IMMUNO-EPIGENETIC MARKERS FOR INFECTIOUS DISEASES

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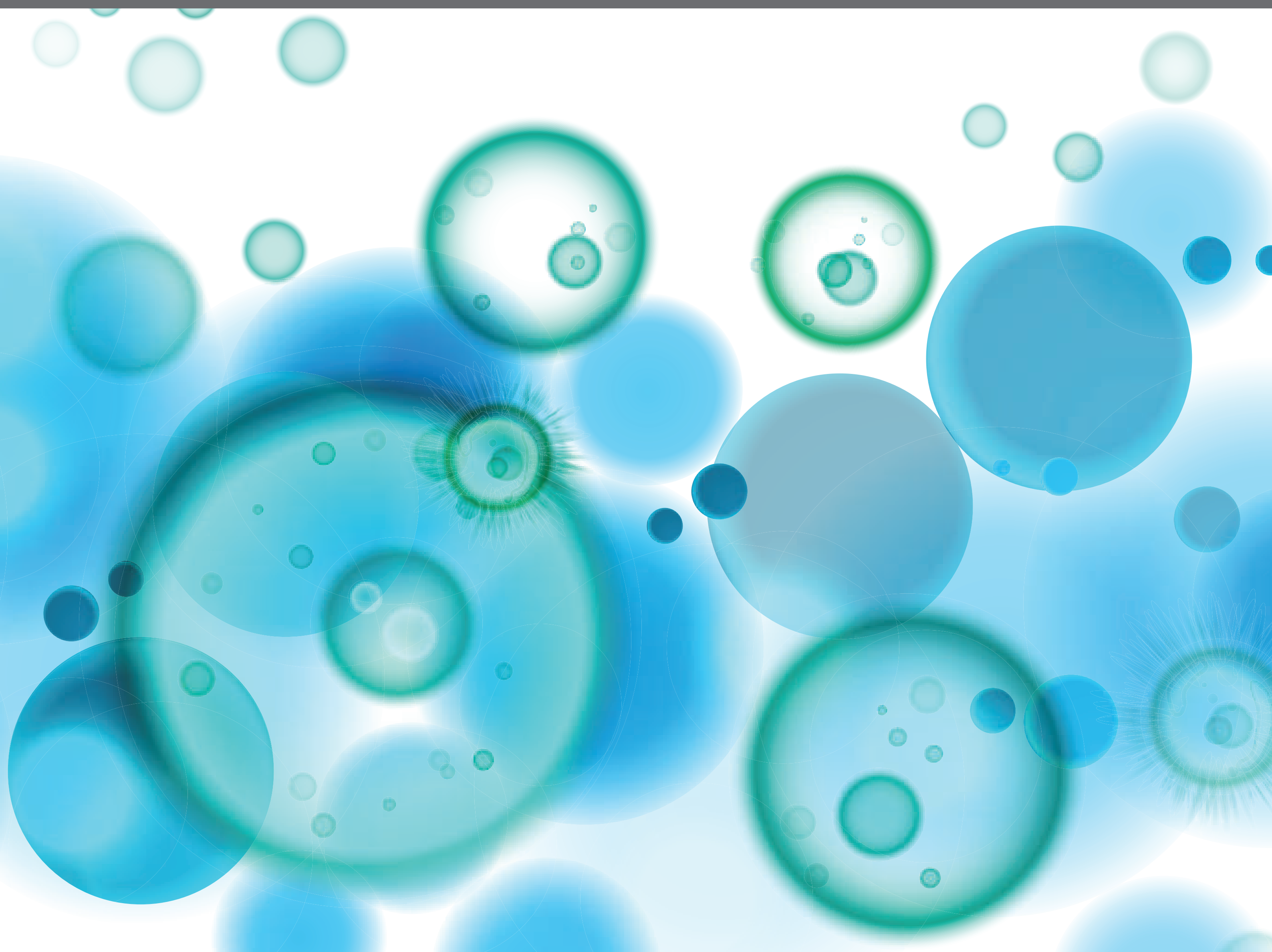

frontiers Research Topics

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IMMUNO-EPIGENETIC MARKERS FOR INFECTIOUS DISEASES

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Table of Contents

05 ***Editorial: Immuno-Epigenetic Markers for Infectious Diseases***
Eduardo José Melo dos Santos, Glen N. Barber, Ricardo Ishak and Antonio Carlos Rosário Vallinoto

07 ***miR-181a-5p Regulates TNF-α and miR-21a-5p Influences Gualynate-Binding Protein 5 and IL-10 Expression in Macrophages Affecting Host Control of* Brucella abortus *Infection***
Patrícia P. Corsetti, Leonardo A. de Almeida, André Nicolau Aquime Gonçalves, Marco Túlio R. Gomes, Erika S. Guimarães, João T. Marques and Sergio C. Oliveira

21 ***Correlation Between the Hepatic Expression of Human MicroRNA hsa-miR-125a-5p and the Progression of Fibrosis in Patients With Overt and Occult HBV Infection***
Nicola Coppola, Lorenzo Onorato, Marta Panella, Giorgio de Stefano, Nicola Mosca, Carmine Minichini, Vincenzo Messina, Nicoletta Potenza, Mario Starace, Loredana Alessio, Nunzia Farella, Evangelista Sagnelli and Aniello Russo

27 ***MicroRNA-Related Polymorphisms in Infectious Diseases—Tiny Changes With a Huge Impact on Viral Infections and Potential Clinical Applications***
Joel Henrique Ellwanger, Francis Maria Báo Zambra, Rafael Lima Guimarães and José Artur Bogo Chies

48 ***Toll-Like Receptor-1 Single-Nucleotide Polymorphism 1805T/G is Associated With Predisposition to Multibacillary Tuberculosis***
Raphaela Honorato Barletta-Naveca, Felipe Gomes Naveca, Vanessa Alves de Almeida, Jorge Ivan Rebelo Porto, George Allan Villarouco da Silva, Mauricio Morishi Ogusku, Aya Sadahiro, Rajendranath Ramasawmy and Antonio Luiz Boechat

55 ***Immune Activation, Proinflammatory Cytokines, and Conventional Risks for Cardiovascular Disease in HIV Patients: A Case-Control Study in Bahia, Brazil***
Clara Brites-Alves, Estela Luz, Eduardo M. Netto, Thalis Ferreira, Ricardo Sohbie Diaz, Celia Pedroso, Kimberly Page and Carlos Brites

61 ***The −3279C>A and −924A>G* polymorphisms *in the FOXP3 Gene are Associated With Viral Load and Liver Enzyme Levels in Patients With Chronic Viral Liver Diseases***
Leonn M. S. Pereira, Ednelza da Silva Graça Amoras, Simone R. S. da Silva Conde, Sâmia Demachki, Jaqueline C. Monteiro, Rosimar N. Martins-Feitosa, Andrea N. M. R. da Silva, Ricardo Ishak and Antonio C. R. Vallinoto

77 ***Variation in the Untranslated Genome and Susceptibility to Infections***
Veron Ramsuran, Rodger Ewy, Hoang Nguyen and Smita Kulkarni

85 ***Association of* Chlamydia trachomatis, C. pneumoniae*, and* IL-6 *and* IL-8 *Gene Alterations With Heart Diseases***
Nubia Caroline Costa Almeida, Maria Alice Freitas Queiroz, Sandra Souza Lima, Igor Brasil Costa, Marco Antonio Ayin Fossa, Antonio Carlos R. Vallinoto, Marluísa de Oliveira Guimarães Ishak and Ricardo Ishak

99 Leishmania-*Host Interactions—An Epigenetic Paradigm*
Farhat Afrin, Inbesat Khan and Hassan A. Hemeg

108 ***Evidence for Host Epigenetic Signatures Arising From Arbovirus Infections: A Systematic Review***
Gabriella Pequeno Costa Gomes de Aguiar, Claudio Manuel Gonçalves da Silva Leite, Beatriz Dias, Silvania Maria Mendes Vasconcelos, Renata Amaral de Moraes, Maria Elisabete Amaral de Moraes, Antonio Carlos Rosario Vallinoto, Danielle Silveira Macedo, Luciano Pamplona de Goes Cavalcanti and Fabio Miyajima

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EDITORIAL
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Editorial: Immuno-Epigenetic Markers for Infectious Diseases

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Keywords: immuno-epigenetic, biomarkers, infectious disease, host, immune response

Editorial on the Research Topic

Immuno-Epigenetic Markers for Infectious Diseases

The ability of microorganisms to infect and cause disease in higher organisms depends on: (i) the nature of the infecting organism(s), (ii) the route of infection, (iii) virulence factors, which enable long-term survival within the organism, and (iv) the immune defense mechanisms of the host. Molecular epidemiological investigations provide evidence regarding the etiology and mechanisms by which microorganisms cause disease, and the results are used to develop strategies for disease prevention. While classic immunogenetic biomarkers (single nucleotide polymorphisms—SNPs) have been shown to be related to resistance or susceptibility to infectious disease, new studies have highlighted alterations in the epigenetic landscape of immune cells as an equally important means of detecting and understanding the progression of infectious diseases. The constant evolutive race between host and infectious agents creates diverse scenarios ranging from differential susceptibility to infection to a large variability at pathogenesis, disease progression, and response to therapy.

