IFN-I protected mice from *P. berghei* infection by reducing blood parasitemia (38). Similar protective responses were observed after treatment with IFN-β, which prevented death related to CM in *P. berghei*-infected mice (39). By contrast, treatment with recombinant IFN-α during the hepatic cycle in mice infected *P. yoelii* sporozoites did not alter the hepatic parasite burden. However, mice showed reduced parasitemia and decreased signs of immunopathology (40).

Plasmodium parasites were reported to induce IFN-I responses. Transcriptomic analysis carried out in mice with blood-stage infection with *P. berghei* revealed that IFN regulatory factors were upregulated during the acute phase (41). Induction of a typical type I IFN signature was also observed in hepatocytes from mice infected with *P. berghei* and *P. chabaudi* sporozoites, where genes such as *Mda*, *Irf3*, *Irf7*, and *Stat1* were upregulated (42–44). Similar results were observed in humans. Patients infected with *P. vivax* and *P. falciparum* showed a predominantly IFN-I transcriptional signature during the mild and the severe phase of infection (44, 45).

Recently, Liehl et al. showed that induction of IFN-I during liver stages of the infection is required for host defence against *P. berghei*. Recognition of *P. berghei* nucleic acids by Mda5 induced IFN-I and consequently, the recruitment of leukocytes necessary for parasite elimination in the liver (42). In *P. yoelii*-infected mice, recruitment and expansion of $CD49b^{+}CD3^{+}$NKT and $CD8^{+}$T cells to the liver were mediated by IFN-I signaling (43). Migration of neutrophils to the liver is also modulated by IFN-I in mice infected with *P. chabaudi* (44). These studies suggest that functionality of the innate immune response in the liver relies on both IFN-I and IFN-II.

In contrast to the protective effects discussed above, a pathogenic role for IFN-I in *Plasmodium* infections has also been described. For instance, impaired IFN-I signaling has been linked to a protective effect in human patients. Polymorphism in the human gene encoding for IFNAR1 are strongly associated with protection against CM (46). This observation is in agreement with results obtained in a murine model, where the lack of IFN-I signaling led to strong resistance to CM and reduced parasite load during *P. berghei* infection (47, 48). Moreover, in *P. chabaudi*-infected mice, IFN-I appear to suppress Th1 responses that are crucial in the control of hyperparasitemia, by modulating dendritic cell functions (49). In addition, IFN-I and Myd88 signaling are responsible for a decreased recruitment of conventional DCs to the spleen during experimental *P. berghei* or *P. yoelii* infection (50).

Perhaps a better approach for truly understanding the role and function of IFN-I during malaria consists in the identification of modulator molecules that could act in the IFN-I signaling cascade. Recently, regulators of IFN-I response have been identified through genome-wide analysis (Trans-species expression quantitative trait locus, ts-eQLT) during *P. yoelii* infection. Eight genes (*Ak3*, *Fcγr1*, *Fosl1*, *Havcr2*, *Sipr5*, *Parp14*, *Selenbp2*, and *Helb*) had an effect on IFN-I activation. For example, $Fc\gamma r1^{-/-}$ mice infected with *P. yoelii* showed significantly higher mRNA and protein levels of IFN-β than wild-type mice, suggesting a negative regulation in the IFN-β response (51).

Future experiments are granted to clarify the spatio-temporal role of IFN-I during malaria.

The role of IFN-I during *Plasmodium* infections is summarized in **Table 1**.

Toxoplasma

Toxoplasma gondii is an obligate intracellular protozoan parasite that can infect a wide range of vertebrates and cause a zoonotic disease called toxoplasmosis. *T. gondii* could be considered one of the most successful parasites worldwide; at least 50% of the human population is infected with *Toxoplasma*. The parasite success is mainly due to its ability to invade any nucleated cell and to survive outside the mammalian host (52). *T. gondii* strains are classified in three main lineages, based on the virulence of the strain in the mouse model. This virulence profile does not necessarily correlate to the degree of human infection. Type I strains of *T. gondii* are the most virulent: less than 10 parasites are able to kill a mouse at the onset of infection. By contrast, type II and III strains are less virulent and lead to the establishment of chronic infection (53). *T. gondii* can undergo both asexual (schizogony) and sexual (gametogony) replication. Gametogony and oocyst formation is restricted to feline species that act as a definitive hosts; sexual reproduction of sporozoites occurs within intestinal epithelial cells. Asexual stages of *T. gondii* are not host-specific. Many mammals and birds can act as intermediate hosts. After ingestion of *T. gondii* oocysts by an intermediate host, the parasite transforms into tachyzoites that rapidly undergo multiplication

TABLE 1 | Role of interferon (IFN)-I in *Plasmodium* infection.

Plasmodium berghei	Mouse serum containing high levels of IFN-I	Protection, ↓ blood parasitemia (38)
	Treatment with rIFN-β	Prevents death to cerebral malaria (CM) (39)
	Induction of IFN-1	Required to control hepatic infection (42)
	Lack of IFN-I signaling	↑ Resistance to CM and ↓ parasite load (47, 48)
	IFN-I	↓ Recruitment of conventional DCs to the spleen
Plasmodium yoelii	Treatment with recombinant IFN-α	No changes in hepatic burden; ↓ parasitemia and immunopathology (40)
	IFN-I signaling	↑ Recruitment of NKT and CD8 T cells to the liver (43)
	IFN-I	↓ Recruitment of conventional DCs to the spleen
Plasmodium chabaudi	IFN-I	↑ Recruitment of neutrophils to the liver (44)
	IFN-I	↓ Protective Th1 responses

within the parasitophorous vacuole inside various cell types. If the infection is controlled, parasites are retained in tissue cysts; if not, they can cause a systemic lethal disease (54, 55).

Humans are considered as an accidental intermediate host for *Toxoplasma*. In immune-competent individuals, the infection with *T. gondii* is mostly clinically silent, but cause severe diseases in immune-suppressed patients in particular with an impaired T cell and IFNγ response (55). Protective immunity is typically achieved by inducing an IL-12-driven Th1 immune response (56, 57).

In the mouse model, IFN-I can already be detected in the serum of *T. gondii*-infected animals during the acute phase (58–60); IFN-I levels gradually increase with the progression of the infection (60). IFN-I was also detected in the brain and spleen of infected mice (61). These results demonstrate that *T. gondii* not only induces IFN-γ, but also IFN-I.

Recently, inflammatory monocytes were identified as the major source of IFN-β in mesenteric lymph nodes. IFN-β production by inflammatory monocytes required three fundamental events: parasite internalization, TLR activation (mainly TLR4 and 2), and efficient MyD88 signaling. Interestingly, heat killed parasites induced higher levels of IFN-β in inflammatory monocytes (62), suggesting that *Toxoplasma* might limit IFN-I responses (62), possibly by blocking STAT1 (63).

As for many other infection models, the first studies carried out during the 1960s on the role of IFN-I in toxoplasmosis evaluated the impact of a treatment with IFN-I on infected cells *in vitro*. Pre-treatment of mouse fibroblast with IFNs conferred protection to *T. gondii* infection (64). In agreement with this observation, human neonatal and adult macrophages treated with IFN-I were able to control parasite multiplication, even if less effectively than IFN-γ treated cells (65). Moreover, human monocyte-derived macrophages treated with human IFN-β in combination with *Escherichia coli* lipopolysaccharides (LPS), but not with murine IFN-β (MuIFN-β) or rHuIFN-β alone (66), were more resistant to *T. gondii* infection (67).

In the mouse model of toxoplasmosis, treatment with HuIFN-β showed a protective effect, which was enhanced by the combination of rHuIFN-β and LPS and was IFN-γ dependent (66). In agreement with these results, it was shown that *Ifnar*$^{-/-}$ mice orally infected with *T. gondii* have an increased parasite load compared to wild-type mice; higher parasite burdens correlated with a decrease in survival (68).

These results suggest that IFN-β may be produced at the onset of infection to enhance the IFN-γ responses.

A study using human fibroblasts as host cells revealed that treatment of *T. gondii*-infected cells with IFN-I had no effect on parasite replication (69), suggesting that the protective effect of IFN-I depends on cell type and/or timing of exposure to the cytokine (prior to or after infection).

During *T. gondii* infection regulation of tryptophan metabolism is a key component for parasite survival. Indeed, tryptophan degradation inhibits parasite replication. In *T. gondii*-infected mice, indoleamine 2,3-dioxygenase (IDO), a tryptophan catalyzer (70, 71), is enhanced by IFN-II (72). However, it has also been reported that IFN-I can regulate IDO in human retinal pigment epithelial cells, inhibiting therefore *T. gondii* replication (73). Together, these results demonstrate that IFN-I also contribute to the regulation of protective immunity against *T. gondii* (**Table 2**).

Leishmania

Leishmania is a complex genus of obligate intracellular protozoan parasites that cause a widespread disease collectively known as Leishmaniasis. The life cycle of these parasites takes place between a mammalian host and a sandfly vector (genus *Lutzomyia* and *Phlebotomus*). Once in the hosts, the promastigote form of the parasite preferentially infects macrophages, but can also be found in other cell types, such as dendritic cells, neutrophils, and fibroblasts. Promastigotes then transform into the non-flagellated form called amastigotes within the host's cell. The *Leishmania* spp. involved and the mammalian host immune status determine the clinical manifestation of the disease. Parasites can either reside in the skin and/or mucosal surfaces, which results in cutaneous (i.e., *Leishmania major*) or mucocutaneous (i.e., *Leishmania braziliensis*) Leishmaniasis; or disseminate to internal organs such as liver, spleen, and bone marrow, causing visceral Leishmaniasis (VL), the most severe form of the disease (i.e., *Leishmania donovani*) (74).

Leishmania immunity is mostly mediated by T lymphocytes. In experimental models, control of infection is mediated by a polarized Th1 response, induced by an initial production of IL-12 by DCs (75). IFN-γ secreting CD4 and CD8 T cells contribute to parasite control by enhancing the ability of phagocytic cells to kill intracellular *Leishmania* (74, 76).

As for many other protozoan models, IFN-II is the main mediators of the cellular immune response. However, IFN-I and IFN-I inducible genes are gradually gaining importance in the *Leishmania* field. One of the pioneer work on the role of IFN-I in Leishmaniasis described the prophylactic treatment with synthetic double-stranded RNA (Poly I:C) prior to *L. donovani* infection. Injection of Poly I:C triggered a burst of IFN-I and led to the control of the hepatic parasite burden (77). The role of endogenous IFN-I was studied for the first time using strains causing cutaneous Leishmaniasis. The induction of IFN-I was observed in macrophages infected *in vitro* with *L. major* promastigotes (78, 79) and in skin macrophages from infected animals (79), showing that promastigotes could enhance IFN-I expression in the host cell. The combination of exogenous IFN-I with *L. major* promastigotes was shown to activate macrophages, inducing type 2 nitric oxide synthase (NOS2). NOS2 is required for parasite elimination; mice deficient in this enzyme are more susceptible to *L. major* infection (80). As for *T. gondii*, the timing of the host cell's exposure to IFN-I determines the effect on parasite control. Indeed, pre-treatment of macrophages with

TABLE 2 | Role of interferon (IFN)-I in *Toxoplasma* infection.

Toxoplasma gondii	IFN-I treatment (*in vitro* infection; mouse fibroblasts; and human macrophages)	↑ Resistance to infection (64–67)
	HuIFN-β treatment (*in vitro*)	↑ Resistance to infection (66)
	Ifnar$^{-/-}$ mice	↑ Parasite load, ↓ survival (68)
	IFN-I treatment, human fibroblasts	No effects on parasite replication (69)

exogenous IFN-I failed to induce NOS2. Similar results were obtained with high doses of exogenous IFN-I, while a low IFN-I dose in combination with *L. major* enhanced leishmanicidal activity (78, 81). These results suggest that the design of *in vitro* experiments greatly influences the outcome of IFN-I treatment in infected macrophages and that the role of IFN-I should be better studied in *in vivo* models.

The protective role of endogenous IFN-I during infection was confirmed by neutralizing IFN-I in mice experimentally infected with *L. major*. In fact, IFN-I neutralization rendered *L. major*-infected mice more susceptible to infection and enhanced parasite multiplication. IFN-I blockade led to abolishment of NOS2 function and reduced cytotoxic activity and IFNγ production by NK cells at early stages of infection (79).

Opposite results were obtained in human macrophages infected *in vitro* with New World *Leishmania* spp. IFN-β treatment of *L. braziliensis* and *Leishmania amazonensis*-infected macrophages enhanced the parasite burden through a superoxide-dependent, NO-independent mechanism (82). In this model, it was shown that IFN-β can regulate the superoxide dismutase SOD1 activity. SOD1 is responsible for catalyzing the disproportionation of superoxide to hydrogen peroxide and dioxygen and is an important constituent in apoptotic signaling and oxidative stress. It has been observed that biopsies from cutaneous Leishmaniasis patients express high levels of SOD1 (82).

The importance of endogenous IFN-I during chronic infection has been investigated using IFNAR-deficient mice in the context of *L. amazonensis* infection. *L. amazonensis* infected *Ifnar*$^{-/-}$ mice developed attenuated cutaneous lesions and displayed a decreased parasite load. This effect appeared to be STAT1 independent, a key protein in the IFN signaling (83). Furthermore, *L. amazonensis*-infected *Ifnar*$^{-/-}$ mice exhibited high levels of neutrophils and lower inflammatory monocytes recruitment at early times post infection. This unique profile was also observed in *L. major* and *L. braziliensis* infections (83). *In vitro* coculture of infected WT macrophages with *Ifnar*$^{-/-}$ neutrophils revealed that IFNAR-deficient neutrophils promote parasite killing (83). This evidence supports the pathogenic role of IFN-I signaling in cutaneous Leishmaniasis caused by New World *Leishmania* species.

We also observed a negative role for IFN-I in an experimental model of VL. *L. donovani* amastigotes were shown to induce IFN-I expression in B cells in an endosomal TLR-dependent manner. This cytokine was involved in a positive regulatory loop that led to the upregulation of endosomal TLRs and to IL-10 production in B cells (84). B cell-derived IL-10 was shown to suppress protective T cells responses and increase disease susceptibility (85). B cells are known to play a detrimental role during VL (86), not only by secreting IL-10 but also for their excessive antibody production (87). Indeed, hypergammaglobulinemia is a hallmark of VL. Interestingly, IFN-I seems to be regulating antibody production during VL. Specific ablation of endosomal TLRs or IFN-I signaling in B cells was shown to severely reduce the Ig titer in the serum of *L. donovani*-infected mice, suggesting that parasite activation of B cells *via* endosomal TLRs and IFN-I are involved in the induction if hypergammaglobulinemia (84). Furthermore, mice with a B cell-specific deficiency in endosomal TLR or IFNAR were more resistant to *L. donovani* infection than their wild-type counterpart.

Very little is known about the function of IFN-I in VL patients. It was reported that human mononuclear phagocytes can be activated by IFN-β, but less efficiently than IFN-γ (88). Exogenous treatments with IFN-I and IFN-II but not IL-2, failed to restore the cytotoxic activity of NK isolated from VL patients (89). Also, treatment of the cutaneous lesion in patients with IFN-I did not improve healing, compared with IFN-γ treatment (72, 90).

Because dendritic cells can also be infected by *Leishmania*, it is important to consider the induction of IFN-I by the parasite and its possible effect in these cells as well. Transcriptomic analysis of human DCs infected *in vitro* with *L. major* or *L. donovani* showed a differential expression pattern for IL-12 associated genes, the NF-KB pathway, and IFN regulatory factors (91). IFN-β produced by *L. major*-infected DCs seems to be required for IL-12 secretion by the infected DC, suggesting that protective Th1 responses, which are IL-12 depended, may also depend on IFN-I (92).

A summary on the role of IFN-I during Leishmaniasis can be found in **Table 3**.

Trypanosomes

Trypanosomes are digenetic protozoan parasites that infect domestic and wild animals, as well humans. Although many species of trypanosomes cause important veterinary disease, mainly two species cause significant human morbidity: *Trypanosoma brucei* and *Trypanosoma cruzi*. These two species are responsible for causing the sleeping sickness (African trypanosomiasis) and the Chagas disease (American trypanosomiasis), respectively.

The life cycle of these parasites takes place between the invertebrate vector and the vertebrate host. *T. brucei* and other African's trypanosomes are transmitted to the mammalian host by a tsetse fly bite. In the blood stream, metacyclic trypomastigotes differentiate into bloodstream trypomastigotes. In humans, trypanosomes proliferate in the blood and lymphatic system at early stages of the infection. This stage is associated with an anti-inflammatory response. At chronic stages, parasites can pass through the blood–brain barrier and enter the central nervous

TABLE 3 | Role of interferon (IFN)-I in *Leishmania* infection.

Leishmania donovani	Treatment with Poly I:C B cell-derived IFN-I	↓ Hepatic parasite burden (77) ↑ IL-10, ↑ hypergammaglobulinemia (84)
Leishmania major	IFN-I treatment of macrophages *in vitro* (78–81)	
	(1) At the time of infection	↑ NOS2
	(2) Before infection	No effect on NOS2
	(3) High dose	No effect
	(4) Low dose	↑ Leishmanicidal activity
	In vivo IFN-I blockade	↓ NOS2, ↓ natural killer functions (79)
Leishmania braziliensis	IFN-β treatment of macrophages *in vitro*	↑ Parasite burden (82)
Leishmania amazonensis	*Ifnar*$^{-/-}$ mice	↓ Lesions, ↓ parasite burden, ↑ neutrophils (83)

system. This stage is associated with inflammatory changes in the brain and is characterized by a neurological disturbance (93, 94).

In *T. cruzi* (American trypanosomiasis), metacyclic trypomastigotes are released in the feces/urine of the triatomine vector after a blood meal. Trypomastigotes can successfully infect the mammalian host if they are able to reach the mucosa or injured skin areas. In contrast to African trypanosomes, *T. cruzi* is an intracellular parasite that has the capacity to invade, differentiate into amastigotes, and replicate within a wide range of nucleated cells. This characteristic is one of the most important features of *T. cruzi* within the host. Amastigotes differentiate into infective bloodstream trypomastigotes, before being released upon cell lysis. The released parasites can then infect neighboring cells or enter the bloodstream (95).

During the acute phase, the innate immune response against *T. cruzi* is characterized by the induction of a cell-mediated response that involves the production of IFN-γ and TNF (by NK and T cells), required for enhancing iNOS activity by phagocytic cells and for priming the adaptive immune response. iNOS activation is critical for controlling parasite growth during the infection (95). *T. cruzi* elicits a prominent IFN-I response at early times of infection (96–99). As mentioned before for *Plasmodium*, the role of IFN-I in *T. cruzi* infection is controversial. Some studies ascribe a protective role to IFN-I; others demonstrate that IFN-I induces pathology. The effect of IFN-I mainly depends on the dose, amount of parasites, and the inoculation route used to set up the infection.

The first studies on the role of IFN-I investigated the outcome of exogenous IFN-I treatment in *T. cruzi*-infected mice. The results showed that administration of IFN-I increased resistance to infection by stimulating T and NK cell activities, which are essential for protection (100, 101).

In an intradermal model of infection, transcriptomic analysis of excised skin from the inoculation site revealed that *T. cruzi* upregulated the expression of ISGs as early as 24 h after infection. Induction of ISGs was dependent on IFN-I signaling, suggesting that IFN-I is an important component of the innate immune response to *T. cruzi* (99). In agreement with the above mentioned literature, studies carried out in *Ifnar*$^{-/-}$ mice infected with *T. cruzi* revealed that efficient IFN-I signaling was required for controlling parasites growth during the acute phase of infection (102, 103). IFN-I was necessary for enhancing NO production in phagocytic cells (102). NO is considered the major effector molecule for intracellular amastigotes elimination within infected cells, being important for the control of parasite multiplication (95).

By contrast, another group reported a potential pathogenic role for IFN-I. In this work, a lethal dose of parasites inoculated intradermally was used to set up the infection in WT and *Ifnar*$^{-/-}$ mice. Surprisingly, *T. cruzi*-infected *Ifnar*$^{-/-}$ mice survived the challenge and were able to control parasite replication (104). Besides the fact that splenocytes from *Ifnar*$^{-/-}$ mice produced higher levels of IFN-II, plasma cytokine profile in *T. cruzi*-infected *Ifnar*$^{-/-}$ mice were not different to control mice (104). Additionally, T cells populations were not inherently different compared with control mice (104), and IFN-γ production by CD8^{+}T cells was not affected by impaired IFN-I signaling (105), suggesting that, in this model, endogenous IFN-I is not the only relevant signal in host defense against *T. cruzi*.

Taken together, the role of IFN-I in *T. cruzii* infection differs from one experimental model to the other, depending on the dose and the route of infection (106). This could explain the controversy about the observations on the role of IFN-I in the *T. cruzi* model of infection (**Table 4**).

The immune response to African trypanosomes is quite different than that to *T. cruzi*. First, parasites never enter the host cell at any stage of their development. The success of these parasites is mainly due to their ability to change the composition of the variant surface glycoprotein (VSG) by switching genes. This confers them the capacity to evade B- and T-cell-mediated immune responses and results in fluctuating waves of parasitemia that characterize African trypanosomiasis (94). VSG is a strong antigen that induces Th1 responses and promotes autoantibody and cytokines production, in particular TNF. Other trypanosome proteins and soluble factors, such as a trypanosome-released triggering factor, also trigger IFN-γ production by T and NK cells and are involved in macrophage activation toward an M1 phenotype, which is required for the control of parasite multiplication during the acute phase of infection. However, sustained activation of M1 macrophages is associated with disease exacerbation. The progression of the infection toward the development of an acute fatal disease or a prolonged chronic infection is determined by the balance between a type I and type II immune responses and the switch from the early type I immune response (dominated by M1 macrophage activation) from a type II (M2 macrophages) regulatory response that controls the inflammation (107).

The literature on the role of IFN-I in African trypanosomiasis is scarce. A study involving *Trypanosoma brucei rhodesiense* reported a beneficial effect of IFN-I during the acute phase of infection. Indeed, *Ifnar*$^{-/-}$ mice displayed delayed control of parasite burden during the first week of infection and died earlier than wild-type controls. Moreover, mice hyperresponsive for IFN-I (*Ubp43*$^{-/-}$) exhibited a significant defect in Th1 responses and IFN-γ production, suggesting that IFN-I plays a role in the early stages of disease. Nevertheless, IFN-I contributes to the downregulation of IFN-γ production and loss of host resistance during chronic infection (108).

No effects of IFN-I signaling were observed in *Trypanosoma brucei brucei*-infected *Ifnar*$^{-/-}$ mice, which showed similar levels of parasitemia to wild-type mice, suggesting that in this model parasite control is independent of IFN-I (109). However, IFN-I

TABLE 4 | Role of interferon (IFN)-I in *Trypanosoma* infection.

Trypanosoma cruzi	IFN-I treatment *in vivo*	↑ Resistance to infection ↑ T and natural killer cell activity (100, 101)
	Ifnar$^{-/-}$ *mice*	Disease exacerbation (102, 103)
	Ifnar$^{-/-}$, lethal dose	↑ Survival (104)
Trypanosoma brucei rhodesiense	*Ifnar*$^{-/-}$, acute phase	↑ Control
	Ifnar$^{-/-}$, later stages	↓ Resistance, IFN-γ ↓ (108)
Trypanosoma brucei brucei	*Ifnar*$^{-/-}$	No effect on parasite control (109)

regulates T cell infiltration to the brain parenchyma at chronic stages of the infection (109).

In conclusion, the contribution of IFN-I to protective immunity against several protozoan parasites is still unclear. Variations in parasite numbers used for infections, the site of inoculation, and the dose of IFN-I all seem to influence the outcome and the interpretation of the results. A spatio-temporal analysis of the role of IFN-I integrated with a more detailed investigation of cell-specific signaling pathways elicited by the cytokine could help to better dissect the involvement of IFN-I in the immune response.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Canonical and Non-Canonical Aspects of JAK–STAT Signaling: Lessons from Interferons for Cytokine Responses

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Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signal transduction mediates cytokine responses. Canonical signaling is based on STAT tyrosine phosphorylation by activated JAKs. Downstream of interferon (IFN) receptors, activated JAKs cause the formation of the transcription factors IFN-stimulated gene factor 3 (ISGF3), a heterotrimer of STAT1, STAT2 and interferon regulatory factor 9 (IRF9) subunits, and gamma interferon-activated factor (GAF), a STAT1 homodimer. In recent years, several deviations from this paradigm were reported. These include kinase-independent JAK functions as well as extra- and intranuclear activities of U-STATs without phosphotyrosines. Additionally, transcriptional control by STAT complexes resembling neither GAF nor ISGF3 contributes to transcriptome changes in IFN-treated cells. Our review summarizes the contribution of non-canonical JAK–STAT signaling to the innate antimicrobial immunity imparted by IFN. Moreover, we touch upon functions of IFN pathway proteins beyond the IFN response. These include metabolic functions of IRF9 as well as the regulation of natural killer cell activity by kinase-dead TYK2 and different phosphorylation isoforms of STAT1.

Keywords: signal transduction, JAK–STAT, non-canonical, interferon, innate immunity

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INTRODUCTION

Since their discovery in the late 1950s (1), interferons (IFNs) have been assigned various functions that extend far beyond the initially observed antiviral activity. Three families of IFNs have been described and are known as type I (IFN-I, mainly IFNα/β), type II (IFN-II or IFNγ), and type III (IFN-III or IFNλ). In canonical IFN signaling, all types of IFNs produce a transcriptionally active signal transducer and activator of transcription 1 (STAT1) through receptor-bound Janus kinase (JAK)-mediated phosphorylation of tyrosine (Y) 701. The IFNγ receptor employs JAK1 and JAK2 to phosphorylate exclusively STAT1, causing its homodimerization. STAT1 dimers, also called gamma interferon-activated factor (GAF), translocate to the nucleus and promote gene expression by binding to gamma interferon-activated site (GAS) of interferon-stimulated genes (ISG). On the other hand, stimulation with IFN-I or IFN-III leads to TYK2- and JAK1-mediated phosphorylation of STAT1 and STAT2. After forming heterodimers, these two proteins associate with interferon regulatory factor 9 (IRF9) to form a transcriptionally active IFN-stimulated gene factor 3 (ISGF3)

that controls gene expression by binding to interferon-stimulated response elements (ISRE) in a different set of ISGs (2, 3). Although a large majority of IFN-induced gene expression is mediated by canonical pathways, it has become clear that the components of these pathways are able to exert non-canonical activity: tyrosine kinase-independent action of JAKs, transcriptional complexes other than ISGF3 and GAF, and pathways building on U-STATs that are not phosphorylated on tyrosine. This review will focus on these non-canonical functions of JAK–STAT signaling components.

IFNs AND THEIR ROLE IN RESISTANCE TO VIRUSES AND BACTERIA

As major components of the innate immune system against viral infections, all type I interferons (IFN-I) stimulate cell-autonomous antiviral activity (4). In addition, they increase cellular immunity through contributions to natural killer (NK) and T cell activation (5). IFN-I act as modulators of cellular immunity by selectively enhancing clonal expansion and survival of CD8+ T cells (6), directing the immune response toward Th1-dominace (7) and activating NK cells (8).

In the context of antibacterial defense, genes activated by IFN-I enhance inflammation and the death of infected cells (9). In addition, they impact on cells at the interface of the innate and adaptive immune systems, such as macrophages and dendritic cells, to increase antigen presentation and trigger the adaptive response (10). Immunostimulatory activities of IFN-I also contribute to immunosurveillance against cancer (11). On the other hand, their proinflammatory activity renders them driving forces behind a group of interferonopathies, such as the Aicardi-Goutieres syndrome (12, 13).

While IFN-I are generally protective against viral infections, they can be both friend and foe in the defense of bacterial pathogens (14, 15). For example, IFN-I exert protective effects in the case of *Chlamydia pneumoniae* (16), *Legionella pneumophila* (17), *Salmonella typhimurium* (18), and both group A and group B *Streptococcus* infections (19–21). However, in infections with *Listeria monocytogenes*, *Francisella tularensis*, and *Mycobacterium tuberculosis*, production of IFN-I is associated with decreased innate immunity (22–27).

Major target cells of IFNγ are macrophages and T cells. Many of the genes induced by IFNγ are transcription factors, which amplify the transcriptional response and, as in Th cells, influence cell differentiation (28–30). IFNγ is particularly important in macrophage biology, where it provides cell-autonomous antimicrobial activity through the upregulation of microbicidal gene products (31). Further, impact of IFNγ on macrophage activation results from its ability to synergize with or to antagonize the effects of different cytokines, growth factors, and pathogen-associated molecular pattern-signaling pathways (e.g., TNFα, IL-4, CSF-1, IFNα/β, LPS, and CpG DNA). Through these mechanisms, IFNγ activates macrophages to express antimicrobial and antitumor effects. It upregulates chemokines and adhesion molecules, directing cells to the sites of inflammation. In the adaptive immunity, IFNγ plays an important role in Th1 responses, repressing the development of Th2 and Th17 T cell responses (30) and acting directly on B cells to promote class switching from IgG2 to IgG3 (32). Mice and humans deficient in IFNγ or IFNGR1 show a decrease in natural resistance to bacterial, parasitic, and viral infections (33–35). Mice and cells lacking IFNγ display compromised tumor rejection, underlining its importance in tumor surveillance (11).

Discovered in the year 2003, IFN-III, better known as IFNλ, are the most recently described members of the IFN family (36). Although signaling through a different receptor complex with IFNλR/IL10R2 chains, IFNλ also stimulate formation of the ISGF3 complex. Given the similarities between the IFN-I and IFNλ signaling pathways, some of the non-canonical signals described below for IFN-I may apply to IFNλ as well. While IFNλ produce similar biological changes of their target cells as IFN-I, including antiviral, antiproliferative, and antitumor activity, the key to different organismic responses to IFN-III lies in their receptor distribution, which is prevalent on cells of epithelial origin (37). In line with that, defects in IFN-III production or signaling cause reduced innate immunity to viral pathogens replicating in epithelia of the lung and the gut. Mice lacking the IFN-I receptor are resistant to all IFN-I subtypes, but retain their sensitivity to IFN-III (38). However, studies performed in IFNAR1$^{-/-}$, IL-28Rα$^{-/-}$, and IFNAR1/IL-28Rα double knockout mice show that, in primary airway epithelia, upon influenza infection, IFN-I and IFN-III mediate parallel amplification loops that lead to the induction of fully overlapping groups of ISGs (39).

KINASE-INDEPENDENT JAK ACTIVITY

Janus kinases are non-receptor tyrosine kinases which have essential roles in cytokine and growth factor signaling (40, 41). There are four different JAKs (JAK1, JAK2, JAK3, and TYK2) that cross-phosphorylate and activate each other when ligand-associated cytokine receptor chains come in close proximity. Canonical, i.e., kinase-dependent functions of JAKs include tyrosine phosphorylation of receptor chains and of STATs at a single tyrosine residue near the C-terminal end. STAT phosphorylation is thought to require SH2 domain-mediated docking to the modified receptor chains, consistent with impaired phosphorylation at mutant receptors lacking the critical tyrosine for JAK-mediated phosphorylation (2).

Recently, reports studying kinase-inactive mutants of TYK2 and JAK2 suggest that these proteins exert functions not requiring their kinase activity and have important non-canonical roles. Elegant studies in mouse models with kinase-dead enzymes allow the demonstration of kinase-independent functions under physiologic conditions and complement studies in human and murine cell lines. Furthermore, description of naturally occurring mutations in JAKs in the human population broadens our understanding of the multifaceted role of JAKs in health and disease.

TYK2

TYK2 is involved in a large number of cytokine signaling cascades as it associates with the IFN-I (IFNAR), IL-12Rβ1,

IL-10R2, gp130, and IL13α1 receptor (42). Early work in human cell lines has demonstrated the important function of TYK2 in IFN-I signaling (43–46), as human cells fail to respond to IFNα in absence of TYK2. Furthermore, such cells display reduced IFNAR expression at the cell surface. Interestingly, this is a consequence of a non-canonical role of TYK2. The scaffold TYK2, but not its kinase or pseudokinase domains are needed for surface expression of IFNAR in human 11,1 cells (47). Specifically, TYK2 masks a tyrosine-based motif found in IFNAR, thereby shielding the receptor from endocytosis and preventing the binding of an enzyme (AP2), which leads to ubiquitin-dependent internalization (48). In this context, TYK2-receptor association requires neither ligand nor ubiquitination. Preventing receptor degradation is a species-specific TYK2 activity, consistent with the lack of the Tyr-based motif in the murine IFNAR. However, TYK2 is not essential for IFN-I signaling in murine cells, and residual IFN-I activity is detected in the absence of this JAK (49, 50).

Mice with a targeted mutation of a critical lysine residue in the ATP-binding pocket of the TYK2 kinase domain (K923E) express a kinase-dead enzyme (51). Kinase-dead TYK2 shows a strongly reduced half-life owing to increased turnover *via* autophagosomal degradation. The reduced levels of TYK2K923E do not increase IFN-induced STAT activation above the level seen in the Tyk2$^{-/-}$ animals. Consistently, TYK2^{K923E} mice are more susceptible to viral infections, comparable to Tyk2$^{-/-}$ mice. In contrast, TYK2^{K923E} expression partially rescued the defect in natural killer cell (NK-cell) maturation and tumor killing that accompanies TYK2 deficiency (**Table 1**). At present, this first observation of a kinase-independent *in vivo* function of TYK2 cannot be assigned to a defined NK signaling pathway. Other attributes of activated NK cells like IL-12 synthesis or activating receptor-stimulated production of IFNγ rely on TYK2 kinase activity (52).

TABLE 1 | An overview of non-canonical Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signaling by components of interferon (IFN) pathways.

Genotype	Non-canonical STAT complexes	Function
WT overexpressing IFN-stimulated gene factor 3 (ISGF3) subunits	Unphosphorylated ISGF3 complex	Prolonged IFN-I responses, resistance to DNA damage
WT expressing high levels of STAT2	pYSTAT1/U-STAT2	Inhibition of STAT1 nuclear translocation, quenching of IFNγ response
STAT1^{Y701F}	STAT1Y701F/STAT2/?	Inhibition of STAT2 nuclear translocation, quenching of type I IFN response
	STAT1Y701F/?	Natural killer (NK) cytotoxicity
STAT1^{S727A}	STAT1S727A dimer STAT1S727A/?	Reduction of IFNγ response NK cytotoxicity
STAT1$^{-/-}$	STAT2/interferon regulatory factor 9 (IRF9)	Flavivirus and *Legionella pneumophila* resistance
STAT2$^{-/-}$	STAT1/IRF9	IFNγ—colitis, IFN-I—*Legionella pneumophila* resistance
TYK2^{K923E}	–	NK cytotoxicity, mitochondrial respiration, IFNAR stability (in humans)
JAK2^{KD}	–	IFN-gamma receptor stability, residual IFNγ response

"?" represents unknown interactors.

Ligation of the IFNAR causes TYK2-dependent activation of the phosphoinositide 3 kinase (PI3K) pathway. Reportedly, this occurs without a need for TYK2 kinase activity (45). Moreover, in murine pro B cells, a part of TYK2's functions in mitochondrial respiration is retained in absence of its kinase activity (53). The cells show a drastic defect in basal oxygen consumption and steady-state cellular ATP levels in the absence of TYK2, which can be reversed after transfection of wild-type or a kinase-dead mutant of TYK2 into these cells. In contrast, the kinase activity of TYK2 is required for other functions of mitochondria like complex I-mediated respiration and the induction of apoptosis after IFNβ treatment. TYK2 has also been linked to the energy expenditure of cells, to the regulation of lipid metabolism, differentiation of brown adipose tissue, and obesity (54–56). In human obese patients and obese mice, decreased TYK2 levels are associated with increased obesity. This effect is regulated *via* Stat3 signaling and prolonged stability of the transcriptional coactivator PRDM16, a master regulator of brown adipose tissue (55). The interaction of TYK2 with STAT3 is most probably a non-canonical event, as tyrosine phosphorylation of STAT3 does not require TYK2. Therefore, the kinase must provide another kind of mechanistic input, either indirectly through other pathways or through another modification of STAT3.

The Tyk2 gene displays many different SNPs in the human population, and GWAS studies have linked mutations in Tyk2 to autoimmune diseases like systemic lupus erythematosus, multiple sclerosis, Crohn's disease, psoriasis, type I diabetes, endometriosis-related infertility, primary biliary cirrhosis, and rheumatoid arthritis (22–25, 57–68). The first patient described with Tyk2 mutation suffered from hyper-IgE syndrome (HIES) and presented with viral, bacterial, and mycobacterial infections. The role of TYK2 in several cytokine signaling pathways leads to these diverse susceptibilities, explained in part by a bias toward Th2 immunity and defective IL-12 and IFN-I pathways (26). Complementing this study, Kreins et al. described seven different patients from four different ethnic backgrounds with different mutations in Tyk2 (27). All patients examined so far had mutations, deletions, or substitutions in the Tyk2 gene which ultimately led to a premature stop codon and no expression of Tyk2 protein. This group of patients did not present HIES. However, similar to the first patient, they suffered from widespread mycobacterial and viral infections. Using microarray analysis, Kreins et al. demonstrated that, similar to TYK2-deficient mice, responses of the patient's cells to various cytokines like IL-12, IFN-I, IL-23, and IL-10 are greatly reduced, but not completely abolished. This observation, made by the use of a sensitive detection method, might resolve the apparent discrepancy regarding the partial or absolute requirement of TYK2 for cytokine signaling in murine versus human cells. Another study described disease-associated human TYK2 variants, which are catalytically impaired, but able

to rescue signaling in response to IFN-I, IL-6, and IL-10 *in vitro* (69). The authors proposed a model for receptors associating with more than one JAK according to which only one JAK needs to be catalytically active in order to convey signals, as long as the second JAK functions as a scaffold.

JAK2

JAK2 is involved in many biological processes, including the growth control, survival, and differentiation particularly of hematopoietic cells. Accordingly, the widespread use of this kinase is reflected by the embryonic lethality of homozygous deletion (70, 71). The kinase activity of JAK2 is also implicated in various lymphoid and myeloid leukemias in which chromosomal translocation generates a hyperactive kinase (72–74). A scaffold function of JAK2 is suggested by the finding that the N-terminal domain of JAK2 alone is sufficient to enhance surface expression of the Epo receptor (EpoR) (75). Like Tyk2, mice expressing kinase-dead JAK2 were generated and resulted in the discovery of kinase-independent functions. Frenzel et al. generated a mouse model expressing a dominant-negative, kinase-inactive JAK2 by mutating residues in the C-terminal kinase domain (W1038G, E1046R) (76). This mouse mimics the complete loss of JAK2 as homozygous embryos die *in utero*. Heterozygous mice containing this dominant-negative form of Jak2 did not show any hematopoietic abnormalities, and it seems that one intact copy of Jak2 can compensate for the loss of kinase activity of the other, inactive form. Keil et al. generated mice with inactive JAK2 by mutation of the activation loop tyrosines [$JAK2^{YY1007/1008FF}$—(77)]. Similar to the mouse described by Frenzel et al., homozygous $JAK2^{YY1007/1008FF}$ alleles caused embryonic lethality and defective EpoR signaling, whereas heterozygous mice appeared phenotypically normal. However, the study revealed a kinase-independent scaffolding function of JAK2 for the heteromeric IFN-gamma receptor (IFNGR) complex. Importantly, $JAK2^{YY1007/1008FF}$ mediated the cell surface expression of the IFNGR indistinguishable from wild-type JAK2. Likewise, the recruitment of JAK1 to the receptor was normal. Contrasting Jak2-deficient cells, JAK1 alone was able to partly compensate for the loss of JAK2 signaling by phosphorylating STAT1 and inducing genes in response to IFNγ.

STAT1-INDEPENDENT RESISTANCE TO VIRUSES AND BACTERIA—THE ROLE OF STAT2 AND IRF9

Experiments performed in cells and mice lacking functional STAT1 revealed its indispensable role in the IFN signaling pathway. To date, mice lacking STAT1 have been challenged with at least 27 different pathogens and proved to be highly susceptible to most of the viruses, with exception of (+) single-strand RNA dengue virus (DENV) and the (−) single-strand RNA measles virus (MV). These mice are also highly susceptible to intracellular bacteria, such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*, and parasites such as *Toxoplasma gondii* and *Leishmania major* (78). Patients with complete autosomal recessive STAT1 deficiency succumb to lethal mycobacterial and viral infections. Heterozygous autosomal recessive STAT1 deficiency also causes impaired IFN responses, but with milder clinical symptoms and more positive prognosis. Patients with autosomal dominant STAT1 loss-of-function mutations suffer from mycobacterial diseases and show an impaired response to IFNγ and IL-27 (78, 79). However, the majority of patients with inborn errors of STAT1 show autosomal dominant gain-of-function mutations leading to an unexpectedly broad clinical phenotype, including mucocutaneous candidiasis and autoimmune disorders (80, 81). In addition, some heterozygous *de novo* acquired mutations of the Stat1 gene, affecting coiled-coil and DNA-binding domains, can be associated with progressive combined immunodeficiency. This kind of Stat1 mutations lead to reduction in overall Stat1 expression and signaling responses and are ultimately fatal due to overwhelming infections and inflammation (82).

While these findings emphasize the critical importance of STAT1 in IFN responses, studies in mice support the idea that residual IFN-dependent activity against some pathogens remains in absence of STAT1. Initial evidence for transcriptional responses to IFNs through non-canonical STAT complexes was provided by studies in $Stat1^{-/-}$ mice infected with Sendai virus, MCMV, or DENV, showing that STAT1-independent responses to IFNs provide some level of resistance against these pathogens (83–85). In contrast, the combined loss of IFNγ and type I IFN receptors or of STAT1 and STAT2 results in early death of infected mice (86, 87). Further, consistent with STAT1-independent responses to IFN, Hahm et al. demonstrated that infection of hematopoietic bone marrow cells with MV or LCMV impaired the maturation of dendritic cells in an IFNβ-dependent and Stat2-dependent, but STAT1-independent, manner (88). Additional studies revealed that STAT2 was required for IFN-I-induced expression of a set of ISGs independently of STAT1 (85, 89, 90). Despite the inability to form ISGF3 complexes, $Stat1^{-/-}$ mice and cells could still induce a subset of ISGs in response to the DENV infection (87). This response was absent in compound $Stat1/2^{-/-}$ mice, directing the main attention toward STAT2 as a component of STAT1-independent ISG expression *in vivo*. STAT2 homodimers alone are known to bind DNA very poorly, owing to the lack of a functional DNA-binding domain (91). This suggests that one or more additional components must contribute to STAT1-independent ISG regulation. IRF9, the DNA-binding subunit of the ISGF3 complex is an obvious candidate. The formation of STAT2–IRF9 complexes after IFN-I stimulation had been proposed by several authors based on studies addressing ISG expression in HEK293 cells with overexpressed STAT2 and IRF9 (91), in U3A cells that lack STAT1 (90), and in Hep3B cells (89). In addition, STAT2 was shown to have a STAT1-independent role in the ability of IFNβ and TNFα to synergistically stimulate the expression of Duox2 NADPH oxidase in epithelial cell lines [(92), reviewed in detail in Ref (93, 94); **Figure 1**]. More recently, the potential of STAT2/IRF9 complexes to stimulate ISG emerged from studies addressing the role of type I IFN in bacterial infections.

As mentioned above, type I IFNs are secreted in response to many bacterial pathogens, such as *Listeria monocytogenes*, *Francisella tularensis*, *Legionella pneumophila*, and others (14, 95–97). The impact of IFN-I on bacterial growth in mammalian

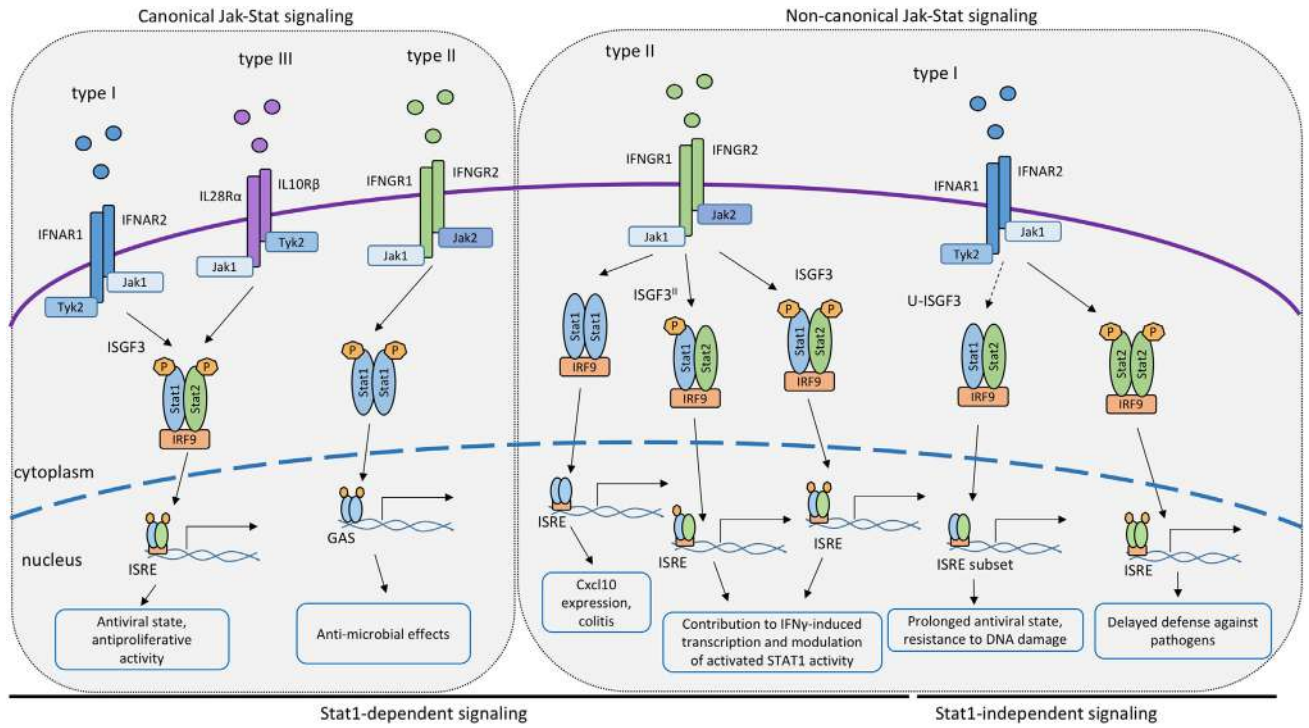

FIGURE 1 | **Canonical and non-canonical STAT signaling by the IFN receptors**. Proposed roles of STAT or STAT/interferon regulatory factor (IRF9) complexes participating in signal transduction and transcriptional activation by the receptors of IFN-I, IFN-II or IFN-III. Complexes containing IRF9 associate with interferon-stimulated response elements promoter sequences whereas dimerized STAT1 binds to GAS elements.

hosts is variable, as described in detail elsewhere (14). In case of *Legionella pneumophila*, IFN-I inhibits its ability to grow inside macrophages. The growth inhibitory effect was retained in cells isolated from Stat1$^{-/-}$, Stat2$^{-/-}$, or Stat3$^{-/-}$ single knockout animals (17). In contrast, macrophages from compound Stat1/2$^{-/-}$ macrophages were not able to limit the bacterial growth after IFN-I stimulation and had higher bacterial loads than single knockouts of STAT1 or STAT2, pinpointing redundant functions of STAT1 and STAT2 in defense against this bacterial pathogen (98). Studies addressing the molecular mechanism of STAT1-independent ISG expression showed that IFN-I stimulated a delayed activation of STAT2 in Stat1$^{-/-}$ cells. Together with IRF9, STAT2 formed a complex able to bind to the ISRE sequence. Data in Stat1$^{-/-}$ macrophages further demonstrated prolonged JAK activation by the IFN-I receptor and a slow but steady accumulation of both STAT2 and pYSTAT2. The data were consistent with a model according to which a threshold of STAT2 phosphorylation needs to be overcome for the formation of transcriptionally active STAT2–IRF9 complexes and for delayed stimulation of ISG transcription (98, 99). This hypothesis has subsequently been validated in cells derived from mice expressing a STAT1Y701F mutant (99). It is further consistent with reports showing that STAT2 constitutively associates with IRF9 (100) and that this preassociation may be required for rapid generation of the ISGF3 in response to IFN signals in wt cells (101). All the findings together strongly suggest that STAT2–IRF9-stimulated ISG expression could serve as a backup or a support mechanism of defense against pathogens that impede STAT1 signaling or serve to integrate the responses to IFN-I and TNFα (17, 84, 87, 92, 98).

NOVEL ASPECTS OF IFNγ SIGNALING BY CANONICAL AND NON-CANONICAL STAT COMPLEXES

Canonical signaling by the IFNγ receptor causes the formation of GAF, the STAT1 homodimer. GAF activates gene transcription by associating with its cognate DNA-binding sequence, the GAS (2, 102). As in the case of IFN-γ, the reality of transcriptional responses to IFNγ adds complexity. In part, this is due to STAT1 modification. The implications of Y701 phosphorylation as a dimerization signal are undisputed. Similarly, the enhancement of IFNγ-induced gene expression through phosphorylation of the C-terminal S727 and increased association with histone acetylase complexes are well documented (103, 104). Sumoylation of K703 was first described by the group of Curt Horvath (105). The implications of this modification for STAT1 activation and activity were initially unclear (105, 106), but subsequent studies, particularly those in cells derived from mice expressing SUMOylation-defective STAT1, clearly linked SUMOylation to decreased IFNγ responsiveness (107, 108). Mechanistically, SUMOylation interferes with the conjugation of a phosphate at the proximal Y701 and increases nuclear tyrosine dephosphorylation by increasing STAT1's solubility. In absence of the SUMO modification, STAT1

molecules assemble into an insoluble, phosphatase-resistant paracrystalline array (108, 109). Other than modification, the ability of STAT1 dimers to interact on DNA is essential for the expression of a large fraction of IFNγ-induced genes. This surprising finding emerged from studies in cells and mice expressing a STAT1F77A mutation that inhibits polymerization of promoter-bound STAT1 dimers (110). Strikingly, responsiveness to type I IFN, hence the activity of the ISGF3 complex, was unaffected by the STAT1F77A mutation.

Further variety is introduced to the IFNγ pathway by association between STAT1 and other proteins, i.e., non-canonical complexes (**Figure 1**). Already in the 1990s, Ifit2, a classical ISRE-regulated gene, was found to be induced by IFNγ in a STAT1- and IRF9-dependent, but STAT2-independent manner (111), suggesting that transcription factors containing both STAT1 and IRF9 are able to control IFNγ-responsive genes. Affirmative observations were made for the expression of the Cxcl10 gene in IFNγ-stimulated 2fTGH cells (112). More recently, work from our lab identified an important role for STAT1/IRF9 in the context of a murine colitis model (113). The Cxcl10 gene, known to contribute to colitogenic inflammation, was shown to be induced downstream of the IFNγ receptor in a STAT1/IRF9-dependent fashion, but independently of STAT2. Molecular analysis confirmed that STAT1/IRF9 complexes form in response to IFNγ and associate with ISRE sequences of enhancer regions 1 and 2 of the Cxcl10 gene promoter.

Other than STAT1/IRF9, STAT2 was proposed to contribute to IFNγ-induced transcription (**Figure 1**). The extent to which this occurs is unclear, owing in part to the fact that the lack of STAT2 reduces STAT1 levels in some cell types, resulting in a mixed Stat1/Stat2$^{-/-}$ phenotype. For example, Stat2$^{-/-}$ fibroblasts express little STAT1 and show impaired inhibition of vesicular stomatitis virus replication when treated with IFNγ (114). In support of a direct role for STAT2 in the IFNγ response, its tyrosine phosphorylation was reported in a study using IFNγ-treated wild-type mouse primary embryonic fibroblasts. This caused the formation of the ISGF3 transcription factor (115). Similar observations were made by Zimmerman and colleagues in MEFs (116). Stimulation of human lung epithelial cells with IFNγ triggered early and delayed peaks of STAT1 Y701 phosphorylation (117). The delayed peak corresponded to the formation of an ISGF3 complex, named ISGF3^{II}, that contained Y701-phosphorylated STAT1 and IRF9, in addition to STAT2 that remained unphosphorylated on Y690. The idea of a STAT2 contribution to the delayed IFNγ response is in line with our recent finding that expression of the Cxcl10 gene is decreased specifically at later stages of the IFNγ response in Stat2$^{-/-}$ macrophages (113). Consistently, we found STAT2 in association with the two Cxcl10 promoter ISREs at the delayed stage of the IFNγ response of wild-type macrophages. Of note, the same study shows that some genes with ISGF3 binding sites did not respond at any time to IFNγ in Stat2$^{-/-}$ macrophages. It appears possible, therefore, that both ISGF3 and ISGF3^{II} complexes contribute to IFNγ-induced transcription in a gene- and stage-specific manner. Of note, however, a stimulatory activity of ISGF3 and ISGF3^{II} complexes is challenged by an entirely different perspective on the role of STAT2 (118). Ho and colleagues show a strong, N-terminal association between unphosphorylated STAT2 and STAT1. This association persists during the IFNγ response and prevents a fraction of STAT1 molecules to enter the nucleus (**Figure 2**). Consistently, mutations disrupting the N-terminal contacts increase the transcriptional IFNγ response. The study thus presents STAT2 as a moderator of IFNγ-activated STAT1. All findings together support both positive and negative regulation of IFNγ-induced transcription by STAT2. Our studies addressing the Cxcl10 gene suggest that this dual function of STAT2 may represent early and late phases of the transcriptional response to IFNγ, with initial repression being followed by stimulatory activity (110).

U-STAT CONTRIBUTION TO THE IFN RESPONSE

According to the original JAK–STAT paradigm, there is a strict correlation between STAT activity and their tyrosine phosphorylation. Defying this notion, numerous reports have meanwhile assigned important tasks to U-STATs lacking a phosphate at the critical tyrosine residue. U-STAT activities include control of organelle metabolism and function in mitochondria or the Golgi apparatus [STAT1, STAT2, and STAT3 and STAT6 in the biology of mitochondria (119–125); STAT5 in the Golgi apparatus (125, 126)]. U-STAT1 was required for TNF-mediated apoptosis of U3A cells. This activity required the protein to be phosphorylated at the C-terminal S727 (127). To address potential functions of U-STAT1 in the immune system, we and our collaborators generated mice expressing a STAT1Y701F mutant and compared immune responses of these animals with Stat1$^{-/-}$ mice (99). Apart from a modest gain of function in antibacterial immunity described below, a notable difference was observed in NK cells. Whereas STAT1 deficiency led to a severe loss in NK cytotoxicity, this is partially retained in Stat1^{Y701F} mice (128). In contrast, U-STAT1 did not rescue the NK maturation defect observed in Stat1$^{-/-}$ mice. Localization to the NK-target cell interface hints at a potential role of U-STAT1 at the immunological synapse. NK cells also demonstrate a further non-canonical activity of STAT1 related to its second phosphorylation site, the C-terminal S727. This site is a target for both p38MAPK and the S/T kinase CDK8 (103, 129–131). Whereas in macrophages or fibroblasts S727 phosphorylation increases the IFNγ-induced expression of a subset of STAT1 target genes, CDK8-mediated S727 phosphorylation in NK cells restricts their cytotoxicity (132). Speculatively, the synaptic pool of STAT1 may be the relevant target, as Stat1^{S727A} mutation in NK cells had little impact on their gene expression. In summary, NK cells reveal several non-canonical activities not only for STAT1, but, as described above, also for TYK2 (**Table 1**). The integration of these activities in cellular signaling networks remains a future challenge.

In addition to cytoplasm or organelle-based roles, nuclear functions were reported involving U-STATs as either gene repressors or activators (133). For example, in *Drosophila*, STAT92E has been shown to associate with heterochromatin protein 1 (HP1) in cells lacking JAK activity, thus maintaining the structure of heterochromatin and gene repression (134, 135). Likewise, mammalian U-STAT5A reportedly binds to HP1α, stabilizing the

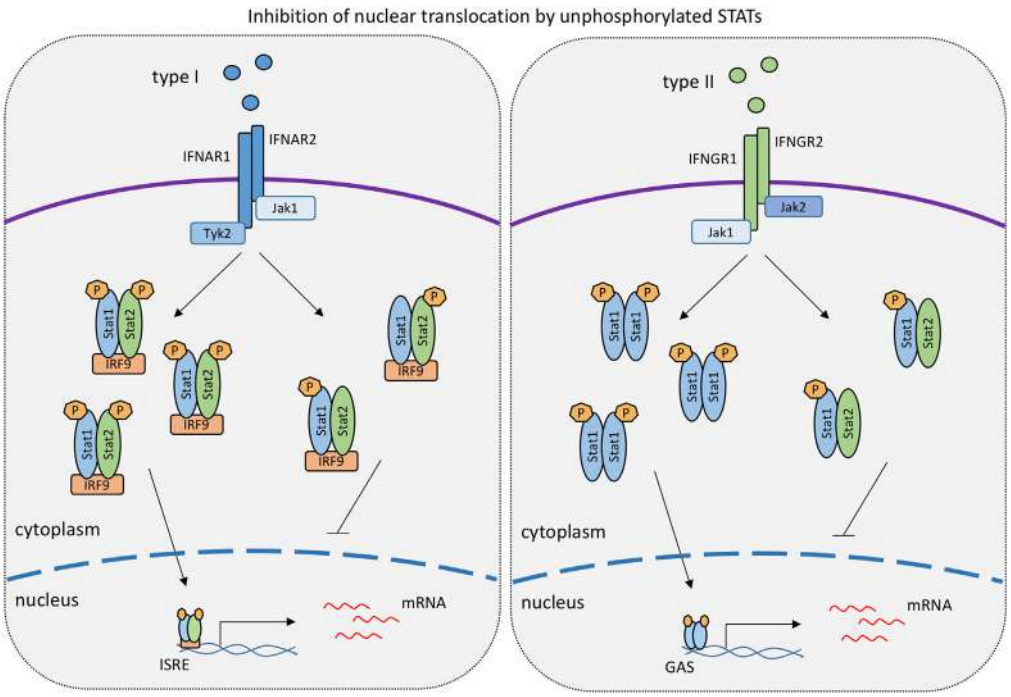

FIGURE 2 | Levels of unphosphorylated signal transducer and activator of transcriptions (STATs) determine the strength of responses to type I IFN and IFNγ. The model is based on work published in references 99 and 118 showing that unphosphorylated STAT1 binds to tyrosine-phosphorylated STAT2 and vice versa. Such hemiphosphorylated STAT dimers are incapable of nuclear translocation (118). In the IFNγ response, unphosphorylated STAT2 thus lowers the formation and nuclear translocation of tyrosine-phoshorylated STAT1 dimers (118). Conversely, unphosphorylated STAT1 inhibits the nuclear translocation of tyrosine-phosphorylated STAT2 in the type I IFN response (99).

heterochromatin in a similar fashion and repressing the genes involved in cancer development (136). In a more recent publication, mouse U-STAT5 was shown to actively repress the transcriptional program required for megakaryocytic differentiation by preventing the binding of the transcriptional activator EGR, acting as a partial antagonist of biological activity of phosphorylated STAT5 (137). U-STAT3 was shown to compete with IκB for binding to unphosphorylated NFκB, translocating to the nucleus and participating in the activation of a subset of NFκB-dependent genes (138). U-STAT6, in cooperation with p300, was suggested to bind to a consensus STAT6 binding site in the promoter of the Cox-2 gene, regulating its constitutive expression (139). The mechanism by which the unphosphorylated STATs enter the nucleus remains to be explored. It is most likely related to the ability of unphosphorylated STATs to shuttle between nucleus and cytoplasm (140). While the nuclear export rate usually localizes most U-STATs in the cytoplasm at steady state, it is conceivable that some of these are trapped in the nucleus by DNA or chromatin association. Other possibilities include the association with non-STAT transcription factors like IRF1, or direct contact with the nuclear pore complex (141–143).

The link between U-STATs and the IFN response was made by George Stark and colleagues. The group initially demonstrated an association of U-STAT1 and IRF1 with the partially overlapping interferon consensus sequence 2 and GAS sites in the promoter of the ISG Lmp2. This constitutive interaction resulted in expression of the Lmp2 gene in U3A cells (141). Additional studies, performed in cells overexpressing subunits of the ISGF3 complex, support the concept that U-STATs prolong the expression of a distinct subset of ISG (144) (**Figure 3**). The authors hypothesize that this occurs as a result of the accumulation of newly synthesized STAT1 and STAT2 following early, canonical type I IFN signaling. According to this hypothesis, prolonged exposure of cells to IFN-I and accumulation of STAT1, STAT2, and IRF9 causes formation of an unphosphorylated ISGF3 complex (U-ISGF3), which in turn maintains the expression of a subset of ISGs that increase resistance to viruses and DNA damage (145).

We attempted to test the U-STAT1 model in Stat1^{Y701F} mice (99). Indeed, some gain of function was noted when cells and animals expressing mutant STAT1 were infected with the bacterial pathogen *Listeria monocytogenes* and compared to STAT1-deficient counterparts. However, Starks U-STAT model could not be tested in these mice owing to the lack of an early, phosphotyrosine-based response to IFN-I that causes an increase of ISGF3 components (**Figure 3**). In fact, Stat1^{Y701F} mutation caused a drastic decrease in basal levels of STAT1 in cells and animals, due to the lack of tonic signaling by the IFN-I receptor (99, 146). Thus, improved animal models expressing increased U-STAT amounts are needed to collect *in vivo* evidence for their function.

Of interest, the low amounts of U-STAT1 expressed in Stat1^{Y701F} mice acted as suppressors of the delayed STAT1-independent, STAT2-dependent expression of ISGs after IFNβ stimulation (see above). This was a consequence of preventing cytoplasmic, tyrosine-phosphorylated STAT2 from entering the nucleus. The

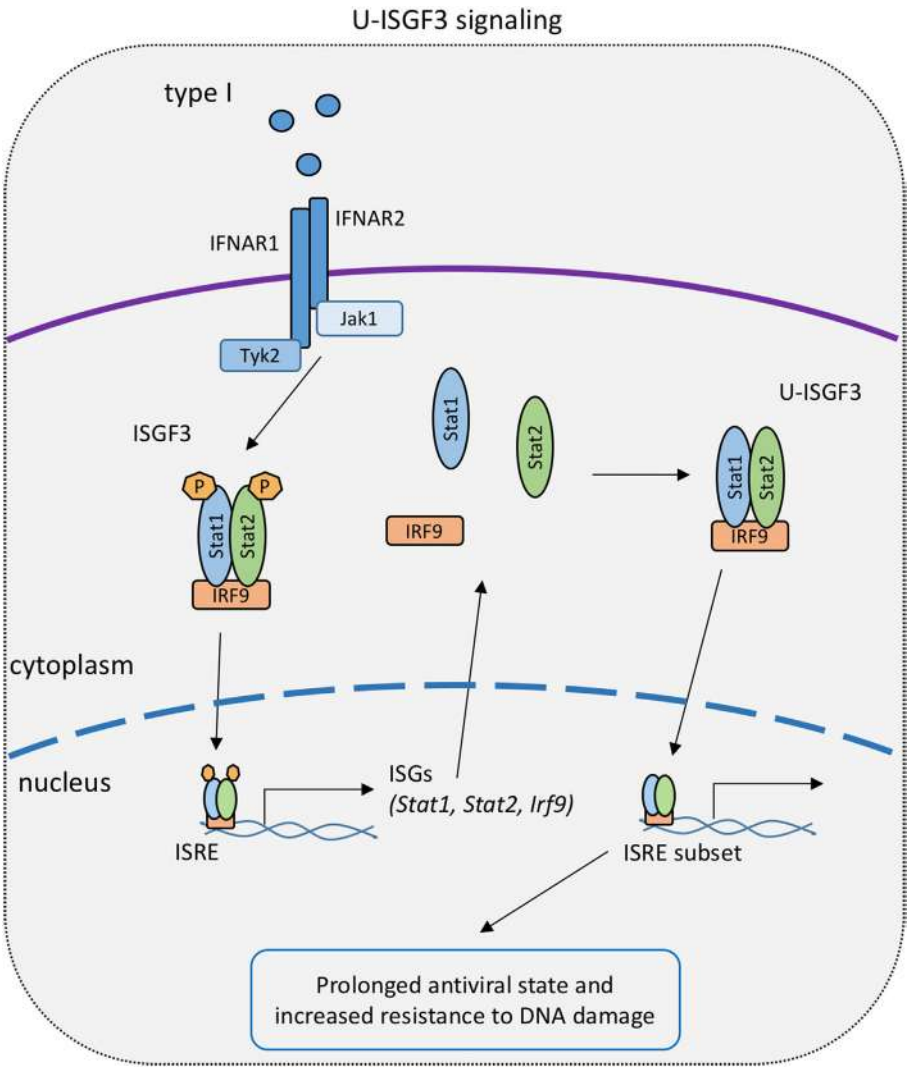

FIGURE 3 | U-STAT signaling in the type I IFN response. Early canonical signaling causes the upregulation of IFN-stimulated gene factor 3 (ISGF3) subunits and the subsequent formation of an ISGF3 complex with unphosphorylated signal transducer and activator of transcriptions 1 and 2 [unphosphorylated ISGF3 complex (U-ISGF3)]. U-ISGF3 stimulates a subset of interferon-stimulated genes to prolong the transcriptional response to IFN-I.

data suggest that hemiphosphorylated STAT dimers do not show the enhanced nuclear translocation of the fully phosphorylated dimers. This notion is in line with the above-mentioned data from the Vinkemeier lab that demonstrate the inability of hemiphosphorylated dimers of wild-type STATs to enter the cell nucleus (118). Therefore, relative ratios of STAT1 and STAT2 and their phosphorylated isoforms may be an important determinant of nuclear signaling by the IFN receptors (**Figure 2**).

FUNCTIONS OF IRF9 BEYOND IFN SIGNALING

The studies described above assign an important function of IRF9 to both IFN-I and IFNγ signaling. In this paragraph, we briefly describe some observations linking IRF9 to different diseases, either protecting from or exacerbating pathology. Notably, although IRF9 is an immune regulator, these data demonstrate additional mechanisms that may either link the immune system with these diseases or reflect IRF9 activities unrelated to the immune system. In most cases, the link to IFN signaling remains to be determined. The studies raise the possibility that IRF9 is capable of interacting with other transcription factors to fulfill a different set of functions (147).

Overexpression of IRF9 has been observed in breast and uterine tumors, where it provides resistance to microtubule-disrupting agents through transcriptional activation of ISGs in a STAT1- and STAT2-independent manner (148). In this situation, the ability of IL-6 to act as an inducer of IRF9 may be of importance, as shown for human prostate cancers (149). The role of IRF proteins in adipocyte biology connects the immune response with metabolic regulation (150, 151). In obese mice IRF7 is increased (152), while the expression of IRF3 (153) and IRF9 (152) is decreased.

Thus, IRFs respond differently to overnutrition stress. In line with this, mice lacking IRF7 show improved hepatic insulin sensitivity and protection from local and systemic inflammation during high-fat diet (152). In contrast, IRF3 and IRF9 play a protective role in high-fat diet-induced obesity (153, 154). IRF9 was shown to interact with peroxisome proliferator-activated receptor α to regulate gene expression in the liver (154). Their target genes are mostly involved in lipid metabolism, thus attenuating insulin resistance in obese mice. This observation suggests a key role for IRF9 in metabolic functions.

Cardiac hypertrophy and pathological remodeling are hallmarks of cardiomyopathy associated with many pathological stressors. Recent reports found that IRF3, IRF7, and IRF9 protect against cardiac hypertrophy (155–157). In murine disease models, IRF9 binds myocardin, an activator of the transcription factor serum response factor (SRF), thereby inhibiting SRF activation and associated proliferative response. Consistently, an aggravated cardiac hypertrophy occurs in $Irf9^{-/-}$ mice (157). Contrasting its protective effect in cardiac hypertrophy, upregulation of IRF9 during myocardial ischemia–reperfusion (I/R) injury contributes to cardiomyocyte death and inflammation through the Sirt1–p53 axis. In this context, the downregulation of the deacetylase SIRT1 by IRF9 promotes apoptotic signaling through p53 (158). Similarly, IRF9 overexpression leads to cell death signaling in neurons in context of a cerebral ischemic stroke. Hence, IRF9 deficiency mitigates neurological deficits upon stroke (159). In arteria, IRF9 mediates neointima formation, a scar that forms upon vascular injury. IRF9 overexpression increases, and its deficiency decreases the proliferation and migration of vascular smooth muscle cells (VSMCs). As in case of cardiomyocytes, IRF9 suppresses SIRT1 by directly binding to an ISRE in the SIRT1 promoter. Thereby, IRF9 prevents the suppression of AP-1 transactivation by SIRT1. AP-1 induces a vascular injury response pathway that promotes VSMC proliferation in the context of neointima formation (160). Although critical roles of IRF9 in immunity, metabolism, and disease have been revealed, many questions regarding the mechanisms by which IRF9 interconnects such a variety of pathways still remain.

CONCLUDING REMARKS

The overwhelming biological impact of canonical JAK–STAT pathways is undisputed. However, as happens often in biological sciences, long shadows of paradigmatic signaling systems obscure alternative installations of their components for distinct purposes. Recent years of JAK–STAT research have begun to uncover some of these undogmatic events and establish them as non-canonical pathways side-by-side with the canonical ones. This provides food for thoughts about the evolution of JAK–STAT pathways, the emergence of non-canonical and canonical functions. Studies in *Dictyostelium* suggest that STATs evolved without the necessity for tyrosine phosphorylation or the ability to activate transcription (161). Thus, it is tempting to speculate that what we now perceive as a deviation from the canonical pathways is in reality closer to the primordial STAT function that had nothing to do with cytokines or the genes they activate. Although this review provides a very brief overview, we hope it allows readers to get an idea of the many different ways by which non-canonical JAK–STAT pathways are established in the mammalian immune system. Their analysis is far from easy, as results from straightforward experimental approaches are likely to be dominated by the pathway's canonical output. In spite of this, future research with an open eye for the unexpected holds the promise of new fascinating insights into the many facets of JAKs and STATs in our immune system.

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REVIEW
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Avian Interferons and Their Antiviral Effectors

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Interferon (IFN) responses, mediated by a myriad of IFN-stimulated genes (ISGs), are the most profound innate immune responses against viruses. Cumulatively, these IFN effectors establish a multilayered antiviral state to safeguard the host against invading viral pathogens. Considerable genetic and functional characterizations of mammalian IFNs and their effectors have been made, and our understanding on the avian IFNs has started to expand. Similar to mammalian counterparts, three types of IFNs have been genetically characterized in most avian species with available annotated genomes. Intriguingly, chickens are capable of mounting potent innate immune responses upon various stimuli in the absence of essential components of IFN pathways including retinoic acid-inducible gene I, IFN regulatory factor 3 (IRF3), and possibility IRF9. Understanding these unique properties of the chicken IFN system would propose valuable targets for the development of potential therapeutics for a broader range of viruses of both veterinary and zoonotic importance. This review outlines recent developments in the roles of avian IFNs and ISGs against viruses and highlights important areas of research toward our understanding of the antiviral functions of IFN effectors against viral infections in birds.

Keywords: interferons, innate immunity, antivirals, viruses, avian, interferon-stimulated genes

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INTRODUCTION

For efficient replication and spread, viruses have to breach a potent and multilayered immune system in the host. Occasionally, either due to defects in host immune responses [e.g., complement system, interferons (IFNs), and adaptive immunity] or due to successful immune-antagonism, viruses overcome these antiviral mechanisms and replicate extensively in the host. This results in the engagement of diverse cascades of cellular signaling pathways (1). One of the most potent and essential events in this host–pathogen battle is the activation of the IFN pathways (1–3).

Three classes of nucleic acid receptors are associated with the activation of the IFN pathways. The first category of intracellular pattern recognition receptors (PRRs) is the family of retinoic acid-inducible gene I (RIG-I)-like helicases (RLH), which includes RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (3). A second class of PRRs is the family of toll-like receptors (TLR) including TLR3, TLR7, and TLR9, which senses extracellular, phagosomal, or endosomal pathogen-associated molecular patterns (1). The third category of PRRs is the family of DNA sensors, which include absent in melanoma 2 (AIM2) and cyclic GMP-AMP synthetase (cGAS) (4). Upon activation, these PRRs recruit downstream signaling molecules and result, directly or indirectly, in the activation of IFN regulatory factors 3 (IRF3) and 7 (IRF7), as well as activating protein 1 (AP-1) and nuclear factor kappa B (NF-κB)

transcription factors (1–4). These are minimally essential events to initiate transcription of type I IFN genes and establishment of an antiviral state by expressing hundreds of IFN-stimulated genes (ISGs) (1, 5) in infected cells.

Extensive structural and functional models have been proposed on the plasticity and dynamics of nucleic acid sensing by intracellular PRRs and on the mechanisms of IFN-induced antiviral states in mammals (1–4, 6). For a detailed description of IFN induction and other innate immune responses in mammals against viruses of diverse genetic backgrounds, we refer to other in-depth reviews (1, 2, 4, 5, 7, 8).

In this article, we offer a review of the IFN pathways and transcriptional activation of ISGs in different avian species. First, we provide an overview of the chicken IFN pathways and highlight areas that differ from mammalian IFN induction and signaling. Then, we convey a comparative genetic and genomic analysis of characterized components of IFN systems among different avian species. We conclude with a description of currently studied antiviral effectors, their implications for avian diseases, and future perspectives.

THE CHICKEN IFN PATHWAYS: SENSING OF VIRAL NUCLEIC ACIDS

RLH-Mediated IFN Induction

The principles of mammalian IFN pathways (exemplified by humans) are in general transferable to chickens. However, there are considerable evolutionary divergences in some of the key elements of the chicken IFN responses to avian viruses if compared to their mammalian counterparts. In mammals, RIG-I primarily senses 5′-triphosphorylated blunt-ended or double-stranded RNA (dsRNA) produced during RNA virus infections. On the other hand, MDA5 can be activated by long dsRNA, whereas LGP2, which differs from RIG-I and MDA5 in lacking caspase activation and recruitment domain domain, can positively regulate MDA5 and negatively regulate RIG-I signaling (9, 10). One of the most striking features of chickens and other members of the order Galliformes (e.g., turkeys) is the absence of RIG-I (11). Despite the absence of this key PRR, chickens respond to highly pathogenic avian influenza virus (HPAIVs) and mount potent type I IFN responses, probably due to cooperative actions of MDA5 and LGP2 (10, 12, 13) (**Figure 1**). Additionally, unlike the mammalian MDA5, which senses only long dsRNA, it appears that chicken MDA5 can also sense short dsRNA implying that chicken MDA5 may compensate, to some extent, the function of RIG-I in chickens (13). Recently, Uchikawa et al. have resolved the structures of dsRNA-bound chicken LGP2 and MDA5 and revealed the plasticity of nucleic acid sensing by these RLH (10). It was shown that chicken LGP2 carries two properties of RLH: an MDA5-like helicase domain and a RIG-I-like C-terminal domain. Chicken LGP2, similar to human RIG-I, is an "end binder," whereas chicken MDA5 is a "stem binder" of dsRNA (**Figure 1**). Based on structural (10) and functional studies (12, 13), it is likely that chicken LGP2-mediated enhancement of MDA5 sensing of dsRNA is dependent on RNA binding. However, it remains to be demonstrated if the mechanisms of LGP2-mediated enhancement of MDA5 signaling are similar to its mammalian counterparts or if the absence of RIG-I in chickens can contribute in the dynamics of cooperative nucleic acid sensing in chickens.

It has been hypothesized that the lack of RIG-I makes chickens highly susceptible to RNA viruses, and therefore chickens continue to play a central role in the emergence of zoonotic influenza viruses (14). However, more research is still required to support this generally accepted concept. Although MDA5 and LGP2 seem to be sufficient to induce a potent activation of the type I IFN pathway, ectopic expression of duck RIG-I in chicken cells potentiated the downstream signaling pathway, including increased induction of several ISGs such as myxovirus-resistance protein (Mx), protein kinase R (PKR), IFN-induced protein with tetratricopeptide repeats 5 (IFIT5), or 2′-5′-oligoadenylate synthetase (2′-5′-OAS) (14, 15). These studies indicate that chickens have acquired mechanisms to compensate the deficiency of the RIG-I signaling molecule; however, it is not possible to assess the outcome of nucleic acid sensing in chickens as it would have been in the presence of endogenous RIG-I. Nevertheless, chickens are one of the most successfully domesticated animal species and are immunologically competent in mounting an effective antiviral type I IFN state against diverse stimuli.

TLR-Mediated IFN Induction

Toll-like receptors are type I transmembrane proteins and have a highly conserved architecture in a variety of species, including insects, fish, amphibians, birds, and mammals (16). Comparative biological approaches revealed that chicken TLRs carry unique properties regarding ligand specificity, formation of TLR receptor complexes, and activation of signaling pathways (17). At least 10 different TLR members (TLR1–10) have been identified in humans (16). Chickens have been shown to have two TLR2 isoforms (chTLR2 types 1 and 2), two TLR1/6/10 orthologs, and single genes for TLR3, TLR4, TLR5, and TLR7. Interestingly, chickens do not possess the viral DNA sensor TLR9. However, TLR-mediated DNA sensing is mediated by a functional ortholog TLR21, which is absent in humans (16). Additionally, it has been proposed that chicken TLR8 is non-functional and that the chicken genome encodes for an additional TLR gene, TLR15, which requires protease-cleavage for activation (18). Beside genomic variations, functional differences exist in the mechanism of TLR-mediated signal induction in chickens. In contrast to humans, lipopolysaccharides failed to stimulate the TLR4–TRAM–TRIF pathway in chicken cells (19). Among all mammalian TLRs, TLR3, TLR7/8, and TLR9 are known to sense viral dsRNA, ssRNA, and DNA molecules, respectively. Since in chicken TLR8 is non-functional and TLR9 is absent, only TLR3 and TLR7 are involved in the recognition of RNA viruses. All TLR family members, with the notable exception of TLR3, signal *via* myeloid differentiation primary response 88 (Myd88). TLR3 recruits TIR-domain-containing adapter-inducing IFN-β (TRIF) through transmembrane, phagosomal, or endosomal compartments (**Figure 1**). Both modes of TLR-dependent signal induction culminate in the activation of the transcription factors required for the transcription of type I IFNs.

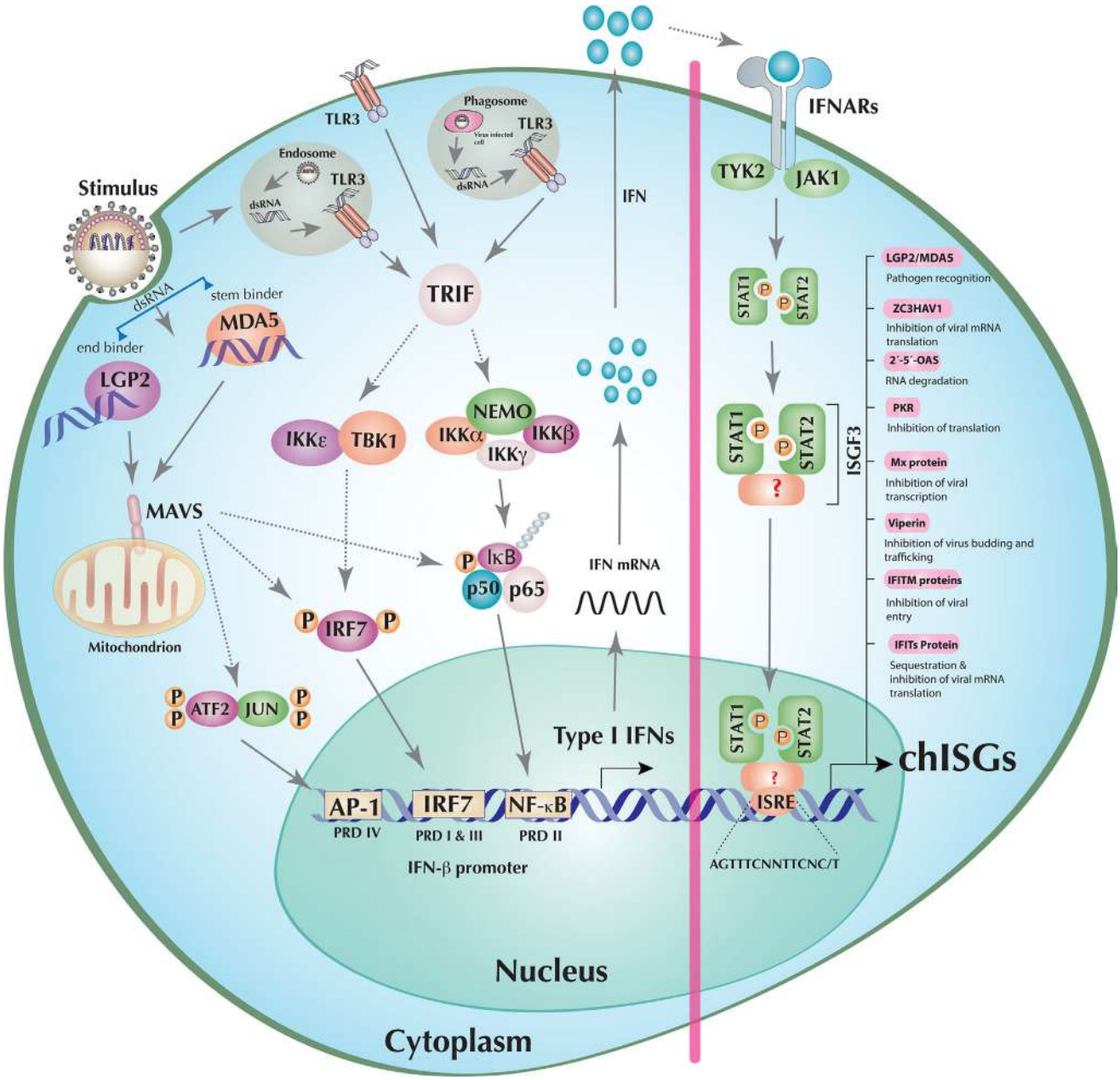

FIGURE 1 | **Induction of interferons (IFNs) and establishment of an antiviral state in a model chicken cell**. The double-stranded RNA (dsRNA), detected by either chicken retinoic acid-inducible gene I (RIG-I)-like helicase (RLH) [melanoma differentiation-associated gene 5 (MDA5) or laboratory of genetics and physiology 2 (LGP2) individually or in cooperation] or toll-like receptor (TLR)3 (endosomal, phagosomal, or transmembrane) initiates downstream signaling mediated through mitochondrial antiviral-signaling protein (MAVS) or TRIF, respectively. These adaptor molecules then activate the transcription factors IFN regulatory factor (IRF)7, nuclear factor kappa B (NF-κB), and activating protein 1 (AP-1) (ATF2/JUN) by orchestrating the assembly of multi-protein complexes. Once activated, IRF7, NF-κB, and AP-1 translocate to the nucleus where they stimulate the transcription of, among others, type I IFNs (e.g., IFN-β). The transcribed, translated, and secreted type I IFNs initiate the JAK–STAT pathway by both autocrine (depicted in the figure) and paracrine signaling through cognate type I IFN receptor recognition. Activated JAK–STAT leads to the phosphorylation of STAT1 and STAT2 molecules, which (together with factors that are currently unknown in chicken) results in the formation of the IFN-stimulated gene factor 3 (ISGF3) transcription factor complex. This multifunctional transcription factor then scans and recognizes unique IFN-stimulated response element (ISRE) sequences to initiate the transcription of hundreds of chicken IFN-stimulated genes (chISGs), which subsequently establish the antiviral state against the invading viruses. Few examples of IFN-stimulated genes (ISGs) along with a summarized description of their functions are enlisted in the right panel of the figure. Abbreviations used in the figure and are not described in the main text are as follows: IκB kinase (IKK) epsilon (IKKε), alpha (IKKα), beta (IKKβ), and gamma (IKKγ); NF-κB essential modulator (NEMO); TANK-binding kinase 1 (TBK1); inhibitors of NF-κB (IκB), NF-κB subunits p50 and p65; activating transcription factor 2 (ATF2); tyrosine kinase 2 (TYK2); Janus kinase 1 (JAK1); signal transducer and activator of transcription 1 (STAT1), and STAT2. "P" represents the phosphorylation state of the protein, and dotted lines indicate the involvement of multiple intermediary steps.

DNA Sensors-Mediated IFN Induction

In addition to TLR9-mediated DNA sensing in mammals, cytosolic DNA, which can be either non-self DNA or results from gross nuclear/mitochondrial damage, can elicit type I IFN responses in mammals (9). Currently two major cytosolic sensors of DNA have been characterized: the PYHIN family member AIM2 and cGAS. Additionally, several proteins have been recognized as DNA receptors, including Z DNA binding protein 1 (ZBP1/DAI), the helicase DDX41, and IFI16, another member of the PYHIN/HIN-200 family (20, 21). Downstream of these DNA sensors, the stimulator of IFN genes (STING) acts as an adapter and stimulates type I IFN production through the activation of IRF3 and NF-κB transcription factors (9). Although DNA sensing in chickens has not yet been explored in greater detail, genetic analysis indicate that the AIM2 gene has been lost independently in several animals, including bats and chickens (22). Even in the latest Ensembl release of the chicken genome, ZBP1 and IFI16 were not identified, suggesting fundamental differences in DNA sensing mechanisms in chickens. However, it has been shown recently that chicken STING can actively sense DNA and in cooperation with the mitochondrial antiviral-signaling protein induces type I IFN responses independent of RIG-I, interfering with the replication of RNA viruses (23). Interestingly, STING-mediated type I IFN induction was synergistically supported by RLHs in chickens (23). This warrants future investigations to understand the molecular mechanisms underlining DNA sensing in chickens.

TRANSCRIPTIONAL ACTIVATION OF IFNs

Signals initiated by the sensing of viral nucleic acids by RLHs, TLRs, or DNA sensors lead to the activation of at least three transcription factors (AP-1, IRF3, and NF-κB) in the mammalian type I IFN enhanceosome (1). There is scarcity in our current understanding of the mechanism and structure of the chicken IFN enhanceosome. Comparative genomics analysis indicates that chickens are IRF3 deficient (detailed below). Currently, it is not known if the presence of functional IRF7 in chickens compensates for the IRF3 deficiency. Components of AP-I and NF-κB transcription factors are encoded in the chicken genome, and it is likely that these signaling cascades are functionally similar to mammals. Thus, a direct functional comparison may be plausible. While inactive, NF-κB, IRF3/IRF7 (in mammals and IRF7 in chicken), and AP-1 remain in the cytoplasm; however, upon stimulation (e.g., nucleic acids) these transcription factors get activated and subsequently translocated to the nucleus of viral-infected cells by unique mechanisms (1). The activation signals result in phosphorylation of IRF7. Conformational changes caused by this post-translational modification result in IRF7 dimerization and exposure of the nuclear localization signal (NLS) (1). This NLS mediates the nuclear translocation of IRF7 (1, 24). The inhibitor of NF-κB (IκB) retains NF-κB molecules in the cytoplasm. However, upon activation by phosphorylation, IκB undergoes ubiquitination and proteasomal degradation. Degradation of IκB exposes the NLS of NF-κB, which leads to its nuclear translocation (7). Phosphorylation of c-jun and activating transcription factor 2, two heterodimeric components of AP-1, also causes nuclear translocation (1). In the nucleus, these three transcription factors assemble in a cooperative manner to build a type I IFN enhanceosome, which binds to its respective positive regulatory domains (PRDs). IRF7, NF-κB, and AP-1 bind to PRD I/III, PRD II, and PRD IV, respectively, where they induce the transcription of type I IFNs and pro-inflammatory cytokines (TNF, IL-6, IL-1β, etc.) (25) (**Figure 1**). These type I IFNs lead to transcriptional activation of several hundreds ISGs to mount an antiviral state in the host (detailed below).

COMPARATIVE GENOMICS AND EVOLUTION BY GENE LOSS

Even in the updated version of chicken Ensembl (Ensembl release 85—July 2016, accessed on September 11, 2016), it appears that chickens lack IRF3 and IRF9 (depicted in **Figure 1**), which are essential components of the type I IFN system in mammals (1). Lately, there have been substantial improvements in the genetic analysis and functional characterization of the avian type I IFN pathway, particularly in chicken. However, the annotation of the chicken genome is not yet completed, leaving open questions on the presence or absence of the mammalian homologs in avian species. Improved annotation of chicken and other avian genomes is required to unambiguously declare the presence or absence of a particular gene in the future. This fact can be exemplified by a recent analysis of IRF3/7 in the chicken genome. The first identified and characterized member in the chicken IRF family (named cIRF3) was classified as IRF3 based on its sequence and overall functional conservation with corresponding IRF3 in other species (26). Availability of updated annotation of chicken genome in the Ensembl has filled the gaps in the chicken chromosome 5, which encodes for the IRF3/7 genes and allowed to reevaluate the IRF locus in the chicken. Based on the analysis of gene loci in different species including human, mouse, dog, and fish (**Figure 2A**) and previous reports (25, 27), it is convincing that the formerly reported cIRF3 is actually IRF7. Furthermore, genetic clustering and sequence divergence analysis indicate that the chicken IRF7 clusters closely with IRF7 of human, mouse, and cattle compared to the IRF3 of corresponding species (**Figure 2B**). Therefore, it is suggested to use the term chicken IRF7 instead of cIRF3 to avoid any misunderstanding in the functional nomenclature between these two transcription factors.

Similar to IRF3/IRF7, the currently annotated chicken IRF9 sequence is both genetically (**Figure 3A**) and phylogenomically (**Figure 3B**) similar to IRF10 in dog and fish. Comparison of the gene orientation and architecture between species in which IRF10 is detected (dog and fish) and species in which IRF10 is lacking (human and mice) provides direct evidence that these crucial elements of the IFN pathways are currently incorrectly annotated. In addition, our analysis on global IRF family members confirms that the chicken genome lacks any significant sequence identity to the mammalian IRF9 orthologs. It remains to be explored how chickens still manage to efficiently trigger the production of ISGs without the need of IRF9 to constitute a functional type I IFN-stimulated gene factor 3

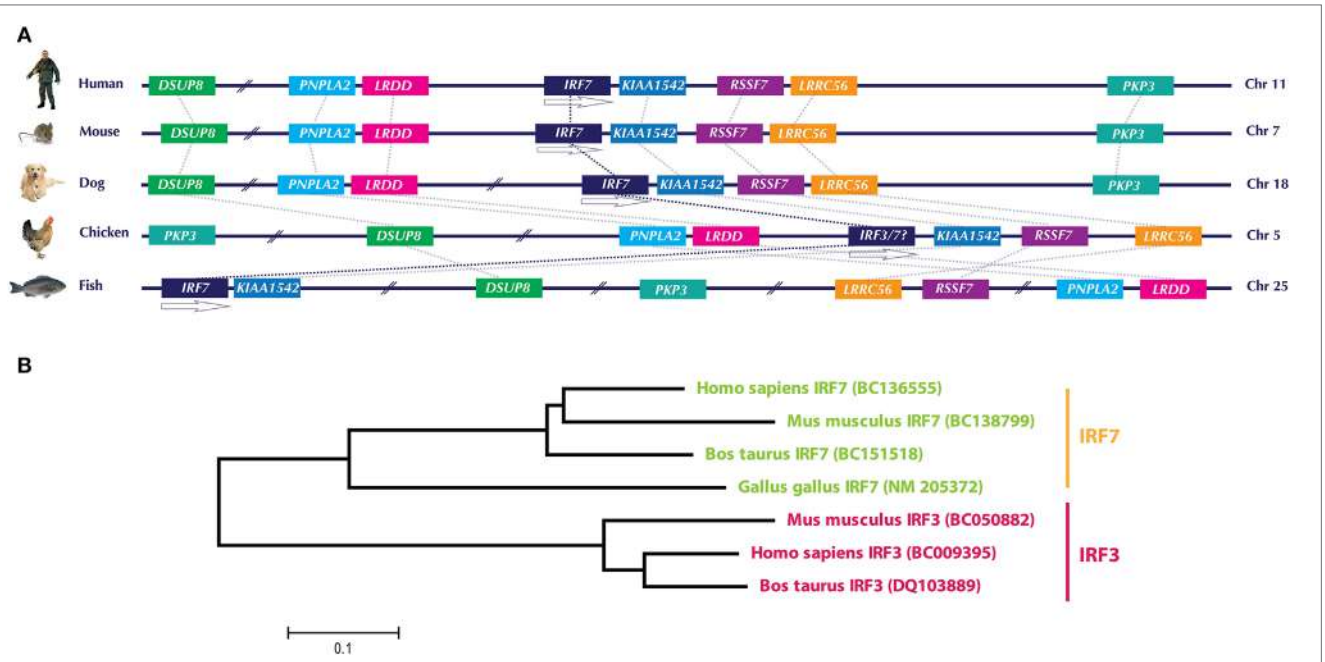

FIGURE 2 | **(A)** Genomic architecture along with relative loci around the IRF7 gene in human, mouse, dog, chicken, and fish. The IRF7 genes in the compared species are flanked upstream with LRDD gene and downstream with KIAA1542 and RSSF7 genes. Direct comparison of previously identified chicken IRF3 with these species indicates that this gene is in fact IRF7. **(B)** Phylogenetic analysis of IRF3 and IRF7 genes in different species. Based on the clustering patterns and sequence homologies, the gene previously identified as "chicken IRF3" clustered closer to IRF7 of other mammals compared to mammalian IRF3. It is therefore proposed to rename "chicken IRF3" to "chicken IRF7." Gene abbreviations used in the figure are dual specificity phosphatase 8 (DSUP8), patatin-like phospholipase domain containing 2 (PNPLA2), leucine-rich repeats and death domain containing (LRDD), interferon regulatory factor 7 (IRF7), CTD-binding SR-like protein rA9 (KIAA1542); Ras association (RalGDS/AF-6) domain family 7 (RSSF7); leucine-rich repeat containing 56 (LRRC56); plakophilin 3 (PKP3).

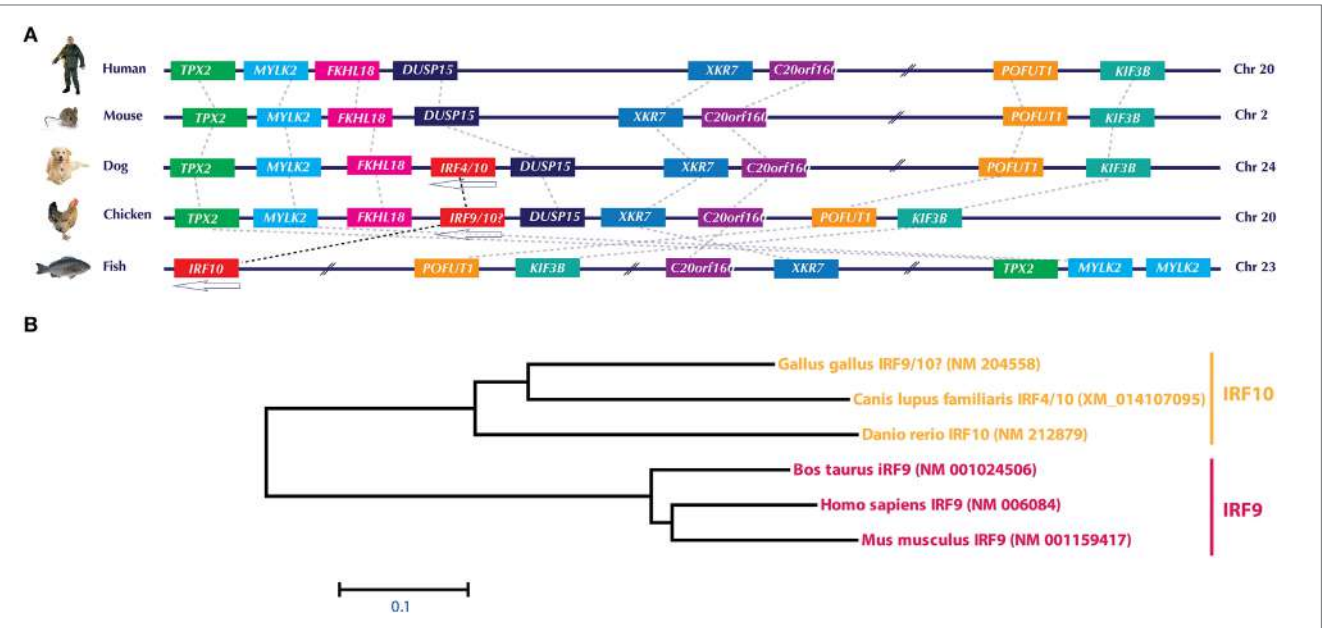

FIGURE 3 | **(A)** Genomic architecture of interferon regulatory factor (IRF)10 loci and phylogenetic analysis of IRF9 and IRF10 in human, mouse, dog, chicken, and fish. Upstream and downstream genes architecture in the IRF10 of chicken, dog, and fish indicate that this locus is similar to the corresponding locus in human and mice, which lack IRF10. Based on this and phylogenetic analysis **(B)**, it is evident that the currently annotated chicken IRF9 is in fact an ortholog of IRF10. Gene abbreviations used in the figure are microtubule-associated protein homolog (*Xenopus laevis*) (TPX2); myosin, light polypeptide kinase 2, skeletal muscle (MYLK2); forkhead-like 18 (*Drosophila*) (FLKHL18); dual specificity phosphatase-like 15 (DUSP15); X Kell blood group precursor-related family member 7 homolog (XKR7); chromosome 20 open reading frame 160 (C20orf160); protein O-fucosyltransferase 1 (POFUT1); kinesin family member 3B (KIF3B).

(ISGF3) complex. However, it is plausible that factor(s) other than IRF9 are involved in the formation of an active ISGF3 complex in chickens. Since type II IFNs-mediate induction of ISGs is IRF9-independent, it may be possible that under virus infection the gamma-activated sequence (GAS) promoter may overwhelm the overall induction of ISGs compared to type I and III IFN-induced expression of ISGs.

From these examples, it is clear that our understanding of the avian genome is still insufficient to accurately annotate the newly identified genes. Efforts have recently become intense through the avian consortium to not only characterize the genetics of endangered and newly sequenced bird species but also to improve the annotation of the existing genome drafts of avian species, especially chicken and duck. As a result of this, a bunch of genome sequences from more than 40 avian species was published recently (28), providing a valuable source for gene mapping. These resources would certainly advance our understanding in exploring genes, which are conserved across avian species, and to confirm existing genes. A special database (AvianBase) has been established to facilitate comparative genomics and immunogenetics in avian species (29).

Beside the fact that genes are incorrectly annotated and important genomic loci are not characterized in the avian species, it is likely that birds have evolutionary lost some genes during their domestication and subsequent division into required phenotypes (egg-laying versus meat-producing) (30). It requires extensive genetic and genomic investigations to confirm gene loss in the evolutionary process of avian species and to identify a minimum number of genes that can be readily lost from avian genomes without compromising the survivability. Although several models can be proposed, loss of genes due to "gene function bias" appears to be operative in chicken and other avian species (31). Gene function bias refers to the gene loss that is preferentially evident in a specific functional category; gene loss in gene ontology category of "immune responses" is highly probable in mammals compared to other vertebrates (32). A similar scenario can be applied to the gene loss in innate immune signaling pathways compared to other gene ontology categories in avian species mainly due to dispensable functional constraints. In this context, different type I IFN-induced proteins with tetratricopeptide repeats (IFITs), including IFIT1, IFIT2, IFIT3, and IFIT5 (33), have been described to play essential roles in nucleic acid sensing, antiviral responses, and protein translation in humans. All these functions of IFIT proteins are redundant, and thus the protein family is likely under selection constraints in chicken where only one IFIT protein (IFIT5) has been identified compared to four in human and mice (34). In summary, understanding the mechanisms and impacts of gene loss would reveal crucial evolutionary aspects of animal domestication and may highlight unexplored ways that could be exploited both for antiviral therapy and disease control.

EVOLUTION AND NOMENCLATURE OF AVIAN IFNs

Phylodynamic analysis of homology-based coding sequences of all three types of IFNs (I, II, and III) indicates that these evolutionary IFN classes are only distantly related and lack apparent sequence homology among each other (**Figure 4A**). However, type I and II IFNs appear to be more closely related to each other compared to type III IFN, despite the fact that type I and III share functional and signaling homologies.

Although chicken IFNs have functional homologies with their mammalian counterparts, gene duplication of each IFN subtype varies markedly among different animal species. In all birds investigated so far, type II and type III IFNs exist as a single gene each (35), whereas in mammals two to four copies of type III IFNs have been identified (35). Compared to fish, where generally only a single type I IFN homolog is detected, 3 to 10 type I IFN copies have been identified on the sex-determining Z chromosome of avian species (36–39). The maintenance of type II IFNs in avian and mammalian speciation indicates their constant function and evolutionary pressures. In both chicken and mammalian genomes, the functional transcript of the single type II IFN gene is encoded by four exons, and the gene architecture resembles that of IL-10-like cytokines.

Direct and parallel comparison of clustering patterns of type I and type II IFNs indicates the divergence of IFN-alpha (IFN-α) and IFN-gamma (IFN-γ) across mammals, rodents, primates, fish, and avian species (**Figure 4B**). It is evident that chicken IFNs and IFN genes of other vertebrates included in this evolutionary tree cluster distinctly from the fish IFNs. However, both fish and chicken type I and type II IFNs formed separate clades with markedly high resolution (bootstrap value of >90%). These clustering patterns may support the evolutionary and structural architecture of at least type I IFNs in different vertebrates, where fish encodes for five exons compared to single exon in birds and mammals. This is postulated to be due to the retrotransposition events in which four exons were lost between divergence of tetrapods and radiation of amniote lineages (37).

Consistent with vertebrate evolution, there are insufficient relationships between type I and type II IFNs in avian and mammalian species (**Figures 4A,B**). Thus, it is concluded that mammalian and avian type I IFNs evolved independently by gene duplication of a progenitor after segregation of mammals and birds (38, 39). Therefore, the avian type I IFNs are no true orthologs of their mammalian counterparts, and the nomenclature used for mammalian type I IFNs is strictly not appropriate for avian species. This is further supported by the level of genetic and functional differences between mammalian and avian type I IFNs (detailed below).