In the center of these challenging diseases is the plasticity of the immune response, along with a myriad of environmental factors. This cross-talk among genes, pathways, and environment, that is carried out mainly by epigenetic actors can be used as biomarkers to predict disease progression and prognosis.

In the present Research Topic, original articles and reviews provided a comprehensive overview of immune and epigenetic biomarkers related to infectious diseases.

Bacterial and viral diseases were approached by the original article from Barletta-Naveca et al. who reported an association of Toll-like receptor 1 polymorphism with multibacillary/paucibacillary tuberculosis, along with sociodemographic and behavioral factors, as well as a classic and novel association study of *Chlamydia trachomatis* and *C. pneumoniae* infections, IL-6 and IL-8 polymorphisms, and heart diseases (Almeida et al.). Moreover, Pereira et al. showed that polymorphisms in the FOXP3 gene regulatory region are associated with viral load and liver enzyme levels in chronic viral hepatitis, and Brites-Alves et al. reported that levels of IL-6 are related to the risk of cardiovascular events in HIV-1-infected patients under antiretroviral therapy.

The plasticity of host-pathogen interaction was addressed by Ramsuran et al., who reviewed the polymorphisms in untranslated genomic regions and their role in the regulatory processes of infectious diseases. In a similar, but more focused approach, Ellwanger et al. reviewed the role of SNP in microRNA genes and/or their binding sites in infection by five major human viral pathogens: hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), Epstein-Barr virus (EBV), and human papillomavirus (HPV), showing the potential clinical applications of this approach.

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Protozoal pathogens were reviewed and highlighted epigenetic processes that occur during *Leishmania* infection, involving histone modifications and non-coding RNAs that modulate the host response to infection. Therapeutic aspects of epigenetics and their possible usefulness as biomarkers were also addressed (Afrin et al.). A systematic review performed by de Aguiar et al. on major epigenetic alterations in arboviruses, predominantly in Dengue, showed the role of microRNA and DNA methylation in secondary Dengue fever.

Finally, two original articles on epigenetic biomarkers showed that hsa-mir-125a-p may be a novel biomarker of liver damage in HBV patients (Coppola et al.), and the role of interference of *Brucella abortus* with microRNA expression affecting the expression of TNF alpha, IL-10, and gualynate-binding protein 5 in host macrophages (Corsetti et al.).

CONCLUDING REMARKS

Epigenetics is commonly defined as gene expression that is inherited but is not dependent on nucleic acid changes. The manuscripts in the present collection focus on some examples of highly important infectious diseases, which are usually associated with immune disorders leading to inflammation. The information presented is interesting and may introduce some new biomarkers of infection, disease progression, and prognosis. Despite our limited knowledge, molecular biology has become a fascinating tool to help unveil the mechanisms explaining the pathology of infection that is mediated by our immunological response. The increasing amount of information generated, which is clearly shown in each manuscript, may seem like unrelated data, but all data are useful, and bioinformatics will soon be able to cope with the enormous amount of information and will be used to investigate the currently available data and direct future studies.

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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miR-181a-5p Regulates TNF-α and miR-21a-5p Influences Gualynate-Binding Protein 5 and IL-10 Expression in Macrophages Affecting Host Control of *Brucella abortus* Infection

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Brucella abortus is a Gram-negative intracellular bacterium that causes a worldwide zoonosis termed brucellosis, which is characterized as a debilitating infection with serious clinical manifestations leading to severe complications. In spite of great advances in studies involving host–*B. abortus* interactions, there are many gaps related to *B. abortus* modulation of the host immune response through regulatory mechanisms. Here, we deep sequenced small RNAs from bone marrow-derived macrophages infected with *B. abortus*, identifying 69 microRNAs (miRNAs) that were differentially expressed during infection. We further validated the expression of four upregulated and five downregulated miRNAs during infection *in vitro* that displayed the same profile in spleens from infected mice at 1, 3, or 6 days post-infection. Among these miRNAs, mmu-miR-181a-5p (upregulated) or mmu-miR-21a-5p (downregulated) were selected for further analysis. First, we determined that changes in the expression of both miRNAs induced by infection were dependent on the adaptor molecule MyD88. Furthermore, evaluating putative targets of mmu-miR-181a-5p, we demonstrated this miRNA negatively regulates *TNF-α* expression following *Brucella* infection. By contrast, miR-21a-5p targets included a negative regulator of IL-10, programmed cell death protein 4, and several guanylate-binding proteins (GBPs). As a result, during infection, miR-21a-5p led to upregulation of *IL-10* expression and downregulation of *GBP5* in macrophages infected with *Brucella*. Since GBP5 and IL-10 are important molecules involved in host control of *Brucella* infection, we decided to investigate the role of mmu-miR-21a-5p in bacterial replication in macrophages. We observed that treating macrophages with a mmu-miR-21a-5p mimic enhanced bacterial growth, whereas transfection of its inhibitor reduced *Brucella* load in macrophages. Taken together, the results indicate that downregulation of mmu-miR-21a-5p induced by infection increases GBP5 levels and decreases *IL-10* expression thus contributing to bacterial control in host cells.

Keywords: microRNA, epigenetics, *Brucella abortus*, guanylate-binding protein 5, inflammation, miR-21a-5p

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INTRODUCTION

Brucellosis is a disease caused by a Gram-negative, facultative intracellular coccobacillus from *Brucella* genus that groups 10 species classified according to host specificity (1). Brucellosis is considered the most widespread zoonosis representing a great public health problem (2, 3). In humans and animals, brucellosis is characterized by a chronic, sometimes lifelong, debilitating infection with serious clinical manifestations leading to severe complications (4). As an intracellular lifestyle bacterium, *Brucella abortus* reaches its replicative niche within phagocytic cells, most prominently macrophages. Despite *B. abortus* is recognized by several innate immune receptors and triggers inflammatory response against this bacterium, it is able to evade killing in phagolysosomes and replicate successively with an endoplasmic reticulum-associated compartment and a modified autophagosome (5, 6). Moreover, we and others demonstrated that *B. abortus* could modulate the immune response through induction of regulatory cytokines such as IL-10 as negative regulation of pro-inflammatory cytokines, suggesting that this interplay between immune responses enables *B. abortus* persistence in the host (7–9). Recently, studies have increasingly reported the involvement of microRNAs (miRNAs) in the regulation of host responses to bacterial pathogens (10). miRNAs are small non-coding RNAs that negatively regulate gene expression by directly binding to the 3′ untranslated region (3′ UTR) of their mRNA targets. Inflammatory and anti-inflammatory responses can induce changes in transcription, processing, or stabilization of mature or precursor miRNA transcripts (11). Several reports have demonstrated the role of host miRNAs during bacterial infection, including *Helicobacter pylori* (12), *Salmonella enterica* (13, 14), *Listeria monocytogenes* (15, 16), *Mycobacterium* species (17–21), or *Francisella tularensis* (22). Those reports used various approaches to determine which miRNAs are differentially expressed during pathogen infection. High-throughput RNA sequencing (RNAseq) allows unbiased analysis of miRNA signatures associated with infection (23). Of note, Zheng et al. (24) described the miRNA expression profile of *Brucella melitensis*-infected RAW264.7 cells by high-throughput sequencing. They observed several differentially expressed miRNAs but did not define putative targets that could be associated with the response to *B. melitensis* infection (24). Here, we describe a panel of miRNAs that are differentially expressed in *B. abortus*-infected macrophages using high-throughput sequencing of small RNA libraries at early times after infection. We further characterize miRNAs whose regulation is MyD88-dependent *in vivo* and *in vitro*. Finally, we show that one of the miRNAs downregulated during infection, miR-21a-5p, affects host control of *B. abortus* infection by negatively regulating guanylate-binding protein (GBP) 5 and inducing *IL-10* expression.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the Brazilian laws 6638 and 9605 in Animal Experimentation. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Minas Gerais (Permit Number: CETEA no. 104/2011).

Mice, Cell Culture, and Bacteria

MyD88 KO mice were kindly provided by Shizuo Akira from the Osaka University in Japan. The wild-type strain C57BL/6 mice were purchased from the Federal University of Minas Gerais animal facility (UFMG, Belo Horizonte, Brazil). Genetically deficient and control mice were maintained at UFMG and used at 6–8 weeks of age. Bone marrow cells were obtained from femora and tibia of mice and they were derived in bone marrow-derived macrophages (BMDMs) as previously described (25). *B. abortus* virulent strain 2308 was obtained from our own laboratory collection. They were grown in *Brucella* broth medium (BD Pharmingen, San Diego, CA, USA) for 3 days at 37°C without CO_2.

Macrophage Infection With *B. abortus*

Bone marrow-derived macrophages were transfected for 24 h with mimics, inhibitors, or scramble controls at a concentration of 5 pmol/well in 24-well plates containing 5×10^5 cells and then infected with virulent *B. abortus* strain 2308 at a multiplicity of infection of 100:1. Bacteria were centrifuged onto macrophages at $400 \times g$ for 10 min at 4°C then incubating the cells for 30 min at 37°C under 7% CO_2. Macrophages were extensively washed with HBSS to remove extracellular bacteria and incubated for an additional 90 min in medium supplemented with 100 µg/mL gentamicin to kill extracellular bacteria. Thereafter, the antibiotic concentration was decreased to 10 µg/mL. At each time point, samples were washed three times with HBSS before processing. To monitor *Brucella* intracellular survival, infected cells were lysed with 0.1% (vol/vol) Triton X-100 in H_2O and serial dilutions of lysates were rapidly plated onto *Brucella* broth agar plates to count the number of CFU.

Real-Time RT-PCR for Pro-Inflammatory Cytokine Expression

Bone marrow-derived macrophages were homogenized with TRIzol reagent (Invitrogen) to isolate total RNA. Reverse transcription of 1 µg from total RNA was performed using illustra™ Ready-To-Go RT-PCR Beads (GE Healthcare, Buckinghamshire, UK). Real-time RT-PCR was conducted in a final volume of 10 µL containing the following: SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), cDNA as the PCR template, and 20 µM of primers. The PCR reaction was performed with ABI 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using the following cycling parameters: 60°C for 10 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min, and a dissociation stage of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s. Primers were used to amplify a specific 100- to 120-bp fragment corresponding to specific gene targets as described in Table S1 in Supplementary Material. All data are presented as relative expression units compared with 0 h post-infection after normalization to the *β-actin* gene ($\Delta\Delta$Ct = ΔCt treatment − ΔCt 0 h post-infection) (26). PCR measurements were conducted in triplicate. The differences in the relative expression were analyzed by analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$ for statistically significant).

Small RNA Library Preparation and Sequencing

For deep sequencing, total RNA was isolated from control uninfected macrophages or 30 min after *B. abortus* infection using TRIzol reagent (Invitrogen). Total RNA was sent to FASTERIS SA (Plan-les-Ouates, Switzerland) for construction and sequencing of strand-specific small RNA libraries. Small RNAs (15–50 nt) purified from polyacrylamide gel were used to construct libraries using the Illumina® TruSeq® Small RNA Library Prep Kit for Illumina HiSeq 2000 sequencing (Illumina Inc., San Diego, CA, USA).

Small Non-Coding RNA Bioinformatics Identification

Raw reads from sequencing of the small RNA libraries were processed to remove adapter sequences using the cutadapt tool[1] and sequencing quality was analyzed using the FastQC tool.[2] Remaining sequencing reads were collapsed to optimize mapping in the reference genomes as previously described (27). Reads were mapped to *Mus musculus* (GRCm38) genome or *B. melitensis* biovar Abortus (strain 2308) genome using the Bowtie Program.[3] Using the BedTool Program,[4] mapped reads were automatic annotated as miRNAs (miRBase version 21), mRNA, snRNA, snoRNA, rRNA (GRCm38.73), or tRNAs.[5] Reads mapping to each annotated miRNA were counted using an *in house* script developed by our group. Only miRNAs that were present in both libraries were used to evaluate differential expression. Each miRNA was normalized (RPM—reads per million) according to the formula:

$$\mathrm{RPM}(\mathrm{miRNA}_i,\mathrm{sample}_j) = \frac{\mathrm{expression}\,(\mathrm{miRNA}_i,\,\mathrm{sample}_j)}{\sum_{k=1}^{n}\mathrm{expression}(\mathrm{miRNA}_k,\mathrm{sample}_j)} \times 1\ \mathrm{million}$$

where i corresponds to a specific miRNA expression and j is the specific library. Differentially expressed miRNAs were chosen by following the criteria of number of reads (minimum of 200) and fold-change between control and infected libraries (at least 1.5-fold). A complete list of miRNAs expressed in control and infected macrophages is in Table S2 in Supplementary Material.

In Vitro or *Ex Vivo* Validation of Differentially Expressed miRNA by Real-Time RT-PCR

The expression of selected miRNAs was validated by qPCR. miRNAs were purified from infected or non-infected (NI) BMDMs and from spleens of NI or 3 days intraperitoneally infected C57BL/6 or MyD88 KO mice using miRNeasy mini kit (QIAGEN). Reverse transcription of miRNAs was performed according to the miScript® II RT kit (QIAGEN) protocol. To access mature miRNA expression, we used miScript SYBR® Green PCR kit (QIAGEN) to detect specific amplification in PCR reaction performed with ABI 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All data are presented as relative expression units compared with 0 min, 0 h, 0 days, or untreated cells after normalization to the *SNORD61* gene (ΔΔCt = ΔCt treatment − ΔCt 0 min, 0 h, 0 days, or untreated cells). PCR measurements were conducted in triplicate. The differences in the relative expression were analyzed by ANOVA followed by Tukey's test ($p < 0.05$ for statistically significant).

Prediction of Putative Targets of Selected miRNAs

Putative targets of nine differentially expressed miRNAs that were validated by RT-qPCR (upregulated: mmu-miR-151-3p, mmu-miR-155-5p, mmu-miR-181a-5p, and mmu-miR-328-3p; and downregulated: mmu-miR-21a-5p, mmu-miR-98-5p, mmu-miR-145a-5p, mmu-miR-146b-5p, and mmu-miR-374b-5p) were obtained from the miRWalk database.[6] Putative targets were filtered based on the following criteria: binding p value ≥0.95, seed fully matched and binding site location of 3′ UTR and CDS. miRNA targets were further analyzed using Reactome V53[7] to select for candidates associated with an immune function (Table S3 in Supplementary Material).

In Vitro Evaluation of TNF-α, IL-10, and GBP5 Targets for Selected miRNAs

To evaluate putative targets of selected miRNAs, BMDMs were transfected for 24 h with small, chemically modified double-stranded RNAs that mimic endogenous mmu-miR-181a-5p or mmu-miR-21a-5p (mimics) to enable miRNAs functional analysis by upregulation of their activities. On the other hand, BMDMs were transfected with small, chemically modified single-stranded RNA molecules designed to specifically bind to and inhibit endogenous mmu-miR181a-5p or mmu-miR-21a-5p (inhibitors) and enable miRNA functional analysis by downregulation of miRNA activity (mirVana™ miRNA mimics or inhibitors—Thermo Fischer Scientific, MA, USA). Non-transfected cells or cells transfected with scramble controls (Thermo Fischer Scientific, MA, USA) were used as negative controls. After transfection, cells were infected with *B. abortus*, as described above, and the total RNA was extracted and reverse transcribed in cDNA from mRNA or miRNA as described above.