AVIAN IFNs

Based on their receptor specificity, sequence homology, and nature of ISG induction, IFNs are divided into those that bind IFNαR1 and IFNαR2 (type I IFNs), those that interact with receptors complexes of IFNγR1 and IFNγR2 (type II IFNs), and those that interact with heterodimeric receptor complex of IL-28Rα and IL-10Rβ (type III IFNs or IL-28/29). Our understanding of the avian IFN pathways is gradually increasing, and recently several significant contributions have been made to characterize existing genes (40–42) and previously identified IFNs, especially in chicken. In the following sections, our current understanding

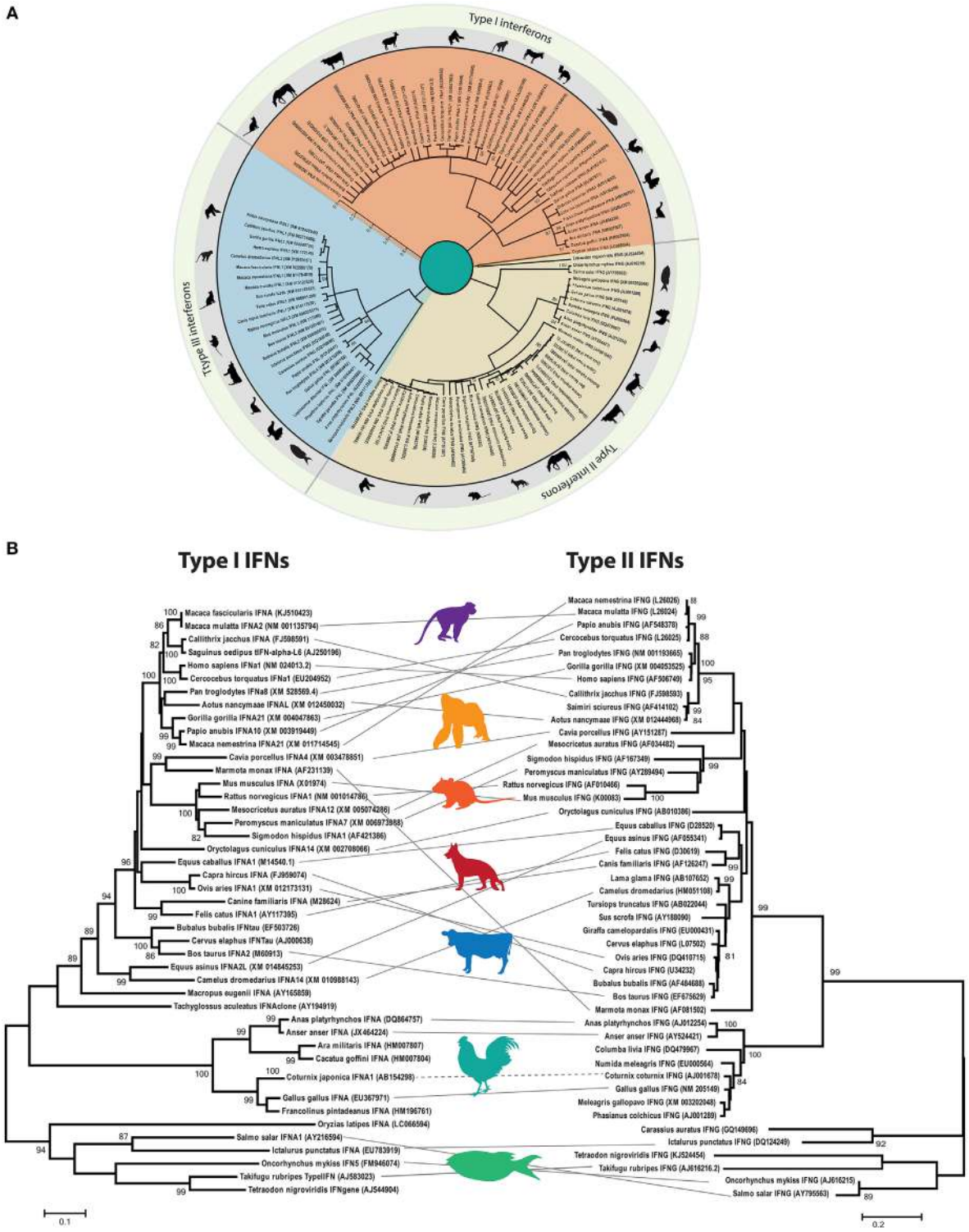

FIGURE 4 | Phylogenetic analysis of interferon (IFN) genes of mammals (including rodents, primates, and domestic animals), avian, and fish species. **(A)** The open reading frames of type I, II, and III IFN genes were manually extracted from public databases and were aligned in BioEdit. The phylogenetic tree was constructed by MEGA6 software using the Kimura-2 model with 1,000 bootstrap replicates. The three types of IFNs clustered distantly and were labeled according to their clustering patterns. Approximate branching position was marked with a representative animal in the class. Only bootstrap values higher than 50 are shown. **(B)** Parallel comparison of type I and type II IFNs. Type III IFNs have been identified in limited numbers of species, and thus direct comparison was avoided. Clustering pattern of type I IFNs were linked to the type II IFN gene of the corresponding species for comparison purposes, and a representative animal image was shown to illustrate the clustering pattern.

on chicken IFNs and comparative genomics in other avian species will be discussed. Several known features of chicken IFNs are summarized in **Table 1**.

Avian Type I IFNs

In contrast to the numerous members of type I IFNs in mammals (IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, and IFN-τ), so far only two serologically distinct, intron-less, acid and heat stable type I IFNs (IFN-α and IFN-β sharing nt homology of 57%) have been identified in avian species on the short arm of Z (sex) chromosome (53). Unlike IFN-β, which is encoded only by a single gene copy, chicken IFN-α exists as a family of several genes (**Table 1**). Although there is low overall amino acid identity between avian and mammalian IFN-α protein sequences (24%), a core region in the chicken IFN-α carries four of six conserved cysteine residues, an α-helix and a high sequence identity (80%) compared to mammalian IFN-α protein (**Figure 5A**).

Moreover, recombinant goose IFN-α has been shown to carry partial cross-species antiviral properties (55, 56). These results indicate that type I IFNs have attained certain levels of functional flexibilities (56). Nevertheless, all type I IFNs are known to be involved in inducing an antiviral state, inhibiting cell proliferation, modulating cell fate, and mediating cell differentiation and migration (57). To accomplish the primary function of IFNs, it is essential for these cytokines to bind to their respective receptors. Receptors for type I IFNs (IFNAR1 and IFNAR2) have been sequenced in chicken (58); however, little information is available about their functional domains and their crucial roles in type I IFN signaling.

Among avian type I IFNs, most of the research has been conducted on chickens, and IFN-α has been identified and more extensively characterized than IFN-β in different avian species (59) (**Figures 5A,B**, respectively). Chicken IFN-α and IFN-β genes were first identified from a cDNA library of aged chicken embryo cells, and subsequent analysis indicated the functionally and evolutionarily conserved properties compared to mammalian type I IFNs (60). Several recent studies have mapped the expression dynamics of chicken type I IFNs triggered by different stimuli (60–63). Collectively, type I IFNs (especially IFN-α) are potent antiviral agents and can ameliorate viral infections including Marek's disease virus (MDV), infectious bursal disease virus (IBDV), infectious bronchitis virus (IBV), and HPAIV in different avian species (43–47). These antiviral properties of type I IFNs are identified not only *in vitro* but also *in ovo* and *in vivo* (43, 47).

Antiviral properties of type I IFNs are essentially mediated by the induction of ISGs. Both chicken IFN-α and IFN-β bind to the same IFN receptors (IFNAR1 and IFNAR2). However, it has been recently found that IFN-α and IFN-β differentially regulate ISGs in chickens (62). The antiviral state induced by chicken IFN-α was observed to be significantly more potent than that induced by chicken IFN-β, although both share genetic and structural similarities (64). These differential effects can be explained by differential binding affinity of IFN-α and IFN-β for the IFNAR1 and IFNAR2 (44). This hypothesis is further supported by a recent ontological study on the development of the chicken type I IFN system in which a markedly stronger upregulation of IFNAR1 as compared to IFNAR2 was observed during embryonic development in chicken lung and spleen cells (65). Since IFN-α and IFN-β differentially regulate the transcriptional activation of ISGs, it is imperative to consider that 5′ upstream regions of the chicken IFN-α genes lack NF-κB-binding sites and carry several binding sites for IRF members in their promoters regions (64). Moreover, observed differences in the ISGs induced by chicken IFN-α and IFN-β could be due to intrinsic functional components of the cell lines under investigation. For instance, type I IFNs induces TLR3 upregulation in the chicken fibroblasts cell line DF-1, whereas this induction was not observed in the chicken macrophages cell line HD11 (66). It cannot be excluded that constitutively primed cells may respond better to IFN-α compared to IFN-β, as has been observed in human lymphocytes that produce IFN-α, without the need to produce IFN-β, by viral infections (67). In conclusion, differential regulation of type I IFN-induced ISG signaling can be multifactorial and represents an interesting area for future investigations on the avian innate immunity.

Type I IFNs, especially IFN-α, have been characterized and assessed for their antiviral activities against IFN-sensitive viruses in various additional avian species. The duck type I IFNs were first detected in duck embryo fibroblasts (DEFs) after infection with high doses of reovirus serotype 3 (strain Dearing). Exogenous expression of this IFN blocked the release of avian RNA tumor virus particles in B77 virus-transformed DEFs (68) and showed

TABLE 1 | Summary of characteristics demonstrated for chicken IFNs.

IFN type	Known variants	Chemical properties	Receptor subunits	Antiviral activities[a]	Primary expression of cytokine	Location	Promoter for ISGs	Reference
I	IFN-α, IFN-β	Acid and heat stable	IFNAR1 IFNAR2	MDV, IBDV, IBV, influenza	Fibroblasts	Z chromosome	ISRE	(43–47)
II	IFN-γ	Sensitive to low pH (2) and heat (65°C)	IFNGR1 IFNGR2	NDV, MDV, influenza	Immune cells	Chromosome 1	GAS	(48, 49)
III	IFN-λ	Heat stable	IL-28Rα IL-10Rβ	NDV, influenza, IBV	Epithelial cells	Scaffold AADN04001262.1	ISRE	(50–52)

[a]*These are few examples of pathogens against which antiviral activities of the cytokine have been demonstrated.*

IFN, interferon; ISGs, IFN-stimulated genes; IFN-α, IFN-alpha; IFN-γ, IFN-gamma; MDV, Marek's disease virus; IBDV, infectious bursal disease virus; IBV, infectious bronchitis virus; NDV, Newcastle disease virus; ISRE, IFN-stimulated response element; GAS, gamma-activated sequence.

FIGURE 5 | **(A)** Structural and amino acid sequence homologies between type I interferons (IFNs) in different avian species. Alignment and sequence homology of avian IFN-alpha (IFN-α) [**(A)**, top panel] and avian IFN-beta (IFN-β) [**(A)**, bottom panel] amino acid sequences. Putative sites for IFN-α binding to IFNAR1 are marked with heart symbol, whereas the sites that are important for binding to IFNAR2 are marked with star sign. In comparison to mammals, sites required for interaction of IFNs with IFNARs are more variable among avian species (54). The previously reported signal peptides are underlined in both IFN-α (top) and IFN-β (bottom) sequences (54). **(B)** A modeled cartoon structure of human and chicken IFN-α. IFN-α protein structures were predicted using I-TASSER online tool and were annotated and aligned in MacPyMOL. Similar to human IFN-α (PBD ID: 1ITF), chicken IFN-α carries five helices and is structurally similar to human IFN-α. Direct structure comparison between human (mammalian) and chicken (avian) IFN-α proteins indicate that the chicken IFN-α protein carries five alpha-helices, which are considered crucial for the functionality of type I IFNs in mammals.

antiviral effects for chronic hepatitis B virus infections (69). Recently, IFN-α has been identified and expressed in cells from the red-crowned crane (70), and an initial bioassay indicated its antiviral activities against vesicular stomatitis virus (VSV) in heterologous chicken fibroblasts. IFN-α has also been cloned from geese and turkeys, and initial functional insights including the antiviral actions have been determined (55, 71).

The results obtained so far on avian type I IFNs indicate that these cytokines are functionally, structurally (**Figure 5B**), and evolutionary related to mammalian IFNs and may have originated from common ancestor genes. However, extensive studies are required to identify other homologs of type I IFNs in all avian species, their mechanisms of action, how they exert individual and cumulative antiviral effects, and their potential for cross-species reactivity.

Avian Type II IFNs

Interferon-gamma is the only member of type II IFN in birds and mammals and serves as a bridge between innate and adaptive immunity. IFN-γ plays a crucial role in regulating the maturation and differentiation process of several immune cells and activates T helper 1-type immune responses (3). Due to these unique properties, significant research has been conducted to map the antiviral potential and mechanistic effects of IFN-γ in chicken, and considerable information is also available for other avian species. Direct gene comparison and evolutionary analysis of avian IFN-γ genes clearly demonstrate the significant identity both at the genome architecture and at the core functional transmembrane domain levels (**Figures 6A,B**). Receptors for type II IFNs have been identified and genetically characterized in chicken (72, 73). It is interesting to observe that unlike IFN-γ receptor β-chain (IFNGR2), the IFN-γ receptor α-chain (IFNGR1) of chicken has a 110 amino acid domain of a fibronectin type III (59). The LPKS and YDKPH motifs in the intracellular domain, required for the interaction with Janus kinase 1 (JAK1) and signal transducer and activator of transcription 1 (STAT1), were found to be conserved between avian and mammalian IFNGR1 (59). From two studies conducted by the same group, it was found that chicken IFNGR1 was highly expressed in spleen, thymus, peripheral blood lymphocytes (PBLs), cecal tonsil lung, and liver, whereas chicken IFNGR2 was highly expressed in spleen, thymus, PBLs, cecal tonsil, and muscle (72, 73). Beside these

FIGURE 6 | Structural and amino acid sequence homologies between type II and type III interferons (IFNs) in different avian species. **(A)** Protein sequence alignment of avian type II IFN (IFN-γ). **(B)** Predicted structure of chicken IFN-γ. **(C)** Protein sequence alignment of avian type III IFN (IFN-λ). **(D)** Predicted structure of chicken IFN-λ. Sequence alignments show that type II and type III IFNs are significantly conserved among avian species and may indicate interspecies cross-reactivity. Previously identified or predicted signal peptides are underlined in both IFN-γ **(A)** and IFN-λ **(C)** sequence alignments (54). These structures were predicted using I-TASSER online tool and were annotated using MacPyMOL. Structurally, these IFNs are well aligned with that of human IFNs (data not shown).

fundamental investigations, our current understanding is limited to the nature and genetics of IFNGRs in avian species, which warrants extensive future research to underpin the mechanisms of the IFN-γ-induced antiviral states.

Chicken IFN-γ was first amplified from a cDNA expression library generated from a T cell line (CC8.1h) in 1995 (74). Chicken IFN-γ is encoded by a single gene located on the chromosome 1 and shares >30% amino acid homology with mammalian IFN-γ genes (74). Genetic and functional studies indicated its actions to be conserved as compared to mammalian IFN-γ-proteins (74). Unlike type I IFNs, IFN-γ is sensitive to low pH (2) and heat (65°C) (74). Several studies demonstrate that small interfering RNA mediated gene silencing of the IFN-γ to ascertain its antiviral effects (75, 76). Likewise, recent studies have clearly defined the antiviral role of IFN-γ and its adjuvant properties against viruses of diverse genetic nature including Newcastle disease virus (NDV), MDV, and influenza viruses (77–79). Similar to its mammalian counterparts, chicken IFN-γ also induces MHC class I and class II molecules and mediates the production of nitric oxide, which is an important inhibitory mechanism for viruses (80). These studies have collectively highlighted the potential and emerging roles of chicken IFN-γ in vaccine-conferred antiviral immunity.

After the initial identification of duck IFN-γ from a cDNA library generated from primary duck hepatocytes and demonstration that duck IFN-γ inhibits duck hepatitis B virus in a dose-dependent manner (81, 82), it has been found that duck IFN-γ shares both structural and functional identities with chicken IFN-γ (83). In contrast to chicken and duck IFN-γ, goose IFN-γ exerts only a weak antiviral state, which may indicate distinct biological activities between these two species (55). It is interesting to observe that the cross-species reactivity of type II IFN has been shown to be considerably higher compared to any other IFN types (48). For instance, recombinant pigeon and turkey IFN-γ was found to be functionally active in chicken cells (48, 49). In conclusion, despite of structural and functional similarities between type II IFNs in different avian species, drivers of differential antiviral activities and molecular mechanisms of diverse immunological responses induced by type II IFNs are yet to be determined in different avian species.

Avian Type III IFN

While at least four IFN-λ genes (IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4) were identified in humans (84), only one functionally conserved IFN-λ copy was identified in chicken (50, 51). Chicken IFN-λ shows high sequence identity with human IFN-λ3. The antiviral activities of type III IFNs are dependent on the heterodimeric IFN-λ receptor, which is composed of the IFN-λ-specific IL-28Rα (IFNLR1) chain and the IL-10Rβ (IFNLR2) chain in mammals. Expression of chicken IL-28Rα was also shown to be indispensable for the antiviral activity of chicken IFN-λ (52). Similar to mammals, expression of chicken IL-28Rα appeared to be highest on epithelial cells and in epithelium-rich organs (52). Moreover, avian type III IFN might be functionally conserved

compared to those of mammalian species, likely playing a predominant role in the antiviral defense of epithelial barriers (85). This view is further supported by antiviral activity of chicken IFN-λ against several respiratory pathogens, including NDV, IBV, and influenza viruses *in vivo*, *in ovo*, and in epithelial cells and in tissue culture systems (52). In contrast, chicken IFN-λ showed only low to moderate antiviral effects on non-epithelial cells, such as primary chicken embryo fibroblasts (CEFs), DF-1 chicken fibroblasts, or the chicken macrophage cell line HD11 (50, 52). This is in line with the assumption that the expression of chicken IL-28Rα is low or absent in most non-epithelial cell types (52).

In contrast to chicken IFN-γ, which induces high levels of nitric oxide in immune cells, IFN-λ as well as IFN-β induces significantly lower levels of nitric oxide in different non-epithelial cell types (50). Recently, it has been shown that chicken IFN-λ inhibits influenza virus replication in CEFs; however, it requires higher doses for achieving effective antiviral activities and to induce ISGs as compared to chicken IFN-γ and IFN-β (63).

In addition to chicken IFN-λ, Yao and colleagues have recently cloned IFN-λ from Pekin ducks and have found that duck IFN-λ is genetically and structurally highly conserved to other avian and mammalian IFN-λ genes (86). Recombinant duck IFN-λ was capable of inducing ISGs (2′-5′-OAS and Mx) in primary duck hepatocytes. Only very little information is available on IFN-λ homologs in other avian species (**Figures 6C,D**).

A CROSS TALK BETWEEN TYPE I, II, AND III IFNs

Following production, IFNs initiate the induction of ISGs by binding to their respective IFN receptors in autocrine and paracrine manners (1). Despite the fact that all types of IFNs play distinct and dedicated roles, a significant functional and regulatory overlap among all types of IFNs has been identified. Type I IFNs (IFN-α/β in the case of chickens) are produced from fibroblasts, whereas the antiviral actions of type III IFNs are mainly restricted to epithelial cells (52). These cell-specific roles are probably linked to the expression of cognate receptors in these organs for their importance in specific system.

It has been shown in mammals that type I and III IFNs initiate the same signaling pathway through phosphorylation of STAT1 and STAT2 heterodimers possibly by tyrosine kinase 2 (TYK2) and JAK1 kinases (1) (**Figure 1**). However, type II IFNs trigger ISGs' induction *via* the activation of STAT1 homodimers by JAK1 and JAK2 kinases (1). Several protein phosphatases and the suppressors of cytokine signaling (SOCS), such as SOCS1 and SOCS3, were found to be involved in negative regulation of STATs phosphorylation (87). Although there are discrete downstream JAK–STAT signaling pathways for different type of IFNs, it has been shown that antibody-based neutralization of type I IFNs, or their receptors, attenuate the type II IFN responses. This may be linked to possible common receptor components or to the priming effect of type I IFNs on the expression of common transcription factors (e.g., STAT1) (1), which could cross-link the signaling between the three types of IFNs. Most components of JAK–STAT signaling pathway have been identified in chickens (**Figure 1**) and ducks, indicating possible functional homologies between mammals and avians.

To initiate the transcriptional activation of ISGs and other cytokines, type I and III IFNs mediate the recruitment and phosphorylation of IRF9 and STAT1/STAT2 heterodimer, to constitute a functional ISGF3 (1, 88). Type II IFNs initiate the formation of a STAT1–STAT1 homodimer to assemble GAF, without the need of IRF9. Upon nuclear translocation, ISGF3 and GAF bind to IFN-stimulated response elements (ISREs) (88) or GAS element, respectively (1). These events consequently lead to the transcriptional activation of hundreds of ISGs (**Figure 1**). In mammals, IRF9 is required for ISRE promoter activation (1). However, as indicated before, this transcription factor has not yet been identified in chickens, raising the question of alternative mechanisms of types I and III IFN-mediated ISG induction.

Regardless of the nature of their induction, ISGs play fundamental roles in a wide range of cellular activities, including transcriptional and translational regulation of immune responses (89, 90). The collective actions of these ISGs counteract viral replication and provide an antagonistic environment to limit virus propagation and spread (detailed below).

AVIAN ANTIVIRAL EFFECTORS

Binding of type I, II, and III IFNs to their respective receptors leads to the initiation of signaling cascades that culminate in the induction of distinct set of >300 ISGs (at least in human, mouse, and rats) (1, 5). These ISGs create an antiviral state and safeguard the host with multilayered, often synergistic, and cumulative actions (91). ISGs act on several stages of the viral replication cycle, ranging from virus entry to virus release (91). Some of these ISGs are PRRs that potentiate virus detection and thus modulate IFN induction through an amplification loop resulting in enhanced IFN production and hence more efficient virus inhibition (1, 91). Some ISGs have direct antiviral roles by acting at the level of host protein translation, post-transcriptional, and post-translational modifications. Significant advancements have been made in screening and mapping the antiviral roles of many ISGs against a broad range of viral pathogens (5). However, similar investigations have just been started in avian species. High throughput host gene expression profiling strategies, such as next-generation sequencing and microarray transcriptome analysis, have provided a snapshot of the ISGs that might have essential roles against avian viruses (92, 93). While the majority of these identified ISGs are still uncharacterized, a comparative knowledge of chicken/avian ISGs with their mammalian counterparts indicates that some of these ISGs are genetically and functionally conserved and are likely crucial for the control of viral infections. Of the hundreds of ISGs identified in mammals, only few have been genetically and functionally characterized in chicken. These include IFN-inducible transmembrane protein (IFITM)3 (94), which can inhibit virus entry; Mx (95), which can block early stages of virus replication; viperin (40), which can inhibit virus release; ZAP (41), which can weaken viral mRNA translation; 2′-5′-oligoadenylate synthetases (2′-5′-OAS/RNaseL) (54), which can cleave viral RNA transcripts; and PKR (62), which

can sense TLR-mediated immune responses (**Figure 1**). To our knowledge, these are the only ISGs that have been, to date, functionally characterized in chickens. A brief description of individually known avian ISGs is provided below.

CCCH-Type Zinc Finger Antiviral Protein (ZC3HAV1)

The antiviral action of ZC3HAV1 (ZAP) in mammals is mediated by its specific binding to the ZAP-responsive element encoded within viral mRNA (96). This binding recruits the host cellular degradation machinery to disable the viral mRNA translation specifically without any damage to host mRNA (96). Recently, chicken ZAP has been genetically characterized, and it appeared that the antiviral role of ZAP is probably evolutionarily conserved among vertebrates (41). In contrast to the presence of a long and a short ZAP isoforms in mammals, only one isoform (tentatively suggested to be the long isoform) has been found in chickens (41). The shorter isoform in mammals has recently been recognized as a positive regulator of the RIG-I pathway (97). While it remains to be finally clarified that chickens lack a shorter ZAP isoform, it may have been coevolutionary lost along with the RIG-I ortholog in chicken (41). The chicken ZAP gene can be prominently induced by polyinosinic:polycytidylic acid (poly I:C, a synthetic dsRNA analog) and type I IFN treatment in avian cells, suggesting that ZAP is an ISG (41). Moreover, the potential relevance of chicken ZAP in viral pathobiology is likely due to its upregulation in influenza H5N1 and IBDV-infected chickens (41). However, future studies are required to investigate whether all avian species have this protein and whether its functions are similar to those of its mammalian counterparts.

IIFITM Members

Several members of the IFITM family including IFITM1, IFITM2, IFITM3, and IFITM5 have been identified in humans (34). They are differentially expressed upon stimulation by type I and type II IFNs, either in the majority of body tissues (IFITM1, IFITM2, and IFITM3) or exclusively in osteoblasts (IFITM5) (34). Recently, functions of these ISGs have been studied extensively against viruses of medical, zoonotic, and veterinary importance (34). IFITM proteins inhibit viral infection by blocking cytoplasmic entry (98). Mechanistically, IFITM proteins suppress viral membrane fusion due to reduced membrane fluidity and thus forming curvature in the outer leaflets of cell membranes (99); or by disturbing the intracellular cholesterol homeostasis by preventing association of vesicle-membrane-protein-associated protein A with oxysterol-binding protein (100). Recently, three chicken IFITM proteins (IFITM1, IFITM2, and IFITM3) have been genetically characterized, and IFITM2 and IFITM3 have been functionally characterized (94). Despite of low sequence homology, human and chicken IFITM2 and IFITM3 are functionally conversed and are potent inhibitors of influenza and lyssaviruses (94). However, it remains to be determined whether the antiviral mechanisms of chicken and mammalian IFITM members are similar. Recently, it has been demonstrated that the duck IFITM3 confers antiviral activities against influenza viruses and that this action is independent of the N-terminal region of IFITM3 (101). Interestingly, several structural divergences were observed in the duck IFITMs probably owing to host–viral coevolution. Different publically available databases clearly indicate the presence of IFITM member proteins in several other avian species with variable levels of sequence and possible functional similarities. This leaves an opportunity to identify and characterize these important effector proteins of the innate immune system and to map their functions in avians.

Myxovirus-Resistance Proteins

Myxovirus-resistance proteins are GTPases that are key antiviral effector proteins of the type I and type III IFN pathways. In mammals, two major forms of Mx protein exist, namely MxA- and MxB-like Mx proteins (102–105). Mammalian MxA-like proteins, such as human MxA or mouse Mx1, are known to be potent inhibitors of influenza and a broad range of other viruses (102–104, 106). In contrast, the human MxB has only recently been shown to inhibit retrovirus infections (107). To date, only one lineage of Mx genes is known in birds (108, 109). Avian Mx proteins appear to be structurally similar to its mammalian counterparts, containing a GTP-binding and a leucine zipper motif, but they possess a unique N-terminal part that lacks significant homology with mammalian Mx proteins (109–111). Chicken Mx is distributed mainly in the cytoplasm (110, 112), while duck Mx has been shown to be located in cytoplasm and nucleus (111). To date, the GTPase activity for chicken Mx has not been demonstrated (113), and conflicting results have been reported on the antiviral activity of avian Mx proteins. In its first description, chicken Mx was reported to lack antiviral functions against a broad range of RNA viruses including influenza A viruses, *Thogotovirus*, VSV, and Sendai virus (110). A subsequent study identified a high degree of genetic diversity in the chicken Mx gene (114). Functional assays demonstrated that chicken Mx alleles carrying an asparagine at amino acid position 631 (Mx-Asn631) possess antiviral activity against VSV and HPAIV H5N1 in transfected mouse cells, whereas alleles carrying a serine at this position (Mx-Ser631) lacked antiviral activity (114, 115). While some studies confirmed the antiviral effects of Mx-Asn631 against VSV and NDV in cell culture (116–118), others failed to demonstrate Mx-mediated resistance of both Mx variants against influenza, NDV, and *Thogotovirus* using comparable approaches (112, 113, 119, 120). Artificial translocation of chicken Mx to the nucleus did not enhance its antiviral activity (112). *In vivo* studies either did not demonstrate an effect of the polymorphism at position 631 on the clinical course of an experimental HPAIV H7N1 infection (121) or reported an association of Mx–Asn631 with slightly reduced mortality and morbidity following HPAIV H5N2 infections of chickens (120). Overexpression of duck Mx in murine cells did not result in enhanced antiviral activity against VSV and HPAIV H7N1 (111).

In summary, the functional characteristics of avian Mx proteins, their role in innate antiviral immunity, and the effect of genetic polymorphisms are still poorly understood and require further investigations. It is possible that, similar to human MxB, avian Mx proteins possess unequivocal antiviral activities against viruses substantially differing from the few RNA virus families, which have been tested so far.

Protein Kinase R

Protein kinase R is a serine/threonine protein kinase and consists of two domains that are functionally independent; the dsRNA-binding N-terminus and the catalytic C-terminus domains (122). PKR was first identified during investigations on the translation inhibition of viral and cellular mRNAs in vaccinia virus (VV)-infected mammalian cells (123). In an inactive form, PKR localizes in the nucleus and upon activation, mediated through viral dsRNA recognition, oxidative stress, growth factors, cytokines, and cellular proteins such as PKR-associated activator, or following the stimulation of TLRs, phosphorylates the eukaryotic initiation factor 2. This action impairs the guanine nucleotide exchange reaction and thus inhibits translation of mRNA in infected cells (124, 125). Although different viruses, including influenza virus, herpes simplex virus type I, and hepatitis C virus, encode for counteracting factors to inhibit PKR actions, this kinase can still surpass and can exert antiviral activities.

It has been demonstrated that chicken PKR carries all features characteristic for RNA-binding proteins and kinase families (126). Similar to the chicken Mx gene, chicken PKR is also polymorphic and confers antiviral effects against VSV (126). However, in an *in vivo* study, transcriptionally upregulated PKR failed to protect chickens from highly pathogenic H5N1 infection (127).

Similar to Mx and several cytokines, it is likely that specific SNPs may define the function of PKR in a specific and understudied avian population. Although PKR is one of the first identified PRRs, our understanding of its function is still incomplete even in mammals. In this regards, a novel role of PKR in specifically maintaining the integrity of newly synthesized IFN mRNAs has been recently described (128), further highlighting the need for future research (124).

2′-5′-Oligoadenylate Synthetase

In an attempt to understand the molecular mechanism of PKR-induced inhibition of protein synthesis during VV replication, another enzyme called 2′-5′-OAS was identified in mouse (129). Interestingly, 2′-5′-OAS mRNA has been detected in erythrocytes and immature red blood cells in several avian species (chicken, goose, and pigeon) (130, 131). The same group also identified the existence of two alleles of the 2′-5′-OAS gene in chickens (132). They found that 2′-5′-OAS A/B allele encodes for 58 and 54 kDa synthetases, whereas chickens carrying 2′-5′-OAS A/A alleles produce only a single 58 kDa protein (133). Expression of each of these two chicken 2′-5′-OAS alleles has been revealed to be age-dependent (133). The stability and persistence of 2′-5′-OAS are determined by the ubiquitin-like domain in the carboxyl-terminus of the 2′-5′-OAS (134). Interestingly, basal 2′-5′-OAS expression was systemically detected in chicken embryos independent of stimuli (130). However, a significant induction of 2′-5′-OAS was observed in IFN-treated chicken embryo cells (135).

More recently, the antiviral activity of chicken 2′-5′-OAS against West Nile virus was demonstrated in a replicon assay in mammalian cells (136). Notably, this assay provides the ability to investigate the effect of allele-specific antiviral actions of 2′-5′-OAS against avian viruses with diverse genetic backgrounds.

Viperin

Viperin is one of the most important IFN effectors in mammals and confers antiviral activity by inhibiting the trafficking of soluble viral proteins in the cytoplasmic compartments. Limited availability of the viral components may restrict viral spread (137, 138). Moreover, several studies have also found that mammalian viperin impairs virus replication and restricted viral budding (139). The recently characterized chicken viperin exhibits mammalian-like domains, including a variable N-terminal variable region spanning 77 amino acids, a central radical SAM domain, and a C-terminal conserved region (40). While chicken viperin was significantly induced by influenza viruses and IBDV as well as by different innate immune receptor ligands both *in vitro* and *in vivo* (40), its antiviral potential requires future investigations. Since chicken viperin carries leucine zipper and radical SAM motifs, which are known to be essential for viperin-induced antiviral activities in mammals, it is conceivable that chicken viperin has functional conservation with the mammalian counterpart.

CONCLUSION AND FUTURE OF AVIAN INNATE IMMUNITY RESEARCH

The currently available information on the immunogenetics of avian IFNs is a basis for future research aimed to understand the molecular mechanisms of IFN induction, associated factors, and to identify uncharacterized IFNs in different avian species, which differ significantly in their IFNs pathways and harbor viruses of both veterinary and medical importance. Because of existing functional and genetic differences, it might be needed to revise the nomenclature of avian IFNs to truly represent their origins and actions. Although IFNs were discovered by Isaacs and Lindenmann in chicken cells (140), knowledge on the dynamics and plasticity of chicken IFNs and their antiviral activities is markedly scarce compared to their mammalian counterparts. An important and evolutionary crucial area of research is to understand the potent innate immune responses in chicken in the apparent absence of essential components of IFN pathways, such as RIG-I, especially in chicken and turkey. Recent availability of genomics data on different avian species has significantly advanced comparative immunogenetics studies. However, extensive efforts are required to improve the current genome annotation of widely used poultry species (chicken, duck, and turkey) and to effectively characterize existing gaps in functionally important genomic loci. Investigations on functional implications of avian ISGs have been started; however, next-generation strategies would be required to map the antiviral or possible proviral roles of these IFN effectors. Most actions of ISGs have been studied using single isoforms of the ISGs exploiting either ectopic expression or silencing methods. Approaches such as CRISPR/Cas9 knockout/knockin will be required for future investigations on effective mapping avian ISG and their functions. One of the aspects that might require future efforts is to identify the overlapping antiviral roles of ISGs and the molecular combinatorial networking in these antiviral, or proviral, properties. Since silencing of individual ISGs leads to observable differences in virus pathobiology, these appear to be valuable targets for the development of potential therapeutics

for a broader range of viruses, and for vaccine production. In this regard, human IFNs have been successfully applied for the treatment of virus-induced human diseases; however, the clinical potential of chicken or other avian IFNs has not yet been exploited. These applications may hold options for future economical antiviral therapy not only in commercial poultry but also in companion birds.

AUTHOR CONTRIBUTIONS

Conception and writing of the manuscript: MM; restructuring and improvement of the contents: DR, LM-S, and DS; and designing the figures: MM and DS.

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Evolutionary Origin of the Interferon–Immune Metabolic Axis: The Sterol–Vitamin D Link

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In vertebrate animals, the sterol metabolic network is emerging as a central player in immunity and inflammation. Upon infection, flux in the network is acutely moderated by the interferon (IFN) response through direct molecular and bi-directional communications. How sterol metabolism became linked to IFN control and for what purpose is not obvious. Here, we deliberate on the origins of these connections based on a systematic review of the literature. A narrative synthesis of publications that met eligibility criteria allowed us to trace an evolutionary path and functional connections between cholesterol metabolism and immunity. The synthesis supports an ancestral link between toxic levels of cholesterol-like products and the vitamin D receptor (VDR). VDR is an ancient nuclear hormone receptor that was originally involved in the recognition and detoxification of xenobiotic marine biotoxins exhibiting planar sterol ring scaffolds present in aquatic environments. Coadaptation of this receptor with the acquisition of sterol biosynthesis and IFNs in vertebrate animals set a stage for repurposing and linking a preexisting host-protection mechanism of harmful xenobiotics to become an important regulator in three key interlinked biological processes: bone development, immunity, and calcium homeostasis. We put forward the hypothesis that sterol metabolites, especially oxysterols, have acted as evolutionary drivers in immunity and may represent the first example of small-molecule metabolites linked to the adaptive coevolution and diversification of host metabolic and immune regulatory pathways.

Keywords: sterol, metabolism, vitamin D receptor, cholesterol, xenobiotics, immunity

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Abbreviations: AluSx, *Alu* Sx subfamily; AHR, aryl hydrocarbon receptor; AMP, antimicrobial peptide; C_{24}, 24-carbon; CAMP, cathelicidin antimicrobial peptide; CAR, constitutive androstane receptor; CBT, cytochrome *b* termination protein; CH25H, cholesterol 25-hydroxylase; CNS, central nervous system; CYP, cytochrome P450; DBD, DNA binding domain; DHR96, *Drosophila* hormone receptor-like in 96; EAE, experimental autoimmune encephalitis; EcR, ecdysone receptor; FXR, farnesoid X receptor; GKO, interferon-gamma knock out; GR, glucocorticoid receptor; HK, hexokinase; JAK/STAT, janus kinase/signal transducer and activator of transcription; IIS, insulin/insulin-like growth factor signaling; IL, interleukin; IFN, interferon; IRF, interferon response factor; ISG, interferon-stimulated gene; ISGF, interferon-stimulated gene factor; LBD, ligand-binding domain; LCA, lithocholic acid; LPS, lipopolysaccharide; LXR, liver X receptor; MAPK, mitogen-activated protein kinase; miR342-5p, microRNA 342-5p; MS, multiple sclerosis; NF-κB, nuclear factor-kappa B; NHR-8, nuclear hormone receptor family member nhr-8; NFAT, nuclear factor of activated T-cells; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RAR, retinoic-acid receptor; ROR, RAR-related orphan receptor; RXR, retinoid X receptor; RSMAD, receptor-regulated SMAD; SMAD, mothers against decapentaplegic homolog; STING, stimulator of interferon genes; TGF-β, transforming growth factor-beta; Th2, type 2 T helper cell; TLR, toll-like receptor; TLR2/1L, toll-like receptor 2/1 ligand; USP, ultraspiracle; UVB, ultraviolet B; VDR, vitamin D receptor; VDRE, vitamin D response element; WGD, whole genome duplication.

INTRODUCTION

Host-protection pathways against foreign harmful exogenous agents, inclusive of biotoxins and pathogens, exist in all branches of life. Pathways that allow the removal of biotoxins and metabolic by-products are considered to be distinct from those that neutralize and eliminate pathogens. For instance, it is understood that the P450 enzymes, which represent an ancient detoxification system, and interferon (IFN) pathways, that are central for immunity against infection in animals, are biologically unrelated. *However, could specific metabolic pathways and metabolites provide an interconnection?*

The substrates for P450 enzymes, while highly diverse, are lipophilic molecules often containing multiple planar ring structures. Notably, the most highly related P450s across the different kingdoms are involved in the metabolism of sterols (constituting multiple planar ring lipophilic molecules) and which further contribute an essential enzymatic role in the production of endogenous lipid metabolites, in particular as part of the sterol biosynthesis pathway (1–3). It has been debated whether the adaptation of P450 enzymes to the biosynthesis of sterols became firmly established in early eukaryotic (or late-stage prokaryotic) evolution with the arrival of atmospheric oxygen leading to the production of cholesterol in animals, ergo-sterol in fungi, and phyto-sterols in plants (4–7). The primary driver for sterol biosynthesis evolution was likely the selective advantage imparted by cholesterol toward modulation of membrane properties. However, too much cholesterol in membranes of cells, especially in the endoplasmic reticulum, the site of biosynthesis, can be highly toxic and accordingly sterol production, storage, and elimination is under stringent homeostatic regulation.

Sterols are not only required for membranes but also for the synthesis of steroid hormones, which regulate diverse physiological functions ranging from reproduction to stress and immunity. Outside the well-known functions of steroids, sterols, and in particular oxidized cholesterol and sterol metabolites, oxysterols, have been more recently found to have key roles in immunity (8, 9). Most importantly, the regulation of metabolic flux in cholesterol biosynthesis is directly linked to immune control through coupling to IFN signaling (10–13). Also see Robertson and Ghazal (14) for a review of our most current understanding of how IFN regulation is molecularly wired to sterol biosynthesis. We, therefore, posit that natural selection may have coadapted sterol metabolism and secondary metabolites as a link between functionally unrelated host-protection pathways in countering harmful chemical and biological agents. *This proposition evokes the question of whether there is evidence for an ancestral gene that supports a link between these distinct host-protection pathways?*

To address this question we sought to systematically review and provide a narrative synthesis of the literature based on investigating the ancestral connections between sterol metabolites, immunity, and xenobiotics. We find evidence supporting an evolutionary course for co-opting the ancestral, xenobiotic binding, vitamin D receptor (VDR) to adaptively recognize a specific non-typical oxysterol molecule, 1,25-dihydroxyvitamin D_3, that in present day mammals governs prominent functions in calcium homeostasis, and immunity. It is important to clarify that vitamin D_3 is a ring-opened version of 7-dehydrocholesterol and hence of the general class of sterols and steroids. For this reason and although vitamin D_3 metabolites are not derived from cholesterol, we consider 25-hydroxyvitamin D_3 (the inactive form found in serum) and 1,25-dihydroxyvitamin D_3 (the active ligand to VDR) as non-typical oxysterols; as they are oxidized forms of the ring-opened cholesterol precursor 7-dehydrocholesterol.

On the basis of evidence presented, we further hypothesize that sterols and their oxidized metabolites have contributed as key evolutionary drivers for repurposing ancestral nuclear hormone receptors, in particular VDR, from protecting against harmful lipids to become important regulators of immunity.

THE NUCLEAR HORMONE RECEPTOR FAMILY CONNECTION

Central to the recognition of sterol-like molecules and activation of detoxification systems and immunity are the nuclear hormone receptors (15, 16). In particular, the subfamily known as NR1I that includes the pregnane X receptor (PXR), constitutive androstane receptor (CAR), and the VDR (15, 17). Although each have important individual functions in humans, these receptors act as important regulators of P450 enzymes and have strong genetic evidence suggesting they originated from a single ancestral nuclear receptor (18).

Notably, VDR that is activated by a specific ligand, 1,25-dihydroxyvitamin D_3, generated from vitamin D that is derived from a precursor of cholesterol, 7-dehydrocholesterol from the sterol biosynthesis pathway and synthesis in humans begins in the skin upon exposure to ultraviolet B (UVB) light emitted from the sun. The vitamin D synthesis pathway is summarized in **Figure 1** (for notation see **Table 1**), and involves the skin, liver, and kidneys (19). Interestingly, animals with fur and feathers are still able to synthesize vitamin D from sunlight despite UVB not reaching the skin (20). Here, vitamin D synthesis occurs through the sebaceous glands producing oily secretions (containing 7-dehydrocholesterol) that cover fur or feathers and ingested after grooming (21–23).

Vitamin D deficiency can result in clinical disorders, the most notable being the characteristic bow-legged musculoskeletal manifestation known as rickets. Additional studies have also linked deficiency to cardiovascular disease, cancer, autoimmune conditions, and decreased antimicrobial protection (26–28). The VDR is known to heterodimerize with retinoid X receptor (RXR) and exerts biological effects as a ligand activated transcription factor by binding to specific vitamin D response element (VDRE) in gene promoters of over 200 genes (29). The active ligand of VDR is 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), also known as calcitriol, although certain bile acids are also capable of inducing transactivation to a lesser degree (**Figure 1**). $1,25(OH)_2D_3$ is the active form of vitamin D, produced by enzymatic hydroxylation of the circulating 25-hydroxyvitamin D_3 by cytochrome P450 (CYP) enzyme 27B1 (see **Figure 1**).

While vitamin D and its receptor have been long regarded as mediators of calcium and phosphate homeostasis, VDR has

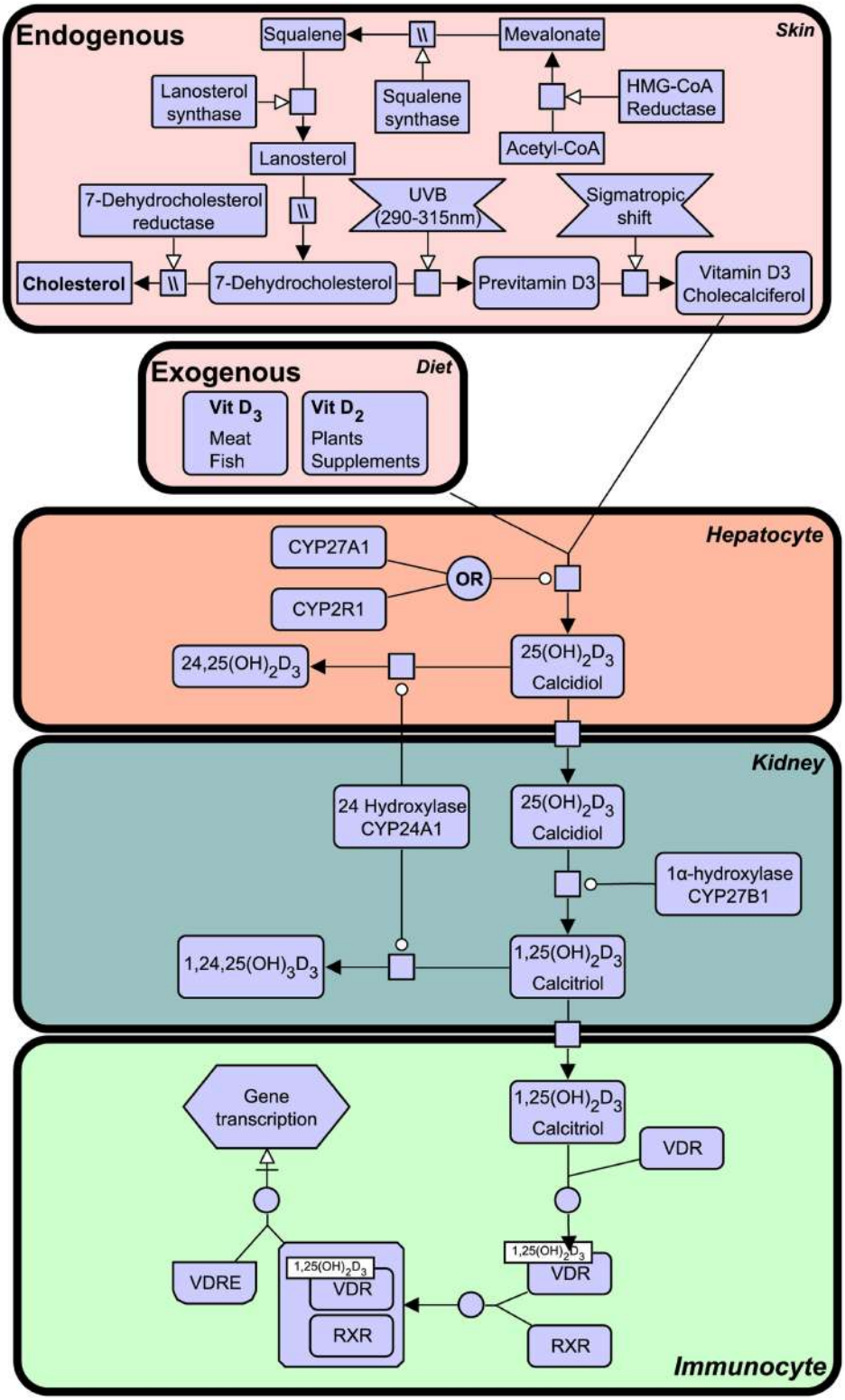
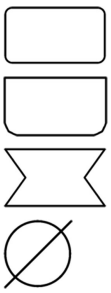
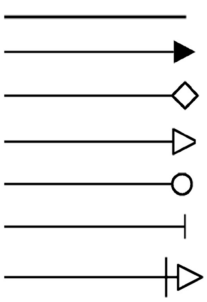


FIGURE 1 | **The vitamin D synthesis pathway**. Vitamin D_3 can be acquired both endogenously from cholesterol in the skin and exogenously through diet (vitamin D_2 and vitamin D_3). In the skin, 7-dehydrocholesterol, a cholesterol precursor, is converted to previtamin D_3 upon ultraviolet B (UVB) exposure. Through a series of cytochrome P450 enzyme-mediated reactions, previtamin D_3 is converted first into $25(OH)_2D_3$ in liver hepatocytes and then activated in the kidney by 1α-hydroxylation, to form $1,25(OH)_2D_3$. The degradation of $1,25(OH)_2D_3$ and intermediate metabolites is mediated by negative feedback mechanisms (24). Figure created using the SBGN format on VANTED (25). See **Table 1** for notation.

TABLE 1 | Systems biology graphical notation legend.

Type	Symbol	Description
Entity pool nodes		Macromolecule Nucleic acid feature Perturbing agent Source and sink
Container nodes		Compartment
Process nodes		Process Omitted process Association Phenotype
Connecting arcs		Consumption Production Modulation Stimulation Catalysis Inhibition Necessary stimulation
Logical operators		AND operator OR operator

additional roles in innate and acquired immunity and xenobiotics (30). Vitamin D-mediated calcium homeostasis has been around since the first terrestrial vertebrates, including amphibians, which have also been observed to suffer from calcium deficient ailments such as rickets (31). Before the calcium endocrine system, ancient VDR functioned as a xenobiotic receptor, mediating the degradation of marine biotoxins (32). It still retains this ability, and in humans VDR is important in detoxifying the toxic secondary bile acid lithocholic acid (LCA) in the colon by activating the CYP3A4 P450 enzyme (30).

Vitamin D is itself an ancient sterol–steroid, present in phytoplankton and zooplankton (33). VDR orthologs have likewise been observed in ancient vertebrates (34) and invertebrates (35). Accordingly, we next examine the origin and early evolutionary progression of VDR and its role in detoxification.

PRIMORDIAL NUCLEAR HORMONE RECEPTOR FAMILY OF THE VDR AND BIOLOGICAL ROLES IN DETOXIFICATION

The VDR is descended from a group of xenobiotic nuclear hormone receptors known as NR1I, as shown in **Figure 2**. The NR1J nuclear receptor subfamily has important roles for xenobiotic detoxification in arthropods and nematodes (15, 16, 36). For instance, the related DHR96 receptor in *Drosophila melanogaster* can bind and detoxify a phenobarbital insult through a CYP transcriptional response (37), and NHR-8, in the *Caenorhabditis elegans* gut, senses colchicine and targets the activation of its cognate detoxification pathway (38). Many of these related nuclear receptors have also shown the ability to bind small lipophilic molecules such as cholesterol or steroid hormones (15, 16, 36). In this way, conservation of function can be seen throughout the evolution of these nuclear receptors. We will first discuss the ancestral xenobiotic role of VDR before considering how evolutionary pressures may have promoted the functional repurposing of this receptor with the acquisition of new roles including detoxification of endogenous compounds (e.g., vitamin D metabolites and bile acids), lipid regulation, and immunity.

The tunicate, *Ciona intestinalis*, represents the closest extant invertebrate relative of vertebrates possessing an ancestral VDR gene (39). Fidler et al. (35) investigated the potential ligand-binding properties of this receptor, named as CiVDR/PXRα for its homology with both the VDR and PXR. Interestingly, vitamin D, or indeed any bile salts, were unable to produce any transactivation potential (40, 41). Despite speculation that the CiVDR/PXRα ortholog may be used for calcium homeostasis, there is evidence for closer functional similarity to PXRs current role in xenobiotics. This possibility is supported by the argument that the ocean is a plentiful source of calcium, making any need for homeostasis redundant. Present day PXR function in humans is to detoxify foreign toxic compounds by sensing and then activating the enzyme CYP3A4. There is good experimental evidence to support a functional role for the *C. intestinalis* CiVDR/PXRα ortholog to be ligand activated by microalgal biotoxins, including okadaic acid and pectenotoxin-2 (35) (**Figure 3**). Filter feeding tunicates like *C. intestinalis* accumulate these biotoxins through the large quantity of microalgae in their diet. As high concentrations of these chemicals are able to kill cells, CiVDR/PXRα's ability to bind and detoxify them would be appropriate and consistent with PXRs current role in humans. Indeed, it has been shown that orthologous NR1Jβ receptors in mollusks likewise respond to xenobiotic insult from okadaic acid by activating detoxification pathways (42).

Similar to CiVDR/PXRα in *C. intestinalis*, NHR-8 and DHR96, members of the NR1J subfamily, in *C. elegans* and *D. melanogaster*, respectively, have also been shown to be essential for mediating xenobiotic resistance by promoting the expression of genes involved in metabolism of endo- and xenobiotics (37, 43, 44). Increased expression of genes involved in xenobiotic metabolism, together with resistance to xenobiotics, are frequently correlated with lifespan extension in *C. elegans*, *D. melanogaster*, and mice, suggesting detoxification of diet-acquired toxins is a host-protection mechanism against accumulation of

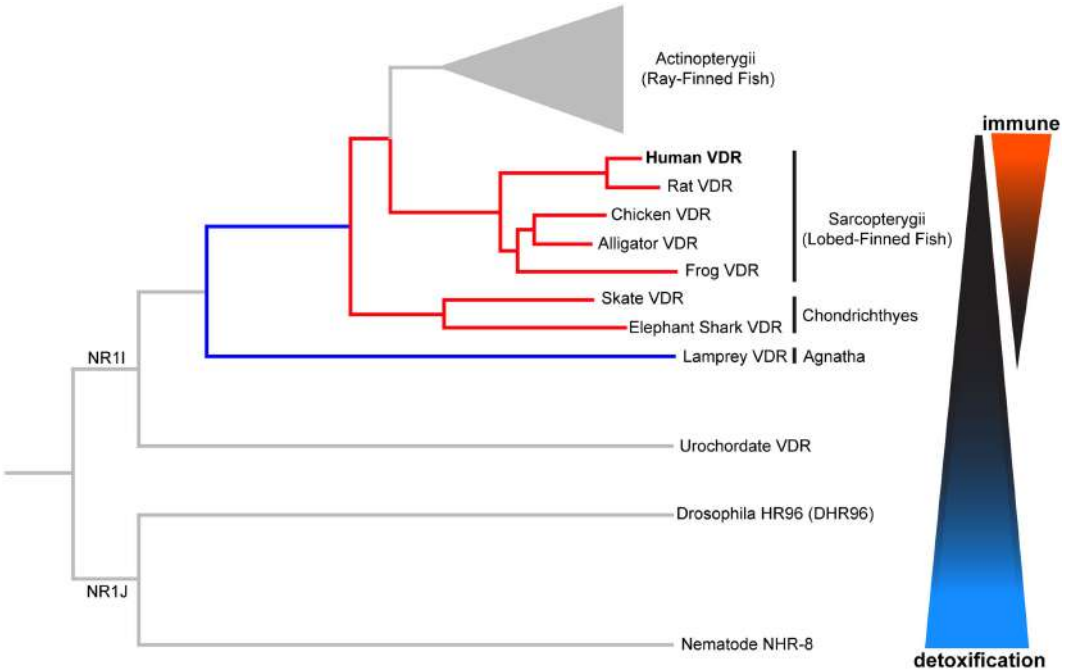


FIGURE 2 | Phylogenetic relationship of vertebrate vitamin D receptor (VDR) with invertebrate orthologs. Both vertebrate and invertebrate VDR, belonging to the closely related NR1I and NR1J subgroups, respectively, regulate the expression of genes involved in xenobiotic metabolism (detoxification). Evolutionary change in the vertebrate VDR has resulted in the acquisition of new immunological functions. Distances between nodes are not to scale.

specifically lipophilic toxins that negatively impact health during aging (The Green Theory of Aging) (16, 45). However, a recent study by Afschar et al. showed that DHR96 is indeed essential for mediating resistance to xenobiotics but not for increasing lifespan of insulin-mutant flies (44), indicating that xenobiotic resistance and longevity may not be causally connected. It has been suggested that the co-occurrence of xenobiotic resistance and lifespan extension may have co-evolved because lowered insulin/insulin-like growth factor signaling (IIS) can also signal the presence of pathogens (44). In line with this concept, in *C. elegans* and *D. melanogaster*, genes involved in xenobiotic metabolism have also been shown to be indirectly activated by toxic microbial by-products that directly cause dysfunction in cellular processes such as an altered metabolism, and decreased host translation and IIS (43, 46, 47).

EVOLUTIONARY REPURPOSING OF PRIMORDIAL VDR FROM EXOGENOUS TO ENDOGENOUS DETOXIFICATION PATHWAYS

Pharmacophore modeling of CiVDR/PXRα ligands revealed specific chemical scaffolds were required for receptor binding, comprising two hydrophobic features (in particular aromatic rings) and one hydrogen bond acceptor in a planar arrangement (35). The structure of activated vitamin D exhibits resemblance to this scaffold presenting a planar conformation with aromatic rings and hydrophobic features (48).

Further sequence identification by Ekins et al. (41) demonstrated a 67.6 and 17.1% similarity between the DNA binding domain (DBD) and the ligand-binding domain (LBD), respectively, between CiVDR/PXR and hVDR. Lower conservation of the LBD suggests evolutionary adaptive changes in ligand affinity. It is perhaps this promiscuous ligand-binding quality that allowed the ancestral VDR to function as a xenobiotic receptor, by binding and detoxifying new toxic chemicals on exposure. The similarities in structure between $1,25(OH)_2D_3$ and exogenous marine biotoxins, alongside the genetic variability of VDRs' LBD, allowed for the eventual binding and regulation of $1,25(OH)_2D_3$ levels, a sterol-derived metabolite. As described below, observations in basal vertebrates provide insight toward understanding how this evolutionary pressure may have been applied.

The lamprey (*Petromyzon marinus*) is the most basal extant vertebrate, and therefore, provides valuable information regarding VDR functional evolution. The lamprey (lampVDR) has an 87 and 60% homology with hVDR DBD and LBD, respectively (18, 34). This differential increase in homology of the LBD in comparison with the tunicate CiVDR/PXR ancestral gene suggests VDRs' ligand activated role may have changed. There have been three rounds of whole genome duplications (WGDs), represented as 1R, 2R, and 3R, since *C. intestinalis*. Humans diverged after the second round while Teleost fish were subjected to a third round and therefore, have an extra copy of the VDR gene (18). It is thought that after 1R, the combined VDR/PXR gene split giving rise to separate VDR and PXR genes with different but also overlapping functions. The lamprey was first to diverge after the 1R WGD event, as shown in **Figure 2**. While, a PXR homolog

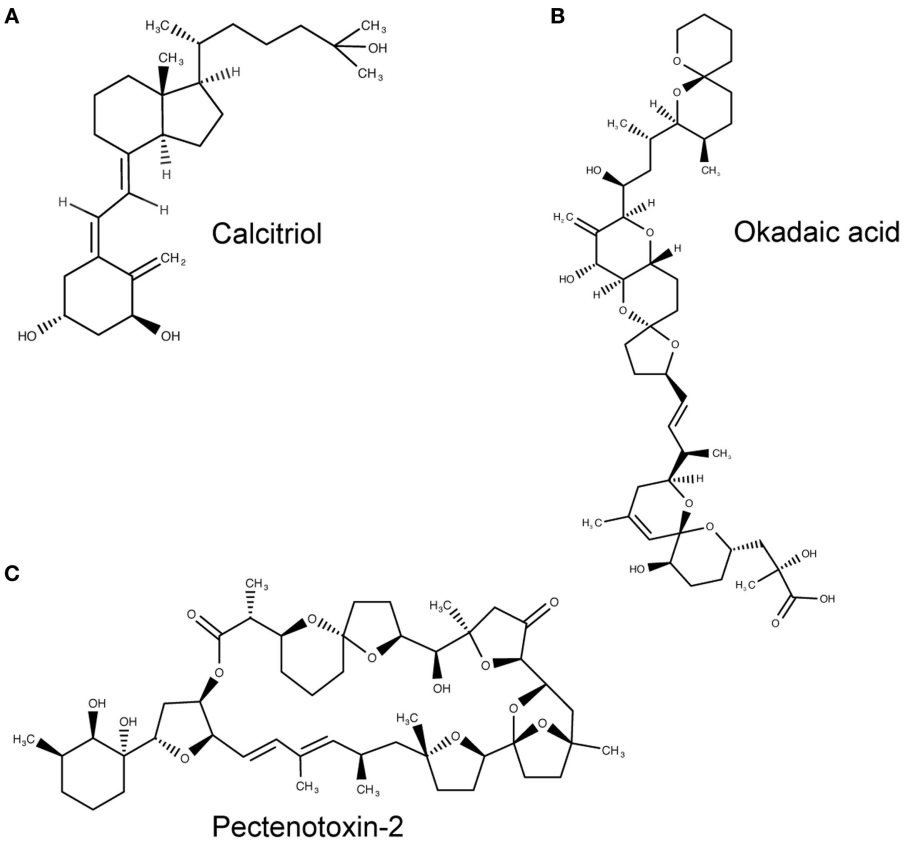


FIGURE 3 | **Molecular structures of calcitriol, okadaic acid, and pectenotoxin-2**. **(A)** Molecular structure of $1,25(OH)_2D_3$, otherwise known as calcitriol (PubChem CID = 5280453), a vitamin D receptor (VDR) agonist. **(B,C)** Atomic structure of two natural *Ciona intestinalis* CiVDR/PXRα analogs, okadaic acid (446512) **(B)** and pectenotoxin-2 (6437385) **(C)**, from left to right. Figures produced using MarvinSketch (http://www.chemaxon.com).

has yet to be identified in the lamprey, lampVDR has been shown to have high affinity binding and transactivation by $1,25(OH)_2D_3$, which functionally activates CYP3A4, and possibly CYP24A1, in order to detoxify high levels of this sterol (34) and providing an opportunity for an extended regulatory role for the VDR in lipid metabolism. Although the lamprey lacks a calcium endocrine system, lampVDR may contribute to the regulation of other processes such as skin differentiation, as an observed increase in VDR presence in mucous glands and keratinized teeth has been reported (34, 49).

Thus, in addition to the metabolism of exogenous xenobiotic compounds, VDR further acquired an ability to detoxify certain lipophilic endogenous molecules such as bile acids. Bile salt pathways are important vertebrate mechanisms by which cholesterol can be removed from the body. There are at least three evolutionary classified bile salt pathways, referred to as early, later, and recent pathways (41) (**Figure 4**). The lamprey uses the early fish pathway, while chondrichthyes, such as the Little skate, use the later pathway. In mammals, the "recent" pathway converts cholesterol to 24-carbon (C_{24}) bile acids, which can subsequently be converted to toxic secondary bile acids in the intestine by resident microorganisms (50). Indeed, one of the major roles of the hVDR is its ability to detoxify the secondary bile acid LCA by transactivation of CYP3A4 (30). As LCA is a product of the most recent C_{24} bile acid pathway, basal vertebrates such as the lamprey, which employ the "early" or "later" fish pathways, are unable to bind this molecule with their VDR (51). In mammals, LCA affinity is almost certainly a more recent evolutionary adaptation to changes in the gut microbiome resulting in the production of toxic secondary bile acids. This ability of VDR to subsequently transactivate the CYP3A4 gene in response to the binding of a toxic chemical traces an evolutionary path from VDRs' ancestral xenobiotic function. In this regard, it is noteworthy that the NHR-8 and DAF-12 nuclear receptors in *C. elegans*, homologs of VDR and part of the NR1J subgroup, have convergently evolved to control and bind dafachronic acids, a bile acid look-alike, important in the life-cycle of this species (52, 53).

FUNCTIONAL DIVERSIFICATION OF VDR IN LIPID METABOLISM AND IMMUNITY

Vitamin D receptor's ancestral roots clearly stem from its ability to recognize and regulate detoxification pathways for environmental toxic chemicals, as observed in both NR1I and NR1J subgroups. From this stemmed, its ability to bind and detoxify endogenous

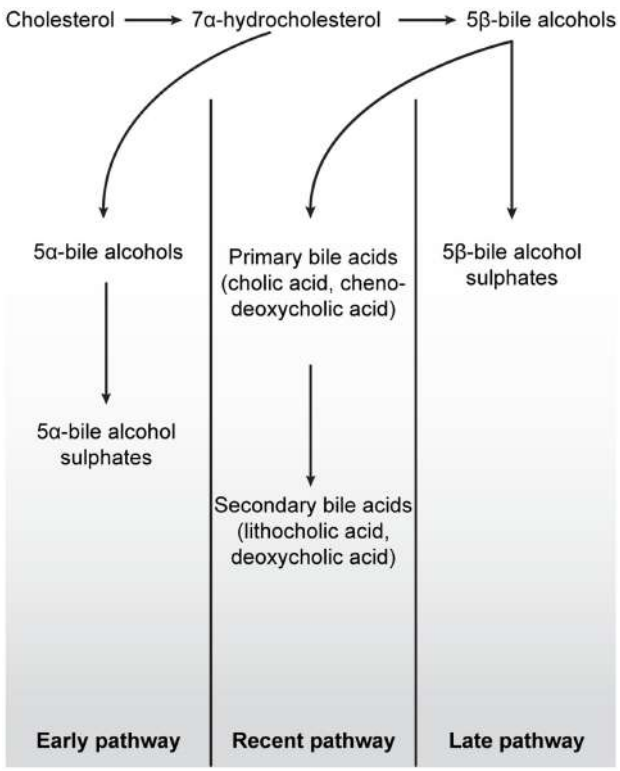


FIGURE 4 | Schematic overview of the three major bile salt pathways throughout vertebrate evolution. All bile acids are derived from cholesterol, a 27-carbon molecule. In early fish, e.g., hagfish and sea lamprey, a 7α-hydrocholesterol is converted to 5α-bile alcohols, followed by conversion to 5α-bile alcohol sulfate. The production of bile salts in mammals, birds, cartilaginous fish, and in some teleost and amphibians is dependent on the conversion of 7α-hydrocholesterol into 5β-bile alcohol. In cartilaginous fish, mainly, but also in some teleost and amphibians, 5β-bile alcohol is further converted to 5β-bile alcohol sulfates ("Later pathway"). In mammals and birds, as depicted in the "Recent pathway," bile salt production involves the conversion of 5β-bile alcohol to 24-carbon bile acids in the liver (cholic acid and chenodeoxycholic acid) by cytochrome P450-mediated oxidation. When these bile acids are secreted into the lumen of the intestine, cholic acid and chenodeoxycholic acid are converted, by colonic bacteria, to the secondary bile acids deoxycholic acid and lithocholic acid, respectively. While secondary bile acids in higher concentrations are potentially toxic to cells, they can, together with primary bile acids, be taken up into the blood stream and liver for re-secretion. In all pathways, the production of 7α-hydroxylation is the rate-limiting step in these reactions. Figure based on Ekins et al. (41).

lipophilic molecules such as $1,25(OH)_2D_3$ and bile acids using P450 enzymes. Sterol metabolites are important biological molecules requiring careful regulation for incorporation into cell membranes and steroid hormones. Accordingly, this opens a new opportunity for VDR to adopt a regulatory role through negative feedback mechanisms conferred by its ligand binding. The next section will discuss how sterol metabolites may have further coadapted VDR for driving the diversification of VDR functionality into lipid regulation, immunity, and bone prior to a role for VDR in calcium homeostasis (34).

As described above, VDR origin is based on recognizing and regulating the levels of lipophilic exogenous and endogenous molecules through the transcriptional regulation of P450 enzymes. To more fully understand the functional relationship by which VDR interacts with various lipid metabolism and immune signaling pathways, we constructed a pathway biology diagram (**Figure 5**, for notation see **Table 1**) from the research synthesis of literature mapping all known interactions. **Figure 5** shows that VDR is deeply embedded in a network of signaling pathways including PPAR-α and PPAR-γ, nuclear factor-kappa B (NF-κB), p38 mitogen-activated protein kinase (MAPK), transforming growth factor-beta (TGF-β), and eicosanoid synthesis. Of note, all of these pathways have instrumental roles in immunity, bone regulation, cell proliferation, and lipid metabolism.

A range of biological functions associated with VDR and targeted signaling pathways are shown in **Table 2**. Notably, most of these pathways not only regulate lipid metabolism, but have direct roles in immunity and bone homeostasis too. All these processes are interconnected. Lipids have a fundamental role in the immune system and directly affect immune cell function. They alter membrane fluidity, lipid peroxidation, gene expression, and eicosanoid production (54). Bone and immunity have a very close relationship, with bone marrow being a "high fat" primary hematopoietic tissue, controlling the production of B cells and other important innate and adaptive immune responses (55). Sterol biosynthesis is at the heart of this control network, using sterol-based interactions with the VDR to effectively regulate lipid metabolism and its associated features within bone and immunity. These pathways provide a link between the seemingly unconnected and multiple divergences of the VDR before additional more specific gene targets became evolutionary fixed and conserved, such as calcium homeostasis. In addition, it was also found that VDR has the ability to directly regulate fatty acid beta-oxidation by interacting with the enzymes hexokinase, CBT1, and CBT2, possibly leading to a role for energy metabolism in adipose tissue (56, 57).

It is worth noting that all these interactions are compartmentalized in terms of tissue specific, time dependent, and multifactorial control levels. This view is consistent with the possibility that cholesterol, as a toxic molecule and precursor sterols to vitamin D, repurposed VDRs' ancestral function to provide a wider regulation over lipid metabolism and immune pathways.

Vitamin D receptor has long been known to promote immune tolerance in the acquired immune system while providing protective innate mechanisms against pathogen infection. The acquired immune functions of the VDR are complicated, involving the regulation of multifaceted signaling pathways such as PPAR-γ and NF-κB. The net outcome of these cross-regulatory responses results in attenuation of the immune adaptive response, involving stimulation of interleukin (IL)-10 and downregulation of IL-12 (75). The most notable case for innate immunity is the VDR function to induce expression of the cathelicidin antimicrobial peptide (CAMP) gene, an important host defense protein. Gombart et al. (76) demonstrated that exaptation of an AluSx short interspersed element provided a perfect VDRE in the *Camp* promoter. Moreover, VDR is upregulated during infection in a toll-like receptor 2/1 (TLR2/1)-dependent manner (77–79). This shows a direct connection to innate immunity and is where the historic

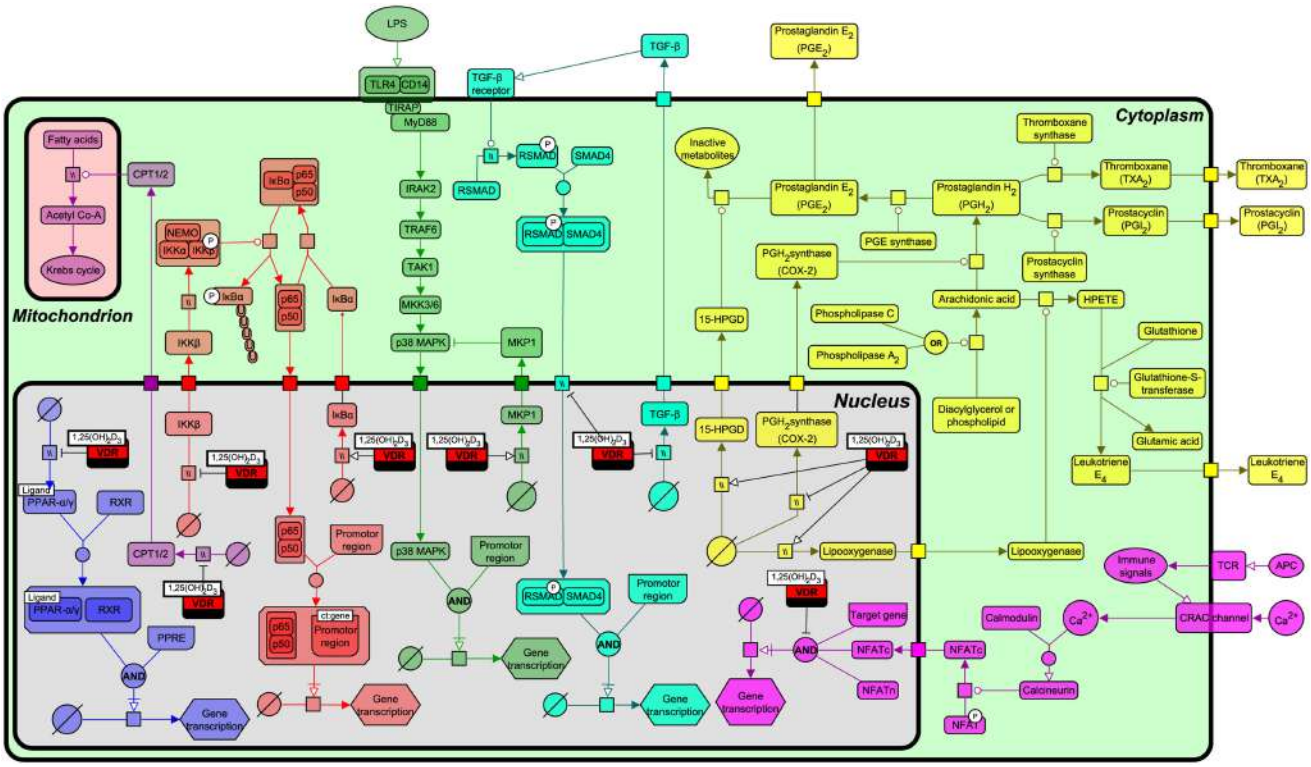


FIGURE 5 | Interactions of the vitamin D receptor (VDR) with PPAR-α, nuclear factor-kappa B (NF-κB), p38 mitogen-activated protein kinase (MAPK), transforming growth factor-beta (TGF)-β, β-oxidation, eicosanoid production, and nuclear factor of activated T-cells (NFAT) pathways. From left to right, the pathways represented are PPAR-α/γ (blue), β-oxidation (light purple), NF-κB (red), p38 MAPK (green), TGF-β (turquoise), NFAT (dark purple), and eicosanoid synthesis (yellow). Figure created using the SBGN format on VANTED with edges representing high and low confidence interactions (25). See **Table 1** for notation.

TABLE 2 | The function of various signaling pathways and their corresponding interaction with the vitamin D receptor (VDR).

Pathway	Functions	Possible VDR interactions	Reference
PPAR-α PPAR-γ	Fatty acid metabolism Energy homeostasis Immune function Bone regulation	Protein–protein interactions between the ligand-binding domains of VDR and PPAR-α	(58) (59) (60) (61)
Nuclear factor-kappa B	Immune response Inflammation Cell cycle Bone regulation	VDR sequesters IKKβ, preventing NF-κB activation VDR modulates IκBα function, thereby controlling the translocation of NF-κB proteins	(62) (63) (64) (65)
P38 mitogen-activated protein kinase (MAPK)	Inflammation Skeletal muscle differentiation	VDR upregulates MAPK phosphatase-1 causing inactivation of p38 MAPK	(66) (67)
Transforming growth factor-beta	Cell growth and differentiation Inflammatory and immunological processes	Reduces TGF-β expression Inhibition of phosphorylated receptor-regulated SMAD Upregulation of SMAD6 Inhibition of SMAD2/3 nuclear translocation	(68) (69) (70) (71)
Eicosanoids	Inflammation Immune function Tissue growth Blood pressure	Inhibits cyclooxygenase-2 Stimulates 15-hydroxy-prostaglandin dehydrogenase Regulates expression of 5-lipoxygenase	(72) (73)
Nuclear factor of activated T-cells	Immune function Cell cycle Cytokine signaling	VDR/retinoid X receptor complex interacts with target gene and prevents NFAT binding.	(74)

treatment of tuberculosis with cod liver oil (high in vitamin D) may have a possible molecular explanation (80). Unsurprisingly, VDR-mediated innate immune responses have become targets of pathogen evasion techniques (81).

THE CONNECTION WITH IFN SIGNALING

Interferons are a group of signaling proteins required for antiviral defense. Released by virally infected cells or leukocytes, they mediate a variety of innate and adaptive immunological responses by upregulating over 300 interferon-stimulated genes (ISGs) (82). IFNs can be classified into three types depending upon the receptor to which they bind and the signal transduction pathways they activate (83). Type I IFNs are split into multiple subtypes including -α, -β, -ω, -ε, -τ, -δ, and -κ, each with independent and redundant functions. Type II IFNs are conserved to just higher mammals and have only one member, IFNγ. Lastly, type III, containing IFN-λ genes (IL-29, IL-28A, and IL-28B), have similar biological properties as type I, but their genetic sequence contains non-coding intron sequences (84–86). The IFN system has displayed remarkable conservation throughout vertebrate evolution, demonstrating its importance for immunological defense (87). It is also inherently linked to cholesterol metabolism, as described elsewhere (8–10, 14).

Interferons appeared to have originated soon after the evolution of vertebrates as IFN homologous genes and their transcription factors have not yet been observed in primitive chordates such as the tunicate and sea urchin (87) or even closely related basal vertebrates such as the jawless fish, lamprey (88). We briefly discuss the evolution of type I and III IFN genes, as their ancestral homologs have coexisted with the evolving metabolic interactions between VDR, cholesterol, and immunity.

Interferon genes are present in many different varieties of fish, including the teleost clade of ray-finned fish that diverged from our evolutionary line 450 million years ago (87). Fish can possess singular or multiple IFN genes depending on the species and it is likely that vertebrate groups have independently evolved a vast array of structurally similar IFN molecules that perform different host protective functions (83, 89). Thus, the expanding role of IFNs coincided with the evolutionary changing roles of sterols and VDR.

Interferons act through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction pathways inducing and suppressing hundreds of genes. In mammals, IFNs are activated through a variety of pathogen pattern recognition systems, notably downstream of toll-like receptor (TLR) activation and by stimulator of interferon genes (STING)-activation that are now known to also target the sterol biosynthesis pathway. IFN signaling *via* STAT1 and IRF1 induces a cholesterol hydroxylase gene, CH25H and its cognate metabolite 25-hydroxycholesterol as well as a microRNA (miR342-5p) that dramatically suppresses the flux in the sterol biosynthesis pathway. A change in the flux of sterol biosynthesis in turn activates STING that further re-enforces the IFN response (14). It is worth mentioning that VDR can interact with STAT1 and curtail the nuclear translocation of ISGF3 and which may contribute to VDRs inherent immunosuppressive attributes (90). Furthermore, as mentioned above, numerous studies have reported on the importance of both IFN-expression and activation of the vitamin D-pathway on the expression of downstream effector molecules [e.g., antimicrobial peptides (AMPs)] that subsequently influence infection and inflammation (78, 91–99). Increased expression of type II IFN (IFNγ) have been correlated with macrophage activation, macrophage-dependent AMP gene expression, as well as with controlled growth of pathogenic intracellular microbes and better disease outcome (96, 97, 99). Fabri et al. reported that IFNγ, released by T cells induce in a vitamin D-pathway-dependent in human macrophages, autophagy, phagosomal maturation, and antimicrobial activity against *Mycobacterium tuberculosis* (97).

The role of IFN responses and the vitamin D-pathway has also been investigated in human leprosy. Teles et al. have revealed an inverse correlation between IFNβ, IL-10, and IFNγ, where IFNβ, in an IL-10-pathway-dependent manner, inhibited the IFNγ-induced and vitamin D-dependent, AMP response in disseminated and progressive lepromatous lesions (99). By contrast, IFNγ-specific genes were enriched in self-healing tuberculoid lesions. Both studies underscore the importance of adequate amounts of vitamin D in human populations for sustaining both innate and acquired immunity against infection. The close connection between IFN and the vitamin D-pathway have also been reported in experimental autoimmune encephalitis (EAE), a model for multiple sclerosis (MS) (100, 101), and diabetes (102), two non-infectious disorders characterized by excessive and uncontrolled inflammation and macrophage foam cell formation (characterized by accumulation of esterified cholesterol) (103, 104). Early studies have shown that IFNγ plays a crucial role in the induction of $1,25(OH)_2D_3$ (105, 106), the active vitamin D metabolite that bind to VDR, in initializing VDR dimerization with RXR and, in VDR–RXR activation of VDRE-containing target genes (107–109). Adams et al. showed that IFNγ induces production of $1,25(OH)_2D_3$ in macrophages and that the effect was abolished by addition of anti-IFNγ to the culture medium (110). The tissue availability of $1,25(OH)_2D_3$ in immune cells is dependent on the expression of the activating enzyme 1α-hydroxylase (*Cyp27b1*) and its catabolic counterpart 25-OHD_3-24-hydroxylase (*Cyp24a1*) (111). In addition to TLR signaling, expression of 1α-hydroxylase can be induced by IFNγ stimulation (112–114). Activation by IFNγ stimulation require, however, the cells to be differentiated, as IFNγ stimulation of undifferentiated monocyte THP1 cells failed to induce *Cyp27b1* expression (1α-hydroxylase) in the absence of a second stimulus [lipopolysaccharide (LPS)] (112). On the other hand, *Cyp24a1* (25-OHD_3-24-hydroxylase) expression is induced by the type 2 T helper cell cytokine IL-4, in toll-like receptor 2/1 ligand-activated monocytes, but not by IFNγ (114). Stoffel et al. revealed that, in cultured monocytes, synergistic induction of *Cyp27b1* gene expression by IFNγ and LPS, required, not only activation of the JAK/STAT pathway and NF-κB binding but also binding of phosphorylated C/EBPβ (by the p38 MAPK pathway) (113).

In addition to regulation of VDR through the transcriptional regulation of *Cyp27b1*, *VDR* gene expression was shown to be

dependent on the *IFNG* gene (101). *Ifng* knockout (GKO) mice exhibited very low *Vdr* gene expression in the central nervous system. Correlating with the low *Vdr* expression, GKO mice also demonstrated an increased pathogenic T cells burden as well as a more severe EAE phenotype, suggesting that the aggressive autoimmune $CD4^+$ T cell phenotype may be a consequence of inadequate *Vdr* gene expression (101). Furthermore, treatment of TLR2/1L-activated monocytes with IFNγ has been shown to not only stimulate *Cyp27b1* expression but also *Vdr* gene expression (114). Collectively, these studies suggest that IFN-regulation of VDR activity is complex and regulated indirectly *via* regulation of 1α-hydroxylase, and possibly directly at the gene level by IFNγ-induced STAT binding.

PRESENT DAY IFN-STEROL METABOLIC LINK

Primitive NR1I and NR1J receptors have inherent affinities for lipophilic molecules, making them useful xenobiotic sensors in vertebrate and invertebrate organisms. **Figure 6** depicts the adaption of this receptor from the detoxification of endogenous compounds including bile acids and vitamin D in vertebrate organisms to acquiring new biological roles associated with the increasing importance of sterols and especially oxysterols for immune cell function. From this foundation, VDR continued to develop further direct and indirect interactions with lipid metabolism pathways, placing VDR in an opportunistic position to influence the development and regulation of other important biological systems including, but not limited to, immunity, bone development, and cell proliferation. The immune roles have been further consolidated with the acquisition of fixed mutations, such as the VDR regulated production of innate AMPs.

In conclusion, our findings support an evolutionary basis to the IFN–sterol–immune metabolic link, arising from the xenobiotic origins of a nuclear hormone receptor, VDR (**Figure 6**). Xenobiotics and immunity are considered separate pathways facilitating the removal of toxic molecules and pathogens, respectively. However, our narrative synthesis suggests that sterol metabolites acted as an evolutionary driver integrating a complex metabolic network under bi-directional IFN and VDR control. We believe this is likely to be a more general evolutionary mechanism for other nuclear hormone receptor functions in immunity such as glucocorticoid receptor, liver X receptor (LXR), and farnesoid X receptor (FXR) and possibly for other distinct immune directed metabolic pathways and associated ligand activated receptor systems.

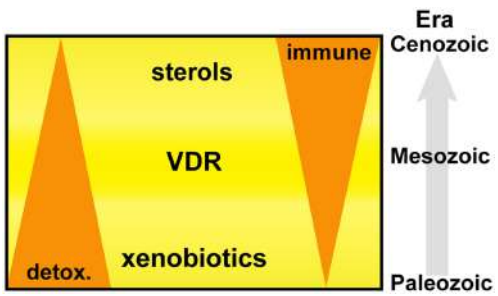


FIGURE 6 | Vitamin D receptor (VDR) is an ancient nuclear hormone receptor that was originally involved in the recognition and detoxification of xenobiotic marine biotoxins exhibiting sterol ring scaffolds present in aquatic environments. Coadaptation of this receptor with the acquisition of sterol biosynthesis and interferons in vertebrate animals set a stage for repurposing and linking a preexisting host-protection mechanism of harmful xenobiotics to become an important regulator in immunity.

CONCLUDING REMARKS ON THE *GENERAL* HYPOTHESIS OF MICROBIAL–HOST SMALL-MOLECULE METABOLITES AS FUNCTIONAL AND EVOLUTIONARY DRIVERS FOR RECOGNIZING AND DEFENDING AGAINST NON-SELF

The question of whether a functional–evolutionary link between planar sterol-like molecules, immunity, and detoxification is specifically unique to VDR would implicate a limited selective role rather than one pertaining to a more central evolutionary principle with broader biological significance. Notably, in this context other evolutionary related subfamily 1 nuclear receptors, including LXR, FXR, PXR, CAR, retinoic-acid receptor (RAR), and RAR-related orphan receptor (32), similarly exhibit, at a number of levels, cross talk between mevalonate-sterol metabolism and immunity, as well as in xenobiotic detoxification [for examples see Ref. (115–118)]. Interestingly, RXRs partner with many of these receptors and which recruits corepressor or coactivator molecules to regulate transcriptional responses. Homologs and orthologs of this highly conserved nuclear receptor have been identified in marine and terrestrial invertebrates (119–123), so it is likely that RXR coevolved with VDR and the many other nuclear receptors it is associated with. RAR and RXR are activated by the vitamin A derived ligand, retinoic acid and its 9-cis conformer, respectively. While this lipophilic molecule is not a steroid-derived molecule, the heterodimerization of RXR provides an important but insufficient role of vitamin A metabolites in integrating with these receptor systems and sterol metabolism (124–127).

Of the related subfamily 1 nuclear receptors, strong conservation across vertebrate species can be found for the LXRs, with approximately 75% sequence identity in the LBD between human and non-mammalian LXRs (128). Consistent with the high degree of sequence conservation, ligand specificities between mammalian and non-mammalian vertebrates homologs, as well as between vertebrate and non-vertebrate LXR orthologs, are very similar (128). While vertebrate LXR agonists do not activate *Ciona* LXR, it is activated by a number of oxysterols as well as some pregnane and androstane steroids. In insects on the other hand, the ecdysone receptor (EcR) has been identified as the ortholog for both LXR and FXR combined (120). Together with its interaction partner,

ultraspiracle (ortholog of RXR), EcR play an essential role in insect development and reproduction, as well as in basic metabolism and immunity (129, 130). In mammals, FXRs, with their high expression in liver, adrenal glands, intestine, and kidney, are activated by farnysol and its metabolites, part of the mevalonate–sterol biosynthesis pathway (131) and by primary bile acids such as chenodeoxycholic acid (132–134). Together with PXRs and VDR, FXR serves as one of the major transcriptional regulators of bile salt synthesis, partly by regulating the expression of CYP7A1 and CYP8B1 (135). Bile salts have to date not been detected in invertebrates, suggesting that regulation of bile salt synthesis is an evolutionary acquired trait specific for the vertebrate nuclear receptors. Two major evolutionary shifts in bile salt structure have been identified (50, 136) and hypothesized that the bile alcohols found in jawless fish (Agnatha) represents the "ancestral" bile salt phenotype from which the more "recent" vertebrate bile acids are derived (51). Secondary bile acids produced as a consequence of microbial metabolism in the gut are further detoxified through recognition by these nuclear receptors. In this regard, it is also notable that the related NRI family member, PXR that is well known for its xenobiotic detoxification role has also been shown to regulate intestinal inflammation by sensing bacterial metabolites (137). Furthermore, another unrelated xenobiotic receptor, the aryl hydrocarbon receptor, has been linked to the antibacterial response through sensing bacterial pigments and in enhancing IL-22 barrier immunity (138, 139).

While a comprehensive evolutionary history of LXR, FXR, and the other nuclear hormone receptors is beyond the present scope of this article; we believe the evolutionary path exemplified by VDR, in selecting dual functional roles in detoxification of harmful planar lipids and immune recognition, represents a central driver for the evolution of these receptors and evokes a general hypothesis for the coevolution of microbial–host metabolism underlying host-protection pathways.

AUTHOR CONTRIBUTIONS

HN, WD, and PG conceived, performed analysis, and wrote the paper.

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Interferon Lambda: Modulating Immunity in Infectious Diseases

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Interferon lambdas (IFN-λs; IFNL1-4) modulate immunity in the context of infections and autoimmune diseases, through a network of induced genes. IFN-λs act by binding to the heterodimeric IFN-λ receptor (IFNLR), activating a STAT phosphorylation-dependent signaling cascade. Thereby hundreds of IFN-stimulated genes are induced, which modulate various immune functions *via* complex forward and feedback loops. When compared to the well-characterized IFN-α signaling cascade, three important differences have been discovered. First, the IFNLR is not ubiquitously expressed: in particular, immune cells show significant variation in the expression levels of and susceptibilities to IFN-λs. Second, the binding affinities of individual IFN-λs to the IFNLR varies greatly and are generally lower compared to the binding affinities of IFN-α to its receptor. Finally, genetic variation in the form of a series of single-nucleotide polymorphisms (SNPs) linked to genes involved in the IFN-λ signaling cascade has been described and associated with the clinical course and treatment outcomes of hepatitis B and C virus infection. The clinical impact of IFN-λ signaling and the SNP variations may, however, reach far beyond viral hepatitis. Recent publications show important roles for IFN-λs in a broad range of viral infections such as human T-cell leukemia type-1 virus, rotaviruses, and influenza virus. IFN-λ also potentially modulates the course of bacterial colonization and infections as shown for *Staphylococcus aureus* and *Mycobacterium tuberculosis*. Although the immunological processes involved in controlling viral and bacterial infections are distinct, IFN-λs may interfere at various levels: as an innate immune cytokine with direct antiviral effects; or as a modulator of IFN-α-induced signaling *via* the suppressor of cytokine signaling 1 and the ubiquitin-specific peptidase 18 inhibitory feedback loops. In addition, the modulation of adaptive immune functions *via* macrophage and dendritic cell polarization, and subsequent priming, activation, and proliferation of pathogen-specific T- and B-cells may also be important elements associated with infectious disease outcomes. This review summarizes the emerging details of the IFN-λ immunobiology in the context of the host immune response and viral and bacterial infections.

Keywords: interferon lambda, immunity, immune cells, virus, infectious diseases, bacteria, fungi, parasites

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IFN-λ EXPRESSION AND SIGNALING PATHWAYS

Patients with infectious diseases often show heterogeneous clinical courses with a range of associated morbidities and variable mortality. This is dependent on a series of factors covering the complex aspects of host–pathogen interactions (1–5). IFNs play a crucial role in these interactions—defining the outcome of many viral, bacterial, fungal, and parasitic infections (6–16) (see **Figure 1**). In addition, IFNs reduce tumor cell proliferation (17, 18) and show important immune regulatory functions in autoimmunity (19, 20). These broad effects are explained through the induction of hundreds of IFN-stimulated genes (ISGs) (21). Three types of IFNs have been described, which can induce ISG expression, and add further complexity: type I with mainly IFN-αs and -βs (22–26), type II with only IFN-γ (27), and type III with IFN-λs (28–31). Although most cells can induce and release various types of IFNs, specialized immune cells are the main producers during an inflammatory process. The effects induced by single or combined IFNs in exposed cells are very heterogeneous and range from differential patterns of ISG expression, regulation of cell proliferation (18), changes in cell surface molecules such as HLA DR (32), to the maturation of monocytes to dendritic cells (33). The effects depend on the plasticity of the various IFNs involved, including the peak concentrations, concentration changes over time, binding affinities of IFNs to the specific receptors, receptor expression, potentially induced feedback mechanisms, and the target cell type itself (34).

Four IFN-λ ligands have been described: IFNL1–4, with each family member having antiviral effects on various viruses within different cell types (28). IFNL1–3 share high amino acid sequence homologies, whereas IFNL4 is more divergent with only 40.8% amino acid similarity to IFNL3 (35). The expression of IFN-λs is induced in a broad range of cell types by pattern recognition receptors including toll-like mediated (36–41), Ku70 (21398614) and RIG-1-like (24952503). Type 2 myeloid dendritic cells have been described as the main producers of IFN-λ (42–48). In mice, commonly used as a model organism for infectious disease and immune function, only IFNL2 and IFNL3 are functional, as IFNL1 and IFNL4 are present as inactive pseudogenes (49).

After release, IFN-λ binds to its heterodimeric IFN-λ receptor (IFNLR). The IFNLR consists of two subunits: α-subunit (IL28RA) and β-subunit (IL10RB) (35, 50–53). Despite high sequence homologies, binding affinities of the different IFN-λs to the IFNLR1 differ greatly. IFNL1 shows the highest binding affinity to IL28RA, and IFNL3 the lowest (54). The dimerization of the receptor subunits leads to activation of Janus Kinase 1 and tyrosine kinase 2 and phosphorylation of STAT-1 and -2, which

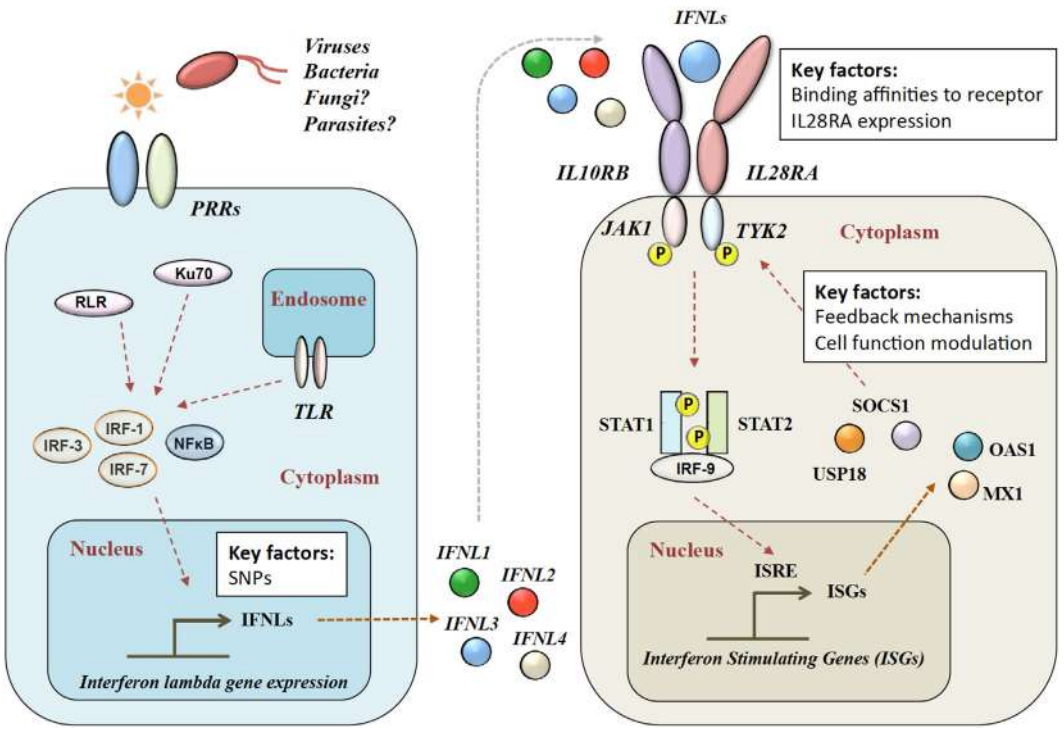

FIGURE 1 | Type III IFN signaling pathway. Viral infection is sensed by pattern recognition receptors (PRRs), which induce IFN-λ production *via* various signaling pathways. IFN-λs bind to the heterodimeric IFN-λ receptor (IFNLR), which consists of IL28RA and IL10RB subunits. Upon binding, a JAK–STAT signaling cascade induces hundreds of IFN-stimulated genes (ISGs). RLR, RIG-1-like receptor; TLR, toll-like receptors; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IL28RA, interleukin 28 receptor alpha; IL10RB, interleukin 10 receptor beta; JAK1, Janus Kinase 1; TYK2, tyrosine kinase 2; STAT, signal transducer and activator of transcription; IRF, interferon regulatory factor; ISRE, interferon-stimulated response element; MX1, interferon-induced GTP-binding protein Mx1; OAS1, 2′-5′-oligoadenylate synthetase.

induces the subsequent downstream signaling with the induction of hundreds of ISGs (31) (see **Figure 1**). IFN-α and IFN-λ both show a complex mechanism of positive and negative feedback loops, mainly modulated *via* the suppressor of cytokine signaling 1 and the ubiquitin-specific peptidase 18 (31, 55).

IFN-λ RESPONSIVENESS TO COUNTERACT PATHOGENS

Two aspects are crucial to understanding the role of IFN-λs in the context of infectious diseases: (i) IFNLR distribution in infected cells and tissues and (ii) single-nucleotide polymorphisms (SNPs) in and around the genes encoding IFN-λs and IFNLR. Both aspects show important differences between humans and mice, which complicate studies and conclusions drawn from infectious disease models (56).

IFNLR Receptor Expression

The IL10RB subunit is expressed in many cell types (57), whereas the IL28RA subunit expression is much more restricted. Expression of IL28RA mRNA has been detected in the lung, intestine, liver tissues, immune cells such as B cells, neutrophils, macrophages, and plasmacytoid dendritic cells (28, 29, 43, 58–62). Human NK cells seem not to express IFNLR (63), whereas mouse NK cells show deficient function in IL28R knockout animals (25901316). The effects of IFN-λ on cells and tissues are often measured *in vitro via* indirect markers, such as downstream expression of ISGs or changes in specific cellular phenotypes. Data on the induction of STAT phosphorylation, as the most direct measurement of signal induction, are still missing for some cell types and tissues. The IFNLR expression is regulated *via* transcription factors (31) and may show variability during an inflammatory process, which adds an additional level of complexity. Primary hepatocytes show relatively low baseline responsiveness to IFN-λs, yet upon IFN-α treatment a marked increase in IL28RA mRNA levels is observed (64, 65). Similarly, during cytomegalovirus (CMV) infection of fibroblasts, IL28RA mRNA levels increase by about twofold, but protein expression levels remain stable (66). A recent paper by Lazear et al. suggested that endothelial cells in the blood–brain barrier may be sensitive to IFN-λs, reducing permeability to West Nile virus in a mouse model (67).

Understanding which immune cells and subsets are responsive to IFN-λs in humans can be experimentally and technically challenging due to low target cell densities and less accessible cell types such as tissue resident cell types. In contrast, peripheral blood mononuclear cells (PBMCs) are relatively easy to access in order to explore responses to IFN-λs; therefore, most literature focuses on hepatocytes (from liver biopsies) and immune cells from the blood. The direct impact of IFN-λs on T-cells *via* surface expression of the specific IFNLR is subject to ongoing debate (58, 68–71). IFN-λs may also induce FOXP3-expressing regulatory T-cells (72), which may impact a series of immunoregulatory aspects during an infection as part of the inflammatory response. Several research groups confirm that IFN-λs influence the T-helper cell balance, which is shifted toward Th1 (70, 71, 73–76). The Th1/Th2 balance might be important for controlling specific infections such as helminths (6, 77, 78). In addition, the B-cell-driven humoral immune responses are also modulated by the presence of Th2 cytokines, e.g., during vaccination. We have recently shown that IFNL3 is a key regulator of the influenza virus-specific B-cell proliferation and antibody production (76). The exact mechanism of how Th1/Th2 balancing and B-cell activation is modulated by IFN-λs and how this impacts infectious disease outcome has to be explored in more detail in the future.

Impact of SNPs

A series of SNPs in IFN-λ ligand and receptor genes have been described (see **Figure 2**). Most importantly, these SNPs have been associated with a series of important clinical phenotypes in the context of infectious diseases (see **Table 1** for more details).

Modulation of IFNLR expression may have a great impact on the effects of a particular IFN-λ ligand, and thereby influence the subsequent signaling pathway and the outcome of infectious diseases. Multiple SNPs in the gene encoding IL28RA have been described (94–97). The rs10903035 SNP is located within the 3′UTR of the IL28RA mRNA sequence, suggesting a potential microRNA binding site. This particular SNP was identified as an independent risk factor for IFN-α treatment failure against hepatitis C virus (HCV) (44, 98). In addition, this SNP has been associated with insulin resistance in HIV/HCV coinfected patients (94). Another SNP in this gene, rs4649203, has been linked to the risk of psoriasis in four independent populations (96), and to the development of systemic lupus erythematosus (97). These observations suggest an important influence of IL28RA on infectious and autoimmune diseases.

Expression of IFN-λ ligands is modulated by SNPs in both transcription factor binding sites and methylation sites of the promoter region, as well as frameshift mutations (99–102). The IFN-λ gene layout is shown in **Figure 2**. The clinical impact of SNPs in the IFNL3/4 locus was originally observed in the context of IFN-α treatment outcomes in patients with chronic HCV (79, 80, 87, 90, 103). SNPs within this locus are in high linkage disequilibrium, e.g., rs12979860 with ss469415590 (103, 104), which complicates the exploration of the effects of individual SNPs. Therefore, the impact of some SNPs on IFN-λ expression is still debated. Most studies have concluded that the minor alleles of SNPs rs12979860 (CT/TT) and rs8099917 (TG/GG) are associated with reduced IFNL3 expression during chronic HCV infection, observed in liver biopsies (80, 105–107), serum, and PBMCs stimulated with polyI:C-, CMV-, and influenza virus (66, 76, 108, 109). However, it has also been shown that the TT allele of rs12979860 in hepatocytes expresses higher levels of IFNL1 and IFNL3 (110). This minor allele genotype of rs12979860 (TT) has also been associated with a higher and prolonged ISG expression in HCV infection (79, 80, 87, 90, 103, 111). Interestingly, the same SNP of the IFNL3 gene is associated with a higher ISG expression in mothers after childbirth, suggesting that postpartum the normalization of physiological control of IFN signaling depends on the IFNL3 genotype (112). Although the rs12979860 SNPs have been specifically associated with IFNL3/L4 expression, these SNPs might also affect the expression of the other IFN-λ genes (80, 87, 113).

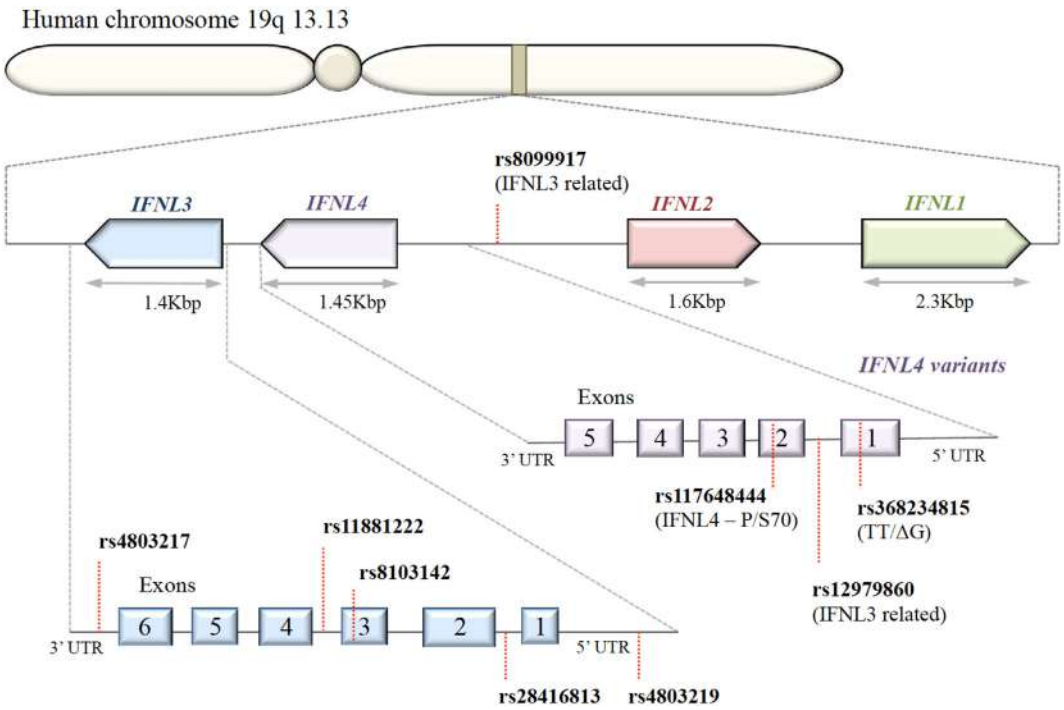

FIGURE 2 | Organization of IFNL genes in the human genome. The IFN-λ genes are located in tandem on chromosome 19. Key single-nucleotide polymorphisms (SNPs) in coding and non-coding regions of IFN-λ genes are shown. IFNL1, IFNL2, and IFNL3 genes are functional; only a subset of the human population possess the SNP rs368234815 with ΔG frameshift mutation in exon 1, producing an in-frame IFNL4.

The impact of the ss469415590 SNP on the expression of IFNL4 is, in contrast, very well described: in the context of a delta-G polymorphism, a frameshift mutation generates a gene containing an alternative reading frame, which causes IFNL4 to be functionally expressed in about 40% of Caucasians (90). An amino acid substitution at residue 70 of IFNL4 (P70S) decreases the antiviral activity *via* a reduction in the ISG expression levels (111).

Beside the impact of SNPs on innate immune signaling *via* differences in ISG expression profiles, an important impact on adaptive immune functions has been noted. We have shown that IFN-λ decreases virus-induced B-cell proliferation and antibody secretion in a dose-dependent manner. In addition, IFN-λ increases influenza-induced Th1 cytokines (IFN-γ, IL6), whereas influenza-induced Th2 cytokines decrease (IL4, IL5, IL9, IL13). These effects can also be reproduced with specific allelic combinations. In particular, the TG/GG allele of rs8099917 shows significantly lower levels of IFN-α, IL2, and IL6 secretion in influenza-stimulated PBMCs. In an influenza vaccine cohort, vaccine recipients with the rs8099917 TG/GG (minor) allele showed significantly higher vaccine-induced humoral immune responses (76). Similarly, in a cohort of children vaccinated against measles, the post-vaccine antibody titers were significantly higher in the group with the rs10853727 SNP AG and GG (minor allele) (89). Both SNPs rs8099917 and rs10853727 lie within the IFNL3 promoter region and have been associated with lower IFNL3 expression (76, 89).

IFN-λ AND INFECTIOUS DISEASES

The dual role of IFN-λs, with direct antiviral effects (innate immunity) and more long-term immunomodulatory effects on T- and B-cell activation and modulation, can result in multiple possible interactions with different types of infectious disease. **Table 2** summarizes the role of IFN-λs in several infectious diseases.

Viral Infections

IFNs protect cells against viral infections. In response, every virus has evolved specific ways to counteract IFN signaling and its effects (139–143). Only a few studies have explored this in the context of IFN-λs. Parainfluenza virus 3 blocks antiviral mediators downstream of the IFNLR signaling by modulation of the STAT1 phosphorylation in BEAS 2B cells, a bronchial epithelial cell line (144). Dengue virus was recently shown to induce IFNL1 *via* its non-structural protein (NS1) in order to facilitate dendritic cell migration (114).