Immunoblotting

Bone marrow-derived macrophages transfected with mmu-miR-21a-5p mimic or inhibitor were lysed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with phosphatase and protease inhibitors (Roche). Equal amounts of proteins were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden). Membranes were blocked for 1 h in TBS with 0.1% Tween-20 containing 5% nonfat dry milk and incubated

[1] https://github.com/marcelm/cutadapt (Accessed: May 14, 2015).
[2] https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Accessed: March 25, 2015).
[3] http://bowtie-bio.sourceforge.net/index.shtml (Accessed: June 21, 2015).
[4] http://bedtools.readthedocs.io/en/latest/ (Accessed: June 25, 2015).
[5] http://gtrnadb.ucsc.edu/Mmusc10/mm10-tRNAs.fa, http://gtrnadb.ucsc.edu/genomes/eukaryota/Mmusc10/mm10-tRNAs.tar.gz (Accessed: November 16, 2017).
[6] http://mirwalk.umm.uni-heidelberg.de/ (Accessed: May 9, 2018).
[7] https://reactome.org/ (Accessed: September 13, 2015).

overnight with primary antibodies [anti-GBP5 dilution 1:500 (ProteinTech, Chicago, IL, USA) or β-actin (dilution 1:5,000, Cell Signaling Technology, Danvers, MA, USA)] at 4°C. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (dilution 1:1,000) and Luminol chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) was used for antibody detection. Densitometry analysis was performed using ImageQuant TL Software (GE Healthcare, Buckinghamshire, UK), and band intensities were normalized to β-actin.

Statistical Analysis

All experiments (except RNAseq) were repeated at least twice with similar results and figures show data from one representative experiment. The number of replicates for each experiment is mentioned in each specific figure legend. Graphs and data analysis were performed using GraphPad Prism 5 (GraphPad Software), using one-way ANOVA or two-way ANOVA (Bonferroni *post hoc* test).

RESULTS

High Pro-Inflammatory Cytokine Gene Expression Occurs in the Initial Phase of Macrophage Infection With *B. abortus*

To evaluate the peak of cytokine gene expression by BMDMs after *B. abortus* infection, total RNA from intracellular-infected cells were transcribed into cDNA and *IL-12*, *TNF-α*, *IL-1β*, or *IL-6* transcripts were accessed at different time-points by real-time PCR (**Figure 1**). The results showed an upregulation of investigated pro-inflammatory cytokines 30 min after BMDM infection with *B. abortus*. *IL-12* expression demonstrated a peak at 30 min and 24 h post-infection (**Figure 1A**). However, at 24 h post-infection, the levels of detected *IL-12* transcripts were much greater compared with the other cytokines tested. *IL-1β* (**Figure 1B**), *TNF-α* (**Figure 1C**), or *IL-6* (**Figure 1D**) present similar patterns of differential expression with a peak at 30 min post-infection when compared with NI cells. These findings demonstrate that *B. abortus* induces pro-inflammatory cytokine expression in BMDMs at 30 min after infection.

High-Throughput Sequencing of Small RNA Libraries Identifies Differential Expression of miRNAs During Macrophage Infection With *B. abortus*

As demonstrated above that BMDMs trigger upregulation of pro-inflammatory cytokines at 30 min after *B. abortus* infection, we chose this time point to prepare small RNA libraries for high-throughput sequencing. RNAseq revealed that more than 80% of sequenced reads corresponded to 18–26 nt small RNAs with a peak at 22 nt, in both libraries (Table S4 in Supplementary Material). These results indicate that both small RNA libraries were enriched for the expected size of mammalian miRNAs. Small RNAs were mapped to mouse (*M. musculus*—GRCm38) and *B. abortus* (strain 2308) reference genomes. In the small RNA library from infected macrophages, we observed that 92.02% of all mapped reads belonged to the *M. musculus* genome, while 7.43% originated from *B. abortus* (Table S5 in Supplementary Material). In the library from control NI cells, 99.95% of mapped reads originated from the *M. musculus* genome. In the library from control macrophages, we identified 819 miRNAs comparable to 800 detected in *B. abortus*-infected cells. In total, 745 miRNAs were detected in both libraries (complete list is shown in Table S2 in Supplementary Material). Less than 10% of mapped reads did not correspond to miRNAs and corresponded to mRNA, snRNA, snoRNA, rRNA, or tRNA. Therefore, vast majority of small RNAs detected in our samples were miRNAs. We next concentrated our analysis on differentially expressed miRNAs. We observed that, in total, 69 miRNAs had at least 1.5 fold-change in expression comparing control and *Brucella*-infected macrophages and at least 200 reads in each sample (**Figure 2**; Table S2 in Supplementary Material). RNAseq results were based on a single sequencing experiment to have a broad picture of the host small RNA response to infection. For further validation, we chose four upregulated (mmu-miR-151-3p, mmu-miR-155-5p, mmu-miR-181a-5p, and mmu-miR-328-3p) and five downregulated (mmu-miR-21a-5p, mmu-miR-98-5p, mmu-miR-145a-5p, mmu-miR-146b-5p, and mmu-miR-374b-5p) miRNAs (Table S6 in Supplementary Material) in infected samples by real-time PCR in macrophages. These results corroborated the differential expression of all nine miRNAs in infected macrophages *in vitro* (**Figure 3**).

To analyze the expression of these miRNAs *in vivo*, C57BL/6 mice were infected intraperitoneally with *Brucella*, and the relative expression of miRNAs were evaluated in mouse spleens at 1, 3, or 6 days post-infection. Consistent with *in vitro* results, we observed that all upregulated or downregulated miRNAs presented this profile in at least one time point of infection analyzed (**Figures 4A–I**).

MyD88 Plays an Important Role in Regulating the Expression of miRNAs During *B. abortus* Infection

Since innate immunity is the first line of host immune defense against bacterial pathogens and our group has previously demonstrated the important role of MyD88 adaptor molecule during *B. abortus* infection (25), we evaluated the influence of MyD88 during differential expression of miRNAs upregulated (mmu-miR-181a-5p and mmu-miR-328-3p) or downregulated (mmu-miR-21a-5p, mmu-miR-98-5p, and mmu-miR-146b-5p) by infection. We observed a dependence of MyD88 in upregulation of mmu-miR-181a-5p (**Figure 5A**), while it was not observed differences of upregulation of mmu-miR-328-3p in the absence of MyD88 (**Figure 5B**). On the other hand, we observed a dependence of MyD88 in the downregulation of all miRNAs we tested during *B. abortus* infection in macrophages (**Figures 5C–E**). We next evaluated the expression of selected miRNAs in spleens from MyD88 KO compared with wild-type mice (**Figures 5F–J**). Among upregulated miRNAs, only mmu-miR-181a-5p showed the same profile of MyD88 regulation *in vitro* and *ex vivo* (**Figure 5F**). Regarding downregulated miRNAs, the dependence on MyD88 was similar *in vitro* as well as *ex vivo*, highlighting the importance of this adaptor molecule in regulating miRNA expression (**Figures 5H–J**).