Using cell culture-based *in vitro* models, IFN-λs have been shown to play a role in controlling viral replication. In most studies, cultured cells were treated with IFN-λs and the impact of viral infection was assessed. These studies investigated human (66) and murine CMV (59), dengue virus (114, 145), encephalomyocarditis virus (28, 29, 146), herpes virus type 2 (120), hepatitis B virus (115), HCV (37, 60, 113, 115, 116, 147), HIV (40, 117, 118), human meta pneumovirus (121), influenza virus (122, 148–152),

TABLE 1 | Single-nucleotide polymorphisms (SNPs) within the IFNL3/IFNL4 gene locus and impact on infectious diseases.

Gene	SNP	Allele type	Effects of the allele on infectious diseases	Reference
IFNL3	rs12979860	C/T and T/T (C-major, T-minor)	HCV: decrease of effective treatment for HCV	(79, 80)
		C/T and T/T (C-major, T-minor)	HTLV1: higher proviral load and higher risk of developing HTLV-1-associated myelopathy and tropical spastic paraparesis (TSP)	(81)
		C/C (C-major)	HBV: higher inflammation and liver fibrosis in chronic hepatitis B patients	(82)
		T/T (T-minor)	EBV: observed higher level of EBV DNA in the plasma of EBV viremia patients	(83)
		T/T (T-minor)	CMV: less CMV replication in solid-organ transplant recipients	(66)
		T/T (T-minor)	CMV: lower incidence of active CMV infection and reduced CMV DNAemia in allogeneic stem cell transplant patients	(84)
		C/T and T/T (C-major, T-minor)	HSV: increased rate of HSV-1-related herpes labialis and more clinical severity	(85)
		T/T (T-minor)	ANDV: associated with mild disease progression	(86)
	rs8099917	T/G (T-major, G-minor)	HCV: lower response to PEG-IFN-α/RBV treatment	(87)
			HTLV1: high risk for developing HTLV-1-associated myelopathy and TSP	(88)
			CMV: trend to show less CMV replication in solid-organ transplant recipients	(66)
		G/G (G-minor)	ANDV: associated with mild disease progression	(86)
		T/G and G/G (T-major, G-minor)	Influenza vaccination: increased Th2 cytokine production and higher rate of seroconversion following influenza vaccination	(76)
	rs4803217	C/T (C-major, T-minor)	HCV: decreased response to PEG-IFN-α/RBV treatment	(80)
	rs10853727	A/G and G/G (A-major, G-minor)	Measles vaccination: increased post-vaccine titers against measles vaccination	(89)
	rs12980275	A/G (A-major, G-minor)	HCV: failure to clear infection (null virological response: NVR)	(80, 87)
IFNL4	ss469415590	ΔG/TT and ΔG/ΔG (frameshift variant from TT genotype)	HCV: creates a new IFNL4 gene and poorer response to PEG-IFN-α/RBV treatment	(90)
	(rs368234815)		CMV: increases susceptibility to CMV retinitis among HIV-infected patients	(91)
			CMV: higher susceptibility to CMV infection in solid-organ transplant recipients	(92)
			HIV: higher prevalence of AIDS-defining illness and lower CD4 lymphocytes levels	(93)
IFNLR1	rs10903035	A/G and G/G (A-major, G-Minor)	HIV/HCV: early treatment failure with HIV/HCV coinfected patients	(94)

IFNL3, interferon lambda 3; IFNL4, interferon lambda 4; IFNLR1, interferon lambda receptor 1; HCV, hepatitis C virus; HTLV-1, human T-lymphotrophic virus type 1; HBV, hepatitis B virus; EBV, Epstein–Barr virus; CMV, cytomegalovirus; HSV, herpes simplex virus; ANDV, Andes virus; HIV, human immunodeficiency virus; PEG-IFN-α/RBV, pegylated-Interferon-α/Ribavirin.

lymphocytic choriomeningitis virus (LCMV) (125), norovirus (124), respiratory syncytial virus (128, 153, 154), sendai virus (155–157), and vesicular stomatitis virus (131, 158, 159).

In vivo, the complexity of the role of IFN-λ within tissues and between various immune cells has been explored using an IL28RA$^{-/-}$ mouse model, leading to the discovery of multiple important aspects of IFN-λ signaling (122, 130, 150).

A recent study by Lin et al. demonstrated that the effects of type III IFNs change with increasing age. Rotavirus was controlled by both type I and III IFN in suckling mice, whereas epithelial cells in particular were responsive. In adult mice, epithelial cells were responsive only to type III and not type I IFNs, suggesting an orchestrated spatial and temporal organization of the IFN-α and IFN-λ responses in the aging murine intestinal tract (160). However, there is some controversy regarding the rotavirus data, as other researchers have shown that rotavirus is specifically controlled by type III and not type I IFN (21518880). Mahlakoiv et al. showed that leukocyte-derived IFN-α/β and epithelial IFN-λ constitute a compartmentalized mucosal defense system to restrict enteric viral infection in mice. The authors concluded that epithelial barriers to IFN-λ may have evolved to reduce frequent triggering of IFN-α/β and thus reduce exacerbated inflammation (161). A study by Baldridge et al. showed that antibiotics could prevent the persistence of enteric murine norovirus infection, but only in the presence of functional IFN-λ signaling. The IL28RA$^{-/-}$ mice showed a high rate of infection, despite the administration of antibiotics. This may suggest cross talk between the gut microbiota and IFN-λ signaling in modulating chronic viral infections (162). Important synergistic effects in the intestine have been described, with IL22-inducing IFN-λ expression in intestinal epithelial cells in a murine rotavirus infection model (163).

The role of IFN-λ during respiratory tract infections has also been explored using the IL28RA$^{-/-}$ mouse model. The studies so far have concentrated on the classical role of IFNs as antiviral cytokines. The IL28RA$^{-/-}$ mouse displayed a significantly higher burden of disease than wild-type mice during infections with influenza virus and SARS coronavirus (122, 130, 150). One study showed the immunoregulatory function of IFN-λ in an LCMV model. The authors noted that in an acute LCMV infection model, the IL28RA$^{-/-}$ mouse showed a greater than normal CD4^{+} and CD8^{+} T-cell response compare to the wild type, whereas in a chronic LCMV infection model, the IL28RA$^{-/-}$ mice showed a greater disease burden and a significantly reduced LCMV-specific T-cell response. The paper showed that germinal center B-cells

TABLE 2 | Described role of IFN-λσ in infectious diseases.

Pathogens	Model	Role of IFN-λ	Reference
Viruses			
Cytomegalovirus (CMV)	*In vitro:* HFF cell line and stimulated peripheral blood mononuclear cells (PBMCs) Clinical study	IFNL3 reduces CMV-induced CD4 T cell proliferation in PBMCs	(66)
Dengue virus	*In vitro*: DC and human lung epithelial cell line A549	IFNL1 induce CCR7 expression and DC migration upon dengue virus infection	(114)
HBV	*In vitro:* murine hepatocyte cell line (HBV-Met)	IFNL induces IFN-α/β-like antiviral response and inhibition of HBV replication in murine heptocyte cell line	(115)
Hepatitis C virus (HCV)	*In vitro: primary* hepatocytes and HUH7 cell lines.	IFNL induces type-1 interferon-like antiviral response and blocks HCV infection in human primary hepatocyte and HUH7 cells	(59, 115, 116)
HIV	*In vitro*: monocyte-derived macrophages *In vitro*: T-cells and clinical study	IFNL3 inhibits HIV infection of macrophage through the JAK-STAT pathway. IFNL induce antiviral state in culture primary T-cells and supress HIV-1 integration and posttranscriptional events	(117, 118)
HSV-1	*In vitro*: human lung epithelial cell line A549 Clinical study	Mediator complex (Med23) interacts with IRF-7 to enhance IFNL production and it inhibits HSV-1 replication	(119)
HSV-2	*In vitro*: human cervical epithelial cells	IFNL contributes to TLR3/RIG-1-mediated HSV-2 inhibition	(120)
Human metapheumovirus (HMPV)	*In vitro*: human lung epithelial cell line A549	Mice treated with IFNL prior to HMPV infection develop lower viral titer and reduced inflammatory responses	(121)
Influenza virus	*In vivo:* mice *In vitro:* cell lines *In vivo:* infected mice	IFNL restricts virus infection in epithelial cells of respiratory and gastrointestinal tracts IFNL reduced Influenza A virus-induced disease, with less inflammatory side effects in comparison to IFN alpha	(122, 123)
Murine CMV	*In vitro:* intestinal epithelial cell lines	IFNL1 mediates antiproliferative and antiviral signals in intestinal epithelial cells	(59)
Norovirus	*In vivo:* infected mice	IFNL cures persistent murine norovirus infection	(124)
Lymphocytic chorimeningitis virus	*In vitro*: human lung epithelial cell line A549	IFNL2 showed more potent antiviral response to lymphocytic choriomeningitis virus than IFNL3	(125)
Rhinovirus	*In vitro:* human bronchial epithelial cell line (BEAS-2B)	Increased IFNL production reduces rhinovirus replication in bronchial epithelial cells	(126)
RSV	*In vitro:* primary human and mouse airway epithelial cells *In vitro:* Hep-2 and Vero cells	TLR-s mediates IFNL production in primary airway epithelial cells and induces the antiviral response IFNL-1 shows prophylactic potential against RSV	(127, 128)
Rotavirus	*In vivo:* infected mice	IFNL reduces viral replication in epithelia cells	(129)
SARS coronavirus	*In vitro*: human lung epithelial cell line A549 *In vivo:* infected mice	Ifnlr1$^{-/-}$ mice exhibit increased susceptibility to SARS corona virus	(122, 130)
VSV	*In vitro:* mouse hepatocyte cell line	IFNL attenuates VSV replication in immortal mouse hepatocytes (MMHD3 cells)	(131)
West Nile virus	*In vitro:* Huh7.5 and HeLa cells *In vivo: infected* mice	IFNL can efficiently prevent West Nile Virus infection in cell line IFNL knockout animals show increased viral load in brain. Treatment with IFNL reduced blood–brain permeability for the virus	(67, 132)
Bacteria			
Staphylococcus aureus and *Pseudomonas aeruginosa*	*In vivo:* infected mice	Ifnlr1$^{-/-}$ mice exhibits less pathology without changes in cell infiltrates	(133)
Mycobacterium tuberculosis	*In vitro:* human lung epithelial cell line A549 Clinical study	Induces IFNL expression on A549 lung epithelial cells Observed increased concentration of IFNL2 in sputum of pulmonary tuberculosis patients	(134, 135)

(*Continued*)

TABLE 2 | **Continued**

Pathogens	Model	Role of IFN-λ	Reference
Listeria monocytogenes	*In vivo:* infected mice	IFNL-mediated immune response may control bacterial colonization	(136)
Salmonella typhimurium	*In vitro:* human monocyte-derived macrophages	The activation of type III interferon by live and heat killed bacteria in phagocytic dentritic cells, but role in pathogenesis is not clear	(137)
Borrelia burgdorferi	*In vitro: stimulated* PBMCs	The ability of IFNL induction correlates with clinical isolates, type III IFN pathway in pathogenesis is yet to be determined	(138)

HSV-1, herpes simplex virus-1; HSV-2, herpes simplex virus-2; RSV, respiratory syncytial virus; VSV, vesicular stomatitis virus; murine CMV, murine cytomegalovirus; SARS, severe acute respiratory syndrome.

were more frequent in peripheral blood in the IL28RA$^{-/-}$ mice than wild-type mice. However, the LCMV-induced memory B-cell response, in terms of frequencies and LCMV-specific antibodies, was comparable (164).

The immunoregulatory actions of IFN-λs have been explored in an ovalbumin (OVA)-induced asthma model. The IL28RA$^{-/-}$ mice showed a clear shift to increased Th2 cytokines and a more severe asthma phenotype. Importantly, IgE antibodies were also significantly increased (73). In this model, the IFNL2 (IL28A) immunoregulatory activity was dependent on lung CD11c^{+} dendritic cells to decrease OX40L, increase IL-12p70, and thereby promote Th1 differentiation (73). The potential role in infection-triggered asthma has also been explored in humans (72, 126).

Although these conclusions from mice studies are very important, a series of important differences to human effects have also been noted. In a human chimeric mouse model using human hepatocytes, the response rates of human and mice hepatocytes toward IFN-λs were very different, specifically in that mouse, hepatocytes did not respond to IFN-λ (56). In addition, the expression of IFNLR in immune cells seems to be strikingly different. Whereas B-cells in humans respond to IFN-λs, in B-cells from mice there seems to be no direct effect from IFN-λs (69, 164).

Studies on the impact of IFN-λs in clinical scenarios have been dominated by the strong association of IFNL3/L4 SNPs with spontaneous clearance of HCV and IFN-α treatment response (79, 80, 87, 90, 103, 111). Details on this important association have been reviewed in detail elsewhere (165–167). The association between IFN-λ SNPs and other infectious diseases is far less well explored. Not many studies have linked the genetic associations with mechanistic immunological assay.

Several studies have explored the association between SNPs in the IFNL3/L4 signaling and CMV replication. Transplant recipients with the rs8099917 GG allele demonstrate significantly less CMV primary replication. This SNP has been associated with reduced ISG expression upon infection (66). We postulate that this phenomenon has two reasons: (i) significant primary CMV replication is less likely due to a higher baseline ISG expression and (ii) naïve CMV-specific T cells from seronegative healthy blood donors show reduced proliferation capacity when pretreated with IFNL3 and stimulated with CMV lysate (66). In contrast, the rs368234815 ΔG SNP shows a higher risk for CMV retinitis in HIV-infected patients (91) and has been associated in a transplant cohort with an increased risk of CMV replication and disease, especially in patients receiving grafts from seropositive donors (92). Non-immunosuppressed patients with chronic periodontitis due to herpes virus infection show significant lower IFNL1 levels in gingival fluid compared to a healthy control group without viral replication (168), suggesting a protective effect of IFNL1 on virus replication, or CMV-induced antagonism of IFN-λ expression. These results highlight the different roles of IFN-λs in acute or chronic infection scenarios and viral reactivation.

The impact of IFN-λs on human T-cell leukemia type-1 virus has also been explored in several independent cohorts. The first evidence came from Kamihira et al. showing that the IFNL3 mRNA expression level was significantly higher in HTLV-1 mono-infection than HTLV-1/HCV coinfection. In addition, the high expression level was associated with the rs8099917 TT SNP (169). The impact of the rs8099917 GG SNP on the risk of HTLV-1 associated myelopathy/tropical spastic paraparesis (TSP) has since been confirmed (88). The impact of the rs12979860 SNP is more controversial. One study on the rs12979860 SNP showed that the CT/TT alleles were more frequent in patients with HTLV-1-associated myelopathy/TSP (81), although this finding was not replicated in two additional studies (170, 171). de Sa et al. reported that the major alleles of IFNL3 SNPs (rs12979860 CC and rs8099917 TT) are associated with a shift in the Th1/Th2 immune response toward a Th1 response (172). The Andes virus causes a hantavirus cardiopulmonary syndrome; in a cohort of Andes virus-infected patients, the minor alleles of rs12979860 and rs8099917 (TT and GG) were linked to milder disease compared to CT/CC and TG/TT (86).

The impact of the IFN-λ signaling on humoral immune function has been described in two vaccine cohorts: immunosuppressed patients vaccinated against influenza (76) and healthy children vaccinated against measles (89). These important observations hold promise for personalized vaccine strategies and adjuvant development (4).

Bacterial Infections

The cytokine microenvironment of a tissue may have an impact on the rate at which a particular infectious bacterium can colonize and also influence the rate of infections. Planet et al. showed that IFN-λs might lead to important changes in the local microbiota during influenza infection. In a mouse model of influenza infection, the authors observed that mice with functional IL28 signaling showed more profound changes in their respiratory microbiota and subsequent higher colonization rates with *Staphylococcus aureus* compared to IL28RA$^{-/-}$ mice (173). These

important findings should be confirmed in a human cohort, as *S. aureus* is an important source of bacterial superinfection after an influenza infection. In addition, microbiota changes upon common clinical scenarios such as antibiotic treatment may be modulated by IFN-λs and their genotypes.

Bacteria including *M. tuberculosis* induce IFN-α/β and IFN-γ; however, little is known about the effects of IFN-λs in epithelial immunity. Gram-positive bacteria such as *S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Listeria monocytogenes* induce IFN-λs, whereas *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, and *Chlamydia trachomatis* do not substantially induce IFN-λs, in intestinal and placental cell lines (134). Others have reported that *S. enterica* serovar Typhumurium can induce IFN-λs in human DCs (137). IFN-λ gene expression can be increased within DCs upon stimulation with bacterial components such as lipopolysaccharide. In particular, during *M. tuberculosis* infection, IFN-α plays an important regulatory role in the pathogenesis (12, 174). *M. tuberculosis* in A549 lung epithelial cells stimulates expression of IFN-λs. In addition, the IFNL2 concentration in sputum of patients with pulmonary tuberculosis is significantly higher than that in the sputum of healthy controls (135). Although the impact of IFN-λs has not been explored in more detail, the cross talk between IFN-α and IFN-λs may play a crucial role in the pathogenesis of *M. tuberculosis*. The modulation of Th1/Th2 toward Th1 may be of additional importance.

Neutrophil functions are crucial in clearing bacterial infections and wound repair (175, 176). A major target of the effects of IFN-λs may be neutrophils (62, 177). A study by Blazek et al. showed that in a collagen-induced arthritis model, IFNL1 showed anti-inflammatory function by reducing the numbers of IL17-producing Th17 cells and the recruitment of IL-1b expressing neutrophils, which is important to amplify the inflammatory process (62). Similar effects on neutrophil recruitment to the lung have been observed in an OVA-based asthma mouse model (73). Although somewhat speculative, this may suggest an important modulatory function of IFN-λs *via* neutrophil recruitment toward sites of bacterial infection.

So far, only one study has linked SNPs in genes involved in the IFN-λ signaling pathway with an increased risk of bacterial infections. Xiao et al. showed that SNP rs10903035 with G allele in the IL28RA was associated with significantly less frequent urinary tract infection (178).

Parasite and Fungal Infections

The role of IFN-λs in parasitic and fungal disease has not yet been explored. Although somewhat speculative, helminth infections in particular might be regulated by SNPs in the IFN-λ system, considering the profound evidence on the importance of Th1/Th2 balance (6, 77, 78). Furthermore, for parasite infections of the liver such as *Plasmodium* spp. there is important evidence on the importance of the IFN-α signaling (13, 179–182). Due the regulatory interactions of IFN-α and IFN-λ and the clinical importance of relevant SNPs (31), it is not unreasonable to postulate an impact.

SUMMARY

IFN-λs, and their modulation *via* SNPs, are increasingly recognized as important players in a broad range of infectious diseases. Although the literature is still dominated by reports on HCV, work especially in mouse models has pointed out the important role in viral, respiratory, and gastrointestinal infections. Bacterial colonization and bacterial infections may also be modulated by IFN-λs. The important diversity in IFNs and the large number of SNPs adds a difficult-to-address layer of complexity. Therefore, further research on IFN-λs outside the HCV field is required to understand their roles and diagnostic and therapeutic potential. Most importantly, predictions of risks associated with infectious diseases have to be confirmed in independent cohorts to allow personalized medicine strategies.

AUTHOR CONTRIBUTIONS

Both the authors (AE and MS) have significantly contributed by writing the manuscript and designing the graphs.

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REVIEW
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Evolution of Interferons and Interferon Receptors

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The earliest jawed vertebrates (Gnathostomes) would likely have had interferon (IFN) genes, since they are present in extant cartilaginous fish (sharks and rays) and bony fish (lobe-finned and ray-finned fish, the latter consisting of the chondrostei, holostei, and teleostei), as well as in tetrapods. They are thought to have evolved from a class II helical cytokine ancestor, along with the interleukin (IL)-10 cytokine family. The two rounds of whole genome duplication (WGD) that occurred between invertebrates and vertebrates (1) may have given rise to additional loci, initially containing an IL-10 ancestor and IFN ancestor, which have duplicated further to give rise to the two loci containing the IL-10 family genes, and potentially the IFN type I and IFN type III loci (2). The timing of the divergence of the IFN type II gene from the IL-10 family genes is not clear but was also an early event in vertebrate evolution. Further WGD events at the base of the teleost fish, and in particular teleost lineages (cyprinids, salmonids), have duplicated the loci further, giving rise to additional IFN genes, with tandem gene duplication within a locus a common occurrence. Finally, retrotransposition events have occurred in different vertebrate lineages giving rise to further IFN loci, with large expansions of genes at these loci in some cases. This review will initially explore the likely IFN system present in the earliest Gnathostomes by comparison of the known cartilaginous fish genes with those present in mammals and will then explore the changes that have occurred in gene number/diversification, gene organization, and the encoded proteins during vertebrate evolution.

Keywords: interferon, interferon receptor, evolution, retrotransposition, gene duplication, fish, vertebrate

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THE INTERFERON (IFN) PATHWAY IN EARLY GNATHOSTOMES

With the recent sequencing of the elephant shark genome (3) it has become apparent that while some differences exist in the antiviral pathways present in cartilaginous fish and mammals, a fully functional IFN system is present in these early vertebrates, as already well established in bony fish (Osteichthyes) (4–7). As outlined below, this includes pattern recognition receptors (PRRs) to detect virus, PRR signaling molecules to effect IFN induction, the IFN genes themselves, their receptors, and associated signaling molecules to trigger antiviral responses, and the IFN-stimulated genes (ISGs) that act to inhibit viral replication in the host.

Sensors

Toll-like receptors (TLRs) are an important family of PRRs that activate IFN responses upon activation by intracellular viral/bacterial oligonucleotide pathogen associated molecular patterns (PAMPs). The TLR family consists of 13 members in mammals, and in the elephant shark, some of the oligonucleotide PRRs present have an apparent orthologous relationship with their mammalian

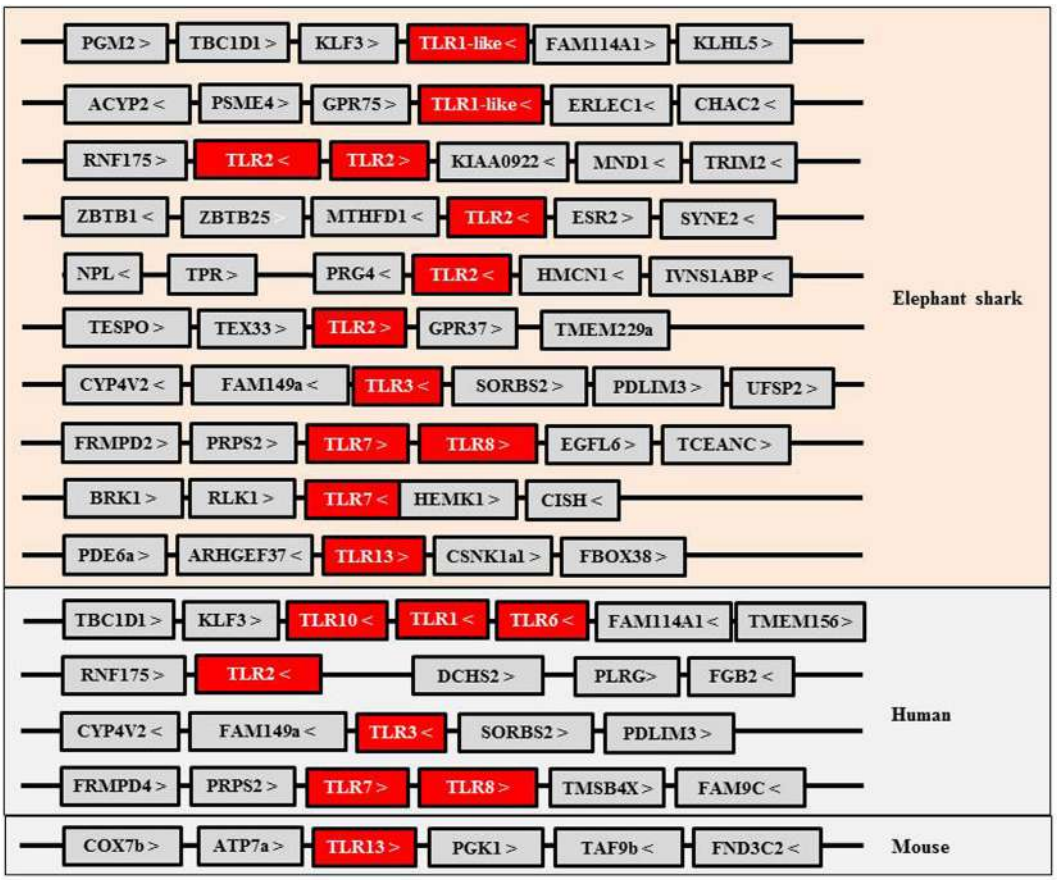

FIGURE 1 | **Gene synteny of Toll-like receptors (TLRs) identified in elephant shark and their homologs in human and mouse**.

counterparts (**Figure 1**). For example, the gene synteny of two loci harboring the TLR3, 7 (two copies), and 8 genes is conserved between elephant shark and humans. However, curiously, PRRs recognizing bacterial PAMPs, such as LPS (by TLR4) and flagellin (by TLR5), and TLR9 which sense CpG PAMPs, are apparently absent in the current version of the elephant shark genome although present in (at least some) Osteichthyes. A fragmented TLR4 gene is present in the elephant shark genome and is likely a pseudogene, suggesting it had evolved early. In bony fish, TLR4 homologs have been described only in cyprinid species and appear to be unresponsive to LPS (8, 9). Some of the PRRs in the elephant shark, including TLR1-like, TLR2, and TLR7, have duplicated copies, and in the case of TLR2, five gene copies are present. These copies are located in four different loci, one of which is the homologous locus of the human TLR2 gene and contains two tandemly linked TLR2 copies. It seems that TLR6 and TLR10 that flank the TLR1 gene in the human genome likely evolved from one of the TLR1-like genes since only a single TLR gene is present in the homologous locus of elephant shark and bony fish (10–12). Interestingly, both TLR1-like and TLR2 genes have been duplicated in the chicken genome (13). A shark TLR13 is also identifiable, suggesting that TLR13 appeared early in Gnathostome evolution but was retained only in certain vertebrates such as some Osteichthyes and mammals (10, 14, 15) (**Figure 1**). TLR13 is a member of the TLR11 family, also consisting of fish TLR19–22 and TLR26 (16, 17). These additional fish members of the TLR11 family are not found in sharks, suggesting they may have diverged from the common ancestor with TLR13. It is worth noting that TLR21 exists in birds, amphibians, and bony fish, and avian TLR21 serves as a functional homolog to mammalian TLR9, sensing microbial CpG DNA (13, 16, 18). Some of the TLR family members have been extensively expanded in teleost fish due to the additional whole genome duplications (WGDs), with up to 19 copies identified in some species (16, 18, 19). In addition, the cytosolic sensors activating IFN genes appear to be present in early Gnathostomes.

Interferons

Type I and II IFN genes, but not type III (also termed IFN-λ), have recently been identified in the elephant shark (3, 20). As in tetrapods, type I IFNs exist as multiple copy genes, while type II IFN is encoded by a single gene. The three type I IFN genes identified in elephant shark are tandemly clustered in the same genomic locus that accommodates the growth hormone and CD79 genes (3), a synteny also seen in bony fish (**Figure 2**). The single copy type II IFN (IFN-γ) resides next to the interleukin

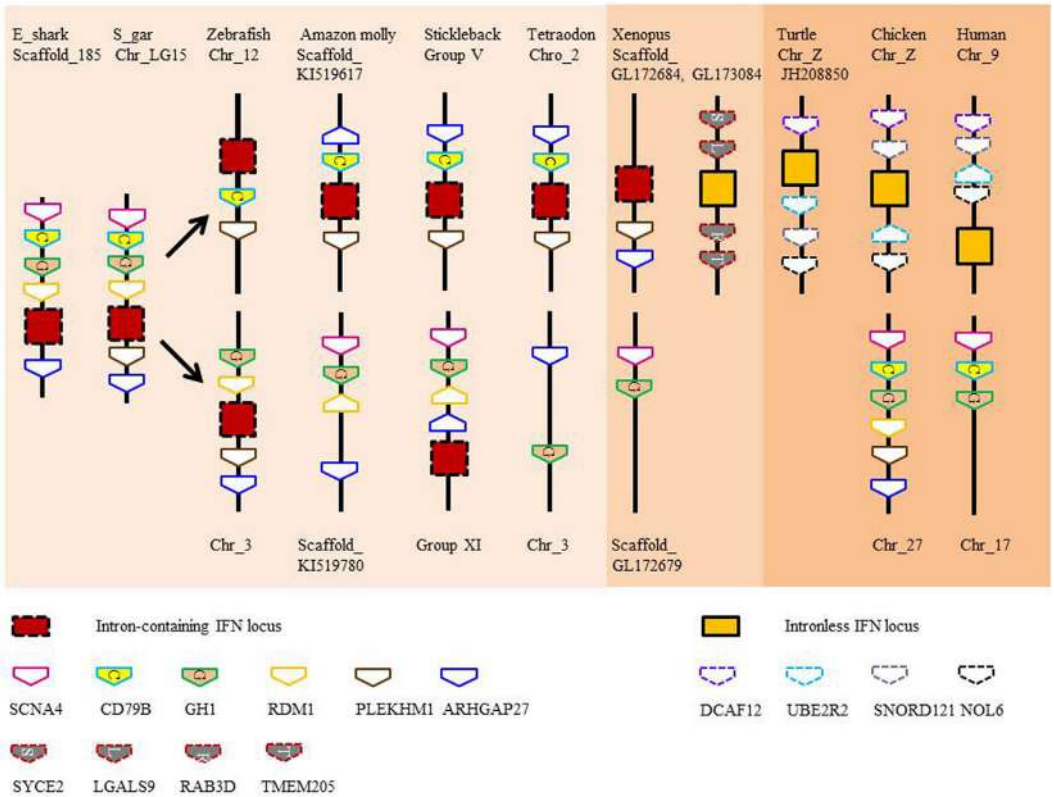

FIGURE 2 | **Gene synteny of type I interferon (IFN) loci in vertebrates**. Note that IFN genes with 2 exons and 1 intron are present in the locus containing intronless genes in scaffold_GL173084 in *Xenopus*.

(IL)-22 gene in the elephant shark genome and is located between the MDM1 and DYRK2 gene (3). This chromosomal arrangement has not changed during the evolution of Gnathostomes although additional duplicated homologs can be seen in this locus in some lineages, as with the so-called IFN-γ related (IFN-γrel) gene (see below) and IL-26 gene (21). When modeled against available crystal structures, the shark type I and II IFNs are predicted to comprise multiple α-helices (unpublished data) as is typical of molecules belonging to the IL-10 family (22). Type III IFNs have not been reported in bony fish to date but are present in all tetrapod groups (23–25). This finding hints at a later appearance of these genes during vertebrate evolution or the loss of these genes in the bony fish lineages. However, the quality of the genome sequences and/or a fast divergence rate, the latter known to contribute to the low sequence homology seen in tetrapods, may have hindered their discovery (3, 25).

Receptors

A complete set of IFN receptors for interaction with IFN ligands are also present in the elephant shark (3). Three putative receptors for type I IFNs, including two copies of IFNAR2/CRFB1–3 and a single copy of IFNAR1/CRFB5, have been reported in the genome cluster also containing the IL-10R2 and IFN-γR2 genes. The expansion of IFNAR2 seems to be common in lower vertebrates, as evidenced in teleosts where up to four copies can be found (26, 27). There are also structural differences of IFNAR1/CRFB5 between fish and tetrapods, notably in the extracellular region where fish IFNAR1/CRFB5 has two rather than four tandem fibronectin-like domains that interact with the IFN ligands and are critical to dictate the actions of individual type I IFNs (4, 7, 27–29). Since the three fibronectin-like domains near the N-terminus (subdomains 1–3) of IFNAR1 are known to be involved in direct binding to the receptors in mammals (30), it will be interesting to determine how fish type I IFN ligands interact with their IFNAR1/CRFB5 receptor. Although the IFN-λ gene has not been found, the existence of its receptor in elephant shark (3) supports the notion that IFN-λ may be present in cartilaginous fish and evolved early.

Regulation

The IFN pathways are coordinated by intracellular signaling molecules. Most of these signaling molecules, including IFN regulatory factors (IRFs), Janus kinases (JAKs), signal transducer and activator of transcription (STAT) proteins, protein inhibitors of activated STAT (PIAS), and suppressors of cytokine signaling (SOCS), are present in cartilaginous fish (**Figure 3**), as well as in Osteichthyes. Among the IRFs, IRF3 and IRF7 are key regulators for initiation of IFN expression, while IRF4 and IRF8 have opposite roles to inhibit or shutdown the IFN response when viruses are cleared from the host. JAKs, STAT1/2, and IRF9 are essential

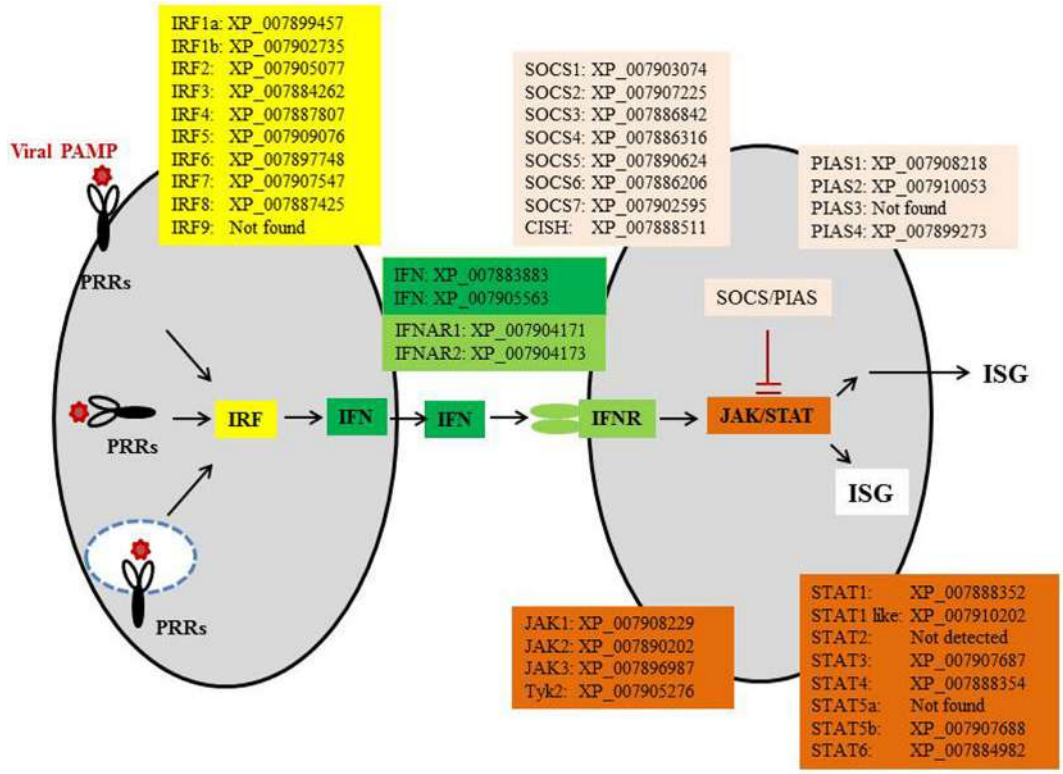

FIGURE 3 | **The IFN pathway of elephant shark**. CISH, cytokine-inducible SH2-containing protein; IFN, interferon; IFNAR, IFN alpha receptor; IRF, IFN regulatory factor; ISGs, IFN-stimulated genes; JAKs, Janus kinases; STAT, signal transducer and activator of transcription protein; PIAS, protein inhibitors of activated STAT; PRR, pattern recognition receptor; SOCS, suppressors of cytokine signaling.

for IFN signaling, which is in turn negatively regulated by PIAS and SOCS. Homologs of these factors can be traced back to the invertebrates where they have a diverse range of physiological roles in addition to antiviral immunity (31, 32). For example, in fruit fly (*Drosophila melanogaster*), JAK/STAT proteins have been shown to be involved in immune responses to viral and bacterial infections (33, 34). These signaling factors have undergone expansion during the two rounds (2R) of WGDs to provide necessary regulation for the IFN system as it emerged in early jawed vertebrates.

IFN GENE STRUCTURE

Molecules within the type II cytokine gene family generally have a 5 exon/4 intron gene organization, as seen in the IL-10 family genes. Within the IFN genes, this gene organization can vary, with examples of intron loss/exon fusion as well as the appearance of intronless genes *via* retrotransposition events (**Figure 4**). One of the benefits of intronless IFN genes is that they do not require RNA intron splicing for synthesizing functional proteins, hence saving time and energy and eliminating the RNA processing step, which could be targeted by viruses. However, whether this provides a selective advantage still needs to be determined.

This ancestral gene organization of 5 exons/4 introns is seen in type I IFNs in bony fish and in some of the amphibian genes. However, in cartilaginous fish, the IFN genes have a 4 exon/3 intron organization, with apparent loss of intron 3. In amphibians both intron-containing and intronless genes are present (35, 36), while in amniotes (reptiles, birds, and mammals) only the intronless genes are present, with apparent loss of the intron-containing genes. The retrotransposition event that gave rise to the intronless type I IFN genes is thought to have occurred independently in amphibians and amniotes (**Figure 5**) and highlights the propensity of IFN genes to undergo this phenomenon. Similarly, the type III IFN (IFN-λ) genes have retained the 5 exon/4 intron organization in tetrapods but can also be found as intronless genes in amphibians and mammals, with most being IFN-λ1 variants in the latter case (37). However, many intron-containing type III genes have an additional intron in the upstream region of the start codon. The type III genes have not been found to date in fish, but the presence of the type III receptor genes (IFN-λR1 and IL-10R2) in cartilaginous fish suggests that they exist/existed in this vertebrate group, and also appeared early in vertebrates as predicted from the above model of IFN gene evolution. Lastly, the type II (IFN-γ) genes have a universal 4 exon/3 intron organization from cartilaginous fish to mammals, with loss of the canonical third intron (37).

The Retrotransposition of Type I and III IFN Genes

Evidence suggests that there were two independent retrotransposition events that led to the appearance of intronless IFN genes in

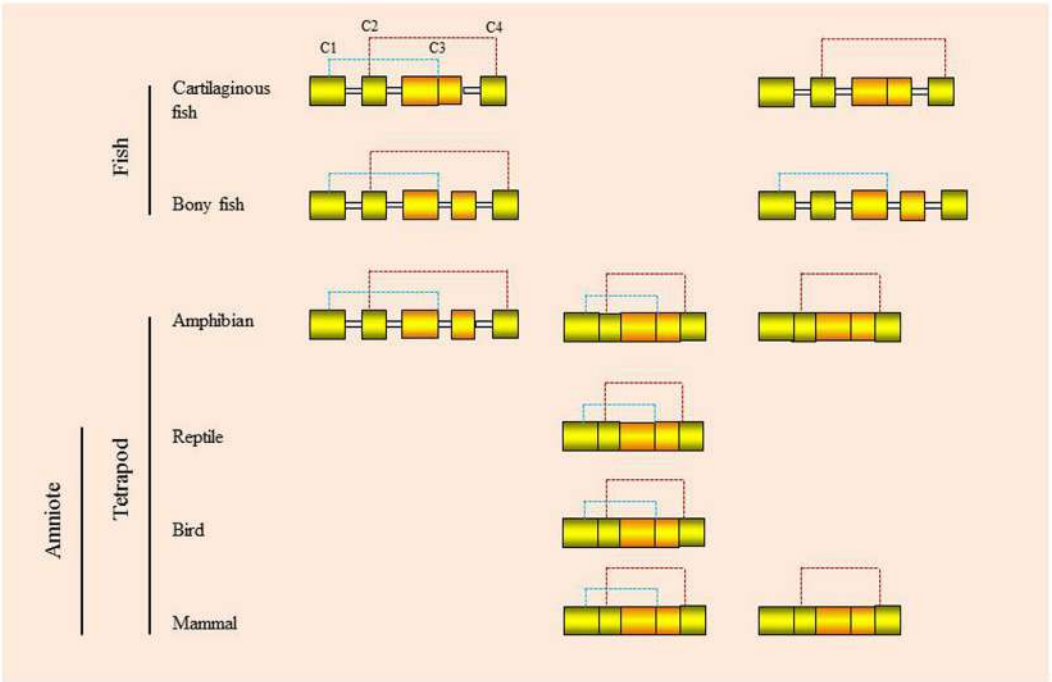

FIGURE 4 | **Genomic organization and putative disulfide bonds of vertebrate type I interferons**.

amphibians and amniotes (**Figure 5**). It is widely believed that one took place in the amniotes, resulting in the insertion of the IFN transcript into the genome locus containing the genes encoding DCAF12 (DB1 and CUL4 associated factor 12), SNORD121A (small nucleolar RNA, C/D box 121A), NOL6 (nucleolar protein 6), and UBE2R2 (ubiquitin conjugating enzyme E2 R2) (**Figure 2**). However, the identity of the primordial gene whose transcript was involved in the retrotransposition is not clear. Phylogenetic tree analysis suggests that it could have been transcribed from the ancestral gene that gave rise to the IFN1 (accession number: BN001167) or IFN2 (accession number: BN001168) in *Xenopus tropicalis* as they have a much closer relationship with the IFN homologs in amniotes than is seen with other *Xenopus* IFN genes (unpublished data). The IFN transcript utilized in retrotransposition probably had to be expressed in the gonad, making it possible to be integrated into the germline. The appearance of the intronless IFN genes in amniotes may be linked in some way to deletion of the locus containing all the clustered intron-containing IFN genes. The timing of this event is not known but could have occurred simultaneously with the retrotransposition event or sometime later.

Several recent studies demonstrate that a retrotransposition event has also happened in amphibians, independent from the one that occurred in the ancestor of amniotes (35, 36, 38). In both *X. tropicalis* and *X. laevis*, a large number of intronless type I IFN genes have been reported in addition to the intron-containing IFN genes, with 32 (Chr. 03 of *X. tropicalis*) and 26 (Scaffold 20 and Chr. 3L of *X. laevis*) intronless type I IFNs identified in each species (36). In addition, multiple type I IFN genes containing a single intron are present in the intronless IFN gene cluster in both species [(36)—although the present authors were unable to verify this] and potentially reflect retrotransposed genes that have acquired an intron. The mechanisms leading to the remarkable diversification of type I IFNs in amphibians are unclear and are postulated to involve multiple processes including polyploidization, chromosomal duplication, local gene duplication, and retrotransposition (36). These findings are fascinating and highlight the diversity of type I IFN genes in amphibians and the complexity of IFN evolution in the vertebrates. Phylogenetic tree analyses indicate that the intronless IFN genes identified in *X. tropicalis* form a clade with the IFN3–5 molecules (accession numbers: BN001169-711) (35), suggesting that the frog intronless IFN genes may be originated from the transcripts of these genes that seem to differ from the putative ancestral genes (IFN1 and IFN2) giving rise to the intronless genes in amniotes.

The retrotransposition events appear to have had profound impacts on the evolution of type I IFN genes in vertebrates and raises many interesting questions. Such events also make it difficult to establish orthologous relationships when undertaking comparative analyses of functions between intron-containing fish/amphibian IFNs with their amniote counterparts. Intriguingly, the intron-containing and intronless type I IFN genes are regulated in a similar manner, governed by the activation of a panel of conserved PRRs (i.e., TLRs and RIG-I family) and IRFs (i.e., IRF3 and IRF7) (6, 7). For example, the binding sites of IRF3 and IRF7 are present in the promoter regions of both intron-containing and intronless type I IFN genes. How the regulatory mechanisms of type I IFN responses have evolved after the retrotransposition

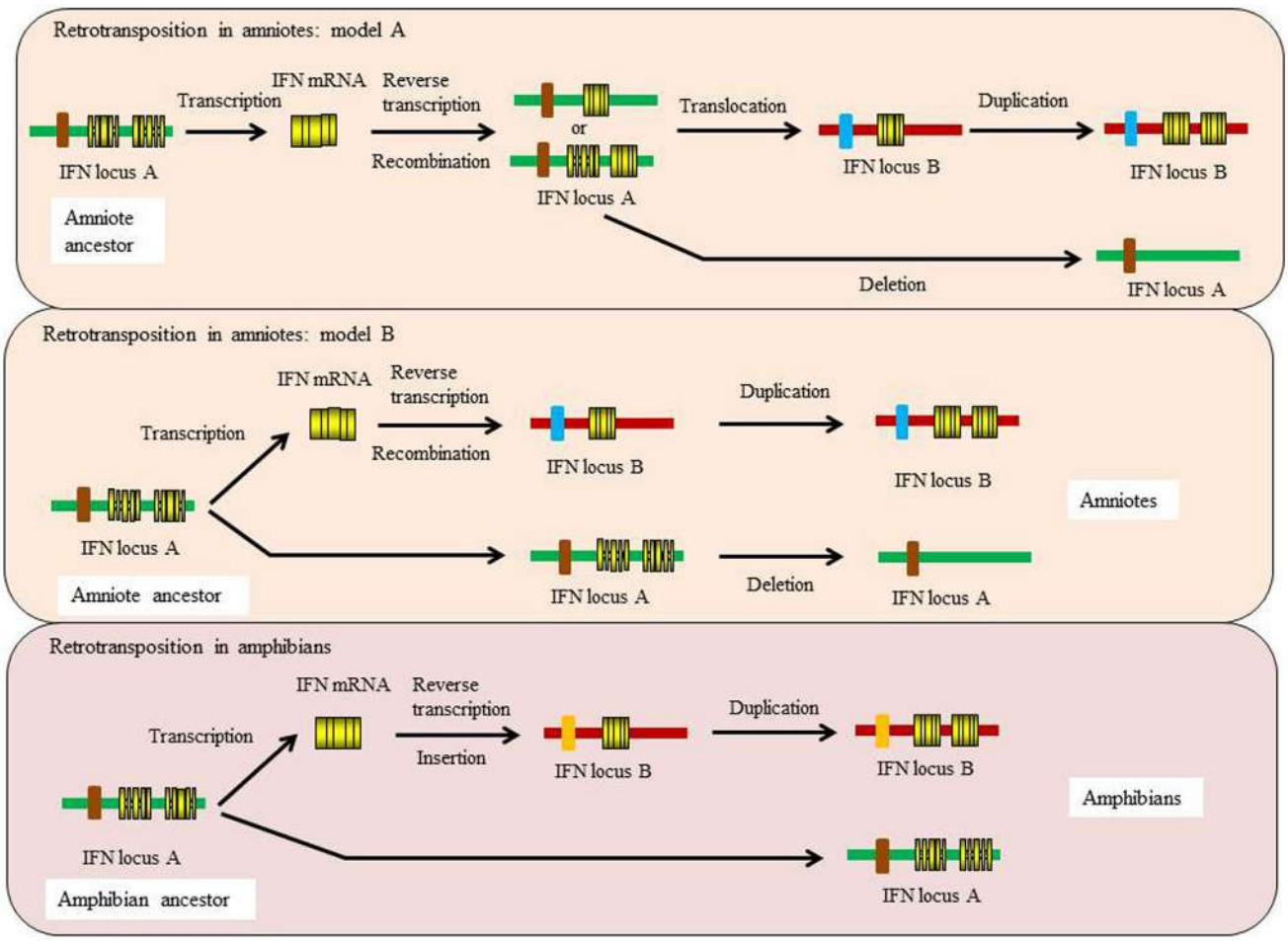

FIGURE 5 | Proposed models for the independent retrotransposition events of type I IFN genes in amphibians and amniotes.

events remains a mystery. Krause proposed a model where the coding exon region (excluding 5′ and 3′ untranslated regions) could be replaced with a double-stranded DNA molecule that is reverse-transcribed from the IFN transcript, leaving the promoter region unchanged (37). It is also possible, as proposed previously, that the DNA recombination could have replaced the entire IFN locus, somehow retaining the promoter region of the most upstream IFN gene, which would contain the necessary regulatory elements (20, 25). Such an event could have taken place prior to the migration of the intronless gene into an alternative locus during genome reshuffling, with subsequent expansion of the intronless genes at this site during evolution (**Figure 5**).

Retrotransposition has also been detected for the type III IFN genes in amphibians and several mammalian species but not in reptiles and birds (36, 39). This led to the integration of intronless type III IFN genes in the genomes. It has been hypothesized that the retrotransposition events are unrelated and could have occurred independently in various lineages during evolution. An interesting observation is that the intronless type III genes are usually associated with retrotransposons in the genome of both amphibians and mammals (36, 37), which have also been speculated to lead to the remarkable expansion of type I IFN genes in rainbow trout (40). In *X. laevis*, two intronless type III IFN genes are located in a region in Chr. 3L that also contains the intronless type I IFN (36). The two genes are constitutively expressed in kidney, skin, and stomach and can be upregulated in a kidney-derived cell line (A6) by polyI:C and infection with swine influenza virus (TX98 strain), suggesting that they are biologically active in regulating antiviral defense in this species. Similar to amphibians, some mammals possess functional intronless type III genes, but they have not been expanded as much as seen for type I IFN genes.

Alternative Splicing

The presence of introns allows the potential for alternative splicing, and in some of the teleost type I IFN genes, this can occur at the 5′ end of the transcript (41, 42). This has been shown to generate intracellular forms of the type I IFN molecule that can elicit IFN signaling and induction of ISG expression *via* intracellular IFN receptors (29), as a unique means to combat viral infection. In rainbow trout, the recombinant proteins of the two intracellular forms of type I IFNs generated from a single IFN

gene (belonging to the IFN-a subgroup) by alternative splicing have been shown to possess similar functions to the secreted IFN and are able to trigger Mx gene expression in a fibroblast cell line (RTG-2 cells) and protect cells against viral infection. In HEK293 cells with over-expressed intracellular type I IFN and its putative intracellular receptors, induced phosphorylation of STAT1 and STAT2 occurs, suggesting an intracellular IFN system mimicking the actions of secreted type I IFNs exists to be deployed for defending host cells against viral infection (29). Production of intracellular type I IFNs does not require secretion, hence reducing the time and energy for the synthesis in the infected cells, especially at the very early stage of infection, to establish an activated antiviral state. In addition, the intracellular IFN system could provide advantages for the host cells to avoid viral blocking of the IFN secretion pathway and interference of extracellular factors on activation of membrane receptors.

IFN GENE/PROTEIN DIVERSITY

Multiple genes are commonly present for both type I and type III IFNs. In mammals the large number of type I IFN genes present can be grouped into subtypes, namely α, β, κ, ε, ω/τ, and δ/ζ. Large numbers of type I IFN genes are also present in teleost fish and amphibians, mainly of intronless forms in the latter case. Most of the encoded mammalian IFN proteins have four conserved cysteines (4C), but some possess only two cysteines (2C), as seen with IFN-β and IFN-ε. 4C-containing IFNs are also seen in fish (cartilaginous and bony), amphibians, reptiles, and birds and are thought to represent the ancestral form. Nevertheless, 2C forms of the IFN protein are also seen in cartilaginous and ray-finned bony fish (i.e., not in lobe-finned bony fish—coelacanth) and amphibians, but the pair of cysteines that is retained can differ (**Figure 4**). Thus, in mammals, amphibians, and cartilaginous fish, it is cysteine 2 and 4 that are retained, while in the 2C subgroups in ray-finned bony fish (holosteans and teleosts), it is cysteines 1 and 3 (20, 28). Interestingly, a recent teleost fish IFN subgroup (termed IFNh) has been described in several perciforme species that groups with the 2C clade but has six cysteines, two of which are aligned in the same position as those in the bony fish group I (2C) type I IFNs (43). Curiously, the perciforme IFNh proteins have an elongated region of approximately 20 aa at the C-terminus and possess similar antiviral functions to the perciforme IFNd previously reported (44, 45). In reptiles and birds only the 4C IFN proteins are known to date, supporting the concept that the 4C form was ancestral and that the 2C forms evolved independently in cartilaginous fish, ray-finned fish, amphibians, and mammals, in the latter two groups following retrotransposition events.

IMPACT OF WGD IN TELEOST FISH

The type I IFN locus present in so-called 2R fish (i.e., the gar—a holostean) has both 4C and 2C genes present in a single genomic locus (**Figures 2** and **4**), and so the ancestor of teleost fish had as a minimal locus one gene of each (4). Hence when the type I IFN locus was duplicated in teleost fish as a consequence of the WGD event that took place at the base of this lineage, two loci were generated as apparent today in species such as zebra fish (28) and stickleback (**Figure 2**). In zebra fish, it is hypothesized that subsequent gene expansion and loss has meant that one locus now has 2 × 4C genes and one 2C gene, while the second has a single 2C gene (4).

Similarly, while there is a single gene for IFN-γ in most vertebrate groups, in salmonids and cyprinids two genes are present (42, 46) likely due to the WGD events that have occurred independently in these fish lineages. Relatively few comparative studies have been performed of the two paralogs (IFN-γ1 and IFN-γ2), but in general they show near identical tissue expression profiles in healthy fish, as seen in homozygous rainbow trout (42). Similarly, following *in vitro* or *in vivo* stimulation, the expression kinetics are typically similar in trout. Thus, following *in vitro* stimulation with polyI:C (42) or rIL-12 (47) both are upregulated with similar kinetics/doses, as is also seen *in vivo* following DNA vaccination [infectious hematopoietic necrosis virus (IHNV) G protein] or infection with IHNV (42). However, the magnitude of upregulation is often higher for IFN-γ2. In contrast, infection with *Saprolegnia parasitica* or *in vitro* stimulation with rIL-4/13 (a Th2 type cytokine in fish) results in down-regulation of both paralogs (15, 48). In cyprinids, the paralogs also have similar expression profiles in healthy tissues, with the exception of gills where IFN-γ1 is dominant (49), and both show antiviral activity when added to GTS9 cells 24 h prior to crucian carp hematopoietic necrosis virus infection (46). They also both have increased expression in scales/epidermis with progression of graft rejection following scale transplantation (50), in kidney cells from allograft-sensitized fish incubated *in vitro* with appropriate allogeneic cells, and following LPS or PHA stimulation of kidney leukocytes *in vitro* (49). Thus, both paralogs of IFN-γ in these species appear to be biologically relevant and have similar regulatory mechanisms.

IFN-γ RELATED

While a single type II IFN gene exists in cartilaginous fish, most bony fish (with the exception of salmonids/cyprinids) and amniotes, in some bony fish a second type II gene is present (21, 51, 52). Since it has not been found in gar and coelacanth, it appears to be a teleost-specific tandem duplication. The gene has been termed IFN-γ related (IFN-γrel) (53) since it has relatively low homology to IFN-γ in the same species, and BLAST analysis does not retrieve IFN-γ genes from other vertebrate lineages. However, it does appear to be a type II IFN since it is adjacent to the authentic IFN-γ gene in the genome, has the same gene organization as IFN-γ, and BLAST analysis does retrieve teleost fish IFN-γ molecules. Initial analysis of the sequence revealed that it was truncated at the 3′ end, such that the translated protein apparently lacks a C-terminal nuclear localization signal (NLS) necessary for IFN-γ function, in teleost fish as in other vertebrates (54). However, subsequent studies in ginbuna crucian carp have found that two isoforms of IFN-γrel exist in carps, with the type called IFN-γ-rel1 containing a form of NLS that can translocate GFP into the cell nucleus (i.e., as GFP-KHHHR) (55). His-tagged recombinant IFN-γrel1 protein can also translocate to the nucleus of GTS9 cells after addition to the culture medium,

as detected by Western blot analysis of nuclear proteins, unlike the IFN-γrel2 protein and strongly suggests these two types of IFN-γrel signal through different intracellular pathways. Studies of IFN-γrel bioactivity in ginbuna crucian carp have revealed that both forms have antiviral activity and are functional as monomers, in contrast to IFN-γ that is a homodimer (55, 56). *In vitro* studies of IFN-γrel in Rohu (a rel1 with a partial NLS) and in goldfish (IFN-γrel2) have shown it can induce IFN-γR and iNOS expression in cultured leukocytes, with additional effects seen on IL-1β, TNF-α, IL-8, and ceruloplasmin expression in goldfish cells (52, 57). Stimulation of tetraodon spleen and head kidney cells *in vitro* with IFN-γrel2 (termed IFN-γ1 in this paper) enhanced their nitric oxide responses and expression of ISG15 (58). Injection of IFN-γrel2 into Japanese pufferfish has been shown to increase phagocyte function in terms of phagocytosis and ROS production, and IFN-γ expression in head kidney cells 1 day post-injection, with a longer term effect seen on IL-6 and IL-12p35/IL-12p40 gene expression (59). It is clear that IFN-γrel is an important immune molecule within the immune system of teleost fish.

IFN RECEPTORS

Six receptor molecules are known to interact with type I, II, and III IFNs in mammals. Although existing as multiple isoforms, type I IFNs bind to the same protein complex consisting of two subunits of the receptor chains IFNAR1 and IFNAR2. Similarly, the type II IFN (IFN-γ) signals through a receptor composed of IFN-γR1 and IFN-γR2, and all the type III IFNs share the same receptor complex of IFN-λR1 (IL-28R1) and IL-10R2. The genes encoding these receptors are found in three genome loci where synteny of these genes has rarely changed during Gnathostome evolution (26, 27). The IFNAR1, IFNAR2, IFN-γR2, and IL-10R2 genes are clustered in a single region except in zebrafish where the IFN-γR2 gene is located in Chr. 9, while the genes encoding IFNAR1, IFNAR2, and IL-10R2 are in Chr. 5 (27). The IFN-γR1 gene is linked with IL-20Ra and IL-22Ra2, which are flanked by the genes encoding Olig3 and SLC35d3 (26, 60, 61). Lastly, the IFN-λR1 gene found in elephant shark and tetrapods resides next to the IL-22Ra1 gene in the genome. It has not been identified in bony fish where the IFN-λ gene is thought to have been lost.

While few receptor-binding studies have been performed out with the mammals, interestingly, in teleost fish type I IFNs bind to distinct receptors in stark contrast to the findings in mammals. While a single IFNAR1 is present in most species (such as zebra fish and tetraodon), multiple forms of IFNAR2 exist, generated by local gene duplications. In zebrafish, it has been shown that the two IFNAR2s (CRFB1 and CRFB2) preferentially bind to group I (containing two cysteines) and II (containing four cysteines) type I IFNs, respectively (28). In Atlantic salmon, interaction of type I IFNs with the receptors is even more complex since both IFNAR1 and IFNAR2 have been multiplied, with four copies of each identified at two different chromosomes (Chr. 21 and 25); namely salmon CRFB5a, 5b, 5c, and x are homologs of IFNAR1, while CRFB1a, 1b, 2, and 3 are homologs of IFNAR2. It has been speculated that the increased copies of IFNAR1 and IFNAR2 are due to the salmonid-specific WGD. Binding to the different IFNAR1 isoforms by the IFN subgroups is possible and may allow differential cellular signaling. For example, salmon IFN-c binds CRFB5a or CRFB5c, while IFN-b may signal through a receptor with CRFB5x (27).

In addition to the above differences in gene number and ligand binding, the protein structure of fish and tetrapod IFNAR1 displays a striking difference. Fish IFNAR1 homologs have only two predicted fibronectin domains in the extracellular region, while tetrapod IFNAR1 possess four fibronectin-like domains, possibly due to a domain duplication that occurred in the tetrapod ancestor. It is worth noting that the structural change of the receptor likely took place before amphibians diverged from the main vertebrate lineage, preceding the IFN retrotransposition events (including those in amphibians). In mammals, all four fibronectin domains are shown to be involved in receptor binding. With only two such domains, how fish type I IFNs form a complex with the receptors is a mystery, especially as crystal structural analyses indicate that fish type I IFNs are structurally similar to that of their mammalian homologs, consisting of six α-helices (22).

As with type I IFNs, in teleost fish, the two members of the type II IFN family that are present (IFN-γ and IFN-γrel) appear to interact with different receptors. In zebrafish, which have a single copy of IFN-γR2/CRFB6, both IFN-γ and IFN-γrel have been shown to induce expression of downstream genes through CRFB13 and CRFB17, respectively (61, 62). However, a recent study demonstrates that tetraodon IFN-γ binds equally to both CRFB13 and CRFB17 expressed in transfected COS cells (63), with weaker binding of IFN-γrel to CRFB13 than to CRFB17. Some cyprinid and salmonid species possess duplicated copies of IFN-γ, IFN-γrel, and the receptor chains, making determination of the pairing relationships between ligands and receptors complicated. For example, two copies of IFN-γR2/CRFB6 as well as IFN-γ and IFN-γrel have been described in Atlantic salmon, rainbow trout, and ginbuna crucian carp (27, 42, 46). In ginbuna crucian carp, it has been shown that the two IFN-γ paralogs exhibit specific binding to different receptors (46). Interestingly, elephant shark also has two copies of the IFN-γR1 gene, which are tandemly arranged in the genome, one of which has a short intracellular region containing well-conserved binding motifs for JAK1 and STAT1. Whether these IFN-γ receptors are functional remains to be investigated.

CONCLUSION

We have learnt a lot about IFN and IFN receptor genes throughout the jawed vertebrate classes, in large part due to the sequencing of the genome of increasing numbers of species. While functional studies lag behind in many cases, studies in fish (especially teleosts) have demonstrated their important role in antiviral defense in early vertebrates as seen in mammals. It is clear that IFN genes have undergone extensive expansion in many lineages, in some cases associated with the generation of intronless genes following retrotransposition, and in other cases following WGD events. The protein cysteine pattern appears to define IFN types in most vertebrate classes, with loss of cysteine 1 and 3 having apparently occurred independently in cartilaginous fish, amphibians, and

mammals. The loss of cysteines 2 and 4 in ray-finned fish appears unique and demonstrates the plasticity of the IFN molecule. It is likely a few surprises regarding IFN gene function in different vertebrate groups are still to be uncovered.

AUTHOR CONTRIBUTIONS

Both authors have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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IFN-λ Inhibits Drug-Resistant HIV Infection of Macrophages

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Type III interferons (IFN-λs) have been demonstrated to inhibit a number of viruses, including HIV. Here, we further examined the anti-HIV effect of IFN-λs in macrophages. We found that IFN-λs synergistically enhanced anti-HIV activity of antiretrovirals [azidothymidine (AZT), efavirenz, indinavir, and enfuvirtide] in infected macrophages. Importantly, IFN-λs could suppress HIV infection of macrophages with the drug-resistant strains, including AZT-resistant virus (A012) and reverse transcriptase inhibitor-resistant virus (TC49). Mechanistically, IFN-λs were able to induce the expression of several important anti-HIV cellular factors, including myxovirus resistance 2 (Mx2), a newly identified HIV post-entry inhibitor and tetherin, a restriction factor that blocks HIV release from infected cells. These observations provide additional evidence to support the potential use of IFN-λs as therapeutics agents for the treatment of HIV infection.

Keywords: IFN-λ, drug-resistant HIV, antiretrovirals, Mx2, tetherin

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INTRODUCTION

Highly active antiretroviral therapy (HAART) has substantially reduced morbidity and mortality in HIV-infected individuals since its introduction in 1996 (1). Although HAART can suppress plasma viral loads to undetectable levels and improve patient life span (2), a substantial fraction of patients fail therapy and/or experience serious side effects from treatment, accompanied by the emergence of drug-resistant viruses (3, 4). More importantly, patients with HIV-1 infection can harbor the virus in latent reservoirs, such as macrophages, one of the key targets of HIV-1 infection. Studies have shown that the intracellular concentrations of antiretrovirals were significantly lower in macrophages than these in T lymphocytes (5, 6). It is known that macrophages play a crucial role in the host defense against HIV-1 infection, as they produce the multiple intracellular HIV restriction factors (7, 8). As HIV-1 latency is the major obstacle in preventing the eradication of the virus, it is necessary to identify agents that can induce intracellular antiviral factors against HIV-1 in macrophages.

Type III interferons (lambda interferons, IFN-λs) or interleukin-28/29 (IL-28/29) display IFN-like activities (9, 10), although they exert their functions through a receptor distinct from type I IFNs (11, 12). IFN-λ subfamily includes three structurally related cytokine members, IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B). IFN-λs could be activated by viral infections or activation of toll-like receptors (13, 14). IFN-λs functionally resemble type I IFNs, the activation of which can trigger antiviral activity *in vitro* (11, 15–18) as well as *in vivo* (19, 20). However, unlike type I IFNs that have receptors expressed on many cell types, including the cells in brain, the expression pattern of IFN-λ receptors is more limited to specific cell types (17, 21–24). Thus, IFN-λs have fewer side effects than type I IFNs. The clinical importance of IFN-λs as novel antiviral

therapeutic agents has recently become apparent. Several studies (12, 25–27) reported that the endogenous IFN-λ system is associated with treatment-induced clearance of hepatitis C virus (HCV). Furthermore, pegylated IFN-λ works as well as pegylated IFN-α for treating chronic hepatitis C (28–31), but with less side effects in several clinical trial studies. While it has been reported that IFN-λs could inhibit HIV replication in macrophages (17, 18) and $CD4^+$ T cells (32), it is unclear whether IFN-λs can inhibit HIV infection with drug-resistant strains. In the present study, we investigated the antiviral effect of IFN-λs on antiretroviral-drug-resistant HIV strains in primary human macrophages. We also determined whether IFN-λs have synergistic effect on anti-HIV activity of antiretroviral drugs in infected macrophages.

MATERIALS AND METHODS

Monocyte and Macrophage Culture

Purified human peripheral blood monocytes were purchased from Human Immunology Core at the University of Pennsylvania (Philadelphia, PA, USA). The Core has the Institutional Review Board approval for blood collection from healthy donors. Monocytes were plated in 48-well culture plates (Corning CellBIND Surface, Corning Incorporated, Corning, NY, USA) at a density of 0.25×10^6 cells/well or 96-well culture plates (Corning CellBIND Surface, Corning Incorporated, Corning, NY, USA) at a density of 10^5 cells/well in the DMEM containing 10% FCS (33, 34). The medium was half-changed every 2 days. Monocytes differentiated to macrophages after *in vitro* cultured for 5–7 days. We used 7-day-cultured macrophages for experiments of this study.

HIV Strains and Other Reagents

Based on their differential use of the major HIV receptors (CCR5 and CXCR4), HIV isolates are classified to R5, X4, and R5X4 strains (35). HIV Bal strain (R5 tropic), AZT-resistant HIV A012 G691-6 strain (R5X4 tropic) (36) and the antiretroviral drugs (AZT, efavirenz, indinavir, and enfuvirtide) were obtained from the AIDS Research and Reference Reagent Program at NIH (Bethesda, MD, USA). Reverse transcriptase (RT) inhibitor-resistant HIV TC49 strain (R5 tropic) was kindly provided by Dr. David Katzenstein (Stanford University, Palo Alto, CA, USA). Recombinant human IFN-λ1 and IFN-λ2 were purchased from PeproTech Inc. (Rocky Hill, NJ, USA). Recombinant human IFN-λ3 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

IFN-λs and/or Anti-HIV Drug Treatment and HIV Infection

For infection with the resistant HIV strains, 7-day-cultured macrophages (10^5 cells/well in 96-well plates) were incubated with or without IFN-λ1, λ2, or λ3 (100 ng/ml each) and/or anti-HIV drugs: azidothymidine (AZT) 10^{-11}M; efavirenz 10^{-10}M; indinavir 10^{-15}M, and enfuvirtide 10^{-8}M for 24 h. Cells were then infected with different strains of HIV (6 ng p24/well) for 2 h. After washed three times with plain DMEM, cells were cultured with fresh 10% DMEM containing IFN-λs and/or antiretroviral drugs. For HIV Bal infection, culture supernatant was harvested at day 8 postinfection for RT and p24 assays. Infected and untreated cells served as controls. HIV Gag gene expression in infected cells was also examined at day 8 postinfection. For anti-HIV drug-resistant virus (A012 G691-6 or TC49) infection, culture supernatant was harvested for HIV p24 protein by ELISA at days 3, 5, 7, and 10 postinfection. The cell cultures were replaced with the fresh media supplemented with IFN-λ1, λ2, or λ3 and/or the antiretrovirals every 2–3 days. The culture supernatant collected at day 10 postinfection was also subjected to RT assay.

HIV RT and p24 ELISA Assays

HIV RT activity was determined based on the technique (37) with modifications (38, 39). For HIV p24 assay, the cultured supernatant was analyzed ELISA as described in the protocol provided by the manufacturer (Chiron Corp., Emeryville, CA, USA).

RNA Extraction and Real-time RT-PCR

RNA was extracted from cell cultures with Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) as previously described

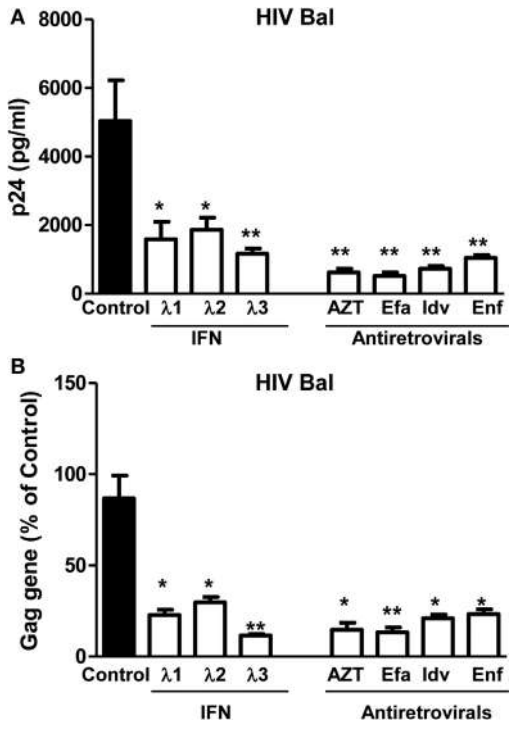

FIGURE 1 | Effect of IFN-λs on HIV (Bal strain) infection of macrophages. Seven-day-cultured macrophages were incubated in the presence or absence of IFN-λs (1, 2, or 3; 100 ng/ml each) or classic antiretrovirals at indicated concentrations (AZT: 10^{-11}M; efavirenz: 10^{-10}M; indinavir: 10^{-15}M; and emfuviride: 10^{-8}M) for 24 h and then infected with HIV Bal strain. HIV-1 p24 production and Gag gene expression was determined at day 8 postinfection. **(A)** The cell culture supernatant was subjected to ELISA assay to detect HIV p24. **(B)** Total RNA from cells was subjected to HIV Gag gene expression by real-time RT-PCR. The data are expressed as RNA levels relative (percent) to the control (without treatment, which is defined as 100%). The results shown are the mean ± SD from three independent experiments with triplicate wells (*$p < 0.05$, **$p < 0.01$, IFN-λ, or antiretroviral vs. control; Efa, efavirenz; Idv, indinavir; Enf, enfuvirtide).

(40, 41). Total RNA (1 μg) was subjected to RTusing the RT system (Promega, Madison, WI, USA) for 1 h at 42°C. The reaction was terminated by incubating the reaction mixture at 99°C for 5 min, and the mixture was then kept at 4°C. The resulting cDNA was then used as a template for real-time PCR quantification. Real-time PCR was performed with 1/10 of the cDNA with the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) as previously described (41–43). The oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA) and sequences will be available upon request. For the Gag gene expression, the specific oligonucleotide primers are listed as follows: Gag gene primer: 5′-ATAATCCACCTATCCC-AGTAGGAGAAA-3′ (SK38) and 5′TTTGGTCCTTGTCTT ATGTCCAGAATGC-3′ (SK39) (44). For the tetherin gene expression, the specific oligonucleotide primers are listed as follows: 5′-AAGAAAGTGGAGGAGCTTTGAGG-3′ (Sense) and 5′-CCTGGTTTTCTCTTCTCAGT-CG-3′ (anti-sense). For the Mx2 gene expression, the specific oligonucleotide primers are listed as follows: 5′-CAGCCACCACCAGGA AACA-3′ (Sense) and 5′-TTCTGCTCGTACTGGCTGTACAG-3′ (anti-sense). The data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, primers are 5′-GGTGGTCTCCTCTGACTTC AACA-3′ for sense and 5′-GTTGCTGTAGCCAAATTCGTT GT-3′ for anti-sense, respectively) and presented as the change in induction relative to that of untreated control cells.

Flow Cytometric Analysis

Cultured macrophages (2.5×10^5 cells/well in 48-well plate) were incubated with or without IFN-λ 1, 2, or 3 (100 ng/ml) for 24 h. Cells were then harvested, washed twice with phosphate-buffered saline containing 1% fetal bovine serum, incubated with PE-conjugated anti-human tetherin (CD317; BioLegend, San Diego, CA, USA) on ice in dark for 30 min. Unstained or isotope-matched mouse immunoglobulin G1-stained cells were included as a negative control. Stained cells were acquired by fluorescence-activated cell sorting (FACSCalibur; BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Western Blotting for Cell Lysates

The expression of the Mx2 and tetherin were evaluated by immunoblot analysis. Following incubation with polyclonal antibodies to Mx2 (Novus, Littleton, CO, USA) or polyclonal rabbit anti-BST-2 (tetherin) serum (AIDS Research and Reference Program,

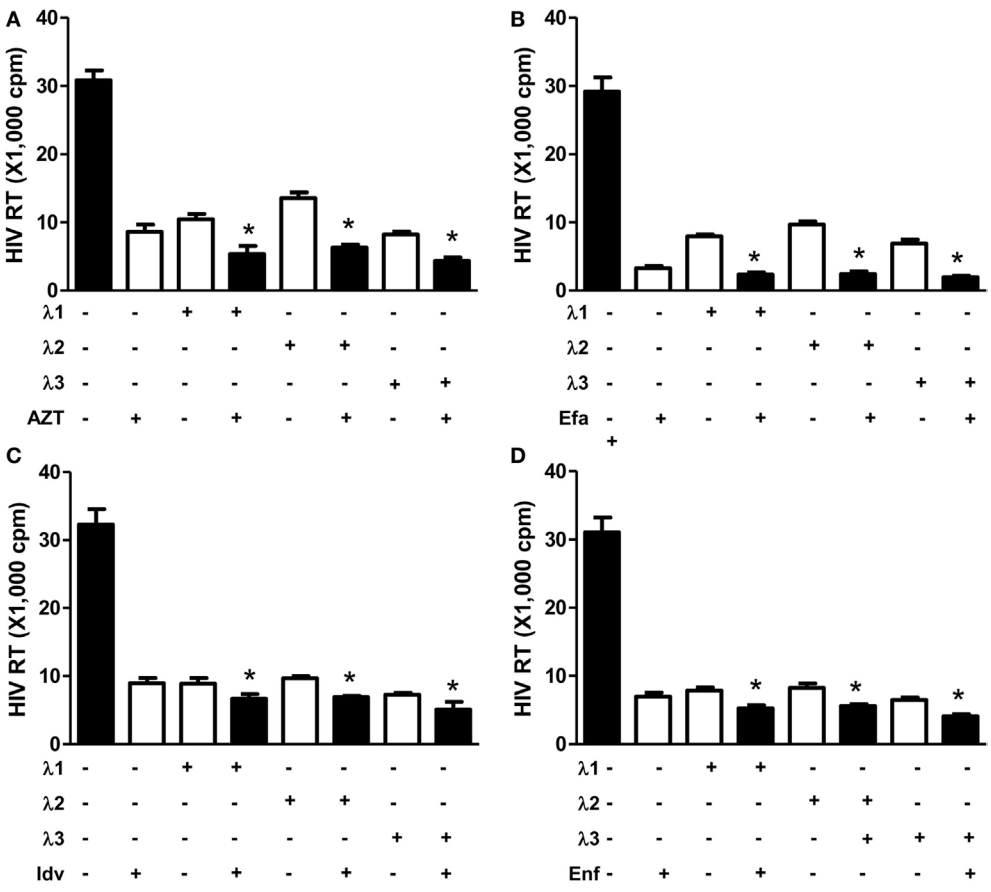

FIGURE 2 | Effect of IFN-λs and/or antiretrovirals on HIV Bal infection of macrophages. Seven-day-cultured macrophages were incubated in the presence or absence of IFN-λs (1, 2, or 3; 100 ng/ml each) or classic antiretrovirals **(A)** AZT: 10^{-11}M; **(B)** efavirenz: 10^{-10}M; **(C)** indinavir: 10^{-15}M; and **(D)** emfuviride: 10^{-8}M) for 24 h and then infected with HIV Bal strain. HIV RT activity was determined at day 8 postinfection. The data shown are the mean ± SD from three independent experiments with triplicate wells (*$p < 0.05$, **$p < 0.01$, IFN-λ+ antiretrovirals vs. antiretrovirals only; Efa, efavirenz; Idv, indinavir; Enf, enfuvirtide).

Bethesda, MD, USA) and extensive washing in PBS containing 0.05% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated IgG (Pierce, Chester, UK) for 1 h at room temperature. The membranes were further washed in PBS. The immunoblots were visualized by enhanced chemiluminescence detection (Amersham, Bucks, UK).

Statistical Analysis

For comparison of the mean of two groups, statistical significance was assessed by Student's *t*-test. One-way ANOVA were used for comparison of result between the different groups (multiple comparisons). All graphs were generated and statistical analyses were performed with GraphPad InStat Statistical Software (GraphPad Software Inc., San Diego, CA, USA), and the data are presented as mean ± SD. Statistical significance was defined as $p < 0.05$.

RESULTS

IFN-λs Enhance Anti-HIV Activity of Antiretrovirals

We first determined the effect of IFN-λs and/or the antiretrovirals (AZT, efavirenz, indinavir, and enfuvirtide) on HIV Bal infection of macrophages. IFN-λs (1, 2, or 3) or the antiretrovirals (AZT, efavirenz, indinavir, and enfuvirtide) significantly inhibited the expression of HIV p24 antigen (**Figure 1A**) and Gag gene (**Figure 1B**) in macrophages. IFN-λs (1, 2, or 3) also enhanced the anti-HIV (Bal) effect of AZT (**Figure 2A**), efavirenz (**Figure 2B**), indinavir (**Figure 2C**), and enfuvirtide (**Figure 2D**).

IFN-λs Inhibit Drug-Resistant HIV Infection of Macrophages

We next examined whether IFN-λs (1, 2, or 3) can inhibit drug-resistant HIV infection of macrophages. While AZT had little effect on AZT-resistant HIV strain (A012) infection (**Figures 3A,C** IFN-λs 1, 2, or 3) potently suppressed infection of macrophages by the AZT-resistant HIV strain (A012) (**Figures 3A,C**). Similarly, IFN-λ 1, 2, or 3) could suppress RT inhibitor-resistant HIV (TC49) infection of macrophages. In contrast, the RT inhibitors (efavirenz) could not inhibit TC49 strain infection of macrophage (**Figures 3B,D**).

IFN-λs Upregulate Tetherin

Tetherin, an important IFN-α inducible cellular restriction factor, has been shown to inhibit HIV infection of host cells by preventing release of virus from an infected cell (45, 46). Thus, we

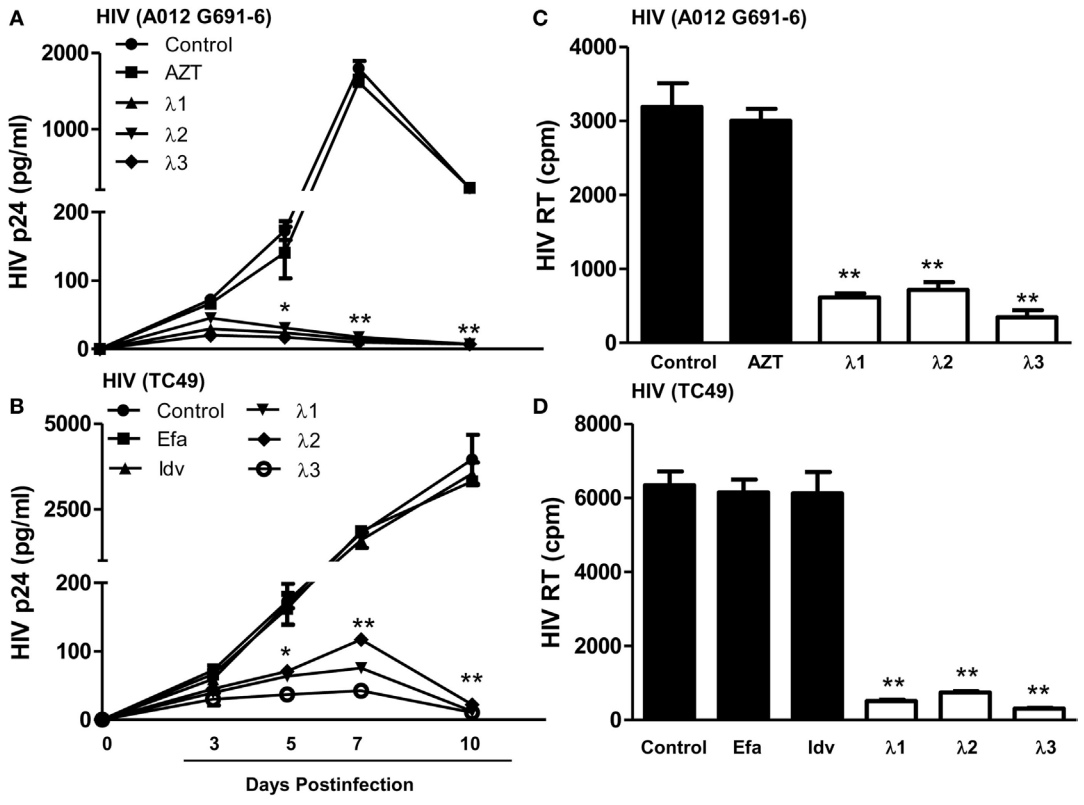

FIGURE 3 | Effect of IFN-λs on drug-resistant HIV infection of macrophages. Seven-day-cultured macrophages were incubated in the presence or absence of IFN-λs (1, 2, or 3; 100 ng/ml each) for 24 h and then infected for 6 h with two drug-resistant viruses (A012 G691-6 or TC49). The HIV p24 antigen **(A,C)** was detected at indicated time points post HIV infection using a commercially available ELISA kit, and HIV RT activity **(B,D)** was assayed from the culture supernatant at day 10 postinfection. The data shown are the mean ± SD from three independent experiments with triplicate wells (*$p < 0.05$, **$p < 0.01$, IFN-λ vs. control; Efa, Efavirenz, 10^{-10}M; Idv, Indinavir, 10^{-15}M; AZT 10^{-11}M).

examined whether IFN-λ treatment of macrophages can induce the tetherin expression. As shown in **Figure 4**, IFN-λ treatment of macrophages significantly increased the tetherin expression at both messenger RNA (mRNA) (**Figure 4A**) and protein (**Figures 4B,C**) levels.

IFN-λs Enhance Mx2

As an IFN-α-inducible cellular factor, Mx2 has recently been identified to inhibit HIV at post-entry level (47–49). Mx2 could abolish capsid-dependent nuclear import of subviral complexes (41–43). We thus examined whether IFN-λs can induce Mx2 expression in macrophages. As shown in **Figure 5**, IFN-λ treatment of macrophages significantly upregulated the Mx2 expression at both mRNA (**Figure 5A**) and protein (**Figure 5B**) levels.

DISCUSSION

To find new antiretroviral agents remains to be an important area of anti-HIV studies. Our earlier studies showed that IFN-λs could inhibit *in vitro* HIV infection/replication (17, 18, 50). IFN-λs are a class of recently identified members of IFN family, including three IFN-λ (lambda) molecules called IFN-λ1, IFN-λ2, and IFN-λ3 (also called IL-29, IL-28A, and IL-28B, respectively) (51). IFN-λs bind to their own distinctive receptor complex, IL-10Rβ and IL-28Rα, which activates janus kinase/signal transducers and activators of the transcription (JAK/STAT) signaling pathway, resulting in the phosphorylation of STAT proteins and forming of IFN-stimulated gene factor 3 complex (11, 15, 52).

While the IL-10Rβ shows a broad expression pattern (53), expression of the IFN-λ receptor subunit IL-28Rα is much more restricted (11, 54, 55). Earlier analysis of the expression pattern of IL-28Rα in human tissues showed that IL-28Rα mRNA levels were highest in the lung, heart, liver, and prostate, while low mRNA levels were detected in the central nervous system, bone marrow, testis, uterus, and skeletal muscle (22, 54, 55). A few immune cells express IL-28Rα especially at the mRNA level (e.g., B cells, macrophages, and plasmacytoid DCs), but conflicting protein expression data are reported in the literature (17, 22, 54–57). Although some evidence indicates that IFN-λ receptor expression on peripheral leukocytes is not functional (22), other evidence shows clear antiviral innate defense in some of these cells, and IFN-λ signals stimulate monocytes and macrophages to produce IL-6, IL-8, and IL-10 (58).

Studies from different investigators have demonstrated that IFN-λs have the ability to inhibit the replication of a number of viruses, including HCV and hepatitis B virus (16, 59), cytomegalovirus (60), Apeu virus (61), herpes simplex virus type 2 (HSV-2) (19), encephalomyocarditis virus (11), vesicular stomatitis virus (60), West Nile virus (62), and dengue virus (63). IFN-λs also had antiviral effect *in vivo* (19, 64, 65). Recent *in vivo* studies with mice showed that IFN-λs had the ability to reduce hepatic viral titer of HSV-2 and completely blocked HSV-2 replication in vaginal mucosa (19). IFN-λs contribute to innate immunity of mice against influenza A virus (66, 67). In addition, their role in direct antiviral effects *in vivo* has also been demonstrated in IL-28RA and STAT1 knockout animals, where a significant increase in influenza A virus replication was

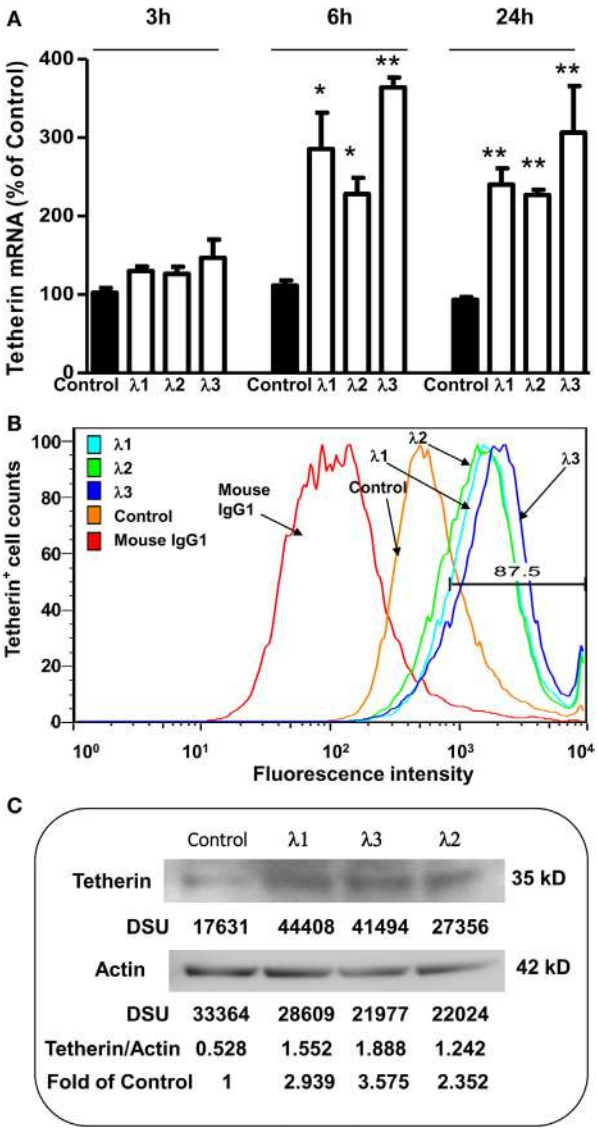

FIGURE 4 | Effect of IFN-λs on tetherin expression. **(A)** Tetherin messenger RNA (mRNA) expression. Seven-day-cultured macrophages were incubated in the presence or absence of IFN-λs (1, 2, or 3; 100 ng/ml) for 3, 6, or 24 h. Total RNA was extracted from cells and then the real-time RT-PCR was performed to determine the induced mRNA expression of tetherin and GAPDH. The data are expressed as mRNA levels for tetherin relative (fold) to the control (without IFN-λ treatment, which is defined as 1). The results shown are the mean ± SD from three independent experiments with triplicate wells (**$p < 0.01$; *$p < 0.05$; IFN-λ vs. control). **(B)** Tetherin protein expression (flow cytometry). Seven day-cultured macrophages were treated with or without IFN-λs (1, 2, or 3; 100 ng/ml each) for 24 h. Cells were stained with fluorescence-conjugated anti-human tetherin (CD317) antibody and analyzed for tetherin expression by flow cytometry. The isotope control is staining with isotope-matched antibody (immunoglobulin G1). A representative histogram graph was shown. **(C)** Tetherin protein expression (Western blot). Seven day-cultured macrophages were treated with or without IFN-λ (1, 2, or 3; 100 ng/ml each) for 24 h. Total protein exacted from macrophages was subjected to Western blot assay using antibody against tetherin and actin. The inserts below the panels show the signal intensities [density scan unit (DSU)] of protein bands of the representative blot, expressed as densitometry scanning units. The results shown are representative of three independent experiments.

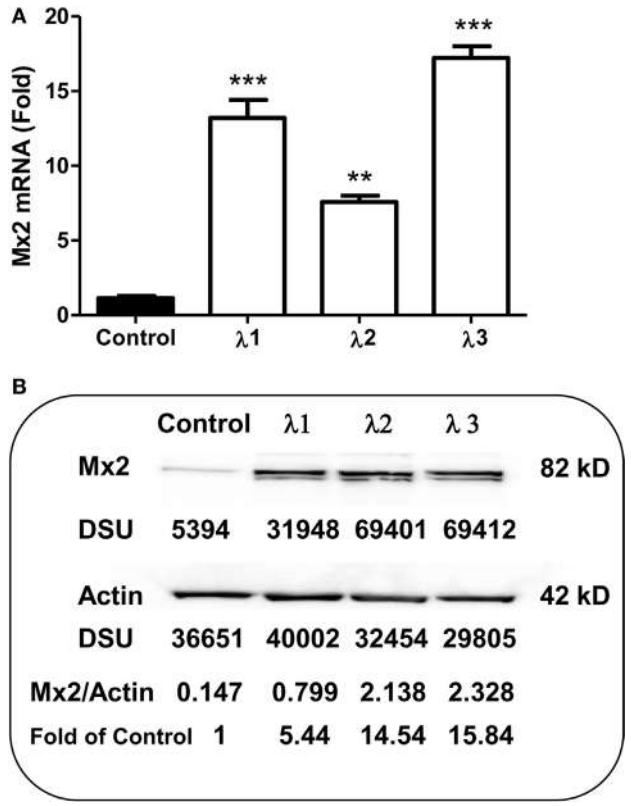

FIGURE 5 | **Effect of IFN-λs on Mx2 expression**. **(A)** Mx2 messenger RNA (mRNA) expression. Seven-day-cultured macrophages were incubated in the presence or absence of IFN-λs (1, 2, or 3; 100 ng/ml each) for 24 h. Total RNA extracted from cells was subjected to the real-time RT-PCR was performed for Mx2 mRNA and GAPDH. The data are expressed as Mx2 mRNA levels relative (fold) to the control (without IFN-λ treatment, which is defined as 1). The results shown are the mean ± SD from three independent experiments with triplicate wells (**$p < 0.01$; *$p < 0.05$; IFN-λ vs. control). **(B)** Mx2 protein expression (Western blot). Seven day-cultured macrophages were treated with or without IFN-λ (1, 2, or 3; 100 ng/ml each) for 24 h. Total protein exacted from macrophages was subjected to Western blot assay using antibody against Mx2 and actin. The inserts below the panels show the signal intensities [density scan unit (DSU)] of protein bands of the representative blot, expressed as densitometry scanning units. The results shown are representative of three independent experiments.

observed (66–68). Others and we have shown that IFN-λs could inhibit HIV infection of CD4+ T cells (32) and macrophages (17, 18, 50). Mechanistically, IFN-λ1 and IFN-λ2 were able to induce the intracellular expression of type I IFN, CC chemokines (the ligands for CCR5), and APOBEC3G/3F, the cellular HIV restriction factors (17). In addition, we demonstrated that IFN-λ3 could induce multiple antiviral cellular factors (ISG56, MxA, OAS-1) (18). We also showed that all three IFN-λs could induce the expression of pattern recognition receptors in macrophages (50). The *in vivo* production of IFN-λ1 also was monitored in HIV-infected patients. Tian et al. found that the plasma IFN-λ1 levels were increased along with the depletion of CD4+ T cells in HIV-1-infected patients, but the elevated IFN-λ1 showed limited repression of viral production (32).

In the present study, we further examined the anti-HIV activity of IFN-λs. We showed that all three IFN-λs not only inhibited drug-resistant virus replication (**Figure 3**) but also enhanced the anti-HIV effect of commonly used antiretrovirals (**Figures 1** and **2**), including zidovudine (AZT, a nucleoside RT inhibitor), efavirenz (a non-nucleoside RT inhibitor), indinavir (protease inhibitor), and enfuvirtide (HIV fusion inhibitor). In addition to the reported mechanisms involved in IFN-λ-mediated HIV inhibition (17, 18, 50): the induction of extracellular factors, e.g., CC chemokines that block HIV entry into macrophages, and the activation of intracellular innate immunity, e.g., the induction of type I IFNs and APOBEC3G/F, we demonstrated that IFN-λ treatment of macrophages induced the expression of tetherin, a cellular factor that can block HIV infection by preventing virus release from infected cells (**Figure 4**). In addition, IFN-λs also enhanced the expression of Mx2 (**Figure 5**), a newly identified HIV post-entry inhibitor that can abolish capsid-dependent nuclear import of subviral complexes (41–43). These anti-HIV cellular factors are the contributors for IFN-λ-mediated anti-HIV activity. These findings in conjunction with our previous observations (17, 18, 50) indicate that IFN-λs are attractive alternative for HIV treatment, as it would be extremely difficult for HIV to develop resistance to IFN-λs that can suppress the virus at various steps of its replication. However, further studies are necessary to determine the impact of IFN-λs on drug-resistant HIV strains in *ex vivo* and *in vivo* systems. These additional studies shall explore the clinical potential for developing IFN-λs-based therapy for HIV/AIDS.

ETHICS STATEMENT

In this *in vitro* study, we obtained primary human monocytes from the Immunology Core at the University of Pennsylvania School of Medicine. The Core has the Institutional Review Board approval for blood collection from healthy donors. Anyone who obtains human cells from the Core is considered as secondary use of de-identified human specimens, which does not subject to human subject review by both NIH and IRB.

AUTHOR CONTRIBUTIONS

XW and W-ZH designed the study. XW, M-QL, YZ, R-HZ, and Y-ZW performed the experiments. J-LL supplied reagents needed for this study. XW, M-QL, and YZ analyzed and interpreted the data and wrote the manuscript. HW, WZ, and W-ZH reviewed and revised the manuscript. All the authors have read, reviewed, and edited the manuscript and agreed for submission to this journal.

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REVIEW
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Type I Interferons as Regulators of Lung Inflammation

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Immune responses to lung infections must be tightly regulated in order to permit pathogen eradication while maintaining organ function. Exuberant or dysregulated inflammation can impair gas exchange and underlies many instances of lung disease. An important driver of inflammation in the lung is the interferon (IFN) response. Type I IFNs are antiviral cytokines that induce a large range of proteins that impair viral replication in infected cells. This cell-intrinsic action plays a crucial role in protecting the lungs from spread of respiratory viruses. However, type I IFNs have also recently been found to be central to the initiation of lung inflammatory responses, by inducing recruitment and activation of immune cells. This helps control virus burden but can cause detrimental immunopathology and contribute to disease severity. Furthermore, there is now increasing evidence that type I IFNs are not only induced after viral infections but also after infection with bacteria and fungi. The pro-inflammatory function of type I IFNs in the lung opens up the possibility of immune modulation directed against this antiviral cytokine family. In this review, the initiation and signaling of type I IFNs as well as their role in driving and maintaining lung inflammation will be discussed.

Keywords: lung, infection, inflammation, type I interferons, pattern recognition receptors

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LUNG INFLAMMATION

Mucosal surfaces such as those found in the intestine and the lungs are the most common targets for invading pathogens. The surface area of the human lung is approximately 70 m^2, and its main function is gas exchange (1). The lung is constantly in direct contact with the environment and the cells of the lung need to be able to tolerate non-harmful stimuli but react appropriately to harmful pathogens. When lung cells respond to invading pathogens, the regulation of inflammation is particularly important since the lung comprises delicate structure crucial for conducting gas exchange.

Interferons (IFNs), discovered in the 1950s, represent a family of cytokines, which induce robust antiviral and immunomodulatory responses to interfere with virus replication and spread (2–4). IFNs can be classified into three main subclasses: type I, II, and III. Type I IFNs consist of several IFN-α isoforms (13 in human and 14 in mice), IFN-β, IFN-ε, IFN-κ, and IFN-ω. In contrast, type II IFNs include only one member, IFN-γ. The most recently discovered group of IFNs are the type III IFNs, including IFN-λ1 (IL-29; non-functional pseudogene in mice), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), and the very recently described human IFN-λ4 (5–7). This review will focus on the role of type I IFNs in the lung, however, many of the effects induced by type I IFNs will also be mediated by the other types of IFNs.

Sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) results in production of many cytokines and chemokines including the type I IFNs. These cytokines have a whole array of functions. First, they elicit an antiviral state in infected and

neighboring, uninfected cells. Second, type I IFNs modulate the immune response by promoting antigen presentation, cytokine production, dendritic cell (DC) and natural killer (NK) cell activity, and macrophage function. Third, they enhance the adaptive immune response by manipulating T cell effector function and antibody production. Overall, the antiviral and immune stimulatory potential of type I IFNs is required for the effective clearance of acute viral infections (2–4, 8). Furthermore, the impact of type I IFNs on the inflammatory response during other types of infections is also starting to be appreciated.

INDUCTION OF TYPE I IFNs

Multiple cell types including leukocytes and structural cells can detect PAMPs, in various cellular compartments and in all tissues. Recognition of PAMPs by the PRRs initiates an intracellular signaling cascade that causes the translocation of transcription factors to the nucleus initiating innate immune gene expression. The PRRs that can couple pathogen detection to type I IFN induction are toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG)-I-like receptors (RLRs), and cGAS/cGAMP/stimulator of IFN genes [stimulator of interferon genes (STING); **Figure 1**].

The endosomally expressed TLRs that induce type I IFN gene expression are TLR-3, -7, -8, and -9, each of which detects different forms of nucleic acids (14). TLR3 recognizes double-stranded (ds) RNA which initiates the TRIF-dependent signaling cascade (15). TLR-7/8 and -9 recognize GU-rich single-stranded (ss) RNA and unmethylated CpG DNA, respectively (9), and they require MyD88 for their signaling. Finally, one cell surface expressed TLR, TLR4, which recognize lipopolysaccharides (LPS), and respiratory syncytial virus (RSV) F protein (16), can induce type I IFNs *via* TRIF-mediated signaling (15) (**Figure 1**).

The three receptors in the RLRs family, RIG-I, melanoma differentiation-associated protein 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) detect viral RNA in the cytosol (10, 11). Due to their localization within the cytoplasm, these receptors are constantly exposed to host RNA, and it is therefore important to distinguish it from that of viral origin. For this reason, the receptors can detect features that are common in many viral genomes and viral replication intermediates but absent from the host (12). RIG-I specifically binds to short uncapped 5′-triphosphate (5′-ppp) and 5′-diphosphate (5′-pp)-bearing base-paired RNA molecules, an RNA motif known to be present in some viral genomes but not in host RNA (17–19). MDA5 binds long stretches of base-paired RNA, which, again, are absent from uninfected cells but often produced as a consequence of viral replication (11). Structurally, RIG-I and MDA5 share many similarities as they both have caspases activation and recruitment domains (CARDs) essential for the downstream signaling (10, 11). LGP2 possesses the RNA-binding domain but it lacks the CARD domains and is therefore not involved in direct signaling. Instead, a role for LGP2 in assisting MDA5-mediated signaling has been suggested (20). For both RIG-I and MDA5, binding of agonists allows downstream signaling through the adaptor molecule mitochondrial antiviral signaling protein (MAVS), leading to the activation of NF-κB and IRF3 and subsequent induction of gene expression of various antiviral mediators including type I IFNs (11, 21, 22) (**Figure 1**).

The first DNA sensor described was DNA-dependent activator of IFN-regulatory factors (DAI), which was shown to induce type I IFNs during *in vitro* infections of fibroblast with either herpes simplex virus 1 or cytomegalovirus (CMV). However, the *in vivo* role for DAI remains unclear (23). Another pathway of recognizing infections with DNA viruses is *via* RNA polymerase III (PolIII), which transforms DNA into a 5′-ppp RNA, the ligand for RIG-I, yet the physiological role of PolIII remains elusive (24, 25). Other proteins belonging to the PYHIN (IFI16; IFN gamma-inducible protein 16, and AIM2; absent in melanoma 2) or DExD/H-box helicase (DDX) protein families were suggested likely DNA sensors, but their specific role *in vivo* is unclear and is being investigated [(26); for an extensive review about DNA sensing, see Ref. (24)]. The discovery of the adaptor protein STING identified a pathway crucial for the recognition of foreign dsDNA *in vivo*. Located at the endoplasmic reticulum, STING activates signaling *via* NF-κB and IRF3, resulting in the production of type I IFNs (27) (**Figure 1**). Recently, guanosine-monophosphate adenosine-monophosphate (cyclic GMP-AMP or cGAMP) synthase (cGAS) was identified as a DNA sensor leading to the downstream activation of STING. cGAS binds dsDNA and catalyzes the synthesis of the second messenger cGAMP from ATP and GTP, which binds to STING and activates the signaling cascade leading to type I IFN production (28–30). Interestingly, cGAMP can be transferred through gap junctions from infected cell to neighboring cells and thereby spread antiviral immunity (31).

Different cells of the lung will respond differently to infections depending on both the tropism of the pathogen (which cells that are infected) and which PRRs that are triggered. Lung epithelial cells are the first and most abundant cell type that will interact with the pathogens, and they have the ability to induce IFN-β production especially after influenza virus infection (32). In addition, plasmacytoid dendritic cells (pDCs) have a constitutive expression of IRF-7, which make them able to respond rapidly to TLR7 ligation and induce type I IFNs (33). This is especially important during influenza virus infection (34). However, during RSV infection, it is the alveolar macrophages (AMs) that are the major source of type I IFNs and they use MAVS-dependent PRRs for sensing the virus (35). Thus, in the lung as well as on other sites, many different pathogens can be recognized by several sets of PRRs expressed on the cell surface, in endosomes, or in the cytosol by different cell types. In combination, this will result in the induction of type I IFNs and an efficient antiviral response.

SIGNALING THROUGH THE TYPE I IFN RECEPTOR

Type I IFNs bind to the heterodimeric transmembrane IFN-α/β receptor (IFNAR), which is expressed on all nucleated cells and composed of the two subunits: IFNAR1 and IFNAR2 (36) (**Figure 2**). *Via* signaling through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, the induction of several hundreds of interferon stimulated genes (ISGs) is initiated (36–40) (**Figure 2**). These ISGs interfere

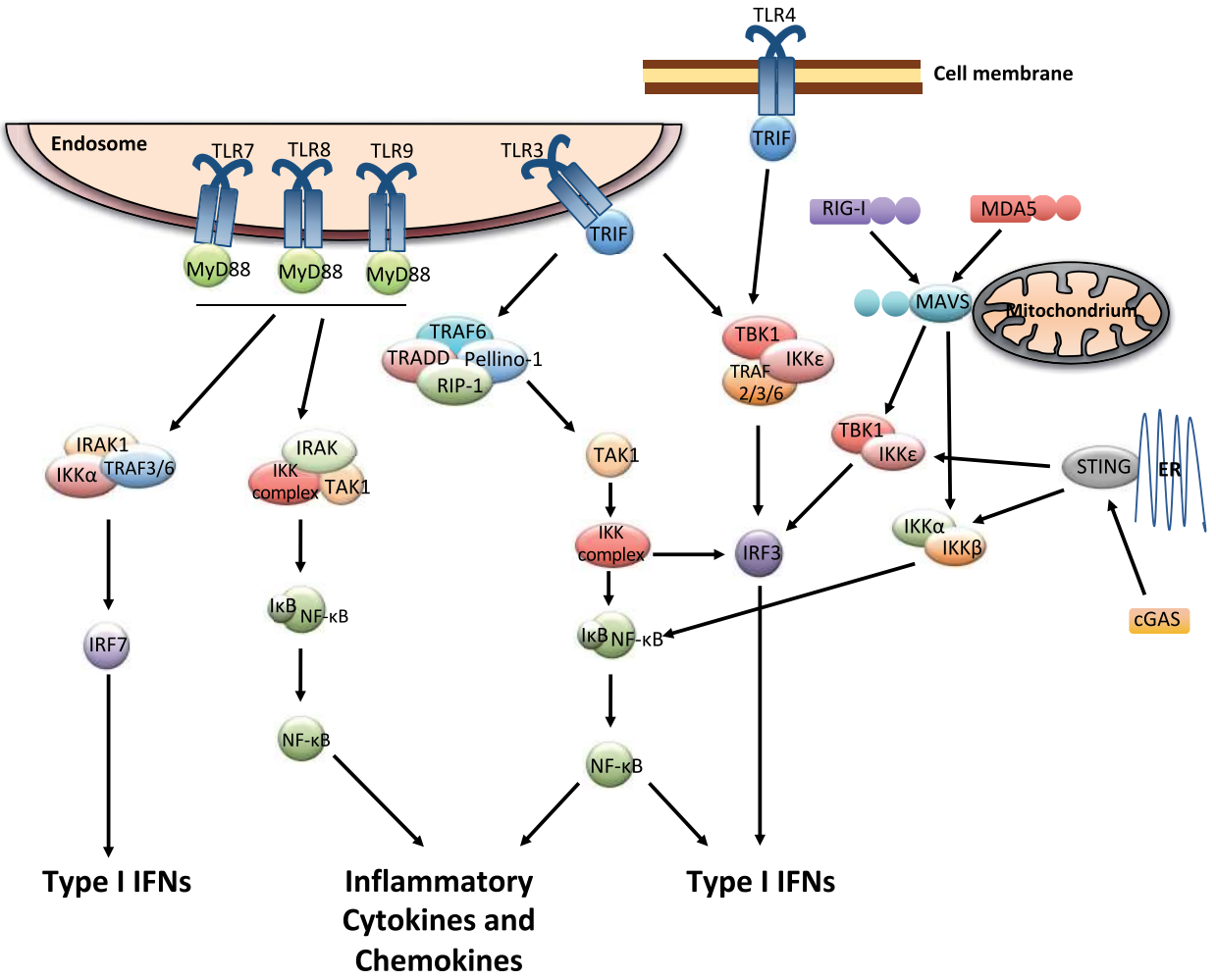

FIGURE 1 | **Pattern recognition receptor signaling that leads to the induction of type I interferons (IFNs)**. The endosomally expressed TLR-3, -7, -8, and -9, cell surface expressed TLR4, the RLRs [retinoic acid-inducible gene I (RIG)-I and MDA-5], and cGAMP synthase (cGAS) can couple pathogen detection to type I IFN induction. TLR3 and TLR4 signal *via* TRIF, which occurs through inhibitor of kappa-B (IκB) kinases (IKKs), tumor necrosis factor (TNF) receptor-associated factor (TRAF) family associated NF-κB activator (TANK)-binding kinase-1 (TBK1), and IKK-ε. This causes the activation of IRF3, which in turn induces the expression of type I IFNs. The activation of TLR3 can also induce the production of inflammatory mediators *via* TRIF by activating a complex formed by TRAF-6, TNF receptor type I DEATH domain-associated protein (TRADD), Pellino-1, and the receptor-interacting kinase (RIP)-1. This causes the activation of NF-κB pathway, which is mediated by the IKK complex and transforming growth factor beta activated kinase (TAK)-1. TLR7, 8, and 9 use MyD88 for downstream signaling and can activate IRF and NF-κB pathways. RIG-I and MDA5 signal through the adaptor molecule mitochondrial antiviral signaling protein (MAVS). cGAS signals *via* the adaptor protein stimulator of interferon genes (STING). MAVS and STING further recruit signaling molecules (involving the IKK complex, TBK1, and several TRAF proteins) and lead to the activation of NF-κB and IRF3, resulting in gene expression of various antiviral cytokines including type I IFNs (9–13).

with various stages of the viral cycle and change the state of the infected and neighboring cells. Type I IFN signaling also drive the immune response to a number of pathogens by, for example, enhancing the production of inflammatory mediators, cell communication, and the induction of apoptosis in infected cells (see below). It is interesting that the type I IFN receptor has so many ligands, and it is still unclear if all type I IFNs bind the receptor in the same way or if binding of certain IFNs results in a functional difference. There is evidence that different IFNs bind to different anchor points resulting in variations to the binding affinities and conformational change of the IFNAR1 (41). In addition, IFN-β can ligate only the subunit IFNAR1 and signal independently of JAK–STAT pathways (42). Type I IFN production and signaling are tightly regulated by a positive feedback loop, with early IFNs (in the mouse IFN-β and IFN-α4) stimulating the expression of the ISG IRF7 and other important signaling molecules (43). This regulates the expression of all IFN-α isotypes (44) resulting in enhanced signaling through the type I IFN receptor (**Figure 2**). While the positive feedback loop is important for enhancing the production and effect of type I IFNs, equally important are the negative regulators that are required to restore cellular homeostasis. The type I IFN response is tightly controlled by a series of mechanism that are dependent on cell-intrinsic factors, ISG-mediated proteins, and miRNA. The activity of STAT proteins can

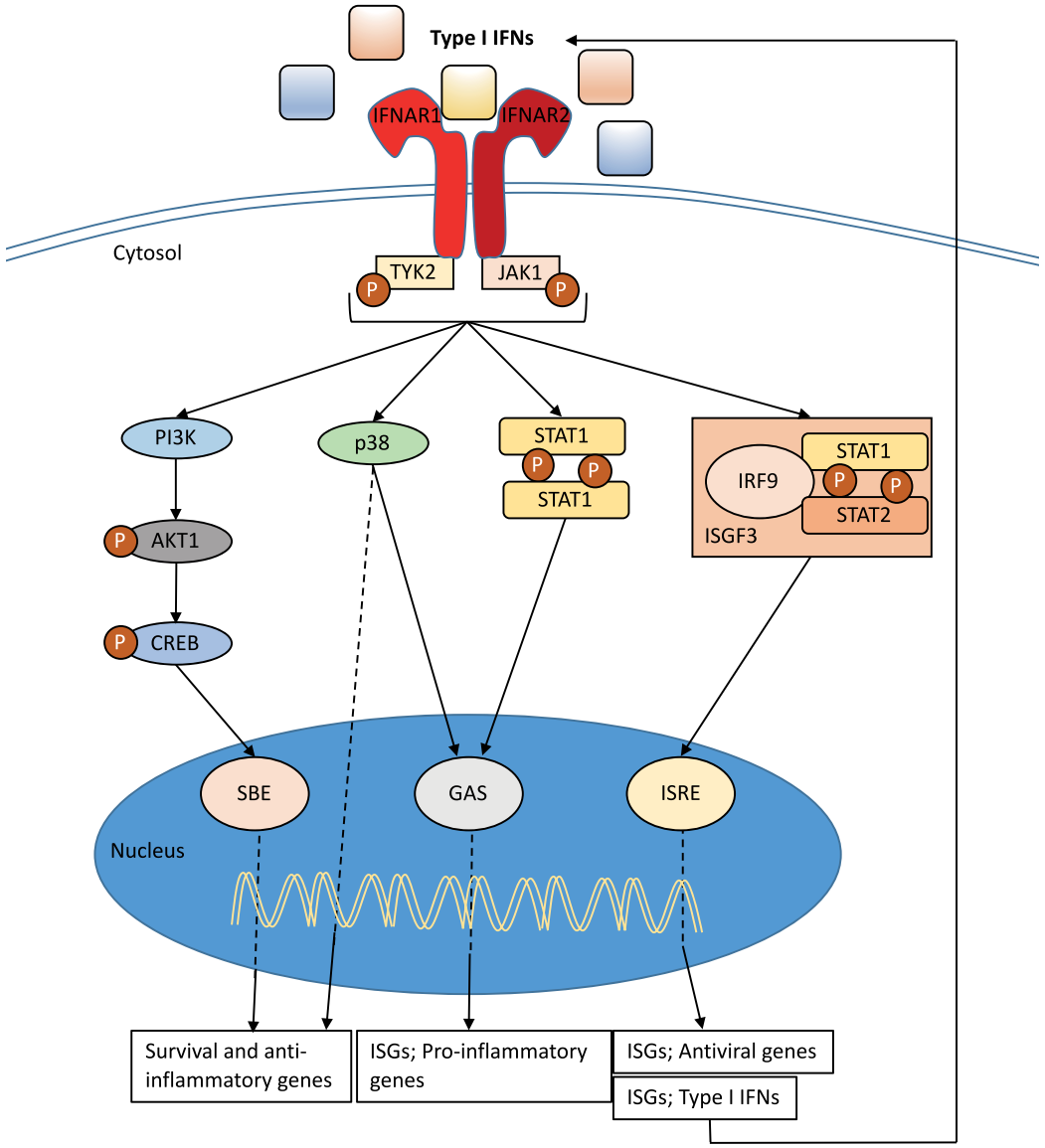

FIGURE 2 | **Type I interferon (IFN) signaling**. Type I IFNs bind to the heterodimeric transmembrane IFN-α/β receptor (IFNAR), which is composed of the two subunits: IFNAR1 and IFNAR2. The c-termini of IFNAR1 and IFNAR2 are associated with the tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1), respectively, and activation of the receptor transduces the phosphorylation of JAK1 and TYK2 by tyrosine phosphorylation. This initiates a signaling cascade composed of proteins of the signal transducer and activator of transcription (STAT) family. The STAT1 and STAT2 proteins are activated upon JAK1 phosphorylation, dimerize and together with IRF9, form the ISG factor 3 (ISGF3) complex. This complex translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) in interferon-stimulated genes (ISGs) promoters to initiate gene transcription. Signaling through IFNAR can also occur independent of IRF9 recruitment through STAT1 homodimers that can bind to IFN-γ-activated sites (GAS) in ISG promoters. Both pathways initiate transcription that promotes the induction of a range of pro-inflammatory mediators and enhance the antiviral state. The JAK–TYK signaling pathway can also promote signaling pathways independent of STAT signaling. One such pathway includes MAPKs, which are important for signals regulating important cellular functions such as gene transcription, post-transcription, apoptosis, and cell-cycle progression. Specifically, the p38 signaling cascade after IFN-stimulation drives transcription of genes that are important for inducing the antiviral effects of type I IFNs and are regulated by ISREs and GAS. Further to MAPK, the type I IFN receptor signaling can also activate the phosphoinositide 3-kinase (PI3K) signaling pathway. The phosphorylation of PI3K causes the activation of the RAC-α serine/threonine-protein kinase (AKT1)/cAMP responsive-element-binding protein that can bind smad binding elements (SBE). This signaling pathway is believed to be important for transcription of genes controlling cellular survival and inflammatory (36, 38–40).

be regulated by protein inhibitors of activated STAT (PIAS) and the suppressor of cytokine proteins (SOCS). Another important ISG for the regulation of type I IFN signaling is the ubiquitin carbol-terminal hydrolase protein, USP18 (40, 45).

Type I IFN responses are difficult to study in humans since by the time patients with severe lower respiratory tract infections are likely to be admitted to hospital several days have passed since the initial infection, at which time the production of type I IFNs

is declining and not easily detectable. However, it is interesting that complete type I IFN deficiency has not been described in humans but mutations in STAT1, tyrosine kinase 2, or NEMO are associated with poor control of viral infections (46, 47). Recently, a mutation in IRF7, leading to impaired type I and type III IFN responses, was shown to result in severe influenza infection in one case (48). In addition, severe RSV disease has been associated with polymorphisms in several innate immune response genes, in particular many that control the type I IFN system (49, 50). Also, several ISGs have been associated with severe influenza infection in mouse and man, for example, IFITM3 and MX1 (51–54). Thus, most data suggest an important role for type I IFNs during respiratory infections.

TYPE I IFNs DRIVING LUNG INFLAMMATION

The type I IFN signaling is especially important for the control of viral infections. However, in many diseases, the usefulness of type I IFNs has been debated (55). Like all immune responses, a balance is required and launching a response is as important as dampening it. As type I IFNs can lead to both cellular recruitment and activation, an imbalance of the type I IFN response can influence the cellular responses to either result in immunosuppression or immunopathology.

Type I IFNs have multiple effects in the lung. They have been shown to result in the production of chemokines such as CCL2, a monocyte chemoattractant, and CXCL10, important for the recruitment of monocytes/macrophages, T cells, NK cells, and DCs, therefore directly influencing inflammation in the lung (35, 56–58). The type I IFNs also drive a multitude of events in DCs including the differentiation of precursors, increased antigen presentation and cross-presentation, expression of costimulatory molecules, and promoting chemokine secretion and migration (8, 59–66). Interestingly, different subsets of DCs have a different degree of sensitivity to type I IFN signaling, which determines the susceptibility to influenza virus infection and thereby how much antigen they can present (67). Type I IFNs are also important for the activation of macrophages and NK cells (8, 68). Also, $CD4^+$ and $CD8^+$ T cells are directly affected by type I IFNs during various infections and the effect of type I IFNs can either be stimulatory or inhibitory by stimulating proliferation, differentiation, survival as well as inducing anti-proliferative and pro-apoptotic responses (63, 69). Type I IFN receptor signaling in T cells is also important for cytokine secretion, cytotoxicity, and memory formation (63). This varied effect of type I IFNs on T cells is partly dependent on the different STATs induced by type I IFNs where for example STAT1 is pro-inflammatory, pro-apoptotic, and anti-proliferative, while STAT4 promotes proliferation and cell survival (63). The different effects are dependent on when and where the T cells get the signal *via* the type I IFN receptor and will therefore be dependent on the site of infection, timing, magnitude, and source of type I IFN responses. B cells are also influenced by type I IFNs, and type I IFN receptor signaling on both B and T cells is important for antibody production (70, 71). Interestingly, it has been suggested that during LCMV infection, type I IFN receptor signaling in $CD8^+$ T cells increase their killing of B cells and therefore decrease the production of neutralizing antibodies (72). If this is also occurring during respiratory infections remain to be elucidated.

In the absence of type I IFNs, there is often less inflammation in the lung. Interestingly, intranasal administration of IFN-α alone can stimulate the expression of pro-inflammatory cytokines (56, 73). Also, IFN-α can potentiate the pro-inflammatory responses after intranasal LPS challenge (56). How this occurs considering most cytokines and chemokines are induced by NF-κβ and not IFNAR signaling is unknown. One possibility is the fact that type I IFNs increase the expression of many PRRs or related signaling molecules, which can enhance signaling *via* other pathways for example those involved in NF-κB activation (74). Nevertheless, all cellular components involved in the mutual influence of NF-κB and type I IFN pathways are not fully investigated and might depend on the cellular context. It is therefore a likely scenario that type I IFNs play a role in NF-κB-induced expression of pro-inflammatory cytokines and chemokines.

Lung Viral Infections

Type I IFNs are rapidly produced during all lung viral infections and contribute to the initial control of viral replication before effective innate and adaptive cellular responses are generated to clear the virus. Signaling *via* the IFNAR activates a cascade of ISGs that directly interfere with viral replication and viral spread. These include proteins that inhibit virus entry (e.g., MX1, IFITM proteins, TRIM proteins), modulate membrane lipids to prevent viral release (e.g., Viperin, Tetherin), induce apoptosis of infected cells [e.g., protein kinase R (PKR)], regulate transcriptional (e.g., Viperin) and posttranscriptional (e.g., OAS/RNaseL, PKR) mechanisms, and posttranslational events (e.g., ISG15) (38, 40). In addition to the ISG-mediated effects, type I IFNs modulate cell viability and function (e.g., apoptosis, inhibiting cell death, differentiation, migration, proliferation) to support antiviral defense (75). The amplification of the primary signal by type I IFNs is achieved by upregulation of many PRR molecules and associated signaling molecules like TLR3, RIG-I, MDA5, MAVS, MyD88, IRF3, and IRF7, which are themselves ISGs and therefore amplify the type I IFN response to viral infections [see above and Ref. (76, 77)]. Without a functioning IFNAR loop the detection of accumulating viral RNA and the further downstream processing of the signal is compromised in infected cells as they lack the feedback-mediated boosted expression of the viral RNA sensors. This imbalance will eventually promote viral replication and spread early during infection and also influence the degree of inflammation. For example, in a severe acute respiratory syndrome-CoV infection, type I IFN induction is delayed resulting in an overwhelming viral burden (78). Interestingly, many viruses have virulence factors that antagonize type I IFN responses [reviewed elsewhere (79–81)], indicating the importance of type I IFN responses during viral infection. It is also important to note that, especially during influenza virus infection, type III IFNs (IFN-λ) are highly induced at the same time as type I IFNs (82, 83). Interestingly, the receptor for IFN-λ is mostly expressed by epithelial cells and therefore these cytokines have a more restricted effect directed to intrinsic antiviral mechanisms (73). The effects of IFN-λ have recently been reviewed (5).

There are also age-related effects of type I IFN production in response to respiratory infections as both cells from infants and neonatal mice show a reduced type I IFN production and ISG induction after RSV exposure (84–86). Furthermore, monocytes from elderly have a diminished type I IFN response after exposure to influenza virus (53). Interestingly, these age groups are also very vulnerable to respiratory infections.

In addition to the cell-intrinsic responses, type I IFNs are known to enhance immune responses especially by activating and recruiting immune cells. During RSV infection, type I IFNs produced by AMs, induced the production of CCL2 and other chemoattractants crucial for monocyte extravasation into the lung during RSV infection (35). These recruited monocytes are important for controlling the virus (35). Interestingly, the expression of pro-inflammatory cytokines and chemokines was significantly reduced in RSV, influenza virus, and human metapneumovirus-infected *Ifnar1*$^{-/-}$ mice (56, 87, 88). This highlights again a synergizing effect of NF-κB and type I IFN pathways in inducing the optimal secretion of cytokines and chemokines. However, NF-κB translocation into the nucleus can be detected as early as 0.5 h post RSV inoculation without the necessity of viral replication (89). This might provide an explanation for the early induction of some of the measured cytokines such as IFN-β, IL-6, TNF-α, or IL-12 in the lungs of RSV-infected *Ifnar1*$^{-/-}$ mice, but perhaps sustained NF-κB activation may be in part type I IFN dependent. In contrast to *Ifnar1*$^{-/-}$ mice, wt mice have the ability to further enhance this "first signal" through the IFNAR-driven autocrine and paracrine amplification loop to maximize the responses. Moreover, the lack of responsiveness to viruses in *Ifnar1*$^{-/-}$ mice could also suppress the recruitment of additional cells to the site of infection, which could otherwise contribute to local cytokine secretion. These usually specifically recruited cells are most likely of non-polymorphonuclear origin, since neutrophil recruitment was comparable or increased in RSV or influenza virus infected *Ifnar1*$^{-/-}$ mice compared to wt mice (56, 90). Furthermore, during influenza or Sendai virus infection type I IFNs can act as messengers from the lung to the bone marrow where they instruct leukocytes to activate an antiviral transcriptional program, resulting in an increased antiviral state of these cells before they migrate to the lung (91).

Additional to T cell expansion, type I IFNs have been shown to promote T effector cell function due to the dependence on IFNAR mediated STAT1 signaling for the cytolytic activity of memory CD8$^+$ T cells during recall responses with respiratory viruses (92). In contrast, after influenza virus infection type I IFNs can induce the secretion of IL-10 from CD8$^+$ T cells (93) and the expression of programmed cell death ligand 1 (PDL-1) on epithelial cells (94). Furthermore, blocking of PDL-1 enhanced CD8$^+$ T cells function and viral clearance (94). Thus, type I IFNs are important both to induce T cell effector functions and also to induce anti-inflammatory mechanisms that can suppress the T cell response.

Despite the essential role for type I IFNs in activating the immune response to successfully combat viral infection and to guarantee survival of the organism, they can have deleterious effects on the host and cause acute immunopathology. High type I IFN production during influenza virus infection mediated by recruited inflammatory monocytes and pDCs cause the upregulation of TNF-related apoptosis-inducing ligand (TRAIL) (8, 95). This "death" ligand binds to the death receptor 5 on epithelial cells, the expression of which is also upregulated by type I IFNs. Thus, the TRAIL-expressing inflammatory monocytes cause the death of the epithelial cells, which in turn increases morbidity or death of the mice (83, 96). Therefore, the potential of type I IFNs to drive and amplify pro-inflammatory responses (56, 83) could, if type I IFNs are produced excessively or for an extended time, cause the increased morbidity and mortality during lung viral infections (78, 83, 96). Thus, the timing and magnitude of type I IFN responses are crucial to obtain an efficient cell-intrinsic response and a balanced cell-extrinsic response that together lead to the clearance of the virus without causing detrimental immunopathology.

Lung Bacterial Infections

Since type I IFNs have an important role in preventing replication and spread during viral infections their role is overall beneficial. During lung bacterial infections, type I IFNs are induced but the role of these cytokines is unclear and their ability to drive inflammation might in these cases be more detrimental. For example, mice deficient in IFNAR1 or TLR9 showed an improved clearance of *Staphylococcus aureus* (97). Also, a lung infection model of *Chlamydia trachomatis* mouse pneumonitis (*Chlamydia muridarum*) showed that deficiency in the IFNAR1 resulted in less bacterial burden and bodyweight loss, and milder pathological changes (98). *Mycobacterium tuberculosis* (MTb) infection of TB-susceptible *Ifnar1*$^{-/-}$ mice showed enhanced protection from death, lower bacterial burden in the lungs, and decreased degree of lung inflammation compared to wt mice (99). In addition, an interferon signature is evident in patients with active MTb disease (100), and mouse studies have shown that the type I IFN responses during MTb infection is tightly regulated by IL-1 and PGE_2 (101). More contradictory data are presented for *Streptococcus pneumoniae* infection, where an invasive strain of *S. pneumoniae* induces type I IFNs in the lungs and blocking the IFNAR decreased the systemic bacteremia (102). In contrast, another study showed that *S. pneumoniae* infection of *Ifnar1*$^{-/-}$ mice or mice treated with an antibody against the type I IFN receptor displayed enhanced bacterial spread and increase bacteremia (103). In addition, if mice were given rIFN-β, this reduced the bacteremia after intranasal *S. pneumoniae* infection (103). Thus, most data suggest that type I IFNs induced during lung bacterial infection are part of the inflammatory response and might be important to initiate immune responses to the infection. However, since these cytokines lead to the recruitment and activation of immune cells, this can enhance inflammation and result in bacterial dissemination and spread.

Interestingly, bacterial secondary infections are common after a severe lung viral infection and the host is more susceptible to infection by *Escherichia coli*, *Pseudomonas aeruginosa*, *S. pneumoniae*, or *S. aureus* after influenza virus infection (104–106). This increased susceptibility is probably due to many factors such as a lower activation threshold of lung cells, inhibition of important signaling pathways and cytokines induction and exhaustion of immune cells resulting in non-appropriate immune responses

elicited to the new pathogen. Noteworthy, this increased susceptibility cease to exist as soon as the virus-induced type I IFNs are decreasing (104) and IFNAR1 deficient mice can effectively clear a secondary *S. pneumoniae* infection (106). Furthermore, the induction of type I IFNs during influenza virus infection attenuates chemokines important for neutrophil recruitment, which can promote secondary bacterial pneumonia (107). A possible mechanism for this is the type I IFN-dependent upregulation of the methyltransferase Setdb2, which can repress chemokines such as CXCL1 at the chromatin level (108). Also, influenza virus infection before or during MTb infection increases the severity of the MTb infection *via* type I IFN signaling (109). Interestingly, type I IFNs have been shown to inhibit inflammasome activation and IL-1 responses and increase IL-10 production (110). Altogether, this suggests that type I elicited during a lung viral infection makes it more possible for a subsequent bacterial infection to establish.

Lung Fungal Infections

The role of type I IFNs during fungal infections has also been investigated using mice deficient in the IFNAR1. During a lung *Pneumocystis* infection, a decreased pro-inflammatory response was detected and even if the pathogen burden was the same, the clearance was delayed in *Ifnar1*$^{-/-}$ mice and this resulted in an exacerbated Th2 response and fibrosis (111). The lack of type I IFN signaling during *Cryptococcus neoformans* infection has been shown both to result in a decreased pathogen burden (112) and increased pathogen burden with increased death (113). However, both studies showed higher Th2 cytokine levels in the *Ifnar1*$^{-/-}$ mice after *C. neoformans* infection (112, 113). Thus, type I IFN responses during lung fungal infection are also part of driving the inflammatory response but it is still unclear in which magnitude or which mechanisms that are used.

SUMMARY

There are obvious benefits of type I IFNs during a lung viral infection as these cytokines have a vital cell-intrinsic antiviral effect limiting viral replication. However, the cell-extrinsic effects of type I IFNs are important during all lung infections as type I IFNs directly drive lung inflammation, most likely by amplifying primary signals initiated by other stimuli. When considering to use type I IFN as a potential antiviral agent, the immune-modulating effects during lung infections needs to be considered as the type I IFN response has to be tightly regulated so that a balance of beneficial (initiation of inflammation) and detrimental (immunopathology) effects is achieved and gas exchange is not impaired.

AUTHOR CONTRIBUTIONS

CJ conceptualized the scope of the review article. CJ, SM, and MP wrote the review.

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REVIEW
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Type I and III Interferon in the Gut: Tight Balance between Host Protection and Immunopathology

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The intestinal mucosa forms an active interface to the outside word, facilitating nutrient and water uptake and at the same time acts as a barrier toward the highly colonized intestinal lumen. A tight balance of the mucosal immune system is essential to tolerate harmless antigens derived from food or commensals and to effectively defend against potentially dangerous pathogens. Interferons (IFN) provide a first line of host defense when cells detect an invading organism. Whereas type I IFN were discovered almost 60 years ago, type III IFN were only identified in the early 2000s. It was initially thought that type I IFN and type III IFN performed largely redundant functions. However, it is becoming increasingly clear that type III IFN exert distinct and non-redundant functions compared to type I IFN, especially in mucosal tissues. Here, we review recent progress made in unraveling the role of type I/III IFN in intestinal mucosal tissue in the steady state, in response to mucosal pathogens and during inflammation.

Keywords: interferon, intestinal mucosa, colitis, enteropathogens, IFN-λ, type 1 IFN, inflammatory bowel diseases, coeliac disease

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INTRODUCTION

The intestinal tract is a major entry site for viruses and bacteria. Mucosal innate and adaptive immune cells are equipped to respond to and fight invading pathogens. At the same time, the intestinal lumen is densely colonized by commensal microflora, which at steady state does not provoke an exacerbated inflammatory response. The intestinal lumen is separated from the underlying sterile lamina propria harboring the body's largest immune cell compartment by a single layer of polarized intestinal epithelial cells (IECs). This epithelial cell layer undergoes rapid and perpetual self-renewal without disrupting the functional integrity of cell–cell junctions. In addition, IECs not only form a passive physical barrier but also participate actively in the immune response against major enteric pathogens and cross talk with the commensal flora (1, 2). However, pathogenic viruses, bacteria, and parasites exploit opportunities for breaching the epithelial barrier.

Upon infection, host cells communicate by means of production and secretion of signaling molecules. Interferons (IFN) are a large family of cytokines with diverse functions during a successful host defense. The family of type I IFN comprises more than 20 members with multiple IFN-α and one IFN-β being the most important. Classically, the most prominent function of type I IFN is to induce antiviral immunity, whereas IFN-γ, the only type II IFN, promotes the response to intracellular bacteria. However, a vast amount of studies has found that type I IFN are also produced during

bacterial infection. In contrast to their action in viral infections, their activity against bacteria can be either favorable or detrimental for the host (3–6).

Recently, a novel family of IFN, the type III IFN or IFN-λ family, was described (7, 8). This family consists of IFN-λ1, IFN-λ2, IFN-λ3 (also called IL-29, IL-28A, and IL-28B), and IFN-λ4 in humans, whereas mice have only two functional genes encoding IFN-λ (*Ifnl2* and *Ifnl3*) and two *Ifnl1* pseudogenes (9). Similar to type I IFN, type III IFN are induced by viral infection and show antiviral activity. However, they are structurally distinct from type I IFN and interact with a heterodimeric class II cytokine receptor consisting of the IFN-λR1 (also called IL-28Rα) chain in complex with the IL-10R2 chain, opposed to the type I IFN receptor (IFNAR).

A number of studies have addressed the functional importance of type III IFN compared to type I IFN in the context of viral infections (10–15). Less is known about the role of type I IFN and almost nothing on the role of type III IFN in the host defense against bacterial enteropathogens, intestinal homeostasis, and colitis. Therefore, we review recent progress made on the importance of type I and III IFN during enteric viral infections and focus on the role of type I IFN in the intestinal mucosal tissue during steady state, in response to bacterial infections and during inflammation.

INDUCTION OF TYPE I AND III IFN

The induction of type I and III IFN has been recently reviewed elsewhere (16), therefore we will only briefly summarize the major mechanism leading to IFN expression. Virtually all cells are equipped with the machinery to recognize viral infection and express type I and III IFN in response. Similar stimuli and pathways lead to the expression of type I and III IFN; however, differences between cell types as well as in magnitude and kinetics have been described (14, 16, 17). Comparable expression patterns of type I and III IFN result from a similar requirement of transcription factors for the expression of their encoding genes, such as IFN regulatory factors (IRFs) and NF-κB. There are however some differences in the promoter region, with IFN-β expression relying on the binding of the constitutively expressed IRF-3 to its promoter, which allows rapid induction. By contrast, IFN-α requires IRF-7 binding, which is an interferon-stimulated gene (ISG) itself and needs to be upregulated in most cell types following infection (3). Type III IFN are more dependent on the activation of NF-κB (18) and require the combined action of IRFs and NF-κB for full induction (19–21).

During systemic viral infections hematopoietic cells are the major source of type I IFN. Plasmacytoid dendritic cells (pDCs), which are designated as being the "professional" type I IFN-producing cells, produce large amounts in response to a wide range of viruses, parasites, and bacteria and are particularly important in the early phase of type I IFN production (22–24). However, depending on the infectious agent, myeloid cells are also involved in systemic type I IFN production. During systemic *Listeria* infection, the vast amount of systemic IFN-β production is independent of pDCs but seems to be produced by LysM-Cre-expressing macrophage/monocyte-like cells including TipDCs but not neutrophils (25–27). In the intestinal lamina propria, dendritic cells (DCs) as well as mononuclear phagocytes produce IFN-β and IFN-α5 in the steady state (14, 23, 28).

Epithelial cells are thought to be the major producer of type III IFN at steady state and during enteric viral infection, while lamina propria leukocytes (LPLs) also produce type III IFN under certain conditions (14, 29). Intraepithelial lymphocytes produce IFN-α and IFN-λ upon TCR activation, which contributes to protection during norovirus infection (30). Moreover, Th17 cells are the main source of IFN-λ in psoriatic lesions of the skin (31).

Bacteria trigger similar intracellular signaling cascades to viral infections and many bacterial infections lead to the production of type I IFN [reviewed in Ref. (32, 33)]. Induction of type III IFN has been demonstrated only for a limited number of bacterial species. A human epithelial colon cancer cell line expresses type III IFN upon infection with Gram-positive bacteria such as *Listeria monocytogenes* (34, 35), *Staphylococcus aureus*, and *Enterococcus faecalis* but fails to produce considerable amounts of type III IFN when infected with Gram-negative bacteria such as *Salmonella enterica* ssp. Typhimurium, *Shigella flexneri*, and *Chlamydia trachomatis* (35). Induction seems to be cell type, species, and gene specific (36, 37).

SIGNALING IN RESPONSE TO IFN

Binding of IFN to their corresponding receptors triggers the stimulation of a Janus kinase (JAK)–signal transducer of transcription (STAT) pathway. The type I IFN receptor (IFNAR) consists of two subunits, IFNAR1 and IFNAR2. Engagement of IFNAR with its ligand ultimately results in the activation of the transcription factor complex ISGF3 comprised of STAT1/STAT2 heterodimers in conjunction with IRF-9 and subsequently the induction of ISGs (3, 38).

The type III IFN receptor consists of the unique IFN-λR1 chain and the IL-10R2 chain, which is shared with the IL-10 receptor. Engagement of this receptor complex results in the activation of a signal transduction cascade in a manner highly similar to that caused by type I IFN signaling. Interestingly, signaling by type III IFN is additionally regulated at the level of receptor expression. Whereas IFNAR is ubiquitously present, the IFN-λR1 chain of the type III IFN receptor is only expressed in a limited number of cell types, preferentially located at mucosal surfaces. Epithelial cells in mucosal tissues are a major target of type III IFN (39, 40). Additional responsiveness to type III IFN has recently been suggested for a restricted panel of immune cells (9). Type III IFN was proposed to have a role in the direct regulation of NK cell effector function (41). A suppressive function of type III IFN in autoimmune and inflammatory diseases was also proposed recently. In a model of collagen-induced arthritis, treatment with type III IFN inhibits the recruitment of IL-1β-expressing neutrophils, which have been shown to express high levels of IFN-λR1 and respond directly to type III IFN (42). In addition, there are controversial data on the responsiveness of T cells, DCs, and monocytes to type III IFN (9). In human cells, expression of the type III IFN receptor seems to be less restricted than in mouse cells and a wider panel of immune cells, including B cells, is responsive to type III IFN (43).

Signaling by IFNs induces the transcription of hundreds of ISGs. These include pattern-recognition receptors, antiviral effectors such as myxovirus resistance (Mx) gene 1 and 2, pro-apoptotic genes, MHC class I genes, inducible nitric oxide synthase, and genes encoding members of the GTPase superfamily which alter the maturation of phagosomes to counteract pathogen strategies based on survival in intracellular compartments. Moreover genes involved in the desensitization to IFNs are also induced, allowing cells to recover from the IFN response (38, 44). The importance of IFNs in the immediate defense against pathogens has been shown by the generation of gene-targeted mice. Mice deficient in components of the type I IFN signal transduction pathway are highly susceptible to a variety of viruses (5, 45). The role of type I IFN in bacterial infections is more complex. Whereas type I IFN protect mice against systemic infection with most extracellular bacteria tested, they exacerbate disease during infection of mice with *L. monocytogenes* or *Mycobacterium tuberculosis* (3–6).

ENTERIC VIRAL INFECTIONS AND IFN

Studies investigating the functional importance of type I IFN versus type III IFN in the context of systemic viral infections found a dominant phenotype for type I IFN and only a small contribution of type III IFN in the absence of type I IFN. The first indication for a tissue-specific role of type III IFN arose from studies with organ-tropic viral infections suggesting that type III IFN are important in enforcing and strengthening the antiviral response at mucosal sites (**Table 1**) (10–12, 46–48). The gastrointestinal tract, lung, vagina, and salivary glands respond strongly to systemic IFN-λ expression (40). In the lung and gastrointestinal tract, epithelial cells were identified to express high levels of the type III IFN receptor and represent the major target of type III IFN (11). These findings explain why mice deficient for both IFN systems are more susceptible to lung-tropic viruses, such as influenza A and B virus, respiratory syncytial virus, and severe acute respiratory syndrome coronavirus than single type I IFN receptor-deficient mice (11). The remaining part of this section focuses on the role of type III IFN in enteric viral infections.

TABLE 1 | Role of type I interferons (IFN) and type III IFN during enteric viral infections.

	Role of type I IFN	Type III IFN
Rotavirus	Type I IFN protect from systemic infection and heterologous oral infection (49, 50)	Type III IFN protect from oral homologous infection by restriction of replication within intestinal epithelial cells (IECs) (12, 29)
Norovirus	Type I IFN protect from systemic spread of acute norovirus infection (48, 51)	Type III IFN protect from persistent norovirus infection; treatment with IFN-λ clears persistent infection (15)
Reovirus	Type I IFN restricts reovirus replication in lamina propria leukocyte (14)	Type III IFN restricts reovirus replication in IECs and fecal shedding (14)
EMCV	IFN-α treatment reduces titer in hearts during systemic infection (47)	Type III IFN does not protect during systemic infection (47); type III IFN protects from oral infection (52)

Rotavirus

Rotavirus belongs to the family of reoviridae and infection of humans leads to severe diarrhea in children younger than 5 years. The susceptibility of infants can be recapitulated in a mouse model, where suckling mice are highly susceptible to infection compared to adult mice. The strict host cell tropism of rotavirus for IECs makes it a clean model to study epithelial-specific effects of IFN.

Mice can be infected with a homologous strain of murine rotavirus or with a heterologous strain such as rhesus or simian rotavirus. Homologous strains are better equipped to evade the host immune response, which generally leads to higher viral titers and a more severe pathology at a lower infectious dose (49).

A protective role of type I IFN and IFN-γ has been questioned, since mice impaired in type I IFN or IFN-γ signaling infected with a murine rotavirus strain do not show differences in viral load, and treatment with either type I IFN or IFN-γ did not result in a clinical benefit (53). However, simian and rhesus rotavirus show enhanced systemic replication in mice deficient for type I IFN and IFN-γ receptor or STAT1.

By contrast, type III IFN were protective in a homologous infection model of suckling and adult mice (12). Of note, a very distinct cell tropism for type III IFN responsiveness in the intestine was reported: IECs were solely activated by type III IFN and are not responsive to type I IFN, whereas cells in the lamina propria respond to type I IFN induced during viral infection (12). Supporting these findings it was shown that IL-22 augments the antiviral effects of type III IFN signaling and contributes to the protective effect during homologous rotavirus infection (29). However, this model has been questioned by another study reporting type I IFN- and type III IFN-mediated protection only for heterologous but not for homologous rotavirus infection of suckling mice (50). Experimental discrepancies between those studies are not apparent suggesting that flora differences between mouse facilities or genetic strategy of the knock-out mouse lines might impact on the efficacy of IFN signaling. Of note, Lin et al. reported age-dependent responsiveness of IECs toward IFNs with neonatal IECs being responsive to both type I IFN and type III IFN, whereas adult IECs were responsive to type III IFN only (50).

Norovirus

Norovirus is the cause of the majority of non-bacterial gastroenteritis in adults. In contrast to rotavirus, the host cell tropism of norovirus is broad and not fully characterized. *Ex vivo* and most *in vivo* studies could not show productive virus replication in IECs (54). Phagocytes allow productive virus replication and during *in vivo* infection, virus was detected in LPLs (54, 55). Although the virus does not replicate in IECs, it has been suggested that it translocates across the epithelium or enters the host *via* M cells (56).

Type I IFN and IFN-γ restrict murine norovirus replication in macrophages and DCs *in vitro* (57, 58). *In vivo*, the antiviral activity of type I IFN mediates some protection from systemic replication of an acute strain (51) and after high-dose oral infection (59). However, local replication in the colon and fecal shedding of a persistent norovirus strain is controlled by type III IFN. Treatment with type III IFN resolves persistent infection,

independent of adaptive immune responses, by acting on non-hematopoietic cells (15). By contrast, type I IFN controls the systemic spread and persistency of the acute norovirus strain CW3 by activation of the host DCs (48). These findings demonstrate the distinct cell-type specificities of type I IFN and type III IFN during infection: local protection in the colon through type III IFN stimulation of epithelial cells and prevention of systemic spread and persistency by type I IFN in myeloid cells.

The commensal bacterial flora was reported to promote norovirus persistency in the intestine and antibiotic treatment of mice prevented persistent infection with norovirus. The protective effect was only observed in the presence of functional type III IFN signaling (13). The antibiotic treatment did not alter type III IFN signaling and therefore the authors concluded that the microflora might render the virus susceptible to the antiviral action of type III IFN. Alternatively, the absence of type III IFN signaling might increase the host's vulnerability to persistent viral infection so dramatically that minor changes by antibiotic treatment do not impact on the overall susceptibility under those conditions.

Reovirus

Reovirus has a broad host cell tropism and replicates in epithelial cells and immune cells of the intestinal mucosa. After oral infection, it enters the host *via* M cells into Peyer's patches and can spread further during infection. Type I IFN produced by hematopoietic cells is essential to limit systemic spread of the virus and to prevent lethality (60). In a study using type I IFN or type III IFN signaling-deficient mice, it was demonstrated that type III IFN signaling specifically prevents replication of the virus in IECs, whereas type I IFN signaling limits replication in lamina propria cells and systemic spread of the virus (14). This study confirms the compartmentalized action of IFN in the intestinal mucosa and provides an explanation by showing that IECs only express low levels of IFNAR (**Figure 1**). Furthermore, it was shown that the production of IFN is cell type specific in that IECs produce higher levels of type III IFN and LPLs predominantly produce type I IFN.

Taken together, the studies of enteric viral models with rotavirus, reovirus, and norovirus show a strong and specific responsiveness of IECs to type III IFN (12, 15, 29). Therefore, type III IFN might specifically enforce the intestinal barrier against enteric viruses and also against viral entry *via* the intestinal route. Additionally, a strong IFN response by type III IFN signaling within the epithelial lining prevents viral spreading (12, 14, 15). Studies showing that type III IFN treatment protects against oral EMCV (52) infection but not from systemic infection (47) support the conclusion that type III IFN protects the host not only from enteric viruses but also from viral entry *via* the oral route. By contrast, the contribution of type I IFN to the epithelial antiviral response in the intestine is less clear and conflicting results suggest it to be context dependent (12, 29, 50).

BACTERIAL INFECTIONS

In contrast to viral infections where type I IFN and/or type III IFN usually provide an efficient host defense by triggering the production of antiviral genes, the role of type I/III IFN in the antibacterial response depends on the pathogen and the route of infection (3, 4, 61). Type I IFN signaling protects against most extracellular bacteria tested but is detrimental in the course of infection with a range of intracellular bacteria [**Table 2**; reviewed in Ref. (61)].

Listeria monocytogenes

Most of the pioneering studies unraveling the potential detrimental action of type I IFN in the antibacterial response have been performed using systemic infection models with *L. monocytogenes* [reviewed in Ref. (4)]. More recent studies, however, have proposed that the impact of type I IFN on the outcome of disease depends on the route of infection (65).

Mice deficient in IFNAR1, IFN-β, IRF-3, or IRF-7 are more resistant to systemic infections with *L. monocytogenes* than wild-type mice (25, 62, 64, 78). A number of mechanistic explanations for this phenotype exist, including increased death of crucial effector cells such as macrophages and T cells in response to type I IFN signaling (64, 78, 79), upregulation of IL-10 in an type I IFN-dependent manner limiting protective inflammatory responses (80, 81), and a negative cross talk between type I IFN and IFN-γ signaling (82).

In contrast to the clear detrimental effects of type I IFN in systemic *L. monocytogenes* infections, the role of type I IFN in oral *L. monocytogenes* infection has only been addressed by a limited number of studies mainly due to the lack of a suitable mouse model. For the uptake into IECs, specific interaction between the *Listeria* virulence factor internalinA (InlA) and E-cadherin expressed on IECs is required. InlA recognizes E-cadherin from human but fails to bind the corresponding domain of murine E-cadherin (83). A knock-in mouse ubiquitously expressing "humanized" E-cadherin provides a model for oral *Listeria* challenge (84). Infected germ-free colonies of this mouse line develop systemic listerosis, which can be dampened by administration of *Lactobacilli* (85). *Lactobacilli* treatment downregulates immune gene expression and in particular ISGs, which are among the most highly induced genes after *L. monocytogenes* infection (85).

Kernbauer et al. used a "murinized" *L. monocytogenes* strain LO28 expressing altered InlA recognizing mouse E-cadherin to show that, in sharp contrast to systemic infection, type I IFN signaling in response to both high-dose and low-dose intragastric *L. monocytogenes* infection is beneficial for the host. Diminished restriction of bacterial growth in the absence of type I IFN signaling resulted in exacerbated hepatic inflammation and damage (65). Different results were obtained by a more recent study using an EGDe derivative strain expressing "murinized" InlA (66). Contrasting systemic infection, which leads to strong type I IFN secretion, oral infection with this strain did not trigger robust type I IFN induction in splenocytes even when comparable bacterial burdens were present in the spleen. Neither major T cell depletion nor increased splenic IL-10 production was observed in this model and the detected infection-induced downregulation of the IFN-γ receptor on DCs and macrophages was not dependent on type I IFN signaling. Consequently, no major differences between wild-type and IFNAR1-deficient mice orally infected with *L. monocytogenes* where detected in this study (66). The discrepancies between the Kernbauer and the Pitts study might

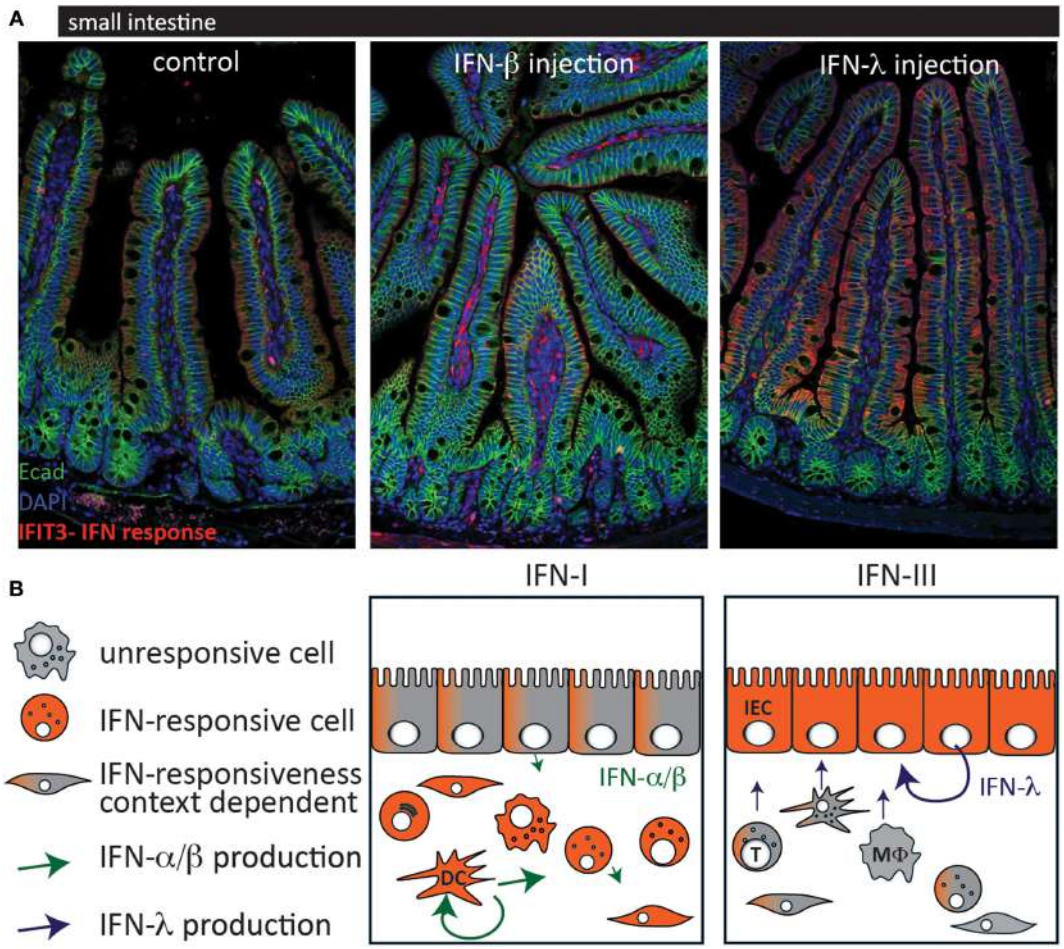

FIGURE 1 | **Cell-type-specific responsiveness to type I interferons (IFN) and type III IFN at the intestinal mucosa**. **(A)** C57BL/6 mice were injected with IFN-β (1,000 U) (middle panel) or IFN-λ (1 μg) (right panel) and 3 h later, the small intestine was processed for histological assessment. Staining was performed for the interferon-stimulated gene IFIT3 (red) as a marker for IFN response and for the epithelial cell marker E-cadherin (green). **(B)** Schematic of the IFN production and responsiveness at the intestinal mucosa. When type I IFN levels are high, lamina propria cells readily respond with a strong IFN response whereas IECs are rather unresponsive but might respond under certain conditions [**(A)** middle panel; **(B)** left panel]. In contrast, IECs are the most responsive cells to type III IFN **(A,B)** right panel. Most virus-infected cells express type I IFN. Hematopoietic cells, such as plasmacytoid dendritic cells and macrophages produce the highest amounts of type I IFN whereas IECs seem to express preferentially type III IFN. T, T cell; DC, dendritic cell; MΦ, macrophage; IEC, intestinal epithelial cell.

be explained by different application methods (intragastric infection compared to natural feeding) or by the use of different strains (type I IFN hyper-inducing LO28 versus EGDe). In addition, differences in the microbiota due to the housing conditions might influence the outcome of infection.

Salmonella enterica Typhimurium

Salmonella enterica serovar Typhimurium causes gastroenteritis and is one of the most frequent causes of bacterial foodborne disease in Western countries, representing a major economic problem (86).

Oral infection of laboratory mice with *S.* Typhimurium causes typhoid-like symptoms without clinical signs of gastroenteritis and can be used to study genetic determinants of systemic infections. IFN-$\beta^{-/-}$ mice show increased resistance to *S.* Typhimurium in lethality and bacterial spread in this model (67, 68).

The natural microbiota of the mouse gut is able to outcompete *Salmonella* to occupy this ecological niche. Thus, a new model of typhlocolitis was developed employing streptomycin treatment to deplete commensal bacteria and to overcome the colonization resistance of mice. This infection model leads to a rapid induction of inflammation in cecum and colon (87, 88).

An influence of type I IFN signaling on the immune response to *S.* Typhimurium infection has been suggested during the analysis of *Usp18*-deficient mice (69, 89). USP18 is a deubiquinating protease with de-ISGylation activity specific for ISG15. It also limits JAK–STAT activation and is thus involved in the negative regulation of type I IFN signaling. During systemic *Salmonella* infection, increased STAT1 activation correlated with impaired STAT4 activation and reduced IFN-γ production, and *Usp18* mutant mice are more susceptible to systemic (i.e., typhoid) *S.* Typhimurium infection (89). By contrast, in the

TABLE 2 | Role of type I interferons (IFN) in intestinal inflammation and bacterial infections.

Mouse strain	Phenotype—type I IFN	Reference
BACTERIAL INFECTION		
Listeria monocytogenes		
IFNAR1$^{-/-}$, IFN-β$^{-/-}$, IRF-3$^{-/-}$, IRF-7$^{-/-}$	Type I IFN signaling is detrimental during systemic infection	(25, 62–64)
IFNAR1$^{-/-}$	Kernbauer et al. showed that type I IFN signaling during oral infection with the potent type I IFN inducing LO28 strain is beneficial for the host. By contrast, Pitts et al. did not observe any role for type I IFN during oral infection with the EGDe strain	(65, 66)
LysM-Cre IFNAR$^{fl/fl}$	Lack of IFN signaling in LysM^{+} cells confers protection during systemic infection most pronounced in early infectious stages	(25)
Salmonella Typhimurium		
IFN-β$^{-/-}$	Lack of IFN-β mediates increased resistance to lethality during oral *S.* Typhimurium infection	(67)
IFNAR$^{-/-}$	IFNAR deficiency leads to increased resistance to oral *S.* Typhimurium characterized by decreased bacterial spread and weight loss but similar intestinal pathology. In accordance, type I IFN induction due to influenza coinfection exacerbates the disease and CFU but decreased intestinal immunopathology	(68)
USP18$^{-/-}$	During *Salmonella* infection, *Usp18*-mutant mice are more susceptible to systemic (i.e., typhoid) *S.* Typhimurium infection. By contrast, in the streptomycin-induced model of typhlocolitis, mutant *Usp18* mice display lower pathology scores, low IFN-γ production but upregulated type I IFN signaling compared to control mice, resulting in earlier systemic dissemination of the bacteria and decreased survival	(69)
Yersinia enterocolitica		
TRIF$^{-/-}$	IFN-β treatment protects TRIF$^{-/-}$ mice from *Y. enterocolitica* lethality	(70)
COLITIS MODELS		
T cell transfer colitis		
IFN-α treatment	Ameliorates T cell transfer colitis	(71)
IFNAR$^{-/-}$host	IFNAR deficiency in the host cells exacerbates colitis; indirect effect on maintenance of Foxp3^{+} Tregs	(23)
IFNAR$^{-/-}$ T cells	Induction of colitis by IFNAR$^{-/-}$ T cells similar to wt T cells, however, boosting type I IFN by poly(I:C) treatment attenuates T cell transfer colitis in a T cell-(IFNAR-)dependent manner	(23, 72)
IFNAR$^{-/-}$ Tregs	Conflicting findings on the role of IFNAR signaling in Tregs for protection from T cell transfer colitis	(23, 71)
Dextran sodium sulfate (DSS) colitis		
CpG ODN treatment IFN-β-expressing *Lactobacillus* (La-IFN-β)	CpG ODN protects against DSS colitis in an IFNAR-dependent manner; by contrast, La-IFN-β treatment exacerbates colitis	(73, 74)
IFNAR1$^{-/-}$	Type I IFN signaling suppress acute DSS colitis but delays the resolution	(73, 75)
Villin-Cre IFNAR1$^{fl/fl}$	IFNAR deficiency in intestinal epithelial cells results in similar susceptibility to DSS colitis as wt; increased tumor burden in DSS + azoxymethane model (due to microbiota alterations)	(76)
IL-28Rα$^{-/-}$	Increased susceptibility in IL-28Rα$^{-/-}$, same as IL-28Rα$^{-/-}$ IFNAR1$^{-/-}$ DKO indicating dominant role of type III IFN	(77)

streptomycin-induced model of typhlocolitis, mutant *Usp18* mice display lower pathology scores, low IFN-γ production but upregulated type I IFN signaling compared to control mice, resulting in earlier systemic dissemination of the bacteria and decreased survival (69).

Interestingly, influenza-induced type I IFN suppress host intestinal immunity leading to increased susceptibility to secondary *Salmonella*-induced colitis (68). Influenza-induced type I IFN strongly inhibited the induction of antimicrobial and inflammatory genes such as IFN-γ, S100A9, and lipocalin-2 in response to secondary *S.* Typhimurium infection causing increased intestinal colonization and increased bacterial dissemination but reduced immunopathology (68).

In summary, these studies suggest a detrimental effect of type I IFN on the growth and dissemination of bacteria in *Salmonella*-induced typhlocolitis whereas it limits intestinal inflammation. These effects are more obivous when type I IFN production is boosted by influenza infection or polyI:C treatment (68). Additional studies are required to determine the direct and indirect effects of type I IFN on *S.* Typhimurium-induced typhlocolitis. Moreover, the role of type III IFN in this model has yet to be addressed.

Other Enteric Bacterial Infections

The role of type I or III IFN in other enteric bacterial infections [e.g., enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *Escherichia coli*, *Citrobacter rodentium*, *Campylobacter jejuni*, or *Yersinia enterocolitica*] has not been extensively addressed in *in vivo* infection models.

Citrobacter rodentium and EPEC developed immune evasion strategies targeting type I IFN signaling, suggesting a protective effect of type I IFN against those pathogens. *C. rodentium* has

been reported to actively inhibit epithelial type I IFN production employing a mechanism depending on the type III secretion system (T3SS) (90). Furthermore, it also reduces type I IFN signaling by decreasing nuclear translocation of phosphorylated STAT1 even after co-stimulation with IFN-β (90).

Infection of a colon cancer cell line with EPEC results in a modest IFN-β production, which regulates tight junction proteins such as claudin 1 and occludin to maintain barrier function (91). Using a distinct mechanism to *C. rodentium*, an EPEC T3SS effector, NleD, reduces IFN-β induction by inhibition of RNase L, ultimately resulting in evasion of antibacterial activities and disruption of barrier function (91).

Type I IFN also play a protective role during oral *Y. enterocolitica* infection. TRIF-deficient mice are highly susceptible to *Y. enterocolitica* infection with increased bacterial spread and lethality. This could be prevented by IFN-β treatment of TRIF-deficient mice and IFNAR blocking of wild-type mice recapitulates increased bacterial burden (70).

Campylobacter jejuni infection of murine DCs leads to TLR4/TRIF-dependent activation of IRF-3 and secretion of type I IFN and cooperative signaling through both TLR4/MyD88 and TLR4/TRIF pathways is required for full Th1 priming ability (92). Interestingly, production of type I IFN and other cytokines in splenic tissue is significantly increased by lipo-oligosaccharide (LOS) sialylation (93).

Whether type I IFN and/or type III IFN responses to sialylated *C. jejuni* have a role in oral infection models of mice awaits further investigation. Recently, novel mouse models have been developed relying on the eradication or modification of the murine gut microbiota using broad-spectrum antibiotic treatment and subsequent re-association with a complex human microbiota. This approach leads to stable colonization with *C. jejuni* and a proinflammatory response in the colons of infected mice (94). Hopefully, these models will provide the necessary tools to answer the questions of an *in vivo* importance of type I IFN and/or type III IFN in the mucosal immune response to *C. jejuni*. This will be of particular importance since type I IFN responses have also been observed in former *C. jejuni*-infected patients suffering from Guillain–Barré syndrome (GBS), an autoimmune neuropathy where infection with *C. jejuni* is the most common predisposing factor (95). *C. jejuni* LOS activates TLR4 and high responsiveness of DCs isolated from former GBS patients was hypothesized to influence the development of GBS. Indeed, although a strong variability in DC responses to LOS was observed, the frequency of CD38, CD40, and type I IFN high responders was significantly increased in the *C. jejuni*-related former GBS patients compared to controls (95).

IMPORTANCE OF TYPE I AND III IFN SIGNALING FOR INTESTINAL HOMEOSTASIS

A Role for Type I and III IFN in Shaping the Microbiota

Intestinal homeostasis is dependent on the tight interplay between the host and commensal flora. The flora directly protects the host from intestinal pathogens by competition for nutrients and space. Furthermore, commensal bacteria are important for the development of the immune system and maintenance of the barrier. The host immune system has also a great influence on the composition of the microbiota (96). Several studies have suggested that IFN signaling influences cross talk between the host and the flora.

It has long been recognized that the microbiota of different inbred mouse strains changes over time despite shared origin, which has been attributed to host genetic factors (97). Gene-expression analysis of the colonic mucosa revealed that IFN-responsive genes are differentially regulated between the different mouse strains and might thus contribute to microbiota regulation (97). Indeed, microbiota analysis of mice with selective ablation of type I IFN signaling in the intestinal epithelium revealed changes of the microbiota composition if littermates were housed separately for 8–12 weeks (76). However, it is not known whether this is a direct effect of type I IFN signaling by the epithelium and what the mechanistic relationship is.

The community stability of the gut microbiota might also depend on IFN signaling (98). IRF-9-deficient mice unable to respond to either type I IFN or type III IFN displayed a significantly higher temporal variation than wild-type mice, which was accompanied by an increased presence of T cells and neutrophils. However, STAT1-deficient mice, which classically are unable to respond to type I IFN, type III IFN, and IFN-γ, were not significantly different from wild-type mice implicating that a previously unrecognized pathway might be involved (98). Indeed, a role for IRF-9 in non-canonical IFN signaling and beyond the IFN response has been suggested (99).

Recently, Deriu et al. reported that systemic influenza-induced type I IFN production significantly alters the intestinal microbiota profile (68). While under their experimental conditions uninfected wild-type and IFNAR1-deficient mice displayed similar fecal microbial communities, influenza infection-induced type I IFN signaling resulted in a depletion of indigenous *segmented filamentous bacteria* and enhanced colonization with *Enterobacteriaceae* (68).

Type III IFN signaling on its own does not have a strong effect on the flora composition, as 16S rDNA sequencing of the V4 region of fecal pellets from wild-type or IFN-λR1$^{-/-}$ mice revealed similar bacterial class composition (13). It is important to note that microbiota studies are difficult to control and generalizations from one specific study should be drawn carefully as major differences have been found between mouse facilities.

A Role for Type I and III IFN in Shaping Intestinal Homeostasis

Upon colonization of germ-free mice with a two-component bacterial community, IFN-responsive genes are strongly upregulated in cecal epithelia (100).

Several studies have also demonstrated a role for commensal-induced tonic type I IFN signaling in the steady state to keep the host in a state of alertness against a systemic viral infection (101–103). Mononuclear phagocytes isolated from non-mucosal lymph nodes of germ-free mice are unable to upregulate type I IFN genes after stimulation with microbial ligands and thus fail

to prime NK cells and antiviral immunity (102). A second study came to similar conclusions demonstrating decreased antiviral gene expression and defective ISG expression of macrophages from antibiotic-treated mice (101). The nature of the commensal bacterial species or microbial product responsible for the tonic type I IFN production is unknown and its identification remains a future challenge.

Recently, a connection between the increased susceptibility of liver cirrhosis patients to bacterial infections and tonic type I IFN signaling was made (81). In a murine model of liver fibrosis, translocated gut bacteria induced the expression of a tonic type I IFN signature in the liver, which in turn conditioned myeloid cells to produce vast amounts of type I IFN upon subsequent systemic infection with *L. monocytogenes*. Type I IFN signaling resulted in the production of IL-10 by myeloid cells, which hampered antibacterial immunity. Key findings of the murine model such as a type I IFN signature in cirrhotic livers and myeloid IL-10 production could also be recapitulated in human patient material. Thus, tonic type I IFN signaling induced by the translocated gut microbiota can also have detrimental effects (81).

Plasmacytoid dendritic cells are known to be important producers of type I IFN after viral infections and treatment with bacterial nucleic acids. Interestingly, pDCs derived from Peyers's patches are incapable of producing significant amounts of type I IFN after stimulation with CpG-enriched oligodeoxynucleotides while producing IL-12 (104). Thus, although tonic type I IFN production by myeloid cells fulfils important functions to maintain basal levels of ISGs (81, 101, 102), the mucosal microenvironment prevents production of vast amounts of type I IFN by pDCs—presumably to prevent harmful immune responses to commensal microorganisms (104). Other sources of type I IFN in the GALT are stromal cells. Indeed stroma-derived type I IFN has been shown to induce APRIL and BAFF expression by pDCs, which facilitates T cell-independent IgA production by mucosal B cells (105).

Of note, the expression of ISGs at steady state seems to be driven by type I IFN and type III IFN in a cell type-specific manner. The cells of the lamina propria are mainly activated by type I IFN whereas IECs respond mainly to baseline levels of type III IFN due to low IFNAR expression on IECs, which is in accordance with the response pattern observed during viral infection (**Figure 1**) (14). Furthermore, the role of altered type I IFN signaling on IECs was addressed by several groups (50, 76, 106).

Tschurtschenthaler and colleagues reported that the lack of type I IFN signaling in IECs leads to a hyperproliferative phenotype (76). Particularly the secretory cell types Paneth and goblet cells are expanded in a setting where IECs are the only cells impaired in type I IFN signaling (Villin-Cre $Ifnar^{fl/fl}$). Although this study suggests that type I IFN signaling occurs in IECs, the proliferative phenotype is secondary due to alterations of the microflora, as cohousing of the mice resolved the differences (76).

Katlinskaya et al. used a model of decreased IFNAR degradation in IECs to study type I IFN signaling outcome in IECs (107). CK1α can phosphorylate IFNAR1, which subsequently leads to its degradation (108). Genetic ablation of CK1α in IECs leads to increased IFNAR levels and elevated type I IFN signaling in the epithelium. CK1α deficiency also results in β-catenin activation, which leads to hyperproliferation of the epithelium and loss of barrier function where type I IFN signaling is additionally blocked. Elevated type I IFN signaling, however, inhibits β-catenin-driven proliferation and induces apoptosis maintaining barrier integrity (107).

In another model of chronically elevated levels of systemic type I IFN, epithelial cell turnover was increased in various tissues including the intestine (106). This effect was not due to direct signaling of type I IFN in epithelial cells but by induction of Apol9a/b in macrophages or stromal cells that subsequently promoted the turnover of epithelial cells (106).

Taken together, epithelial type I IFN signaling seems to have a pleiotropic effect depending on levels of receptor expression, ligand abundance, microflora, and tissue context.

IMPORTANCE OF TYPE I/III IFN SIGNALING UNDER INFLAMMATORY CONDITIONS

Experiments employing different murine models of colitis such as dextran sodium sulfate (DSS) colitis or the T cell transfer model have provided a complex picture of the role of type I/III IFN in intestinal inflammation. Administration of DSS in the drinking water leads to disruption of the epithelial barrier and an inflammatory reaction to microbial patterns and food antigens. The T cell transfer model on the other hand relies on the transfer of naive $CD4^+$ T cells into immune-deficient mice (e.g., $RAG^{-/-}$ mice), which undergo activation and proliferation in response to microbial products to provoke inflammatory colitis when a suppressive T cell population ($Foxp3^+$ Tregs) is absent (109, 110).

Katakura et al. investigated the role of type I IFN induction after administration of CpG ODN in experimental colitis (73). Mice deficient in type I IFN signaling are resistant to the CpG ODN-mediated effect and are more susceptible to DSS treatment than wild-type mice, suggesting a protective effect of type I IFN signaling.

By contrast, a therapeutic approach employing a transgenic *Lactobacillus acidophilus* strain constitutively expressing IFN-β (La-IFN-β) failed to protect against DSS-induced colitis but exacerbated the disease (74). Colitic mice pretreated with La-IFN-β displayed increased production of proinflammatory cytokines and decreased numbers of Tregs in their small intestine. *In vitro*, maturation of bone-marrow-derived DCs with La-IFN-β resulted in a threefold reduction of IFNAR1 and an impaired ability to induce Tregs (74). Thus, although a correlation between downregulation of IFNAR1 on DCs and exacerbation of colitis was observed, pretreatment of colitic mice with La-IFN-β surprisingly also resulted in increased intestinal damage (74). These results suggest that a tight regulation of type I IFN signaling is important for the balance of intestinal homeostasis.

The effect of type I IFN in experimental colitis might depend on the severity of inflammation and opposing roles in specific phases of intestinal damage and inflammation have been proposed (75, 111). At high DSS concentrations, type I IFN signaling protected

against acute intestinal damage presumably by suppressing the release of IL-1β from colonic MHC class II$^+$ cells (75). In the recovery phase after DSS treatment, type I IFN signaling resulted in delayed recovery from intestinal inflammation accompanied by increased cell apoptosis as well as an increase in chemokine production and subsequent infiltration of neutrophils and inflammatory monocytes (75). The potential of type I IFN signaling to either suppress acute colitis or delay the recovery might provide an explanation for the varying effects of type I IFN treatment on inflammatory bowel diseases (IBD) patients.

Several groups have analyzed the effect of type I IFN signaling on T cells (23, 71, 72, 112).

CD69 is suggested to be a regulator of intestinal homeostasis and is highly expressed on intestinal CD4$^+$ T cells, which at steady state is driven by the microflora but can be further induced by type I IFN signaling (72, 113). Poly(I:C) treatment during T cell transfer colitis attenuates colitis by IFNAR-dependent CD69 induction on T cells, which leads to downregulation of proinflammatory cytokine levels (72).

Furthermore, Lee et al. identified a protective role of T cell type I IFN signaling by regulating the expression of Foxp3 and the suppressive effect of Tregs (71). Whereas Tregs isolated from wild-type mice suppressed colitis when cotransferred with naïve CD4$^+$ T cells, the same cell population derived from IFNAR1$^{-/-}$ mice failed to do so. Although Tregs undergo normal development in IFNAR1$^{-/-}$ mice, the cells acquire a dysfunctional phenotype accompanied with reduced Foxp3 expression when cotransferred with naive CD4$^+$ T cells into RAG1$^{-/-}$ recipients. Administration of recombinant IFN-α ameliorated T-cell-dependent colitis by augmenting the number of Foxp3$^+$ Tregs suggesting a potential therapeutic application of type I IFN in intestinal inflammation (71, 72, 113).

By contrast, several studies suggested an indirect effect of TLR9 ligands and type I IFN signaling for protection in T cell-mediated experimental colitis (23, 99, 112). While a role for B cells was excluded, colitis-reducing effects of CpG ODN were mediated by CD11c$^+$ cells and required functional type I IFN signaling in a model of T cell transfer colitis (112).

Kole et al. showed that colon mononuclear phagocytes deficient in type I IFN signaling failed to produce regulatory cytokines such as IL-10, IL-27, and IL-1RA in response to TLR activation. Furthermore in the T cell transfer colitis model, IFNAR signaling of host hematopoietic cells was important to limit effector cell expansion and to promote the stabilization of Foxp3$^+$ Tregs (23).

Intestinal epithelial cells and in particular Paneth cells have also been proposed to be a target of type I IFN signaling in the intestine (76, 114). Paneth cells are epithelial cells located at the bases of small-intestinal crypts specialized in secretion of antimicrobial peptides and factors to sustain epithelial stem cells and progenitor cells (115). Mice with a specific deletion of IFNAR1 in IECs display expansion of Paneth cell numbers and epithelial hyperproliferation when compared with wild-type littermates. Although epithelial-specific deletion of IFNAR1 did not impact on the severity of spontaneous or DSS-induced intestinal inflammation, they exhibited increased tumor burden in the azoxymethane/DSS model of colitis-associated colon cancer (76), Both spontaneous epithelial hyperproliferation and tumor promotion are dependent on the microbial flora, since differences between wild-type and IEC-specific IFNAR1-deficient mice were only apparent if the mice were housed separately (76).

Human and murine IECs display high responsiveness to type III IFN treatment (12, 116). A recent study has demonstrated a protective role of type III IFN signaling in DSS-induced colitis (77). Mice deficient for the type III IFN receptor lost significantly more weight and suffered from significantly increased intestinal damage after DSS treatment when compared to WT controls. Additional loss of the type I IFN receptor did not change the pathology scores compared to the single loss of type III IFN receptor, emphasizing a prominent role of type III IFN signaling in this model. The protective effect of type III IFN signaling is independent of potential changes in the microbiota since the same results were obtained when wild-type and type III IFN signaling-deficient mice were cohoused for 3 weeks (77).

TYPE I IFN AND IBD

The IBD, comprising Crohn's disease (CD), and ulcerative colitis (UC) are chronic debilitating inflammatory disorders of the gastrointestinal tract. IBD affects about 0.2% of Western populations and there is no current cure, typically requiring long-term treatment with immune suppressive agents and, in many cases, surgical intervention. Although the etiology remains unclear, IBD is thought to arise due to aberrant immune responses to components of the commensal bacterial microbiota (117). Recent genome-wide association studies have identified more than 160 genetic susceptibility loci for IBD, with affected genes involved in immunity and in barrier function (118). Many of those single-nucleotide polymorphisms (SNPs) are found in genes associated with pathogenic cytokine circuits, such as the Th17/IL23 circuit, IL-10, and type I IFN-I signaling (119). The majority of signaling mediators are shared between different cytokine signaling cascades and therefore exact determination of the relevant pathways is impossible from the genetic data only. Interestingly, several of the IBD-associated genes are also involved in the type I IFN signaling pathway. The rs2284553 SNP is commonly associated with the *IFNGR2* gene but could also affect the *IFNAR1* gene (118). *JAK2*, *TYK2*, *STAT1*, and *STAT3* genes harbor identified SNPs and are signaling mediators in many cytokine pathways such as IL-22, IL-10, and also type I/III IFN. Furthermore, polymorphisms in the *MDA5* or *IRF5* gene might alter the production of type I/III IFN (118). Although the type I IFN signaling network is not one of the major players in IBD pathology, slight alterations may contribute to the imbalanced immune response at the lamina propria, as suggested by mouse studies.

Indeed, Giles and colleagues analyzed the responsiveness of T cells from healthy controls and IBD patients to IFN-β and found that IFN-β signaling modulates colonic T cell responses in a context-dependent manner. Human colonic T cells were responsive to exogenous IFN-β and endogenous IFN-β influenced the cytokine profile of *ex vivo* cultured T cells. T cells from healthy controls produced decreased levels of IL-10 in the absence

of IFN-β signaling whereas T cells from IBD patients produced elevated levels of proinflammatory cytokines (120).

Interferons-β has been approved for the treatment of multiple sclerosis (MS); however, a subset of patients does not respond to the treatment. Axtell and colleagues analyzed the effect of IFN-β on different Th subsets and found that IFN-β treatment in a mouse model of EAE attenuates disease development in a Th1-driven pathology, but had no effect or even exacerbates pathology in Th17-driven disease. Furthermore, they could correlate high IL-17-F serum levels in MS patients to non-responsiveness toward IFN-β treatment. These findings confirm the immunomodulatory role of IFN-β but also demonstrate the diverse consequences it has in different context with opposing effects within a Th1 and Th17 setting (121).

Despite the varying results from mouse studies on the role of type I IFN in colitis and the discrepancy between type I IFN effects on suppressing acute colitis and delaying recovery (74, 75), type I IFN have been suggested for the treatment of IBD. Several small studies have evaluated the consequences of IFN-β1a in IBD patients with varying results (122–128). Although small pilot studies suggested a beneficial outcome of type I IFN treatment of IBD patients (123, 124), a placebo-controlled, double-blind study on Crohn's patients in remission did not find any improvement by IFN-β1a treatment on the maintenance of remission (127). Also two randomized placebo-controlled studies in UC patients with active disease could not show a beneficial effect of IFN-α or IFN-β1 treatment on disease remission (125, 126). A small study analyzing cytokine levels before and after treatment with IFN-β1a found a correlation between responsiveness and reduction of IL-13 levels in UC patients. The unresponsiveness to IFN-β1 treatment correlated with elevated levels of IL-17 in accordance with the findings in MS patients (121, 128).

Taken together, these studies do not support a beneficial outcome of type I IFN treatment during IBD. This conclusion was also drawn in a recent intervention review analyzing all trial data published on the effectiveness of type I IFN treatment on remission in UC patients (129). However, considering the analysis of IFN-β non-responsiveness of patients with MS (121), context-specific responsiveness of T cells toward type I IFN (120), and controversial findings in mouse studies (23, 74, 75), the effect of the treatment might vary between Th profiles of patients and a careful pre-selection of patients would be required. Further studies with sufficient patient numbers and thorough analysis of immunological and disease parameters are required.

TYPE I IFN AND CELIAC DISEASE

Celiac disease is a small-intestinal enteropathy characterized by an aberrant T cell-mediated immune response of susceptible individuals to dietary gluten. The pathogenic adaptive immune response is initiated by the interplay between gluten and the MHC class II molecules HLA-DQ2 and DQ8 and is characterized by a potent Th1 response. The excessive tissue destruction is further driven by a severe IEL hyperplasia targeting IECs. Histologically, celiac disease is characterized by villous flattening, crypt hyperplasia, and IEL infiltration. Affected individuals can present with very variable symptoms ranging from asymptomatic to severe symptoms ascribed to impaired absorption of nutrients (130).

The strongest genetic factor for the disease is HLA-DQ2 and DQ8; however, it is now recognized that further immune-regulatory or activating factors are required for disease establishment. Several celiac disease susceptibility loci in genes associated with innate immune responses have been identified, suggesting a role for innate immunity in the development of the disease (131).

An influence of type I IFN on the development of celiac disease has been widely discussed. Indeed, a number of case studies reported on the development of diarrhea and the onset of celiac disease during treatment with IFN-α for chronic hepatitis C patients (132–137). A retrospective study of 534 hepatitis C patients with or without symptoms of celiac disease showed an activation of silent celiac disease in the majority of patients positive for transglutaminase antibodies while on IFN therapy (138). The immunomodulatory properties of type I IFN might worsen underlying autoimmune disorders and monitoring of hepatitis C patients for celiac disease before starting an IFN therapy has been suggested. A potential role of cotreatment with ribavirin, which promotes a Th1-mediated immune response while suppressing Th2 responses, has also been discussed (133).

The high prevalence of celiac disease in HCV patients treated with IFN-α was investigated in a study including 210 chronic hepatitis C patients. This study failed to detect a significant association of celiac disease and HCV infection and in addition came to the conclusion that IFN-α therapy *per se* does not trigger celiac disease in patients negative for endomysium (EMA) and tissue transglutaminase (139). It does not however rule out that IFN-α treatment might trigger the development of celiac disease in susceptible individuals.

To investigate the underlying mechanisms, explant cultures of human fetal gut were analyzed after activation of T cells with anti-CD3 and IFN-α. While single treatment with either anti-CD3 or IFN-α alone did not trigger any profound changes, the combination of both resulted in enhanced Th1 responses and crypt cell hyperplasia associated with enhanced STAT1, STAT3, and FYN phosphorylation. IFN-α treatment might thus facilitate activation of Th1-reactive cells and trigger immunopathology (135, 140).

Onset of celiac disease-like symptoms have also been observed in a case of chronic myeloid leukemia treated with IFN-α again suggesting a role of type I IFN in promoting Th1 responses to gluten (135). Also, IFN-α protein was detected in duodenal tissue of celiac disease patients but not in control samples (135).

Further studies are required to determine whether a direct link exists between type I IFN signaling and celiac disease.

CONCLUDING REMARKS

Although important progress has been made in recent years, additional studies are required to deepen our understanding of the role of type I IFN and type III IFN in the gut. Type I IFN signaling in enteric viral infections is mostly protective, whereas it can be detrimental in certain enteric bacterial infections. The

clinical data using type I IFN for colitis treatment are partly contradictory and a larger number of patients are required to obtain conclusive results.

With the exception of some viral infection and colitis models, where type III IFN signaling is mostly protective, the data on illuminating the role of type III IFN signaling in the gut are generally scarce. It is now well established that epithelial cells are especially responsive to type III IFN, which enforces the mucosal barrier and prevents viral entry and infection. Less clear is the non-responsiveness of the epithelium to type I IFN observed under several conditions (**Figure 1**). What benefit do epithelial cells gain from the decreased responsiveness? Can receptor expression levels and localization fully explain reduced type I IFN responsiveness in the epithelium?

The narrow range of cells responding to type III IFN makes it an attractive target for future clinical studies with increased specificity and fewer side effects than type I IFN.

AUTHOR CONTRIBUTIONS

JP and SST wrote the manuscript.

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REVIEW
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The Impact of the Interferon/TNF-Related Apoptosis-Inducing Ligand Signaling Axis on Disease Progression in Respiratory Viral Infection and Beyond

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Interferons (IFNs) are well described to be rapidly induced upon pathogen-associated pattern recognition. After binding to their respective IFN receptors and activation of the cellular JAK/signal transducer and activator of transcription signaling cascade, they stimulate the transcription of a plethora of IFN-stimulated genes (ISGs) in infected as well as bystander cells such as the non-infected epithelium and cells of the immune system. ISGs may directly act on the invading pathogen or can either positively or negatively regulate the innate and adaptive immune response. However, IFNs and ISGs do not only play a key role in the limitation of pathogen spread but have also been recently found to provoke an unbalanced, overshooting inflammatory response causing tissue injury and hampering repair processes. A prominent regulator of disease outcome, especially in—but not limited to—respiratory viral infection, is the IFN-dependent mediator TRAIL (TNF-related apoptosis-inducing ligand) produced by several cell types including immune cells such as macrophages or T cells. First described as an apoptosis-inducing agent in transformed cells, it is now also well established to rapidly evoke cellular stress pathways in epithelial cells, finally leading to caspase-dependent or -independent cell death. Hereby, pathogen spread is limited; however in some cases, also the surrounding tissue is severely harmed, thus augmenting disease severity. Interestingly, the lack of a strictly controlled and well balanced IFN/TRAIL signaling response has not only been implicated in viral infection but might furthermore be an important determinant of disease progression in bacterial superinfections and in chronic respiratory illness. Conclusively, the IFN/TRAIL signaling axis is subjected to a complex modulation and might be exploited for the evaluation of new therapeutic concepts aiming at attenuation of tissue injury.

Keywords: interferon, interferon-stimulated genes, tumor necrosis factor-related apoptosis-inducing ligand, acute lung injury, innate immunity, influenza, respiratory syncytial virus, coronavirus

INTRODUCTION

In 1957, Isaacs and Lindenmann (1) first recognized the potential of a soluble and probably cell-derived factor to combat influenza virus infection and named this factor interferon [(IFN) from latin *interferre*, to interfere]. Since then, three subgroups of IFNs have been defined, primarily by their differential receptor usage. While the groups of type I IFN and type III IFN comprise largely agents directly limiting pathogen spread by improving cellular counter measurements, IFN-γ, the sole type II IFN, has been mainly implicated in the modulation of innate and also adaptive immune responses (2, 3). Accordingly, type I and III IFNs are key signaling molecules in viral control, and lack of both signaling pathways results in increased viral loads and disease severity. Still, there is accumulating evidence that not only lack of an antiviral response but that also an unbalanced overshooting activation of IFNs contributes to an exaggerated inflammatory reaction, tissue injury, reduced proliferative capacity, and thus enhanced disease severity (**Table 1**). Especially in viral infections, this effect has not only been tracked down to IFN signaling in general but specifically to the exaggerated production of key effector IFN-stimulated genes (ISGs) (4). A prominent example is the TNF-related apoptosis-inducing ligand (TRAIL) that displays an ambivalent role in viral infection (5–7) (**Table 1**). Whereas first identified as factor produced by immune cells in non-respiratory infection (8, 9), TRAIL is now especially well studied in influenza A virus (IAV) infection, where it is released in high amounts from bone marrow-derived macrophages upon pathogen-associated molecular patterns (PAMPs) recognition and type I IFN production (10). Macrophage-released soluble TRAIL, but also membrane-bound cell-associated TRAIL, acts *via* distinct receptors on infected but also on non-infected, neighboring cells. In viral infection, its preliminary role is to drive infected cells into apoptosis to limit virus spread. However, studies performed within the last decade demonstrate that TRAIL's antiviral activity seems to be outweighed by the functional and structural damage it induces not only in infected but also in bystander cells such as uninfected cells of the alveolar epithelium (10, 11). This process is not only relevant in promoting viral disease progression but has further implications in bacterial superinfection and probably also in chronic diseases. The recognition of the ambivalent role of IFN-driven signaling *in vivo* is a first important step to better understand disease progression and to envision novel treatment options for primary viral respiratory infection targeting distinct host-derived signaling mediators such as TRAIL.

FROM PATTERN RECOGNITION TO ISGs—BASIC PRINCIPLES OF IFN SIGNALING

IFN Induction upon Virus Recognition

It is a commonly accepted concept that—as Janeway (12) already proposed in 1989—immune activation toward invading

TABLE 1 | Major effects of the interferon (IFN)/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) signaling axis on host cells in respiratory viral infection.

	Effect	Virus	Reference
IFN	Virus control by antiviral interferon-stimulated genes induction	Influenza A virus (IAV)	(58–60)
		Coronaviruses (CoV)	(63, 64)
		Respiratory syncytial virus (RSV)	(62)
	e.g., *via*		
	Interferon-induced transmembrane proteins	IAV, West Nile virus	(50, 51)
	Myxovirus resistance protein A	Vesicular stomatitis virus, IAV	(52–54)
	ISG20	IAV	(57)
	Restriction of immunopathology	IAV	(88)
		CoV	(63, 64)
		RSV	(62)
	Enhanced inflammatory response contributing to tissue damage, morbidity, and mortality	CoV	(76)
		IAV	(74, 95, 98)
		RSV	(67)
		Sendai virus	(73)
	Cell death induction, e.g., Bcl-2-associated X protein, caspase-8, Fas-associated protein with death domain, Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL)	dsRNA, polyI:C	(4, 110)
		IAV	(4, 5, 10, 115)
		Sendai virus	(110)
TRAIL	Virus control by apoptosis induction in infected cells	IAV	(6, 170, 171)
	Tissue injury by apoptosis of both infected and non-infected alveolar epithelial cells, lung macrophages	IAV	(5, 7, 10)
		RSV	(137)
	Necrosis of fibroblasts, dendritic cells, and epithelial cells	IAV	(146, 147, 168)
	Increased cellular infiltration	CoV	(175)
	Decreased expression of Na,K-ATPase, impaired epithelial fluid reabsorption	IAV	(11)

pathogens is mounted upon recognition of PAMPs. PAMPs are evolutionary conserved biomolecules such as proteins, lipids, nitrogen bases, sugars, and complexed biomolecules such as lipoglycans that are essential to the survival of a given pathogen (13). PAMPs are recognized by distinct pattern recognition receptors (PRRs) that are germ-line encoded and—similar to PAMPs—usually show a high evolutionary conservation. The first recognized and probably most intensely studied family of PRRs are the toll-like receptors [TLRs; reviewed in Mogensen (14); Leifer and Medvedev (15)]. In viral infection, both host cell membrane-localized TLRs (TLR2, TLR4, detecting viral envelope proteins) and endosomal TLRs (TLR3, TLR7, TLR8, and TLR9, nucleic acid sensors) initiate signal transduction cascades leading to IFN production (**Figure 1**). TLR activation results in either myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adaptor protein-inducing IFN-β (TRIF) recruitment that both trigger various downstream signaling events, eventually leading to IFN regulatory factor (IRF)3, IRF7, and NFκB nuclear translocation as well as MAP kinase and activator protein 1 (AP-1) activation (16, 17).

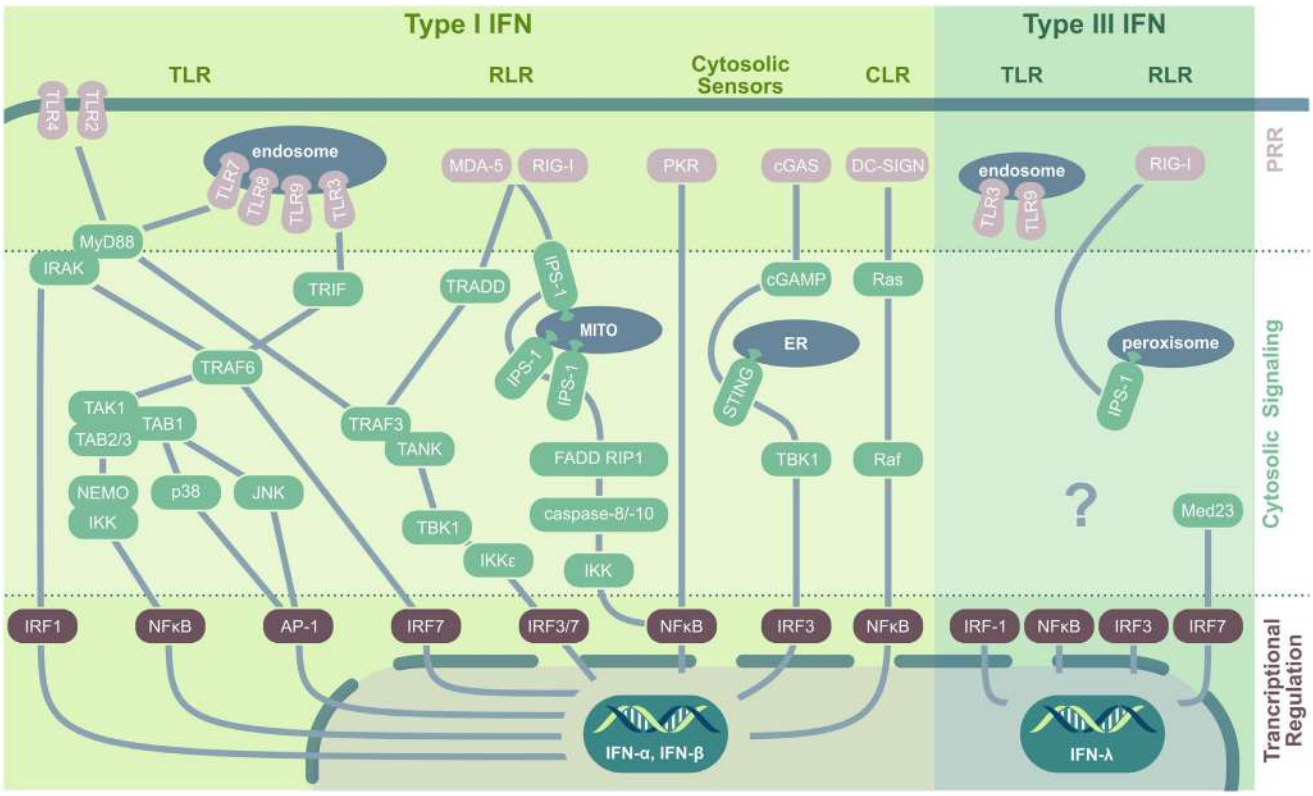

FIGURE 1 | **PRRs and their downstream signaling pathways in virus-induced IFN induction**. In viral infection, type I IFNs are induced by TLR, RLR, CLR, and cytosolic nucleic acid sensors. Cell membrane-located *TLRs* ligate to viral envelope proteins (e.g., TLR2/herpes simplex virus), upon which they recruit MyD88. MyD88 interacts with IRAK kinase (IRAK-1, -2, or -4) that either directly activate IRF1 or interact with TRAF6, which induces IRF7 or assembles with TAK1. TAK1 forms a complex with TAB-1/-2 and -3 and subsequently either activates the MAPK kinases p38 and JNK, leading to AP-1 phosphorylation and nuclear translocation, or induces ubiquitination of NEMO followed by IκB degradation and NFκB activation. Endosomal TLRs recognizing viral nucleic acid and signal *via* the adaptor protein MyD88 (for TLR7/8/9) or interact with TRIF (for TLR3) followed by TRAF3, TANK, and TBK1 activation. TBK1 and IKKε then phosphorylate and activate IRF3 and IRF7. Additionally, TRIF can interact with TRAF6 to initiate TAK1 signaling. Both *RLRs*, RIG-I and MDA-5, recognize nucleic acid contents in the cytoplasm and stimulate the mitochondrial anchored IPS-1 for dimerization followed by TRADD recruitment that acts *via* TRAF3 on IRF3 and IRF7. Additionally, IPS-1 can interact with FADD and RIP1 to activate NFκB *via* IKK activated by caspase-8 and -10. Also *PKR* signaling results in NFκB activation and nuclear translocation. The dsDNA sensor *cGAS* produces cGAMP that activates ER-located STING that *via* TBK1 induces IRF3 translocation and type I IFN production. *CLRs* play a minor role in viral recognition; however, DC-SIGN activates the small GTPase Ras and Raf protein kinase, followed by NFκB activation. *Type III IFN* are induced by TLR3, TLR9, and *via* RIG-I and peroxisomal-resident IPS-1. Especially IRF1, but also NFκB, IRF3, and IRF7 are implicated in IFN-λ production, with the latter being stabilized by Med23. *Abbreviations*: TLR, toll-like receptor; RIG-I, retinoic acid-inducible gene; RLR, RIG-I-like receptors; CLR, C-type lectin receptors; PRR, pattern recognition receptor; MITO, mitochondrium; ER, endoplasmatic reticulum; IFN, interferon; MyD88, myeloid differentiation factor 88; IRAK, interleukin-1 receptor-associated kinase; IRF, IFN regulatory factor; TRIF, TIR-domain-containing adaptor protein-inducing IFN-β; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; TAK, transforming growth factor β-activated kinase 1; TAB, TAK1-binding protein; NEMO, essential modulator; IKK, inhibitor-κB kinase; JNK, c-Jun N-terminal kinase; AP-1, activator protein 1; TANK, TRAF family member-associated NF-κB activator; TBK, TANK-binding kinase; MDA-5, melanoma differentiation antigen 5; TRADD, TNF receptor type 1-associated death protein; IPS-1, IFN-β promoter stimulation 1; FADD, Fas-associated protein with death domain; RIP1, receptor-interacting serine/threonine-protein kinase 1; PKR, protein kinase R; cGAS, cyclic GMP–AMP synthase; cGAMP, cyclic guanosine monophosphate–adenosine monophosphate; STING, stimulator of IFN genes; DC-SIGN, dendritic cell-specific ICAM3-grabbing non-integrin; AIM-2, absent in melanoma 2; Med23, mediator complex subunit 23; TRAF3, TNF receptor-associated factor 3.

Similar to endosomal TLRs, the cytosolic retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) are specialized to recognize viral nucleic acid contents and are central PRRs relevant to mount an antiviral response, providing resistance to most RNA (e.g., orthomyxoviruses) and some DNA (e.g., reoviruses) viruses [reviewed in Ref. (18, 19)]. Both melanoma differentiation-associated gene 5 (MDA-5) and RIG-I recognize dsRNA, 5′-triphosphate RNA, or the synthetic analog to dsRNA, polyI:C (20, 21). Both drive the dimerization of the mitochondria-associated adaptor protein IFN-β promoter stimulation 1 (IPS-1) (also named MAVS, VISA, CARDIF). A subsequently activated cascade including TRADD (TNF receptor type 1-associated death domain protein), TRAF3 (TNF receptor-associated factor 3), and TANK (TRAF family member-associated NF-κB activator) induces the phosphorylation of IRF3 and IRF7, resulting in type I IFN production (22). The third RLR, LGP2, so far has primarily been implicated to regulate RIG-I or MDA-5 as a cofactor; however, a recent study by Stone et al. (23) demonstrated a novel, non-redundant, and independent role of LGP2 in West Nile virus infection. Another class of PRR, the nucleotide oligomerization domain (NOD)-like receptors (NLRs), has mainly been implicated in bacterial recognition (24), still several NLRs are activated as well upon virus infection. Especially, NLRP3 is known to recognize RNA of different viruses including hepatitis C virus, measles virus, influenza, and vesicular stomatitis virus (VSV) (25–28), resulting in inflammasome formation and caspase-1-dependent activation of IL-1β and IL-18 (29–31). In addition, virus infections are sensed also by a structurally diverse group of viral RNA and DNA sensors residing in the cytoplasm. These include the cyclic GMP–AMP synthase that synthesizes the second messenger cGAMP. cGAMP in turn activates stimulator of IFN genes (STING), TANK-binding kinase 1, and IRF3, triggering IFN production (32–34). Moreover, STING itself acts as a PRR and has been implicated in DNA virus recognition including HSV, adenovirus, vaccinia virus, and papilloma virus and in sensing of retroviral RNA–DNA hybrids (35) and RNA viruses after being activated by RIG-I (36, 37). Another cytosolic nucleic acid sensor, PKR, is well known for its phosphorylation of eukaryotic initiation factor 2 α (eIF2α) in response to viral dsRNA. Phosphorylation of eIF2α results in its deactivation, host translational shut-off, and the limitation of viral replication. Of note, the PKR–eIF2α-driven inhibition of protein synthesis can contribute to an IPS-1-dependent IFN-β induction (38). Furthermore, PKR has been implicated in efficient type I IFN activation by TLR3 in response to dsRNA (39) and can mediate—at least partially—activities of IRF1 (40).

In addition to type I IFNs, also type III IFNs exert antiviral activity and are widely expressed after viral recognition, being produced by most cell types including epithelial, endothelial, fibroblast, and polymorphonuclear cells [reviewed in Ref. (41, 42)]. Like type I IFNs, type III IFNs are induced in viral infection by the PRR RIG-I as well as TLR3 and TLR9 and rely on the activation of the same transcriptional activators, including IRF3, IRF7, and NFκB. These observations initially led to the conclusion that type I and type III IFN comprised two completely redundant systems to induce ISGs in response to PAMP recognition. However, more recent data suggest distinct selection mechanisms for either type I or type III IFN expression. As such, IPS-1 specifically induces IFN-λ, but not type I IFN, when located at the peroxisomal membrane instead of the mitochondrial membrane in response to RIG-I activation by reovirus, Sendai virus, or dengue virus challenge (43). Interestingly, type III IFN induction is largely independent toward AP-1 translocation, which facilitates an instantaneous induction of IFN-λ after viral recognition, highlighting it as an important immediate factor driving innate microbial defense mechanisms.

JAK/STAT-Dependent Induction of ISGs

Release of IFNs upon pathogen recognition is a highly conserved mechanism—found from teleost fish to insects and mammals—to prepare the surrounding cells as well as the host defense against the invading threat (44, 45). Whereas often high-level IFN production relies on specialized sentinel cells such as macrophages or dendritic cells (DCs), mostly all cells of the multicellular organisms are able to respond to at least one type of IFN by expression of respective receptors. Receptor binding then induces a signal transduction cascade relying on the Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT), which results in efficient transcription of a plethora of different ISGs in infected as well as bystander cells (46, 47). IFNs engage a classical canonical signal transduction cascade employing JAK/STAT molecules after binding to their respective receptors. Herein, type I IFNs ligate to their common heterodimeric receptor consisting of the IFN-α receptor (IFNAR)1 and IFNAR2 subunits, whereas type III IFNs act *via* a interleukin-10 receptor 2 (IL-10R2)/IFN-λ receptor 1 (IFNLR1) heterodimer that to date has been reported to be restricted in its expression to epithelial cells (48). In type I IFN signaling, IFNAR engagement leads to the activation of the receptor-associated protein tyrosine kinases JAK1 and tyrosine kinase 2 followed by the recruitment or repositioning of already associated but elsewise latent cytoplasmatic transcription factors STAT1 and STAT2. Consequently, STAT1/STAT2 are phosphorylated on conserved tyrosine residues, they disassemble, undergo conformational changes enabling their heterodimerization as well as the exposure of a nuclear localization sequence. Subsequently, the STAT1/STAT2 heterodimer translocates into the nucleus where it interacts with the IRF9 to form the trimeric IFN-stimulated factor 3 (ISGF3). ISGF3 binds cognate DNA sequences, the IFN-stimulated response elements (ISRE), finally leading to ISG induction. Also type III IFN interaction with the IL-10R2/IFNLR1 receptor complex triggers STAT1/STAT2 heterodimerization, nuclear translocation, and ISGF3 assembly (49).

IFNs IN ACUTE RESPIRATORY VIRAL INFECTION

Interferon signaling results in the induction of ISGs evoking different cellular responses against viral infection, both in infected as well as in non-infected cells, including direct antiviral, immune-modulatory, or cell death-inducing effects to enable an immediate and robust response to a pathogen challenge. Many ISGs directly interfere with viral replication on an intracellular level. Well-studied examples of antiviral ISGs comprise

IFN-induced transmembrane proteins (IFITMs) effective in IAV, West Nile virus, and dengue virus infection (50, 51), the myxovirus resistance protein A (MxA) that interferes with VSV mRNA production and binds the IAV nucleocapsid to prevent nuclear translocation of viral genetic material (52–54), the 2-5-oligoadenylate synthase (OAS), which activates RNAse L triggering viral RNA degradation, or the PRR PKR, which besides activating the IFN response has a major impact on viral protein translation by inhibiting the eIF2α (55). More recently identified ISGs include the plasminogen activator inhibitor 1 that blocks IAV infection by inhibiting glycoprotein cleavage executed by extracellular airway proteases (56) or the antiviral ISG20 that limits IAV viral replication *via* its exonuclease activity most likely by interfering with the viral NP (57).

Accordingly, IFN pretreatment usually results in the establishment of an antiviral state that limits viral replication and spread from the start of infection and thus favors milder disease outcomes. IFN-α pretreatment has been demonstrated to limit viral spreading of seasonal IAV strains and thus decrease morbidity and mortality in mice, guinea pigs as well as ferrets (58–60). As shown by a study by Tumpey et al. (61), this effect can be attributed to the early induction of antiviral ISGs including MxA. Importantly, type I IFN pretreatment also dampens early replication of highly pathogenic avian influenza in ferrets (58). Also in respiratory syncytial virus (RSV) infection, treatment with recombinant IFN-α results in significantly decreased lung viral titers, alveolar inflammatory cell accumulation, and clinical disease in RSV-infected mice (62). In addition, respiratory infections caused by emerging coronaviruses (CoV) can be ameliorated by type I IFN pretreatment strategies. In an *in vivo* macaque model (*Macaca fascicularis*) of severe acute respiratory syndrome (SARS)-CoV infection, it could be demonstrated that pretreatment with pegylated IFN-α significantly diminished CoV replication and excretion and resulted in reduced pulmonary damage (63). Macaques also serve as a preclinical model for Middle East respiratory syndrome (MERS)-CoV, and similar to SARS-CoV, IFN-α in combination therapy with ribavirin reduces viral replication and severe histopathological changes (64).

In line, genetic alteration leading to an enhanced type I IFN signaling has been demonstrated to limit IAV-induced disease outcomes, as a recent study by Xing et al. (65) reported that deletion of TRIM29, a negative regulator of NEMO, which leads to NFκB induction and therefore enhanced type I IFN production, is protective *in vivo* in IAV-infected mice. Conversely, the genetic depletion of IFN signaling in IFN receptor-deficient mice can result in a lack of viral control, resulting in enhanced viral titers in different viral infections including RSV or IAV (66, 67). Still, it must be noted that this effect is often mild in IFNAR- or IFNLR-deficient animals, which is probably related to a certain redundancy between type I and type III IFN signaling in limiting viral spreading in epithelial cells (68). In contrast, IFNAR/IFNLR-double knockout or STAT1 knockout animals that are deficient in both type I and type III IFN signal transduction succumb more readily to infection due to excessive viral replication (69–71). *Vice versa*, mutations in key ISGs such as IFITM3 are associated with increased IAV disease severity in mice and humans (72).

However, IFN pretreatment and genetic loss-of-function approaches generally are not relevant to human respiratory virus-induced hospitalizations, where patients already present with ongoing respiratory infection and inflammation, and preclinical studies underline that type I IFN signaling in an already inflamed organ is rather detrimental and enhances tissue injury, and lack of type I IFN *in vivo* may even ameliorate disease outcome. Accordingly, in cases where the antiviral defense was not compromised (e.g., in animals with efficient type III IFN signaling) IFNAR-deficient mice infected with Sendai virus or IAV were reported to be more resistant to infection-induced morbidity and mortality (73, 74). Similarly, in Sendai virus *in vivo* infection, Wetzel et al. (75) showed that increased IFN-β levels in the lung homogenate correlates to increased morbidity and mortality, and also for SARS-CoV, a recent study demonstrates that high type I IFN induction in an already ongoing viral infection contributes to mortality in SARS-CoV-infected mice (76). Also for IAV infection, type I IFN application after infection has been proven to drive disease severity (74). Of note, the detrimental effects of type I IFNs were especially pronounced in mice lacking central antiviral factors, namely the IFIT protein in Sendai virus infection and MxA in IAV. Interestingly, Beilharz et al. (77) demonstrated that application of low doses of IFN-α reduces viral load, which to a certain degree led to attenuated disease progression, whereas high dose application of type I IFN contributed to morbidity (77). In line, high expression levels of ISGs have been shown to correlate to worse outcomes in ARDS patients (78). This observation corresponds to reports stating that the IFN threshold needed to induce antiviral ISGs—showing a beneficial effect in acute respiratory viral infection—is by at least 10-fold lower than the IFN dose necessary to trigger ISGs that show immunomodulatory, death-inducing, or anti-proliferative effects and thus can contribute to disease progression (79–82). Altogether, these data demonstrate that IFNs may significantly contribute to unbalanced inflammation and tissue injury during respiratory viral infection depending on expression levels and duration of IFN-related signaling events.

To date, the underlying mechanisms leading to the IFN-dependent enhanced disease progression are not fully understood but often result from a dysregulated IFN signaling response. One mode of action of IFN and IFN-stimulated ISGs is to stimulate negative feedback loops on IFN signaling. For example, suppression of JAK1 or STAT1 *via* specific phosphatases, expression of suppressor of cytokine signaling (SOCS)1 and SOCS3, or ubiquitination and endocytosis of the IFN receptors (83–86) desensitize cells to IFN signaling and allow recovery and the return to homeostasis after microbial challenge. As demonstrated by Bhattacharya et al. (87), the lack of IFNAR downregulation and thus the failure to initiate IFN-desensitization contributes to increased inflammatory signaling, extensive lung injury and, importantly, also impaired tissue regeneration (87). Moreover, IFNs are immunomodulatory and shape the specific responses of cells of the immune system, which has been implied to influence disease progression both positively and negatively. In a recent study, type I IFNs have been associated in the regulation of innate lymphoid immune cells (ILC)2 in IAV infection, where they—in

concert with IFN-γ and IL-27—promote an ILC2-dependent restriction of immunopathology (88). Moreover, type I IFNs play an important role in stimulating the immune response driven by DCs; they stimulate the expression of MHC molecules as well as the co-stimulatory ligands CD80 and CD86 and thus activate T cell responses (89, 90). Additionally, ligand-driven activation of IFNAR enhances the proliferation of CD8 positive T cells, especially early in infection. However, late in infection, type I IFNs were also implied in decreasing T cell expansion upon SARS-CoV and arenavirus infection (76, 91), which might potentially be related to the above described desensitization upon prolonged IFN signaling and might be detrimental if initiated too early in infection. In line, Pinto et al. (92) reported an impairment of T cell responses upon type IFN induction in West Nile virus infection. In B cells, the lack of IFNAR has been demonstrated to result in enhanced release of neutralizing antibodies in IAV infection (93) implying a repressive role for type I IFN in B cell antibody production. However, immunization studies by Le Bon et al. (94) reported the necessity of IFNAR on B cells for efficient IgM and IgG production, underlining the need for further studies to understand the detailed effects of IFN-dosage and timing adaptive immunity activity upon respiratory viral infection.