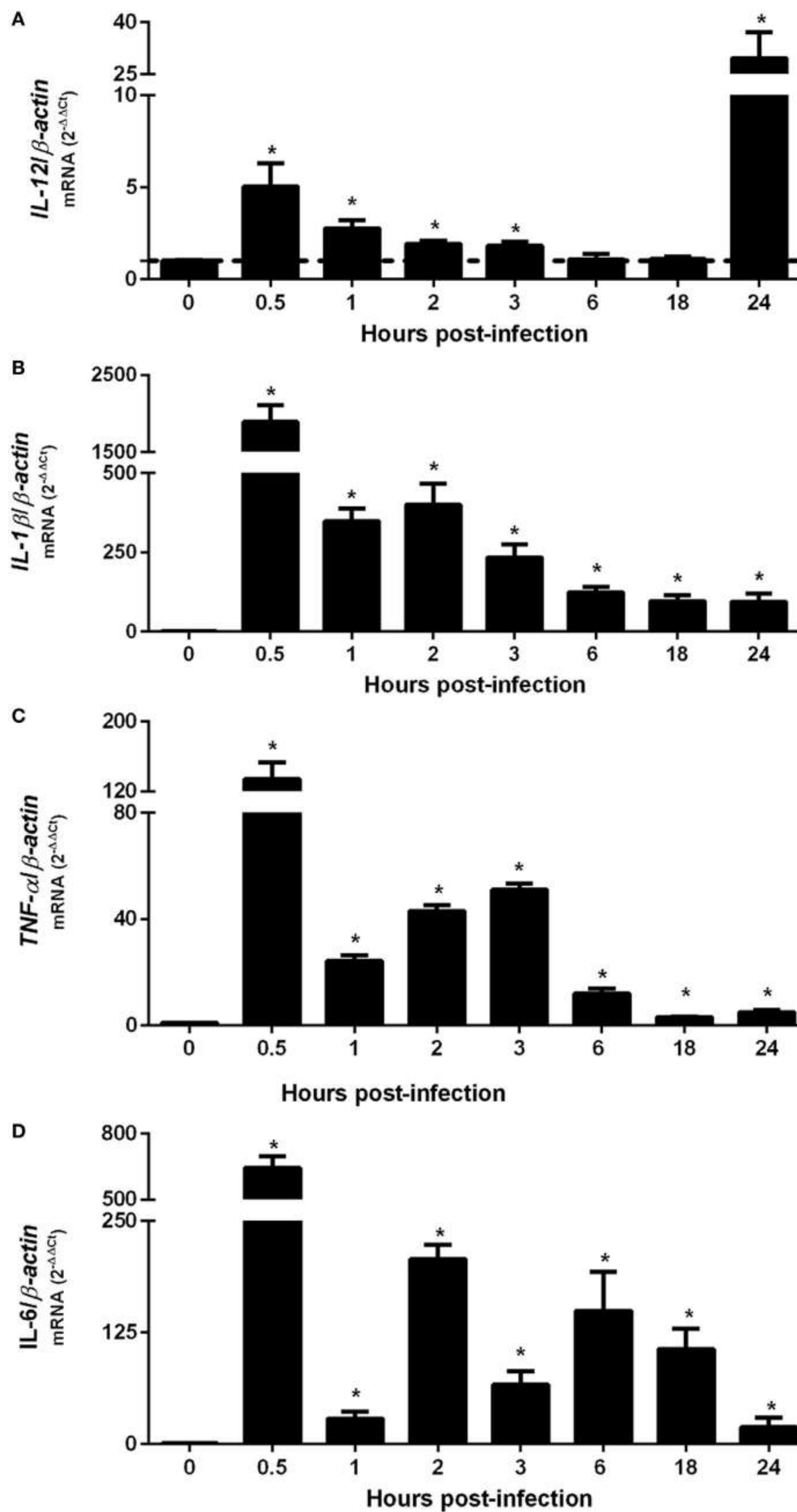

FIGURE 1 | Continued

FIGURE 1 | Upregulation of pro-inflammatory cytokine genes occurs 30 min after *Brucella abortus* infection of macrophages. Bone marrow-derived macrophages from C57BL/6 mice were obtained and infected with *B. abortus* strain 2308 for 0.5, 1, 2, 3, 6, 18, or 24 h. Total RNA were extracted and cDNA for IL-12 **(A)**, IL-1β **(B)**, TNF-α **(C)**, or IL-6 **(D)** were assessed by real-time PCR to determine the level of differential expression compared with non-infected (NI) cells (0 h post-infection). The results were normalized to β-actin. Error bars represent the mean ± SD. Similar results were obtained in three independent experiments. Statistically significant differences of gene expression from infected compared with NI cells are denoted by an asterisk ($p < 0.05$).

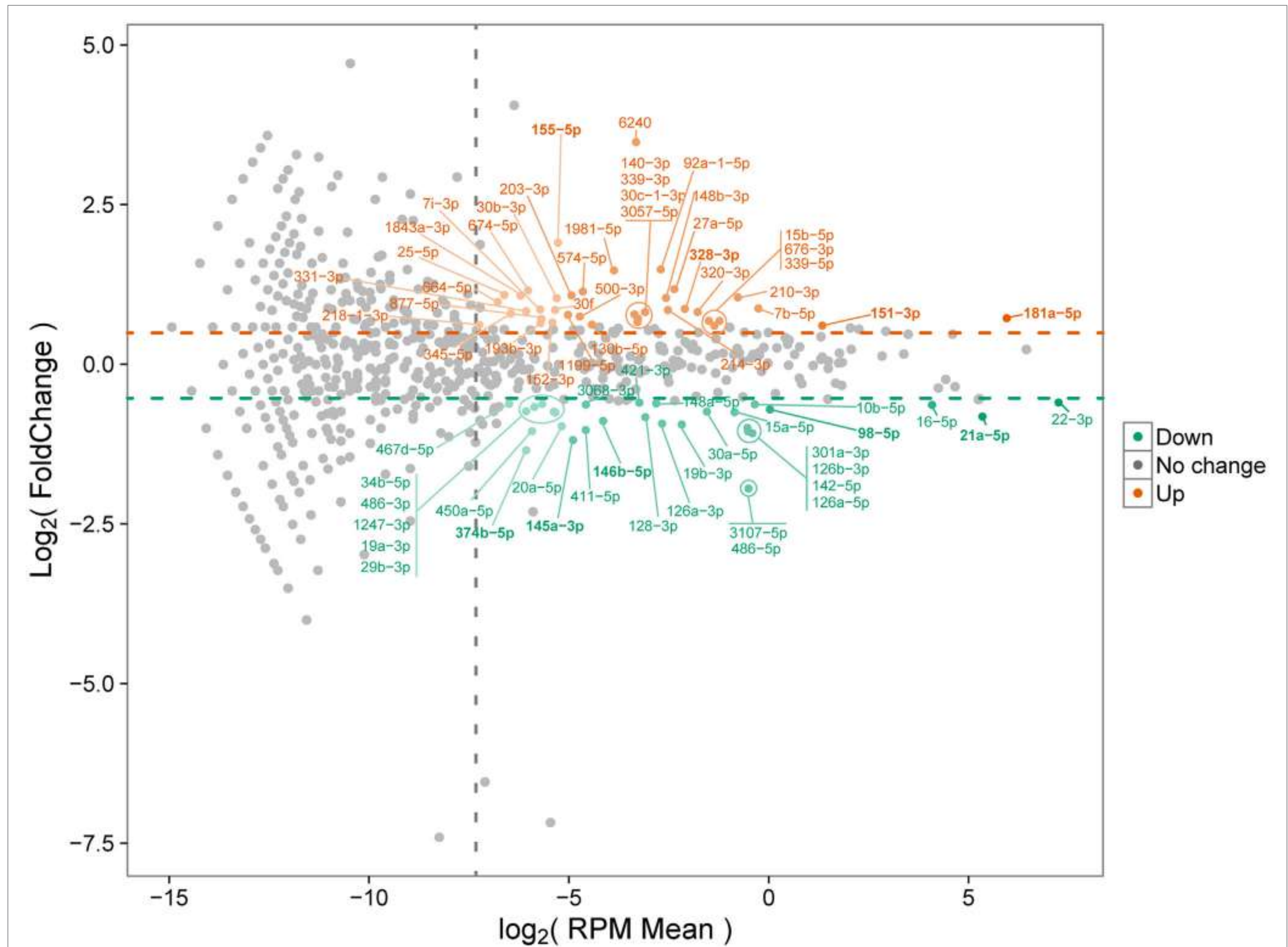

FIGURE 2 | MA plot of differentially expressed microRNAs (miRNAs). Differential miRNA expression between control and infected macrophages is shown. Upregulated and downregulated miRNAs in infected samples compared with controls are indicated. Only miRNAs with at least 200 reads in each sample were considered. Orange and green circles represent upregulated and downregulated miRNAs, respectively. Horizontal dashed lines represent the upper and lower limits of at least 1.5 fold-change. Vertical dashed line shows the normalized miRNA expression corresponding to 200 reads per sample.

miR-181a-5p and miR-21a-5p Regulate Important Immune Pathways During *B. abortus* Infection

mmu-miR-181a-5p and mmu-miR-21a-5p showed differential expression during *B. abortus* infection *in vivo* and *in vitro* in a MyD88-dependent manner. Furthermore, these were the most expressed miRNAs in macrophages that showed differential expression (**Figure 2**). Therefore, we chose these miRNAs for further studies. First, we analyzed the kinetics of expression for mmu-miR-181a-5p or mmu-miR-21a-5p during *B. abortus* infection in macrophages. As demonstrated in **Figures 6A,B**, both miRNAs were differentially expressed throughout the time course of infection. Expression of mmu-miR-181a-5p increased during all time-points evaluated when compared with 0 h (**Figure 6A**). Nevertheless, we observed a slight reduction in mmu-miR-181a-5p expression at 3 h when compared with 1 or 6 h post-infection. We also observed that mmu-miR-21a-5p was downregulated during the first 6 h post-infection (**Figure 6B**). To characterize the role of these miRNAs during *B. abortus* infection, we transfected macrophages with miRNA mimics or inhibitors for mmu-miR-181a-5p or mmu-miR-21a-5p. We specifically