Type I IFNs additionally induce the production of high levels of pro-inflammatory cytokines that have been closely linked to worsened outcomes of acute respiratory viral infection. Especially in IAV, disease severity and disease progression are linked with an overshooting, IFN-driven inflammatory response, in which further exogenous supplementation with type I IFN in fact correlates with increased morbidity and mortality (74, 95). In non-human primates, IAV infection with a highly pathogenic H5N1 isolate evokes a strong induction of type I IFN, resulting in severe lung injury by a necrotizing bronchiolitis and alveolotis (96). IFN levels in turn have been demonstrated to cause elevated pro-inflammatory cytokine levels after *in vivo* IAV infection and additionally, in human alveolar macrophages, the release of pro-inflammatory cytokines (e.g., MCP-1) are preceded by a robust type I IFN response (97). Importantly, also in human infection with H5N1, levels of pro-inflammatory cytokines are strongly elevated in bronchoalveolar lavage fluid, and cytokine levels have been associated with organ damage and worsened disease outcomes (98, 99). Still, it should be noted that due to strain differences in virus-elicited PRR activation and, importantly, IFN antagonism by the IAV non-structural (NS)1 protein, IFN levels and disease severity do not always directly correlate; actually, the extent to which NS1 can suppress the IFN response relates to prolonged viremia and thus can also be a determinant of virus pathogenicity both in human bronchial epithelial cells and in an *in vivo* model of IAV infection (100, 101). Alongside IAV, also in RSV infection the induction of high levels of pro-inflammatory cytokines has been directly related to type IFN, as RSV-infected but IFNAR-deficient mice presented with significantly diminished pro-inflammatory cytokine release, which translated into an attenuated disease course (67). Also in SARS-CoV, the late phase type I IFN induction relates to accumulation of inflammatory macrophage populations and elevated lung cytokine levels (76).

TRAIL IN ACUTE RESPIRATORY VIRAL INFECTION—LIMITATION OF PATHOGEN SPREADING VERSUS INDUCTION OF TISSUE INJURY

Cell Death Pathways in IFN Signaling

In addition to antiviral, immunomodulatory, and pro-inflammatory ISGs, IFN signaling results in the transcription and translation of cell death-inducing ISGs. In the context of viral infection, these factors provide a mode to block viral spreading and reinfection by killing those infected cells, in which the internal activation of antiviral ISGs is not sufficient to restrict viral replication. Thus, the infected cell is sacrificed to prevent the release of infectious progeny virions to limit viral spreading. However, especially in the lung, the disruption of the alveolar epithelial barrier by cell death of infected cells, but importantly also non-infected bystander cells induced by factors such as TRAIL, significantly contributes to worsened disease outcomes.

Controlled cell death or apoptosis can be induced by intrinsic and extrinsic signals. The intrinsic apoptosis pathway is initiated by diverse intracellular stimuli that influence the expression and activation of B cell lymphoma (Bcl)-2 family proteins that govern the permeabilization status of the outer mitochondrial membrane. Once cytochrome *c* is released from the mitochondria, it binds to the intracellular adaptor protein, apoptotic peptidase activating factor 1, forming the so-called apoptosome that in turn recruits pro-caspase-9 (102). Caspases (cysteine-aspartic proteases) exert their action by cleaving other proteins and substrates. Herein, initiator caspases such as caspase-8 and caspase-9 target other downstream caspases, whereas effector caspases, including caspase-3, -6, and -7, directly cause apoptosis by cleaving and thus inactivating or disassembling a vast array of cellular integral proteins and complexes (103). The extrinsic apoptosis pathway relies on an extracellular signal exerted by ligands of the tumor necrosis factor (TNF) receptor (TNFR) superfamily, including TRAIL, TNF-α, and Fas ligand (FasL) (104). Their ligation to their respective cell surface-expressed death receptors (DR) leads *via* the signal transmission by Fas-associated protein with death domain (FADD) to the activation of the initiator caspases-8 or -10, finally stimulating effector caspases including caspase-3 (105).

To date, several type I and type III IFN-induced, proapoptotic factors have been identified (106). Both caspase-4 and caspase-8 have been shown to be upregulated upon type I IFN signaling (4, 107); caspase-8 enhances the FADD-driven extrinsic apoptosis pathway, whereas the less-studied caspase-4 may promote pro-IL-1β cleavage and inflammasome-driven cell death (pyroptosis) in macrophages (108, 109). Chattopadhyay et al. (110) demonstrated that Sendai virus infection and polyI:C treatment resulted in Bcl-2-associated X protein (Bax) activation and apoptosis induction *via* one of the key transcription factors of IFN genes, IRF3. In addition IRF5 was reported to enhance TRAIL-dependent extrinsic apoptosis by nuclear translocation resulting in the translation of to date undefined factors that increase cell death upstream of caspase-8 activation (111). Furthermore, both RLRs, RIG-I and MDA-5, trigger the proteins Puma and Noxa that induce Bcl and

thus activate the intrinsic mitochondrial apoptotic cascade (112). Also, PKR influences a cell's susceptibility to apoptotic signals, as it was demonstrated to sensitize to the FADD/caspase-8 apoptosis pathway upon type I IFN signaling after challenge with IAV or dsRNA (4) and the OAS-RNAseL system has been suggested to contribute to IFN-α-related cell death induction, but the exact mechanisms remain to be elucidated (113). Finally, also two classical initiators of the extrinsic apoptosis cascade are induced as ISGs. Both FasL and its receptor Fas are upregulated on mRNA levels by IFN-α (114), and FasL was reported to be induced by type I IFN in IAV infection in the murine lung *in vivo* (115). Also, the proapoptotic factor TRAIL (or TNFSF10, Apo2L) is induced by IFN-mediated and ISGF3-executed transcriptional activation, as has been shown by Sato et al. (116), who revealed the presence of the ISRE sequence within the TRAIL promoter region (116). In IAV infection, TRAIL is released in high amounts from infected alveolar macrophages depending on a PKR- and IFN-β-driven autocrine signaling loop. Binding of IFN-β to macrophage-expressed IFNAR activates a JAK/STAT-dependent release of TRAIL, which then acts through its receptor DR5 on the alveolar epithelial cells (5, 10).

However, certain prerequisites may decrease the ability of a cell to undergo apoptosis, including a shortage in pro-caspase-8 availability, expression of cellular FADD-like IL-1β-converting enzyme-inhibitory proteins (c-FLIPs) that block FADD-driven caspase activation, inactivation, or degradation of FADD itself, or expression of CYLD, which acts as a receptor-interacting serine/threonine-protein (RIP)1 kinase de-ubiquitinase and thus stabilizes RIP1. However, in these cases IFN signaling can still promote a caspase-independent, programmed inflammatory cell death by activating the necroptosis pathway (117, 118). Necroptosis is induced by a complex formation by RIP1 and RIP3 kinases that activate both poly-ADP-ribose (PAR) polymerase 1 (PARP-1) and/or mixed lineage kinase domain-like (MLKL), leading to ATP depletion, calpain activation, PAR polymer accumulation or cell membrane permeabilization, and release of damage-associated molecular patterns, respectively [reviewed in Ref. (119, 120)]. Both a type I IFN-dependent JAK/STAT-driven activation of PKR as well as signaling by the PRR DAI (DNA-dependent activator of IRFs) initiates necroptosis *via* RIP1/RIP3 activation, respectively (117, 121).

Importantly, the activation of proapoptotic and pro-necroptotic pathways in respiratory infection can result in a structural disruption of the airway and the alveolar epithelial barrier, which is a major hallmark of respiratory disease and its progression to the acute respiratory distress syndrome (122, 123). In virus-induced lung injury, especially expression of TRAIL, which can initiate both apoptosis as well as necroptosis has been correlated with more severe outcomes.

TRAIL-Induced Cellular Stress and Death Pathways

TRAIL and Its Receptors

As described earlier, TRAIL belongs to the superfamily of TNF ligands and has been reported to be inducible by both type I and type III IFNs. TRAIL has been found to be present in various cells of the immune system, among them natural killer (NK) cells, T cells, NK T cells, DC subsets such as IFN-γ-producing killer DCs and macrophages, and can be displayed in large amounts on the cell surface or be shed upon IFN- and/or pro-inflammatory cytokine signaling (124–126). In addition to cells of the immune system, fibroblasts have been shown to produce TRAIL after IFN-γ treatment or viral challenge. Also, club cells and the alveolar epithelium have been reported to produce TRAIL (127–130). Similar to other ligands of the TNF superfamily, TRAIL is a homotrimeric type II transmembrane protein with a conserved C-terminal extracellular domain that mediates receptor binding and can be cleaved by metalloproteinases to generate a soluble mediator (131). However, TRAIL can induce cell death also in its membrane-bound form, that is, similar to TRAIL expression levels and TRAIL shedding, upregulated by type I IFN (126). Direct cell-to-cell TRAIL–DR interactions have been demonstrated to play a role in macrophage, NK as well as $CD4^+$ T cell-mediated induction of cellular death (132, 133).

In humans, five different binding partners for TRAIL are present: the membrane-bound DR4 (TRAIL-R1) and DR5 (TRAIL-R2) that both induce a proapoptotic signaling cascade, the membrane-bound anti-apoptotic decoy receptors (DcR)1 and DcR2, and the soluble interaction partner osteoprotegerin (134). In the murine system, only DR5 has been identified to ligate to TRAIL (135). In the human respiratory compartment, both DR4 and DR5 have been demonstrated to be present under steady-state conditions (136, 137). However, upon viral infection, cell-sensitivity to TRAIL-induced apoptosis is enhanced, which has been attributed to increased TRAIL receptor expression especially on infected cells, as DR levels are markedly increased in IAV-, adenovirus-, and paramyxovirus-infected cells in contrast to non-infected bystander cells (10, 138, 139). Of note, studies on the dependency of DR upregulation upon type I IFN signaling after IAV infection have yielded conflicting results in different strains of mice (10, 74), highlighting the complex interplay of IFN-induced cascades in a host- and tissue-specific context, whereas the exact virus- and host-specific mechanisms for DR regulation remain less well defined. Moreover, previous assumptions that also DcR expression would correlate with cell-sensitivity to TRAIL-induced cell death could not be experimentally verified (125).

TRAIL-Induced Signaling Cascades

Tumor necrosis factor-related apoptosis-inducing ligand ligation to the proapoptotic receptors DR4 or DR5 triggers a trimerization of the receptors. Subsequently, depending on additional stimuli, presence or absence of adaptor molecules or inhibitory proteins, different signaling pathways can be activated (**Figure 2**). In the classical TRAIL-dependent extrinsic apoptosis induction, the proteins RIP, TRADD, and FADD are subsequently recruited to the DR cytoplasmic domain upon TRAIL ligation (140, 141). These factors and the proapoptotic DRs all share a cytoplasmic death domain (DD), which is lacking or truncated and thus inactive in the DcR. The DD plays a central role in the concerted formation of the death-inducing signaling complex (DISC). DISC formation exposes a second functional domain of FADD, the death effector domain that is directly able to recruit pro-caspase-8

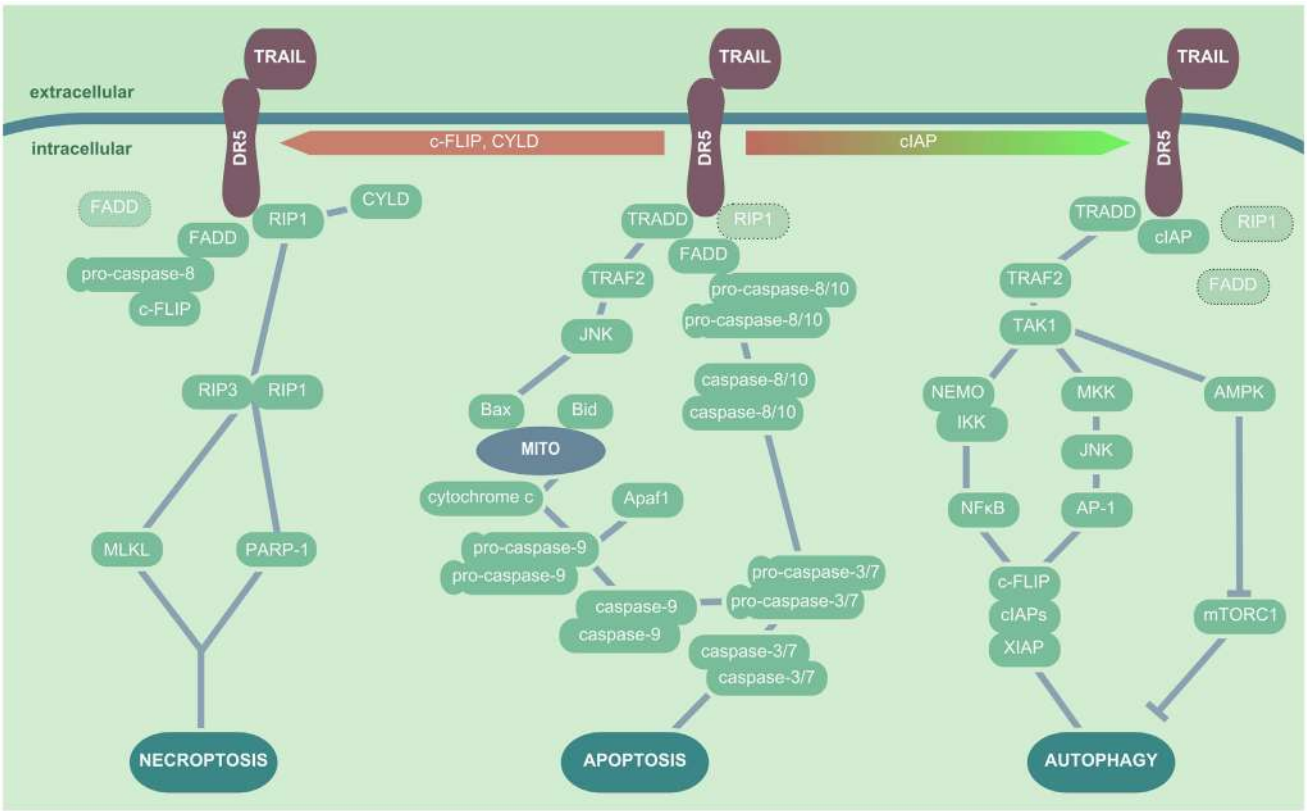

FIGURE 2 | **TRAIL/DR5-mediated cellular signaling pathways**. In presence of RIP1, TRADD, and FADD, TRAIL ligation to DR5 results in apoptosis induction, which is initiated by recruitment of the pro-caspase-8 or -10 to FADD. These in turn activate the effector caspases-3 and -7, which leads to DNA fragmentation and apoptosis induction. In addition, TRADD can trigger a TRAF2- and JNK-dependent activation of Bax and subsequent release of mitochondrial cytochrome *c*, inducing the pro-caspase-9 activation. In the presence of CYLD, c-FLIP or absence of sufficient amounts of FADD or pro-caspase-8, TRAIL ligation to DR triggers the interaction of RIP1 and RIP3 kinase, which in turn cause cell death *via* induction of MLKL and/or PARP-1. In the presence of cIAPS, FADD is not recruited to DR5 upon TRAIL ligation, and TAK1 is activated by TRADD/TRAF2 interactions. TAK1 induces NEMO followed by IκB degradation and NFκB activation, as well as MKK and JNK activation leading to AP-1 nuclear translocation; both events promote the production of cytoprotective factors such as XIAP, cIAPs, and c-FLIP. Additionally, TAK1 triggers AMPK activation and thus mTORC inhibition, which results in enhanced autophagic activity. *Abbreviations*: AP-1, activator protein 1; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; DR5, death receptor 5; RIP1, receptor-interacting serine/threonine-protein kinase 1; TRADD, TNF receptor type 1-associated death protein; FADD, Fas-associated protein with death domain; TRAF, TNF receptor-associated protein; JNK, Janus kinase; Bax, Bcl-2-associated X protein; c-FLIP, cellular FADD-like IL-1β-converting enzyme-inhibitory proteins; RIP, receptor-interacting serine/threonine-protein; MLKL, mixed lineage kinase domain-like; PARP-1, poly-ADP-ribose (PAR) polymerase 1; cIAP, cytoprotective factors including inhibition of the autophagic machinery; XIAP, X-linked inhibitor of apoptosis protein; AMPK, AMP-activated protein kinase; mTORC, mammalian target of rapamycin complex; TRAF2, TNF receptor-associated protein 2; MKK, mitogen-activated protein kinase.

and pro-caspase-10. How exactly DISC formation induces caspase activation is still under debate. The most probable scenarios include either an autocatalytic cleavage of caspase-pro-domains enabled by the spatial proximity between pro-caspases (generated by their recruitment to DISC), by pro-caspase dimerization, or by pro-caspase conformational stabilization (125). Removal of the pro-domain of caspase-8 and caspase-10 results in the activation of the effector caspases-3 and -7, which cleave DNA fragmentation factor 45 and lead to apoptosis (142, 143). Moreover, TRAIL-binding to DR4 and DR5 can induce the JNK either *via* caspase-8 or recruitment of TNF receptor-associated protein 2 (TRAF2) to the DISC complex, which results in the activation of the intrinsic apoptotic cascade by Bax-dependent mitochondrial cytochrome *c* release (144). In addition, TRAIL signaling is also able to induce necroptosis by both activating the RIP1/RIP3 kinase downstream effectors PARP-1 and MLKL, contributing to epithelial cell death and tissue injury (145–147).

It has become apparent in recent years that TRAIL signaling is closely linked to induction of autophagy, a process generally associated with the blockade of apoptosis and necrosis. Indeed, autophagy has been reported to improve cellular survival in cell stress by catabolic removal of cytoplasmic long-lived proteins and damaged organelles. It also contributes to viral clearance and the transfer of viral material to endosomal-/lysosomal-located TLR7 or MHC class II compartments for the activation of adaptive immunity (148). Several studies outline that TRAIL ligation to DR5 can result in a TRAF2-dependent activation of TAK1 (MAP3K7) that has been attributed a central role in

TRAIL-induced autophagy activation (149). TAK1 modulates the IKK-dependent translocation of NFκB, and it also induces JNK activation *via* mitogen-activated protein kinase. Both events lead to expression of autophagy-related factors including inhibition of the autophagic machinery (cIAP)1, cIAP2, X-linked inhibitor of apoptosis protein, and c-FLIP (150, 151). Especially, c-FLIP has been associated with desensitization of cells to TRAIL-induced apoptosis, favoring autophagy-related cascades (152). Another study revealed that upon TRAIL signaling the AMP-activated protein kinase (AMPK) is activated. AMPK in turn inhibits the mammalian target of rapamycin complex 1 that itself is an inhibitor of autophagy, thus the activation of the autophagic machinery is promoted (153). The decision if TRAIL signaling results rather in necroptotic or apoptotic cell death or in activation of autophagy seems to be dependent on the presence of cIAPs that promote RIP kinase ubiquitination and degradation (146), but also on the balance between active caspases and autophagic proteins such as Beclin-1 (154, 155). This suggests a scenario where autophagy is activated as cell protective mechanism until cell stress—as executed by enhanced TRAIL signaling or additional viral infection—increases over a threshold to favor cell death induction. Accordingly, as TRAIL signaling is not restricted to infected cells, excessive cell death activation might be limited by autophagy induction in non-infected bystander cells. However, autophagy is not only related to cell survival but can also positively affect apoptosis and induce—even if the exact mechanisms are still under debate—autosis, the autophagy-related cell death, another mode of TRAIL to trigger cell death (156, 157). Of note, autophagy activation needs to be placed into its virus-specific context, as some viruses, including Dengue virus, poliovirus, and Coxsackie B virus (158), can exploit autophagic pathways for their own replication and thus promote apoptosis and tissue injury.

TRAIL in Acute Respiratory Viral Infection

As discussed above, TRAIL is a potent activator of cell death. However, its signaling outcomes can differ largely depending on its delivered form (e.g., membrane-bound versus soluble), the availability of DRs on the target cell membrane, alternate intracellular pathways that might be activated and finally the pathogen itself, as it might exploit TRAIL-induced pathways for its own survival and replication. In acute respiratory infection, TRAIL signaling is often part of an IFN-driven overshooting inflammatory reaction that promotes unspecific tissue injury and thus disease severity by increasing functional and structural changes in infected but also non-infected cells, as will be outlined below.

Influenza A Virus

The release and effects of TRAIL have been especially well studied in IAV infection in the last decade. Earlier studies reported that within 3 days after infection, bronchial, bronchiolar, and alveolar epithelial cells undergo apoptosis (159). This early induction of cell death is mainly attributed to direct apoptosis induction by the virus itself, as IAV actively promotes apoptosis for efficient viral replication (160). Herein, the viral NS1 and PB-F2 proteins not only play a crucial role (161, 162) but also the viral M2 protein has been implicated in this process as it inhibits autophagy in infected cells (163). In addition, our own data revealed that later in IAV *in vivo* infection, the recruitment of bone marrow-derived macrophages *via* the CC chemokine receptor type 2 (CCR2)–CC-chemokine ligand 2 (CCL2) axis significantly contributes to alveolar cell apoptosis and structural damage of the alveolar epithelium (5). Studies by Wurzer et al. (164) had previously demonstrated that IAV promotes the production of proapoptotic factors in an auto- and paracrine fashion *via* NFκB transcriptional activation by IAV (164). Subsequently, Brincks et al. (6) elucidated that human peripheral blood mononuclear cell treated with IAV released TRAIL and that increased TRAIL levels correlated with type I as well as II IFN induction. Additionally, TRAIL sensitivity was increased in influenza virus-infected cells. In line, our investigations could elucidate that IAV triggers a PKR-dependent translocation of NFκB that results the production of type I IFNs. These in turn induce, *via* ligation to the IFNAR receptor complex, expression and shedding of TRAIL by bone marrow-derived macrophages (10). In addition, Davidson et al. (74) demonstrated that type I IFN application to IAV-infected mice increased morbidity and lung injury, which could be attributed to both DR5 and TRAIL upregulation inducing epithelial cell apoptosis. Importantly, Högner et al. also reported that the IAV strain used in these studies, A/PR8 (H1N1), which is highly pathogenic for mice, induced an approximately 800-fold induction in macrophage TRAIL expression, whereas the lower pathogenic virus A/X-31 (H3N2) only stimulated TRAIL by a factor of eight. Of note, the relation between TRAIL induction and IAV strain-specific pathogenicity also translates to the highly pathogenic avian H5N1 IAV, causing severe pneumonia in mice as well as in humans (165, 166). Moreover, human infection with both the highly pathogenic H5N1 as well as the pandemic 1918 H1N1 IAV strains are characterized by a massive influx of mononuclear phagocytes into the alveoli, which is correlated with extensive alveolar epithelial cell apoptosis (97, 167). Additionally, macrophages gained from bronchoalveolar lavages of patients presenting with ARDS caused by the pandemic H1N1/2009 virus strain showed high surface expression and release of TRAIL (10). Another recent report demonstrates that in highly pathogenic avian influenza, in addition to macrophages also the alveolar epithelium might be involved in causing elevated levels of TRAIL in the alveolar space (130). Besides its role in apoptosis, TRAIL signaling upon IAV infection has also been implicated in the induction of necroptosis in fibroblasts, DCs, and lung epithelial cells (146, 147, 168). Rodrigue-Gervais et al. (146) demonstrated that lack of cIPA2 promotes RIP3 kinase-mediated necroptosis in response to TRAIL—but also the proapoptotic factor FasL—released from hematopoietic cells. This contributed to severe lung epithelial degeneration and increased mortality, even though viral control was not compromised. Nogusa et al. (147) further elucidated that IAV-induced necroptosis depends on RIP3 kinase activation of MLKL, and that RIP3 kinase deficiency, similar to cIAP2-deficiency, increased IAV-susceptibility *in vivo*.

In IAV infection, as mentioned earlier, DR5 expression is elevated on infected alveolar epithelial cells, but not in non-infected cells *in vivo*, which might impact on TRAIL susceptibility to apoptosis induction (10). However, both infected as well as neighboring bystander cells were found to be targeted for apoptosis induction by macrophage-released TRAIL.

Nonetheless, we could recently show that specifically in non-infected cells within the IAV-infected lung, TRAIL severely compromises the function of the ion channel Na,K-ATPase, which was mediated *via* induction of the stress kinase AMPK (11), thereby potentially revealing a cross-link to TRAIL-induced autophagic cell stress pathways in bystander cells both *in vitro* and *in vivo*. The TRAIL-induced and AMPK-mediated downregulation of the Na,K-ATPase, a major driver of vertical ion and fluid transport from the alveolar airspace toward the interstitium, resulted in a reduced capacity of IAV-infected mice to clear excessive fluid from the alveoli. Thus, TRAIL signaling contributes to intensive edema formation, a hallmark of disease in virus-induced ARDS (123). Notably, this effect of TRAIL on Na,K-ATPase expression was induced independently of cell death pathways elicited by caspases, as treatment of cells and mice with a specific caspase-3 inhibitor diminished apoptosis in alveolar epithelial cells but still allowed for the reduction of the Na,K-ATPase (11). Conclusively, treatment of IAV-infected mice with neutralizing antibodies directed against TRAIL or the abrogation of recruitment of TRAIL$^+$ bone marrow-derived macrophages inhibited apoptosis of both non-infected and bystander cells. Thus, lung leakage due to loss of alveolar barrier function was reduced, whereas alveolar fluid clearance capacity was enhanced, resulting in reduced edema, improved survival, and outcome upon IAV challenge *in vivo*. However, TRAIL has also been shown to be upregulated on NK, DC, and on CD4$^+$ and CD8$^+$ T cells after IAV infection (169). Studies by Brincks et al. demonstrated that especially CD8$^+$ T involved in cytotoxic T cell responses toward IAV and drive IAV-infected cells into apoptosis *via* TRAIL, thus contributing to efficient virus clearance (6, 170). In addition, both FasL and TRAIL are involved in DC-mediated CTL activation and cytotoxicity against IAV-infected cells (6, 171). Furthermore, studies showed delayed viral clearance upon neutralizing anti-TRAIL antibody administration (169, 172). Our data, however, demonstrate that the transfer of TRAIL-deficient bone marrow into irradiated wild-type mice, resulting in loss of TRAIL production by bone marrow-derived macrophages upon IAV infection, does not impact on the capacity to fully clear viral particles from the lung at day 7 after infection, suggesting that other compensatory mechanisms are recruited to guarantee viral clearance (10). Taken together, in IAV infection, TRAIL acts both as an important mediator of infected cell killing but particularly as a detrimental factor contributing to tissue injury and impaired inflammation resolution when released in excessive amounts by recruited immune cells.

Respiratory Syncytial Virus

Respiratory syncytial virus is an important cause of respiratory tract infections especially in children worldwide. Generally, there seem to be virus-elicited anti-apoptotic mechanisms active in the lung epithelium, as RSV-infected primary human airway cells show a minimal cytopathic effect (173). However, several cell lines including small airway cells, primary tracheal-bronchial cells, and A549 and HEp-2 showed increased expression of TRAIL and its ligands DR4 and DR5 in an *in vitro* RSV infection model (174). Moreover, soluble TRAIL released from leukocytes was elevated in the bronchoalveolar lavage fluid of patients with RSV-associated respiratory failure, suggesting that similar to IAV, TRAIL contributes to RSV-induced epithelial injury and disease progression (137).

Coronaviruses

Also in CoV respiratory tract infection, TRAIL levels, but less so FasL, have been reported to be markedly elevated (175, 176). For SARS-CoV that presents with a severe damage to both the upper and lower respiratory tract (177), especially DCs respond with a strong induction of TRAIL production, which was suggested to correlate to increased cellular lung infiltrations present in SARS-CoV patients (175). Interestingly, SARS-CoV infection drives cells into apoptosis by a PKR-driven but eIF2α-independent pathway (178), which might—similarly as seen in IAV infection—suggest a PKR-induced and autocrine/paracrine executed activation of apoptosis.

Also MERS-CoV, which causes pneumonia and respiratory failure, has been demonstrated to induce profound cell death within 24 h of infection, irrespective of viral titers produced by the infected cells. However, type I IFN expression is strongly reduced in MERS-CoV in comparison to seasonal human CoV in *in vitro* infection models, including human monocyte-derived macrophages, Calu-3, and human lung fibroblasts (179, 180), which might also dampen downstream TRAIL induction. Therefore, the exact mechanism by which MERS-CoV promotes cell death remains to be investigated.

THE IFN/TRAIL AXIS IN BACTERIAL SUPERINFECTION AFTER VIRAL INJURY

Recurrently, viral infections of the respiratory tract are followed by outgrowth of colonizing Gram-positive bacteria that aggravates the course of illness. This is well documented for IAV, where "super" infections with *Streptococcus pneumoniae* and *Staphylococcus aureus* are the most frequent and increase viral pneumonia-associated morbidity and mortality (181). During the 1918 IAV pandemic, bacterial pneumonia was evident in most cases (182) and also during the recent 2009 H1N1 pandemic, coinfections were a relevant factor for severe disease in a young patient population without comorbidities (183). Interestingly, virus-induced elevation of the type I IFN response levels might promote secondary bacterial outgrowth by several mechanisms [reviewed in Ref. (184)]. In line, it has been repeatedly demonstrated that lack of type I IFN signaling results in better bacterial clearance and increased survival rates in IAV- and *S. pneumoniae*-superinfected mice (185–187). Herein, IFN-induced apoptosis induction as well as depletion or impaired recruitment of lymphocyte subsets necessary for bacterial control play a critical role (188, 189). Bacterial clearance from the lung has been reported to rely on sufficient phagocyte generation, recruitment, and survival. Type I IFN has been demonstrated to cause apoptosis in bone marrow-derived granulocytes, affecting the numbers of recruited neutrophils (189), but also to impair expression of the cytokines CXCL1 (or KC) and CXCL2 (or MIP-2), thus inhibiting neutrophil recruitment to the lungs with severe effects on survival of superinfected mice (185). A recent report

by Schliehe et al. (190) elucidated the mechanistic background for impaired CXCL1 expression and secretion and demonstrated that type I IFNs activate the histone methyltransferase Setdb2, which in turn represses the Cxcl1 promoter and thus impairs neutrophil recruitment and bacterial clearance. Moreover, type I IFN production decreases CCL2 production, thus inhibiting macrophage recruitment, which as well has been reported to have detrimental effects on bacterial clearance and disease progression in bacterial superinfection after viral insult *in vivo* (186). In addition, type I IFNs also impair γδ T cell function and IL-17 release, which was shown to increase susceptibility to *S. pneumoniae* superinfection after IAV challenge (187). Also in *S. aureus* pneumonia, a robust type I IFN response is correlated to excessive morbidity and tissue injury (191). In a model of polyI:C, *S. aureus* (methicillin-resistant strain, MRSA) superinfection, polyI:C treatment prior to bacterial infection enhanced type I IFN levels and decreased bacterial clearance and survival (192). Furthermore, Shepardson et al. (193) demonstrated that late type I IFN induction rendered mice more susceptible to secondary bacterial pneumonia in a model of IAV–MRSA superinfection.

Only limited data are available on a direct role of TRAIL in respiratory disease progression due to bacterial superinfections. In a model of IAV–*Haemophilus influenza* infection, neither deficiency for CC chemokine receptor type 2, inhibiting bone marrow-derived macrophage recruitment, nor deficiency of Fas or TNFR1 impacted outcome (194). Yet, during *S. pneumoniae* single infection, early cell death of macrophages is thought to limit an exuberant inflammatory reaction and accordingly, a study by Steinwede et al. (195) revealed that neutrophil-derived TRAIL limits tissue injury by inducing cell death in DR5-epressing lung macrophages in bacterial mono-infection (195). In contrast, in the IAV–*S. pneumoniae* superinfection mouse model, IAV-induced TRAIL has a detrimental effect on overall mortality (7), as TRAIL-induced epithelial injury enhanced bacterial outgrowth of *S. pneumoniae*—administered at day 5 after IAV infection—markedly. Importantly, administration of anti-TRAIL neutralizing antibodies enhanced bacterial control by the host organism. Thus, the activation of IFN/TRAIL-mediated signaling in viral infection has detrimental implication for outcome of secondary bacterial infection following viral insult, rendering the IFN/TRAIL signaling axis an interesting therapeutic target not only in respiratory viral infections but also in complicating bacterial superinfection.

IFN/TRAIL AXIS IN CHRONIC LUNG DISEASES

An increasing number of reports connect progression of chronic respiratory disease to acute respiratory virus infection or proapoptotic signaling events. In fact, TRAIL has been reported to be a critical determinant for promoting the development of chronic lung disease in early life (196); targeting TRAIL by genetic deletion or neutralizing antibody application in early-life respiratory infections ameliorated infection-induced histopathology, inflammation, as well as emphysema-like alveolar enlargement and lung function. Furthermore, TRAIL was also shown to play a role in the development of allergy and asthma. TRAIL is not only elevated in the sputum of asthmatic patients but has also been reported to be highly expressed in an experimental mouse model of asthma, where it induces CCL20 secretion by bronchial epithelial cells, thus promoting T_H2 cell responses and airway hyperreactivity (197).

In COPD, acute exacerbations driven by viral and bacterial infection are a major factor increasing both mortality and morbidity, and both influenza and *S. pneumoniae* have been identified among the most common causes of COPD exacerbations (198). Indeed, primary bronchial epithelial cells isolated from subjects with COPD show an impaired production of type I IFN (199), which has been implied in the enhanced susceptibility of COPD patients to respiratory infections; however, even in absence of high IFN induction, both an abnormally elevated loss of alveolar epithelial cells due to apoptosis as well as elevated TRAIL and DR5 levels were reported (200), implying a possible link between viral/bacterial induction of TRAIL and acute exacerbations in COPD. TRAIL induction has also been directly linked to cigarette-smoke exposure, a common cause of COPD, and TRAIL deficiency resulted in decreased pulmonary inflammation and emphysema-like alveolar enlargement *in vivo* (201). Moreover, increased levels of both TRAIL and DR5 were associated to impaired lung function and increased systemic inflammation in human COPD patients (202). While alveolar epithelial cell death is closely connected to idiopathic pulmonary fibrosis (IPF), TRAIL and its receptors DR4 and DR5 in AEC were shown to be upregulated in IPF lungs (129). Also, in pulmonary arterial hypertension virus infection is considered to be a possible risk factor (203), and pulmonary hypertension has been reported to be a side effect of prolonged treatment with type I IFN (204, 205). In line, TRAIL has been closely linked to disease progression in pulmonary hypertension. TRAIL has been found to be increased within pulmonary vascular lesions of patients with pulmonary hypertension (206) and also in a mouse model of hypoxia-induced pulmonary hypertension, levels of soluble TRAIL correlated with right ventricular systolic pressure, right ventricular hypertrophy, and pathologic alterations (33, 34). Importantly, neutralizing antibody-treatment against TRAIL showed positive effects on survival while reducing pulmonary vascular remodeling (207). Notably, the extent to which infection-induced TRAIL release causes or exacerbates chronic lung disease or in how far TRAIL production in chronic lung diseases affects susceptibility to respiratory viral and complicating bacterial infection remains to be elucidated.

OUTLOOK: THERAPEUTIC CONCEPTS TARGETING TRAIL IN ACUTE RESPIRATORY VIRAL INFECTION

Respiratory viral infections are major causative agents for lung injury and ARDS; however, in many cases antivirals are not sufficient to limit disease (208). Besides the fact that most viruses are subject to strong selective pressures that favor quickly evolving, drug-resistant virus variants, recent advances in understanding the processes that contribute to tissue injury and ARDS highlight

a crucial role of immune-related, IFN-driven events. Therefore, novel therapeutic strategies often aim to improve the outcome of severe respiratory infection by modulating host cell responses; however, to date, clinical trials trying to improve severe viral infections or ARDS outcomes by targeting host pathways have not resulted in approval of new drugs (122).

Of note, for establishment of such therapies it has to be considered that the timing and intensity of induction and amplification as well as of dampening and termination of the IFN-driven immune response needs to precisely match the pathogen- and organ-specific requirements of a given infection. A non-controlled regulation of these processes may lead to either an unrestricted pathogen spreading or, on the other extreme, to an overshooting inflammatory response, including the increased production of pro-inflammatory and proapoptotic mediators, elevated levels of recruited immune cells, and/or aberrant repair processes. Notably, both too low and too high levels of IFN-induced effects facilitate disease progression with a possible increase of fatal outcomes in ARDS patients (78). Accordingly, preclinical *in vivo* studies of IFN-directed therapies yielded seemingly adverse results, depending on the context, timing, and dosage of IFN modulation. However, in multiple settings of acute respiratory viral infection, studies demonstrate that an exaggerated signaling derived from type I IFN in an already inflamed tissue contributes to worsened outcomes, and importantly, might favor secondary bacterial superinfection [e.g., Ref. (75, 76, 209)]. Interestingly, Davidson et al. (209) demonstrated that type III IFN release upon influenza challenge—in contrast to type I IFN induction—does not trigger an unbalanced inflammatory response that critically contributes to respiratory disease progression *in vivo*, highlighting it as a possible therapeutic option in IAV-induced lung injury. Most likely, this effect derives from the lack of the IFN-λR1/IL-10R2 receptor complex, but presence of IFNAR, on immune cells, including bone marrow-derived macrophages. Nonetheless, other reports identify IFN-λ as a driver of macrophage polarization to an inflammatory M1 phenotype (41) that has been attributed to further promote an overshooting inflammatory response, highlighting the need for further studies of type III IFN biology in pathogen-associated disease progression.

As generally IFN-directed therapeutic approaches target various downstream signaling events that might both act beneficially as well as detrimentally on viral replication and pathogenesis, a further approach is to address specific ISGs that primarily show detrimental effects on disease progression. As outlined above, TRAIL or its downstream signaling events might comprise a suitable target for adjunct therapies in addition to antivirals. Accordingly, our own data in a preclinical mouse model of IAV infection demonstrate a clear benefit of the systemic application of neutralizing antibodies against TRAIL at days 3 and 5 postinfection for lung injury, morbidity, and mortality (10, 11). Targeting TRAIL as a major determinant of disease severity in respiratory viral infections including IAV, but also RSV and CoV, may yield therapeutic approaches that are superior to IFN-directed strategies, as they seemingly do not bear the risk of compromising host defense. Yet, it should be thoroughly excluded that blocking TRAIL-induced cell death of infected cells will not lead to an overwhelming viral spreading, especially as reports on viral loads upon TRAIL inhibition in preclinical models of IAV are controversial (6, 10, 170). Accordingly, additional studies are needed to understand how and to which extent virus-infected cells can be killed or viral spreading can be controlled by other means in the absence of TRAIL. Moreover, targeting pathways and signaling hubs downstream of TRAIL/DRs, such as AMPK (11), in a well-timed and lung compartment-specific way, may open new therapeutic avenues but requires more detailed preclinical studies on efficacies and side effects. A valid approach might be the use of a combination therapy of such a treatment together with a classical antiviral drug therapy limiting viral replication; however, exact dosage, timing, kinetics, and application routes remain to be defined.

AUTHOR CONTRIBUTIONS

CP and SH performed bibliographic research and drafted the manuscript.

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Grass Carp Laboratory of Genetics and Physiology 2 Serves As a Negative Regulator in Retinoic Acid-Inducible Gene I- and Melanoma Differentiation-Associated Gene 5-Mediated Antiviral Signaling in Resting State and Early Stage of Grass Carp Reovirus Infection

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Laboratory of genetics and physiology 2 (LGP2) is a key component of RIG-I-like receptors (RLRs). However, the lack of the caspase recruitment domains (CARDs) results in its controversial functional performance as a negative or positive regulator in antiviral responses. Especially, no sufficient evidence uncovers the functional mechanisms of LGP2 in RLR signaling pathways in teleost. Here, negative regulation mechanism of LGP2 in certain situations in retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5)-mediated antiviral responses was identified in *Ctenopharyngodon idella* kidney cells. LGP2 overexpression inhibits synthesis and phosphorylation of interferon regulatory factor 3/7 (IRF3/7), and mRNA levels and promoter activities of IFNs and NF-κBs in resting state and early phase of grass carp reovirus (GCRV) infection. Knockdown of LGP2 obtains opposite effects. Luciferase report assay indicates that LGP2 works at the upstream of RIG-I and MDA5. LGP2 binds to RIG-I and MDA5 with diverse domain preference and which is independent of GCRV infection. Furthermore, LGP2 restrains K63-linked ubiquitination of RIG-I and MDA5 in various degrees. These differences result in disparate repressive mechanisms of LGP2 to RIG-I- and MDA5-mediated signal activations of IFN-β promoter stimulator 1 and mediator of IRF3 activation. Interestingly, LGP2 also inhibits K48-linked RIG-I and MDA5 ubiquitination to suppress proteins degradation, which guarantees the basal protein levels for subsequently rapid signal activation. All these results reveal a mechanism that LGP2 functions as a suppressor in RLR signaling pathways to maintain cellular homeostasis in resting state and early phase during GCRV infection.

Keywords: laboratory of genetics and physiology 2, innate immunity, grass carp (*Ctenopharyngodon idella*), grass carp reovirus, RLRs, interferon regulatory factor 3, IRF7

HIGHLIGHTS

1. LGP2 interacts with RIG-I and MDA5 independent of GCRV infection.
2. LGP2 suppresses both K63- and K48-linked ubiquitination of RIG-I and MDA5.
3. LGP2 restrains activation of IRF3/7 *via* repressing their Ser and Thr phosphorylation.
4. LGP2 inhibits production and promoter activities of IFNs and NF-κBs in resting state and early stage of GCRV infection.
5. Knockdown of LGP2 enhances the immune responses of IFNs, NF-κBs, and IRF3/7.

INTRODUCTION

The host possesses intrinsic antiviral immune system that binds viral components and inhibits viral replication (1). Pattern recognition receptors (PRRs) directly sense the presence of pathogen components, so called pathogen-associated molecular patterns (PAMPs) (2, 3). RIG-I like receptors (RLRs) are a family of cytoplasmic PRRs that sense viral PAMPs in cytosol (1, 3, 4). Three members have been identified in this family: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2), and all of them belong to DExD/H box RNA helicases family (5). RIG-I and MDA5 have three domains: two tandem N-terminal caspase recruitment domains (CARDs), a DExD/H-box helicase, and a C-terminal repressor domain (RD). LGP2 has DExD/H-box helicase and RD domains, but lacks the CARD (6). To date, RIG-I and MDA5 have been well characterized: RIG-I mainly recognizes RNAs with 5' PPP or short dsRNA (~20 bp), while RNA web can induce the activation of MDA5 (1, 7). Both RIG-I and MDA5 can sense a wide variety of RNA or DNA viruses (1, 7). Upon viral recognition, RIG-I and MDA5 activate nuclear factor κB (NF-κB) and interferon regulatory factor 3 (IRF3) through the adaptor proteins IFN-β promoter stimulator 1 [(IPS-1), also known as MAVS, VISA, or Cardif] and mediator of IRF3 activation [MITA, also named as STING] (8, 9). IPS-1 is a CARD domain-containing protein that drives the expression of type I interferons (IFN-Is) and inflammatory cytokines (1). MITA functions downstream of RIG-I and IPS-1, which is necessary for efficient induction of IFN-Is and IFN-stimulated genes (ISGs) (8, 10).

As for the third member of RLRs, LGP2 lacks the CARDs, which implies the different functions from RIG-I and MDA5. Up to now, the role of LGP2 in antiviral signaling is controversial. Accumulating data report the antithetical roles of LGP2 as a negative or positive regulator in antiviral responses (11–13). A study indicates that LGP2 RD is necessary and sufficient for inhibition of RIG-I, but not MDA5, by interacting *in trans* with RIG-I to ablate self-association and signaling (14). Moreover, LGP2 can inhibit antiviral signaling by competing with the kinase IKKi for a common interaction site on IPS-1 (15). However, other groups support the positive role of LGP2 in antiviral responses (16–19). Direct evidence suggests that LGP2 assists MDA5–RNA interaction and regulates MDA5 filament assembly to enhance MDA5-mediated antiviral signaling (16). LGP2 can synergize with MDA5 to potentiate IFN-β transcription *in vivo* during encephalomyocarditis virus infection or polyinosinic–polycytidylic potassium salt [poly(I:C)] transfection *via* ATP-enhanced RNA recognition (17). LGP2 also facilitates viral RNA recognition by both RIG-I and MDA5 through its ATPase domain (18). In the Chinese tree shrew, LGP2 synergizes with MDA5 to sense Sendai virus infection for IFN-I induction along with the loss of RIG-I (19).

Fish harbor more complicated innate immune systems than those in mammals (20). Nearly all the counterparts of vertebrate PRRs and their downstream signaling components have been identified in teleost (20, 21). RLRs are evolutionarily conserved from fish to mammals (21). Generally, teleost RLRs also consist of three members: RIG-I, MDA5, and LGP2, although RIG-I is absent in some fish species (20). So far, RLRs have been identified in many teleosts such as zebrafish (*Danio rerio*) (21–23), grass carp (*Ctenopharyngodon idella*) (24–26), rainbow trout (*Oncorhynchus mykiss*) (12), Atlantic salmon (*Salmo salar*), and Japanese flounder (*Paralichthys olivaceus*) (27). The roles of RLRs in mediating downstream signal pathways have been preliminarily studied in some fish species (28–31). Nevertheless, the antithetical functions of fish LGP2 as a positive or negative regulator in antiviral responses are still controversial in fish (12, 32–34).

Grass carp is an important freshwater economic fish in China. However, hemorrhagic disease caused by grass carp reovirus (GCRV), a dsRNA virus, seriously affects the grass carp cultivation industry (20). To uncover the definite role of grass carp LGP2 in antiviral immune responses, the regulation mechanisms of LGP2 in RLR signaling pathways in response to GCRV infection were investigated in *Ctenopharyngodon idella* kidney (CIK) cells. Our results demonstrated that grass carp LGP2 function as a negative regulator in RIG-I and MDA5-mediated antiviral immune responses under resting state and early phase of GCRV infection. The findings provide a molecular mechanism on LGP2 in maintaining cellular homeostasis and preventing the host from the uncontrolled innate immune responses.

MATERIALS AND METHODS

Cells and Virus Infection

Ctenopharyngodon idella kidney cells, obtained from China Center for Type Culture Collection, were cultured according to previous description (35). Fathead minnow (FHM) cell line (36) was kindly provided by Dr. Junfa Yuan, which was maintained in M199 (Gibco) supplemented with 10% FBS (Gibco), 100 U/ml of penicillin (Sigma), and 100 U/ml of streptomycin (Sigma). Both cells were incubated at 28°C with 5% CO_2 humidified atmosphere.

For virus infection, CIK or FHM cells were plated for 24 h in advance and then infected with GCRV 097 stain at a multiplicity of infection of 1. After 2 h, the virus inoculum was removed, the cells were washed with PBS, and further incubated with new medium (DMEM for CIK, M199 for FHM, and no FBS). The control group was treated with PBS.

Plasmid Constructions and Transfections

pCMV-CMV-GFP was employed as original plasmid (28) for constructing the following expression plasmids: LGP2-Flag, RIG-I-Flag, MDA5-Flag, RIG-I-HA, RIG-I-CARD-HA, RIG-I-Helicase-HA, RIG-I-RD-HA, MDA5-HA, MDA5-CARD-HA, MDA5-Helicase-HA, MDA5-RD-HA, RIG-I-CARD-Flag, and MDA5-CARD-Flag. The ORFs or the domains of the relevant genes were amplified from grass carp spleen tissue cDNA and then inserted behind the first CMV promoter. The Flag or HA tag was introduced by the reverse primer. The primers were listed in Table S1 in Supplementary Material. To construct the luciferase reporter vectors of grass carp IPS-1, MITA, IRF3, IRF7, IFN1, IFN2, IFN3, IFN4, IFNγ1, IFNγ2, NF-κB1, and NF-κB2, the 5′-flanking fragments of these genes were obtained from the grass carp genome (37). The core promoter regions were predicted by WWW Promoter Scan,[1] GPMiner,[2] and Promoter 2.0 Prediction Server.[3] To verify the promoter activities, the predicted promoters of the related genes were introduced to the pCMV-GFP vector by replacing the CMV promoter (38). The primers were shown in Table S1 in Supplementary Material. Then, these vectors were transfected into CIK cells by FuGENE 6 Transfection Reagent (Promega), respectively. The promoter activity was reflected by promoting green fluorescent protein expression, observed under a fluorescence microscope (Nikon). The promoter activities of RIG-I, MDA5, and MITA were verified in the previous studies (39–41). For dual-luciferase reporter assay, the valid promoters were cloned into pGL3-basic luciferase reporter vector (Promega), respectively. For transient transfection, CIK or FHM cells were plated in 24-well plates, 6-well plates, or 10 cm^2 dishes with 70–90% confluency. Approximately 24 h later, transfection was performed with FuGENE 6 Transfection Reagent (Promega) according to the manufacturer's instructions. LGP2 stable transfected cell line was obtained by G418 selection as previously reported (38). It is worth noting that the vector (pCMV-CMV-EGFP) used for protein overexpression in the present study contains two CMV promoters, which promote the expressions of target protein and EGFP, respectively, and the later is used to monitor the transfection efficiency. Hence, we employed empty vector-transfected cells rather than normal cells as control in the present study, which can make a better demonstration of LGP2 functions in RLR signaling pathways, not EGFP or other components in the vector skeleton. To assess the influence of empty vector on dual luciferase reporter assay, transcription level, and protein expression, we compared the promoter activities, mRNA expressions, and protein levels between empty vector-transfected cells and normal cells, and the results demonstrated that empty vector has no significant influence on the promoter activity, mRNA level, and protein synthesis (Figures S1 and S2 in Supplementary Material; **Figure 3E**).

Dual Luciferase Reporter Assays

Fathead minnow cells were seeded in 24-well plates and cotransfected with the indicated luciferase reporter plasmid and overexpression plasmid. pRL-TK vector (Promega) was used as an internal control to normalize the expression level of the transfected plasmid. At 16 h post-transfection, the cells were infected with GCRV or treated with PBS for 12 or 24 h, then washed with PBS, and lysed by Passive Lysis Buffer (Promega). Dual-luciferase reporter assay was conducted in 96-well luminometer plates with Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). Luciferase activity was measured by Multiscan Spectrum (PerkinElmer). Data represent relative firefly luciferase activity normalized to Renilla luciferase activity. The results were obtained from four independent experiments, and each was performed in triplicate.

Immunoprecipitation (IP) and Western Blotting (WB) Analyses

For transient transfection and Co-IP experiments, FHM cells in 10 cm^2 dishes were co-transfected with the indicated plasmids for 24 h, then infected with GCRV for 12 or 24 h according to test requirements. The cells were lysed in western and IP lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1mM EDTA, 1mM Na3VO4, 0.5 μg/ml leupeptin, 2.5 mM sodium pyrophosphate) (Beytotime) added with 1 mM PMSF for 30 min on ice, and then centrifuged at 12,000 rpm for 30 min at 4°C. For each IP, 1 mg of cell lysate was incubated with 1 μg of the indicated antibody (Ab) overnight at 4°C, adding 35 μl of protein A + G-agarose (Beyotime) for 4 h. The sepharose beads were washed three times with 1 ml lysis buffer, then eluted with 20 μl 2 × SDS loading buffer by boiling for 10 min at 95°C. The precipitates were detected by IP with indicated Ab.

For WB, protein extracts were separated by 8% SDS-PAGE gels and transferred onto NC membranes (Millipore). The membranes were blocked in fresh 3% non-fat dry milk dissolved in TBST buffer for 2 h at room temperature, then incubated with the following primary Ab for 2 h at room temperature: anti-Flag (monoclonal, 1:1,000) (Abcam), anti-HA (monoclonal, 1:1,000) (Abcam), anti-β-Tubulin (monoclonal, 1:5,000) (Abcam), respectively. Rabbit polyclonal antiserum of IRF3 was kindly provided by Prof. Yibing Zhang. Anti-IRF7 rabbit antiserum was prepared in our laboratory. Purified rabbit polyclonal anti-phosphoserine (anti-pSer), anti-phosphothreonine (anti-pThr), and anti-phosphotyrosine (anti-pTyr) Ab were purchased from IMMUNECHEM (Canada). Calf intestinal alkaline phosphatase (CIP) used for dephosphorylation was purchased from BioLabs. The results were obtained from three independent experiments.

In vivo ubiquitination assay was performed in FHM cells. The transiently transfected cells were infected with GCRV at indicated time points and treated with 25 μM MG132 (Selleckchem) for 6 h before harvest, then lysed with NP-40 lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 1mM EDTA, 1mM Na_3VO_4, 0.5 μg/ml leupeptin, 2.5 mM sodium pyrophosphate] (Beytotime) added with 1 mM PMSF and 1% SDS for 30 min on ice. Before centrifugation, the samples were diluted with lysis buffer to ensure the final concentration of SDS with 0.1%. IP and immunoblotting (IB) examinations were conducted as above descriptions.

[1] http://www-bimas.cit.nih.gov/molbio/proscan/.
[2] http://gpminer.mbc.nctu.edu.tw/index.php.
[3] http://www.cbs.dtu.dk/services/Promoter/.

siRNA-Mediated Knockdown

Transient knockdown of endogenous LGP2 in CIK cells were achieved by transfection of siRNA targeting on LGP2 mRNA. Three siRNA sequences (s1: AAAGUGCUGGUCUACCAGG, s2: CCUGGUAGACCAGCACUUU, s3: AUCUUCAAAGGUCUU CUCC) targeting different regions of LGP2 gene were synthesized by RiboBio. The silencing efficiencies of the three LGP2 siRNA candidates were evaluated by qRT-PCR and WB, comparing with those in the negative control siRNA provided by the supplier. Our preliminary experiment indicated that s3 possesses the best silencing efficiency at a final concentration of 100 nM in mRNA level. For WB, LGP2-Flag overexpression CIK cell line was plated in 6-well plates and transfected with s3 using FuGENE 6. The cells were lysed for WB at 48 h post-transfection.

qRT-PCR

Total RNAs were isolated using RNAiso Plus (TaKaRa) according to manufacturer's instructions and incubated with RNase-free DNase I to eliminate contaminated genomic DNA. Reverse transcription was performed using random hexamer primers and M-MLV Reverse Transcriptase (Promega). Roche LightCycler® 480 system was used to quantify the mRNA expressions of related genes. EF1α was employed as an internal control gene for cDNA normalization (42). The qRT-PCR amplification was carried out in a total volume of 15 μl, containing 7.5 μl of BioEasy Master Mix (SYBR Green) (Hangzhou Bioer Technology Co., Ltd.), 5.1 μl of nuclease-free water, 2 μl of diluted cDNA (200 ng), and 0.2 μl of each gene specific primer (10 μM). The relative mRNA abundances were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to EF1α. All data were subjected to one-way analysis of variance (one-way ANOVA), followed by unpaired, two-tailed *t*-test (35). The results were obtained from four independent experiments, and each was performed in triplicate.

RESULTS

Identification of LGP2 As a Negative Mediator in IFNs and NF-κBs Induction

IFNs and NF-κBs are effector molecules mainly involved in virus-triggered innate immune responses. To identify the roles of LGP2 in GCRV-mediated IFNs and NF-κBs induction, we examined the promoter activities of all the members of grass carp IFNs (type I IFNs: IFN1, IFN2, IFN3, IFN4; type II IFNs: IFNγ1, IFNγ2) (43) and NF-κBs (NF-κB1, NF-κB2) upon LGP2 overexpression. In all these effector molecules, IFN2, IFN4, and IFNγ2 are early induced, so their promoter activities were examined at 12 h post-GCRV inoculation, and the others were investigated at 24 h. As shown in **Figures 1A,B**, except for IFN2, the promoter activities of all the examined genes were significantly suppressed in LGP2 overexpression cells under mock and GCRV-infected conditions. The promoter

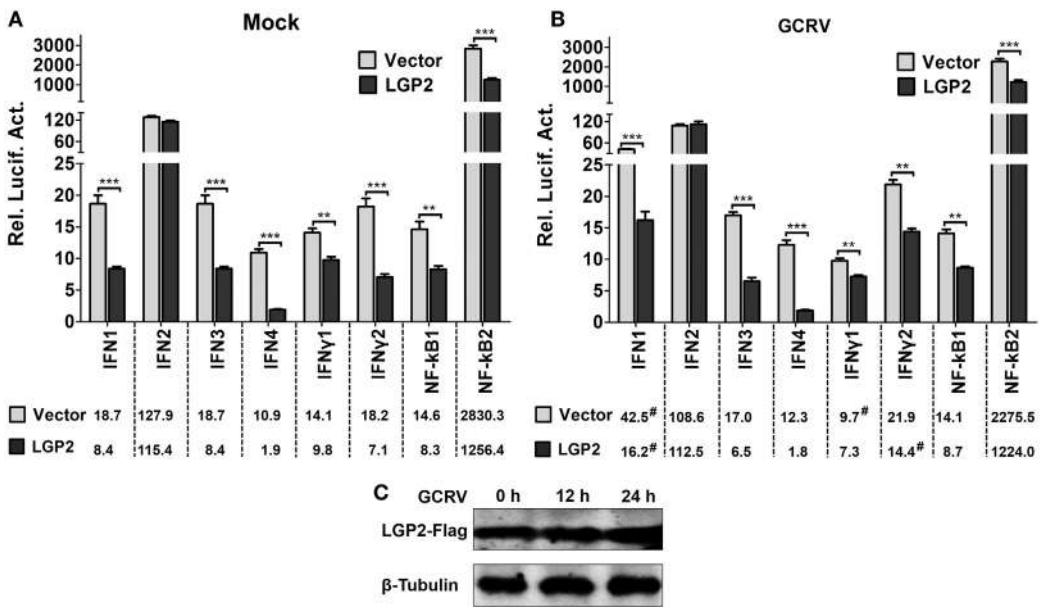

FIGURE 1 | **Identification of laboratory of genetics and physiology 2 (LGP2) as an inhibitor in IFNs and NF-κBs activation**. **(A,B)** LGP2 overexpression suppresses the promoter activities of IFNs and NF-κBs. Fathead minnow (FHM) cells were cotransfected with 300 ng of LGP2-Flag overexpression plasmid, 30 ng of pRL-TK, and 300 ng of IFN1pro-luc, IFN2pro-luc, IFN3pro-luc, IFN4pro-luc, IFNγ1pro-luc, IFNγ2pro-luc, NF-κB1pro-luc, NF-κB2pro-luc in 24-well plates. Control was transfected with 300 ng of empty vector (pCMV-CMV-GFP), same amount of the corresponding report vectors, and pRL-TK. At 16 h post-transfection, the cells were infected with grass carp reovirus (GCRV) or uninfected. Dual-luciferase report assays were conducted at 12 h (IFN2, IFN4, IFNγ2) or 24 h (IFN1, IFN3, IFNγ1, NF-κB1, NF-κB2) after GCRV infection. Time-matched mocks were treated with PBS. Error bars indicate SD ($n = 4$). Asterisks indicate significant differences from control (**$0.001 < P < 0.01$; ***$P < 0.001$). Digitals under histograms show the average values. Symbol "#" indicates significant difference between mock and GCRV-infected conditions. **(C)** Examination of exogenous LGP2 in FHM cells upon GCRV infection. FHM cells were transfected with 1 μg of LGP2-Flag overexpression plasmid in 6-well plates. GCRV infection was preformed at 12 and 24 h post-transfection. The cell lysates were prepared for WB using anti-Flag and anti-β-Tubulin Abs.

activities of IFN1, IFNγ1, and IFNγ2 were remarkably changed upon GCRV infection in empty vector or LGP2 transfected cells. GCRV infection had no influence on the protein level of exogenous LGP2 in FHM cells (**Figure 1C**). These results indicate that LGP2 plays a negative role in both IFNs and NF-κBs pathways.

LGP2 Inhibits the Expressions of IFNs and NF-κBs at the Early Phase Post-GCRV Infection

First, we investigated the expression patterns of IFNs, NF-κBs, and IRF3/7 in mock cells (empty vector transfected cells) post-GCRV infection. The results indicate that GCRV infection significantly upregulated the mRNA levels of IFN4, IFNγ1, IFNγ2, NF-κB1, NF-κB2, IRF3, and IRF7 (Figure S2 in Supplementary Material). To explore the influence of LGP2 overexpression on IFNs and NF-κBs expressions in response to GCRV infection, LGP2-Flag stable transfected CIK cell line was infected with GCRV at different time points. qRT-PCR showed that LGP2 overexpression markedly decreases the transcriptions of IFNs and NF-κBs expect for IFN3. However, mRNA expression levels of these genes were mostly recovered to levels of control cells at 12 or 24 h after GCRV infection (**Figures 2A–G**). No significant change of exogenous LGP2 was detected in CIK cells upon GCRV infection (**Figure 2H**). Furthermore, we also examined the expressions of IRF3 and IRF7, which mediate IFN production. The results indicated that both IRF3 and IRF7 were decreased at early time points but recovered to control levels at 24 h post-GCRV infection (**Figures 3A,B**). These data suggested that LGP2 inhibits induction of IFNs and NF-κBs at the early phase of GCRV infection.

LGP2 Suppresses Synthesis and Activation of IRF3 and IRF7 at the Early Phase of GCRV Infection

Interferon regulatory factor 3 and IRF7 are essential for virus-induced IFN-I activation and development of the innate antiviral responses (44). To investigate regulation of LGP2 to IRF3 and IRF7, dual-luciferase report assay was performed. As shown

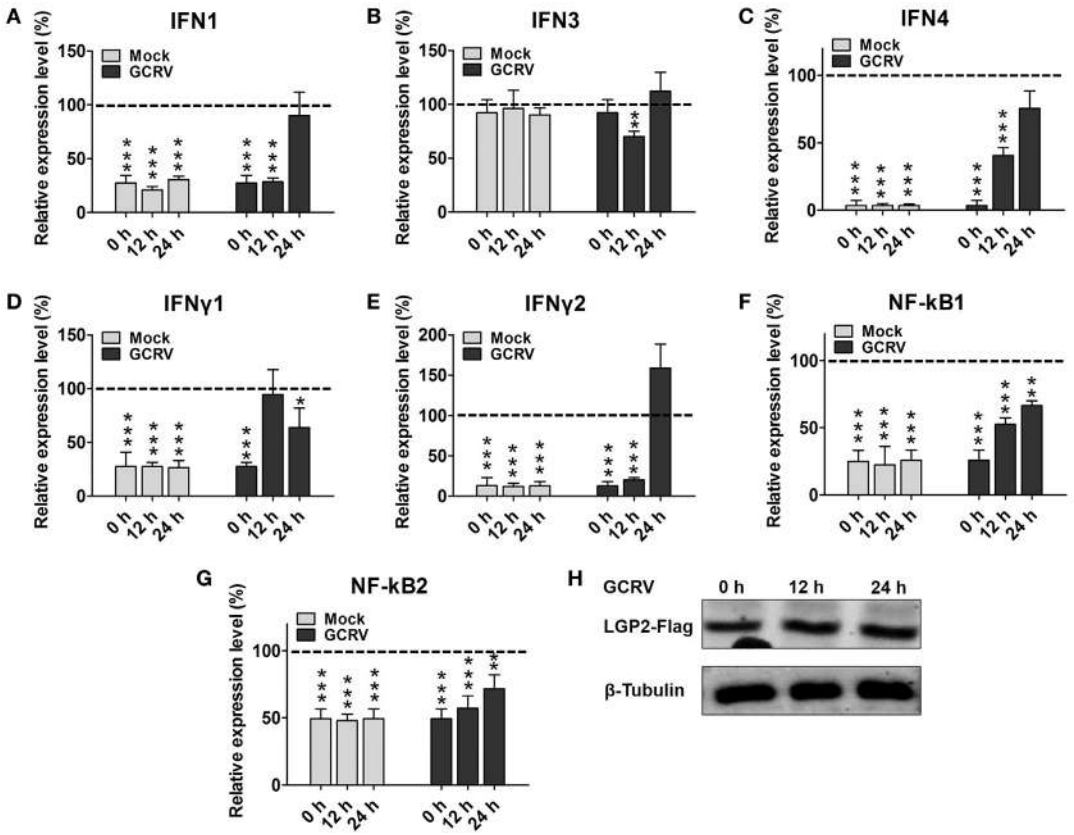

FIGURE 2 | Laboratory of genetics and physiology 2 (LGP2) overexpression decreases grass carp reovirus (GCRV)-induced IFNs and NF-κBs transcriptions at early stage. LGP2-Flag and empty vector (pCMV-pCMV-GFP) stable transfected *Ctenopharyngodon idella* kidney (CIK) cells were seeded in 12-well plates with about 70–90% monolayer confluency. Twenty-four hours later, the cells were infected with GCRV for 12 and 24 h or time-matched mock treatment. Then the cells were harvested for qRT-PCR to quantify the relative expression levels of IFN1 **(A)**, IFN3 **(B)**, IFN4 **(C)**, IFNγ1 **(D)**, IFNγ2 **(E)**, NF-κB1 **(F)**, and NF-κB2 **(G)**, respectively. Fold change was determined relative to corresponding treatment group in empty vector (dash line). Error bars indicate significant differences from control (*$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$). **(H)** Analysis of LGP2-Flag post GCRV infection. LGP2-Flag stable transfected CIK cells were seeded in 6-well plates. Then the cells were treated with or without GCRV for 12 and 24 h. The cell lysate was used for WB analysis with anti-Flag and anti-β-Tubulin Abs.

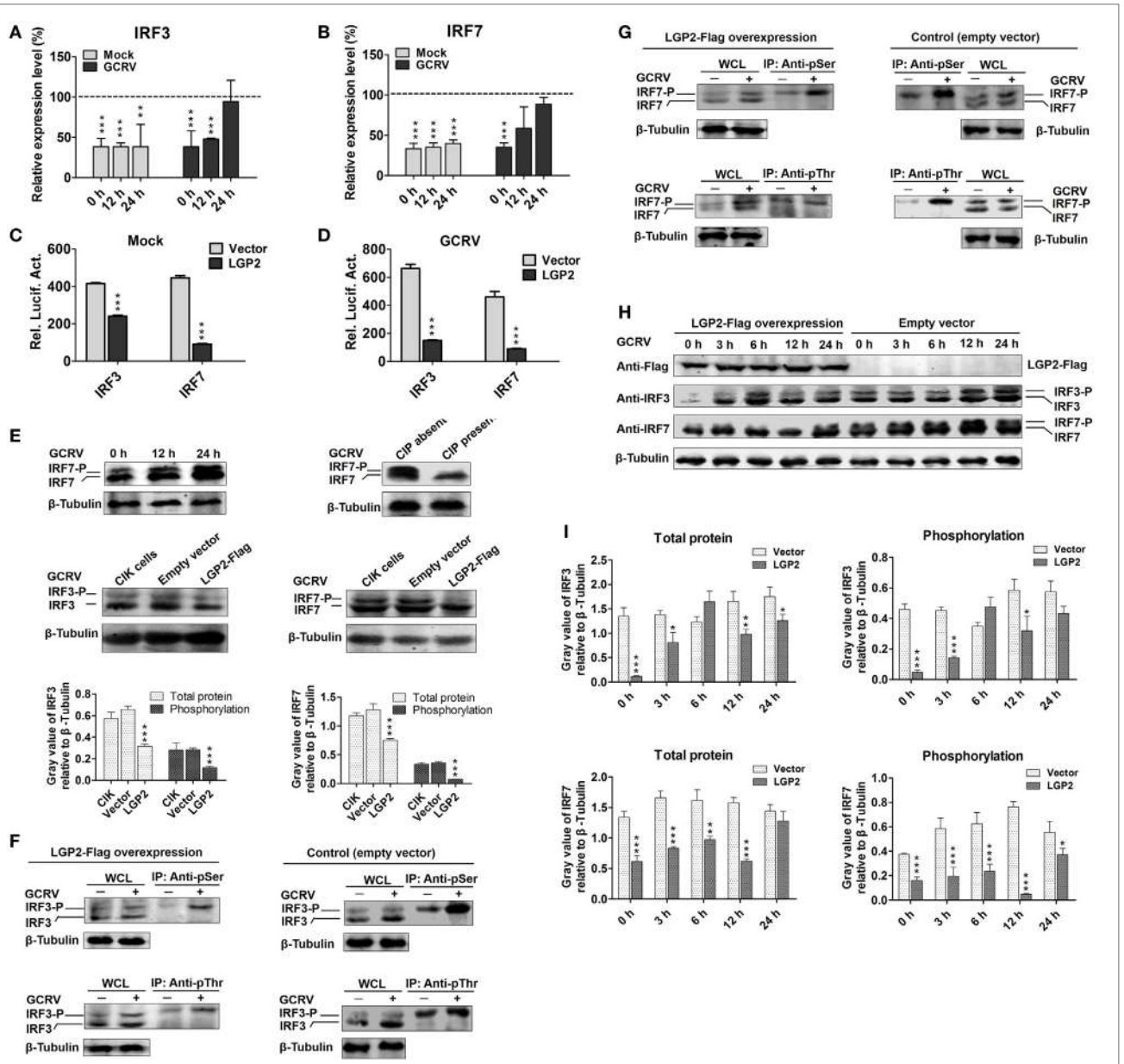

FIGURE 3 | **Laboratory of genetics and physiology 2 (LGP2) suppresses interferon regulatory factor 3 (IRF3) and IRF7 signal activation *via* downregulating their synthesis and phosphorylation**. **(A,B)** LGP2 overexpression inhibits the transcriptions of IRF3 and IRF7. mRNA levels of IRF3 and IRF7 were measured by qRT-PCR in LGP2-Flag and empty vector stable transfected *C. idella* kidney (CIK) cells at 12 and 24 h post grass carp reovirus (GCRV) infection or mock treatment. Fold change was determined relative to corresponding treatment group in empty vector (dash line). Error bars indicate SD ($n = 4$). Asterisks indicate significant differences from control (***$P < 0.001$). **(C,D)** LGP2 overexpression inhibits the promoter activities of IRF3 and IRF7. FHM cells were cotransfected with 300 ng of LGP2-Flag, 30 ng of pRL-TK, and 300 ng of IRF3pro-luc or IRF7pro-luc plasmids. At 16 h post-transfection, the cells were infected with or without GCRV for 24 h, and then they were collected for luciferase report assay. Error bars indicate SD ($n = 4$). Asterisks indicate significant differences from control (***$P < 0.001$). **(E)** Upper: verification of the phosphorylation band of IRF7 antiserum. CIK cells were infected with GCRV for 12 or 24 h in 6-well plates for Western blotting (WB). A portion of the whole cell lysis (WCL), which was infected with GCRV for 24 h was incubated with or without 10 U of CIP for 30 min. IRF7 was examined by WB with IRF7 antiserum. β-Tubulin was served as an internal control. Middle: LGP2 involves in the inhibition of both IRF3 and IRF7 protein levels. CIK cells, empty vector, and LGP2-Flag-transfected cells were infected with GCRV for 12 h, and the cells lysate was used for WB. The histograms (below) display the relative expression levels, which were quantified by using ImageJ software. Error bars indicate SD ($n = 3$). Asterisks indicate significant differences from control (***$P < 0.001$). **(F,G)** LGP2 overexpression inhibits GCRV-induced IRF3 and IRF7 Ser and Thr phosphorylation. LGP2-Flag and empty vector stable transfected CIK cells were seeded in 10 cm^2 dishes for GCRV infection. At 24 h post-GCRV infection, the cells were lysed for immunoprecipitation (IP) with anti-pSer and anti-pThr Abs (Ab), respectively. The IP samples and WCL were subjected to immunoblotting (IB) with anti-IRF3 and anti-IRF7 antiserum, respectively. **(H)** LGP2 overexpression represses the protein and phosphorylation levels of IRF3 and IRF7. LGP2-Flag and empty vector stable transfected CIK cells were infected with GCRV and samples were collected at 0, 3, 6, 12, and 24 h postinfection. WCL was subjected to IB with IRF3, IRF7, and β-Tubulin Ab, respectively. **(I)** The relative protein expression levels were quantified by using ImageJ software. Error bars indicate SD ($n = 3$). Asterisks indicate significant differences from control (*$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$).

in **Figures 3C,D**, LGP2 overexpression significantly inhibits the promoter activities of IRF3 and IRF7 under both basal and GCRV infection conditions. In LGP2 overexpression CIK cells, transcription levels of IRF3 and IRF7 were notably inhibited at early stage of GCRV infection (**Figures 3A,B**). A previous study indicated that C-terminal phosphorylation induced by virus infection is essential for activation of IRF3 and IRF7 (44). To uncover whether LGP2 can regulate phosphorylation of IRF3 and IRF7, we first verified the recognition of phosphorylation specificity of anti-IRF7 antiserum, which has been confirmed to specifically bind recombinant IRF7 protein. CIK cells infected with GCRV at different time points (0, 12, and 24 h) were collected for WB analysis with anti-IRF7 antiserum. Two bands between 43 and 55 kDa (marker not shown) were induced by GCRV infection. The lower band was IRF7 and the upper band may be the phosphorylated form of IRF7 (**Figure 3E**, upper left). Then, we treated the whole cell lysis of CIK cells infected by GCRV with or without CIP. The results indicate that CIP treatment led to the disappearance of the upper band and had no influence on the basal band (**Figure 3E**, upper right). This result indicates that the upper band is the phosphorylated form of IRF7 indeed. Specificity and phosphorylated band of anti-IRF3 antiserum have been verified in the previous report (45). In **Figure 3E**, middle and below, LGP2 overexpression significantly inhibits the protein levels of IRF3 and IRF7 compared with those in CIK or empty vector-transfected cells, and empty vector has no influence on the protein expressions of IRF3 and IRF7.

Phosphorylation of the C-terminal serine (Ser) and threonine (Thr) residues is important for IRF3 and IRF7 activation following viral infection (46). To this end, IP with anti-pSer and anti-pThr Ab were performed in the LGP2-Flag stable transfected CIK cells, followed by IB with IRF3 and IRF7 antiserums, respectively. Compared with those in control (empty vector-transfected cells), LGP2 overexpression significantly inhibited GCRV-induced Ser and Thr phosphorylation of IRF3 and IRF7 (**Figures 3F,G**). To better understand the influence of LGP2 on the protein synthesis and activation of IRF3 and IRF7 induced by GCRV infection, LGP2-Flag and empty vector stable-transfected CIK cells were infected with GCRV at different time points. As showed in **Figures 3H,I**, LGP2 overexpression not only suppressed phosphorylation but also downregulated the basal protein levels of IRF3 at early phase of GCRV infection. As for IRF7, LGP2 overexpression mainly inhibited the phosphorylation levels at early time points, but had no notable effect on the basal protein levels. These results implied that LGP2 inhibits GCRV-induced activation of IRF3 and IRF7 at early phase through diverse manners: LGP2 inhibits the synthesis and Ser/Thr phosphorylation of IRF3, but mainly decreases GCRV-induced Ser/Thr phosphorylation of IRF7. Considering overall, LGP2 overexpression significantly inhibits the total protein levels (phosphorylation + basal protein) of both IRF3 and IRF7 (**Figures 3H,I**). We also examined tyrosine (Tyr) phosphorylation of IRF3 and IRF7. However, no Tyr phosphorylation was detected in IRF7, and IRF3 possessed Tyr residue phosphorylation but was unable to be induced by GCRV infection (Figure S3 in Supplementary Material). So, Tyr phosphorylation of IRF3 may not involve in antiviral immunity.

Knockdown of LGP2 Enhances GCRV-Mediated Signal Induction at Early Stage

To verify the results obtained from above experiments, LGP2 in CIK cells (endogenous) or in LGP2 stable overexpression CIK cells was silenced by LGP2-specific siRNA. Among three candidate siRNA sequences, s3 showed the best interference efficiency in LGP2 mRNA level in CIK cells (**Figure 4A**). Consistently, s3 induced significant knockdown in LGP2 protein level in LGP2 stable overexpression CIK cells (**Figure 4B**), so, s3 was selected for the following experiments. Compared with the untransfected or transfected with control siRNA, LGP2 knockdown significantly increased GCRV-induced basal protein and phosphorylation levels of IRF3 and IRF7 (**Figures 4C,D**). To further detect the influence of LGP2 knockdown on virus-triggered immune genes, IFN1, IFN4, IFNγ2, and NF-κB2, which were chosen

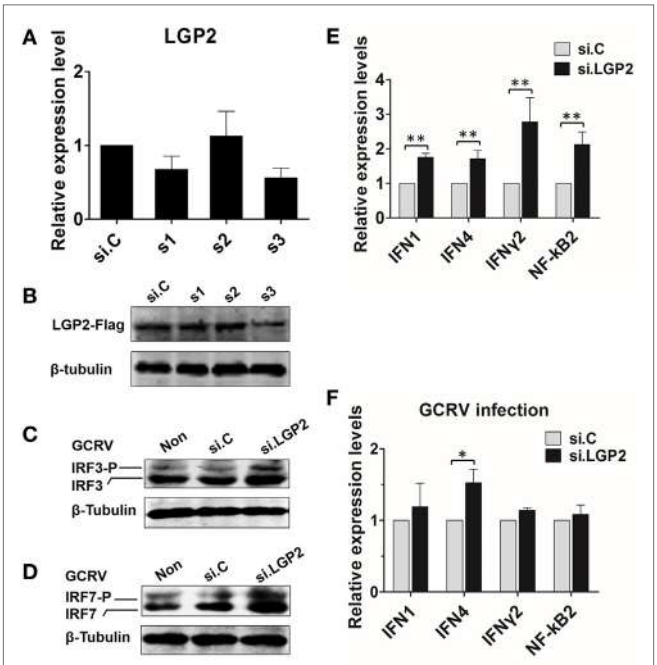

FIGURE 4 | Knockdown of laboratory of genetics and physiology 2 (LGP2) potentiates grass carp reovirus (GCRV)-mediated activation of innate immune responses in *Ctenopharyngodon idella* kidney (CIK) cells. **(A)** Screening LGP2 interference sequences. Three siRNA sequences (s1, s2, and s3) along with the negative control si.C were transiently transfected into CIK cells. The cells were harvested for qRT-PCR at 24 h post-transfection to detect the transcription level of LGP2. **(B)** Examining the interference efficiency of the three siRNA in protein level. LGP2-Flag stable transfected CIK cells were transiently transfected with s1, s2, s3, and si.C in 6-well plates. Twenty-four hours later, cell lysates were prepared for IB using anti-Flag Ab. **(C,D)** Knockdown of LGP2 upregulates the protein levels of IRF3 and IRF7 induced by GCRV infection. CIK cells were transfected with s3 in 6-well plates. Twelve hours later, cells were infected with GCRV for 12 h and WB was conducted with anti-IRF3 and anti-IRF7 antiserums, respectively. **(E,F)** CIK cells were transfected with s3 and si.C, respectively, and treated or untreated with GCRV for 12 h. The cells were prepared for qRT-PCR to test the transcription levels of IFN1, IFN4, IFNγ2, and NF-κB2, respectively. Error bars indicate SD ($n = 4$). Asterisks indicate significant differences from control (*$0.01 < P < 0.05$; **$0.001 < P < 0.01$).

as representations for IFNs and NF-κBs, respectively, were examined by qRT-PCR. Inversely correlated with the results in **Figure 2**, knockdown of LGP2 remarkably upregulated the basal inductions of these genes (**Figure 4E**). However, upon GCRV infection, mRNA expressions of these genes showed a trend to recover to control levels (**Figure 4F**). These results further confirm the negative role of LGP2 in antiviral immune responses at early stage.

LGP2 Interacts with RIG-I and MDA5 Independent of GCRV Infection

A previous report indicated that LGP2 functions upstream of RIG-I and MDA5 (18). Consistently, LGP2 overexpression significantly inhibited the promoter activities of RIG-I and MDA5 (**Figures 5A,B**). To determine whether LGP2 can directly interact with RIG-I or MDA5, flag-tagged LGP2 was co-transfected with HA-tagged RIG-I or MDA5 into FHM cells. Co-IP assay was carried out with anti-HA, and IB analysis was performed with anti-HA or anti-Flag Ab. As showed in **Figures 5C,D**, LGP2 efficiently interacted with RIG-I and MDA5 no matter under basal condition or GCRV infection. Meanwhile, similar results were obtained from the reverse Co-IP assay (Figure S4 in Supplementary Material). These results demonstrate that LGP2 interact with RIG-I and MDA5 independent of GCRV infection.

LGP2 Restrains RIG-I- and MDA5-Mediated IPS-1 and MITA Activation

Upon activation, RIG-I and MDA5 induce downstream signaling *via* interaction with IPS-1 and MITA (10, 20). To determine whether LGP2 can restrain RIG-I-, MDA5-mediated IPS-1, MITA promoter activities upon GCRV infection, dual-luciferase reporter assays were performed in FHM cells. The results indicate that LGP2 overexpression inhibits RIG-I- and MDA5-mediated basal and GCRV-induced IPS-1 promoter activities (**Figures 6A,C**). However, LGP2 overexpression just inhibits RIG-I-mediated basal, but not GCRV-triggered activity of MITA promoter, and had no influence on MDA5-mediated MITA promoter activity (**Figures 6B,D**). As well known, RIG-I and MDA5 activate IPS-1 *via* a CARD–CARD-mediated interaction (1). To identify the domain specificity of the interaction between LGP2 and RIG-I or MDA5, HA-tagged RIG-I or MDA5 domains (CARDs, helicase, and RD) expression plasmids were constructed (**Figures 6E,F** upper). IP assay indicates that LGP2 specifically interacts with RIG-I helicase and RD domains, but not the CARDs domain (**Figure 6E** below). However, LGP2 interacts with all the three domains of MDA5 (**Figure 6F**, below). These observations imply the difference of LGP2 in regulating RIG-I and MDA5. Do the different interactions of LGP2 with RIG-I or MDA5 CARDs domain affect IPS-1 activation? To this end, we tested the influence of LGP2 on the promoter activities of IPS-1 and MITA mediated by RIG-I CARDs or MDA5

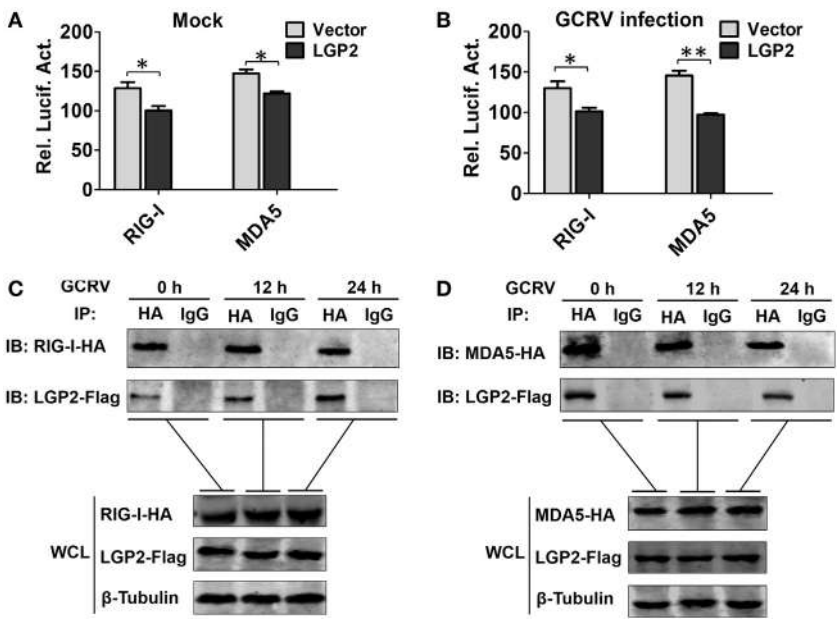

FIGURE 5 | Laboratory of genetics and physiology 2 (LGP2) interacts with melanoma differentiation-associated gene 5 (MDA5) and retinoic acid-inducible gene I (RIG-I) independent of grass carp reovirus (GCRV) infection. **(A,B)** LGP2 overexpression inhibits RIG-I and MDA5 promoter activities. Fathead minnow (FHM) cells were transiently transfected with 300 ng of LGP2-Flag overexpression plasmid or empty vector, 30 ng of pRL-TK, and 300 ng of report vector (RIG-Ipro-luc or MDA5pro-luc) for 16 h, and then the cells were infected with GCRV or uninfected. Dual-luciferase report assays were conducted at 24 h after GCRV infection. Error bars indicate SD ($n = 4$). Asterisks indicate significant differences from control (*$0.01 < P < 0.05$; **$0.001 < P < 0.01$). **(C,D)** LGP2 interacts with RIG-I and MDA5. FHM cells were transfected with LGP2-Flag and RIG-I-HA or LGP2-Flag and MDA5-HA for 16 h, and then infected with GCRV for 12 or 24 h. Co-IP was performed with anti-HA monoclonal antibody (Ab). Mouse IgG was used as control. WCL of each time point was subjected to IBs with anti-Flag, anti-HA, and β-Tubulin Ab, respectively.

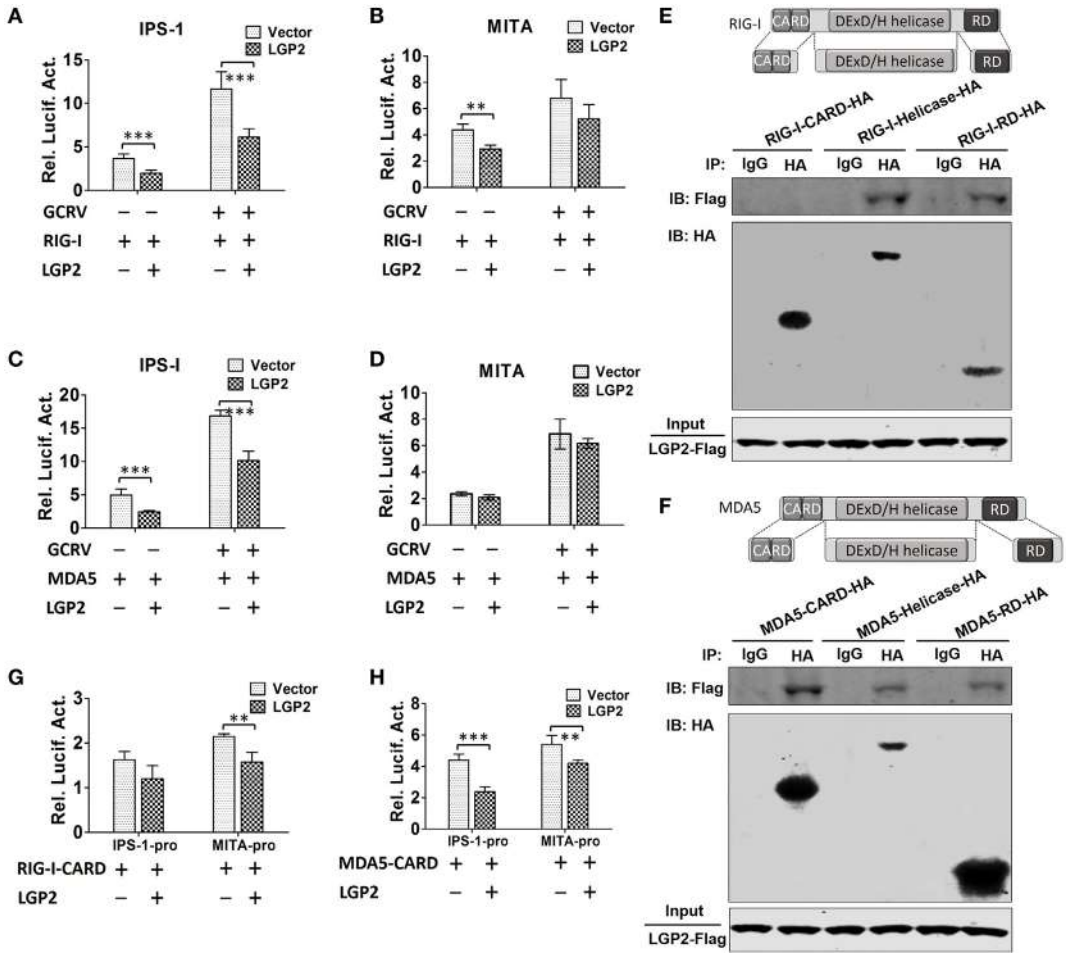

FIGURE 6 | Laboratory of genetics and physiology 2 (LGP2) inhibits downstream signaling of retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). **(A,B)** LGP2 inhibits RIG-I-mediated IFN-β promoter stimulator 1 (IPS-1) and mediator of IRF3 activation (MITA) promoter activities. Fathead minnow (FHM) cells were transiently transfected with 200 ng of LGP2-Flag or empty vector, 200 ng of RIG-I expression plasmid, 30 ng of pRL-TK plus 200 ng of IPS-1pro-luc or MITApro-luc for 16 h and then infected with grass carp reovirus (GCRV). Luciferase activities were conducted at 24 h after GCRV infection. **(C,D)** LGP2 inhibits MDA5-mediated IPS-1, but not MITA promoter activity. FHM cells were transiently transfected with 200 ng of LGP2-Flag or empty vector, 200 ng of MDA5 expression plasmids, 30 ng of pRL-TK plus 200 ng of IPS-1pro-luc or MITApro-luc in 24-well plates. At 16 h post-transfection, the cells were infected with GCRV for 24 h and then subjected to luciferase activities analysis. **(E)** Upper: schematic representations of full-length RIG-I and the three domains constructed in the present study. Below: LGP2 interacts with RIG-I-Helicase, RIG-I-RD, but not RIG-I-CARDs domain. FHM cells were cotransfected with 4 μg LGP2-Flag and 4 μg RIG-I-CARD-HA or RIG-I-Helicase-HA or RIG-I-RD-HA for 24 h in 10 cm^2 dishes. Co-IP was performed using anti-HA antibody (Ab), and mouse IgG was used as control. IPs were analyzed by IBs with anti-HA and anti-Flag, respectively. Expression of LGP2-Flag (input) was examined with anti-Flag. **(F)** Upper: full-length MDA5 and its domain structures. Below: LGP2 interacts with MDA5-CARDs, MDA5-Helicase, and MDA5-RD. FHM cells were transfected with the indicated plasmids (4 μg each). Twenty-four hours later, cells were lysed, Co-IP and IB analyses were performed with the indicated Abs. **(G)** LGP2 inhibits RIG-I-CARDs-mediated MITA, but not IPS-1 promoter activity. FHM cells were transfected with 200 ng of LGP2-Flag or empty vector, 200 ng of RIG-I-CARD-HA expression plasmid, 30 ng of pRL-TK plus 200 ng of IPS-1pro-luc or MITApro-luc. Luciferase assays were performed at 24 h post-transfection. **(H)** LGP2 inhibits MDA5-CARDs-mediated IPS-1 and MITA promoter activities. FHM cells were transfected with 200 ng of LGP2-Flag or empty vector, 200 ng of MDA5-CARD-HA expression plasmid, 30 ng of pRL-TK plus 200 ng of IPS-1pro-luc or MITApro-luc. Luciferase assays were performed at 24 h post-transfection. Error bars indicate SD ($n = 4$). Asterisks indicate significant differences from control (**$0.001 < P < 0.01$; ***$P < 0.001$).

CARDs. Interestingly, LGP2 overexpression suppresses both RIG-I CARDs- and MDA5 CARDs-mediated MITA promoter activities (**Figures 6G,H**). For IPS-1 promoter, LGP2 overexpression significantly inhibits MDA5 CARDs-mediated promoter activity of IPS-1 ($P < 0.001$), but had no notable inhibition in RIG-I CARDs-mediated IPS-1 activity ($P > 0.05$) (**Figures 6G,H**). These results collectively demonstrate that LGP2 inhibits RLRs signaling *via* direct protein–protein interaction with RIG-I and MDA5. Difference in interaction of LGP2 with RIG-I CARDs or MDA5 CARDs also implies the distinguishable regulation strategies of LGP2 to RIG-I and MDA5.

LGP2 Inhibits K63-Linked Ubiquitination of RIG-I and MDA5

Previous studies have demonstrated that K63-linked ubiquitination positively regulates downstream signaling of RIG-I and

MDA5 in antiviral innate immune responses (6, 47). Given the negative regulation of LGP2 in RIG-I- and MDA5-mediated downstream signaling, whether LGP2 can affect the K63-linked ubiquitination of RIG-I and MDA5? To this end, *in vivo* ubiquitination assay was performed in FHM cells. Our results indicated that GCRV infection enhanced the K63-linked ubiquitination of both RIG-I and MDA5 (**Figures 7A,B**). Meanwhile, LGP2 overexpression inhibited the K63-linked ubiquitination of both RIG-I and MDA5 in a dose-dependent manner (**Figures 7C,D**). In mammals, binding to K63 ubiquitin chain in CARDs domain is essential for activation of RIG-I and MDA5 (47). In order to gain more insights into the impact of LGP2 on RIG-I and MDA5 activation, K63-linked ubiquitination of RIG-I CARDs and MDA5 CARDs were further examined. Interestingly, LGP2 overexpression significantly inhibited the K63-linked ubiquitination of RIG-I CARDs but had no influence on the ubiquitination of MDA5 CARDs (**Figures 7E,F**). These results indicate that LGP2 represses MDA5 activation by way of inhibiting the K63-linked ubiquitination of MDA5 helicase or RD domains, but not CARDs. However, suppression of RIG-I CARDs K63-linked ubiquitination is important for LGP2-inhibited activation of RIG-I. Sequence alignment indicated that K154, K164, K169, and K172 residues, which bind K63-linked polyubiquitin chain are conserved in grass carp RIG-I CARDs (Figure S5A in Supplementary Material) (48–50), so the ability to bind K63 polyubiquitin chain of grass carp RIG-I CARDs is similar with that in mammals.

LGP2 Suppresses K48-Linked Ubiquitination of RIG-I and MDA5 at Early Stage during GCRV Infection

To investigate whether LGP2 is involved in the proteasome-mediated degradation of RIG-I and MDA5, the K48-linked ubiquitination of RIG-I and MDA5 was examined upon LGP2 overexpression. Surprisingly, LGP2 overexpression did not promote the degradation of RIG-I and MDA5, but significantly depressed the K48-linked ubiquitination of MDA5 in a dose-dependent manner and slightly inhibited that of RIG-I at steady state (**Figures 8A,B**). These results raise a question that what is the biological significances of LGP2-triggered inhibition of RIG-I and MDA5 degradation? Then, MDA5 was selected for further examination of the K48-linked ubiquitination at different time points post-GCRV infection. Comparatively, the K48-linked

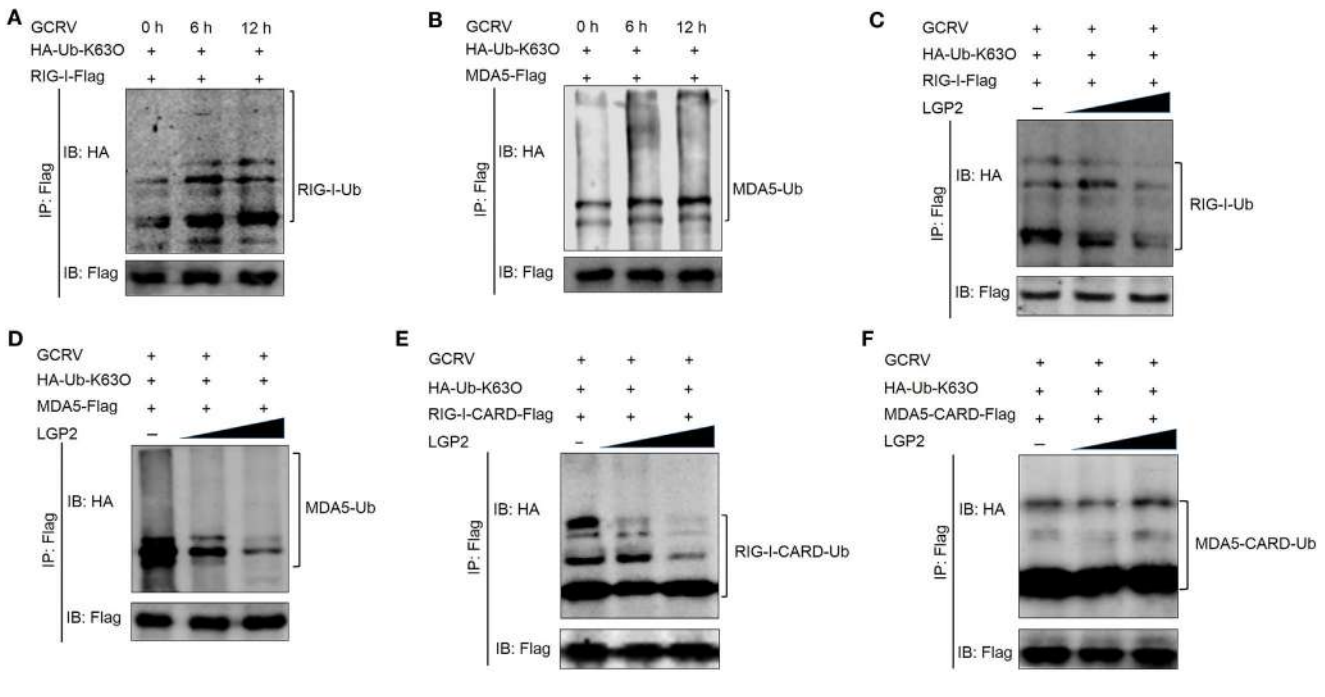

FIGURE 7 | Laboratory of genetics and physiology 2 (LGP2) inhibits the K63-linked ubiquitination of retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). **(A,B)** Grass carp reovirus (GCRV) infection upregulates the K63-linked ubiquitination of RIG-I and MDA5. Fathead minnow (FHM) cells were transfected with 2 μg HA-Ub-K63O, 6 μg RIG-I-Flag **(A)**, or 6 μg MDA5-Flag **(B)** in 10 cm^2 dishes, respectively. At 24 h post-transfection, the cells were treated with MG132 and GCRV for 6 and 12 h. Then, the cells were harvested for immunoprecipitation (IP) with anti-Flag antibody (Ab) and IBs with anti-HA and anti-Flag Ab. **(C,D)** LGP2 inhibits the K63-linked ubiquitination of RIG-I and MDA5 in a dose-dependent manner. FHM cells were seeded in 10 cm^2 dishes for 24 h and transfected with 1 μg HA-Ub-K63O, LGP2 (0, 1.5, and 3 μg) together with decreasing amounts of empty vector (3, 1.5, and 0 μg), 4 μg RIG-I-Flag **(C)**, or 4 μg MDA5-Flag **(D)**. At 24 h post-transfection, the cells were treated with MG132 and GCRV for 6 h. Then, the cells were harvested for IP with anti-Flag Ab and IBs with anti-HA and anti-Flag Ab, respectively. **(E,F)** LGP2 represses the K63-linked ubiquitination of RIG-I CARDs, but not MDA5 CARDs. FHM cells were transfected with 1 μg HA-Ub-K63O, LGP2 (0, 1.5, and 3 μg), empty vector (3, 1.5, and 0 μg), 4 μg RIG-I-CARD-Flag **(E)**, or 4 μg MDA5-CARD-Flag **(F)** in 10 cm^2 dishes. At 24 h post-transfection, the cells were treated with MG132 and GCRV for 6 h. Then, the cells were harvested for IP with anti-Flag Ab and IBs with the indicated Abs.

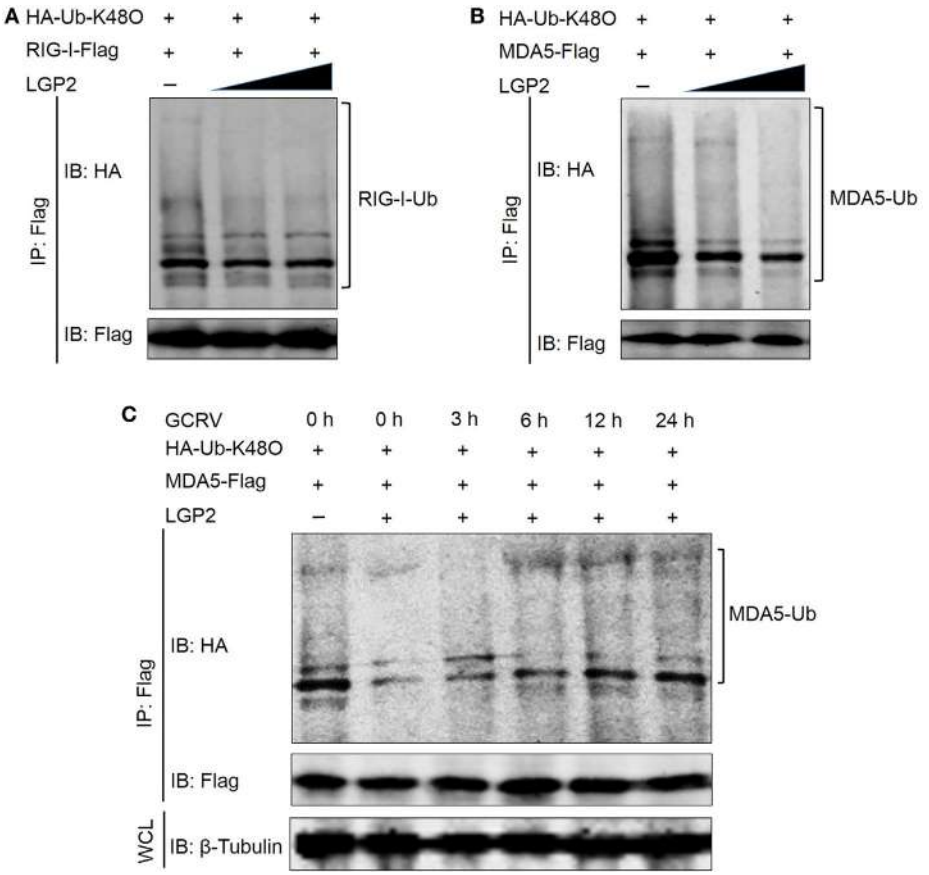

FIGURE 8 | Laboratory of genetics and physiology 2 (LGP2) mediates the K48-linked ubiquitination of retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). **(A,B)** LGP2 significantly inhibits the K48-linked ubiquitination of MDA5 but slightly reduces that of RIG-I. Fathead minnow (FHM) cells were transfected with 1 μg HA-Ub-K48O, LGP2 (0, 1.5, and 3 μg) together with decreasing amounts of empty vector (3, 1.5, and 0 μg), 4 μg RIG-I-Flag **(A)**, or 4 μg MDA5-Flag **(B)**. At 24 h post-transfection, the cells were treated with MG132 for 6 h, and the cells were harvested for immunoprecipitation (IP) with anti-Flag antibody (Ab) and IBs with anti-HA and anti-Flag Ab, respectively. **(C)** LGP2 suppresses the K48-linked ubiquitination of MDA5 at early stage of grass carp reovirus (GCRV) infection. FHM cells were transfected with 1 μg HA-Ub-K48O, 2 μg LGP2, 1 μg empty vector, and 4 μg MDA5-Flag. Control cells were transfected with 1 μg HA-Ub-K48O, 3 μg empty vector, and 4 μg MDA5-Flag. At 24 h post-transfection, the cells were infected with GCRV for 3, 6, 12, and 24 h and treated with MG132 for 6 h. After that, the cells were lysed and subjected to IP with Flag Ab and subsequent IB with anti-HA and anti-Flag, respectively. WCL was used for IB with anti-β-Tubulin.

ubiquitination of MDA5 was significantly inhibited by LGP2 overexpression at early phase of GCRV infection, but gradually recovered to the control level at later time (**Figure 8C**). These results collectively demonstrate that LGP2-induced inhibition of MDA5 and RIG-I K48-linked ubiquitination just occurs at resting state and early stage post-GCRV infection.

DISCUSSION

In contrast to previous reports of LGP2 as a positive regulator of MDA5- and RIG-I-mediated viral recognition (16, 18, 51), our present study demonstrates that grass carp LGP2 is a negative regulator in RIG-I- and MDA5-mediated antiviral signaling pathway at resting state and early phase during GCRV infection. Previous investigations found the negatively regulatory role of LGP2 in IFN signaling: overexpression of LGP2 strongly inhibits IRF3 activation and IFN-stimulated regulatory element and NF-κB signaling pathways post-Newcastle disease virus infection (13). LGP2 can inhibit antiviral signaling independent of dsRNA or virus infection (15). IRF family has been demonstrated to contain 9 members in mammals, 10 members in avian, and 13 members in fish (43). IRF3 and IRF7, two structurally homologous members, are able to activate fish IFN promoters and upregulate fish IFNs and ISGs (52). Here, the inhibition of IRF3, IRF7, IFNs, and NF-κBs promoter activities and mRNA expression levels caused by LGP2 overexpression provides direct proofs for the negative role of LGP2. This conclusion is also verified by LGP2 knockdown assay.

In the resting state cells, IRF3 and IRF7 localize in cytoplasm, whereas poly(I:C) stimulation or virus infection induces their cytoplasmic-nuclear translocation (20, 45). Phosphorylation is the prerequisite for activation and nuclear import of IRF3 and IRF7. In line with the result from luciferase report assays, LGP2 overexpression inhibits both Ser and Thr phosphorylation of

IRF3 and IRF7, which is the substance that LGP2 suppresses activation of IRF3 and IRF7. IRF3 and IRF7 are proposed to synergistically induce the expressions of IFNs (52). However, compared with these abundant data about the relationship between LGP2 and IRF3, no evidence reflects regulation model of LGP2 to IRF7 (13). Our results first identified the negative regulation of LGP2 to IRF7 in the promoter activity, mRNA, and protein level in response to dsRNA virus infection in fish cells. We also revealed the different mechanisms of LGP2 in regulating IRF3 and IRF7: in resting state and early stage of GCRV infection, LGP2 overexpression inhibits both basal protein and phosphorylation levels of IRF3 but just reduces the phosphorylation level of IRF7.

In Huh7 cells, Saito et al. found that LGP2 overexpression can form a stable complex with RIG-I or MDA5 (14). Our Co-IP experiments demonstrated that LGP2 interacts with RIG-I or MDA5 independent of GCRV infection. Saito et al. also described that LGP2 just represses RIG-I signaling, but is not sufficient for MDA5 signaling inhibition (14). However, the direct interactions of grass carp LGP2 with RIG-I or MDA5 functionally inhibit RIG-I- or MDA5-induced IPS-1 promoter activities. Classical model supports that RIG-I adopts a closed autoinhibited conformation where CARDs are sterically masked and unavailable for signal transduction in resting cells (1). In our domain interaction assay, no direct interaction was observed between LGP2 and RIG-I CARDs, meanwhile, LGP2 fails to block RIG-I CARDs-mediated promoter activity of IPS-1 at resting state. These results indicate that LGP2 inhibits RIG-I-mediated signal transduction independent of direct binding with RIG-I CARDs, which is in line with the previous report that LGP2 controls RIG-I signaling through *in trans* interaction between RIG-I helicase domain and LGP2 RD domain (14). The present study proposes the following model of LGP2 in mediating RIG-I signaling: under normal condition, LGP2 invertedly binds to RIG-I (LGP2 helicase domain interacts with RIG-I RD and LGP2 RD binds to RIG-I helicase domain); upon viral infection, cooperative ATP and viral dsRNA binding to RIG-I helicase domain leads to a conformational switch to a closed form with dsRNA, and the CARDs are released to interact with IPS-1 concomitantly (53). Unlike RIG-I, MDA5 is thought to adopt an open conformation with exposed CARDs in the absence of ligand (1). Our interaction study provided an efficient interaction between LGP2 and MDA5 CARDs. Importantly, LGP2 indeed significantly represses MDA5 CARDs-mediated IPS-1 promoter activity (**Figure 6H**). A reasonable mechanism may be that strong interaction between LGP2 and MDA5 CARDs inhibits MDA5 to establish an intramolecular interaction. In other words, LGP2 binds to MDA5 CARDs, which fails to make MDA5 form a self-inhibited state. Simultaneously, this interaction efficiently restrains MDA5 CARDs-mediated signaling to IPS-1 (**Figure 9**). The exact interaction relationship between domains of LGP2 and MDA5 are still unknown. Comparatively, LGP2 shows more preference to restrain RIG-I- rather than MDA5-modulated MITA promoter activity. In zebrafish, MITA associates with RIG-I–IPS-1 complexes, but not with that involving MDA5–IPS-1. It is likely that fish MITA is a key scaffolding protein of RIG-I rather than MDA5 (8). However, in some RIG-I-null species, such as chicken and Chinese tree shrew, MITA can interact with MDA5 to mediate the corresponding signaling. Knockdown of MITA inhibits MDA5-mediated IFN-β activation (9, 19). Therefore, experimental evidence needs to be proposed to identify whether MITA is essential for MDA5 signaling pathway in RIG-I-existed species and compare the difference between RIG-I- and MDA5-mediated MITA downstream signals.

The ubiquitin system is responsible for regulating almost all the host cellular processes. Numerous studies have highlighted the important insights into the regulation of protein stability, immune activation, and host–pathogen interplay by protein ubiquitination (1, 54, 55). In the present study, LGP2 overexpression significantly inhibited the K63-linked ubiquitination of full-length RIG-I and MDA5. But for CARDs domain, LGP2 just suppressed the K63-induced ubiquitination in RIG-I, not in MDA5. These results suggest that LGP2 utilizes different mechanisms to modulate the K63-linked ubiquitination of RIG-I and MDA5. Tripartite motif 25 (TRIM25, also called Efp) and Riplet (also called Reul or RNF135) are two important E3 ubiquitin ligases for the K63-linked ubiquitination of RIG-I (56). A study has demonstrated that the K63-linked ubiquitination mediated by TRIM25 in K172 residue in RIG-I CARDs is indispensable for IPS-1 recruitment (57). Meanwhile, K154, K164, and K172 residues of RIG-I CARDs are critical for Riplet-mediated K63-linked ubiquitination and antiviral signal transduction of RIG-I (48). Interestingly, these residues are conserved in grass carp RIG-I (Figure S5A in Supplementary Material). In all probability, LGP2 inhibits the K63-linked ubiquitination of RIG-I through regulating these ubiquitin E3 ligases. For MDA5, even though the present study has demonstrated that MDA5 CARDs can bind to K63 ubiquitin chain, the specific ubiquitin ligase and residue remain unresolved (47). LGP2 restrains the K63-linked ubiquitination of MDA5 independent of CARDs domain.

Unlike the K63-linked ubiquitination, the K48-conjugated ubiquitination chain delivers the substrates to the proteasomes for degradation. A study has demonstrated that RNF125, an E2 ubiquitin-conjugating enzyme, mediates the degradation of RIG-I and signaling impairment of MDA5 *via* the K48-linked ubiquitination (56). Surprisingly, grass carp LGP2 did not promote but inhibited the K48-linked ubiquitination, especially for MDA5, which suggests that LGP2 functions as a "positive" regulator for RIG-I and MDA5. For these seemingly contradictory results, an optimal interpretation may be that: at resting state and early phase of virus invasion, LGP2, on one hand, restrains the K63-linked ubiquitination of RIG-I and MDA5 to inactivate downstream signaling, on the other hand, inhibits the K48-linked ubiquitination to suppress RIG-I and MDA5 degradation to guarantee the basal protein levels, which are crucial for subsequently rapid signal activation. Our subsequent results further supported this hypothesis that upon GCRV infection, the K48-linked ubiquitination of MDA5 induced by LGP2 gradually recovers to normal level in time-dependent manner. As we know, uncontrolled antiviral responses have deleterious effects on the host (52). Therefore, to control excessive immune responses and maintain cellular homeostasis, LGP2 may function as a balancer for RLR signal transduction: under resting state, make immune

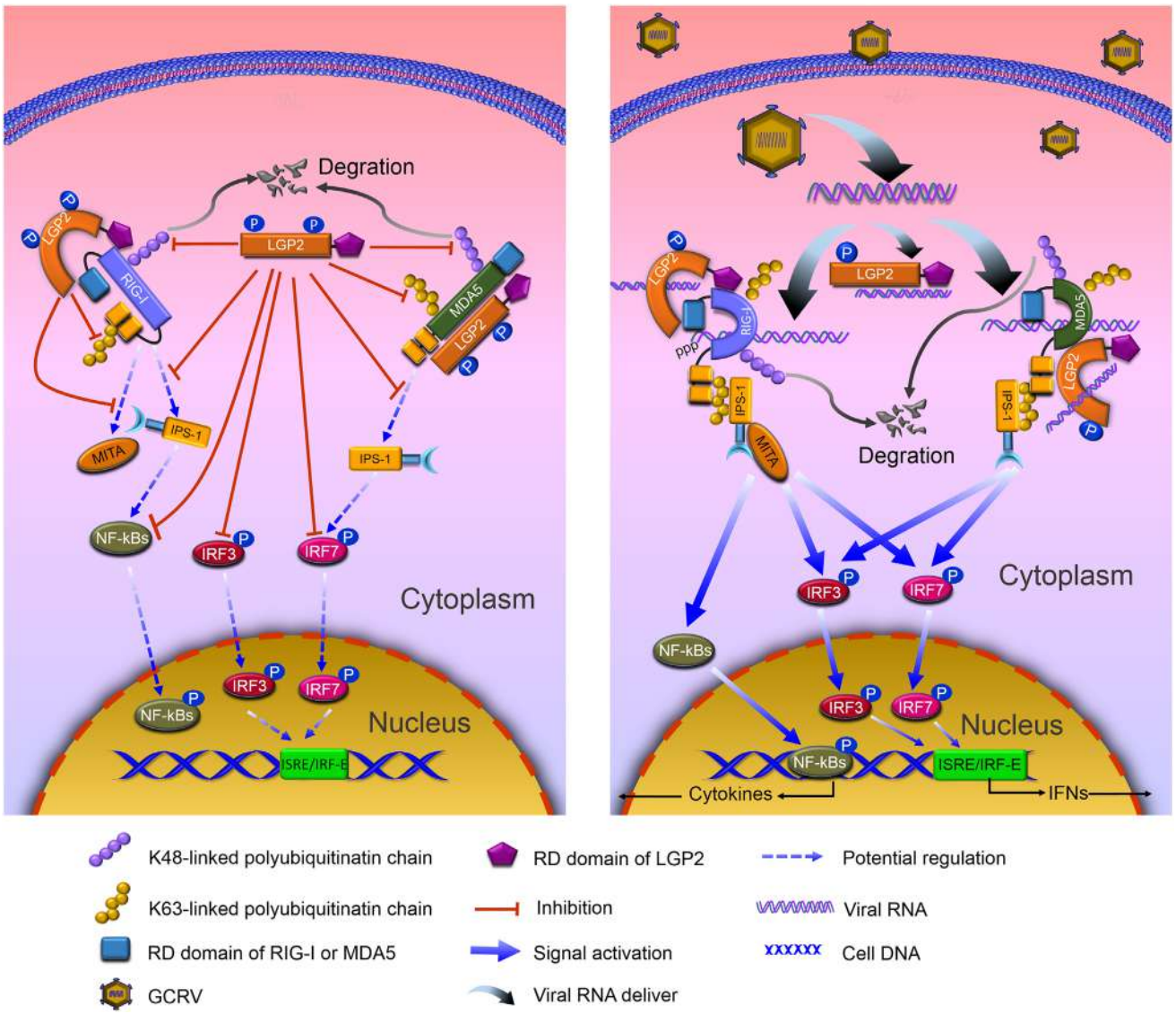

FIGURE 9 | Model of negative role of laboratory of genetics and physiology 2 (LGP2) in modulating retinoic acid-inducible gene I (RIG-I)- and melanoma differentiation-associated gene 5 (MDA5)-mediated antiviral signaling in grass carp. Left: in resting state, LGP2 binds Helicase and repressor domains (RDs) of RIG-I, but leaves the CARDs to form an anti-inhibited state with Helicase, which weakens binding with downstream adaptor IFN-β promoter stimulator 1 (IPS-1). Besides binding to Helicase and CARDs domains, LGP2 competitively interacts with the CARDs of MDA5 that represses MDA5 to form anti-inhibited comformation and interaction with IPS-1. Meanwhile, LGP2 suppresses K63-linked polyubiquitination of RIG-I CARDs and MDA5 Helcase or RD domains. Consequently, signal transductions from RIG-I and MDA5 to IPS-1 and mediator of IRF3 activation (MITA) are inhibited. Furthermore, LGP2 restrains phosphorylation and expressions of IRF3 and IRF7, and the subsequent signals of NF-κBs and IFNs. Concomitantly, LGP2 suppresses the degradation of RIG-I and MDA5 through inhibiting K48-linked polyubiquitination of RIG-I and MDA5, which ensures the basal levels of RIG-I and MDA5 for subsequent antiviral activation. Right: grass carp reovirus (GCRV) infection induces the activation of RLR signals, yet vanishes LGP2-induced inhibiton. dsRNAs derived from GCRV facilitate conformational change of RIG-I and MDA5. Activated RIG-I and MDA5 release the CARDs, which interact with the CARD of IPS-1. IPS-1, then associates with MITA, and activates downstream signals *via* NF-κBs and IRF3/IRF7-IFNs pathways. However, in GCRV invading cells, LGP2 remains to interact with RIG-I and MDA5, disappears Thr-phosphorylation, which may contribute to the derepression for RLR-mediated activation.

system "keep silence" and "activate rapidly" upon virus infection (**Figure 9**). Interactions between LGP2 and RIG-I or MDA5 are independent of GCRV infection. How does LGP2 transform its function from inhibition to derepression? Possible explanation may owe to binding viral RNA, which results in modification change of LGP2. Our study found that LGP2 possesses Thr and Tyr phosphorylation and GCRV infection leads to dephosphorylation of LGP2 Thr residue (Figure S6 in Supplementary Material). Probably, phosphorylation is involved in the regulation of LGP2 function. In addition, sequence of fish LGP2 holds low similarity with mammalian, although LGP2 is structurally conserved in vertebrate (Figure S5B in Supplementary Material). Therefore, fish LGP2 may possess peculiar modifications, which are different from those in mammals.

In conclusion, our findings provide novel insights into the negative role of LGP2 in RLR signal modulation. Grass carp LGP2 directly interacts with RIG-I and MDA5 and suppresses downstream signal activations of IPS-1 and MITA *via* dual regulations of RIG-I and MDA5 by the K48- and K63-linked ubiquitination, then represses expressions and phosphorylation of IRF3 and IRF7. All of these finally inhibit productions of IFNs and NF-κBs. Upon GCRV infection, LGP2, first, undergoes gradual disinhibition and then allows the robust antiviral immune responses (**Figure 9**). However, additionally, experimental proofs are required to illuminate the precise mechanisms of LGP2 in regulation of RIG-I and MDA5 ubiquitination, and the essential role of LGP2 Thr phosphorylation in its functional regulation.

AUTHOR CONTRIBUTIONS

JS and YR conceived and designed the experiments. YR, QW, and CY performed the experiments and analyzed these data. YR and JS wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00352/full#supplementary-material.

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REVIEW
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Type I Interferons and Natural Killer Cell Regulation in Cancer

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Type I interferons (IFNs) are known to mediate antitumor effects against several tumor types and have therefore been commonly used in clinical anticancer treatment. However, how IFN signaling exerts its beneficial effects is only partially understood. The clinically relevant activity of type I IFNs has been mainly attributed to their role in tumor immune surveillance. Different mechanisms have been postulated to explain how type I IFNs stimulate the immune system. On the one hand, they modulate innate immune cell subsets such as natural killer (NK) cells. On the other hand, type I IFNs also influence adaptive immune responses. Here, we review evidence for the impact of type I IFNs on immune surveillance against cancer and highlight the role of NK cells therein.

Keywords: type I interferon, interferon signaling, natural killer cells, tumor surveillance, innate immunity, tumor microenvironment, anticancer therapy

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INTRODUCTION

Type I interferons (IFNs) have been initially identified 60 years ago as antiviral substances (1). They are a family of monomeric cytokines consisting of 14 IFNα subtypes, IFNβ, IFNε, IFNκ, and IFNω. While IFNα and IFNβ have been extensively studied during the past decades, the functions of IFNε, IFNκ, and IFNω remain poorly understood (2, 3). The term type I IFNs in this review therefore refers to the well-characterized forms IFNα and IFNβ, whereas the other type I IFN subtypes have been reviewed elsewhere (4, 5).

Type I IFNs can be secreted by most cell types in the body in response to activation of host pattern recognition receptors such as toll-like receptors (TLRs) and retinoic acid inducible gene-I-like RNA helicases that are triggered by bacterial or viral components (6–8). IFNα and IFNβ signal through the interferon α/β receptor (IFNAR), a heterodimeric transmembrane receptor that is composed of the two subunits IFNAR1 and IFNAR2. Following receptor binding, downstream signals lead to the phosphorylation and translocation of signal transducer and activator of transcription (STAT) factors to the nucleus to drive the expression of IFN-regulated genes (IRGs). For type I IFNs, the main STAT signaling complex is formed by IFN-stimulated gene factor 3 consisting of STAT1, STAT2, and IFN regulatory factor (IRF)-9 (3, 9, 10) (**Figure 1**), however, alternative pathways of IRG stimulation have been described as well (11–13).

It has become well-accepted that functions of IFNα and β reach far beyond antiviral and microbial defense and include the regulation of physiological processes such as cell survival (12), immune cell homeostasis and functions (14), cell cycle, and differentiation (15–17). Many years back, it came as a surprise that constitutively released endogenous IFNα and IFNβ contribute to tissue homeostasis and inhibit malignant cellular transformation (14, 18, 19). Consequently, the finding that type I IFNs have antineoplastic functions stimulated the clinical development of type I IFN anticancer therapies for certain neoplasias.

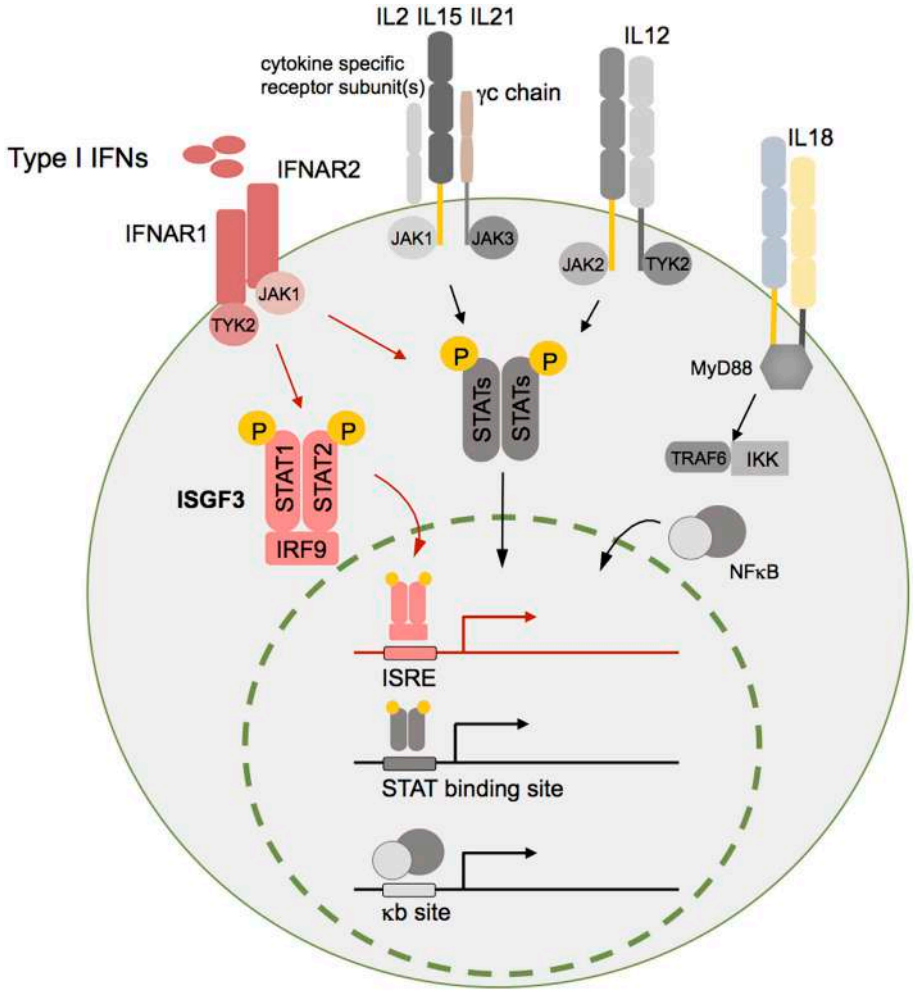

FIGURE 1 | Type I interferons (IFNs) and different other cytokines are essential for natural killer (NK) cell homeostasis and function. Although type I IFNs are in focus of this review, additional cytokine pathways such as interleukin (IL)-2, IL12, IL15, IL18, and IL21 are schematically indicated here as important mediators of NK cell function. Cytokine receptor binding triggers downstream signaling pathways such as the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) or nuclear factor kappa B (NFκB) pathway. The respective activated transcription factor complex—IFN-stimulated gene factor 3 (ISGF3) (type I IFNs), STAT dimers (IL2, IL15, IL21, and IL12), and NFκB (IL18)—translocates into the nucleus and induces target gene transcription leading to expression of genes that are crucial for survival, proliferation, differentiation, and cytotoxic function of NK cells. For reasons of simplicity, IL2R, IL15R, and IL21R were summarized in this graph. The receptor-specific subunit(s) in case of IL2R and IL15R refer to the β- and high-affinity α chain and for IL21R only to one specific subunit. Of note, IL15Rα chain is mainly expressed by other cells such as DCs, which is not displayed here. Abbreviations: ISRE, interferon stimulated response element; γc, common gamma chain; MyD88, myeloid differentiation primary response 88; TRAF6, TNF receptor associated factor 6; IKK, I kappa B kinase; NFκB, nuclear factor kappa B.

However, unraveling molecular key mechanisms underlying the antitumor function of type I IFNs remained very challenging for a long time. Recent advances in the development of genetically engineered mouse models have provided useful tools for investigating these mechanisms and continuously improved our understanding of how IFN signaling interferes with tumor development.

Type I IFNs in Tumor Development

Type I IFNs have been shown to prevent cellular transformation in premalignant cells *in vitro* by sustaining the expression of the tumor suppressor gene p53 (20). Moreover, cell-intrinsic roles for type I IFN signaling in negatively regulating tumor cell proliferation and in triggering apoptosis in different human cancer cell lines have been suggested as well (21). *In vitro* generated findings on direct antineoplastic effects of type I IFNs were substantiated by more recently performed *in vivo* studies, where tissue-specific deletion of IFNAR1 from intestinal epithelial cells increased tumor formation in mice treated with dextran sodium sulfate and the carcinogen azoxymethane to induce colitis (22).

However, a growing number of studies during the past decades provided solid evidence that type I IFNs execute antitumor functions mainly indirectly via stimulating immune cells to rapidly eliminate malignant cells. Owing to the ubiquitous IFNAR expression, type I IFNs have been shown to have crucial

regulatory effects on immune cells in the context of inflammatory and viral diseases (2, 23). Thus, cellular mediators of the innate as well as the adaptive immune response may be regulated by type I IFNs in the protection of the host against malignant diseases. Indeed, an increasing number of studies performed during the past decades have supported the idea of an anticancer immune response analogous to the reaction of the host against pathogens.

A study performed by Dunn and colleagues elegantly demonstrated for the first time an essential role of endogenously produced type I IFNs in a process widely known as tumor immune surveillance (24). Unexpectedly and in contrast to IFNγ, type I IFNs were found in bone marrow transfer experiments to act on host hematopoietic cells and not on the tumor cell itself during the formation of a protective antitumor immune response.

The knowledge on how type I IFNs impact on cells of the innate and adaptive immune system in the context of tumor surveillance has been refined in numerous subsequent studies [reviewed in Ref. (21, 25)]. Some of the earliest studies identified an essential role of type I IFNs, particularly, for the function of host antigen presenting cells (26–28). Early produced type I IFNs act on the level of $CD8\alpha^+$ dendritic cells (DCs) that are required for the successful activation of tumor antigen-specific cytotoxic $CD8^+$ T lymphocytes (CTLs). Based on *in vitro* data, it was demonstrated that type I IFN signaling specifically enhances the ability of $CD8\alpha^+$ DCs to cross-present antigens (27), most likely by promoting survival of DCs and enhancing antigen persistence on the cell surface during cross-presentation (21, 29, 30). Moreover, type I IFNs have been shown to promote DC maturation, differentiation, and migration (28).

Finally, type I IFNs induce the release of interleukin 15 (IL15) by DCs (31), thus promoting the survival of $CD8^+$ memory cells and NK cells (32), which will be discussed in more detail later on. In response to type I IFNs, CTLs have also been shown to acquire full effector functions (26, 33). Also by impacting on other innate immune cell subsets such as neutrophils (34–38), NKT, and γδ T cells (39), type I IFNs exhibit tumor-growth limiting properties.

In addition, type I IFNs promote a protective antitumor response by inhibiting cells of the tolerogenic tumor microenvironment such as myeloid-derived suppressor cells (MDSCs) (40, 41) and regulatory T cells (Tregs) (42–45) that might interfere with the host tumor immune response.

Type I IFNs are released very early during infections (46), thus it was not surprising that they are important regulators specifically of innate immune cell subsets such as DCs and NK cells in anticancer host responses. For NK cells, type I IFNs have already been demonstrated in viral infection to be critical for early responses and are thought to enhance NK cell cytotoxicity and cytokine production (47, 48). However, how type I IFNs regulate NK cell function in the context of tumor development will be outlined in detail in the following sections.

NK CELLS AND TYPE I IFNs

The importance of NK cells in tumor immune surveillance was initially demonstrated via depletion of NK cells from mice rendering them more susceptible to transplanted tumor cells or methylcholanthrene (MCA)-induced sarcomas (49, 50). Furthermore, NK cells have been shown to control the development of B cell lymphomas that arise in mice deficient for perforin, and NK cells were able to recognize and eliminate some of the tumors in the absence of major histocompatibility complex class I (MHC I) (49, 51, 52). Importantly, impaired type I IFN signaling in NK cells leads to a substantial loss of mature NK cell functions that are essential for efficient tumor cell killing. Initially, the effect of type I IFNs on NK cell homeostasis and development has been studied in mice deficient for IFNAR1 or IFNAR2. In the spleens of those mice, NK cell proportions were significantly decreased and mature NK cells of both genotypes expressed lower levels of the surface molecules CD122, CD11b, and Ly49 C + I (53). Thus, IFNAR-deficient NK cells are reduced in numbers and exhibit impaired cytotoxic capacity (24, 53). The cellular and molecular mechanisms of how type I IFN signaling impacts on NK cells and their effector functions are discussed in detail in this and the following section.

NK Cell Development and Type I IFN Signaling

Murine NK cells develop in the bone marrow and at alternative sites such as thymus and liver (54–57). However, the majority of NK cells detected in the periphery is likely to have developed in the bone marrow. There, common lymphoid progenitor cells lose their potential to develop into precursor cells of other lineages and differentiate toward an NK cell-restricted precursor cell (NKP) via intermediate stages (58–60). Based on the expression of cell-specific markers and the acquisition of functional competence, NK cell differentiation is subdivided into distinct developmental stages. Natural killer cell-restricted precursor cells express CD122 that enables the cell to respond to IL15, which is the hallmark cytokine of NK cell lineage specification. Natural killer cell-restricted precursor cells progress to a transitory immature NK cell (iNK) stage that is characterized by the upregulation of the pan-NK cell marker NK1.1. The terminal maturation step from iNK cells to mature NK cells (mNK) involves the upregulation of Ly49 receptor family members together with CD11b and DX5. Following their complete maturation, mNK cells egress from the bone marrow and reside in the blood, spleen, liver, lung, and various other organs, where they continue to mature to tissue-specific and functionally distinct NK cell subsets (54). In the periphery, classical stages of NK cell maturation are described based on the expression of CD11b and killer cell lectin-like receptor subfamily G, member 1 as well as loss of CD27 and TNF-related apoptosis inducing ligand (TRAIL) expression (61–64).

We have previously identified an unexpected role for type I IFNs in NK cell development. In IFNAR-deficient mice, type I IFN signaling was dispensable for NK cell maturation in the bone marrow, but lack of IFNAR1 expression on NK cells significantly abrogated peripheral maturation in the spleen. Of note, late stage deletion of *Ifnar1* in mature NK cells (*Ifnar1*$^{f/f}$ Ncr1-iCre mice) did not interfere with splenic NK cell maturation indicating that type I IFNs are required at an earlier stage or by other cells for

full NK cell maturation in the spleen (65). The impact on NK cell maturation by systemic type I IFNs was also evidenced by Guan and colleagues (66). By generating mixed bone marrow chimeric mice from *Ifnar*$^{-/-}$ and wild-type animals, they showed an intrinsic effect of IFNAR signaling on early NK cell maturation in the bone marrow and also in the liver. In line with results from our study (65), mature NK cell numbers remained unchanged in spleen and blood.

Memory NK Cells and Type I IFNs

Similar to T cells, NK cells as part of the innate immune system are also able to form an immunological memory and terminally differentiate into memory NK cells. Different educational routes have been described that lead to the formation of NK cell memory by antigen-dependent (hapten- and virus-induced) or antigen-independent (cytokine-induced) mechanisms (67, 68).

Sensitization of mice with haptens in the presence of the pro-inflammatory cytokines IL12, IFNγ, and IFNα leads to hapten-specific memory NK cells in the liver (67, 68). Type I IFNs play an important role herein as hepatic NK cells in hapten-sensitized *Ifnar1*$^{-/-}$ (and *Il12*$^{-/-}$, *Ifng*$^{-/-}$) mice failed to induce contact hypersensitivity after adoptive transfer to the challenged host (69).

Interestingly, in a murine cytomegalovirus (MCMV) infection model, type I IFNs have been proposed to play a role in the differentiation of antigen-dependent memory NK cells. Acute MCMV infection stimulates the production of type I IFNs and other pro-inflammatory cytokines (IL12, IL18, IFNγ, IL21) (70, 71). These pro-inflammatory signals drive the expression of the BTB-ZF transcription factor *Zbtb32* (also known as ROG, FAZF, TZFP, PLZP) in antigen-specific NK cells, which is essential for their proliferation and protective function during MCMV infection (72). By using NK cells deficient for IFNAR1 in mixed bone marrow chimeric mice, Madera et al. demonstrated that direct type I IFN signaling in NK cells promotes their optimal activation and function during MCMV infection. However, type I IFNs were shown to be dispensable for the survival of NK cells and NK memory formation (73).

Also in other virus infection models, type I IFNs and NK cells play important roles. In mice, lytic infection in macrophages with gammaherpesvirus was restricted by NK cells independently of type I IFNs, but spreading of virions to the spleen was only possible in the absence of both, type I IFNs and NK cells (74).

Of note, NK cell memory against tumors has not been observed under physiological conditions. Receptors such as NKG2D that are involved in the recognition of tumor cells by NK cells may not be capable of efficiently generating memory. Moreover, it is also conceivable that host-derived factors such as cytokines in addition to specific ligands for activating NK receptors are needed for the generation of memory NK cells against tumors and that these factors are under-represented in the tumor microenvironment (68). Still, memory NK cells bear the potential to be further manipulated to target tumor cells (see section "Type I IFNs and Anticancer Therapies—A Role for NK Cells Therein?").

INTERPLAY OF TYPE I IFNs AND NK CELLS AS PART OF THE TUMOR IMMUNE SURVEILLANCE SYSTEM

Direct Type I IFN Effects on NK Cell Cytotoxicity

As mentioned above, IFNAR1 as well as IFNAR2-deficient NK cells are diminished in numbers and exhibit considerably reduced cytotoxic capacity. These defects finally translate into severely impaired tumor surveillance in *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice, which succumb earlier to carcinogen-induced fibrosarcoma and RMA-S lymphoma (24, 53). These findings were substantiated by the importance of type I IFN signaling on NK cell-mediated *v-Abl* oncogene-driven B cell leukemogenesis (65). In this context, mice with impaired type I IFN signaling (i.e., *Ifnar1*$^{-/-}$ and *Ifnb*$^{-/-}$ mice) had an increased susceptibility to *v-Abl*-induced leukemia/lymphoma and B16F10 melanoma. Increased tumor incidence in these models is linked to defects in NK cell-mediated tumor surveillance, which is dependent on their reduced cytotoxic capacity. Indeed, NK cells derived from *Ifnar1*$^{-/-}$ and *Ifnb*$^{-/-}$ animals display impaired cytotoxic effector function against their target cells *in vitro* (24, 53, 65).

In line with reduced cytotoxicity observed in NK cells lacking either IFNAR1 or IFNβ expression, a similar effect has been reported for NK cells deficient for downstream components of the type I IFN pathway, such as TYK2 (75) or STAT1 (47, 76).

Similar to NK cells derived from IFNAR1-deficient mice, NK cells isolated from mice lacking type I IFN signaling only at the mature NK cell stage (*Ifnar1*$^{f/f}$ Ncr1-iCre mice) (65, 77) display a substantial defect in cytolytic capacity against hematopoietic tumor cell lines (YAC-1, RMA-S) *in vitro*. However, challenging these *Ifnar1*$^{f/f}$ Ncr1-iCre mice with the *v-Abl* oncogene revealed that IFN signaling in mature NK cells is dispensable for the surveillance of leukemia (65). This result might be explainable by the complex cytokine milieu *in vivo* compensating for the obvious defects under IL2-dependent *in vitro* culturing. Previous studies showed that *Ifnar1* deficiency severely curtails NK cell cytotoxicity even in the presence of high doses of IL2 (53). Interestingly, exogenous IL12 stimulation significantly enhances the cytotoxicity of *Ifnar1*$^{-/-}$ and *Stat1*$^{-/-}$ NK cells. Moreover, IL15 stimulation completely restores cytotoxic activity of *Stat1*$^{-/-}$ NK cells *in vitro* (47). These findings clearly show that NK cell defects in *Ifnar1*$^{-/-}$ or *Stat1*$^{-/-}$ animals cannot be overcome by IL2 stimulation, but might be partially compensated by other cytokines *in vivo*. This underscores the importance of other cytokines in NK cell biology such as IL15 and IL21 that are known to increase the cytolytic activity of NK cells *in vivo* (52, 78, 79) (**Figure 1**). An additional possible explanation is that in *Ifnar1*$^{-/-}$ mice other cell types that do require type I IFNs are critically involved in tumor surveillance.

Indirect Type I IFN Effects on NK Cells via Other Immune Cells

Natural killer cells do not possess immediate and permanent effector functions. A process called "priming" is required to induce the establishment of the entire NK cell competence (80,

81). Natural killer cell priming is dominated by type I IFNs, which provide essential signals for DCs to produce IL15, the master cytokine for promoting NK cell development, proliferation, and function (54, 80, 82–84).

The activation of NK cells can be induced by DCs through pathways that require cell–cell contact (NKG2D-MICA and/or MICB) and cytokines such as IFNα, IFNβ, IL2, IL12, IL15, and IL18 (82) (**Figure 1**). Dendritic cell-derived signals elicit both NK-cell-mediated cytolysis as well as cytokine production. Resting and activated DCs are capable of activating NK cells, however, the latter far more potently. The interaction between activated DCs and NK cells has been shown to augment the efficiency of NK cell antitumor effector function in different *in vitro* and *in vivo* models (85, 86). Upon type I IFN signal recognition, DCs produce IL15 and trans-present IL15 to resting NK cells (80). Thus, the interaction with DCs equips NK cells for full effector function. In turn, NK cells are also capable of affecting DC functions through their involvement in DC maturation and DC elimination (82).

More recently, myeloid cells came again into focus as a mechanism was proposed on how cells such as DCs and macrophages could assist NK cell-mediated tumor control (87). Dectin-1 expressed on myeloid cells is critical for NK cell-mediated killing of tumor cells that express high levels of N-glycan structures. Receptor recognition of such tumor cells led to activation of IRF5, an IRF best known for its function in pathogen-induced immunity via activation of MyD88-dependent TLR pathway. This Dectin-1-IRF5 pathway activation in myeloid cells led to activation and efficient tumoricidal function of NK cells. The interaction of myeloid cells and NK cells here may be partially dependent on the expression of the IRF3-dependent NK activating molecule, a membrane-bound protein known to activate NK cells via its homophilic interaction (88).

Apart from effects elicited by type I IFNs on myeloid cells, the following mechanisms could also affect NK cell-mediated tumor surveillance. Although most of those mechanisms have been identified in the context of viral infections, they might be of significant importance in the tumor setting.

The interaction of NK and T cells is also influenced by type I IFN signaling. Type I IFNs keep NK cells from eliminating antigen-activated CTLs by modulating the expression of NK cell receptor ligands (89, 90). In the context of lymphocytic choriomeningitis virus infection, Crouse et al. demonstrated that direct sensing of type I IFNs by T cells prevents them from NK cell-mediated killing by keeping the expression of NCR1 ligands on the CTLs low (89). With the same viral infection setting, Xu et al. showed that the elimination of virus-activated T cells by NK cells was inhibited by type I IFN-induced expression of selected inhibitory NK cell receptor ligands, i.e., classical and nonclassical MHC molecules (MHC I and Qa-1b) (90). An effect of type I IFN signaling on MHC I expression and therefore antigen presentation was reported already earlier, however, the differences in MHC I expression on IFNAR1-deficient cells appeared to be of minor extent (91–93).

Another NK cell surface molecule, TRAIL, was reported to be critical for NK antitumor function in mice and humans (94–96). For example, murine liver NK cells contribute to natural antimetastatic function against TRAIL sensitive tumor cells and constitutive TRAIL expression on these NK cells is IFNγ dependent (96, 97). During viral infection, type I IFNs were also described to enhance antiviral response by NK cell cytotoxicity through induction of TRAIL on NK cells (98).

Another aspect of tumorigenesis influenced by type I IFN signaling is oncogene-induced senescence. In this context, DNA-damage-induced production of type I IFNs enhances cellular senescence (99). In addition, type I IFNs produced by senescent cells indirectly stimulate NKG2D ligand expression on senescent malignant cells, thus promoting the elimination by NK cells (100). However, IFNα has been shown to downregulate the expression of NKG2D ligand H60 in MCA-induced tumors in 129/Sv mice resulting in reduced effectiveness of NK target recognition and NK-dependent killing (101). This indicates that depending on the tumor model, type I IFNs differentially regulate NKG2DL expression.

Finally, ligands for receptors of immune checkpoints such as those of the programmed cell death protein 1 (PD1) family are induced by type I IFNs (102, 103). This immunoregulatory function of type I IFNs is of great relevance and needs to be taken into consideration for the design of clinical anticancer treatments. Recently, targeting of PD1-ligand (PDL1), which is recognized by its inhibitory receptor PD1 expressed on NK cells and other immune cell subsets gained a lot of attention in oncology and will be discussed in more detail later on.

The mechanisms involving NK cells and type I IFN signaling in tumor surveillance are summarized in **Figure 2**.

TYPE I IFNs, NK CELLS, AND METASTASIS

Metastasis as the dreadful consequence of tumorigenesis has recently been shown to be controlled by antitumor immune responses. In this context, NK and $CD8^+$ T cells as the main cellular mediators of tumor immune surveillance have been described to be capable of restricting metastatic tumor growth. Therefore, depletion of $CD8^+$ T cells or NK cells increased metastasis formation in a breast cancer mouse model without affecting primary tumor growth (104). One mechanism proposed for the metastasis surveillance function of NK cells relies on the inhibition of the MERTK (also known as TAM; TYR3, AXL, and MER) family tyrosine kinase receptors that suppress NK cell activation (105, 106). Of note, the protective function of NK cells against metastases can be also linked to and is partially dependent on type I IFN signaling. In a syngeneic mouse model of mammary tumor metastasis using 4T1.2 cells, Bidwell and coworkers identified a number of IRF7 target genes that are suppressed in bone metastases (104). Consequently, metastasis formation in spontaneous (MMTV-PyMT) and orthotopic mammary tumorigenesis models was accelerated in mice deficient for IFNAR1, NK cell, or $CD8^+$ T cell responses (104, 107). Conversely, enforced expression of IRF7 in tumor cells or treatment with type I IFNs enhanced the immune activity and suppressed bone metastasis, thus prolonging survival of the diseased mice. Of note, depletion of both $CD8^+$ T and NK cells significantly accelerated metastasis and shortened survival time in mice harboring 4T1.2 tumors

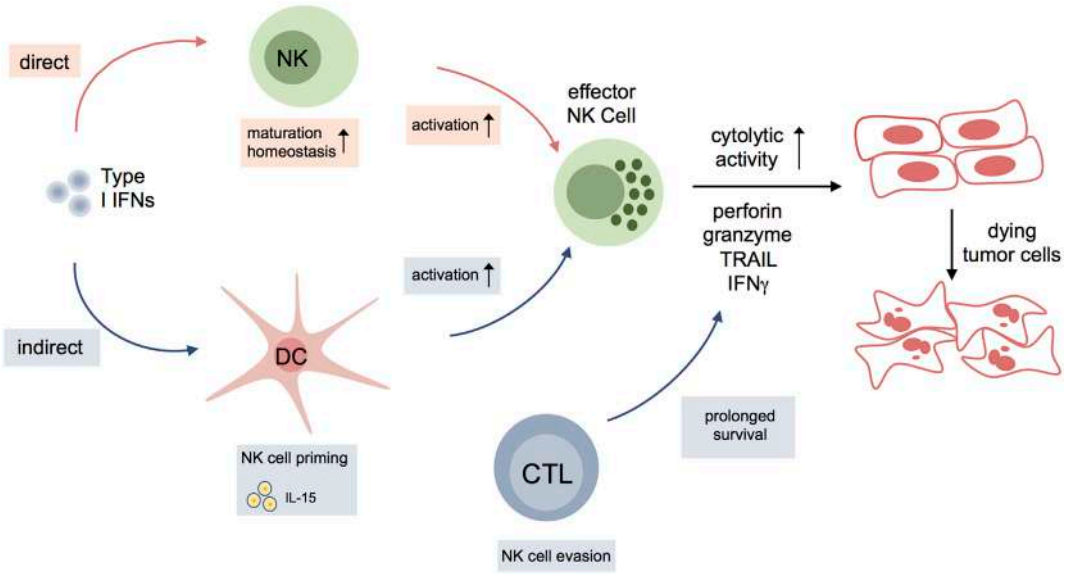

FIGURE 2 | Interplay of type I interferons (IFNs) and natural killer (NK) cell activation during antitumor response. Type I IFNs either impact on maturation, homeostasis, and activation of NK cells, or indirectly influence NK cells to kill tumor cells via other immune cells or cells of the tumor microenvironment. Dendritic cells (DCs), in particular, are essential for NK cell priming via production of IL15. Another indirect effect of type I IFNs on NK cell function in cancer might result from modulation of surface molecules on $CD8^+$ cytotoxic T lymphocytes (CTLs) [NCR1 ligands; classical and nonclassical major histocompatibility complex class I (MHC I)] leading to evasion of CTLs from NK cell-mediated elimination.

ectopically expressing IRF7. This indicates that IRF7-induced and type I IFN-dependent inhibition of bone metastasis was mediated by $CD8^+$ T and NK cells (104). In line with the data obtained from metastasis studies in mice, loss of IRF7-associated gene signature in primary tumors of breast cancer patients predicted an increased risk of bone metastasis and also additional studies suggest a suppressive role for type I IFN signaling on breast cancer progression (25).

However, tumor cells use different immune evasive strategies to survive at distinct metastatic sites. The recruitment of immunosuppressive cells is one major mechanism to overcome the immune surveillance system (108). For example, systemic factors from hypoxic breast cancer cells increase myeloid $CD11b^+$ cell accumulation and reduce the cytotoxic functions of NK cells in the premetastatic lung (109). Myeloid cells, especially MDSCs, have the capacity to suppress immune responses, thus it is conceivable that recruited myeloid cells establish a premetastatic immune-suppressive niche to promote tumor metastasis. Moreover, platelet activation and the resulting fibrin clot formation support survival of tumor cells that are nested at metastatic sites by protecting them from NK cells (108, 110).

In mice engrafted with mammary tumor cell lines, type I IFN treatment has been shown to reduce metastasis to bone. Interestingly, while MDSC accumulation was substantially decreased, there was an increase in numbers of NK cells present in the bone marrow of these mice (104). Hence, the authors proposed that type I IFNs specifically inhibit bone metastases of mammary cancer by a selective modulation of MDSCs and NK effector cells in the bone marrow (104).

A consecutive study demonstrated that endogenous type I IFN signaling in the host hematopoietic system is indispensable for the responsiveness of circulating NK cells and therefore essential for metastasis-free survival. Consistently, *in vivo* stimulated NK cells derived from *Ifnar1*$^{-/-}$ mice but not from wild-type counterparts failed to eliminate the 4T1 and 66cl4 mammary tumor cell lines *in vitro* (107).

In summary, these studies clearly highlight an essential role for IFN signaling and NK cells during metastasis formation and could pave the way for type I IFNs for new therapeutic means in metastatic cancer.

TYPE I IFNs AND ANTICANCER THERAPIES—A ROLE FOR NK CELLS THEREIN?

As outlined above, ample evidence substantiates the importance of type I IFN signaling in NK cell-mediated tumor surveillance. Interferons mainly function by modulating the immune system rather than executing direct anticancer effects. In the clinics, type I IFNs have been used for decades as anticancer therapy, however, the exact mechanism of action of type I IFNs has not been clarified yet (111, 112). IFNα has been and is still used mainly for the treatment of hematopoietic neoplasms. Especially, before the advent and breakthrough of the BCR-ABL inhibitor imatinib as therapy for chronic myeloid leukemia (CML), IFNα was the treatment of choice for patients not suitable for bone marrow transplantation. Interestingly, in chronic myeloproliferative neoplasms the positive effect of IFNα coincided with a substantially higher frequency of circulating $CD56^{bright}$ NK cells that produced increased levels of IFNγ (113). Recently, IFNα has gained attention for further use as

therapeutic option in CML, preferably in combination with imatinib or its next generation inhibitors (114).

Trials with IFN therapies in solid malignancies have met with varied success. However, besides virus-related cancers at least in melanoma as one type of solid tumors, IFNα is clinically used (21). In high-risk melanoma patients, high-dose IFNα treatment leads to an extension of relapse-free survival and is therefore considered a valid therapeutic option. Interestingly, IFN therapy is more effective at targeting disseminated cancer cells and minimal residual disease before they form large proliferative metastases, emphasizing again that promotion of antitumor immunity rather than direct antiproliferative effects is the predominant mechanism of action (25).

Data obtained mainly from tumor studies in mice strongly suggest that the success of conventional chemotherapeutics (such as anthracyclines, cyclophosphamide), targeted anticancer agents, radiotherapy, and immunotherapy depends on type I IFN signaling (21, 115). Under certain circumstances, this mode of action of IFN signaling involves NK cells. For example, some immunogenic chemotherapeutics lead to the activation of TLR3 in malignant cells by cancer-cell derived RNA which results in type I IFN production. Subsequently, IRGs such as CXC-chemokine ligand 10 (CXCL10) are expressed, which in turn are crucial for recruitment in NK cell-mediated tumor control (116, 117).

The concept of tumor immune surveillance has triggered an increasing interest in immunomodulatory treatment strategies. However, immune-activating therapies are likely to induce the expression of immunosuppressive ligands and receptors such as PDL1, PD1, and CTLA4. Since type I IFNs have immunostimulatory functions, they can promote the upregulation of such surface molecules (102, 118), thus preventing prolonged antitumor immune responses. In this case, a sustained therapeutic antitumor response could be achieved by the combination of type I IFN therapy with other therapeutic means targeting the PD1–PDL1 axis to block secondary immune suppression. Programmed cell death protein 1- and CTLA4-targeted therapeutics have been proven in some cancers to significantly prolong survival of the patients. Combining these agents with type I IFNs could be a suitable strategy to overcome immunosuppression and raise patient responsiveness. Programmed cell death protein 1 is well documented in the context of T-cell responses and has recently been shown to be upregulated on NK cells, which leads to downregulation of anticancer function (110, 119, 120).

On the contrary, IFN signaling seems to be also important for the success of checkpoint immunotherapy, which is illustrated by a recent study on late relapses of PD1 blockade treatment in metastatic melanoma. Here, a loss-of-function mutation in the Janus kinase 1 has been identified in one patient, suggesting that disruption of type I and type II IFN signaling might be involved in preventing the success of checkpoint immunotherapies (121). If this turns out to be a more frequent observation, the combination of type I IFNs with checkpoint inhibitors would be desirable for an improved treatment outcome. Furthermore, in anticancer virotherapy, type I IFNs play a key role, as intratumoral injection of the oncolytic Newcastle disease virus combined with systemic CTLA4 blockade leads to regression of murine B16 melanomas. Interestingly, this effect has been reported to be dependent on CTLs, NK cells, and IFNAR signaling (122).

As outlined above, there are a number of reasons pleading for type I IFNs as tools in anticancer treatment. However, one big disadvantage are dose-limiting side effects, including influenza-like symptoms (fatigue, fever, headache, and muscle aches), nausea, anorexia, dizziness, depression, and leukopenia. To avoid these side effects of IFN therapy, strategies are now being developed to deliver type I IFNs directly to the tumor microenvironment (21). Different types of cells can be manipulated to express type I IFNs to augment their own antitumor activity or to promote the activity of other immune effector cells of the host. This has been also assessed with NK cells: a genetically engineered NK cell line expressing human IFNα displayed improved cytotoxicity functions against hepatocellular carcinoma cells *in vitro*, as well as in xenograft tumor models (123). Moreover, mesenchymal stem cells modified to express mouse IFNα efficiently decreased the growth of murine B16 melanomas *in vivo*, an effect that was shown to be dependent on NK and T cells (90). However, translating this strategy to the clinics might be difficult and other means, such as the usage of modulators of specific immune cell subtypes and/or pathways might be preferred.

As described above, memory NK cells against tumors have not been observed yet, but would be highly appreciable if those could be generated *in vitro* by different manipulations such as transduction of proliferating NK cells with chimeric antigen receptors, or enhanced antibody dependent cellular cytotoxicity (ADCC) using newly identified human FcεRIγ-deficient adaptive NK cells (68). As antigen-dependent memory NK cell formation relies also on type I IFN signaling, this could be another strategy for type I IFNs and NK cells in cancer control.

The studies on type I IFNs and breast cancer metastasis (see section "Type I IFNs, NK cells and metastasis") may provide a rationale for targeting the endogenous type I IFN pathway as an antimetastatic strategy. As IFN signaling modulates the tumor immune response, targeting type I IFNs to a specific cellular compartment of the tumor mass may mediate optimal therapeutic effects for some cancer types. Type I IFN signaling within tumors is essential for both natural and therapy-induced immune surveillance. Thus, downstream effectors of type I IFN signaling would be suitable candidates for further investigation as prognostic and predictive biomarkers in cancer diagnosis and progression (21).

The high potential and importance of type I IFNs and NK cells in cancer is also illustrated by a glimpse on current clinical trials. Searching for IFNα, NK cells, and cancer at ClinicalTrials.gov resulted in 16 studies, half of them dealing with type I IFNs and NK cells for cancer patients (https://clinicaltrials.gov; November 2016). Already in 1997, Nagler et al. combined type I IFNs with NK cell-stimulating molecules such as IL2 and indeed showed increased survival in lymphoma patients after stem cell transplantation (124). Recently, a more specific approach using adoptive transfer of autologous or allogeneic NK cells is frequently tested for cancer treatment (125). Here, even synergistic or additive effects of type I IFNs applied in this context could be imagined.

The combination of type I IFNs with other immunostimulatory agents such as immune checkpoint blockers, cytokines, or other inhibitors that target different immunosuppressive circuits is likely to result in optimal NK cell anticancer function and tumor control.

CONCLUSION AND PERSPECTIVES

Type I IFNs are essential in antitumor control and execute their function predominantly by modulating the activity of other immune cells. Although type I IFNs affect various immune cell subsets, the impact of type I IFNs on NK cells is especially crucial for efficient tumor immune surveillance. Type I IFNs not only positively regulate NK cell maturation and memory, but also NK cell priming and NK cell-mediated tumor surveillance by various mechanisms. Detailed knowledge about underlying mechanisms of immunoregulatory cell recruitment and their suppressive functions in primary tumors and at metastatic sites should lead to more effective immunotherapies.

Thus, therapeutic approaches will need to include the evaluation of immune cell profiles in individual cancers, so that drug targeting can be precisely tailored to maximize the response. In addition, tumor-type specific treatments of type I IFNs and other therapeutic concepts might extend the pharmacological armament to combat diverse cancer types.

AUTHOR CONTRIBUTIONS

LM, PA, and DS have written the review article. LM has compiled graphics for this review article.

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REVIEW
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The Protective Role of Type I Interferons in the Gastrointestinal Tract

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The immune system of the gastrointestinal (GI) tract manages the significant task of recognizing and eliminating pathogens while maintaining tolerance of commensal bacteria. Dysregulation of this delicate balance can be detrimental, resulting in severe inflammation, intestinal injury, and cancer. Therefore, mechanisms to relay important signals regulating cell growth and immune reactivity must be in place to support GI homeostasis. Type I interferons (IFN-I) are a family of pleiotropic cytokines, which exert a wide range of biological effects including promotion of both pro- and anti-inflammatory activities. Using animal models of colitis, investigations into the regulation of intestinal epithelium inflammation highlight the role of IFN-I signaling during fine modulation of the immune system. The intestinal epithelium of the gut guides the immune system to differentiate between commensal and pathogenic microbiota, which relies on intimate links with the IFN-I signal-transduction pathway. The current paradigm depicts an IFN-I-induced anti-proliferative state in the intestinal epithelium enabling cell differentiation, cell maturation, and proper intestinal barrier function, strongly supporting its role in maintaining baseline immune activity and clearance of damaged epithelia or pathogens. In this review, we will highlight the importance of IFN-I in intestinal homeostasis by discussing its function in inflammation, immunity, and cancer.

Keywords: interferon, intestine, inflammation, microbiome, epithelium

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INTRODUCTION

The gastrointestinal (GI) tract has the greatest mucosal surface area of any organ system shared with the environment, interacting with a wide array of microbes and chemical irritants. These interactions with colonizing bacteria, especially early in life, are fundamental in developing proper gut health (1). The intestines of newborns are initially sterile (2), but become colonized immediately after birth, upon exposure to their new environment. The establishment of healthy intestinal microbiota can be hindered due to lack of exposure to commensal bacteria or upon treatment with antibiotic medications (3). This appears to be very important as there is mounting evidence that resident microbiota play an important role in shaping the function of the GI tract. The initiation and progression of human inflammatory bowel diseases (IBDs) are reliant on the dysregulation of complex interactions among genetic, environmental, and immune factors, as well as physical barriers within the intestinal mucosa. The physical barrier between the external environment and internal tissue is the first line of defense against microbial pathogens, toxins, and other environmental factors (4). This protective barrier is provided by the inner lining of the intestine, a single-cell layer of intestinal epithelial cells (IECs), and their specialized subtypes (e.g., Paneth, goblet, or enteroendocrine cells) (5). IECs serve an essential role as regulators of mucosal immune responses (6) and as cohabitants within

the intestinal environs, which can be colonized by commensal or pathogenic bacteria, fungi, and viruses (7). Paneth cells, in particular, play a vital role in gut homeostasis (8–10) at least *via* expression of IFN-I and interferon-stimulated genes (ISGs) (11, 12). Ingested antigens and constituents of commensal bacteria are constantly testing the immune system of the gut. Responses to antigens can be either positive or negative, inducing an antigen-specific state of immunity (13). Cytokines like IFN-I act as initial signaling mechanisms within this innate immune system determining the durability and specificity of the response. Together, a series of direct responses and feedback loops are in place for maintaining gut homeostasis—preventing tissue damage, hyperplasia, malignancy, and ultimately cancer.

TYPE I INTERFERONS (IFN-I)

The innate immune system is a remarkable network that has evolved to protect the host against disease. It has the ability to detect a wide range of microbial markers and, in response, rapidly activate a number of inflammatory and antimicrobial pathways. Part of this sophisticated system involves the family of IFN-I (IFN-α or IFN-β). These immunomodulatory cytokines are broadly expressed as α-helical cytokines transcribed from 13 homologous IFN-α genes (IFN-α1 and -α13 are the same) and a single IFN-β gene (14). They play a critical role as first line of defense by promoting and shaping antiviral and antibacterial immunity. Constitutive, baseline expression of IFN-I is very low in the intestines, typical of most tissues (12, 15–18). IFN induction is a rapid event that can be triggered in response to viral attack (*via* recognition of cytosolic viral double-stranded RNA, 5′triphosphate single-stranded RNA, or viral DNA) and bacterial infections (*via* recognition of lipopolysaccharide, lipoprotein, or flagellin, for example) (19). Each response is activated by specific pattern-recognition receptors (PRRs), like RIG-like helicases and toll-like receptors (TLRs), expressed by different cell types (20). Secreted IFN-I then activates autocrine and paracrine signaling cascades *via* the heterodimeric IFN-I receptor complex (14). IFN-I bind to and activate the cognate cell surface receptor consisting of the IFNAR1 and IFNAR2 chains, which induce downstream signaling *via* tyrosine phosphorylation of JAK kinases (JAK1 and TYK2). Activated JAKs then phosphorylate the transcription factors STAT1 and STAT2 in the cytoplasm that in association with IRF9 from the heterotrimeric complex ISGF3. ISGF3 translocates to the nucleus and binds to the promoters of IFN target genes and activates the transcription of many ISGs (21). These ISGs drive immunomodulatory antiviral (22), antiproliferative (23), antibacterial (24), and antitumor actions (15) throughout the body, including the GI tract (18).

IFN-I AS ANTI-INFLAMMATORY IMMUNOMODULATORS

Type I interferons not only function as signaling molecules of innate immunity but also promote the activation of adaptive immunity. It is well-established that systemic IFN-I can influence $CD4^+$ T cell differentiation and function *via* their effects on dendritic cells (DCs). IFN-I drive DC activation and maturation (25), MHC II expression, and production of IL-12 (26, 27), to augment T helper (Th)1 cell responses. In addition, IFN-I can act directly on T cells to inhibit their expansion from lymph nodes, thus promoting DC–T cell interactions (28). Several studies also show that IFN-I enhance natural killer (NK), B, and $CD8^+$ T cell activity (29, 30). By contrast, other studies present a different side of IFN-I—as key factors in the attenuation of an active immune response. Primarily, IFN-I increase the susceptibility of lymphocytes and macrophages to apoptosis (24, 31–34). IFN-I also inhibit the expression of IL-8, a chemotactic cytokine responsible for recruiting neutrophils and leukocytes to areas of inflammation (35, 36), and of IL-17, *via* inhibition of Th17 differentiation (37, 38). IFN-I antagonize the effects of local IL-17 by downregulating the expression of IL-1β, IL-23, and osteopontin, and by inducing the production of the anti-inflammatory cytokine IL-27 in DCs (38, 39). Induction of IFN-I in macrophages by bacterial infection reduces IL-17A/F variant expression, followed by a decrease in IL-17A(+) γδ T cells, further highlighting the role of IFN-I on T cell populations during infection (40). Further, IFN-I can inhibit the secretion of IL-1β, both by inhibiting production of pro-IL-1β and blocking pro-IL-1β cleavage to mature IL-1β *via* impeding inflammasome activation (41). To suppress inflammation, IFN-I also induce the secretion of anti-inflammatory cytokines (e.g., IL-10, IL-27, and IL-1RA) from phagocytes *via* expression of inhibitory feedback SOCS and PIAS proteins in T cells and phagocytes (42–44). Additionally, IFN-I suppress IFN-γ-induced MHC II expression by downregulating IFNGR1 levels as a negative feedback mechanism (45, 46), and high levels of IFN-I can inhibit IL-12 production during certain viral infections (47). IFN-I also inhibit inflammatory responses by inducing tristetraprolin, a strong suppressor of TNF-α and IFN-γ (48, 49).

Alterations to the IFNAR1 gene have been linked to susceptibility for IBD and changes to microbiome populations (50, 51), thus providing supporting evidence that IFN-I contribute to immune defenses against conditionally pathogenic microbiota and intestinal inflammation (52). In a T cell adoptive transfer model of colitis, signaling through host hematopoietic cell Ifnar1 was necessary to deter development of colitis. *Ifnar1*$^{-/-}$-recipient mice developed severe colitis, compared with *Ifnar1*$^{+/+}$ mice, when inoculated with $CD4^+$ T cells from a WT mouse (18). Phagocytes collected from the colonic lamina propria (LP) of *Ifnar1*$^{-/-}$ mice produced less IL-10, IL-1RA, and IL-27 than did cells from WT mice (18) demonstrating an important role for IFN-I signaling driving the expression of anti-inflammatory cytokines by gut phagocytes and maintenance of intestinal T cell homeostasis. Oral administration of the colonic irritant, dextran sulfate sodium (DSS) is another well-established model of acute colitis as it produces submucosal inflammation and ulceration in the gut thereby providing a "leaky" epithelial cell-lining ideal for translocation of luminal microbiota into the LP (53). *Ifnar1*$^{-/-}$ mice are found more susceptible to DSS-induced colitis pointing to conventional DCs as critical players in attenuating inflammation (16, 18, 54, 55). However, a later study found that deletion of *Ifnar1* in $LysM^+$ myeloid cells, but not in conventional DCs exacerbated DSS-induced colitis (56). These differing results could be attributed to the mouse model employed. In the first study, Abe et al. used transgenic DTR mice with intact Ifnar1 to deplete $CD11c^+$ DCs

via administration of diphtheria toxin. By contrast, Rauch et al. used mice with conditional deletion of Ifnar1 in DCs or in myeloid cells. Nevertheless, both studies agree on the protective effect of IFN-I by suppressing IL-1 production during inflammation of the gut. Altogether, IFN-I activate and orchestrate different programs to keep inflammation under control.

IFN-I ARE INSTRUMENTAL IN MAINTAINING HOMEOSTASIS IN THE GUT

Balance of the microbiome within the small and large intestine is important for not only maintenance of the intestinal epithelium, proper digestion, and nutrient uptake but is also strongly tied to immunity, inflammation, and cancer risk (57, 58). Both pro- and anti-inflammatory cytokines are chief among these immunomodulatory agents, including IFN-I, in regulating the growth and renewal of IECs (59–61). IFN-I are constitutively expressed in the intestines by LP CD11c$^+$ DCs (16, 18, 62). The LP is the layer of connective tissue underneath the intestinal epithelium, enriched in immune cells. In the colon, CD11c$^+$ DCs cells express mRNA for IFN-α5-, IFN-α9-, and IFN-I/ISGS3-induced genes thus indicative of active IFN-I production and signaling. Proper regulation of epithelial cell turnover in the intestinal lining is important for balance between replacement of damaged/sloughed cells and hyperplasia, which leads to pre-cancerous polyp formation (61, 63). Secretion of IFN-α has been shown as an important regulator of epithelial apoptosis. IFN-α administration prevented epithelial cell apoptosis in an *Escherichia coli*-induced mouse model of disease (64). Basolateral IFN-α also polarized monolayers of IECs, protected these cells against apoptosis, and promoted disruption of epithelial tight junctions (54). Moreover, IFN-α can induce the expression of GBP-1 (64), shown to prevent apoptosis, and promote intestinal epithelial barrier integrity (65). Prevention of apoptosis by IFN-α-induced GBP-1 subsequently inhibited endothelial cell angiogenesis (66, 67). In a study conducted in mice with deleted *Ifnar1* in the intestines, loss of IFN-I signaling increased the number of Paneth cells and hyperproliferation of epithelial cells with no signs of spontaneous inflammation or enhanced susceptibility to DSS, when compared to littermate controls (50, 51). Most recently, Fuchs et al. reported that increased protein levels of IFNAR1 *in vivo* [*via* deletion in the intestine of casein kinase 1α (CK1α), which controls the ubiquitination and degradation of both β-catenin and the IFNAR1] led to an increased ISG transcriptional signature (52) highlighting baseline IFN-I signaling in the intestinal epithelium. Deletion of CK1α in the intestines of *Ifnar1*$^{-/-}$ mice resulted in decreased levels of p21, inhibited p53 activation, and unrestricted IEC proliferation resulting in loss of gut barrier function and prompt animal death. Hence, IFN-I enable enhanced maturation, differentiation, and establishment of the cohesive epithelial barrier in the gut highlighting the contribution of IFN-I signaling to the control of IEC proliferation and function. As such, IFN-I are vital in maintenance of the host-microbiota equilibrium and constraining IEC proliferation and viability.

The microbiome in the gut plays an important role in the pathogenesis of IBD. This is evidenced by a variety of animal models in which development of intestinal inflammation is completely abolished under germ-free conditions (68). In healthy individuals, the gut microenvironment exists in a continuous state of controlled inflammation, despite the presence of potent antigen-presenting cells, like DCs. DCs are important for controlling T cell-mediated antigen response (69) and are the major source of TLR-driven IFN-I production (70). Conventional DCs have been attributed with inhibition of DSS-induced colitis, in part, to IFN-I production (14, 55). IFN-I regulated colonic recruitment of neutrophils and monocytes, as well as activation of pro-inflammatory macrophages (55). Additionally, *Ifnar1* loss in myeloid cells promoted colitis *via* increased IL-1 production (56), a pro-inflammatory cytokine produced by activated macrophages (71). Interestingly, in celiac disease (an IBD driven by strong T cell activation toward gluten), the role of IFN-I appears reversed. In humans, mucosal DC populations are increased in celiac disease patients (72). Activated mature DCs from these patients maintained higher IFN-α transcripts, as well as for IL-18 and IL-23, two cytokines responsible for Th1 polarization and subsequent IFN-γ production. Furthermore, IFN-α blockade inhibited IFN-γ transcripts in *ex vivo*-organ culture of celiac biopsy specimens challenged with gluten (72). Yet in mouse models of colitis pretreated with synthetic bacterial DNA, increased anti-inflammatory IL-10 and decreased IFN-γ production were reported (73). Along these same lines, a human ulcerative colitis (UC) study showed a correlation between IFN-I response and Th17 differentiation and suppression of IL-17 production (74). Th17 cells are central effectors that produce pro-inflammatory cytokines, particularly IL-17 in the gut (75, 76). IL-17 then induces the secretion of chemokines and antimicrobial peptides to create a mucosal barrier to eliminate pathogens; however, excessive IL-17 production exacerbates inflammation thereby promoting pathogen colonization (77).

T regulatory (Treg) cells play a central role in suppressing the development of intestinal inflammation and IBD (78–80). Tregs maintain intestinal homeostasis under conditions of continuous challenge with inflammatory microbes. Induction of Treg populations by recombinant bacterial DNA analogs was TGF-β- and IFN-I-dependent in a mouse model of IBD (81). Maintenance of the Treg population in the gut is mediated by IFN-I signaling driving the expression of Foxp3 in colonic Tregs (82). Continuous Foxp3 expression is necessary for the development and regulatory function of Tregs (83, 84). IFN-I limit inflammation by eliciting production of the regulatory cytokine IL-10 or by enhancing the activity of Treg cells (79, 85). Additionally, apoptotic resident intestinal DCs help regulate the populations of Tregs in the intestine *via* production of IFN-β (86). In IBD patients, Th1 and Th17 constitute a major driving force in the disease process in the inflamed mucosa characterized by high surface expression of activated CD69 (87, 88). Expression of CD69 is strongly induced by IFN-I (28). Several studies in mice indicate a role of CD69 in the regulation of arthritis (89), asthma (90), myocarditis (91), pathogen clearance (92), and tumor immunity (93). Commensal bacteria in the intestinal tract are shown to induce CD69 expression in CD4$^+$ T cells. Secretion of the regulatory cytokine TGF-β1 by CD4$^+$ T cells decreased, whereas production of the pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-21)

increased, upon deletion of CD69. $CD69^{-/-}$ cells showed impaired IFN-β1 induction by TLR3 ligand polyI:C. $CD4^+$ T cells lacking CD69 expression were hindered in their ability to mature into Tregs ($Foxp3^+$) leading to accelerated colitis (94).

IFN-I CONFER PROTECTION AGAINST COLITIS

Toll-like receptors play an important role in innate immunity by recognizing structurally conserved bacterial and viral components. TLRs are important transmembrane-signaling PRRs involved in inducing inflammation and are pivotal in the establishment of adaptive immunity. In addition to innate immune cells such as macrophages and DCs, IECs express a spectrum of TLRs (95). TLR signaling can induce strong production of several inflammatory cytokines, including IFN-I (96). TLR2 and TLR4 recognize bacterial cell wall components at the cell surface, while TLR3, TLR7, and TLR9 recognize bacterial or viral nucleic acids in endosomes after phagocytosis of bacteria or viruses (97). Activation of DCs *via* TLRs contributes to both rapid anti-pathogen responses and maintenance of homeostatic protective immunity (98). This is partly mediated by the direct production of cytokines necessary for the development of downstream humoral and cell-mediated immunity. Imiquimod, a TLR7 agonist, has been shown to ameliorate DSS-induced acute colitis by inducing the expression of IFN-I in the colonic mucosa (99). When administered as a preventive measure, ligands for TLR9 (CpG) or TLR3 (polyI:C) also induced IFN-I and lessened disease severity of DSS-induced colitis (54, 100). Administration of neutralizing antibodies against IFN-I also impeded these downstream anti-inflammatory effects *via* TLR9, thus highlighting the importance of IFN-I signaling in maintaining intestinal homeostasis and providing avenues for future therapeutics (54, 101). The activation of TLR9 by CpG dinucleotides initiates a cascade of innate and adaptive immune responses, at least partially mediated by secretion of IFN-I and IFN-γ, that results in cell-mediated Th1 and humoral immune reactions (102). The TLR9 signaling pathway can induce the production of inflammatory cytokines through nuclear factor κB and interferon regulatory factor (IRF)-5, and IFN-I through IRF7 (96). In other studies, comparison of transcriptome profiles from gnotobiotic mice, which lack commensal bacteria that constitute the microbiome, to three bacterial colonization models—specific pathogen-free mice, ex-germ-free mice with bacterial reconstitution at the time of delivery, and ex-germ-free mice with bacterial reconstitution at 5 weeks of age—showed that TLR-driven expression of *Irf3*, a crucial rate-limiting transcription factor in the induction of IFN-I, was essential for normal development of the host immune system (103). Commensal bacteria triggered the production of IFN-β *via* recognition of dsRNA by TLR3, which in turn protected mice from experimental colitis (104).

Inflammatory bowel disease is a group of intestinal chronic inflammatory conditions mainly UC and Crohn's disease (CD) that affects part or the entire GI tract. The precise cause is unknown, but evidence overwhelmingly suggests symptoms arise from either pathogenic or commensal intestinal bacteria triggering an abnormal immune response. IFN-α-secreting DCs in gut-associated lymphoid tissues (GALTs) regulate differentiation of Tregs (105). GALTs are primary locations of host encounter with exogenous antigens and pathogens. Interaction of GALT with microbiota regulates both the size and duration of systemic immune responses (106, 107). The commensal microflora constituting the microbiome of the intestinal tract is strictly entwined in the well-being of the host. In particular, the balance of bacterial populations is directly related to IBD, though additional host-driven genetic predispositions are also suspected. Genome-wide association studies have implicated the locus containing IFNAR1 as a genetic risk factor for developing human IBD (50). In patients with IBD, chronic inflammation is a major risk factor for the development of GI malignancies (108). Patients suffering from IBD typically use non-specific medications to manage the symptoms and include steroids, 5-aminosalacylic acid derivative, immunosuppressants, or antibodies against TNF-α (109). Systemic administration of IFN-I to treat IBD patients has been evaluated and the results vary in suppressing disease burden (110–113). UC is associated with increased expression of IL-13 in NK T cells from the mucosa of the GI tract (114–116). IFN-I have been shown to deter IL-4/IL-13 transcription and secretion (117) by, as well as blocking of signaling in, human $CD4^+$ T cells (118). In one small study, the majority of UC patients treated systemically with interferon-β-a1 showed reduced disease burden using rectal bleeding as a clinical measure. In the responder group, the clinical effect of IFN-I therapy correlated with decreased IL-13 production by LP mononuclear cells. By contrast, the non-responders had significantly higher production of IL-17 and IL-6 compared to responders (119). In cases where IFN-I therapy exacerbated the disease, parallel diseases in the patient may have complicated the correlated observations (111). Initial studies in an experimental model of colitis depicted the benefits of IFN-I in regulating intestinal growth, *via* apoptotic turnover of old cells or constitution of the hematopoietic cell population in the gut (54), but subsequent studies could not produce a therapeutic effect from IFN-I in IBD patients (120, 121). In an animal study, the therapeutic potential of IFN-β-secreting *Lactobacillus* (La-IFN-β) by delivering IFN-β in the gut prior to the induction of colitis was evaluated (122). Unexpectedly, this preventive measure heightened sensitivity to DSS when compared to mice pretreated with control *Lactobacillus*. Colitic mice that received La-IFN-β had increased intestinal secretion of TNF-α, IFN-γ, IL-17A, and IL-13 and decreased Treg populations in their small intestine. Intestinal DCs from La-IFN-β-treated mice and bone marrow-derived DCs exposed La-IFN-β showed decreased IFNAR1 expression. The underlying causes for the differing results of these various studies have yet to be identified. Further muddying the waters, conventional DCs can either enhance or inhibit DSS-induced colitis, independently of T cells, contingent on their manner of activation (55), emphasizing again the importance of IFN-I-driven immunoregulation in the gut.

IFN-I IN COLORECTAL CANCER

Like many other cancer types, colorectal cancer development has an inflammatory component. In fact, the risk of patients

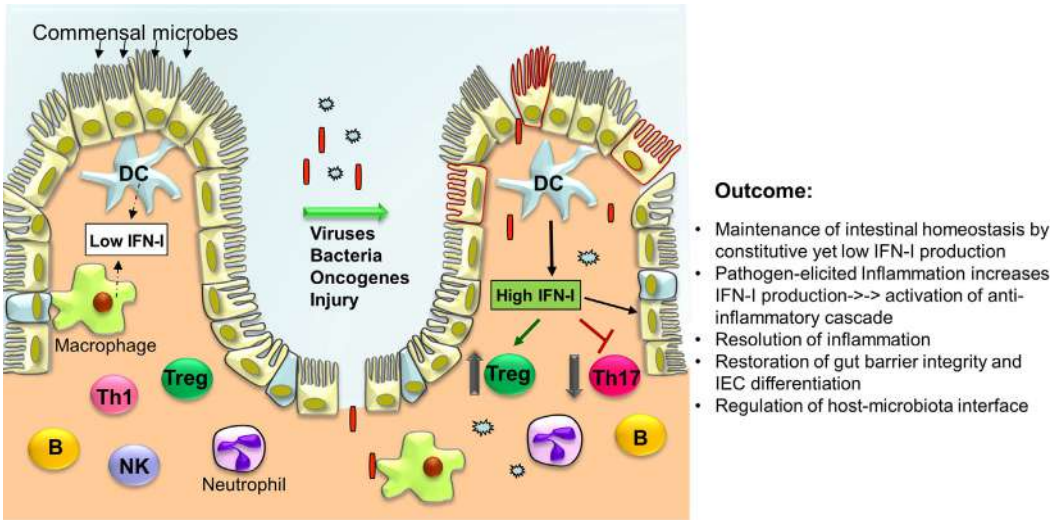

FIGURE 1 | Type I interferons (IFN-I) orchestrate a series of intracellular events in immune cells and intestinal epithelial cells (IECs) to cease inflammation resulting in the regeneration of the intestinal epithelium and restoration of the gut barrier. Under normal conditions, low levels of IFN-I are secreted by lamina propria dendritic cells (DCs) and other phagocytes. In response to microbial attack and/or tissue injury, production of IFN-I by these cells is increased that in turn act on T cells to suppress Th17 cell differentiation while promoting Treg expansion thereby limiting inflammation. IFN-I also inhibit the production of pro-inflammatory cytokines (i.e., IL-1β, IL-8, IL-23) and induce the production of anti-inflammatory mediators (IL-1RA, IL-10, IL-27). Furthermore, IFN-I act on IEC and Paneth cells to restrict proliferation and favor their differentiation to establish gut barrier integrity.

with IBD to develop CRC is strongly linked to the duration of the disease, anatomical extent, and severity of colonic inflammation (123). It is estimated that as much as 15% of all IBD patients will die of colitis-associated cancer (CAC), although early diagnosis and proper treatment of IBD symptoms can reduce the risk of CAC (124). IFN-I promote the recruitment and activation of tumor-parallel immune cells, the presence of which is believed to improve the prognostic pathological assessments of CRC (125). Aside from the immune-compartment-driven inflammation referenced earlier, genetic alterations within IFN-I signaling cascades have been implicated in CRC. To model CAC in rodents, the axozymethane (AOM)/DSS protocol was developed and is widely used to study colorectal cancer (126). Mice are given a single intraperitoneal injection of the carcinogen AOM, which is known to cause activating mutations in β-catenin, Kras and upregulation of Cox2, and iNOS (127). Addition of DSS given in multiple cycles generates a chronic inflammatory environment that reliably accelerates the carcinogenic effect of a single dose of AOM by dramatically shortening the duration of time for tumors to arise. Using this model, loss of Ifnar1 in IECs was reported to increase inflammation and severity of colitis. This poses cancer risk as evidenced by *Ifnar1*-expressing mice displaying decreased GI tumor burden corresponding with decreased mucosal inflammation (51). However, findings from our lab employing the same CAC model revealed a distinct and unexpected phenotype, in that loss of *Stat2* (an essential component in IFN-I signaling) reduced tumor burden and inflammation in the colon (128). To further establish the role of IFN-I in CRC, additional animal studies are warranted using the sporadic model of CRC, which also has a strong link to inflammation (129).

Another link to consider in CRC is the study of single-nucleotide polymorphisms (SNPs) in IFN-I-related genes that include STAT1, JAKs, IRFs, IFN-γ, and IFN-γR, which have been associated with increased CRC risk and disease progression (130, 131). In stark contrast, SNPs in IFNA7 and IFNA14 genes have been found associated with overall survival, more specifically in CRC patients without distant metastasis at time of diagnosis (132). These genes are located nearby several transcription factor-binding sites, but remains unknown how IFNA7 and IFNA14 directly influence overall survival, though they may still be regarded as potential CRC patient biomarkers. SNPs in IFNAR1 were also found associated with CRC risk (132), but how they affect IFN-I signaling and inflammation as a whole in the gut remains to be evaluated.

In humans, CRC tumor specimens show elevated mRNA expression of TLR9, IFNAR1, and IL-6, indicating that IFN-I-signaling components and effectors may be good predictors for overall survival (133). Other contrasting studies, however, find that TLR9 expression is decreased in hyperplastic and villous polyps from patients who develop CRC, further supporting a possible protective role for TLR9 expression against malignant transformation in colorectal mucosa (134). To add to the complexity of the role of signaling components of IFN-I and gene products of IFN-I, a recent study reported that *in vitro* formation of colorectal tumor spheroids, in the absence of IFN-I treatment, induced transcription of ISGs *via* IRF9/STAT2 (135). *In vitro*-tumor spheroids are characterized by non-proliferating, metabolically stressed cells in the hypoxic inner core, surrounded by actively proliferating cells in the outer layers. Knockdown of STAT2, but especially IRF9 inhibited accumulation of three ISGs: IFI27, IFITM1, and OAS1, whereas STAT1 knockdown had no

effect. In addition, expression of IRF9 in this 3D model resulted in a significant decrease in the sensitivity of CRC cells to multiple chemotherapeutic drugs (135). Another ISG, ISG15, functions as a ubiquitin-like modifier, able to form covalent conjugates called "protein ISGylation" on many cellular proteins leading to a cellular stress response and increased inflammation. Elevated ISGylation has recently been proposed to promote intestinal inflammation and CAC in mice (136). In other studies, ATG16LI, which is not an ISG, has been found to regulate autophagy as well as innate immunity. A non-synonymous ATG16LI polymorphism carrying a T300A amino acid substitution is implicated in CD. Paradoxically, this SNP in CRC patients was found associated with increased overall survival and reduced metastasis. Data show an elevated IFN-I transcriptional signature and mitochondrial antiviral signaling suggesting that ATG16L1 T300A could be regulating IFN-I production (137). Further investigation is warranted to fully understand the contributions of IFN-I-related signaling pathways in CRC.

CONCLUDING REMARKS

Type I interferons are broadly expressed cytokines that drive innate immunity, responding to pathogenic attack or injury with both pro- and anti-inflammatory responses (summarized in **Figure 1**). This remarkable and well-orchestrated task is facilitated by the production of other cytokines and chemokines to eradicate the invading microorganism and begin the process of wound healing. IFN-I are the bridge between innate and adaptive immunity *via* promotion of DC maturation leading to disease-specific education and expansion of T cells. This process removes pathogenic microbes while promoting beneficial commensals. Similarly, IFN-I regulate epithelial cell apoptosis to promote intestinal barrier function. Deregulated immune responses to commensal bacteria that penetrate the intestinal epithelium barrier is believed to be the main cause of IBD, primarily UC and CD, which leave patients more vulnerable to CAC. Intensive research has been performed in experimental mouse models of colitis, however, to better understand the complex IFN-I-driven immunological effects, more studies are needed to better explain the diverse clinical results of IFN-I when evaluated in the setting of IBD. In fact, some patients appeared to respond better than others to IFN-I treatment, implying that additional factors must be identified to determine their regulatory role in IFN-I signaling. Most IECs appear capable of producing sufficient IFN-I, as well as other cytokines, and considering the important observations already made with *Ifnar1*$^{-/-}$ mice, the influences of STAT proteins and IFN-I-related proteins and parallel signaling pathways, will need to be taken into account and studied in more depth in future studies of intestinal immunity and homeostasis.

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KK and AG wrote and edited the review. BT assisted with literature searches and organization of review.

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The Role of IFN-β during the Course of Sepsis Progression and Its Therapeutic Potential

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Sepsis is a complex biphasic syndrome characterized by both pro- and anti-inflammatory immune states. Whereas early sepsis mortality is caused by an acute, deleterious pro-inflammatory response, the second sepsis phase is governed by acute immunosuppression, which predisposes patients to long-term risk for life-threatening secondary infections. Despite extensive basic research and clinical trials, there is to date no specific therapy for sepsis, and mortality rates are on the rise. Although IFN-β is one of the most-studied cytokines, its diverse effects are not fully understood. Depending on the disease or type of infection, it can have beneficial or detrimental effects. As IFN-β has been used successfully to treat diverse diseases, emphasis has been placed on understanding the role of IFN-β in sepsis. Analyses of mouse models of septic shock attribute a pro-inflammatory role to IFN-β in sepsis development. As anti-inflammatory treatments in humans with antibodies to TNF-α or IL1-β resulted disappointing, cytokine modulation approaches were discouraged and neutralization of IFN-β has not been pursued for sepsis treatment. In the case of patients with delayed sepsis and immunosuppression, there is a debate as to whether the use of specific cytokines would restore the deactivated immune response. Recent reports show an association of low IFN-β levels with the hyporesponsive state of monocytes from sepsis patients and after endotoxin tolerance induction. These data, discussed here, project a role for IFN-β in restoring monocyte function and reversing immunosuppression, and suggest IFN-β-based additive immunomodulatory therapy. The dichotomy in putative therapeutic approaches, involving reduction or an increase in IFN-β levels, mirrors the contrasting nature of the early hyperinflammatory state and the delayed immunosuppression phase.

Keywords: IFN, IFN-β, sepsis, macrophages, monocytes, immunosuppression, M1–M2 polarization, p21

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INTRODUCTION

Sepsis is a systemic inflammatory syndrome caused by massive microbial infections and is a major cause of death worldwide. Sepsis is defined as an "organ dysfunction caused by a dysregulated host response to infection," while septic shock is associated to a greater mortality risk, caused by "underlying circulatory, cellular and metabolic abnormalities" (1, 2). Although survival of sepsis patients with

overwhelming pro-inflammatory responses is greatly improved in intensive care units (ICU), most patients develop delayed sepsis with severely suppressed immune responses and succumb to secondary infections (3, 4).

IFN-β is an essential cytokine in promoting and regulating innate and adaptive immune responses; its potential as an antimicrobial agent has been studied extensively. Data from murine models have assigned IFN-β a role in septic shock development, and its neutralization is proposed as a therapeutic strategy for human sepsis (5). As recent findings show low IFN-β expression by non-responsive monocytes in delayed sepsis patients, we discuss the therapeutic value of blocking or enhancing the levels of this cytokine to modulate immunosuppression.

Sepsis Progression from Hyperinflammation and Early Death to Immunosuppression and Delayed Death

Following massive microbial infection, highly produced inflammatory cytokines, mainly TNF-α and IL-1β, drive hyperinflammation in sepsis patients (6, 7). Patients can suffer early death several days after systemic infection, due to sepsis and septic shock (8). Improvement in ICU care and compliance with the "Surviving Sepsis Campaign," which provides clinical practice guidelines for the recognition and management of sepsis and septic shock, has reduced death incidence of sepsis patients (9–11). Early sepsis patients that survive ICU can nonetheless develop delayed sepsis and immunosuppression (12, 13). Patient death can be prolonged after initial sepsis diagnosis, due to a deactivated immune response (14, 15).

Hyporesponsiveness is considered a counteracting mechanism that regulates hyperinflammation and alleviates the deleterious effects of primary infection (16, 17). This state correlates with sepsis progression and death, as it is associated with increased risk for secondary nosocomial infections (3). In a recent review, Delano and Ward (18) present the evolution of mortality as early and late deaths and introduce a third modality of sepsis, long-term death, which can be delayed for years. As the incidence of early deaths in the ICU has diminished over the years (4), the burden of sepsis-related deaths is linked to the hyporesponsive phase of the syndrome, and late and long-term deaths are on the rise (19). The progression from initial sepsis to the prolonged syndrome is not clearly defined and the host response to sepsis might consist of concurrent inflammatory and immunosuppression processes (8, 20). Patients were recently identified that develop persistent inflammation-immunosuppression and catabolism syndrome (PICS), which leads to ongoing organ injury and death (13, 18, 21).

Mouse Models for Analysis of the Biphasic Aspects of Sepsis

Mouse models are valuable tools with which to dissect the mechanisms of human disease, and aid in discovering innovative therapeutic advances. In sepsis, there is nonetheless a disparity at the molecular level between mouse models and the human syndrome (22–25), and human and mouse inflammatory diseases show low gene correlation (26). Findings from these models must thus be evaluated critically for applicability to human sepsis.

Injection of lipopolysaccharide (LPS, also termed endotoxin), a constituent of Gram-negative bacteria, leads to septic shock in mice. Infection models or cecal ligation and puncture (CLP) (27) also lead to septic shock. Compared to the widely used LPS model, the CLP model is a more clear approximation of polymicrobial sepsis as it leads to bacteremia, a feature shared with human sepsis (27).

Mouse systems that emulate hyporesponsiveness and delayed sepsis in humans are limited due to the inherent complexity of sepsis and to its heterogeneity. Non-lethal CLP models adapted to restrict death of treated mice can also be used to study delayed sepsis [see review by Dejager et al. (27)]. Mice subjected to mild CLP induction or CLP models treated with antibiotics survive early hyperinflammation and show long-term immune dysfunction. Exposure of these mice to secondary bacterial infection establishes the "two-hit" model that allows the study of compromised responses (28).

Endotoxin tolerance is a convenient model for analysis of macrophage hyporesponsiveness; it is induced after exposure of mice to a non-lethal LPS dose that induces hyperinflammation (29, 30). In a few hours, macrophages from treated mice undergo functional reprogramming from activated M1 status to an M2 hyporesponsive phenotype, and epigenetic modifications could explain this polarization (31–34). Endotoxin-tolerant macrophages are hyporesponsive to subsequent LPS challenge, and produce low amounts of TNF-α, IFN-β, and inducible nitric oxide synthase (iNOS) (34). This system deviates from the CLP model and from basic human sepsis features, as it is limited to the effects of LPS and not bacterial infection, and the initial LPS treatment does not lead to trauma and death.

As *in vitro* LPS stimulation induces endotoxin tolerance in human monocytes (31, 35) and their refractory state shows a certain analogy to monocyte hyporesponsiveness in sepsis patients (6, 15, 20), data from this model may be useful, but are considered preliminary (16, 24) and should be verified in CPL models and in human sepsis.

TREATMENT STRATEGIES FOR SEPSIS: PAST AND PRESENT

To date, intense research in the field has provided effective approaches for early sepsis treatment that have increased survival in the ICU (3, 21); there have nonetheless been no therapeutic advances for long-term sepsis-related immunosuppression.

To minimize the pro-inflammatory condition of sepsis patients, it seemed logical to antagonize hyperinflammation and to treat sepsis by neutralizing hyperinflammation through anti-TNF-α or -IL-1β specific antibodies (7). In mice, anti-TNF-α delivery protected from septic shock when delivered before or simultaneously with LPS, although patients treated with anti-TNF-α or -IL-1β antibodies failed to show sepsis improvement (7, 36). Perhaps, therapeutics directed to the early physio-pathological conditions that derive from this initial pro-inflammatory response would be more efficient in preventing early death in sepsis patients. One such condition is the activation of procoagulant pathways (27).

The ideal treatment would be based on an approach that could remedy both phases of sepsis (37). Because of the contrasting nature of early and delayed sepsis, distinct therapeutic approaches are currently considered to control hyperinflammation or immunosuppression. Before treatment, the state of each patient should thus be taken into strict account and tested, for example, by measuring HLA-DR expression in myeloid cells and evaluating overall immune cell status (3, 18).

As early sepsis survivors eventually develop immunosuppression, there is particular interest in establishing interventions for the late sepsis phase and a debate as to whether treatment for such patients should focus on boosting the pro-inflammatory response (7, 37). Macrophages are directly associated with sepsis development since Gram-negative bacteria, major constituents of infection, promote their activation through the TLR4 receptor; macrophages can then acquire a deactivated status (16). Other immune components participate in immunosuppression development in delayed sepsis. For example, T cell numbers decline due to apoptosis and attain an exhaustion state or impaired function, whereas T regulatory cells (Treg) are associated with mortality of delayed sepsis patients. NK cells and neutrophils have altered signaling functions. Moreover, a population of immature myeloid-derived suppressor cells (MDSC) arises and promotes immunosuppression (8, 18, 21). Dendritic cells undergo apoptosis in sepsis (38–41) and subsequently reemerge, but their activity is compromised due to the production of anti-inflammatory IL-10 (42–45). Because of these diverse immunosuppression features, it was suggested that intervention should not be limited to targeting a single affected immune component, but rather implement combination approaches to improve critical immune defects of sepsis-affected individuals (18). Such methods would imply delivery of immune modulators such as G-CSF, GM-CSF, IFN-γ, or IL-15 (7, 18), to reconstitute specific immune deficiencies that depend on sepsis stage and the patient's needs.

THE ROLE OF IFN-β IN IMMUNE DISEASE AND IN SEPSIS

IFN-β in Infection and Disease: Beneficial and Harmful Effects

The interferons are cytokines that modulate the immune response and antimicrobial infection; they are classified as types I, II, and III. IFN-γ is the only type-II cytokine, and IFN-α and -β (IFNα/β) of the broad IFN I family are the most studied. After microbial infection, endothelial, epithelial, and immune cells detect through their pattern-recognition receptors (PRRs), which include TLR, pathogen-associated molecular patterns (PAMPs). This interaction promotes IFN-β, which is produced by most nucleated cells (46, 47). All type-I IFNs bind to the same cell surface IFN-α/β receptor (IFNAR). Signaling through IFNAR initiates a cascade of events, which activates innate cells, and elicits chemokine/cytokine production and activation of adaptive immunity (47). IFNα/β was initially found to have antiviral activity, as their defective signaling increases viral susceptibility (48–53). Accordingly, IFN-α is effective for treatment of viral infections such as hepatitis C (54), and IFN-β is also used in cancer treatment (55). IFNα/β can nonetheless have detrimental immunosuppressive effects during chronic infection with certain viruses (56, 57). These contrasting roles of IFNα/β in controlling or exacerbating disease are a central feature of these pleiotropic cytokines (58). Similarly, IFNα/β might contribute to the development of autoimmune diseases such as lupus or psoriasis (59–61), whereas IFN-β can be beneficial in a large proportion of multiple sclerosis patients (62). In another setting, although IFNα/β are critical in the defense against bacteria (63–67), they could also impede antibacterial immunity by inducing apoptosis of immune cells, by suppressing inflammatory cytokine release, by responsiveness to IFN-γ, or by promoting IL-10 production (68–71).

In general terms, IFNα/β boosts pro-inflammatory cytokine/chemokine production and activates adaptive immunity, but also has diverse roles in immunity and, depending on context, might also suppress immune responses. IFN-α and IFN-β share a common receptor with apparently redundant functions. The two cytokines are used differently for treatment, and IFN-α is highly produced by plasmacytoid dendritic cells (47). The differences between IFN-α and IFN-β probably derive from the weak IFN-α binding to their common receptor (72).

IFN-β Neutralization in Treating Hyperinflammation in Acute Sepsis

Several studies in IFN-β- and IFNAR-deficient mouse models of LPS- or TNF-α-induced septic shock suggest a pro-inflammatory

TABLE 1 | Role of IFN-β in modulating hyperinflammatory and immunosuppressive responses in mouse models and humans.

	Models of IFN-β in sepsis	Reference
Hyperinflammation	IFN-β and IFN-α/β receptor (IFNAR) deficiency protect mice from LPS-induced septic shock	(73, 74)
	IFN-β and IFNAR deficiency protect mice from TNF-α-induced lethal shock	(75)
	IFNAR blockade protects mice in cecal ligation and puncture model	(76)
	IFN-α protects from LPS-induced lethality in mice	(77)
	IFN-β protects from LPS-induced septic shock in mice	(78)
	LPS treatment induces IFN-β expression in human monocytes	(31, 83)
	Pseudomonas aeruginosa infection induces IFN-β expression in human whole blood	(76)
Immunosuppression	IFN-β stimulation increases inflammatory response during endotoxin tolerance	(83)
	IFN-β is downregulated during endotoxin tolerance	(34, 83)
	IFNAR deficiency increases lethality in mouse model of delayed sepsis	(96)
	IFN-β is downregulated in immunosuppressed monocytes from sepsis patients	(83)

role for IFN-β in septic shock (**Table 1**) (73–75). These observations support the idea of IFN-β or IFNAR neutralization as an adjunctive immunomodulatory therapy for sepsis (5). This view was corroborated by a subsequent report showing that IFNAR is needed for CLP-triggered sepsis development (76). An anti-IFNAR antibody also reduced sepsis symptoms and was functional even when injected after CLP induction, thus reinforcing the potential of IFN-β signaling inactivation as a therapeutic approach for sepsis (76). Sepsis improvement by the anti-IFNAR antibody precludes doubts about the relevance of data from mice in which IFN signaling is genetically abolished, based on the supposition that knockout mice might not reproduce the exact function of the eliminated gene. Studies in which septic shock was reduced by IFN-α (77) or IFN-β delivery (78) that suggest an anti-inflammatory effect for these cytokines need to be reinterpreted. Perhaps, the injected cytokine dose in these two studies elicits non-physiological effects that increase survival, as there is a striking difference between physiological IFN-β levels and those used for treatment (79). The role of IFN-β appears to lie more in propagating the inflammatory response than in initiating it, as its effect differs from that of TNF-α, a potent pro-inflammatory agent that causes septic shock in mice (75). In accordance with this view, TNF-α induces IFN-β production (58).

TLR4 stimulation elicits hyperinflammation and IFN-β signaling in monocytes and macrophages as illustrated in **Figure 1** (left). TLR4 triggering recruits MyD88 (myeloid differentiation primary-response protein 88) in order to activate both NF-κB and protein kinases (MAPK), which drives nuclear translocation of p65/p50 NF-κB and phospho-AP-1 and transcription of inflammatory cytokines. TLR4 stimulation also results in phosphorylation of IFN-regulatory factor 3 (IRF3), which in conjunction with NF-κB, induces expression of IFN-β (80, 81). Secretion of IFN-β activates the IFNAR complex in an autocrine manner. Subsequent STAT1 phosphorylation induces IFN-responsive elements such as iNOS, and chemokines such as CXCL9, CXCL10, and CXCL11 (**Figure 1**), which are potent white blood cell (WBC) attractors that further potentiate the immune response (31, 32, 82).

We recently showed that neutralization of IFNAR reduces iNOS and NO production as well as WBC-attracting chemokines in a mouse model of increased response to LPS (83). Neutralization of IFN-β signaling could thus reduce the

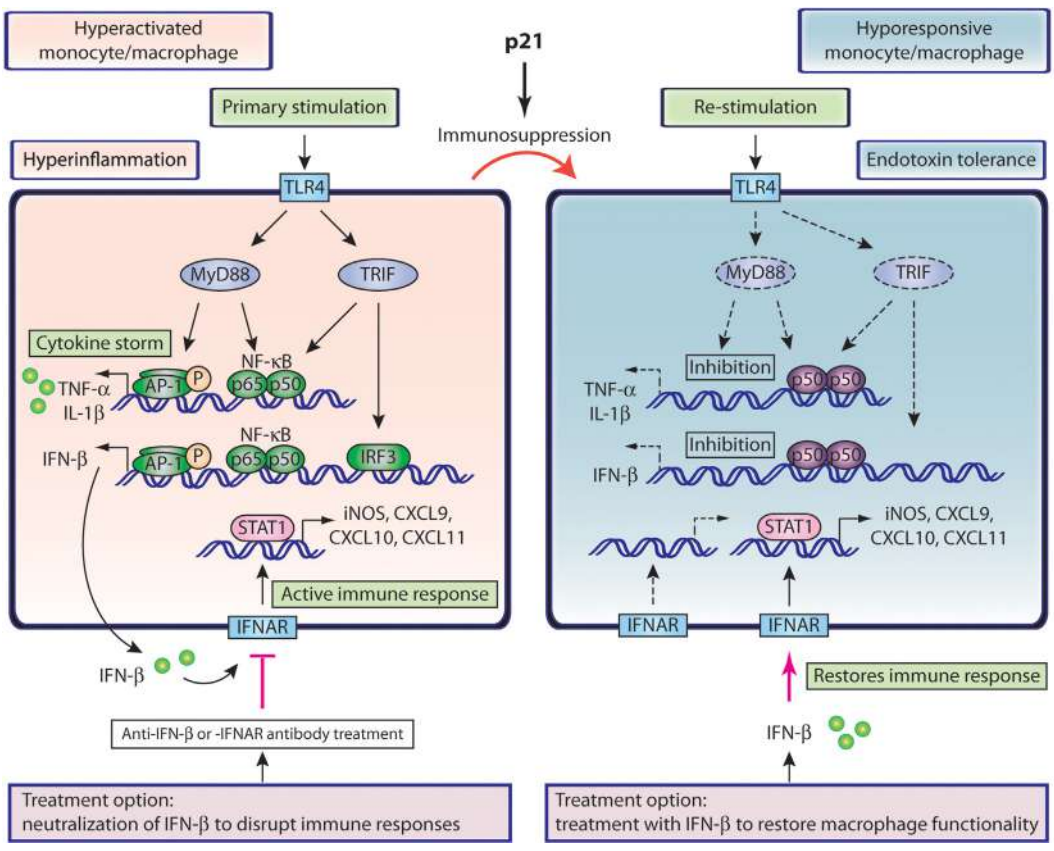

FIGURE 1 | Role of IFN-β in hyperactivated and hyporesponsive monocytes/macrophages. Left, hyperactivated macrophages or monocytes present hyperinflammatory status and elevated IFN-β production. Secreted IFN-β interacts with its receptor and propagates the immune responses through iNOS and chemokine production. Neutralization of the IFN-β pathway interrupts these responses and could be beneficial in sepsis treatment. Right, hyporesponsive macrophages or monocytes associated with delayed sepsis or endotoxin tolerance arise from their hyperactivated counterparts, as a result of immunosuppression, driven by p21. TLR4 restimulation of such cells shows compromised activation pathways and inflammatory cytokine production, including IFN-β. IFN-β treatment could restore compromised monocyte functions and benefit immunosuppressed delayed sepsis patients.

propagation of inflammation and harmful physiological effects that depend on persistent NO production (83). These findings corroborate the idea that IFN-β does not act as an immediate hyperinflammatory factor, and its neutralization could be an attractive option that allows a greater margin for intervention, as the interval of hyperinflammation induction might be too short for effective treatment.

These data reinforce the idea that neutralizing IFN-β signaling could be a therapeutic option for acute sepsis patients. As suggested by Mahiou et al. (5), such an approach might be applied to relieve acute hyperinflammation, and caution should be taken to exclude patients with delayed sepsis to avoid aggravated immunosuppression.

IFN-β in Restoring Functions of Compromised Immune Components

Boosting monocytes and other immune components to recover function is a prospective therapeutic approach for immunocompromised late sepsis patients. This idea is based on data showing that immunosuppressed monocytes from late sepsis patients recover HLA-DR levels and inflammation modulators after IFN-γ treatment (84). Although IFN-γ delivery to trauma or sepsis patients in clinical studies had some positive effects, it does not cure sepsis (7, 18). It is therefore suggested that a combination of IFN-γ treatment with GM-CSF, another monocyte booster, might prove more effective in treating immunosuppression in sepsis (7). The prevailing idea is that key cytokines or other pharmacological agents could revert immunosuppression of monocytes and other immune cells (37). Here we evaluate whether IFN-β could be included in the chart of promising factors to alleviate immunosuppression (7, 18).

IFN-β is essential for human monocyte inflammation (31), but is downmodulated in endotoxin-tolerized monocytes (34, 83). Importantly, IFN-β is also downregulated in immunosuppressed monocytes from sepsis patients (**Table 1**) (83), which implies that IFN-β downregulation could be critical for immunosuppression of monocytes in human sepsis, and that IFN-β treatment could reverse monocyte deactivation.

p21 was initially identified as a cell cycle and cyclin-dependent kinase 2 inhibitor (CDK2) (85). Other functions have been attributed to p21 (86, 87), and several studies designate it as an immune response modulator. p21 inhibits development of autoreactive T cells and autoimmunity (88–91). Moreover, it controls macrophage activation in septic shock and rheumatoid arthritis (92–94) and inhibits LPS-induced NF-κB activation, as well as endotoxin hyporesponsiveness of macrophages and monocytes (83). p21 regulates IFN-β levels in human monocytes, and monocytes from sepsis patients show high p21 levels, which correlate with low IFN-β expression.

These data suggest a model in which monocyte immunosuppression is controlled by p21, which promotes inhibitory p50–p50 over active p65–p50 NF-κB products (**Figure 1**, right). This p21 effect compromises production of inflammatory cytokines and IFN-β, and reduces iNOS induction and chemokine upregulation, which impairs WBC attraction and activation of innate and adaptive immunity (34, 47, 55, 83, 95). These IFNAR-dependent effects could theoretically be reestablished by an exogenous supply of IFN-β and thus restore monocyte functions and counteract immunosuppression (**Figure 1**, right). This model is further reinforced by our recent work showing that IFN-β treatment of endotoxin hyporesponsive macrophages increases expression of iNOS and CXCL11 (83). IFN-β can thus reestablish critical functional properties in immunosuppressed monocytes.

The role of IFN-β in controlling infection in long-term sepsis is supported by a mild CLP sepsis model. In such settings, WT mice survive the initial inflammatory shock, control bacteremia, and elude delayed death, whereas IFN-β-deficient mice, also unaffected by early inflammation, succumb to infection, and undergo late death (96). The data from this model, which in a way resembles delayed sepsis, show association of IFN-β expression with production of CXCL10, a chemokine that promotes homing of immune cells such as neutrophils (95, 96). In this mild CLP model, immunosuppression is possibly partial and IFN-β is produced. The results, however, support our view (**Figure 1**, right) that IFN-β, which must be supplied exogenously in severe immunosuppression, is essential for chemokine-mediated WBC attraction and antimicrobial action.

The model in **Figure 1** (right) shows the possible effect of exogenous IFN-β in reversing monocyte hyporesponsiveness. This IFN-β effect can be extended to other immunosuppressed immune cells, as it can increase effector T cells, antibody responses in B cells, and activate innate immunity and antigen presentation (47, 95). As IFN-β can induce dendritic cell maturation (95), this cytokine could enhance the generation of dendritic cells, which are reduced by apoptosis in sepsis patients. Similarly to monocytes, exogenous IFN-β could reverse immunosuppressive aspects of dendritic cells, including IL-10 production, which inhibits IL-12 synthesis and T cell stimulation (45, 97).