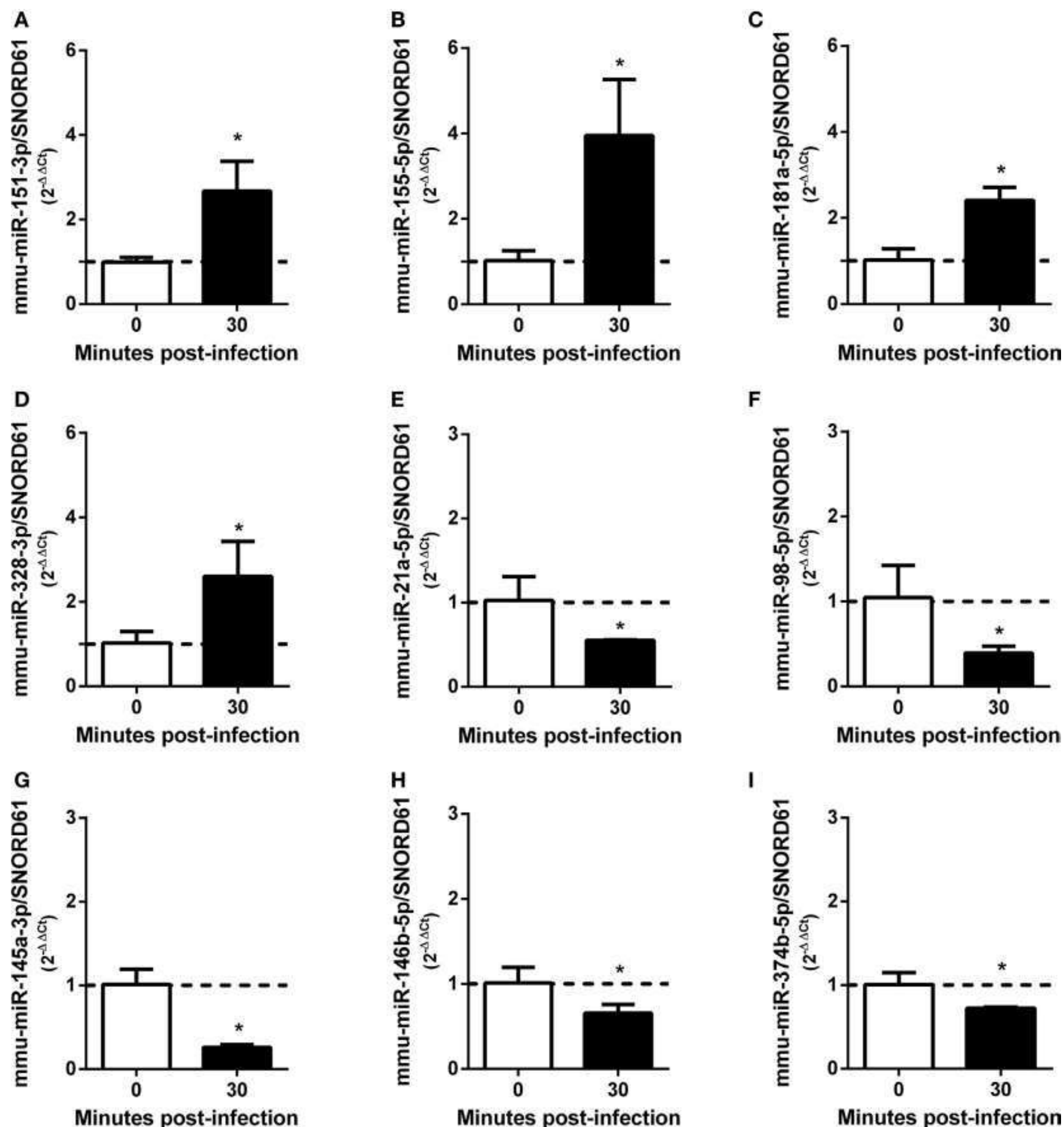

FIGURE 3 | MicroRNAs (miRNAs) differentially expressed during *Brucella abortus* infection in macrophages identified by RNA sequencing were validated by real-time PCR. Four miRNAs were validated by real-time PCR as upregulated: **(A)** mmu-miR-151-3p, **(B)** mmu-miR-155-5p, **(C)** mmu-miR-181a-5p, and **(D)** mmu-miR-328-3p. By contrast, five miRNAs were validated as downregulated: **(E)** mmu-miR-21a-5p, **(F)** mmu-miR-98-5p, **(G)** mmu-miR-145a-3p, **(H)** mmu-miR-146b-5p, and **(I)** mmu-miR-374b-5p. miRNAs expression was assessed by real-time PCR and were normalized to *SNOR61*. Error bars represent the mean ± SD. Similar results were obtained in three independent experiments. Statistically significant differences of miRNAs expression after 30 min bone marrow-derived macrophage infection compared with non-infected cells are denoted by an asterisk ($p < 0.05$).

observed reduction of miRNA expression by the inhibitor or increased levels by the mimic (**Figures 6C,D**).

Each of these miRNAs, mmu-miR-181a-5p and mmu-miR-21a-5p, could regulate several mRNA targets that could affect the host immune responses to *B. abortus* (Table S3 in Supplementary Material). mmu-miR-181a-5p was reported to be important in the regulation of NF-κB activation and TNF-α production (28). More recently, Luo et al. (29) have demonstrated that *Brucella suis* upregulated miR-181a that correlated with decreased TNF-α in Raw264.7 macrophage cell line. To further investigate whether miR-181a-5p can influence TNF-α expression in BMDMs during *B. abortus* infection, we transfected macrophages with the specific mimic or inhibitor for mmu-miR-181a-5p before infection. As observed in **Figure 6F** when the miR-181a-5p mimic was used, there was a decrease in *TNF-α* transcripts in *B. abortus*-infected macrophages. By contrast, when the miR-181a-5p inhibitor was transfected in BMDMs, we observed an increase in *TNF-α* mRNA levels. These data suggest that upregulation of miR-181a-5p prevents further increase in TNF-α levels during *B. abortus* infection.

One well defined target of miR-21a-5p is programmed cell death protein 4 (PDCD4), which is a pro-inflammatory protein that suppresses IL-10 expression (30). To better understand if the downregulation of miR-21a-5p observed during *B. abortus*

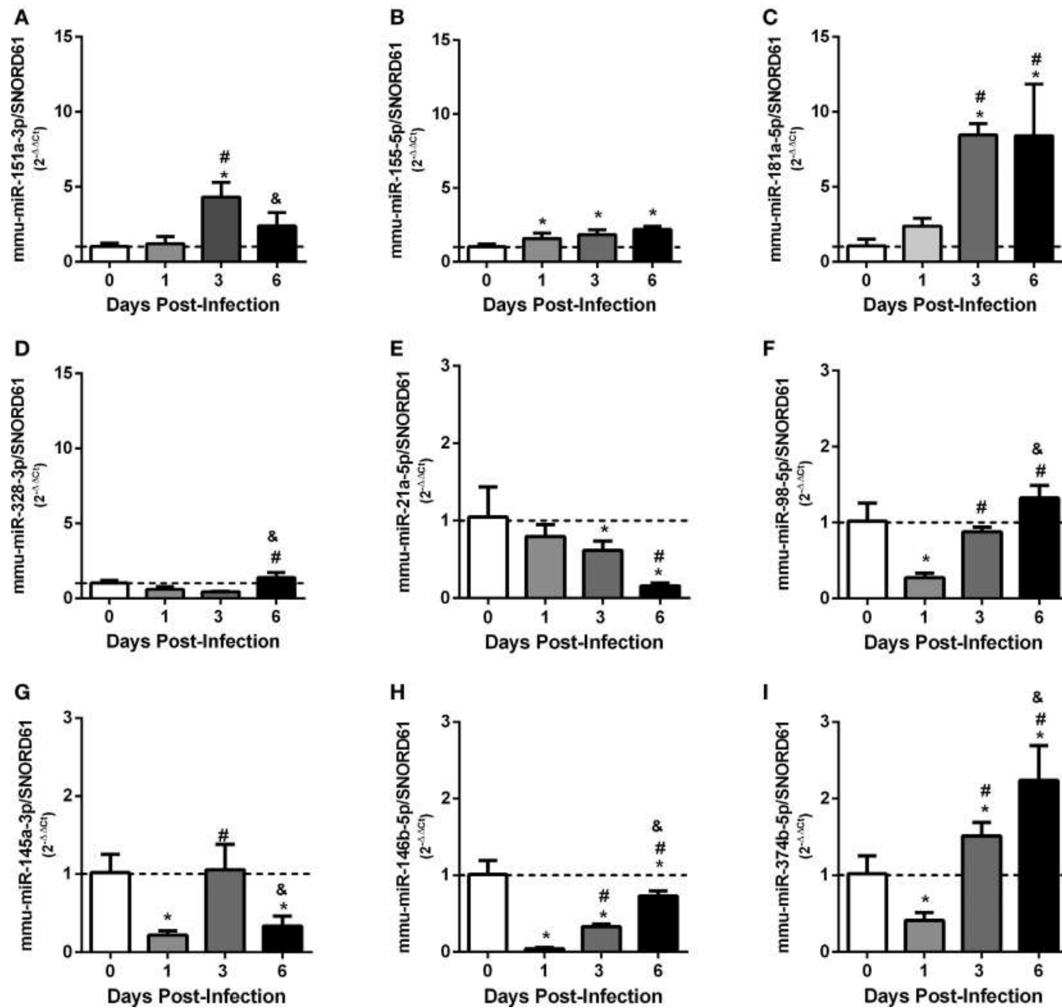

FIGURE 4 | *Ex vivo* analysis of differentially expressed validated microRNAs (miRNAs) in mouse spleens. C57BL/6 mice were infected intraperitoneally at 1, 3, or 6 days post-infection, and the relative expression of miRNAs: **(A)** mmu-miR-151-3p, **(B)** mmu-miR-155-5p, **(C)** mmu-miR-181a-5p, **(D)** mmu-miR-328-3p, **(E)** mmu-miR-21a-5p, **(F)** mmu-miR-98-5p, **(G)** mmu-miR-145a-3p, **(H)** mmu-miR-146b-5p, and **(I)** mmu-miR-374b-5p were evaluated in mouse spleens. miRNAs expression were assessed by real-time PCR and were normalized to *SNOR61*. Error bars represent the mean ± SD. Similar results were obtained in two independent experiments. Statistically significant differences of miRNAs expression from infected mice compared with non-infected mice (0) are denoted by an asterisk ($p < 0.05$), # ($p < 0.05$) represents statistically significant differences compared with 1 day post-infection, and & represents statistically significant differences compared with 3 day post-infection with *B. abortus*.

infection could modulate *IL-10* mRNA in BMDMs, we used specific miRNA mimics or inhibitors. We observed that the miR-21a-5p mimic induced higher levels of *IL-10* mRNA in infected macrophages while its inhibitor reduced *IL-10* transcripts compared with controls (**Figure 6E**). These results suggest that the reduction of mmu-miR-21a-5p during *B. abortus* infection helps increase *IL-10* mRNA levels by inhibiting PDCD4 in macrophages.

miR-21a-5p Regulates GBP5 Expression and Partially Influences Intracellular *B. abortus* Growth

In addition to these previously characterized targets, we also observed that the mRNA for several GBPs including GBP2, 4, 5, and 8 had binding sites for mmu-miR-21a-5p (Table S3 in Supplementary Material). GBPs are interferon-inducible GTPases that exert direct anti-microbial effects (31). GBPs encoded by genes on mouse chromosome 3 (GBP1, GBP2, GBP3, GBP5, and GBP7) promote recognition of the vacuolar bacterium *Salmonella typhimurium* leading to the escape of the bacteria into the cytosol (32). More recently, our group showed that the same GBPs are critical for *B. abortus* control in mouse macrophages and *in vivo* (33). Since GBPs are putative targets of miR-21a-5p, we decided to evaluate whether this miRNA could also regulate *GBP5* expression in macrophages infected with *B. abortus*. Transfection of the miR-21a-5p mimic decreased levels of the *GBP5* mRNA in macrophages (**Figure 7A**). By contrast, cells transfected with miR-21a-5p inhibitor showed increased levels of *GBP5* transcripts (**Figure 7A**). We also assessed GBP5 protein levels in BMDMs treated with miR-21a-5p mimic or inhibitor. As shown