Apart from its activating effect in monocytes and consistent with its pleiotropic antimicrobial responses, IFN-β could thus abrogate a wide range of sepsis-associated immunosuppressive responses. As little is known about its positive immunomodulatory effects in sepsis, establishing a role for IFN-β in antagonizing immunosuppression requires experimental evidence. Testing the effect of exogenous IFN-β in the "two hit" mouse model (28) could impart some early answers on its suitability for treatment of sepsis, which should be evaluated in human sepsis.

IFN-β has adverse immune effects that hinder microbial clearance in some systems (47, 98), such as proapoptotic effects on innate cells and T cells, inhibition of the IFN-γ pathway in macrophages, as well as generation of IL-10-producing Treg cells (58). The idea that IFN-β delivery might benefit immunosuppressed patients with delayed sepsis thus needs to be assessed meticulously. As the negative immune impact of IFN-β in infection is manifested in the context of certain but not all microbial infections, sepsis treatment might not be affected by these discordant IFN-β effects.

Much experimentation remains in order to elucidate the potential immunomodulatory effect of IFN-β in sepsis therapeutics and to estimate whether, in addition to IFN-β, any other

stimulus could aid in efficient immune response reactivation in patients with delayed sepsis.

CONCLUDING REMARKS

The failure of anti-TNF-α- or IL-1β-based therapies to decrease the death toll in sepsis patients has generated doubts as to whether cytokine-based treatments can be effective. Recent research has given new impetus to the field, and appropriate cytokine combinations are being considered for restoring suppressed immune functions in delayed sepsis patients. IFN-β-based therapeutic approaches such as neutralization could be used during the hyperinflammation phase of sepsis, but also during the immunosuppression phase, with IFN-β delivery to boost suppressed immunity. A thorough analysis of individual sepsis patients is needed before applying such radically opposed treatments for hyperinflammation or hyporesponsiveness. Patients with established immunosuppression might thus be a clearer target for IFN-β treatment to refurbish immune responses. Research is needed in mouse models and in humans to determine the precise mechanistic aspects and effectiveness of IFN-β-based treatments.

AUTHOR CONTRIBUTIONS

GR composed the figures and contributed in writing the manuscript; RS composed the final versions of the figures and organised the argument and the manuscript; MM and CM-A contributed to the intellectual content of the manuscript. DB conceived and wrote the manuscript; all authors read and approved the final version of the manuscript.

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REVIEW
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The Role of Interferons in Inflammation and Inflammasome Activation

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Inflammation is an essential physiological process, which enables survival during infection and maintains tissue homeostasis. Interferons (IFNs) and pro- and anti-inflammatory cytokines are crucial for appropriate response to pathogens, damaged cells, or irritants in inflammatory response. The inflammasom is multiprotein complex, which initiates cleavage of pro-inflammatory cytokines IL-1β and IL-18 into active forms. In addition, inflammasomes initiate pyroptotic cell death. In the present review, I summarize and analyze recent findings regarding the cross talk of IFNs and inflammasomes.

Keywords: caspase-1, caspase-11, cyclic GMP-AMP synthase, guanylate-binding protein, interferon, inflammasome, macrophages, pyropotosis

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INTRODUCTION

Inflammation is a complex immune response to response to pathogens, damaged cells, or irritants and enables survival during infection or injury and maintains tissue homeostasis (1). In response to an infection, a cascade of signals leads to the recruitment of inflammatory cells (neutrophils and macrophages), which produce cytokines and chemokines (2). The sustained robust inflammation may lead to serious disorders due to the overproduction of inflammatory cytokines and tissue damage (2). However, cytokine secretion from neutrophils and macrophages is tightly regulated on the transcriptional level, and several pro-inflammatory cytokines also have posttranscriptional level of regulation (3). A typical inflammatory response consists of four components: inflammatory inducers, the sensors that detect them, the inflammatory mediators induced by the sensors, and the target tissues that are affected by the inflammatory mediators (1, 3). The innate immune response is involved in various inflammatory processes and has a particularly important role in bacterial and viral infections. Interferons (IFNs) and inflammatory cytokines are crucial molecules in this process, influencing cellular, tissue, and global physiological functions. Immune cells (macrophages, dendritic cells) recognize pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) (4, 5). Bacterial and viral PAMPs are detected by pattern recognition receptors (PRRs), which are also able to recognize DAMPs—endogenous molecules, released by dying or damaged cells (5–7). PRRs have distinct subcellular localization: toll-like receptors (TLRs) and C-type lectin receptors are transmembrane proteins found in the plasma membrane and endosomes, where they can survey PAMPs and DAMPs in the extracellular milieu. Intracellular PRRs are the retinoic acid-inducible gene I (RIG-I)-like receptor, the AIM2-like receptor (ALR), and the nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins (8). In addition, PRRs that sense cytosolic DNA and trigger the production of type I interferon were described (9). In this review, we discuss recent advances in understanding the role of IFNs in inflammatory response and inflammasome activation.

INTERFERONS

Interferons were first described as an antiviral factor that interferes with viral replication in mammalian cells (10). They are secreted from infected cells and activate innate immune response that promotes not only cytokine production but also natural killer cell functions and antigen presentation (11, 12). On the basis of the structural homology and the specific receptor they associate with, three classes of IFNs have been described (Type I, II, and III) (12). Type-I IFN family includes numerous IFN-α variants (13 in human and 14 in mouse), a single IFN-β; in addition, several other IFNs were reported (IFN-ε, -k, -ω, and -δ) (11, 13). IFN-γ, is the sole type II interferon, is structurally different from the type I and III IFNs, and signals through a different receptor: the IFN-γ receptor (3). IFN-γ can potentiate pro-inflammatory signaling by priming macrophages for anti-microbial actions, since it induces nitric oxide (NO) production and inhibit NLRP3 inflammasome activation (14, 15).

Type I-IFN expression is induced by activation of PRRs and by cytokines (9, 16). While, several different cell types express IFN-β, IFN-α is secreted only by hematopoietic cells, predominately plasmacytoid dendritic cells (17). Type I-IFNs are protective in acute viral infections; however, in bacterial infections, they could have either protective or deleterious roles (18). Type I-IFNs are induced by ssRNA, dsRNA, and cytosolic DNA from viruses or bacteria (19, 20). Type-I and type-II IFNs were reported to promote the expression of over 2,000 IFN-stimulated genes (ISGs), and the ISGs-induced proteins were demonstrated to act by enhancing pathogen detection and restrict the replication of pathogens (21). Several environmental factors, as well as host and pathogen factors, regulate responses of cells to IFN signaling (11).

Toll-like receptors are a family of 13 receptors known as PRRs and play a key role in the innate and adaptive immune response (22). Viral nucleic acids are recognized by endosomal TLR-3 (double-stranded RNA), TLR-7, -8 (single-stranded RNA), and TLR-9 (unmethylated CpG DNA) (4, 19). While TLR7 and TLR9 are expressed in B cells, macrophages, and DCs, TLR8 is expressed in macrophages and DCs In addition, TLR3 is broadly expressed also in non-hematopoietic cells, in humans. Triggering of PRR results in signaling pathways that activate gene transcription by nuclear factor (NF)-κB, as well as interferon regulatory factors (IRFs) and leads to production of type I IFNs and cytokines and chemokines (4, 23, 24). Endosomal TLR3 signals solely *via* the adaptor TIR domain-containing adaptor inducing IFN-β (TRIF), while TLR7, 8, and 9 depend on myeloid differentiation factor-88. Both pathways subsequently activate the IκB kinase (IKK) complex leading to nuclear translocation of the transcription factor NF-κB to upregulate the expression of inflammatory cytokines and chemokines (25). IRF transcription factors, crucial for the induction of type I IFNs (IFN-α and IFN-β), are also activated by endosomal TLRs. Signaling of TLR receptors and their adaptors result in transcription factors IRF3 and IRF7 activation, while IRF3 is expressed in many different cell types, plasmacytoid dendritic cells are the only cell type that constitutively express IRF7 (11). PRRs also induce activation of pro inflammatory caspases, leading to production of processed mature cytokines. Recently, also epigenetic mechanism that determines cell type-specific differences in IFN and IFN-stimulated gene (ISG) expression in response to exogenous signals was described (26).

Cytosolic DNA sensor proteins include cyclic GMP-AMP synthase (cGAS) (27) and ALR inflammasomes: Aim-2 and IFN-γ-inducible protein 16 (IFI16). Both Aim2 and IFI16 contain HIN200 domain that bind directly to DNA and a pyrin domain (28–30). Moreover, an endoplasmic reticulum-associated molecule referred to as stimulator of interferon genes (STING) was reported to control a signaling pathway important for the detection of cytosolic DNA and type I IFN expression (31, 32). Microbial RNAs are recognized by melanoma differentiation-associated gene 5 and (RIG-I), both of which are expressed in macrophages and non-hematopoietic cells (4, 19). Downstream signaling pathways are transmitted by mitochondrial antiviral mitochondrial antiviral signaling (MAVS), also known as IFN-β promoter stimulator-1 (IPS-1)/virus-induced signaling adaptor (VISA)/Cardif, a transmembrane protein on mitochondria (33). Recently, several excellent reviews describe the mechanism of nucleic acid sensing and signaling in the cytosol (34–36).

Interferon (IFN)-α and IFN-β bind to IFN-α receptor (IFNAR), a heterodimeric transmembrane receptor, which consist of two subunits: IFNAR1 and IFNAR2. Type I IFN-induced canonical signaling pathway IFNAR engagement activated the receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2, which in turn phosphorylated the signal transducer and activator of transcription 1 (STAT1) and STAT2 (37). The activated STAT1 and STAT2 dimerize and rapidly translocate to the nucleus, where they together with IFN-regulatory factor 9 form a trimolecular complex called IFN-stimulated gene factor 3 (ISGF3) (11). ISGF3 binds to DNA sequences, which are known as IFN-stimulated response elements and directly activating the transcription of ISGs. Within a period of hours, however, the signal decays and the STATs are exported back to the cytoplasm for the next round of signaling (38, 39). Interestingly, the affinity of the IFNAR receptor varies between the different type I IFN ligands, due to the activation of different regulatory elements (40). However, the other cytokines activate STAT homodimers that recognize different gamma-activated sequence. Therefore, canonical type I IFN signaling induces a distinct subset of several hundred ISRE-driven ISGs. Cellular responses to IFNAR ligation vary during the course of an immune response and are cell type- and context-dependent (41).

Several different mechanisms were described that suppress type I IFN-mediated responses: downregulation of IFNAR expression on cell surface, induction of negative regulators like ubiquitin carboxy-terminal hydrolase 18 (USP18), and suppressor of cytokine signaling (SOCS). SOCS proteins compete with STATs for binding to IFNAR, while USP18 displaces JAK1 from IFNAR2 (42, 43). In addition, type I IFN responses are regulated by miRNAs (44, 45). During PRR and inflammatory signaling, miR-155 is highly induced (46, 47). It was reported that miR-155 suppressed the expression of IFNAR–JAK–STAT pathway in $CD8^+$ T cells and the consequence of this suppression was enhanced $CD8^+$ T cell responses to viral and bacterial pathogens (47).

Type-I and type-II IFNs are known to promote the expression of over 2,000 ISGs, and the products of ISGs have been shown to

act by enhancing pathogen detection and innate immune signaling or restricting intracellular replication of viruses, bacteria, and parasites (21). Protein modification by the ubiquitin-like modifier interferon (IFN)-stimulated gene 15 (ISG15) is strongly induced by type I IFNs and represents one of the major antiviral IFN effector systems (48, 49). Conjugation of ISG15 to its substrates is counteracted by the activity of ubiquitin-specific protease 18 (USP18/UBP43) (50).

Another important group of proteins is superfamily of IFN-induced GTPases. Based on biochemical and structural studies, IFN-induced GTPases are grouped into four families of IFN-inducible, dynamin-like GTPases: the myxovirus resistance proteins (Mx), the immunity-related GTPases, the guanylate-binding proteins (GBPs), and the very large IFN-inducible GTPases (51). IFN-induced GTPases are transcribed in response to type-I, type-II, and type-III IFNs, while the Mx proteins are expressed only in response to type-I and type-III IFNs. TNF-α signaling was proposed to act as an alternative induction route for the GTPase; therefore, IFNs are not the only factors acting as GTPase inducers (52, 53). However, type-II IFNs and type-I IFNs are the strongest inducers, while TNFα and LPS are relatively weak stimuli.

INFLAMMASOMES

Activation of the inflammasome is a key event in inflammatory immune response. The inflammasomes are cytosolic multiprotein complexes that are composed of an inflammasome-initiating sensor, apoptosis-associated speck-like protein containing a CARD (ASC) acts as an adaptor protein and the protease-caspase-1. Inflammasome-initiating sensors include members of the NLRs the pyrin and HIN domain-containing (also known as PYHIN, Aim 2-like receptors, or ALRs; e.g., Aim2), or the TRIM (e.g., pyrin) family (54). Complex assembly leads to caspase-1-dependent cleavage of cytokines pro-interleukin 1β (pro-IL-1β) and pro-IL-18 into secreted mature forms (55–57). In addition, inflammasomes initiate pyroptotic cell death (52, 57, 58). Pyroptosis involves cell swelling, membrane rupture, and release of the cytoplasmic content into the extracellular space (58–60). Pyroptotic cell death is induced by caspase-1 or mouse caspase-11 (human caspase-4/5) cleavage gasdermin D (GSDMD), a pore-forming protein that normally exists in the auto inhibited state (58, 61, 62). Interestingly, since mature IL-1β lacks target sequences, secretion may require pyroptosis of the macrophages (60). However, other mechanisms of IL-1β secretion might also exist, human monocytes were reported to release IL-1β without pyroptosis (63).

Recently, several excellent reviews described mechanism of inflammasome activation (52, 56, 64, 65). Several NLR family members have been described as components of inflammasomes: Nlrp1b inflammasome (66, 67), Naip-Nlrc4 inflammasome (68, 69), the Nlrp6 inflammasome (70), the Nlrp12 inflammasome, the Aim2 inflammasome (28, 71), the RIG-I inflammasome (72), and the IFI16 inflammasome (73). Particularly, the activation of Nlrp3 inflamamsome is well characterized (55, 64, 74, 75). Since it responds to variety of stimuli, many different mechanisms of its activation have been proposed, including the release of oxidized mitochondrial DNA, production of reactive oxygen species and mitochondrial dysfunction, lysosomal destabilization, changes in intracellular calcium levels, the formation of large non-specific membrane. The Nlrp3 inflammasome activation in macrophages requires 2 steps: the first, priming step is provided by TLR signaling that upregulates NLPR3 and pro-IL-1β gene expression. This process is tightly controlled by signals culminating in the activation of NF-κB (76). Moreover, Nlrp3 activation can be regulated through direct posttranslational modifications, such as ubiquitination (77). Recently, several independent studies reported non-canonical inflammasome activation (78–80). While canonical inflammasome activation results in caspase-1 cleavage and activation, the activation of a non-canonical inflammasome results in activation of procaspase-11 (56). The mouse caspase-11 has high similarities to caspase-1 and is orthologous to human caspases-4 and -5 (81, 82). Both caspase-1 and caspase-4/11 could induce pyroptosis, while only caspase-1 processes proforms of IL-1β and IL-18 into secreted mature forms (78, 83). Only caspase-11-deficient mice, but not caspase-1-deficient mice were partially protected from septic death (78, 84). Recent reports showed that caspase-11 was involved in the response to cytosolic LPS, independently of TLR4 and was integral to the pathology of LPS-mediated endotoxic shock in mice (61). Moreover, it was shown that human caspase-4 and caspase-5 and mouse caspase-11 bound directly to LPS in the cytosol (85). With the difference to canonical inflammasome activation were the receptor (Nlrp3) and ASC form a scaffold on which caspase-1 can oligomerize, in non-canonical infalmmasome activation, caspase-11 oligomerization occurs directly upon binding to LPS (85). Human caspase-4/5/or mouse caspase-11 cleave GSDMD, a pore-forming protein that normally exists in the auto inhibited state (58, 61, 62). Furthermore, GSDMD N-terminal domain was found to associate with membranes, including the plasma membrane (86–89). It was reported that canonical Nlrp3 inflammasome activation downstream of caspase-4 and caspase-11 activation was dependent on potassium efflux (90–92). Yang et al. reported that cytosolic LPS stimulation induced caspase-11-dependent cleavage of the pannexin-1 channel followed up by potassium efflux and ATP release (92).

AIM2-like receptor inflammasomes are another class of inflammasomes that function to induce caspase-1 activation and IL-1β cytokine maturation. However, unlike NLR inflammasomes, ALR inflammasomes directly bind their ligand, dsDNA (28–30). While IFI16 recognizes dsDNA in the cytosol and nucleus, while Alm2 is localized only in the cytosol (93). In addition, IFI16 could induce type I IFN expression (30).

Inhibition of inflammasome activation by decoy proteins uses proteins structurally related to components of inflammasome and competing for the same adaptors. The CARD-only proteins and PYD-only proteins (POPs) function as endogenous dominant negative proteins that modulate the activity of inflammasomes and protect from excessive inflammation (94, 95). The genes encoding these decoy proteins, POPs, are located on the same chromosome, in the proximity of genes that encode their ligands: the gene encoding POP1 is located on human chromosome 16 next to the gene encoding ASC (96). POP3 has significant

sequence similarity to the PYRIN domain of AIM2 (its target protein), encoded by a neighboring gene (97). Recently, it was demonstrated that POP2 not only prevented inflammasome assembly by binding to ASC but also impaired macrophage priming by inhibiting the activation of non-canonical IKK ε and IκBα (98).

CROSS TALK OF IFNs AND INFLAMMASOMES

Interferons could contribute to inflammasome activation through several different mechanisms (**Figure 1**). It was reported that type I IFNs are required for the caspase-11 expression, which contributes to activation of non-canonical inflammasome (79). Several recent studies have shown that IFN-inducible endogenous proteins could act also as negative regulators and thus inhibit inflammasome activation (97, 99). Among others, interferon-inducible GBPs not only mediate host resistance to pathogens but also promote inflammasome activation in bacterial infections (100, 101). Also, small proteins that are composed of either a CARD or a PYD only, emerged as important inflammasome regulators (94, 95). It was demonstrated that POP3, which is induced by type I IFNs, interacted with the PYD domain of AIM2 and competed with ASC to inhibit AIM2 inflammasome activation in response to dsDNA, mouse CMV, and modified vaccinia virus Ankara infection (97). Silencing of POP3 in human macrophages enhanced DNA and DNA virus-induced ALR inflammasome formation and hence the maturation and release of IL-1β and IL-18 (97). Not only POPs but also metabolites like 25-hydroxycholesterol, an oxysterol and is derived from cholesterol, suppress inflammasome activation (99). At least in macrophages, IFN-β strongly induced cholesterol 25-hydroxylase, the enzyme that

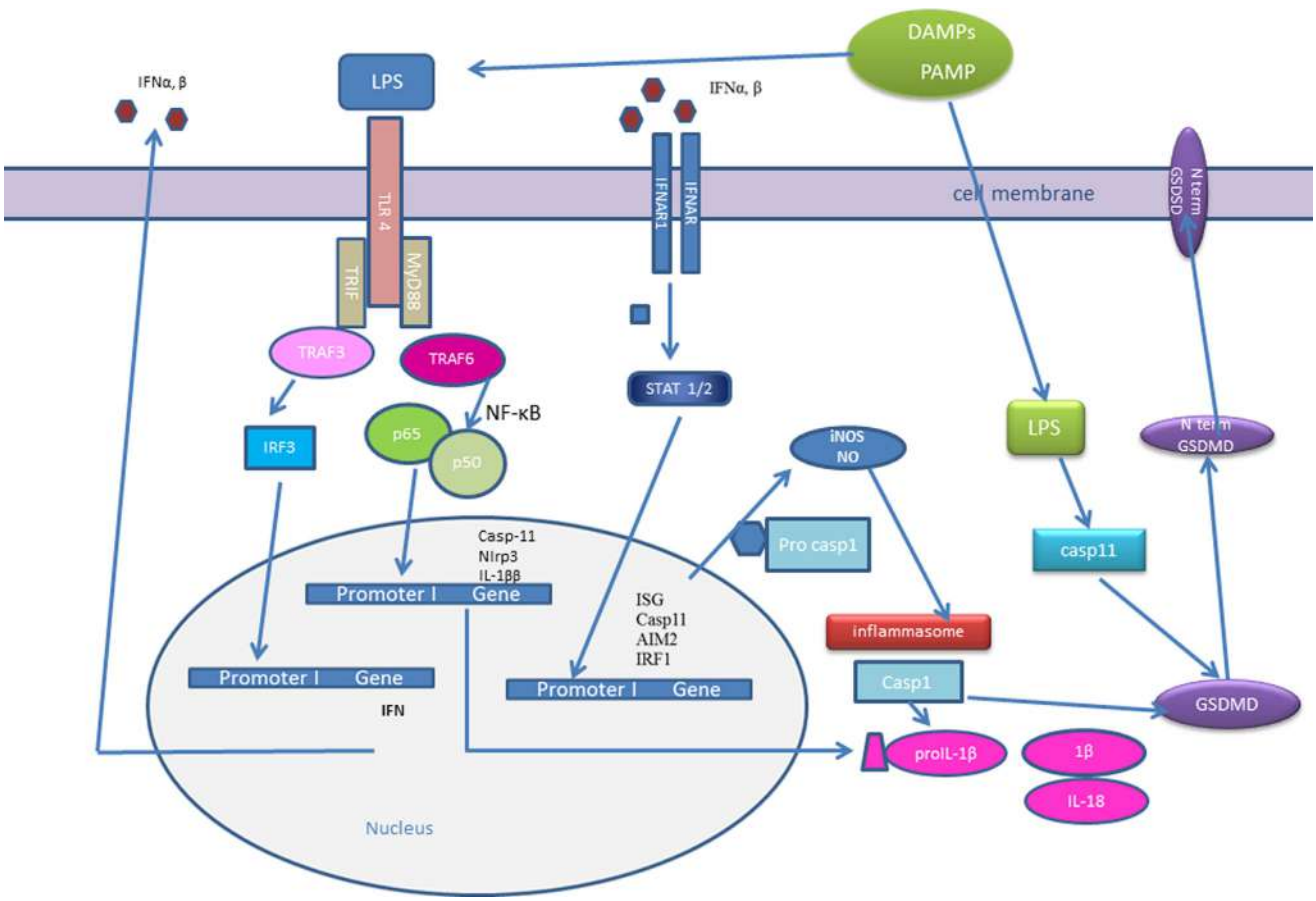

FIGURE 1 | Type I interferons (IFNs) and inflammasome activation. Initial pathogen-associated molecular patterns (PAMPs) recognition by pattern recognition receptors induces IFN-β expression. IFNs could signal in an autocrine or paracrine manner and trigger expression of IFN-stimulated genes (ISGs): interferon regulatory factor (IRF)1, AIM2, caspase-11. Caspase-11 recognizes cytosolic LPS and induces IL-1β processing in an Nlrp3-dependent manner and triggers pyroptosis through gasdermin D (GSDMD) cleavage. Active caspase-1 and caspase-11 cleave GSDMD and the released gasdermin-N domain binds to phosphoinositides in the plasma membrane, oligomerizes to generate membrane pores, and initiates cell death-pyroptosis. IRF induce the expression of guanylate-binding proteins (GBPs), which target vacuolar and cytosolic bacteria, compromise the integrity of bacterial cells, and expose PAMPs like LPS and dsDNA to cytosolic sensors, caspase-11, and AIM2. IFN signaling triggers the expression of inducible nitric oxide synthase (iNOS), which upregulates cellular nitric oxide (NO) levels leading to NLRP3 S-nitrosylation.

transforms cholesterol into 25-hydroxycholesterol (102, 103). Work of Reboldi et al. showed that 25-hydroxycholesterol inhibited not only pro-IL-1β gene transcription but also the inflammasome activation (99). The authors proposed that 25-hydroxycholesterol antagonized the sterol response element-binding protein processing (99). Moreover, cholesterol 25-hydroxylase-deficient mice showed increased sensitivity to LPS-induced septic shock (99).

Both type-I IFNs and IFN-γ could promote inducible nitric oxide synthase (iNOS), which increases the amount of endogenous NO, expression in macrophages (15, 104). NO plays an important role in a defense against pathogens, it could be oxidized to reactive nitrogen oxide species, that *S*-nitrosate thiols in proteins (15, 104). Mishra et al. reported that NO inhibited NLRP3 oligomerization by means of direct S-nitrosylation of the NLRP3 protein, preventing full inflammasome assembly (15). Also study by Mao et al. demonstrated that NO prevented the activation of the NLRP3 inflammasome (14). In line with the above results, in iNOS-deficient macrophages, NLRP3 inflammasome activation was enhanced, iNOS-deficient mice had increased mortality from LPS-induced sepsis (14).

In addition, type I IFN signal *via* STAT1 decreased the activity of Nlrp3 inflammasome that induce caspase-1 to process the IL1-β precursor in response to a large variety of intracellular PAMPs (105). Different mechanisms could contribute to diminished IL1-β processing in IFN-stimulated cells. STAT1 target gene products directly repress NLRP3 inflammasome. Moreover, the IFN-I/STAT1 pathway increases IL-10 synthesis, IL-10-mediated STAT3 activation, and the suppression of IL1-β precursor synthesis by activated STAT3 (106). Guarda et al. showed that IL-1α and IL-1β were downregulated in mice pretreated with poly(I:C), a synthetic RNA analog that strongly induces type-I IFNs (106). In addition, they demonstrated that the recruitment of inflammatory cells (neutrophils and monocytes) into peritoneal cavity was significantly lower in poly(I:C) pretreated mice, than in control animals injected only with LPS. Moreover, they demonstrated that IFN-β suppress not only inflammasome activation and IL-1β secretion but also it rendered the mice more susceptible to *Candida albicans* infection (106).

Several recent studies reported cross talk between IFNs and inflammasome activation in bacterial infections (79, 100, 101, 107, 108). An early study showed that caspase-11 gene expression in response to LPS and IFN-γ was dependent on NF-κB and STAT-1 signaling (109). Rathinam et al. demonstrated that transcriptional induction of caspase-11 by IFN-β signaling was enough to induce both its expression and auto activation (79). Gurung et al. reported that TLR4–TRIF–IFNβ-induced caspase-11 synthesis is crucial for non-canonical Nlrp3 inflammasome activation in macrophages infected with enteric pathogens *Escherichia coli* and *Citrobacter rodentium* (110). IFN-γ could also upregulate caspase-11 expression. Aachoui et al. showed that caspase-1 activity is required upstream of caspase-11 to control infection by cytosolic bacterium *Burkholderia thailandensis*. Caspase-1-activated IL-18, which further induced IFN-γ to prime caspase-11 and rapidly clear *B. thailandensis* infection. Whereas IFN-γ was essential, endogenous type I IFNs were insufficient to prime caspase-11 and cleared *B. thailandensis* (111). Oficjalska et al. reported that IFN-γ-dependent, type I IFN–TRIF-independent signaling pathway was required for *in vivo* caspase-11 production in intestinal epithelial cells during DSS-induced colitis (112). However, LPS-stimulated macrophages from TRIF-deficient mice had impaired caspase-11 expression, implying a context-dependent role for type I or II IFN in the regulation of caspase-11 activity (79, 112). In addition, IFN-γ induced upregulation of Nlrp3, ASC, and procaspase-1 expression (100, 113, 114). IFN-γ enhanced Aim2-induced IL-1β release or Nlrp3-dependent pro-IL-18 cleavage during HSV-1 and *Chlamydia muridarum* infections (115, 116).

Upon bacterial infection, IFN-inducible GTPases—GBPs target vacuolar and cytosolic bacteria and compromise the integrity of bacterial cells, thus exposing the microbial ligands LPS and DNA to cytosolic sensors caspase-11 and Aim2 (100, 101). GBPs have also been shown to regulate the entry of LPS into the cytosol by, as yet, poorly defined mechanisms (100). Significant reduction in NLRP3 inflammasome activation was reported in GBP5-deficient macrophages infected with *S. typhimurium* or treated with potassium efflux agonists (117). However, studies on different mouse strain of GBP5-deficient mice could not confirm the initial results (108, 114). Despite the uncertainty surrounding the role of GBP5 in Nlrp3 inflammasome activation, studies using mice lacking the entire cluster of GBP genes on chromosome 3, have firmly confirmed a functional link between GBPs and the activation of the canonical NLRP3 and AIM2 inflammasomes, as well as the non-canonical caspase-11 inflammasomes. Recently, GBP2 emerged as a critical activator of AIM2 and caspase-11 inflammasomes (100, 101). GBP2 is induced by type I or II IFNs and exposes Gram-negative bacteria-derived LPS to caspase-11 (114). In addition, it was shown that IFN-β boosts canonical AIM2-dependent IL-1β secretion to *Francisella tularenis* or *Listeria monocytogenes* (71, 118) and helps to control caspase-11-dependent pyroptosis by Gram-negative bacteria (79, 107).

Type I-IFN signaling is also essential in response to *Francisella novicida* infection (101, 119). *F. novicida* DNA is detected by DNA sensor cGAS, which induced STING-dependent production of type I-IFNs (71). Type I-IFNs in act *via* the transcription factor IRF1, which regulates expression of GBPs and IRG (108, 120) (**Figure 2**). Interferon response gene B10 together with GBP2, GBP5 work synergistically to rupture *F. novicida* that have entered the cytoplasm, and their action result in the exposure of *F. novicida* DNA for sensing by DNA sensor AIM2 (52, 101, 114).

Another IFN-inducible protein, Z-DNA-binding protein 1 (ZBP1), also known as DNA-dependent activator of IFN-regulatory factors (DAI), has been known as a cytosolic DNA sensor for almost a decade (121). However, a recent work demonstrated that ZBP1 could sense the RNA virus, influenza A virus (IAV) proteins: nucleoprotein and polymerase subunit 1. Kuriakose showed that in IAV-infected cells, ZBP1 regulated NLRP3 inflammasome activation, as well as induction of apoptosis, necroptosis, and pyroptosis (122) (**Figure 2**). ZBP1-deficient mice were protected from mortality during IAV infection, due to reduced inflammatory response (122).

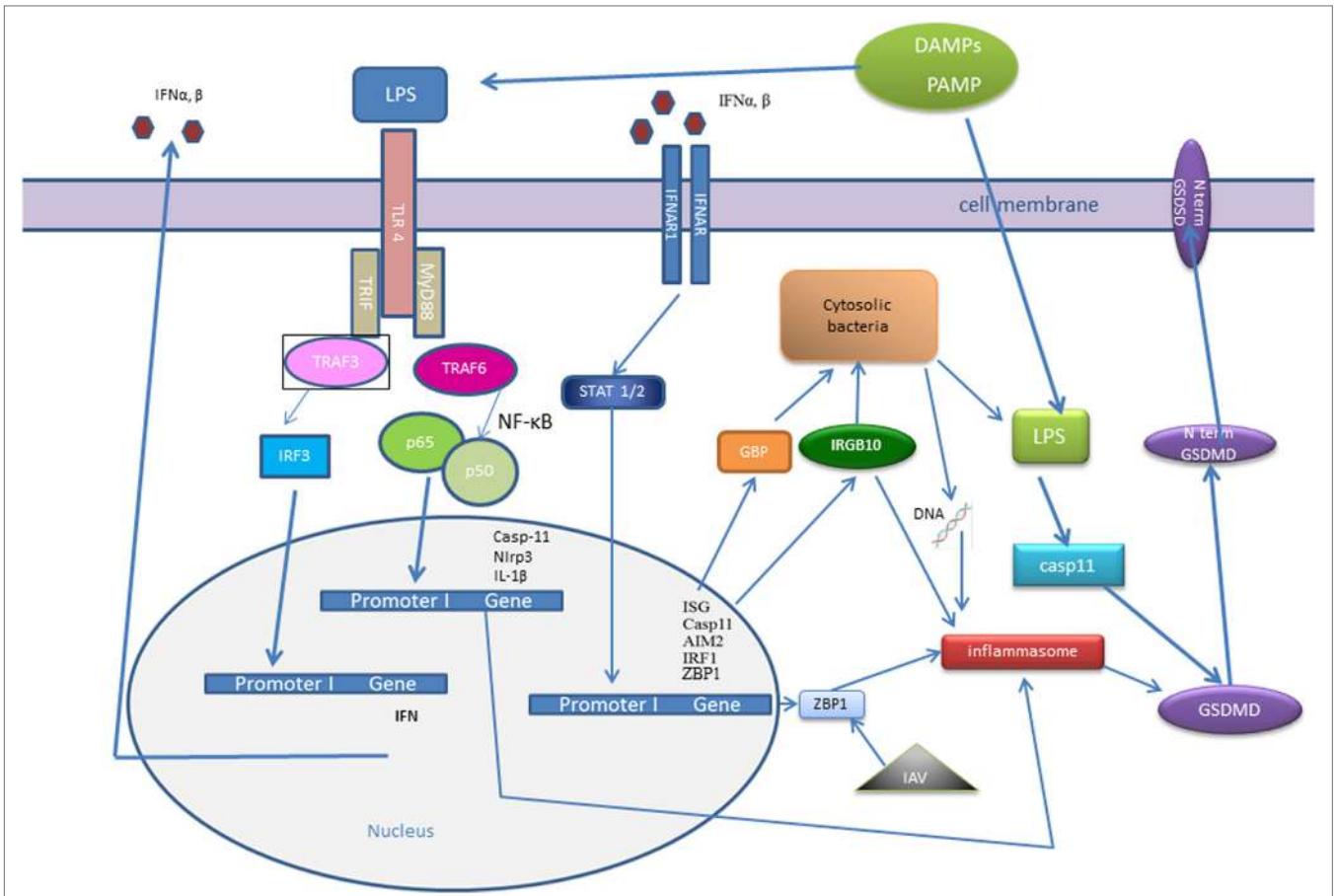

FIGURE 2 | Interferon (IFN) signaling influence recognition of intracellular pathogens—cytosolic bacteria and influenza A virus (IAV). IFNs signaling trigger the transcription factor interferon regulatory factor (IRF)1, which promotes expression of guanylate-binding proteins (GBPs) and interferon response gene B10 (IRGB10). IRGB10, together with GBPs permeabilizes the membrane of Gram-negative bacteria, an action that results in release of bacterial DNA and LPS. Bacterial cytosolic DNA is sensed by Aim2 inflammasome and LPS directly interacts with caspase-11. Type I IFN signaling mediates upregulation of interferon-inducible protein Z-DNA-binding protein 1 (ZBP1), which recognizes the IAV proteins and triggers NLRP3 inflammasome activation, as well as induction of apoptosis, necroptosis, and pyroptosis in IAV-infected cells.

CONCLUDING REMARKS

I have summarized considerable, but by no means all evidence documenting the role of IFNs in inflammasome activation and inflammation. Several recent studies reported the essential role of type I IFNs in non-canonical Nlrp3 inflammasome activation and pyroptosis. Different levels of regulation are involved in the cross talk of IFNs in inflammasome. Not only type I-IFNs but also IFN-γ influence caspase-11 expression and consequently pyroptosis. Dysregulated type I-IFN production could lead to a cell death. However, a recent study reported that in the absence of active proapoptotic caspases-3 and -7, mitochondrial outer membrane permeabilization by Bax and Bak resulted in the expression of type I-IFNs. The process was mediated by mitochondrial DNA-dependent activation of the cGAS/STING (123). Particularly, the role of STAT and other protein modification in IFN signaling pathways could give us important insight into the regulatory mechanisms. IFN-induced GBPs were reported to have an important role in caspase-11 activation and pyroptotic cell death. How does the polymorphisms of GBPs influence inflammasome activation and inflammation is yet to be determined. Future research should explore the detailed molecular mechanisms that are responsible for type I IFN-dependent cell death and inflammasome activation in inflammatory response. Moreover, recently, several studies determined the role of cytokines in metabolic reprograming and inflammasome activation (124). The role cross talk of IFNs, inflammasomes, and metabolism could be a future frontier for the cutting edge research. Identification of the factors involved in inflammasome regulation and signaling will lead to the identification of novel targets for therapeutic intervention.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Type I Interferon Supports Inducible Nitric Oxide Synthase in Murine Hepatoma Cells and Hepatocytes and during Experimental Acetaminophen-Induced Liver Damage

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Cytokine regulation of high-output nitric oxide (NO) derived from inducible NO synthase (iNOS) is critically involved in inflammation biology and host defense. Herein, we set out to characterize the role of type I interferon (IFN) as potential regulator of hepatic iNOS *in vitro* and *in vivo*. In this regard, we identified in murine Hepa1-6 hepatoma cells a potent synergism between pro-inflammatory interleukin-β/tumor necrosis factor-α and immunoregulatory IFNβ as detected by analysis of iNOS expression and nitrite release. Upregulation of iNOS by IFNβ coincided with enhanced binding of signal transducer and activator of transcription-1 to a regulatory region at the murine iNOS promoter known to support target gene expression in response to this signaling pathway. Synergistic iNOS induction under the influence of IFNβ was confirmed in alternate murine Hepa56.1D hepatoma cells and primary hepatocytes. To assess iNOS regulation by type I IFN *in vivo*, murine acetaminophen (APAP)-induced sterile liver inflammation was investigated. In this model of acute liver injury, excessive necroinflammation drives iNOS expression in diverse liver cell types, among others hepatocytes. Herein, we demonstrate impaired iNOS expression in type I IFN receptor-deficient mice which associated with diminished APAP-induced liver damage. Data presented indicate a vital role of type I IFN within the inflamed liver for fine-tuning pathological processes such as overt iNOS expression.

Keywords: type I interferon, inducible nitric oxide synthase, signal transducer and activator of transcription-1, acetaminophen, liver damage

INTRODUCTION

High-output nitric oxide (NO) production achieved by inducible NO synthase (iNOS) is key to efficient innate host defense but also involved in pathological inflammation at diverse organs including the liver (1–4). There, iNOS is detectable in several cell types including Kupffer cells and hepatocytes. Accordingly, expression and biological activity of iNOS has been related to the pathogenesis of acute and chronic liver diseases, among others drug-induced liver injury, non-alcoholic fatty liver disease/steatohepatitis, alcoholic liver disease, liver fibrosis, viral hepatitis, and carcinogenesis (3, 4).

Especially under conditions of glutathione depletion, hepatotoxicity by xenobiotics is frequently mediated at least partly by the highly reactive NO metabolite peroxynitrite (5).

Regulation of iNOS expression and activity occurs foremost on the level of gene transcription with pathways activating nuclear factor (NF)-κB and signal transducer and activator of transcription (STAT)-1 being of particular importance. Depending on the biochemical and cellular context STAT1 is able to support iNOS expression as STAT1 homodimer or as part of a protein complex together with STAT2 and IRF9 known as interferon (IFN)-stimulated gene factor (ISGF)-3 (1, 2, 6–9). Particularly in non-leukocytic cells such as renal mesangial cells and hepatocytes, iNOS mRNA is amplified by NO-driven feed-forward mechanisms (10, 11). Whereas a crucial role for IFNγ concerning iNOS induction is established in diverse cell types including hepatocytes (12), information on the role of immunoregulatory type I IFN (including IFNα/β) as cofactor for induction of hepatocyte iNOS is currently lacking. Of note, IFNα/β is reported to efficiently drive monocyte/macrophage-derived iNOS in humans and mice (9, 13–15). Moreover, in combination with interleukin (IL)-22, IFNα upregulates expression of iNOS in human DLD1 colon carcinoma cells (16). Herein, we set out to further characterize *in vitro* and *in vivo* the role of type I IFN for murine hepatic iNOS regulation by using the cellular model of IFNβ-stimulated hepatoma cells (Hepa1-6, Hepa56.1D) and hepatocytes and by investigating murine acetaminophen (paracetamol, APAP)-induced sterile liver inflammation in the context type I IFN receptor-deficient mice.

MATERIALS AND METHODS

Reagents

Human IL-1β and murine tumor necrosis factor (TNF)-α were from Peprotech, Inc. (Frankfurt, Germany). Murine IFNβ was purchased from PBL (New York, USA) and APAP from Sigma-Aldrich (Taufkirchen, Germany). Inhibitor-κB kinase (IKK)-VII inhibitor was from Calbiochem/Merck Millipore (Darmstadt, Germany).

Cultivation of Murine Hepa 1-6 and Hepa56.1D Cells

Hepa1-6 cells (LGC Standards, Wesel, Germany) and Hepa56.1D (CLS GmbH, Eppenheim, Germany) were maintained in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated FCS (Thermo Fisher Scientific, Langenselbold, Germany). For experiments, cells were seeded on six-well polystyrene plates (Greiner, Frickenhausen, Germany) in the aforementioned medium.

Isolation of Primary Murine Hepatocytes

C57Bl/6 mice were sacrificed and obtained livers were perfused *post mortem*. The isolation procedure was adapted from Godoy et al. (17). Briefly, perfusion was performed with 37°C warm HBSS without Ca^{2+} and Mg^{2+} (supplemented with 15 mM HEPES, 2.5 mM EGTA, 1 g/l glucose, 100 U/ml penicillin, and 100 µg/ml streptomycin) using a roller pump (10 ml/min) for 10 min. Thereafter, the liver was perfused with HBSS with Ca^{2+} and Mg^{2+} [supplemented with 15 mM HEPES, 5 mM $CaCl_2$, 0.13 mg/ml collagenase IV (Sigma-Aldrich, Darmstadt, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin] for additional 10 min. The liver was carefully removed from the abdominal cavity, placed in a Petri dish on ice in DMEM (supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin) and opened with a forceps. Liver cells were resuspended and put over a 100 µm cell strainer (Becton Dickinson, Heidelberg, Germany). After two rounds of centrifugation (5 min at 50 g and 4°C) and resuspension, cell viability was determined by trypan blue dye exclusion, and cells were seeded in DMEM (supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin) on collagen G-coated plates (Biochrom, Berlin, Germany). Adherent hepatocytes were washed after 4 h with PBS and fresh Williams Medium E [supplemented with 10% FCS, 2 mM L-Alanyl-L-Glutamin (Biochrom), 2 ng/ml insulin, 100 ng/ml dexamethasone, 100 U/ml penicillin, and 100 µg/ml streptomycin] was added. Stimulation with recombinant cytokines for analysis of iNOS expression was performed 16 h thereafter. Dexamethasone was not removed from standard supplemented Williams Medium E because this glucocorticoid is known to inhibit hepatocyte apoptosis (18, 19) thereby supporting viability. This protocol was adhered to despite the prospect that dexamethasone at this concentration is capable of reducing NF-κB thus partly affecting hepatocyte iNOS (20).

Detection of CXCL9, IFNα, IFNβ, iNOS, MIP2, and STAT1 mRNA

Total RNA isolation was performed as described (16). Briefly, RNA isolated by Tri-Reagent (Sigma-Aldrich) was transcribed using random hexameric primers (Qiagen, Hilden, Germany) and Moloney virus reverse transcriptase (Thermo Fisher Scientific). During realtime PCR, changes in fluorescence are caused by the Taq polymerase degrading a probe containing a fluorescent dye (GAPDH: VIC; all others: FAM). Pre-developed reagents (Thermo Fisher Scientific): GAPDH (4352339E), CXCL9 (Mm00434946_m1), IFNα2 (Mm00833961_s1), IFNα4 (Mm00833969_s1), IFNα5 (Mm00833976_s1), IFNβ (Mm00439552_s1), STAT1 (Mm00439531_m1), iNOS (Mm00440502_m1), and MIP2 (Mm00436450_m1). Assay mix was from Thermo Fisher Scientific. Realtime PCR (AbiPrism7500 Fast Sequence Detector, Thermo Fisher Scientific): two initial steps at 50°C (2 min) and 95°C (20 s) were followed by 40 cycles at 95°C (3 s) and 60°C (30 s). Detection of the dequenched probe, calculation of threshold cycles (C_T values), and data analysis were performed by the Sequence Detector software. Relative changes in mRNA expression compared with unstimulated control and normalized to GAPDH were quantified by the $2^{-\Delta\Delta CT}$ method. As IFNα and IFNβ gene loci lack introns, all RNA isolates were digested with RNase-free DNase I (Thermo Fisher Scientific) before reverse transcription.

Interferonα was analyzed by standard PCR using universal primers that target all α-subtypes: forward, 5′-ATGGCTAGR CTCTGTGCTTTCCT-3′; revers, 5′-AGGGCTCTCCA GAYTT CTGCTCTG-3′. GAPDH: forward, 5′-CTGGCATTGCTCTCA

ATGAC-3′; revers, 5′-TCTTACTCCTTGGAGGCC-3′. PCR conditions: 95°C for 10 min (1 cycle), 95°C for 30 s, 62°C (IFNα) or 55°C (GAPDH) for 30 s, and 72°C for 45 s (with 37 cycles for IFNα and 25 cycles for GAPDH), and a final extension phase at 72°C for 7 min. Amplicon length: IFNα, 524nt; GAPDH, 110nt. Amplicons was confirmed by sequencing (Eurofins, Ebersberg, Germany).

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as previously described (21). For immunoprecipitation, an IgG control or a specific STAT1 antibody was used (rabbit polyclonal antibody; Santa Cruz Biotechnology, Heidelberg, Germany). To amplify murine iNOS promoter regions enclosing relevant STAT1-binding sites [−951 to −912 bp relative to the transcriptional start site (TSS) (8)], the following primers were used for PCR (9): forward, 5′-ccaactattgaggccacacac-3′ (−1,098 to −1,078 bp); reverse, 5′-gcttccaat aaagcattcaca-3′ (−889 to −869 bp). Conditions: 95°C for 10 min (1 cycle), 95°C for 30 s, 56°C for 30 s, 72°C for 45 s (40 cycles), and final extension (72°C, 7 min). Amplicons were confirmed by sequencing (Eurofins).

Murine Model of Experimental APAP-Induced Liver Injury

C57BL/6 mice were maintained under SPF conditions at the "Zentrale Tierhaltung" (Paul-Ehrlich-Institut, Langen, Germany). Type I IFN-receptor (IFNAR) chain 1-deficient mice, lacking a functional receptor for type I IFN ($IFNAR^{-/-}$ mice), were approximately 20× backcrossed on the C57BL/6 background (22). All animal experiments using C57Bl/6 mice [male, 9–10-week old, wild-type (wt), and $IFNAR^{-/-}$ mice] were carried out in accordance with the recommendations of the Animal Protection Agency of the Federal State of Hessen (Regierungspräsidium Darmstadt, Germany). The protocol was approved by the Regierungspräsidium Darmstadt (Germany).

Murine APAP (500 mg/kg)-induced liver injury was performed as described (23). Briefly, fasted male mice obtained i.p. injection of either warm 0.9% NaCl (B. Braun, Melsungen, Germany) or 500 mg/kg (dissolved in warm 0.9% NaCl) APAP. Mice that obtained NaCl are depicted as control mice (ctrl) throughout the manuscript. Mice had free access to food and water. After 6 h (only wt-mice) or 24 h (wt- and $IFNAR^{-/-}$ mice), mice underwent isoflurane (Abbott, Wiesbaden, Germany) anesthesia and were sacrificed thereafter. Blood was taken from the retroorbital venous plexus. Serum alanine aminotransferase (ALT) activity was quantified according to manufacturer's instructions (Reflotron, Roche Diagnostics, Mannheim, Germany). Serum was stored at −80°C. For RNA and protein analysis, liver tissue was snap frozen in liquid nitrogen and stored at −80°C. For histological analysis, liver tissue was perfused with PBS via the portal vein followed by overnight incubation in 4.5% buffered formalin. Thereafter, tissue was embedded in paraffin for histologic analysis. Paraffin-embedded liver sections (4 µm) were stained with hematoxylin (Applichem, Darmstadt, Germany). The degree of histopathological liver injury was quantified by Keyence BZ-II Analyzer software (Neu-Isenburg, Germany). Specifically, computer-aided analysis of tissue necrosis was performed by using similarly located liver sections obtained from 9 wt- and 9 IFNAR-deficient individual mice ($n = 9$ per genotype) treated with APAP (500 mg/kg, 24 h). One complete liver section per individual mice underwent analysis. The software quantifies the degree of liver necrosis by identifying necrotic areas based on differences in hematoxylin staining. Results are presented as text-only and expressed as (%-reduction) of liver necrosis observed in IFNAR-deficient mice compared with wt-mice.

Immunohistochemical Detection of iNOS

Paraffin-embedded liver sections (4 µm) were used for detection of iNOS. Briefly, sections were deparaffinized and unmasked by heat treatment (Target Retrieval Solution; Dako, Glostrup, Denmark). Sections were stained using either a self-made in-house (24, 25) or a commercially available (Enzo Life Sciences, Lörrach, Germany) rabbit polyclonal antimurine iNOS antibody overnight at 4°C. Notably, both iNOS detecting antibodies generated analogous iNOS staining in livers of APAP-treated mice. For detection, goat antirabbit ABC staining system (Santa Cruz Biotechnology) and the 3,3′-diaminobenzidine Substrate Kit for Peroxidase (Sigma-Aldrich) were used. Sections were counterstained with hematoxylin.

Immunoblot Analysis

Tissue homogenates were generated as previously described (23). Briefly, cells or liver homogenates were generated using lysis buffer [150 mM NaCl, 1 mM $CaCl_2$, 25 mM Tris-Cl (pH 7.4), 1% Triton X-100], supplemented with protease inhibitor cocktail (Roche Diagnostics) and DTT, Na_3VO_4, PMSF (each 1 mM), and NaF (20 mM). Thereafter, SDS-PAGE and immunoblotting were performed. To detect iNOS or pSTAT1 together with GAPDH on the same blot, the blot was cut. Antibodies: iNOS, rabbit polyclonal antibody (Enzo Life Sciences); GAPDH, rabbit polyclonal antibody (Trevigen, Gaithersburg, USA); pSTAT1 (Tyr-701), rabbit polyclonal antibody (Cell Signaling, Frankfurt, Germany). Quantifications of immunoblots were performed by Quantity-One analysis software (Bio-Rad, Munich, Germany). As a "positive control" for iNOS expressing murine C57BL/6 tissue, cutaneous wound lysates obtained 3 days after skin wounding were used. At that time point iNOS protein expression in the wounded skin peaks (26). Wound lysates of iNOS-deficient mice from the same time point were analyzed as "negative control." Those cutaneous wound lysates were kindly provided by Dr. Itamar Goren and Prof. Stefan Frank (University Hospital, Goethe University Frankfurt, *pharmazentrum frankfurt*). All animal experiments were approved by the Regierungspräsidium Darmstadt (Germany).

Analysis of Nitrite Production

Griess assays (Merck, Darmstadt, Germany) were performed as described (10). Briefly, nitrite, a stable NO metabolite, was determined in cell-free supernatants using the Griess reagent (Merck, Darmstadt, Germany). Supernatants were mixed with equal volume of Griess reagent. The absorbance was measured at 540 nm using a microplate reader and nitrite concentrations were calculated using a sodium nitrite calibration curve.

Statistics

Data were checked with the Kolmogorov–Smimov test for parametric distribution and are shown as mean ± SD (*in vitro*) or SEM (*in vivo*) (fold-induction or raw data relative to GAPDH, percent of input, Adj. Vol. INT*mm^2, units/liter, or micromolar). Statistics was performed on raw data as indicated by either one-way analysis of variance with *post hoc* Bonferroni correction or by unpaired Student's *t*-test (Prism 5.0, GraphPad, La Jolla, CA, USA).

RESULTS

IFNβ Amplifies Hepatocyte iNOS Expression in Cell Culture

Whereas type I IFN is an established inducer of iNOS in monocytes/macrophages (9, 13–15), information on effects of IFNα/β on hepatocyte iNOS is scarce. Notably, one earlier report demonstrated that iNOS in human hepatoma Huh7 cells is not induced by IFNα as sole stimulus. Interactions with other cytokines were not investigated in that earlier report (27). To further investigate this matter, murine Hepa1-6 hepatoma cells were exposed to IFNβ in the presence or absence of NF-κB-activating cytokines IL-1β and TNFα (28). The combination IL-1β plus TNFα was employed since pilot experiments in Hepa1-6 cells (data not shown) demonstrated that this combination synergizes for induction of the prototypic hepatocyte-derived NF-κB-dependent chemokine MIP2 (29). Herein, we demonstrate that IFNβ in cooperation with aforementioned pro-inflammatory cytokines potentiates iNOS expression (**Figure 1A**) and activity as detected by nitrite release (**Figure 1B**). Potentiation of nitrite release by coincubation with IFNβ was stable over a 48 h time period (**Figure 1C**). Notably, in accord with the aforementioned report (27), IFNβ

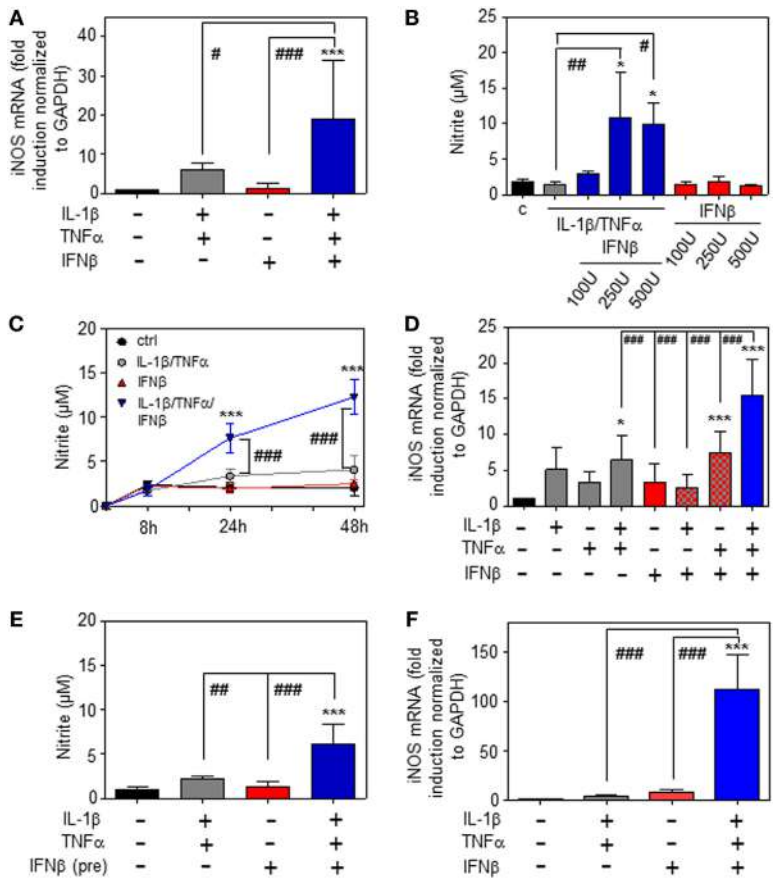

FIGURE 1 | Inducible nitric oxide synthase (iNOS) upregulation by interferon (IFN)β in murine hepatoma cells. **(A–E)** Hepa1-6 cells were either kept as unstimulated control or stimulated with interleukin (IL)-1β (2 ng/ml), tumor necrosis factor (TNF)α (2 ng/ml), IL-1β/TNFα (each 2 ng/ml), IFNβ [at 250 U/ml **(A,C–E)** or the indicated concentrations **(B)**], or with IL-1β/TNFα (each 2 ng/ml)/IFNβ [at 250 U/ml **(A,C–E)** or the indicated concentrations **(B)**]. **(E)** Before addition of IL-1β/TNFα, cells were pre-incubated with IFNβ for 1 h. After 16 h **(A)**, 24 h **(B,E)**, 8 h **(D)**, or the indicated time periods **(C)** cells and culture supernatants were harvested. **(A,D)** iNOS mRNA determined by realtime PCR was normalized to that of GAPDH and is shown as fold-induction compared with unstimulated control [mean ± SD, $n = 5$ **(A)**, $n = 4$ **(D)**; $^{*}P < 0.05$, $^{***}P < 0.001$ versus unstimulated control, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$]. **(B,C,E)** Nitrite production was determined using the Griess-assay [mean ± SD, $n = 4$; $^{*}P < 0.05$, $^{***}P < 0.001$ versus unstimulated control (at the indicated time point **(C)**), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$]. **(F)** Hepa56.1D cells were either kept as unstimulated control or stimulated with IL-1β/TNFα (each 2 ng/ml), IFNβ (250 U/ml), or IL-1β/TNFα (each 2 ng/ml)/IFNβ (250 U/ml). After 16 h cells were harvested and mRNA determined by realtime PCR was normalized to that of GAPDH and is shown as fold-induction compared with unstimulated control (mean ± SD, $n = 3$; $^{***}P < 0.001$ versus unstimulated control, $^{\#\#\#}P < 0.001$). **(A–F)** Statistical analysis, raw data were analyzed by one-way ANOVA with *post hoc* Bonferroni correction.

failed to induce iNOS as sole stimulus (**Figures 1A–C**). Detailed analysis furthermore revealed that triplet stimulation by IL-1β/TNFα/IFNβ is superior to that by either IL-1β/IFNβ or TNFα/IFNβ (**Figure 1D**) and that potentiation of iNOS is likewise detectable in the context of IFNβ preincubation (**Figure 1E**). Amplification of hepatocyte iNOS by IFNβ was not confined to Hepa1-6 cells. Synergism between IL-1β/TNFα and IFNβ for iNOS expression was actually even more pronounced in alternate murine Hepa56.1D hepatoma cells (**Figure 1F**). Moreover, IFNβ likewise potentiated iNOS in murine primary C57BL/6 hepatocytes which was readily detectable on mRNA (**Figure 2A**) and nitrite level (**Figure 2B**).

In order to characterize molecular mechanisms of murine hepatic iNOS gene induction by IFNβ, we chose to focus herein on Hepa1-6 hepatoma cells. Activation of the transcription factor STAT1 is key to immunoregulation by IFNα/β (30, 31). STAT1 activation, assessed by analysis of Tyr-701 phosphorylation (pSTAT1), was readily detectable in Hepa1-6 cells under the influence of IFNβ (**Figure 3A**). Within the murine iNOS promoter, a specific region (−912 to −1,029 bp relative to the TSS) was found to mediate STAT1-induced iNOS transcriptional activation which may be achieved by STAT1-homodimers (in response to IFNγ or type I IFN) or by the type I IFN/ISGF3 axis. In this region (**Figure 3B**, upper panel), adjacent STAT1-binding elements are located. Namely, a dual GAS/IFN-stimulated response element (ISRE) sequence (−951 to −935 bp)—binding STAT1 homodimers or ISGF3—and an additional ISRE site (−924 to −912 bp)—likewise potentially binding ISGF3 (8, 9, 31). STAT1 binding to this region was investigated herein to further characterize mechanisms mediating IFNβ potentiation of iNOS in Hepa1-6 hepatoma cells. In the presence of IL-1β/TNFα, ChIP analysis revealed STAT1 binding to this site in response to IFNβ which suggests enhanced STAT1-dependent transcriptional activity at the iNOS promoter. Unexpectedly, IFNβ as single stimulus failed to initiate STAT1 binding in Hepa1-6 cells. This was in contrast to IFNγ and may propose pivotal action of ISGF3 signaling in the context of stimulation by IFNβ. Data indicate that, in Hepa1-6 cells, signaling by IL-1β/TNFα enforces IFNβ-induced STAT1 binding to the iNOS promoter thereby enabling synergistic gene induction. As expected, IL-1β/TNFα-stimulation, without IFNβ, did not mediate STAT1 binding to this promoter region (**Figure 3B**, lower panel). In order to further deepen the connection between IL-1β/TNFα and IFNβ-related STAT1 binding to the hepatocyte iNOS promoter, NF-κB activation was inhibited by exposing cells to the IKK inhibitor IKK-VII (32). As detected by ChIP analysis, STAT1 binding to the aforementioned region of the iNOS promoter (−912 to −1,029 bp relative to the TSS) was significantly impaired under the influence of IKK-VII (**Figure 3C**). Data indicate that NF-κB activation by IL-1β/TNFα, likely mediated by an active proximal NF-κB site at −85 to −76 bp relative to the TSS (8), supports STAT1 binding to the iNOS promoter in murine Hepa1-6 hepatoma cells.

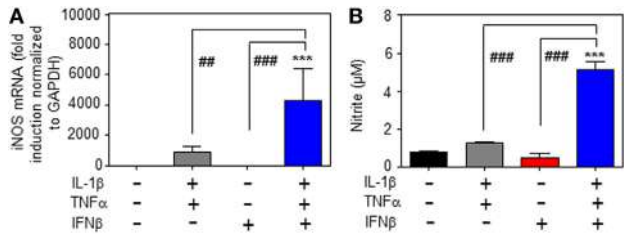

FIGURE 2 | Inducible nitric oxide synthase (iNOS) upregulation by interferon (IFN)β in primary murine hepatocytes. **(A,B)** Primary murine hepatocytes were either kept as unstimulated control or stimulated with interleukin (IL)-1β/tumor necrosis factor (TNF)α (each 2 ng/ml), with IFNβ (at 250 U/ml), or with IL-1β/TNFα (each 2 ng/ml)/IFNβ (at 250 U/ml). After 8 h **(A)** and 16 h **(B)** cells and culture supernatants were harvested. **(A)** iNOS mRNA determined by realtime PCR was normalized to that of GAPDH and is shown as fold-induction compared with unstimulated control (mean ± SEM, $n = 3$; $^{***}P < 0.001$ versus unstimulated control, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$). Statistical analysis, raw data were analyzed by one-way ANOVA with *post hoc* Bonferroni correction. **(B)** Nitrite production was determined using the Griess-assay (mean ± SEM, $n = 3$; $^{***}P < 0.001$ versus unstimulated control, $^{\#\#\#}P < 0.001$). Statistical analysis, data were analyzed by one-way ANOVA with *post hoc* Bonferroni correction.

Type I IFN Supports Expression of iNOS during APAP-Induced Liver Injury

To investigate regulation of hepatic iNOS by type I IFN *in vivo*, the model of moderate APAP-induced acute liver inflammation was used. Liver injury and thus associated necroinflammation in this model is at its peak at around 24 h after APAP administration. Notably, 48 h after onset of intoxication serum ALT levels, indicative of liver necrosis, drop to approximately or below 20% of those detectable at 24 h with liver morphology displaying regeneration and recovery from injury (33–38). In light of these characteristics, we chose to focus herein on the 24 h time point after APAP administration. Notably, this APAP toxicity is associated with a cytokine response that includes upregulation of IL-1β and TNFα (23, 39). Previous reports demonstrated that, during murine APAP intoxication, iNOS protein is well detectable (33, 40) in hepatocytes at regions with centrilobular injury (41, 42). Evaluation of iNOS knockout mice indicated that iNOS-derived NO may promote injury during early intoxication (detected by serum ALT) (43, 44). In contrast, hepatotoxicity was found to be independent from iNOS analyzed histochemically after 24 h. The role of iNOS in APAP-induced liver injury appears complex since NO also inhibits generation of poisonous *N*-acetyl-p-benzoquinone imine from APAP and reduces superoxide anion-dependent lipid peroxidation (43).

In the present study, we confirm increased hepatic iNOS protein in APAP-treated mice (**Figure 4A**; with densitometric quantification, right panel). Immunohistochemistry likewise corroborated iNOS protein expression by hepatocytes during APAP intoxication which was absent in ctrl-mice (**Figure 4B**).

To determine the relevance of type I IFN for iNOS expression in the context of APAP-induced liver injury, experiments were performed by using IFNAR$^{-/-}$ mice. Those mice are unable to respond to type I IFN (22, 31). In fact, induction of hepatic iNOS protein was impaired in IFNAR-deficient mice (**Figure 5A**; with densitometric quantification, right panel). In a next step, expression of hepatic IFNα/β was determined in order to further assess

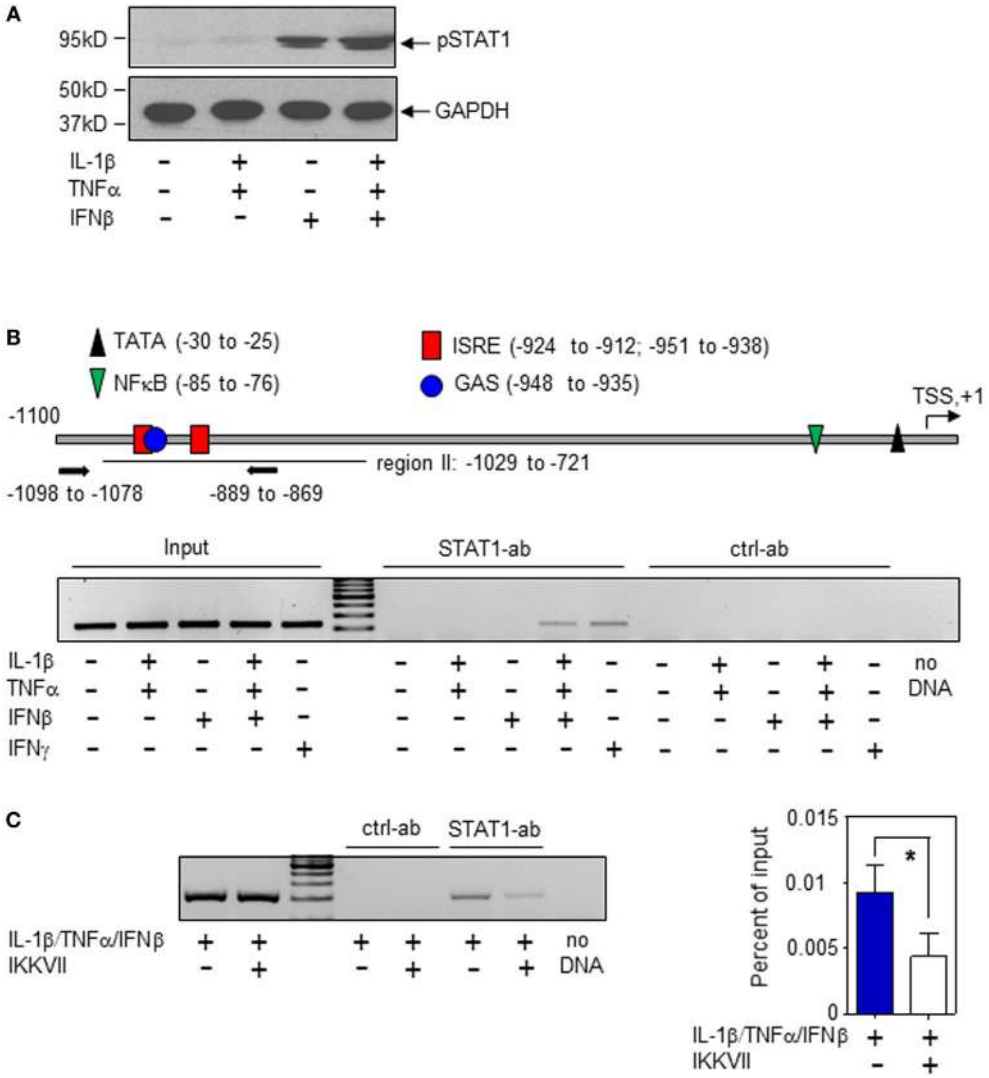

FIGURE 3 | Interferon (IFN)β-induced signal transducer and activator of transcription (STAT)1 activation and inducible nitric oxide synthase (iNOS) expression in murine Hepa1-6 cells. **(A)** Cells were either kept as unstimulated control or stimulated with interleukin (IL)-1β/tumor necrosis factor (TNF)α (each 2 ng/ml), IFNβ (250 U/ml), or IL-1β/TNFα (each 2 ng/ml)/IFNβ (250 U/ml) for 1 h. pSTAT1 and GAPDH were determined by immunoblotting. One representative of three independently performed experiments is shown. [**(B)**, upper panel)] Schematic of the murine iNOS promoter (8). Critical regions of STAT1 (GAS/ISRE-sites) and nuclear factor (NF)-κB binding as well as the TATA-Box and the transcriptional start site (TSS) are indicated. Primers used for chromatin immunoprecipitation (ChIP)-PCR are depicted. [**(B)**, lower panel)] Cells were either kept as unstimulated control or stimulated with IL-1β/TNFα (each 2 ng/ml), IFNβ (250 U/ml), IL-1β/TNFα (each 2 ng/ml)/IFNβ (250 U/ml), or with IFNγ (10 ng/ml). After 2 h, ChIP analysis was performed for detection of STAT1 binding to the illustrated GAS/ISRE-promoter region (−1,098 to −869 bp). One representative of three independently performed experiments is shown. **(C)** Cells were either kept as unstimulated control or stimulated with IL-1β/TNFα (each 2 ng/ml)/IFNβ (250 U/ml). Where indicated, cells were pre-incubated with inhibitor-κB kinase-VII inhibitor (10 μM) for 30 min. After 2 h, ChIP analysis was performed for detection of STAT1 binding to the illustrated GAS/ISRE-promoter region. One representative of three independently performed experiments is shown (left panel). Right panel, densitometric quantification of the PCR signals from the left panel ($n = 3$; $^*P < 0.05$). Statistical analysis, data were analyzed by Student's *t*-test.

type I IFN action during APAP intoxication. Notably, we did not observe upregulation of hepatic IFNα/β mRNA as detected 6 h (data not shown) or 24 h after administration of APAP. However, basal constitutive type I IFN was readily detectable in all liver specimens investigated. **Figure 5B** demonstrates constitutive hepatic IFNα expression as detected by standard PCR using primers targeting the entire panel of murine α-subtypes. Those results were confirmed by realtime PCR for detection of IFNα2 (**Figure 5C**, left panel), IFNα4 (**Figure 5C**, middle panel), and IFNα5 (**Figure 5C**, right panel). Likewise, hepatic IFNβ was expressed constitutively (**Figure 5D**).

Besides iNOS, IFNAR-deficient mice exposed to APAP likewise displayed decreased hepatic mRNA of STAT1-dependent CXCL9 (**Figure 5E**). We and others have previously reported that STAT1 gene expression is regulated by IFN/STAT1-driven positive feedback regulation (16, 31, 45–47). Accordingly, decreased

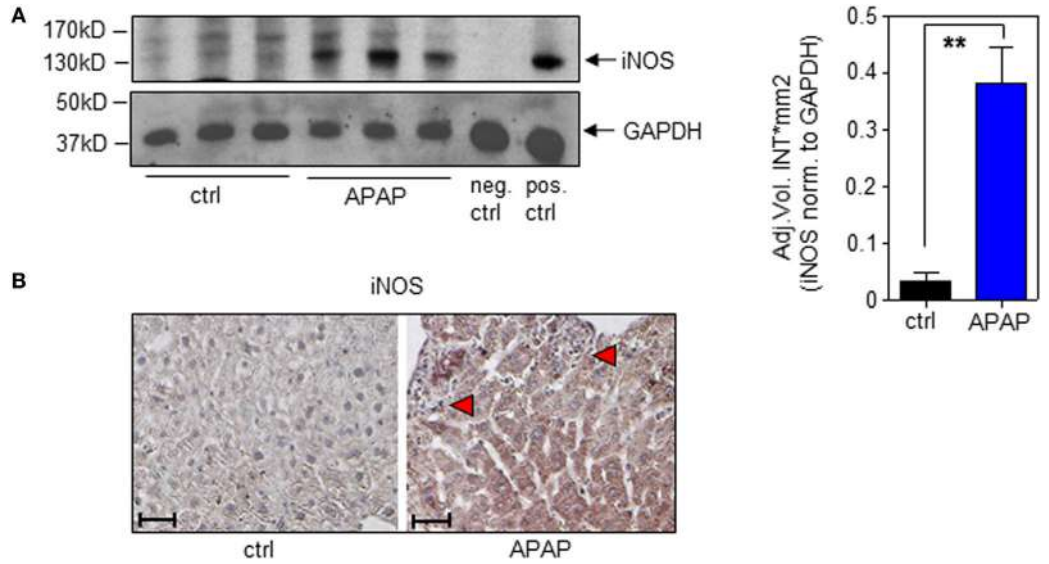

FIGURE 4 | Hepatic inducible nitric oxide synthase (iNOS) expression in acetaminophen (APAP)-induced liver injury. Wild-type mice received either NaCl-ctrl solution or APAP (500 mg/kg) and were maintained for 24 h. **(A)** Left panel, hepatic iNOS was determined by immunoblotting. Right panel, densitometric quantification of iNOS from left panel (mean ± SEM, $n = 3$ individual mice per group; $^{**}P < 0.01$). Statistics, Student's *t*-test. pos.-ctrl., iNOS-positive murine 3-day-after-wounding skin; neg.-ctrl., murine 3-day-after-wounding skin from iNOS$^{-/-}$ mice (see Materials and Methods). **(B)** Paraffin sections were stained immunohistologically for iNOS with hematoxylin counterstaining. Shown are representative liver sections of four control specimens and six specimens obtained from APAP-treated mice. Antibody, rabbit polyclonal antimurine iNOS antibody (Enzo Life Sciences); magnification, 10×; scale, 100 µm; red triangles indicate areas of necrosis.

hepatic STAT1 mRNA was likewise detected in IFNAR-deficient mice (**Figure 5F**) which likely contributes to downregulation of STAT1-inducible genes such as iNOS.

Finally, the role of IFNAR concerning the severity of APAP-induced liver injury was assessed. This issue is in fact controversially discussed. Whereas a previous study reported no effects of IFNAR deficiency on intoxication (35), another study observed pathological action of type I IFN. Specifically, administration of IFNAR-neutralizing antibodies diminishes murine APAP-induced liver damage. Moreover, intoxication is enhanced in genetically engineered mice displaying impaired IFNAR degradation but ameliorated when degradation is enforced by pharmacological means (48). In support of this latter view, herein, reduced APAP toxicity connected to IFNAR deficiency which was observed by analysis of serum ALT (**Figure 6A**) and histological software-aided evaluation 24 h after administration of 500 mg/kg APAP (29.6 ± 5.3% reduction of liver necrosis in IFNAR-deficient versus wt mice; $n = 9$, $P < 0.01$ by unpaired Student's *t*-test). **Figure 6B** displays histochemistry of representative APAP-induced hepatic injury in wt and IFNAR$^{-/-}$ mice, respectively.

DISCUSSION

Type I IFN is a key cytokine component of innate immunity supposed to affect course of disease particularly in viral but also during bacterial infections and, due to a substantial immunoregulatory potential, likewise in sterile inflammation (30, 49–52).

Herein, type I IFN is characterized *in vitro* and *in vivo* as significant determinant of hepatic iNOS expression having the potential to determine disease outcome during liver inflammation. On a cellular level, we demonstrate that IFNβ potently synergizes with the prototypic inflammatory cytokines IL-1β/TNFα for induction of iNOS in primary murine hepatocytes and murine Hepa56.1D as well as Hepa1-6 hepatoma cells. As determined in this latter cellular model, IFNβ directs STAT1 binding to a critical regulatory site within the murine iNOS promoter (−912 to −1,029 bp relative to the TSS) (8, 9), a process that, in Hepa1-6 hepatoma cells, demanded simultaneous pro-inflammatory signaling by IL-1β/TNFα. Notably, a recent report identified in murine bone marrow-derived macrophages (BMDM) an additional distal STAT1 binding region 30 kB upstream of the iNOS TSS which may be able to regulate gene expression (53). However, using IL-1β/TNFα/IFNβ-stimulated Hepa1-6 cells we confirm evident binding of STAT1 to the aforementioned proximal promoter region. Combined with previous studies (8, 9), present data thus emphasize the relevance of this proximal STAT1-binding region for iNOS induction. Interestingly, IFNγ but not IFNβ as single stimulus mediated STAT1 binding to this site—suggesting a role for ISGF3. In contrast to the present observations using Hepa1-6 cells, IFNβ as single stimulus induces STAT1 binding to this proximal binding region in BMDM (9) which indicates cell type-specific mechanisms at work. The unexpected requirement of IL-1β/TNFα signaling for STAT1 binding to the iNOS promoter observed herein is a further cell-type specific facet of the well-described STAT1/NF-κB synergism that drives inflammatory/antimicrobial gene expression (53). Regulation of CXCL9 in murine NIH3T3 fibroblasts may serve as a leading case in that context. There, efficient STAT1 binding to the CXCL9 promoter demands TNFα costimulation and downstream STAT1/NF-κB

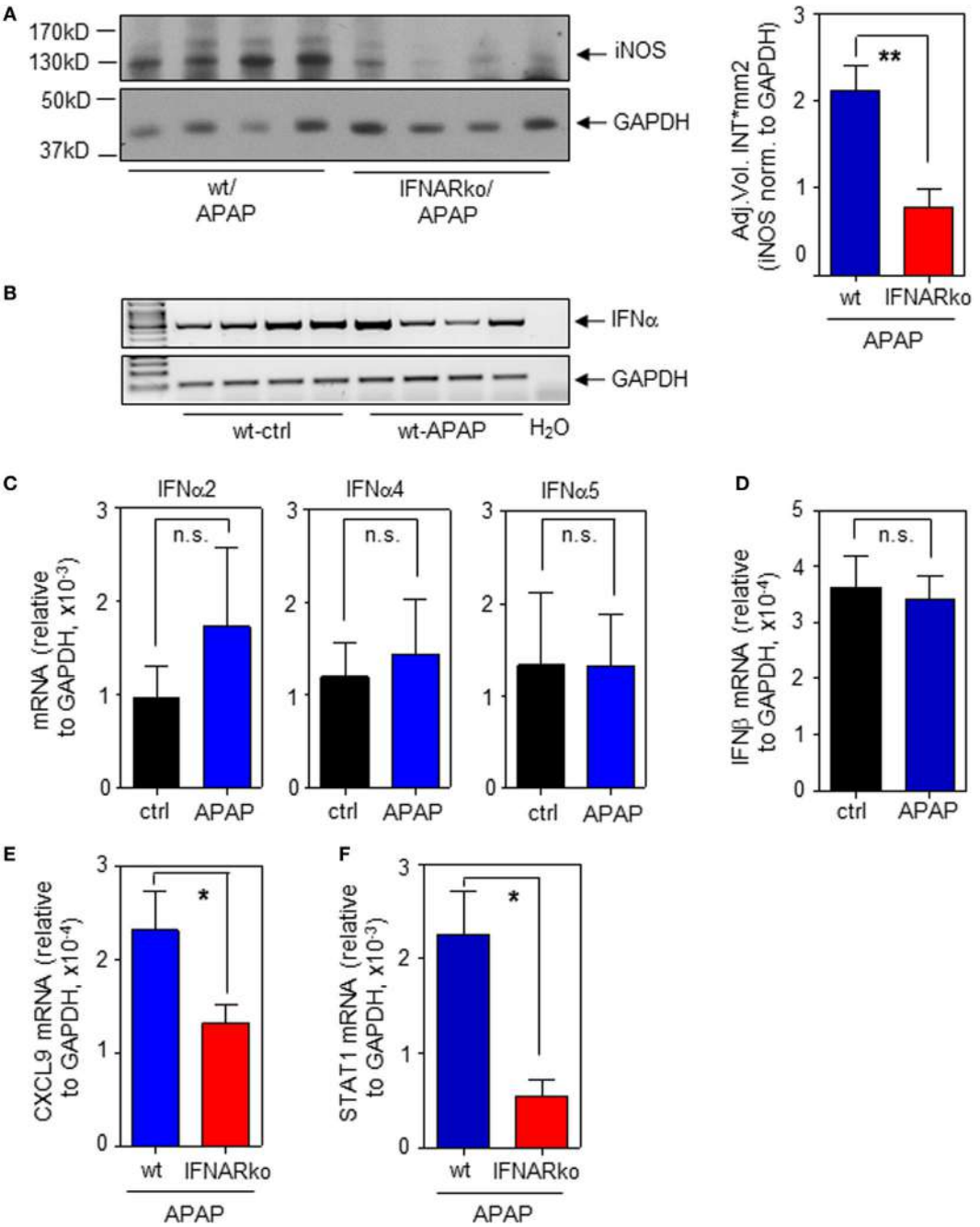

FIGURE 5 | Impaired hepatic inducible nitric oxide synthase (iNOS) expression in interferon-receptor (IFNAR)$^{-/-}$ mice during acetaminophen (APAP)-induced liver injury. Wild-type (wt) and IFNAR$^{-/-}$ mice received NaCl-ctrl solution or APAP (500 mg/kg) and were maintained for 24 h. **(A)** Left panel, hepatic iNOS was determined by immunoblotting (shown are four individual mice per genotype). Right panel, densitometric quantification of iNOS from the left panel with two additional mice per genotype (mean ± SEM, $n = 6$; $^{**}P < 0.01$). **(B)** Hepatic IFNα mRNA was determined by standard PCR using universal primers that target all α-subtypes ($n = 4$ individual mice per group). **(C)** Hepatic IFNα2 (left panel), IFNα4 (middle panel), and IFNα5 (right panel) mRNA were determined by realtime PCR and normalized to GAPDH (mean ± SEM; all subtypes, $n = 4$). **(D)** Hepatic IFNβ was determined by realtime PCR and normalized to GAPDH (mean ± SEM; ctrl, $n = 4$; APAP, $n = 5$). **(E)** Hepatic CXCL9 mRNA was determined by realtime PCR and normalized to GAPDH (mean ± SEM; wt, $n = 8$ individual mice; IFNAR$^{-/-}$, $n = 9$; $^{*}P < 0.05$). **(F)** Hepatic STAT1 mRNA was determined by realtime PCR and normalized to GAPDH (mean ± SEM; $n = 9$ individual mice per genotype; $^{*}P < 0.05$). **(A,E,F)** Statistical analysis, raw data were analyzed by Student's *t*-test.

bridging by CREB binding protein—though STAT1 homodimers are involved in this case (54). The notion of cooperative transcription factor binding, specifically NF-κB enforcing STAT1 binding to the iNOS promoter in IL-1β/TNFα/IFNβ-stimulated Hepa1-6 cells, was confirmed herein by pharmacological inhibition of NF-κB.

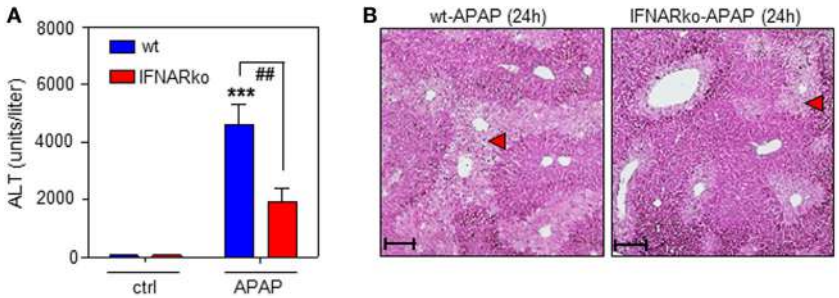

FIGURE 6 | Acetaminophen (APAP)-induced liver injury in wild-type (wt) and interferon-receptor (IFNAR)$^{-/-}$ mice during APAP-induced liver injury. **(A,B)** wt or IFNAR$^{-/-}$ mice received NaCl-ctrl solution ($n = 4$ individual mice per genotype) or APAP (500 mg/kg; $n = 9$ individual mice per genotype). **(A)** After 24 h, serum ALT was determined and is depicted as units/liter (mean ± SEM; $^{***}P < 0.001$ versus NaCl-ctrl solution-treated mice of the same genotype, $^{\#\#}P < 0.01$). Statistical analysis, raw data were analyzed one-way ANOVA with *post hoc* Bonferroni correction. **(B)** Representative liver sections (H&E stain) 24 h after the onset of APAP intoxication. 10×; scale, 200 µm; red triangles indicate areas of necrosis.

Upregulation of iNOS *in vivo* during murine APAP-induced liver injury and sterile inflammation in fact largely depended on type I IFN signaling which associated with pronounced liver injury. Whereas hepatic type I IFN, as assessed by analysis of IFNα/β expression, was not upregulated during APAP intoxication, basal type I IFN was well detectable in murine liver tissue, an observation that agrees with previous reports on constitutive murine hepatic IFNα (55) and IFNβ (56), respectively. Notably, the liver is regarded a major target for constitutively produced type I IFN in healthy mice (57) and low-level "physiological" expression of type I IFN also applies to human liver tissue and hepatocytes (58, 59). By generally promoting signal transduction mechanisms related to cellular activation, constitutive low-level expression of type I IFN is supposed to prime diverse tissues for immunological alertness (45) which may in particular apply to the liver as crucial host/environment-interface serving "firewall" functions (60).

It must, however, be emphasized that regulatory properties of type I IFN during hepatic inflammation are multilayered and context dependent. This is exemplified by the general ability of type I and II IFN to potently upregulate anti-inflammatory IL-1 receptor antagonist (IL-1Ra) (61) which, by inhibiting IL-1 biological activity, enables protection in murine models of nucleic acid (virus)-induced liver damage (22, 62). Interestingly, the role of IL-1 in APAP-induced liver injury is actually discussed controversially with disease aggravating action (63, 64), no significant role (65), or even protective functions (66) being ascribed to this cytokine. Of note, administration of IL-1Ra did not affect disease in the current protocol of APAP-induced liver injury (Bachmann and Mühl, unpublished data), an observation supporting aforementioned previous report (65). Data thus indicate that putative upregulation of potentially protective IL-1Ra by surplus type I IFN falls short in the current pathophysiological context.

Data presented not only relate to sterile inflammation as seen in APAP intoxication but likewise connect to infectious diseases such as viral hepatitis. Interestingly, hepatocytes express iNOS protein during chronic hepatitis C virus (HCV) infection (67) which, according to data presented herein, should be supported by induction of endogenous type I IFN in response to the virus (68). Moreover, HCV patients responding most efficiently to IFNα therapy likewise display increased serum nitrite/nitrate levels (69), an established surrogate marker of iNOS activation during infectious diseases (70).

Taken together, current knowledge and data presented herein suggest that IFNβ supports hepatocyte iNOS by dual complementary action. That is, IFN signaling directly triggers STAT1 biological activity, a process further enhanced by feed-forward upregulation of STAT1 gene expression. Data also suggest a pathogenic role for constitutive type I IFN during the course of APAP intoxication which is regarded a prototypic model for drug-induced injury and sterile inflammation at the liver compartment.

ETHICS STATEMENT

All animal experiments using C57Bl/6 mice (male, 9–10-week-old, wt, and IFNAR$^{-/-}$ mice) were carried out in accordance with the recommendations of the Animal Protection Agency of the Federal State of Hessen (Regierungspräsidium Darmstadt, Germany). The protocol was approved by the Regierungspräsidium Darmstadt (Germany).

AUTHOR CONTRIBUTIONS

HM analyzed the data, designed the study, wrote the paper, and performed manuscript editing. MB performed all experiments, analyzed the data, and contributed to manuscript writing and editing. ZW analyzed the data, provided mice, and technical support. TP provided crucial technical support. JP analyzed the data, provided reagents (antibodies), and contributed to manuscript editing.

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Double-Stranded RNA Derived from Lactic Acid Bacteria Augments Th1 Immunity *via* Interferon-β from Human Dendritic Cells

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Lactic acid bacteria (LAB) are one of the major commensal species in the small intestine and known for contributing to maintenance of protective immunity and immune homeostasis. However, currently there has been no evidence regarding the cellular mechanisms involved in the probiotic effects of LAB on human immune cells. Here, we demonstrated that LAB double-stranded RNA (dsRNA) triggered interferon-β (IFN-β) production by human dendritic cells (DCs), which activated IFN-γ-producing T cells. Interleukin-12 (IL-12) secretion from human DCs in response to LAB was abrogated by depletion of bacterial dsRNA, and was attenuated by neutralizing IFN-β, indicating LAB dsRNA primarily activated the IFN-β/IL-12 pathway. Moreover, the induction of IL-12 secretion from DCs by LAB was abolished by the inhibition of endosomal acidification, confirming the critical role of the endosomal digestion of LAB. In a coculture of human naïve $CD4^+$ T cells and $BDCA1^+$ DCs, DCs stimulated with LAB containing dsRNA induced IFN-γ-producing T cells. These results indicate that human DCs activated by LAB enhance Th1 immunity depending on IFN-β secretion in response to bacterial dsRNA.

Keywords: lactic acid bacteria, double-stranded RNA, human dendritic cells, interferon-β, interleukin-12, Th1

INTRODUCTION

Exposure to bacterial or viral components is critical for the functional maturation of host immunity, including innate and acquired cell populations such as IFN-γ-producing Th1 cells and anti-inflammatory regulatory T cells. Therefore, microorganisms in the intestine are essential for the development of each cellular mechanism, and suppress aberrant Th2 responses at the same time (1–5). Allergic diseases or pollinosis caused by excessive Th2 immune responses may be improved by the induction of Th1 immunity, or by the anti-inflammatory effects of regulatory T cells (6–9).

The recognition of a variety of components from microorganisms by innate immune receptors triggers robust immune responses such as cytokine production (10). Toll-like receptors (TLRs) play a critical role in the recognition of structurally conserved bacterial and viral components, termed pathogen-associated molecular patterns, and signal transduction *via* TLRs induces rapid

anti-infectious responses and sequentially promotes the development of acquired immunity, resulting in the maintenance of long-term homeostatic protective immunity (11–13). TLR2 and TLR4 recognize cell wall components of bacteria, while TLR3/8/9 recognize nucleic acids in endosomes (10, 14). In humans, two subsets of myeloid dendritic cells (DCs), BDCA1$^+$ DCs (mDC1) and BDCA3$^+$ DCs (mDC2), and plasmacytoid DCs (pDCs) are present in peripheral blood mononuclear cells (PBMCs), and mDC1 and pDCs are more abundant compared with mDC2 among these subsets (15–17). mDC1 expressing a variety of TLRs secrete high levels of interleukin-12 (IL-12), while mDC2 expressing high levels of TLR3 secrete IFN-λ, a type III IFN (18). pDCs express TLR7 and TLR9, and robustly secrete IFN-α in response to viral infection (19–21).

Lactic acid bacteria (LAB) are a major microbial species in the small intestine, and are often utilized for fermented food to prolong the preservation period and produce a variety of flavors (22, 23). Probiotic strains of LAB exert immunomodulatory effects, such as anti-infection, anti-allergy, or anti-inflammation in humans and experimental animals (24–28). Recently, it has been reported that endosomal recognition of ssRNA in *Lactococcus lactis* contributes to its allergy-protective effects (29). We previously discovered that LAB contain a large amount of double-stranded RNA (dsRNA) compared with pathogenic bacteria and can induce TLR3-mediated IFN-β production (28). Here, we elucidate the immunomodulatory role of bacterial dsRNA that induce IFN-β and IL-12 production from human DCs. Furthermore, how bacterial dsRNA promotes Th1 differentiation and that the induction of IFN-γ-producing T cells is partially dependent on IFN-β.

MATERIALS AND METHODS

Preparation of LAB

Lactic acid bacteria were purchased from the Japan Collection of Microorganisms (JCM) or isolated from fermented foods (Table S1 in Supplementary Material). *Pediococcus acidilactici* strain K15, *Lactobacillus plantarum* ATCC14197^T, *Lactobacillus pentosus* ATCC8041^T, and *Lactococcus lactis* subsp. *lactis* ATCC19435^T were cultured at 30°C for 24 h in MRS broth (BD). *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC11842^T and *Lactobacillus rhamnosus* ATCC53103^T (LGG) were cultured at 37°C for 24 h in MRS broth. Four strains of *Bacteroides* sp. were cultured at 37°C for 24 h in GAM broth (Nissui Pharmaceutical Co. Ltd.). Then, they were heat-killed at 95°C for 10 min, washed twice with saline, and suspended in saline. For the nuclease treatment of heat-killed bacteria, RNase A (from bovine pancreas, Sigma) treatment was performed under low salt conditions (10 mM Tris–HCl, pH 8.0) or high salt conditions (10 mM Tris–HCl, 0.3 M NaCl, pH 8.0) at 37°C for 2 h. RNase A-treated bacteria were washed twice with each buffer and used for subsequent experiments.

Cell Preparation

Blood was provided from consenting, healthy donors in accordance with the Ethics Committee of Kikkoman Corporation (Chiba, Japan), and PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare). mDC1 were isolated from PBMCs by CD1c$^+$ (BDCA1$^+$) Dendritic Cell Isolation Kit (Miltenyi Biotec). Cell purity was >98% as assessed by staining with FITC-conjugated anti-CD11c antibody (Ab), BV421-conjugated anti-CD1c Ab and APC-conjugated anti-HLA-DR Ab (BioLegend). Naïve CD4$^+$ T cells were isolated by Naïve CD4$^+$ T Cell Isolation Kit II (Miltenyi Biotec). Cell purity was >98% as assessed by staining with FITC-conjugated anti-CD45RA Ab and APC-conjugated anti-CD4 Ab (BioLegend). Monocyte-derived DCs (moDCs) were prepared by culturing CD14$^+$ monocytes isolated from PBMCs using CD14 Microbeads (Miltenyi Biotec) for 7 days in culture medium including IL-4 and GM-CSF (PeproTech).

Cytokine Analysis

Peripheral blood mononuclear cells were cultured in 96-well round-bottomed plates at 5×10^5 cells/well/200 µl in the presence or absence of 2×10^7 bacteria for 24 h. moDCs were cultured at 1×10^5 cells/well/200 µl with 2×10^7 bacteria for 24 h. mDC1 were cultured at 5×10^4 cells/well/200 µl with 1×10^7 bacteria for 24 h. For the analysis of T cell cytokines, PBMCs were cultured at 1×10^5 cells/well/250 µl with 1×10^7 bacteria, plate-bound anti-CD3 Ab and IL-2 for 5 days. The level of cytokines in culture supernatants was measured by specific ELISA Sets (eBioscience).

Flow Cytometric Analysis

Naïve CD4$^+$ T cells purified from PBMCs were stimulated with mDC1 in culture medium containing anti-CD3/CD28 Dynabeads (Invitrogen) and cytokines [IL-2 under neutral conditions; and IL-2, IL-4, and anti-IFN-γ monoclonal Ab (mAb) under Th2 conditions] in the absence or presence of K15 for 4 days, and then cells were cultured in IL-2 containing medium without Dynabeads for 3 days. Thereafter, cells were suspended in PBS supplemented with 2% FBS at a concentration of $1–10 \times 10^6$ cells/ml and stained with the optimal concentration of FITC-conjugated anti-CD4 Ab (eBioscience). Then, the cells were fixed in 3% paraformaldehyde buffer (BD PharMingen) for 30 min and cell membranes were permeabilized prior to staining with BV421-conjugated anti-IFN-γ Ab (BioLegend) and PE-conjugated anti-IL-4 Ab (BioLegend) for 30 min. Cells were analyzed on a FACSAria II and FlowJo software.

Reagents

To neutralize IFN-β, anti- IFN-β mAb (BioLegend, mouse IgG1 Ab) was added at 10 µg/ml. Mouse IgG_1 Ab (BioLegend) was used as an isotype control Ab. Poly(I:C) and LPS (both purchased from InvivoGen) were added at 50 and 10 µg/ml for each TLR ligand. To inhibit endosomal acidification, chloroquine (Sigma) was added at 5 µM.

Analysis of Bacterial dsRNA

Nucleic acid was extracted from untreated or heat-killed bacteria. The concentration of bacterial dsRNA was determined by sandwich ELISA using mAb K1 and biotinylated mAb J2 (English and Scientific Consulting) for detection, followed by streptavidin peroxidase (Zymed) (28). The concentration of dsRNA was calculated using poly(I:C) as a standard.

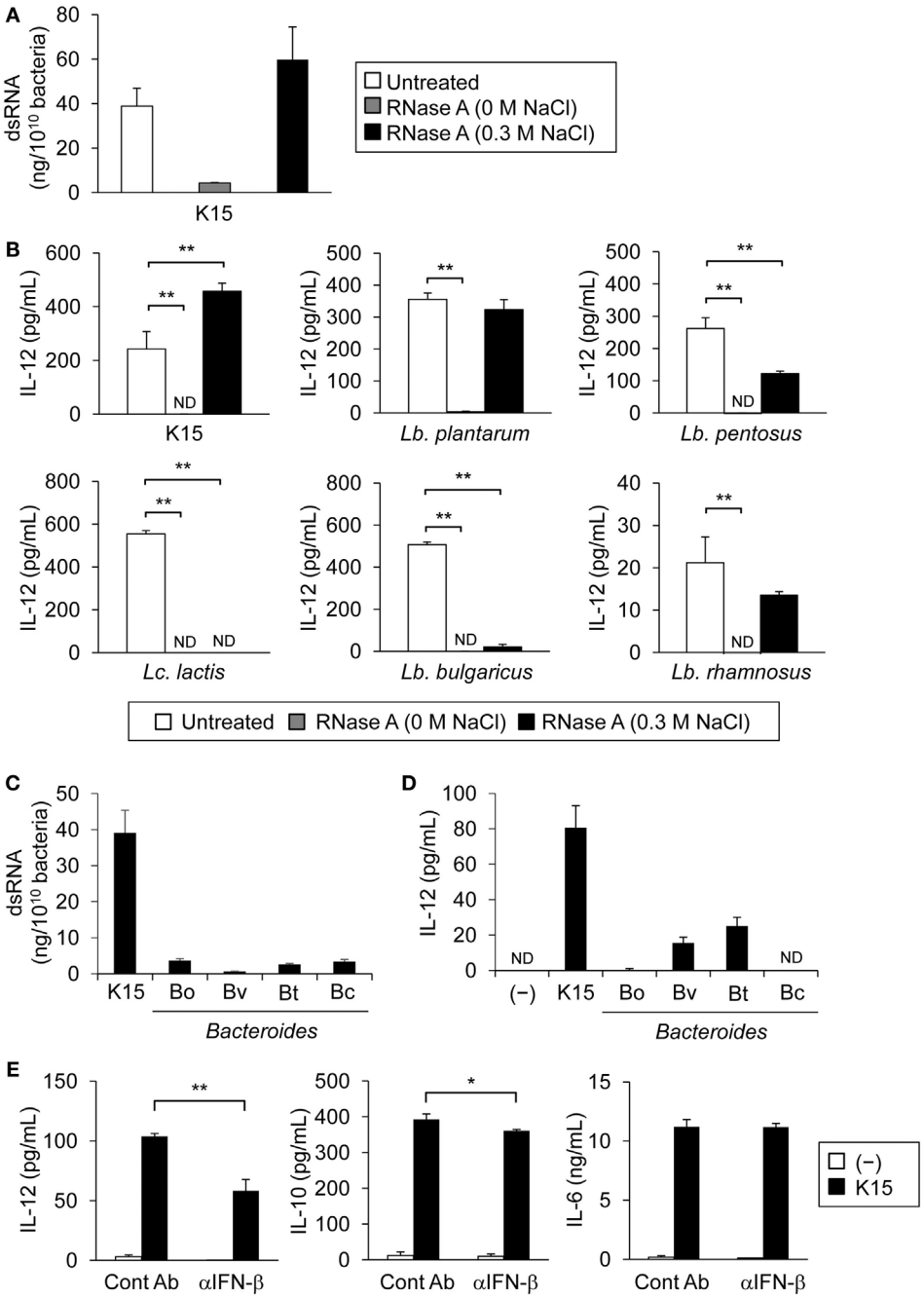

FIGURE 1 | Double-stranded RNA (dsRNA) in lactic acid bacteria (LAB) induce interleukin-12 (IL-12) secretion through IFN-β from peripheral blood mononuclear cells (PBMCs). **(A)** dsRNA in *P. acidilactici* strain K15 was quantified by sandwich ELISA. Heat-killed K15 was treated with RNase A in the absence of NaCl (0 M NaCl) to degrade ssRNA and dsRNA or under 0.3 M NaCl for the degradation of ssRNA alone. Data are the mean ± SD of triplicates and are representative of two independent experiments. **(B)** PBMCs were cultured in medium alone (–) or stimulated with untreated or RNase A-treated heat-killed LAB strains for 24 h. Tested strains are described in **Table 1**. IL-12 concentration in culture medium was quantified by ELISA. Data are the mean ± SD of triplicates and are representative of two different donors (ND: not detected). $^{**}p < 0.01$ (vs untreated, Student's *t*-test). **(C)** dsRNA in heat-killed K15 and *Bacteroides* sp. was quantified by sandwich ELISA. Data are the mean ± SD of triplicates and are representative of two independent experiments. **(D)** PBMCs were cultured in medium alone (–) or stimulated with heat-killed K15 or *Bacteroides* sp. for 24 h. Tested strains are described in **Table 1**. IL-12 concentration in culture medium was quantified by ELISA. Data are the mean ± SD of triplicates and are representative of two different donors (ND: not detected). **(E)** PBMCs were cultured in medium alone (–) or stimulated with heat-killed K15 in the presence or absence of 20 μg/ml anti-human IFN-β monoclonal Ab (mAb) (αIFN-β) for 24 h. Mouse IgG1 Ab was used as the isotype control (Cont Ab). IL-12, IL-10, and IL-6 concentrations in culture medium were quantified by ELISA. Data are the mean ± SD of triplicates and are representative of two different donors. $^{*}p < 0.05$, $^{**}p < 0.01$ (Student's *t*-test).

Quantitative RT-PCR

Total RNA was extracted from cells with a NucleoSpin RNA Kit (Takara) following the manufacturer's instructions. An equal amount of total RNA (300 ng) corresponding to each priming dose was reverse-transcribed using PrimeScript RT Reagent (Takara). The cDNA obtained after reverse transcription was amplified using specific primers (purchased from Takara) and SYBR Premix Ex Taq II (Takara) following the protocols provided.

Statistical Analysis

Error bars indicate the SD of triplicate samples of experiments in cell culture assays. The statistical significance was determined with a two-tailed Student's *t*-test for unpaired data, and *p* values <0.05 were considered significant ($^*p < 0.05$, $^{**}p < 0.01$).

RESULTS

IL-12 Secretion from PBMCs in Response to LAB-Derived dsRNA Is Partially Dependent on IFN-β

We previously reported a higher amount of dsRNA present in LAB compared with pathogenic bacteria, which contributed to anti-inflammation *via* induction of IFN-β secretion from murine DCs (28). Here, we investigated whether dsRNA in LAB triggers IFN-β production in human cells as well. RNase A digest ssRNA only in the presence of 0.3 M NaCl, but digest ssRNA and dsRNA in the absence of NaCl (30). Importantly, after treatment with RNase A with 0.3 M NaCl, the amount of dsRNA in a heat-killed LAB, *P. acidilactici* strain K15, was comparable to that in untreated K15 (**Figure 1A**). When PBMCs were stimulated with untreated LAB strains described in **Table 1**, IL-12 secretion from PBMCs was induced by various strains. However, it was abolished when both ssRNA and dsRNA were digested (**Figure 1B**), indicating RNA is essential for LAB to induce the secretion of IL-12. Among them, K15, *Lb. plantarum*, *Lb. pentosus*, and *Lb. rhamnosus* retained their ability to induce IL-12 under the condition that only ssRNA was digested. On the other hand, *Lc. lactis* and *Lb. bulgaricus* lost their ability to induce IL-12 after digestion of their ssRNA (**Figure 1B**; Figure S1A in Supplementary Material). These results indicate that dsRNA contained in LABs largely contributes to IL-12 induction in most of LAB strains tested while in some LAB strains ssRNA is involved in the induction of IL-12. Different effects of dsRNA and ssRNA observed among different species of LAB are brought by yet unknown mechanisms. The secretion of IL-10 and IL-6 from PBMC induced by LABs was not attenuated by digesting RNAs (Figure S1B in Supplementary Material). We also confirmed that dsRNA contained in other commensal species of bacteria, *Bacteroides*, was detected at very low level (**Figure 1C**), and that they induced less amount of IL-12 secretion from PBMCs compared with a representative LAB strain, K15 (**Figure 1D**; Figure S1C in Supplementary Material).

In our previous experiments using murine cells, dsRNA in LAB uniquely stimulated innate immune system and induced IFN-β production via TLR3 pathway (28). We next evaluated the involvement of IFN-β in IL-12 secretion by human PBMCs, in response to LAB. In a coculture of PBMCs with heat-killed K15 in the presence of neutralizing Ab to IFN-β, IL-12 secretion induced by K15 was significantly reduced, whereas production of IL-10 was slightly reduced and IL-6 was not affected, respectively (**Figure 1E**; Figure S1D in Supplementary Material). These results indicate that IFN-β production by PBMCs in response to LAB augments IL-12 secretion.

TABLE 1 | Strain number and organism names of bacteria.

Organism name	Strain No.	Abbreviation
Lactic acid bacteria		
Pediococcus acidilactici	K15	K15
Lactobacillus plantarum	ATCC14197^T	*Lb. plantarum*
Lactobacillus pentosus	ATCC8041^T	*Lb. pentosus*
Lactococcus lactis subsp. *lactis*	ATCC19435^T	*Lc. lactis*
Lactobacillus delbrueckii subsp. *bulgaricus*	ATCC11842^T	*Lb. bulgaricus*
Lactobacillus rhamnosus	ATCC53103^T	*Lb. rhamnosus*
***Bacteroides* sp.**		
Bacteroides ovatus	ATCC8483^T	Bo
Bacteroides vulgatus	ATCC8482^T	Bv
Bacteroides thetaiotaomicron	ATCC29148^T	Bt
Bacteroides caccae	ATCC43185^T	Bc

IL-12 Secretion from moDCs in Response to LAB-Derived dsRNA Is Partially Dependent on IFN-β

To evaluate the response of DCs to K15, moDCs were stimulated with K15 in the presence or absence of neutralizing Ab to IFN-β. As observed for PBMCs, IL-12 secretion from moDCs was induced by stimulation with K15 and was suppressed by IFN-β neutralization. IL-10 secretion was slightly attenuated by anti-IFN-β mAb and IL-6 was not affected (**Figure 2A**; Figure S2A in Supplementary Material). A previous study reported two subsets of moDCs: $CD1a^+$ moDCs activated by CD40L produce a higher level of IL-12 than $CD1a^-$ moDCs, whereas IL-10 secretion was higher in $CD1a^-$ moDCs (31). When we stimulated each subset of moDCs with K15 after sorting (Figure S2B in Supplementary Material), both $CD1a^+$ and $CD1a^-$ moDCs produced IL-12 but more efficiently by $CD1a^+$ cells. IL-6 and IL-10 $CD1a^-$ were preferentially secreted by $CD1a^-$ moDCs (Table S1 in Supplementary Material).

Next, we evaluated the involvement of dsRNA in the response of moDCs to K15. In a coculture of moDCs with RNase A-treated K15, the secretion of IL-12, but not IL-6 and IL-10, was strongly impaired by the depletion of ssRNA and dsRNA (**Figure 2B**; Figure S2C in Supplementary Material). The enhancement of mRNA expression of IFN-β and IL-12 by K15 stimulation was also impaired by the degradation of bacterial dsRNA (**Figure 2C**; Figure S2D in Supplementary Material). The expressions of genes encoding IRF families, such as IRF1, IRF7, and IRF8, which control the transcription of IL-12 (32, 33), were enhanced by K15 stimulation, and depletion of ssRNA and dsRNA impaired the induction of IRFs, especially IRF7 and IRF8 (**Figure 2C**; Figure S2D in Supplementary Material). Depletion of ssRNA alone in K15 did not affect IL-12 production or the mRNA expression of

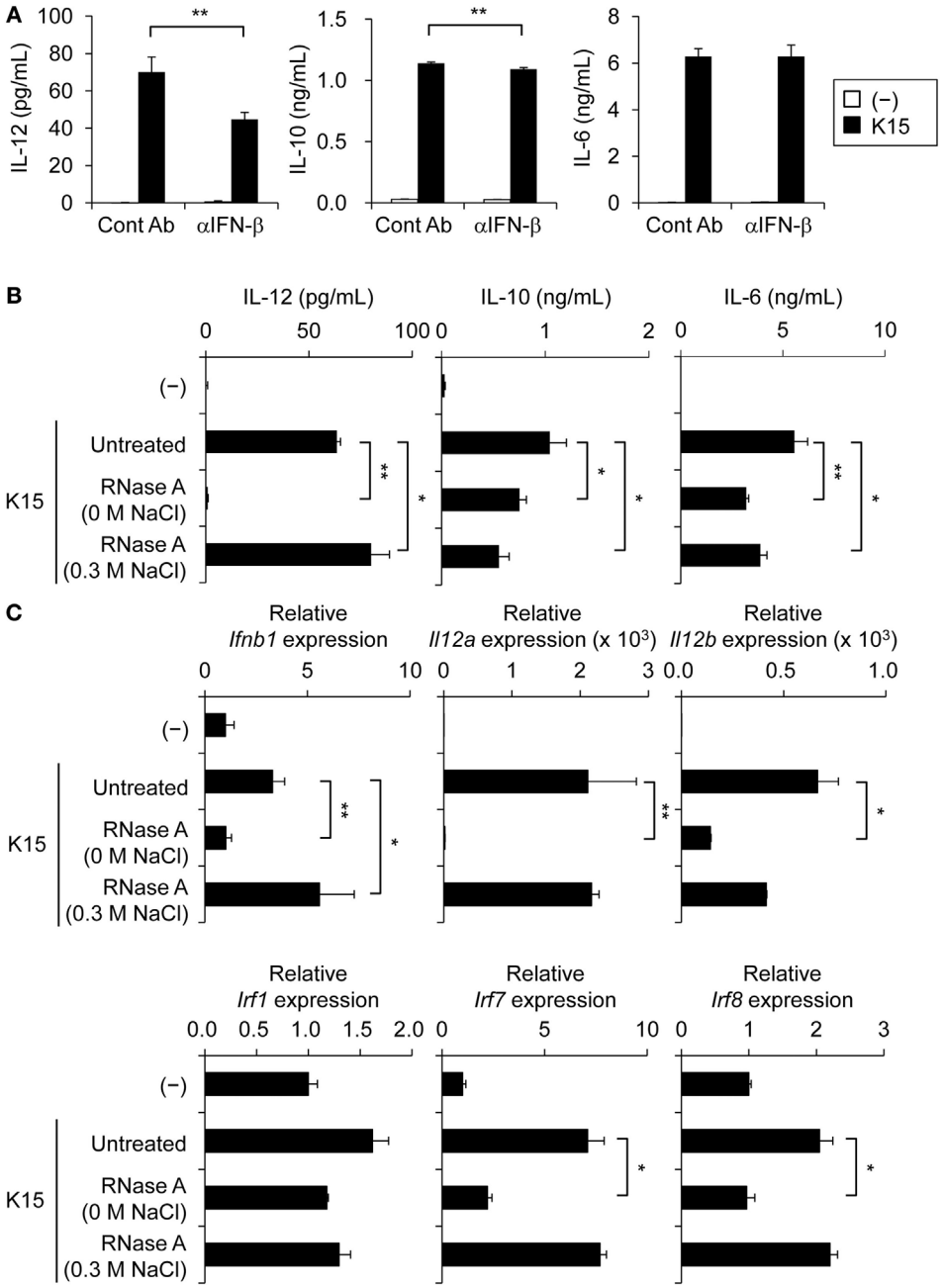

FIGURE 2 | IFN-β is involved in interleukin-12 (IL-12) secretion by monocyte-derived DCs (moDCs) stimulated with bacterial double-stranded RNA (dsRNA). **(A)** moDCs were cultured in medium alone (–) or stimulated with heat-killed K15 in the presence or absence of 20 μg/ml anti-human IFN-β monoclonal Ab (mAb) (αIFN-β) for 24 h. Mouse IgG1 Ab was used as the isotype control (Cont Ab). IL-12, IL-10, and IL-6 concentrations in culture medium were quantified by ELISA. Data are the mean ± SD of triplicates and are representative of two different donors. $^{**}p < 0.01$ (Student's *t*-test). **(B)** moDCs were cultured in medium alone (–) or stimulated with untreated or RNase A-treated heat-killed K15 for 24 h. IL-12, IL-10, and IL-6 concentrations in culture medium were quantified by ELISA. Data are the mean ± SD of triplicates and are representative of two different donors. $^{*}p < 0.05$, $^{**}p < 0.01$ (vs untreated K15, Student's *t*-test). **(C)** Levels of IFN-β, IL-12, and IRF mRNA expressions were determined by quantitative RT-PCR. Total RNA was extracted at 9 h after stimulation with heat-killed K15. Expression is represented as relative expression compared with unstimulated moDCs. Data are the mean ± SD of triplicates and are representative of two different donors. $^{*}p < 0.05$, $^{**}p < 0.01$ (vs untreated K15, Student's *t*-test).

IRF7 and IRF8, but slightly attenuated IL-6, IL-10 production and IRF1 mRNA expression (**Figures 2B,C**; Figure S2C,D in Supplementary Material). These results indicate that dsRNA is essential for the induction of IFN-β secretion and IRF7/8 mRNA expression in moDCs in response to K15, and that IL-12 secretion partially depends on this IFN-β response.

Endosomal Processes Are Essential for IL-12 Secretion in Response to LAB

TLR3, 8, and 9, which recognize nucleic acids, are localized to the endosome of DCs. As we showed in the previous study that dsRNA in LAB is recognized by endosomal TLR3 and induce IFN-β production in murine DCs (28), endosomal processes are likely to be essential for the response of human DCs to LAB. We investigated the process by stimulating moDCs with K15 in the presence of hydroxychloroquine (also called chloroquine), which blocks the signal transduction of endosomal TLRs by inhibiting endosomal acidification (34). Poly(I:C) is used as a control that stimulates endosomal TLR3, and LPS binds to TLR4 on the cell surface.

We observed that the production of IL-12 by moDCs in response to K15 was significantly higher than control TLR ligands, and it was totally blocked in the presence of chloroquine. The same treatment did not attenuate the production of IL-6 and IL-10 (**Figure 3**; Figure S3 in Supplementary Material). In stimulation with *Bacteroides* sp., IL-12 secretion was suppressed by chloroquine in some strains but this inhibitory effect was partial and strain dependent (**Figure 3**; Figure S3 in Supplementary Material). These results indicate that the signal transduction of endosomal TLRs is essential for the induction of IL-12 against LAB in moDCs. By contrast, the production of IL-6 and IL-10 are partially induced by RNAs but largely dependent on other recognition mechanisms.

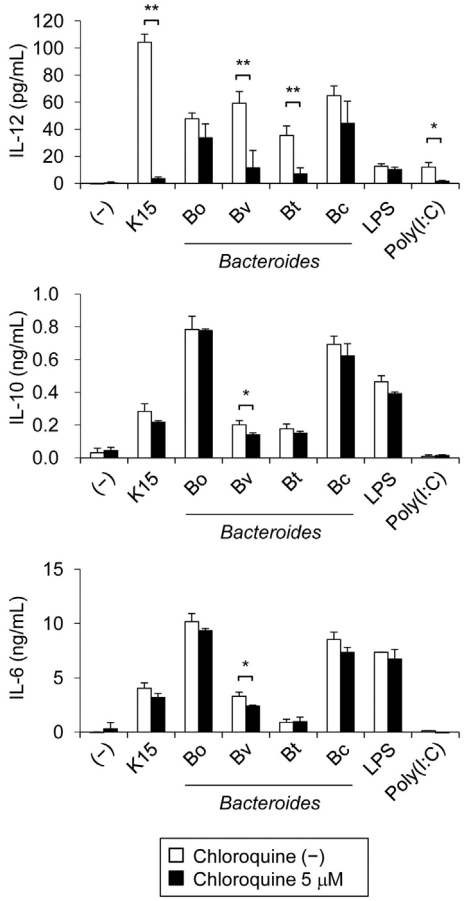

FIGURE 3 | Endosomal processing is essential for interleukin-12 (IL-12) secretion by monocyte-derived DCs (moDCs) in response to lactic acid bacteria (LAB). moDCs were cultured in medium alone (–) or stimulated with heat-killed K15, *Bacteroides* sp., LPS or poly(I:C) in the presence or absence of chloroquine (5 μM) for 24 h. IL-12, IL-10, and IL-6 concentrations in culture medium were quantified by ELISA. Data are the mean ± SD of triplicates and are representative of two different donors. $^{*}p < 0.05$, $^{**}p < 0.01$ (Student's *t*-test).

dsRNA in LAB Promotes the Differentiation of IFN-γ-Producing T Cells

Next, we assessed the IL-12 response of myeloid DCs expressing BDCA1, termed mDC1, in PMBC to evaluate the potential activity of LAB in contact with peripheral DCs under steady-state conditions. As also indicated from **Figure 1B**, sorted cell population of mDC1 secreted IL-12 in response to K15, which was impaired by the degradation of ssRNA and dsRNA, but not by the degradation of ssRNA alone (**Figure 4A**; Figure S4A in Supplementary Material). We concluded that mDC1 should be functional antigen-presenting cells to T cells in the presence of LAB, as they secrete IL-12 in response to dsRNA in LAB similar to moDCs. We also confirmed that K15 stimulation upregulate the expression of HLA-DR and CD86 (Figures S4B,C in Supplementary Material) that is required for efficient co-stimulation to T cells upon antigen stimulation. We cocultured mDC1 and naïve $CD4^+$ T cells from PBMCs in the presence of anti-CD3 Ab and IL-2, and analyzed T cell differentiation in response to K15 under neutral and Th2 conditions. K15 stimulation promoted the differentiation of IFN-γ producing T cells under both conditions, and strongly suppressed IL-4-producing T cell differentiation under Th2 conditions (**Figures 4B,C**; Figures S4D,E in Supplementary Material). RNase A treatment that digested dsRNA attenuated the ability of K15 to induce IFN-γ producing T cells under both conditions (**Figures 4B,C**; Figures S4D,E in Supplementary Material). The reduction of IFN-γ producing T cells by the digestion of dsRNA is probably caused by the attenuated IFN-β production and consequent reduction of IL-12 production.

Similar results were obtained in the whole assay of PBMCs including DCs and T cells when stimulated with K15 in the presence of anti-CD3 Ab and IL-2. K15 stimulation enhanced the secretion of IFN-γ produced by Th1 cells, and this induction was attenuated in the presence of neutralizing Abs to IFN-β (**Figure 4D**; Figure S4F in Supplementary Material). IL-4 and IL-17 produced by Th2 and Th17 cells, respectively, were suppressed by K15 but neutralization of IFN-β did not affect these cytokine productions (**Figure 4D**; Figure S4F in Supplementary Material). These results indicate that K15 induce robust IL-12 secretion from DCs *via* IFN-β production, resulting in the secretion of IFN-γ. Together these data confirm that bacterial dsRNA is involved in the recognition of K15 and contributes to the secretion of IL-12 from DCs and the differentiation of T cells toward IFN-γ producing cells, providing the molecular mechanisms to support the idea that probiotic LAB enhance Th1 immunity under steady-state conditions.

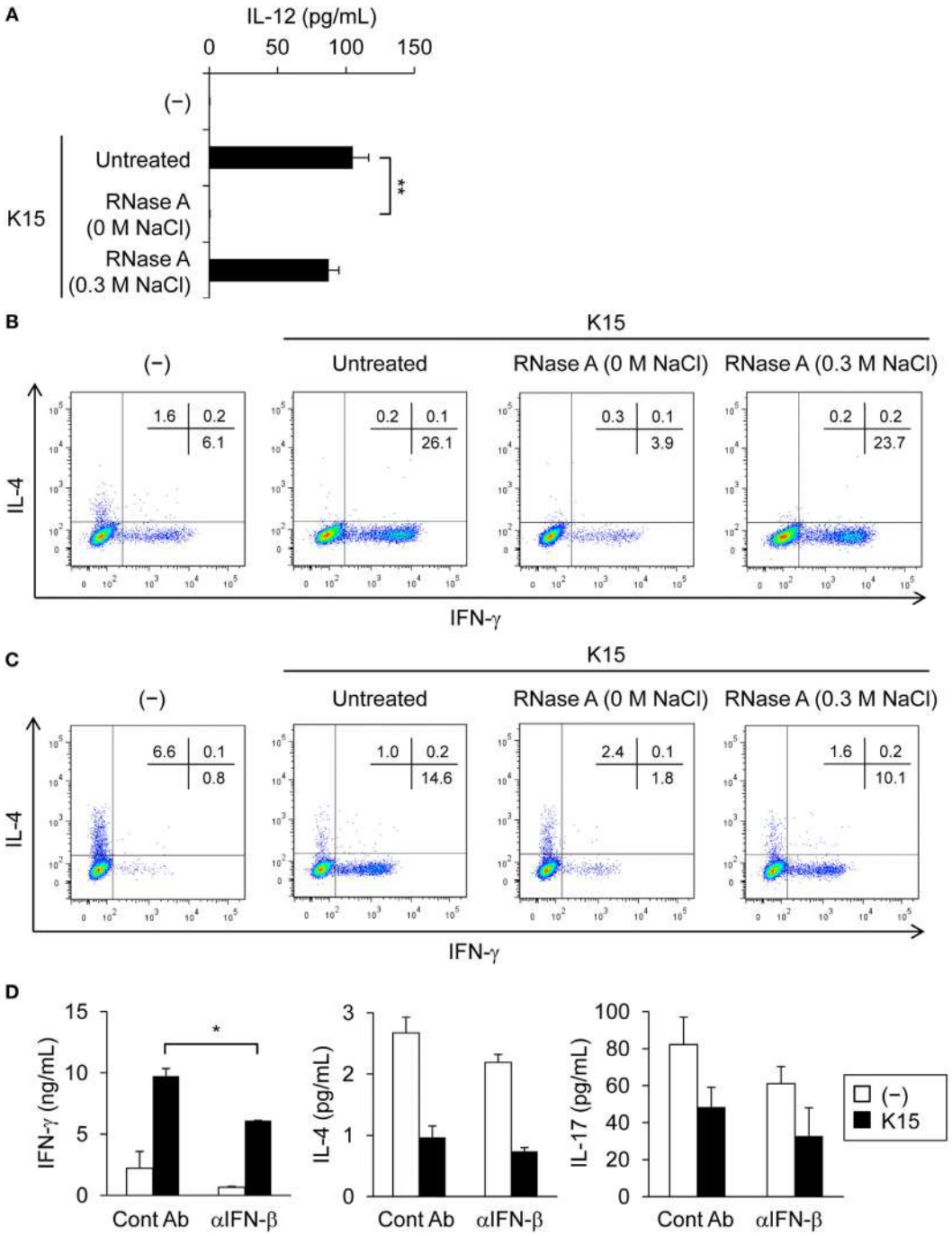

FIGURE 4 | Th1 cell differentiation is induced by dendritic cells (DCs) in response to double-stranded RNA (dsRNA) in lactic acid bacteria (LAB). **(A)** BDCA1$^+$ DCs (mDC1) were cultured in medium alone (–) or stimulated with untreated or RNase A-treated (0 M or 0.3 M NaCl) heat-killed K15 for 24 h. Interleukin-12 (IL-12) concentrations in culture medium were quantified by ELISA. Data are the mean ± SD of triplicates and are representative of two different donors. $^{**}p < 0.01$ (vs untreated K15, Student's *t*-test). **(B,C)** Naïve CD4$^+$ T cells purified from peripheral blood mononuclear cells (PBMCs) were stimulated with mDC1 in the absence (–) or in the presence of untreated or RNase A-treated (0 M or 0.3 M NaCl) K15 under neutral conditions containing only IL-2 **(B)** or Th2 conditions containing IL-2, IL-4, and anti-IFN-γ monoclonal Ab (mAb) **(C)**. After 7 days, the percentage of cells producing cytoplasmic IFN-γ and IL-4 was determined by flow cytometry. Numbers indicate the percentage of the total cells present in each quadrant. Data are representative of two different donors. **(D)** PBMCs were cultured in medium alone (–) or stimulated with heat-killed K15 in the presence or absence of 20 μg/ml anti-human IFN-β mAb (αIFN-β) for 5 days. IFN-γ, IL-4, and IL-17 concentrations in culture medium were quantified by ELISA. Data are the mean ± SD of triplicates and are representative of two different donors. $^{*}p < 0.05$ (Student's *t*-test).

DISCUSSION

Here, we demonstrated that bacterial dsRNA in most strains of LAB tested was essential for IL-12 secretion from human DCs. Moreover, it was shown that IL-12 induction by stimulation with heat-killed LAB was enhanced by IFN-β through type I IFN receptors and upregulation of IRF gene transcription. Currently, there has been no evidence regarding the cellular mechanisms involved in the probiotic effects of LAB on human immune cells, we investigated the mechanisms on this issue using DCs and T cells from human peripheral blood for the first time. IL-12 and IFN-γ are major cytokines that drive T cell immunity toward Th1 response (35, 36). Other cytokines, such as IL-18 and IL-27, are also important for the efficient induction of Th1 responses *in vitro* and *in vivo* (26, 37). The enhancement of Th1 immunity by LAB, which is induced by IL-12 production from antigen-presenting cells, was observed in experimental animals (25, 26, 38, 39). Major components of the bacterial cell wall from Gram-positive bacteria enhanced IL-12 production by DCs or macrophages *via* TLR2 and/or TLR4 signaling (40, 41). However, in addition to those knowledge, we have shown that bacterial RNA is strongly involved in the induction of IL-12 production upon stimulation with LAB. A previous report indicated the involvement of bacterial ssRNA (or total RNA) in IL-12 production or type I IFNs by DCs (29, 42, 43), which, in our experiments, was observed in two species, *Lc. lactis* and *Lb. bulgaricus*, among all tested LAB. As we reported previously, a large amount of dsRNA as cellular components is a characteristic of LAB (28). Our present data now revealed that elimination of RNA abolish the production of IL-12 from PBMCs and moDCs in response to LAB. Thus RNA, especially dsRNA, is essential for the induction of IL-12. Combination effects with other molecules than IFN-β induced by the recognition of nucleic acids in LAB seems to be important to fully explain this interesting observation. Collectively, probiotic or commensal LAB seems to induce IL-12 efficiently through both the canonical pathway *via* TLR2/TLR4 and the nucleic acid sensing pathway *via* TLR3/TLR8 (28, 29, 44–46), and its synergistic effects should be uncovered in the near future.

Importantly, the IFN-β-IL-12 pathway in response to LAB was demonstrated to bridge the IL-12/IFN-γ axis in T cell immunity. Although IFN-β production is triggered by stress induced by pathogens, such as viral and bacterial infection (44–46), it was also induced by stimulation with LAB-derived dsRNA *in vitro* and *in vivo* (28). Thus, dsRNA of LAB in microbiota or fermented foods might be functional ligands for endosomal TLR3 of intestinal DCs and lead to the maintenance of T cell immune homeostasis. We also tested Bacteroides species among other commensal species which mostly reside in large intestine, and showed the clear difference from LAB. Only small amount of dsRNA was detected in Bacteroides species; therefore, the role of TLR3 seems to be marginal in the responsiveness to those commensal bacteria. This observation matches well with our previous finding that TLR3 is important to maintain the level of IFN-β in the small intestine where LAB is the major commensal bacteria species. The IFN-β-IL-12 pathway in DCs that enhance Th1 immunity seems to be unique to LAB as small intestinal commensal bacteria.

Toll-like receptor signal-mediated type I IFNs induce the expression of IFN-regulated genes including IRF7 (47, 48) and consequently contribute to the production of inflammatory cytokines, such as IL-12p35 (IL-12A) (49–51). Thus, in the regulation of IL-12p70 production, IL-12p40 (IL-12B) is induced by NF-κB activation (32) whereas IL-12p35 gene expression is induced by IRF family molecules (33, 52). We observed that the production of IL-12p70 by moDCs in response to K15 was significantly higher than control TLR ligands and it was totally blocked in the presence of chloroquine, confirming the critical role of the endosomal digestion of LAB and the recognition of their nucleic acids *in situ*. The same treatment also attenuated the production of IL-6 and IL-10 but only partially. Our data show that IRF7 mRNA expression is strongly induced by K15 and that the degradation of bacterial dsRNA suppressed IRF7 and IFN-β mRNA induction. Therefore, IFN-β production induced by bacterial dsRNA, recognized in endosome, is important for IRF7 mRNA induction and the subsequent gene transcription of IL-12p35. Since IL-12p70 was barely produced from moDCs with poly(I:C) stimulation alone at the time point of 24 h (53), it is suggested that the combination of TLR ligands in LAB contribute to induce IL-12p70, i.e., both IL-12p35 and IL-12p40, resulting in the development of Th1 immune responses. There are many reports for the immunomodulatory effects of LAB on allergic diseases, and the cellular mechanisms we showed in the present study may involve such probiotic effects.

ETHICS STATEMENT

This study was carried out in accordance with the Ethics Committee of Kikkoman Corporation (Chiba, Japan, No. KC-RD1 and KC-RD9), and blood samples were acquired from healthy volunteers under informed written consent in compliance with the Declaration of Helsinki (2013).

AUTHOR CONTRIBUTIONS

TK and NI equally contributed to this work. TK, SM, NS, and NMT conceived and designed experiments. TK, NI, YW, and YK performed experiments and analyzed data. SY and TK contributed reagents/materials/analysis tools. TK, NI, and NMT wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00027/full#supplementary-material.

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Genetic Regulation of Guanylate-Binding Proteins 2b and 5 during Leishmaniasis in Mice

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Interferon-induced GTPases [guanylate-binding proteins (GBPs)] play an important role in inflammasome activation and mediate innate resistance to many intracellular pathogens, but little is known about their role in leishmaniasis. We therefore studied expression of *Gbp2b/Gbp1* and *Gbp5* mRNA in skin, inguinal lymph nodes, spleen, and liver after *Leishmania major* infection and in uninfected controls. We used two different groups of related mouse strains: BALB/c, STS, and CcS-5, CcS-16, and CcS-20 that carry different combinations of BALB/c and STS genomes, and strains O20, C57BL/10 (B10) and B10.O20, OcB-9, and OcB-43 carrying different combinations of O20 and B10 genomes. The strains were classified on the basis of size and number of infection-induced skin lesions as highly susceptible (BALB/c, CcS-16), susceptible (B10.O20), intermediate (CcS-20), and resistant (STS, O20, B10, OcB-9, OcB-43). Some uninfected strains differed in expression of *Gbp2b/Gbp1* and *Gbp5*, especially of *Gbp2b/Gbp1* in skin. Uninfected BALB/c and STS did not differ in their expression, but in CcS-5, CcS-16, and CcS-20, which all carry BALB/c-derived *Gbp* gene-cluster, expression of *Gbp2b/Gbp1* exceeds that of both parents. These data indicate *trans*-regulation of *Gbp*s. Infection resulted in approximately 10× upregulation of *Gbp2b/Gbp1* and *Gbp5* mRNAs in organs of both susceptible and resistant strains, which was most pronounced in skin. CcS-20 expressed higher level of *Gbp2b/Gbp1* than both parental strains in skin, whereas CcS-16 expressed higher level of *Gbp2b/Gbp1* than both parental strains in skin and liver. This indicates a *trans*-regulation present in infected mice CcS-16 and CcS-20. Immunostaining of skin of five strains revealed in resistant and intermediate strains STS, CcS-5, O20, and CcS-20 tight co-localization of Gbp2b/Gbp1 protein with most *L. major* parasites, whereas in the highly susceptible strain, BALB/c most parasites did not associate with Gbp2b/Gbp1. In conclusion, expression of *Gbp2b/Gbp1* and *Gbp5* was increased even in organs of clinically asymptomatic resistant mice. It suggests a hidden inflammation, which might contribute to control of persisting parasites. This is supported by the co-localization of Gbpb2/Gbp1 protein and *L. major* parasites in skin of resistant and intermediate but not highly susceptible mice.

Keywords: *Leishmania major*, recombinant congenic strains, guanylate-binding proteins, a hidden inflammation, genetic control

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INTRODUCTION

Guanylate-binding proteins (GBPs) are components of cell-autonomous immunity playing a key role in response to intracellular infections [reviewed in Ref. (1–3)]. Besides their role in defense against pathogens, they influence cellular proliferation, adhesion, and migration [reviewed in Ref. (4)], and some members have direct anti-tumorigenic effect on tumor cells (5). GBPs and Gbps were first detected as a 67 kDa protein fraction after stimulation of different human and mouse cell lines with IFN (6) and further characterized as a GBP after stimulation of human and mouse fibroblasts with IFNα, IFNβ, and IFNγ (7). There are currently seven GBPs known in humans (encoded by genes located on the chromosome 1) [reviewed in Ref. (3, 8)] and 11 Gbps in mouse. *Gbp2b*/*Gbp1*, *Gbp2*, *Gbp3*, *Gbp5*, and *Gbp7* map to chromosome 3, whereas *Gbp4*, *Gbp6*, *Gbp8*, *Gbp9*, *Gbp10*, and *Gbp11* are localized on chromosome 5 (9). These proteins are highly conserved and belong to dynamin superfamily—multidomain mechano-chemical GTPases, which are implicated in nucleotide-dependent membrane remodeling events (10, 11).

Guanylate-binding proteins consist of an N-terminal α, β globular large GTPase domain and a α-helical finger-like C-terminal regulatory domain. The domains are connected by a short intermediate region consisting of one α-helix and a short two-stranded β-sheet (12, 13). A GTPase-domain binds guanine nucleotides with low affinities. This induces nucleotide dependent GBP multimerization and hydrolysis of GTP via GDP to GMP [reviewed in Ref. (3)]. Human GBP1, GBP2, and GBP5 and murine Gbp2b/Gbp1, Gbp2, and Gbp5 have at the C-terminus a CaaX sequence (C—cysteine, aa two amino acids, X—terminal amino acid), which directs isoprenylation—the addition of lipid moiety to the protein, which targets proteins to intracellular membranes and facilitates protein-protein interaction (4). Recruitment of proteins to parasitophorous vacuoles harboring pathogens can lead to restriction of pathogen proliferation (14).

GBPs are involved in regulation of inflammasomes—a high-molecular-weight complexes present in the cytosol of stimulated immune cells that mediate the activation of inflammatory caspases resulting in pathogen clearance and/or death of infected cell [reviewed in Ref. (1, 3, 15)]. Gbps can also attack parasites directly via supramolecular complexes (16) and interfere with virus replication (17) or virion assembly (18). Type of effective defense depends on pathogen involved.

A wide range of studies revealed an important role of GBPs in response to different infections including viral (17–20), bacterial (21–24), and protozoan pathogens (14, 16, 25), both vacuolar (14, 16, 21, 24, 25) and cytosolic (17–20).

Abbreviations: Gbp, guanylate-binding protein (murine); GBP, guanylate-binding protein (human); *Gbp2b*/*Gbp1*, murine gene coding this guanylate-binding protein was originally named *Gbp1* and later renamed *Gbp2b*; RCS, recombinant congenic strains; CcS—BALB/c-c-STS, series of recombinant congenic containing random 12.5% of genome of the donor strain STS/A (STS) on 87.5% genome of the background strain BALB/cHeA (BALB/c); OcB—O20-c-C57BL/10-*H-2pz* (B10.O20/Dem), series of recombinant congenic containing random 12.5% (or 6.25% or less) of genome of the donor strain B10.O20/Dem (B10.O20) on the background strain O20/A (O20).

For example, in human GBP1 influences resistance to vesicular stomatitis virus (19), encephalomyelocarditis virus (19), influenza A viruses (17), and *Chlamydia trachomatis* (22), GBP3 reduces virus titers of influenza A viruses (17) and GBP5 prevents processing and incorporation of the viral glycoprotein Env of HIV-1 (18).

Murine Gbp2b/Gbp1 plays role in defense against *Listeria monocytogenes* and *Mycobacterium bovis* BCG (23), Gbp2 inhibits replication of vesicular stomatitis virus and encephalomyelocarditis virus (20), *Toxoplasma gondii* (14), and *Salmonella typhimurium* (24), and Gbp5 protects against *S. typhimurium* (21) and *M. bovis* BCG (23). Moreover, several Gbps can cooperate for more effective defense. Gene specific-silencing using siRNA established that murine Gbp2b/Gbp1, Gbp5, Gbp7, and Gbp6/10 protect against *M. bovis* BCG and *L. monocytogenes*. A combination of siRNAs exacerbated the loss of function, which indicated that protective Gbps functioned cooperatively (23). Similarly, mutual molecular interactions of murine Gbp2b/Gbp1, Gbp2, Gbp3, Gbp5, and Gbp6 protected against *T. gondii* (16).

Leishmania is an obligatory intracellular mammalian pathogen that enters skin by the bite of female phlebotomine sand flies and infects so-called professional phagocytes (neutrophils, monocytes, and macrophages), as well as dendritic cells and fibroblasts. The major host cell is the macrophage where parasites reside inside parasitophorous vacuole, multiply, eventually rupturing the cell and spread to uninfected cells. Infected cells can spread to lymph nodes, spleen, liver, bone marrow, and sometimes lungs [reviewed in Ref. (26)]. The infection can remain asymptomatic or result in one of three main clinical syndromes: the cutaneous form of the disease in dermis, which can be localized or diffuse; mucocutaneous leishmaniasis in the mucosa and the visceral leishmaniasis characterized by splenomegaly and hepatomegaly that results from the metastatic spread of infection to the spleen and liver (27, 28). Manifestations of the disease depend on the infecting species, environmental and social factors, and the genotype of the mammalian host [reviewed in Ref. (26)].

There is very little known about a possible role of GBPs in *Leishmania* infection. Analysis of global gene expression of bone marrow derived macrophages from BALB/c mouse demonstrated upregulation of expression of *Gbp2b*/*Gbp1*, *Gbp2*, *Gbp3*, *Gbp6*, and *Gbp7* after 24 hours of infection with *Leishmania major* promastigotes (29). Dendritic cells generated from blood of healthy human donors exhibited increased expression of *GBP1* and *GBP2* 16 hours after infection by *L. major* promastigotes, whereas dendritic cells infected by *Leishmania donovani* had increased expression of *GBP1* (30). Comparison of global gene expression in skin lesions of *Leishmania braziliensis*-infected patients with skin of normal skin biopsies revealed upregulation of *GBP5* mRNA (31).

For our analysis, we selected two murine *Gbps* with the C-terminal CaaX sequence enabling targeting proteins to parasitophorous membranes (4). We studied expression of *Gbp2b*/*Gbp1* and *Gbp5 in vivo* before and 8 weeks after *L. major* infection in 10 mouse strains from two genetically distant but internally related groups: CcS/Dem (BALB/c, STS, CcS-5, CcS-16, CcS-20) and OcB/Dem (O20, C57BL/10 (B10), C57BL/10-*H2*^{pz}

(B10.O20), OcB-9, OcB-43). Each CcS/Dem strain contains a different, random set of approximately 12.5% genes of the donor strain STS and approximately 87.5% genes of the background strain BALB/c (32, 33). OcB/Dem strains were derived from strains B10.O20 and O20. Strains OcB-43 and OcB-9 contain different 4 and 12.5% of B10 genome on O20 background, respectively; strain B10.O20 contains 4% of O20 genome on B10 background (32, 33). The limited and defined genetic differences between strains in each group (33) make it possible to identify the differences in *Gbp* expression that are controlled by genes outside the *Gbp* coding gene-complex on chromosome 3. Incidence and size of skin lesions indicate that BALB/c and CcS-16 are highly susceptible and B10.O20 is susceptible to *L. major*; whereas CcS-20 is intermediate and STS, CcS-5, O20, B10, OcB-9, and OcB-43 are resistant to this parasite (34) (this study).

We found that the levels of *Gbp2b/Gbp1* and *Gbp5* mRNAs are influenced by *L. major* infection and by genome of the host. The infection caused a large increase of *Gbp2b/Gbp1* and *Gbp5* expression, but *Gbp*s levels in both uninfected and infected mice differ among mouse strains indicating influence of genetic factors. These genetic influences are different in uninfected and infected mice and in some strains there is a clear evidence for a regulation by genes other than the *Gbp* genes (*trans*-regulation). We also show that Gbp2b/Gbp1 protein and *L. major* parasites co-localize in resistant strains STS, CcS-5, and O20 and in the intermediate strain CcS-20 but not in the highly susceptible strain BALB/c.

MATERIALS AND METHODS

Mice

mRNA Expression Experiments

A total of 275 (152 infected and 123 uninfected) female mice of strains BALB/c (33 infected and 22 uninfected), STS (20 infected and 13 uninfected), CcS-5 (11 infected and 10 uninfected), CcS-16 (10 infected and 11 uninfected), CcS-20 (12 infected and 12 uninfected), O20/A (O20) (12 infected and 12 uninfected), C57BL/10Sn (B10) (17 infected and 10 uninfected), B10.O20/R164/Dem (B10.O20) (17 infected and 12 uninfected), OcB-9 (7 infected and 7 uninfected), and OcB-43 (13 infected and 14 uninfected) were tested in 15 independent experiments. The age of mice was 8–23 weeks (mean = 11.9 weeks, median = 11 weeks) at the time of infection (start of experiment in control mice). A total of 81 infected mice of strains BALB/c ($n = 9$), STS ($n = 10$), CcS-5 ($n = 11$), O20 ($n = 12$), B10 ($n = 16$), B10.O20 ($n = 16$), and OcB-9 ($n = 7$) from these experiments were used for estimation of parasite load in skin and/or spleen. 40 infected female mice of the strains BALB/c ($n = 5$), STS ($n = 10$), CcS-5 ($n = 4$), CcS-16 ($n = 12$), and CcS-20 ($n = 9$) from additional four experiments were also used for the estimation of parasite load in skin and/or spleen.

Immunohistochemistry Experiments

97 (48 infected and 49 uninfected) female mice of strain BALB/c (9 infected and 9 uninfected), STS (9 infected and 9 uninfected), CcS-5 (8 infected and 8 uninfected), CcS-20 (11 infected and 11 uninfected), and O20 (11 infected and 12 uninfected) were tested in two independent experiments. The age of mice was 8–18 weeks (mean 13 weeks, median 14 weeks) at the time of infection.

Ethics Statement

All experimental protocols utilized in this study comply with the Czech Government Requirements under the Policy of Animal Protection Law (No. 246/1992) and with the regulations of the Ministry of Agriculture of the Czech Republic (No. 207/2004), which are in agreement with all relevant European Union guidelines for work with animals and were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics AS CR and by Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of Sciences of the Czech Republic (permissions Nr. 190/2010; 232/2012).

Parasite

Leishmania major LV 561 (MHOM/IL/67/LRCL 137 JERICHO II) was maintained in rump lesions of BALB/c females. Amastigotes were transformed to promastigotes using SNB-9 (35). 10^7 promastigotes from the passage two cultivated for 6 days were inoculated in 50 µl sterile saline s.c. into mouse rump (36). Control uninfected mice were injected by 50 µl sterile saline.

Disease Phenotype

The size of the skin lesions was measured every week using the Profi LCD Electronic Digital Caliper Messschieber Schieblehre Messer (Shenzhen Xtension Technology Co., Ltd. Guangdong, China), which has accuracy 0.02 mm. The mice were killed 8 weeks after inoculation. Skin, spleen, liver, and inguinal lymph nodes were collected for later analysis. Preparation of skin samples: approximately 3 mm of border skin surrounding lesion was taken. Hair was removed with scissors. A half of each skin sample was snap-frozen in liquid nitrogen for further RNA and DNA isolations. Another half was fixed in 4% formaldehyde for further paraffin embedding and immunohistochemical analysis. Samples from uninfected mice were obtained from the same rump area and used as a negative control.

Quantification of Parasite Load by PCR-ELISA

Parasite load was measured in DNA from frozen skin and spleen samples using PCR-ELISA according to the previously published protocol (37). Briefly, total DNA was isolated using a TRI reagent (Molecular Research Center, Cincinnati, OH, USA) standard procedure (https://www.mrcgene.com/wp-content/uploads/2014/06/TRI-LSMarch2017.pdf) or a modified proteinase K procedure (37). For PCR, two primers (digoxigenin-labeled F 5′-ATT TTA CAC CAA CCC CCA GTT-3′ and biotin-labeled R 5′-GTG GGG GAG GGG CGT TCT-3′; VBC Genomics Biosciences Research, Austria) were used for amplification of the 120-bp conservative region of the kinetoplast minicircle of *Leishmania* parasite, and 50 ng of extracted DNA was used per each PCR reaction. For a positive control, 20 ng of *L. major* DNA per reaction was amplified as a highest concentration of standard. A 33-cycle (expression experiments) or 26-cycle

(immunostaining experiments) PCR reaction was used for quantification of parasites. Under these conditions, the amount of PCR product is linearly proportional to number of parasites (37). PCR product was measured by the modified ELISA (Pharmingen, San Diego, CA, USA). Concentration of *Leishmania* DNA was determined using the ELISA Reader Tecan and the curve fitter program KIM-E (Schoeller Pharma, Prague, Czech Republic) with least squares-based linear regression analysis.

RNA Isolation

Mouse spleens, skins, liver, and inguinal lymph nodes were snapped frozen by liquid nitrogen immediately after dissection and stored at −80°C until total RNA extraction. At the time of RNA isolation tissue were homogenized in TRI Reagent (Sigma-Aldrich, Inc., St. Louis, MO, USA) using Polytron PT 2100 homogenizer (Kinematica Ag, Luzern, Switzerland) and immediately followed by total RNA isolation according to the manufacturer's protocol. RNA concentration was measured with Nanodrop (NanoDrop Technologies, LLC, Wilmington, DL), and quality of RNA was estimated also using Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). The isolated RNA was stored at −80°C.

Real-time PCR

One microgram of total RNA was diluted in 8 µl of sterile RNase- and DNase-free water, was treated with 1 µl DNase I (1 U/µl) and 1 µl DNase I reaction buffer (Promega Corporation, Madison, WI, USA), and used for subsequent reverse transcription. Single-strand cDNA was prepared from total RNA using Promega first-strand synthesis method. DNase I-treated RNA was incubated for 10 minutes at 65°C, then cooled quickly on ice for 5 minutes, and then treated with 1 µl DNase I stop solution (Promega Corporation, Madison, WI, USA). For the next step, a mixture containing 1 µl of random hexamers primers (100 ng/1 µl) (Invitrogen, Carlsbad, CA, USA), 5 µl (50 ng/µl) of dNTP mix (Invitrogen, Carlsbad, CA, USA), 5 µl of the reaction buffer (Promega Corporation, Madison, WI, USA), 2.5 µl of RNase/DNase-free water (Invitrogen, Carlsbad, CA, USA), and 0.5 µl of M-MLV Reverse Transcriptase RNAase H Minus Point Mutant (100 U/1 µl) (Promega Corporation, Madison, WI, USA) was added and followed by 60 minutes at 37°C. Single-strand cDNA was kept at −80°C until RT-PCR analysis. Real-time PCR was performed using a BioRad iQ iCycler Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primers were designed using Roche Universal ProbeLibrary, ProbeFinder version 2.45 for mouse (*Gbp2b*/*Gbp1*-F AAACCAGGAGGCTACTACCTTTTT, *Gbp2b*/*Gbp1*-R GTATTTTCTCAGCATCACTTCAGC; *Gbp5*-F TTCACCCAATCTAAGACCAAGAC, *Gbp5*-R AGCACCAG GCTTTCTAGACG; *Gapdh*-F AGAACATCATCCCTGCAT CC, *Gapdh*-R ACATTGGGGGTAGGAACAC). Reaction was performed in total volume of 25 µl, including 12.5 µl of 2× SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 1 µl of each primer of *Gbp2b*/*Gbp1* and *Gbp5* genes (final concentration 6.6 µM), 7.5 µl of water (Invitrogen, Carlsbad, CA, USA), and 3 µl of the cDNA template. Six different samples from each experimental group were used, and all samples were tested in triplets. The average Ct values (cycle threshold) were used for quantification, and the relative amount of each mRNA was normalized to the housekeeping gene, *Gapdh* mRNA. Using the delta Ct value, relative expression was calculated [ratio (reference/target) = 2 Ct (reference) − Ct (target)] × 10,000. All experiments included negative controls containing water instead of cDNA.

Genotyping of *Gbp* Cluster in OcB Series

DNA was isolated from tails using a standard proteinase procedure. Strains O20, B10, B10.O20, OcB-9, and OcB-43 were genotyped using microsatellite markers D3Mit160 (size of B10 allele 137 bp, size of O20 allele 127 bp) and D3Mit17 (B10 allele 200 bp, O20 allele 174 bp) (Generi Biotech, Hradec Králové, Czech Republic): The DNA genotyping by PCR was performed as described elsewhere (38).

Immunostaining

After deparaffinization and rehydration, the 3 µm thick slices of skin tissue were 15 minutes heat induced in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.5) for antigen retrieval. For fluorescent labeling of *Leishmania* parasite was used anti-*Leishmania* lipophosphoglycan mouse monoclonal antibody (cat. no. CLP003A; Cedarlane, Hornby, Canada) and TRITC-labeled IgM (115-025-020; Jackson ImmunoResearch, West Grove, PA) all diluted 1:500. Gbp2b/Gbp1 protein was stained with rabbit anti-Gbp1 Polyclonal antibody (PA5-23509; Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:100 and anti-rabbit-AlexaFluor-647 (cat. no. 711-605-152; Jackson ImmunoResearch, West Grove, PA) diluted 1:500. Nuclei of the cells were stained with bisBenzimide H33258 (Sigma-Aldrich, St. Louis, MO, USA) 10 mg per 1 ml diluted 1:1,000. Images were captured with microscope Leica DM6000 objective HCX PL Apo 40×/0.75 PH2 and color camera Leica DFC490. Evaluation of images was done with Fiji ImageJ 1.51n. 10 fields (320.66 × 239.57 µM) from each mouse were analyzed.

Statistical Analysis

The differences among strains within each of the two groups in expression of *Gbp2b*/*Gbp1* and *Gbp5* and the differences between uninfected and infected mice were evaluated by Mann–Whitney test using the program Statistica for Windows 12.0 (StatSoft, Inc., Tulsa, OK, USA). The results were corrected for multiple testing by Bonferroni correction. The correction factor was 10× both for intragroup differences and differences between infected and uninfected mice of the same strain.

RESULTS

Mouse Strains Differ in Expression of Both *Gbp2b*/*Gbp1* and *Gbp5* in Uninfected Mice

We observed strong genetic influence on mRNA levels of tested *Gbp*s. We have examined expression of *Gbp2b*/*Gbp1* (**Figure 1**) and *Gbp5* (**Figure 2**) in skin, inguinal lymph nodes, spleen, and liver of uninfected mice belonging to two genetically different series of strains—CcS/Dem (BALB/c, STS, CcS-5, CcS-16, CcS-20) and OcB/Dem (O20, B10, B10.O20, OcB-9, OcB-43). We have compared expression in parental strains BALB/c and STS with the

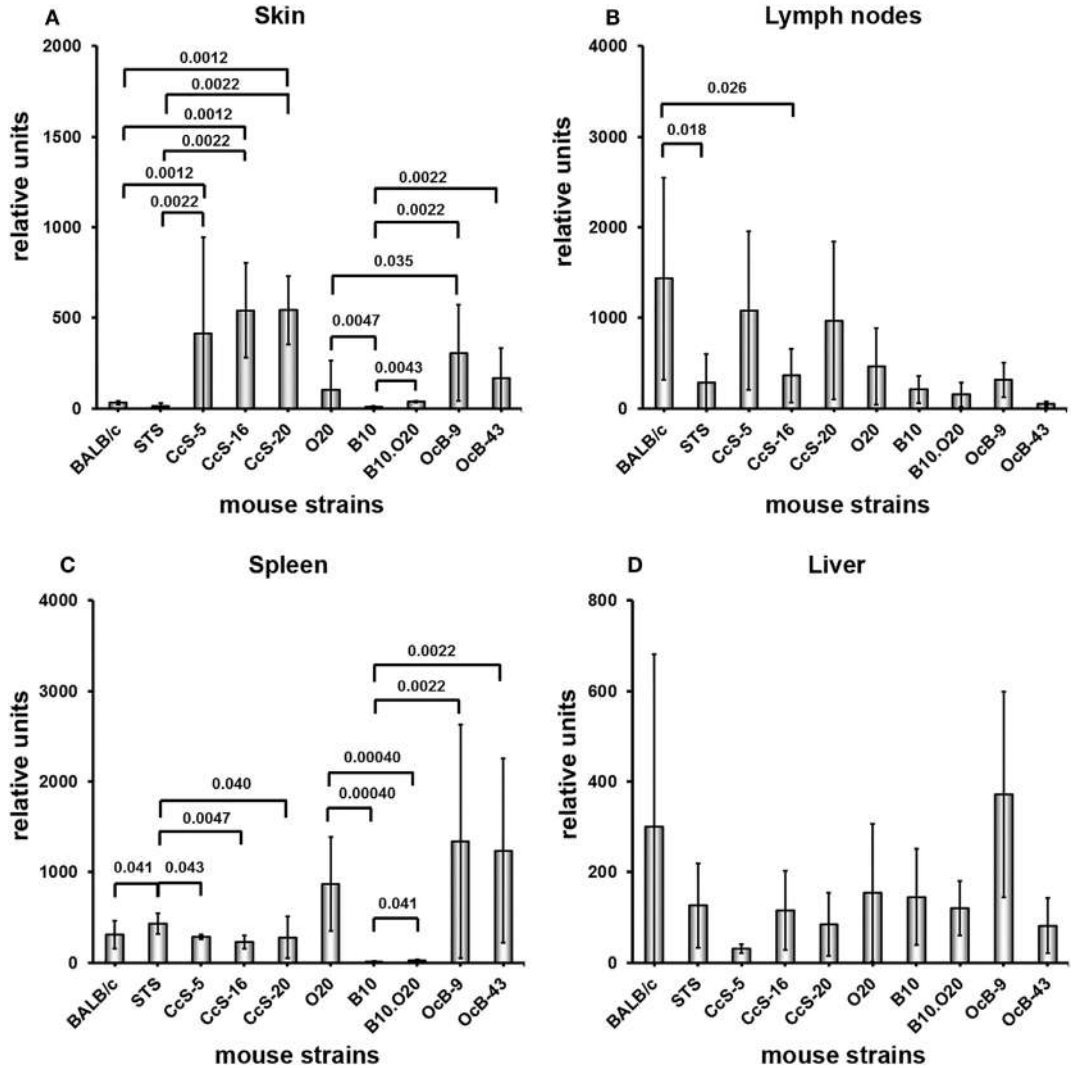


FIGURE 1 | Differences in expression of *Gbp2b/Gbp1* in organs of uninfected mice. Expression of *Gbp2b/Gbp1* in skin **(A)**, lymph nodes **(B)**, spleen **(C)**, and liver **(D)** of uninfected female mice of strains BALB/c (n = 7 skin, 9 lymph nodes, 11 spleen, 9 liver), STS (6 skin, 9 lymph nodes, 8 spleen, 6 liver), CcS-5 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-16 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-20 (6 skin, 6 lymph nodes, 7 spleen, 6 liver), O20 (7 skin, 6 lymph nodes, 9 spleen, 6 liver), B10 (6 skin, 7 lymph nodes, 6 spleen, 6 liver), B10.O20 (5 skin, 7 lymph nodes, 6 spleen, 8 liver), OcB-9 (6 skin, 6 lymph nodes, 6 spleen, 5 liver), and OcB-43 (6 skin, 6 lymph nodes, 6 spleen, 7 liver) were compared. The data show the means ± SD. Only the differences between parental strains BALB/c and STS and strains of CcS/Dem series and parental strains O20 and B10 and strains of OcB/Dem series are shown. Nominal P values are shown.

strains of CcS/Dem series, and expression in parental strains O20 and B10 with the strains of OcB/Dem series (**Figures 1** and **2**), as well as expression of the strains within CcS/Dem and OcB/Dem series in skin (**Tables 1A,C**), lymph nodes (**Tables 2A,C**), spleen (**Tables 3A,C**), and liver (**Tables 4A,C**).

Expression of *Gbp2b*/*Gbp1* in background strain BALB/c and donor strain STS in skin (**Figure 1A**; **Table 1A**) does not differ, whereas strains CcS-5, CcS-16, and CcS-20, each carrying a different set of 12.5% genes of STS on BALB/c background, exhibit higher expression than either parent (**Figure 1A**; **Table 1A**). Expression of *Gbp2b*/*Gbp1* in parental strains of OcB/Dem series O20 and B10 in skin differed (P = 0.0047); strains B10.O20, OcB-9, and OcB-43 exceeded in *Gbp2b*/*Gbp1* expression of the parental strain B10 but not O20 (**Figure 1A**; **Table 1A**).

We have observed differences in the expression of *Gbp2b*/*Gbp1* among mouse strains also in other tested organs (**Figures 1B–D**; **Tables 2A**, **3A**, and **4A**). Strains OcB-43 and OcB-9 differed in the expression of *Gbp2b*/*Gbp1* in lymph nodes (**Table 2A**), CcS-16 exhibited lower expression than STS in spleen (**Figure 2C**; **Table 3A**), B10 and B10.O20 exhibited lowest expression in spleen and differed from strains O20, OcB-9, and OcB-43, CcS-5 exhibited lower expression than CcS-16 in liver (**Figure 1D**; **Table 4A**); however, expression in none of the CcS or OcB strains exceeded the range of expression in both parental strains.

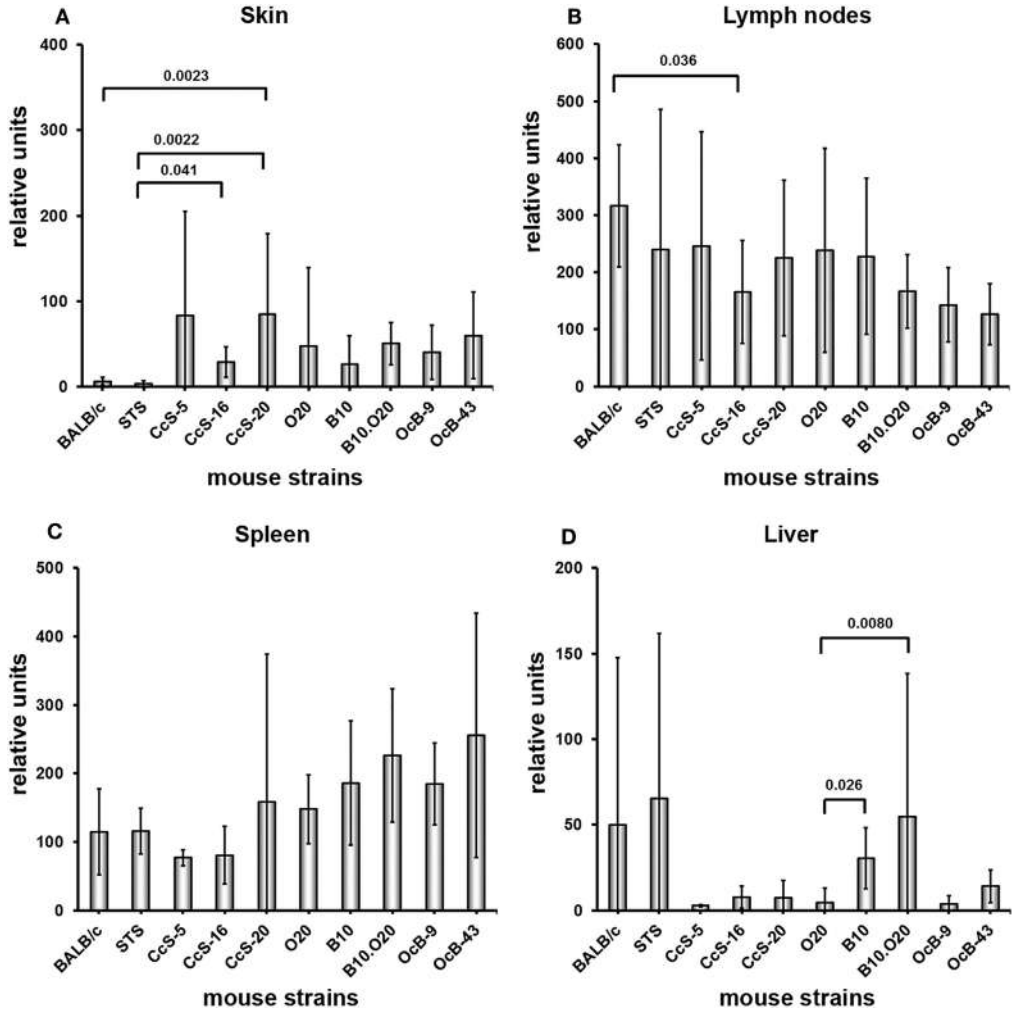


FIGURE 2 | Differences in expression of *Gbp5* in organs of uninfected mice. Expression of *Gbp5* in skin **(A)**, lymph nodes **(B)**, spleen **(C)** and liver **(D)** of uninfected female mice of strains of strains BALB/c (n = 7 skin, 9 lymph nodes, 11 spleen, 9 liver), STS (6 skin, 9 lymph nodes, 8 spleen, 6 liver), CcS-5 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-16 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-20 (6 skin, 6 lymph nodes, 7 spleen, 6 liver), O20 (7 skin, 6 lymph nodes, 9 spleen, 6 liver), B10 (6 skin, 7 lymph nodes, 6 spleen, 6 liver), B10.O20 (5 skin, 7 lymph nodes, 6 spleen, 8 liver), OcB-9 (6 skin, 6 lymph nodes, 6 spleen, 5 liver), and OcB-43 (6 skin, 6 lymph nodes, 6 spleen, 7 liver) were compared. The data show the means ± SD. Only the differences between parental strains BALB/c and STS and strains of CcS/Dem series and parental strains O20 and B10 and strains of OcB/Dem series are shown. Nominal P values are shown.

Expression of *Gbp5* in skin did not differ in parental strains of CcS/Dem series BALB/c and STS (**Figure 2A**; **Table 1C**), and expression of *Gbp5* in CcS-20 exceeded the expression of both parental strains (**Figure 2A**; **Table 1C**). There was no difference in *Gbp5* expression among strains of OcB/Dem series (**Table 1C**).

We did not find any significant differences in expression of *Gbp5* among the strains of both CcS/Dem and OcB/Dem series in the lymph nodes, spleen, and liver; none of the strains in CcS/Dem or in OcB/Dem series differed from either parent (**Figures 2B–D**; **Tables 2C**, **3C**, and **4C**).

Upregulation of *Gbp2b*/*Gbp1* and *Gbp5* mRNAs after Infection

In susceptible mice, pathology started as a nodule at the site of *L. major* infection appearing between weeks 2 and 4, which progressed in susceptible strains into a skin lesion (**Figure 3A**) (34). Strains BALB/c and CcS-16 are highly susceptible and develop large skin lesions (**Figures 3A,B**), B10.O20 develops moderate skin lesions (**Figures 3A,C**), CcS-20 is intermediate (**Figures 3A,D**) (34), and STS, CcS-5, O20, B10, OcB-9, and OcB-43 are resistant with no or minimal skin lesions (**Figure 3A**). All tested strains contain parasites in skin (**Figure 3D**) and spleen (**Figure 3E**), although the parasite load in resistant strains STS, CcS-5 and O20 (skin and spleen), and OcB-9 (spleen) is low.

Infection increased the expression of *Gbp2b*/*Gbp1* and *Gbp5* in organs of tested mice, the highest increase was observed in skin (**Figures 4**–**7**). All tested strains except CcS-5 and OcB-9 exhibited significantly higher expression of *Gbp2b*/*Gbp1* and *Gbp5* in skin after infection, irrespective of their susceptibility or resistance status (**Figure 4A**). Similarly as in uninfected mice,

TABLE 1 | Comparison of expression of *Gbp2b/Gbp1* or *Gbp5* among mouse strains of CcS/Dem and OcB/Dem series in skin.

CcS/Dem series					OcB/Dem series				
Strain	**BALB/c**	**STS**	**CcS-5**	**CcS-16**	**Strain**	**O20**	**B10**	**B10.O20**	**OcB-9**
A. *Gbp2b/Gbp1* uninfected									
BALB/c					**O20**				
STS	0.11				**B10**	0.0047			
CcS-5	0.0012	0.0022			**B10.O20**	1	0.0043		
CcS-16	0.0012	0.0022	0.24		**OcB-9**	0.035	0.0022	0.0043	
CcS-20	0.0012	0.0022	0.31	0.82	**OcB-43**	0.23	0.0022	0.052	0.18
B. *Gbp2b/Gbp1* infected									
BALB/c					**O20**				
STS	0.54				**B10**	0.043			
CcS-5	0.86	0.73			**B10.O20**	0.0012	0.19		
CcS-16	0.0076	0.0012	0.0022		**OcB-9**	0.54	0.40	0.0047	
CcS-20	0.0048	0.0012	0.0022	0.59	**OcB-43**	0.91	0.036	0.00012	0.30
C. *Gbp5* uninfected									
BALB/c					**O20**				
STS	0.37				**B10**	0.73			
CcS-5	0.30	0.13			**B10.O20**	0.20	0.25		
CcS-16	0.051	0.041	0.82		**OcB-9**	0.45	0.39	0.79	
CcS-20	0.0023	0.0022	0.31	0.093	**OcB-43**	0.14	0.18	0.79	0.59
D. *Gbp5* infected									
BALB/c					**O20**				
STS	0.54				**B10**	0.83			
CcS-5	0.18	0.37			**B10.O20**	0.21	0.076		
CcS-16	0.088	0.0012	0.0022		**OcB-9**	0.088	0.15	0.000026	
CcS-20	0.036	0.0023	0.0022	0.49	**OcB-43**	0.50	0.24	0.51	0.0012

A. Differences of Gbp2b/Gbp1 expression in uninfected skin; B. Differences of Gbp2b/Gbp1 expression in skin after 8 weeks of infection; C. Differences of Gbp5 expression in uninfected skin; D. Differences of Gbp5 expression in skin after 8 weeks of infection. Green: nominal (uncorrected) P value < 0.05; red: difference significant after correction for multiple testing at P < 0.05.

levels of *Gbp2b*/*Gbp1* mRNA in CcS-16 and CcS-20 exceeded those in both parental strains BALB/c and STS (Figure S1A in Supplementary Material; **Table 1B**); *Gbp5* expression in infected CcS-20 also exceeded that in both BALB/c and STS (Figure S2A in Supplementary Material; **Table 1D**).

Infection also induced increase of *Gbp2b*/*Gbp1* in inguinal lymph nodes of all strains except BALB/c and CcS-20, the highest expression was observed in CcS-5 (**Figure 5A**), which differed from all tested strains except STS (Figure S1B in Supplementary Material; **Table 2B**), but only increase of expression of B10.O20 and OcB-43 was significant after correction for multiple testing; we did not observe significant increase of *Gbp5* mRNA in lymph nodes (**Figure 5B**).

Four strains (BALB/c, STS, CcS-5, and CcS-16) show significantly increased expression of *Gbp2b*/*Gbp1* in spleen (**Figure 6A**). Expression of *Gbp5* was increased in CcS-5 (**Figure 6B**).

In liver, infection induced significant increases of *Gbp2b*/*Gbp1* mRNA in strains of CcS/Dem series, CcS-5, and CcS-16 (**Figure 7A**). Level of *Gbp2b*/*Gbp1* mRNA in CcS-16 is highest from all tested strains (Figure S1 in Supplementary Material; **Table 4B**). Expression of *Gbp5* was significantly increased in CcS/Dem strains CcS-5 and CcS-16 and decreased in OcB/Dem strain OcB-43 (**Figure 7B**; **Table 4D**).

Gbp2b/Gbp1 Protein Tends to Co-Localize with *Leishmania* Parasites in Skin of Resistant and Intermediate Strains but Not in the Highly Susceptible Strain BALB/c

Expression of *Gbp2b*/*Gbp1* mRNA was highest in skin of infected mice (Figure S1 in Supplementary Material; **Figure 4**), we have therefore analyzed by immunohistochemistry a presence of Gbp2b/Gbp1 protein in the skin of selected strains BALB/c, STS, CcS-5, CcS-20, and O20 and its relationship to *L. major* parasite in infected mice. **Figure 8** shows the presence of Gbp2b/Gbp1 protein in the skin of uninfected strains. The comparison of position of *L. major* and Gbp2b/Gbp1 in the skin of chronically infected highly susceptible strain BALB/c showed few Gbp2b/Gbp1 in the vicinity of *L. major* parasites, but a large part of parasites was free of Gbp2b/

TABLE 2 | Comparison of expression of *Gbp2b/Gbp1* or *Gbp5* among mouse strains of CcS/Dem and OcB/Dem series in inguinal lymph nodes.

CcS/Dem series					OcB/Dem series				
Strain	**BALB/c**	**STS**	**CcS-5**	**CcS-16**	**Strain**	**O20**	**B10**	**B10.O20**	**OcB-9**
A. *Gbp2b/Gbp1* uninfected									
BALB/c					**O20**				
STS	0.018				**B10**	0.53			
CcS-5	0.69	0.065			**B10.O20**	0.53	0.62		
CcS-16	0.026	0.59	0.18		**OcB-9**	0.70	0.37	0.051	
CcS-20	0.61	0.24	1	0.39	**OcB-43**	0.31	0.10	0.035	0.0022
B. *Gbp2b/Gbp1* infected									
BALB/c					**O20**				
STS	0.18				**B10**	0.021			
CcS-5	0.0049	0.13			**B10.O20**	0.038	0.28		
CcS-16	0.40	0.18	0.0022		**OcB-9**	0.30	0.081	0.13	
CcS-20	0.96	0.31	0.0087	0.49	**OcB-43**	0.15	0.25	0.019	0.64
C. *Gbp5* uninfected									
BALB/c					**O20**				
STS	0.39				**B10**	0.95			
CcS-5	0.61	0.82			**B10.O20**	0.37	0.62		
CcS-16	0.036	1	0.59		**OcB-9**	0.24	0.23	0.63	
CcS-20	0.22	1	1	0.39	**OcB-43**	0.18	0.051	0.30	0.59
D. *Gbp5* infected									
BALB/c					**O20**				
STS	0.96				**B10**	0.14			
CcS-5	0.53	0.59			**B10.O20**	0.37	0.69		
CcS-16	0.010	0.31	0.0022		**OcB-9**	0.92	0.24	0.48	
CcS-20	0.53	0.94	0.18	0.31	**OcB-43**	0.060	0.44	0.22	0.34

A. Differences of Gbp2b/Gbp1 expression in uninfected lymph nodes; B. Differences of Gbp2b/Gbp1 expression in lymph nodes after 8 weeks of infection; C. Differences of Gbp5 expression in uninfected lymph nodes; D. Differences of Gbp5 expression in lymph nodes after 8 weeks of infection. Green: nominal (uncorrected) P value < 0.05; red: difference significant after correction for multiple testing at P < 0.05.

Gbp1 (**Figure 9A**); the comparison of parasite load in the skin of the tested strains is shown in Figure S3 in Supplementary Material. In resistant strains STS (**Figure 9B**), CcS-5 (**Figure 9C**), and O20 (**Figure 9E**) and in intermediate strain CcS-20 (**Figure 9D**), Gbp2b/Gbp1 co-localized with clusters of parasites (**Figures 9B–E**) that in some places formed large clusters or long stretches. Gbp2b/Gbp1 either surrounded these clusters (**Figures 9B–D**) or formed stretches consisting of *L. major* parasites and Gbp2b/Gbp1 (**Figures 9C,E**). The tightest co-localization was observed in strains CcS-20 (**Figure 9D**) and O20 (**Figure 9E**).

DISCUSSION

Genetic Influence on Expression of *Gbp2b/Gbp1* and *Gbp5*

Tested strains exhibited genetic differences in *Gbps* expression both before and after *L. major* infection (**Figures 1**, **2**, **4** and **7**; **Tables 1–4**). Our study extends analysis of genetic influence by Staeheli and coworkers on Gbp2b/Gbp1 expression (39), who injected forty six mouse strains by poly(I);poly(C) in order to induce interferon production and tested their spleen cells for guanylate-binding activity. Tested strains were divided into Gbp2b/Gbp1 inducible and Gbp2b/Gbp1 noninducible groups. BALB/c was in the inducible group, whereas STS, O20, and C57BL/6J belonged to noninducible one (39). Our data confirm strong genetic influence on expression of *Gbp2b/Gbp1*; however, a direct comparison of outcome of study of Staeheli et al. (39) with our results is impossible due to different experimental designs. They induced Gbp2b/Gbp1 expression by poly(I);poly(C) that is structurally similar to double-stranded RNA present in some viruses, whereas we stimulated *Gbp2b/Gbp1* expression by the chronic infection with parasite *L. major*.

Comparison of Genotypes in *Gbp* Cluster on Mouse Chromosome 3 Indicates *Trans*-Regulation

Our data surprisingly showed that in several organs expressions levels of *Gbps* in recombinant congenic strains were outside the range of their parents. In skin of uninfected mice, expression

TABLE 3 | Comparison of expression of *Gbp2b/Gbp1* or *Gbp5* among mouse strains of CcS/Dem and OcB/Dem series in spleen.

CcS/Dem series					OcB/Dem series				
Strain	**BALB/c**	**STS**	**CcS-5**	**CcS-16**	**Strain**	**O20**	**B10**	**B10.O20**	**OcB-9**
A. *Gbp2b/Gbp1* uninfected									
BALB/c					**O20**				
STS	0.041				**B10**	0.00040			
CcS-5	0.66	0.043			**B10.O20**	0.00040	0.041		
CcS-16	0.40	0.0047	0.24		**OcB-9**	0.69	0.0022	0.0022	
CcS-20	0.25	0.040	0.23	0.84	**OcB-43**	0.53	0.0022	0.0022	0.94
B. *Gbp2b/Gbp1* infected									
BALB/c					**O20**				
STS	0.35				**B10**	0.000022			
CcS-5	0.40	0.018			**B10.O20**	0.00080	0.26		
CcS-16	0.44	0.018	0.49		**OcB-9**	0.53	0.00025	0.0022	
CcS-20	0.22	0.049	0.93	0.54	**OcB-43**	0.96	0.00025	0.0022	0.39
C. *Gbp5* uninfected									
BALB/c					**O20**				
STS	0.55				**B10**	0.46			
CcS-5	0.15	0.059			**B10.O20**	0.18	0.70		
CcS-16	0.30	0.14	1		**OcB-9**	0.27	1	0.49	
CcS-20	0.60	0.19	0.73	0.95	**OcB-43**	0.33	0.94	0.94	0.94
D. *Gbp5* infected									
BALB/c					**O20**				
STS	0.031				**B10**	0.32			
CcS-5	0.66	0.032			**B10.O20**	1	0.22		
CcS-16	0.08	0.00011	0.0022		**OcB-9**	0.61	0.26	0.59	
CcS-20	0.62	0.0061	0.18	0.247	**OcB-43**	0.61	0.26	0.94	0.24

A. Differences of Gbp2b/Gbp1 expression in uninfected spleen; B. Differences of Gbp2b/Gbp1 expression in spleen after 8 weeks of infection; C. Differences of Gbp5 expression in uninfected spleen; D. Differences of Gbp5 expression in spleen after 8 weeks of infection. Green: nominal (uncorrected) P value < 0.05; red: difference significant after correction for multiple testing at P < 0.05.

of *Gbp2b*/*Gbp1* in CcS-5, CcS-16, and CcS-20 exceeded those of both their parents BALB/c and STS (**Figure 1A**) and expression of *Gbp2b*/*Gbp1* in B10.O20 exceeded expression in parental strain B10 (**Figure 1A**). Such pattern of inheritance has been considered to be caused by *trans*-regulatory effects of non-linked or distant genes (40). The differences between parental strains and CcS/Dem strain CcS-20 persist after *L. major* infection, whereas the differences between expression of parents and CcS-5 and CcS-16 and between parent B10 and the strain B10.O20 disappear after infection (**Figure 1A**; Figure S1A in Supplementary Material; **Tables 1A,B**). Expression of *Gbp5* in skin of uninfected CcS-20 exceeded level of both parents (**Figure 2A**; **Table 1C**) but was significantly higher only than the parental strain STS after 8 weeks of infection (Figure S2A in Supplementary Material; **Table 1D**). CcS-5 and CcS-16 highly differed in the expression of both *Gbp1*/*Gbp2b* and *Gbp5* in lymph nodes and liver of infected mice; these strains also differed in expression of *Gbp5* in spleen (**Tables 2B,D**, **3D**, and **Tables 4B,D**).

Comparison of genotypes of the tested strains (33, 41, 42) (this study) in the *Gbp* cluster on the mouse chromosome 3 (**Figure 10**) revealed that strains CcS-5, CcS-16, and CcS-20 exhibiting higher expression of *Gbp* had *Gbp* genotype identical to that of BALB/c (C). Similarly, highly differing CcS-5 and CcS-16 strains carry the same *Gbp* allele. The presence of the same allele of *Gbp* gene cluster on chromosome 3 in strains that differ in other genes suggests that their differences in expression of *Gbp2*/*Gbp1* and/or *Gbp5* from one or both parents or from other RC strain are due to regulatory influence of non-*Gbp* gene(s) of STS origin carried on other genetic segments (*trans*-regulation). In the OcB/Dem series, B10.O20 carried in *Gbp* cluster B10 genotype (B), which similarly indicated *trans*-regulation of expression from O20 genome situated outside *Gbp* cluster (**Table 1A**; **Figure 10**). This *trans*-regulation can be partly overlaid by other regulatory events appearing after infection. Further genetic studies will be needed to elucidate nature of regulatory events observed in our studies.

The observations of progeny having a phenotype, which is beyond the range of the phenotype of its parents, are not rare. For example, analysis of gene expression from livers in chromosome substitution mouse strains revealed that only 438 of the 4,209 expressed genes were inside the parental range (40).

TABLE 4 | Comparison of expression of *Gbp2b/Gbp1* or *Gbp5* among mouse of CcS/Dem and OcB/Dem series strains in liver.

CcS/Dem series					OcB/Dem series				
Strain	**BALB/c**	**STS**	**CcS-5**	**CcS-16**	**Strain**	**O20**	**B10**	**B10.O20**	**OcB-9**
A. *Gbp2b/Gbp1* uninfected									
BALB/c					**O20**				
STS	0.46				**B10**	0.94			
CcS-5	0.088	0.24			**B10.O20**	0.85	0.66		
CcS-16	0.53	0.82	0.0022		**OcB-9**	0.052	0.13	0.045	
CcS-20	0.18	0.39	0.39	0.70	**OcB-43**	0.63	0.45	0.19	0.010
B. *Gbp2b/Gbp1* infected									
BALB/c					**O20**				
STS	0.072				**B10**	0.43			
CcS-5	0.21	0.31			**B10.O20**	0.66	0.98		
CcS-16	0.000074	0.0022	0.0022		**OcB-9**	0.054	0.0018	0.035	
CcS-20	0.0085	0.14	0.073	0.0012	**OcB-43**	0.49	0.042	0.40	0.014
C. *Gbp5* uninfected									
BALB/c					**O20**				
STS	0.33				**B10**	0.026			
CcS-5	0.86	0.18			**B10.O20**	0.0080	0.85		
CcS-16	0.46	0.94	0.041		**OcB-9**	0.79	0.052	0.011	
CcS-20	0.86	0.59	0.59	0.49	**OcB-43**	0.073	0.14	0.19	0.030
D. *Gbp5* infected									
BALB/c					**O20**				
STS	0.00030				**B10**	0.069			
CcS-5	0.0047	0.31			**B10.O20**	0.026	0.45		
CcS-16	0.77	0.0022	0.0022		**OcB-9**	0.78	0.54	0.33	
CcS-20	0.011	0.84	0.95	0.0023	**OcB-43**	0.23	0.13	0.062	0.95

A. Differences of Gbp2b/Gbp1 expression in uninfected liver; B. Differences of Gbp2b/Gbp1 expression in liver after 8 weeks of infection; C. Differences of Gbp5 expression in uninfected liver; D. Differences of Gbp5 expression in liver after 8 weeks of infection. Green: nominal (uncorrected) P value < 0.05; red: difference significant after correction for multiple testing at P < 0.05.

These observations are due to multiple regulatory interactions, which in new combinations of these genes in recombinant congenic or chromosomal substitution strains can lead to the appearance of new phenotypes that exceed their range in parental strains.

Increased Expression of *Gbp2b/Gbp1* and/or *Gbp5* in Resistant Mice Suggests Hidden Inflammation

We and others have demonstrated that *Leishmania* parasites are present not only in organs of infected susceptible mice with clinical manifestations of the disease but also in clinically asymptomatic mice of resistant strains (37, 43–46). This is also shown in **Figures 3D,E** and **9B,C,E**. **Figures 4–7** show that the expression of *Gbp2b/Gbp1* and/or *Gbp5* has increased after infection in at least one organ of each of the tested mice, including the resistant ones (STS, CcS-5, O20, B10, OcB-9, and OcB-43), which had no or only minimal and transient clinical symptoms. This strongly suggests that persistent parasites can contribute to the maintenance of protective immunity, which was manifested in our experiments by the increased levels of *Gbp2b/Gbp1* and *Gbp5* in resistant mice. It was demonstrated previously that this latent infection is controlled by inducible nitric oxide synthase (43) and phagocyte NADPH oxidase (46). It remains to be established, whether defense mechanisms including Gbps that were found to act against other pathogens (16, 23), operate also in *Leishmania*-infected mammalian host. In defense against *M. bovis*, Gbp2b/Gbp1 and Gbp7 could promote NADPH oxidase activity after the recruitment of gp91phox and gp22phox components to bacteria vacuoles (23), whereas parasite *T. gondii* was directly attacked via Gbp supramolecular complexes (16). The observed association (**Figure 9**) of Gbp2b/Gbp1 with *L. major* parasites in the skin of resistant and intermediate strains but not the highly susceptible strain BALB/c may suggest a role of this protein in response against the *L. major* pathogens.

Importantly, persistent parasites, besides stimulating protective immune reactions, can also represent a danger for hosts (45). The increased expression of *Gbp2b/Gbp1* and *Gbp5* in clinically asymptomatic mice reveals the price exacted from the

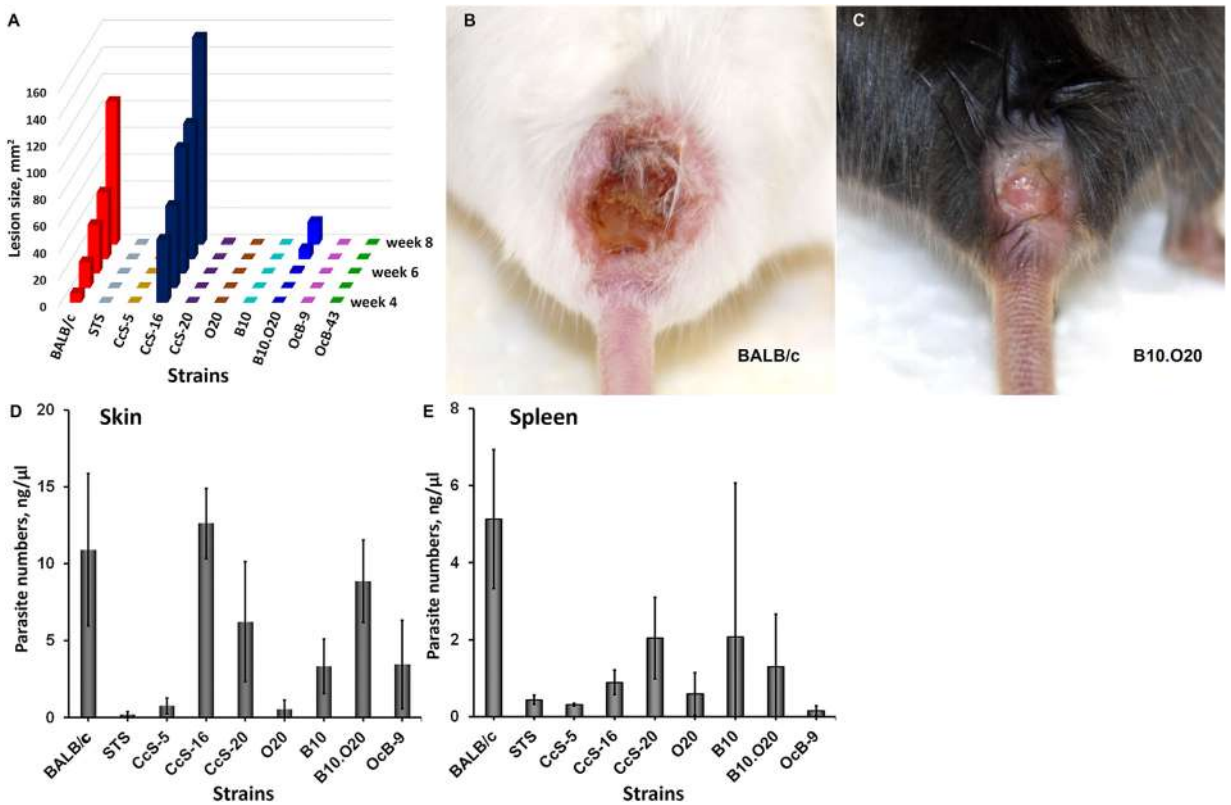


FIGURE 3 | Skin lesion development and parasite load in skin and spleen of infected mice. Kinetics of lesion development from week 4 (appearance of lesions in highly susceptible strains) till week 8 (end of experiment) **(A)**. Median values of skin lesions of mice tested in expression experiments are shown. Skin lesion caused by *Leishmania major* in female mouse of BALB/c strain at week 8 after infection **(B)**. Skin lesion caused by *L. major* in female mouse of B10.O20 strain at week 8 after infection **(C)**. Parasite load in skin **(D)** and spleen **(E)** of infected female mice of strains BALB/c (n = 5 skin, 11 spleen), STS (13 skin, 7 spleen), CcS-5 (11 skin, 4 spleen), CcS-16 (6 skin, 6 spleen), CcS-20 (7 skin, 9 spleen), O20 (6 skin, 12 spleen), B10 (9 skin, 13 spleen), B10.O20 (7 skin, 15 spleen), and OcB-9 (7 skin, 7 spleen). The data show the means ± SD.

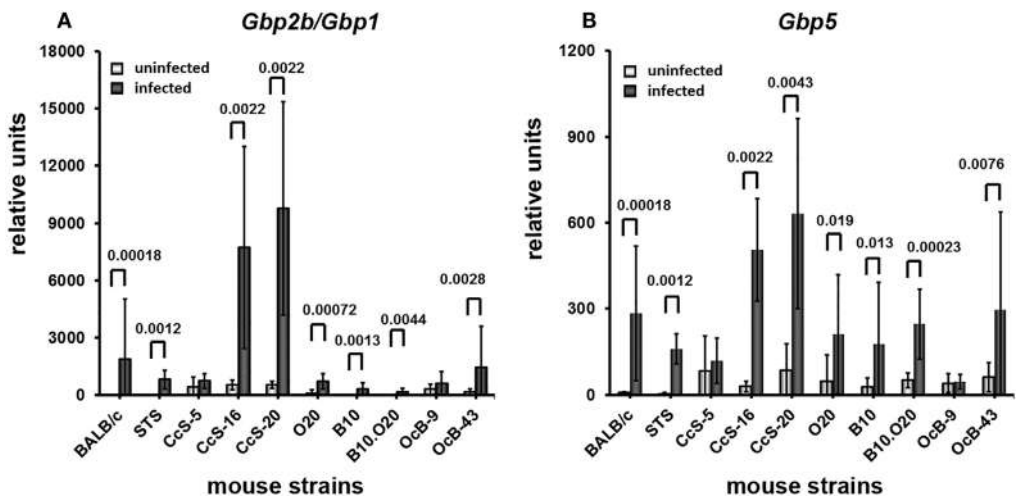


FIGURE 4 | Differences in expression of *Gbp2b/Gbp1* and *Gbp5* in skin of uninfected and infected mice. Expression of *Gbp2b/Gbp1* **(A)** and *Gbp5* **(B)** in skin of uninfected and infected female mice of strains BALB/c (9 infected and 7 uninfected), STS (7 infected and 6 uninfected), CcS-5 (6 infected and 6 uninfected), CcS-16 (6 infected and 6 uninfected), CcS-20 (6 infected and 6 uninfected), O20 (10 infected and 7 uninfected), B10 (8 infected and 6 uninfected), B10.O20 (13 infected and 5 uninfected), OcB-9 (7 infected and 6 uninfected), and OcB-43 (9 infected and 6 uninfected) were compared. Animals were subcutaneously inoculated with 10^7 promastigotes of *Leishmania major.* Control, uninfected mice were kept in the same animal facility. Both groups were killed in the same time—after 8 weeks of infection or start of experiment. The data show the means ± SD. Nominal P values are shown.

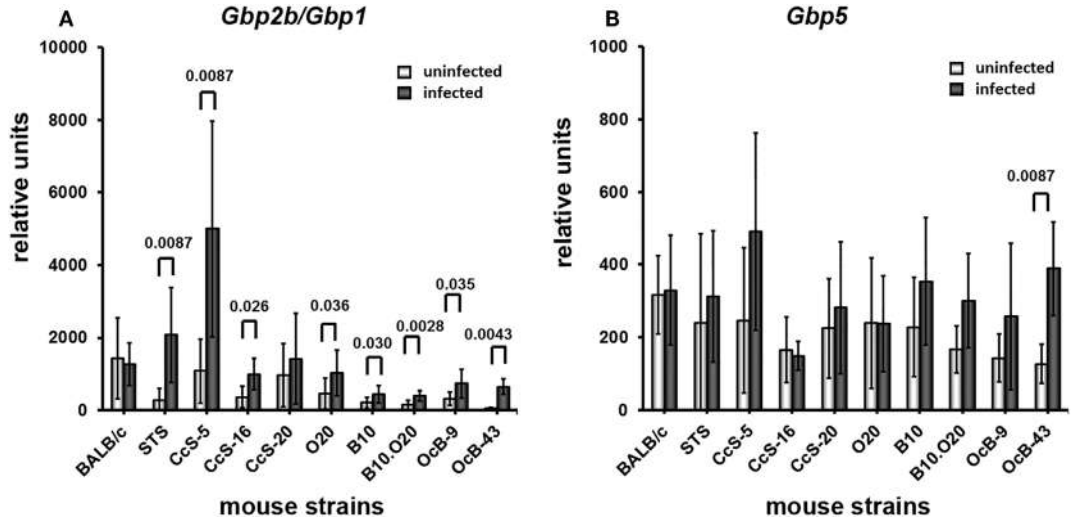


FIGURE 5 | Differences in expression of *Gbp2b/Gbp1* and *Gbp5* in inguinal lymph nodes of uninfected and infected mice. Expression of *Gbp2b/Gbp1* **(A)** and *Gbp5* **(B)** in inguinal lymph nodes of uninfected and infected female mice of strains BALB/c (11 infected and 9 uninfected), STS (6 infected and 6 uninfected), CcS-5 (6 infected and 6 uninfected), CcS-16 (6 infected and 6 uninfected), CcS-20 (6 infected and 6 uninfected), O20 (9 infected and 6 uninfected), B10 (13 infected and 7 uninfected), B10.O20 (11 infected and 7 uninfected), OcB-9 (7 infected and 6 uninfected), and OcB-43 (5 infected and 6 uninfected) were compared. Animals were subcutaneously inoculated with 10^7 promastigotes of *Leishmania major.* Control, uninfected mice were kept in the same animal facility. Both groups were killed after 8 weeks of infection. The data show the means ± SD from 12 independent experiments. Nominal *P* values are shown.

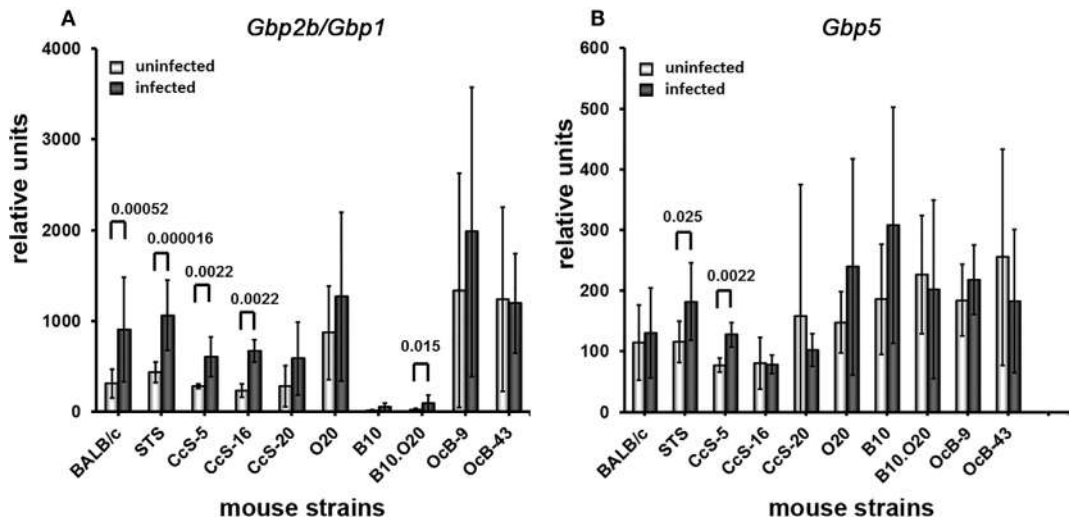


FIGURE 6 | Differences in expression of *Gbp2b/Gbp1* and *Gbp5* in spleen of uninfected and infected mice. Expression of *Gbp2b/Gbp1* **(A)** and *Gbp5* **(B)** in spleens of uninfected and infected female mice of strains BALB/c (14 infected and 11 uninfected), STS (12 infected and 8 uninfected), CcS-5 (6 infected and 6 uninfected), CcS-16 (6 infected and 6 uninfected), CcS-20 (5 infected and 7 uninfected), O20 (9 infected and 9 uninfected), B10 (10 infected and 6 uninfected), B10.O20 (6 infected and 6 uninfected), OcB-9 (6 infected and 6 uninfected), and OcB-43 (6 infected and 6 uninfected) were compared. Animals were subcutaneously inoculated with 10^7 promastigotes of *Leishmania major.* Control, uninfected mice were kept in the same animal facility. Both groups were killed after 8 weeks of infection. The data show the means ± SD from 12 independent experiments. Nominal *P* values are shown.

organism by a dormant infection. This finding deserves attention, because elevated levels of human GBP1 are directly involved in the endothelial dysfunction and the regulation of endothelial progenitor cells activity in patients with the autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and systemic sclerosis (47). In mice, elevated levels of Gbp3 and Gbp6 were linked with the pathogenesis of atherosclerosis (48). In humans with colorectal cancer, the anti-angiogenic effect of increased levels of GPB1 was beneficial in colorectal carcinoma patients, where it was associated with sustained reduction of intratumoral angiogenic activity and improved cancer-related survival (49).

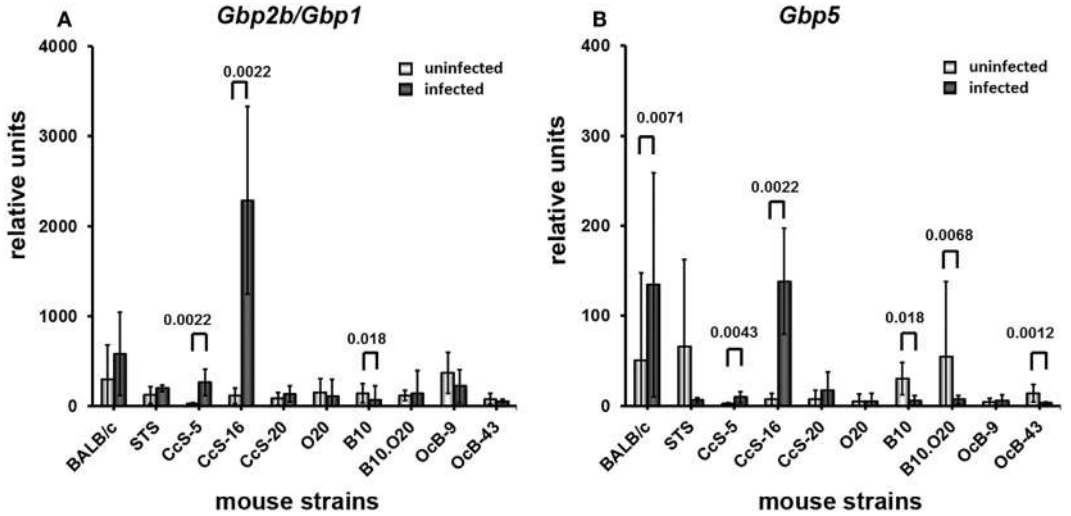


FIGURE 7 | Differences in expression of *Gbp2b/Gbp1* and *Gbp5* in liver of uninfected and infected mice. Expression of *Gbp2b/Gbp1* **(A)** and *Gbp5* **(B)** in liver uninfected and infected female mice of strains BALB/c (13 infected and 9 uninfected), STS (6 infected and 6 uninfected), CcS-5 (6 infected and 6 uninfected), CcS-16 (6 infected and 6 uninfected), CcS-20 (7 infected and 6 uninfected), O20 (8 infected and 6 uninfected), B10 (12 infected and 6 uninfected), B10.O20 (11 infected and 8 uninfected), OcB-9 (7 infected and 5 uninfected), and OcB-43 (7 infected and 6 uninfected) were compared. Animals were subcutaneously inoculated with 10^7 promastigotes of *Leishmania major.* Control, uninfected mice were kept in the same animal facility. Both groups were killed after 8 weeks of infection. The data show the means ± SD from 12 independent experiments. Nominal *P* values are shown.

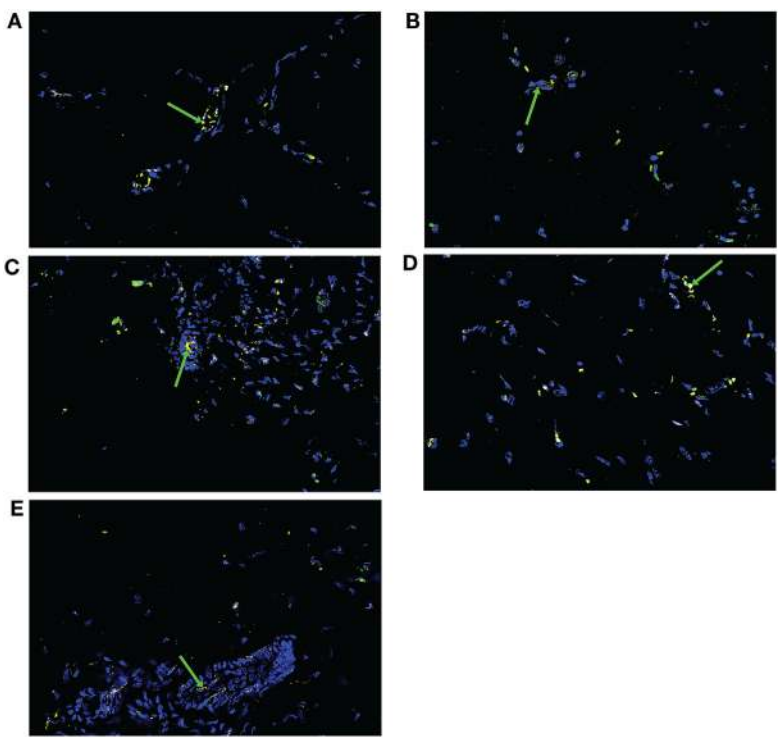


FIGURE 8 | Gbp2b/Gbp1 protein in skin of uninfected mice. Slices of skin tissue of females of BALB/c **(A)**, STS **(B)**, CcS-5 **(C)**, CcS-20 **(D)**, and O20 **(E)** mice were stained with the rabbit anti-Gbp1 Polyclonal antibody (PA5-23509, Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:100 and anti-rabbit-AlexaFluor-647 (cat. no. 711-605-152; Jackson ImmunoResearch, West Grove, PA) diluted 1:500. Nuclei of the cells were stained with bisBenzimide H33258 (Sigma-Aldrich, St. Louis, MO, USA) 10 mg per 1 ml diluted 1:1,000. Images were captured with microscope Leica DM6000 objective HCX PL Apo 40x/0.75 PH2 and color camera Leica DFC490. Evaluation of images was done with Fiji ImageJ 1.51n. Figures are representatives of data from 8 to 12 mice (see Materials and Methods) in 3 of them 10 fields (320.66 × 239.57 μM) from each mouse were analyzed, in the rest one field was analyzed to verify the results. Green arrows show Gbp2b/Gbp1 protein (yellow color), cell nuclei are stained in blue.

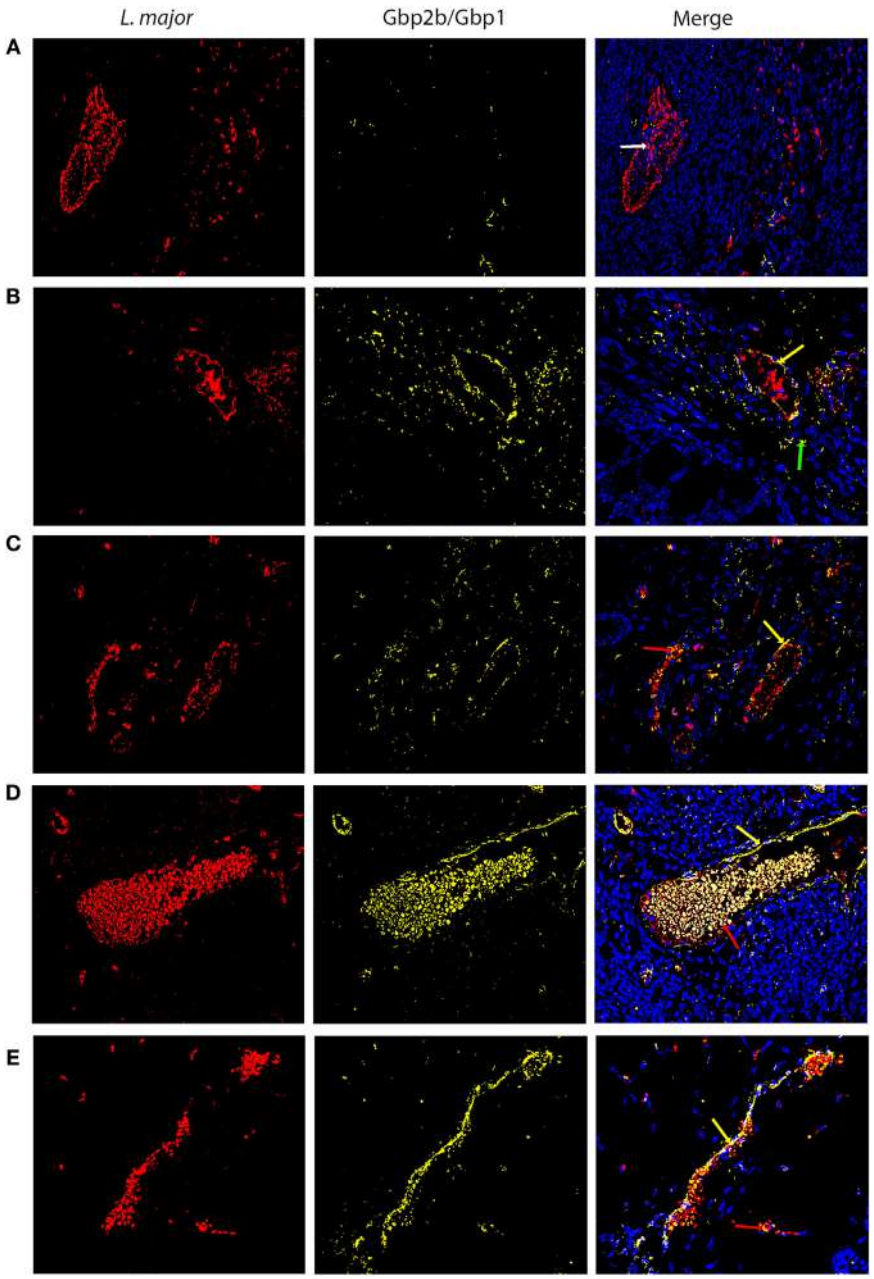
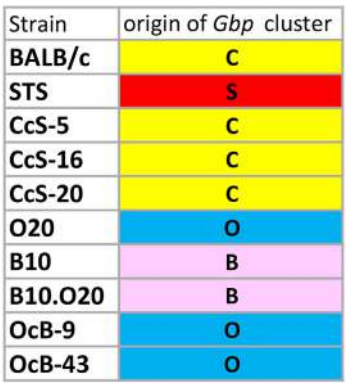


FIGURE 9 | Gbp2b/Gbp1 protein and *Leishmania major* parasites in skin of infected mice. Slices of skin tissue of females of BALB/c **(A)**, STS **(B)**, CcS-5 **(C)**, CcS-20 **(D)**, and O20 **(E)** mice infected for 8 weeks with *L. major* were stained with the anti-*Leishmania* lipophosphoglycan mouse monoclonal antibody (cat. no. CLP003A, Cedarlane, Hornby, Canada) and TRITC labeled IgM (115-025-020, Jackson ImmunoResearch, West Grove, PA) all diluted 1:500 and the rabbit anti-Gbp1 Polyclonal antibody (PA5-23509, Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:100 and anti-rabbit-AlexaFluor-647 (cat. no. 711-605-152; Jackson ImmunoResearch, West Grove, PA) diluted 1:500. Nuclei of the cells were stained with bisBenzimide H33258 (Sigma-Aldrich, St. Louis, MO, USA) 10 mg per 1 ml diluted 1:1,000. Images were captured with microscope Leica DM6000 objective HCX PL Apo 40×/0.75 PH2 and color camera Leica DFC490. Evaluation of images was done with Fiji ImageJ 1.51n. Figures are representatives of data from 8 to 11 mice (see Materials and Methods) in 3 of them 10 fields (320.66 × 239.57 μM) from each mouse were analyzed, in the rest one field was analyzed to verify the results. White arrow shows *L. major* amastigotes (red color), green arrows show Gbp2b/Gbp1 protein (yellow color), red arrows point to amastigotes co-localized with Gbp2b/Gbp1, whereas yellow arrows show either Gbp2b/Gbp1 surrounding parasite clusters or stretch of parasites and Gbp2b/Gbp1. Cell nuclei are stained in blue.

The immune reactions accompanying persistent *Leishmania* infection might be very important, because in addition to 12 million people presently suffering from the clinical manifestations of leishmaniasis (50), there are at least 120 million people with asymptomatic infection (45). It needs to be elucidated, whether such clinically asymptomatic people harboring persistent *Leishmania* parasites are more prone to immune-related diseases.



Strain	origin of *Gbp* cluster
BALB/c	C
STS	S
CcS-5	C
CcS-16	C
CcS-20	C
O20	O
B10	B
B10.O20	B
OcB-9	O
OcB-43	O

FIGURE 10 | Genetic origin (alleles) of *Gbp* cluster on chromosome 3 of tested strains. C—genotype of BALB/c origin, S—genotype of STS origin, O—genotype of O20 origin, B—genotype of B10 origin.

CONCLUSION

Our results represent the presently most comprehensive information about expression of *Gbps* in leishmaniasis *in vivo*.

We found that expression of *Gbp2b*/*Gbp1* and *Gbp5* is under strong genetic control involving in some strains also *trans*-regulation both in uninfected and *L. major*-infected mice.

We have observed that in several organs, expression of *Gbps* in recombinant congenic strains was outside the range of their parents. Tests of different strains that carry the same *Gbp* cluster genotypes on chromosome 3 indicate a *trans*-regulation of *Gbp2b*/*Gbp1* and *Gbp5* by genes that are not closely linked to *Gbp* genes. This finding may open way to identification and manipulation of these presently unknown genes.

Our results also point out that expression of *Gbp2b*/*Gbp1* and *Gbp5* was increased even in organs of resistant mice, which might suggest a hidden inflammation. It remains to be established whether the clinically asymptomatic infection might represent danger in predisposing organism to other diseases.

Co-localization of Gbp2b/Gbp1 protein with most *L. major* parasites in skin of resistant and intermediate strains STS, CcS-5, O20, and CcS-20 but not in highly susceptible BALB/c mice suggests that this molecule might play role in defense against leishmaniasis and opens new research direction in analysis of control of persistent parasites.

ETHICS STATEMENT

All experimental protocols utilized in this study comply with the Czech Government Requirements under the Policy of Animal Protection Law (No. 246/1992) and with the regulations of the Ministry of Agriculture of the Czech Republic (No. 207/2004), which are in agreement with all relevant European Union guidelines for work with animals and were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics AS CR and by Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of Sciences of the Czech Republic (permissions Nr. 190/2010; 232/2012).

AUTHOR CONTRIBUTIONS

YS planned and performed parasitology and expression experiments and contributed to the writing of the manuscript. VV performed parasitology experiments, analyzed the data, and contributed to the writing of the manuscript. TK contributed to the writing of the manuscript, parasitology experiments, estimation of parasite numbers, and data analysis. HH designed and performed immunohistochemistry analysis and analyzed the data. IK contributed to the estimation of parasite numbers. MS performed parasitology experiments. PD analyzed the data and contributed to the writing of the manuscript. ML conceived the study, interpreted data, and wrote the manuscript. All authors reviewed the manuscript.

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YS and IK are PhD students of Faculty of Science, Charles University, Prague, Czech Republic. MS is a PhD student of Faculty of Science, The University of South Bohemia, České Budějovice, Czech Republic. We thank J. Bartůněk and T. Pokorná for technical help.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00130/full#supplementary-material.

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