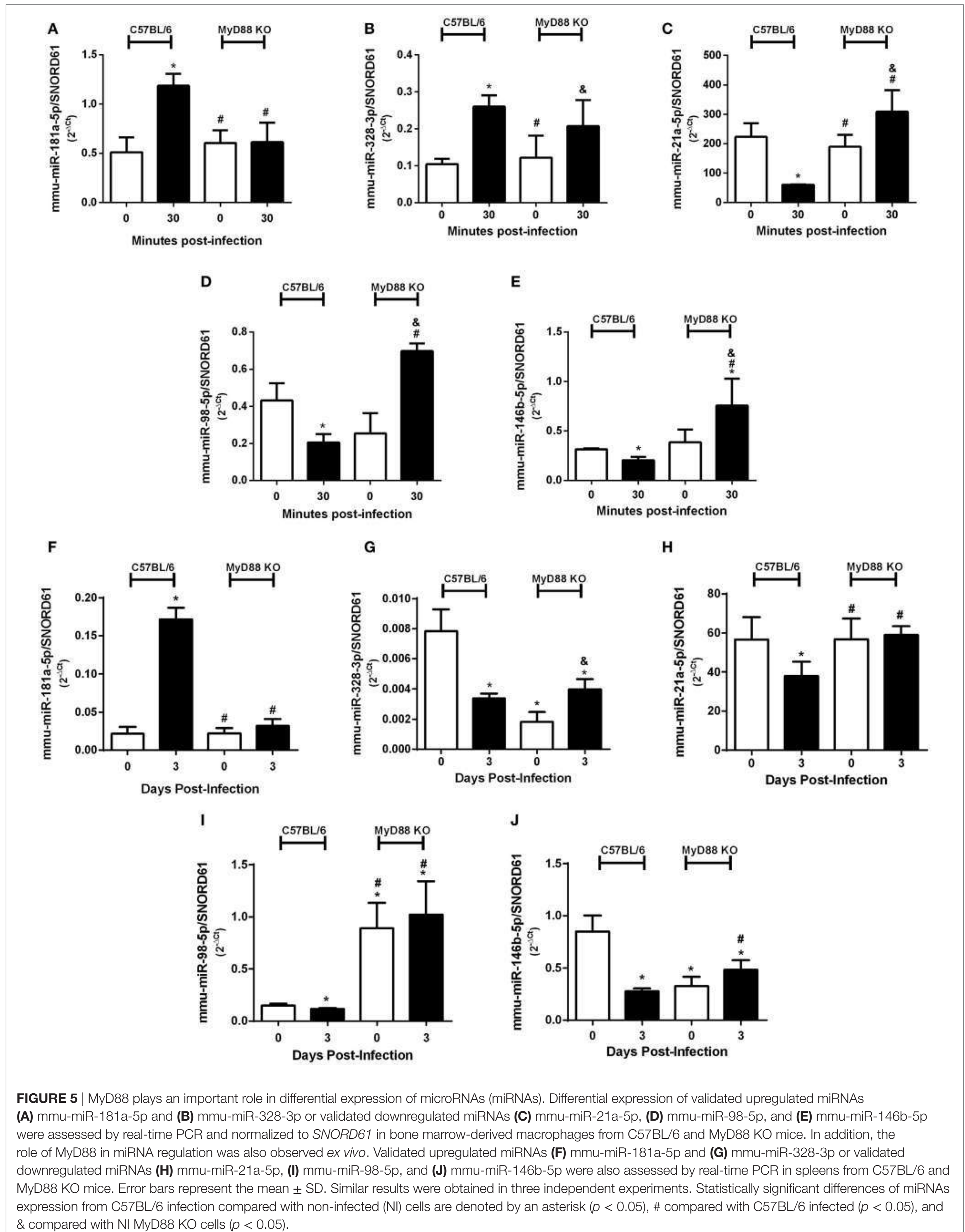

FIGURE 5 | MyD88 plays an important role in differential expression of microRNAs (miRNAs). Differential expression of validated upregulated miRNAs **(A)** mmu-miR-181a-5p and **(B)** mmu-miR-328-3p or validated downregulated miRNAs **(C)** mmu-miR-21a-5p, **(D)** mmu-miR-98-5p, and **(E)** mmu-miR-146b-5p were assessed by real-time PCR and normalized to *SNORD61* in bone marrow-derived macrophages from C57BL/6 and MyD88 KO mice. In addition, the role of MyD88 in miRNA regulation was also observed *ex vivo*. Validated upregulated miRNAs **(F)** mmu-miR-181a-5p and **(G)** mmu-miR-328-3p or validated downregulated miRNAs **(H)** mmu-miR-21a-5p, **(I)** mmu-miR-98-5p, and **(J)** mmu-miR-146b-5p were also assessed by real-time PCR in spleens from C57BL/6 and MyD88 KO mice. Error bars represent the mean ± SD. Similar results were obtained in three independent experiments. Statistically significant differences of miRNAs expression from C57BL/6 infection compared with non-infected (NI) cells are denoted by an asterisk ($p < 0.05$), # compared with C57BL/6 infected ($p < 0.05$), and & compared with NI MyD88 KO cells ($p < 0.05$).

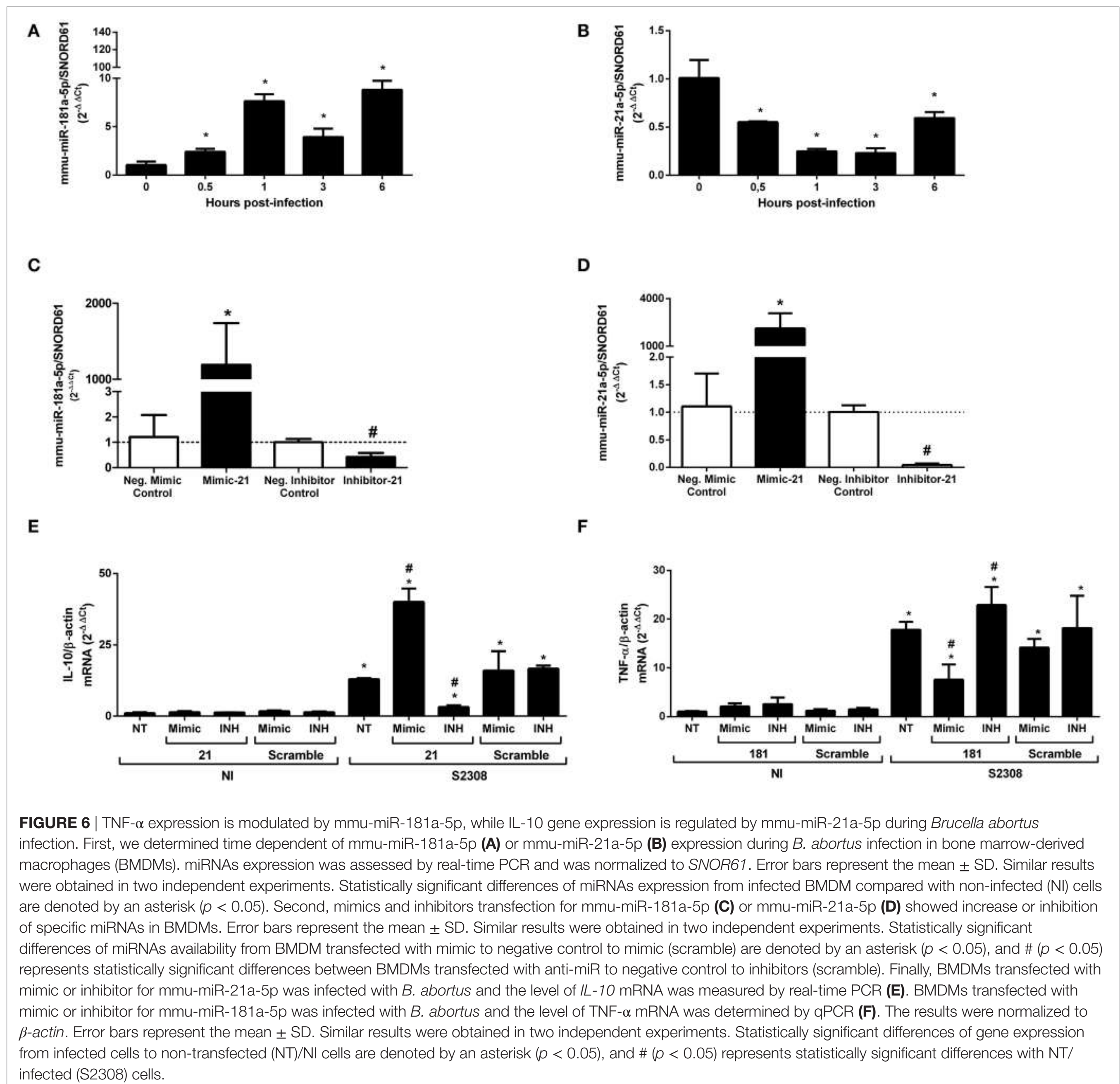

FIGURE 6 | TNF-α expression is modulated by mmu-miR-181a-5p, while IL-10 gene expression is regulated by mmu-miR-21a-5p during *Brucella abortus* infection. First, we determined time dependent of mmu-miR-181a-5p **(A)** or mmu-miR-21a-5p **(B)** expression during *B. abortus* infection in bone marrow-derived macrophages (BMDMs). miRNAs expression was assessed by real-time PCR and was normalized to *SNOR61*. Error bars represent the mean ± SD. Similar results were obtained in two independent experiments. Statistically significant differences of miRNAs expression from infected BMDM compared with non-infected (NI) cells are denoted by an asterisk ($p < 0.05$). Second, mimics and inhibitors transfection for mmu-miR-181a-5p **(C)** or mmu-miR-21a-5p **(D)** showed increase or inhibition of specific miRNAs in BMDMs. Error bars represent the mean ± SD. Similar results were obtained in two independent experiments. Statistically significant differences of miRNAs availability from BMDM transfected with mimic to negative control to mimic (scramble) are denoted by an asterisk ($p < 0.05$), and # ($p < 0.05$) represents statistically significant differences between BMDMs transfected with anti-miR to negative control to inhibitors (scramble). Finally, BMDMs transfected with mimic or inhibitor for mmu-miR-21a-5p was infected with *B. abortus* and the level of *IL-10* mRNA was measured by real-time PCR **(E)**. BMDMs transfected with mimic or inhibitor for mmu-miR-181a-5p was infected with *B. abortus* and the level of TNF-α mRNA was determined by qPCR **(F)**. The results were normalized to *β-actin*. Error bars represent the mean ± SD. Similar results were obtained in two independent experiments. Statistically significant differences of gene expression from infected cells to non-transfected (NT)/NI cells are denoted by an asterisk ($p < 0.05$), and # ($p < 0.05$) represents statistically significant differences with NT/infected (S2308) cells.

in **Figure 7B**, it was possible to observe that *B. abortus*-infected cells transfected with the inhibitor for mmu-miR-21a-5p showed an enhanced amount of GBP5 when compared with untreated cells. Surprisingly, we also detected an increased in GBP5 in cells treated with the mimic for mmu-miR-21a-5p but in much lower levels compared with macrophages transfected with the inhibitor. Since GBPs are important mediators of anti-bacterial immunity, we decided to investigate whether miR-21a-5p affects *Brucella* replication in macrophages. To evaluate the influence of miR-21a-5p in intracellular *B. abortus* growth in macrophages, BMDMs were transfected with mimic or inhibitor and infected with *B. abortus* for 24 hrs. CFU analysis demonstrated that the miR-21a-5p mimic increased numbers of intracellular bacteria while the inhibitor had the opposite effect (**Figure 7C**). Taken together, these results suggest that miR-21a-5p modulates *GBP5* expression, thus affecting the ability of the host to control *B. abortus* infection.

DISCUSSION

MicroRNAs play important role in the regulation of immune response to different infectious agents (10). *B. abortus* is a facultative intracellular bacterium that can adapt to environmental stressor and replicates in phagocytic cells. Many strategies are

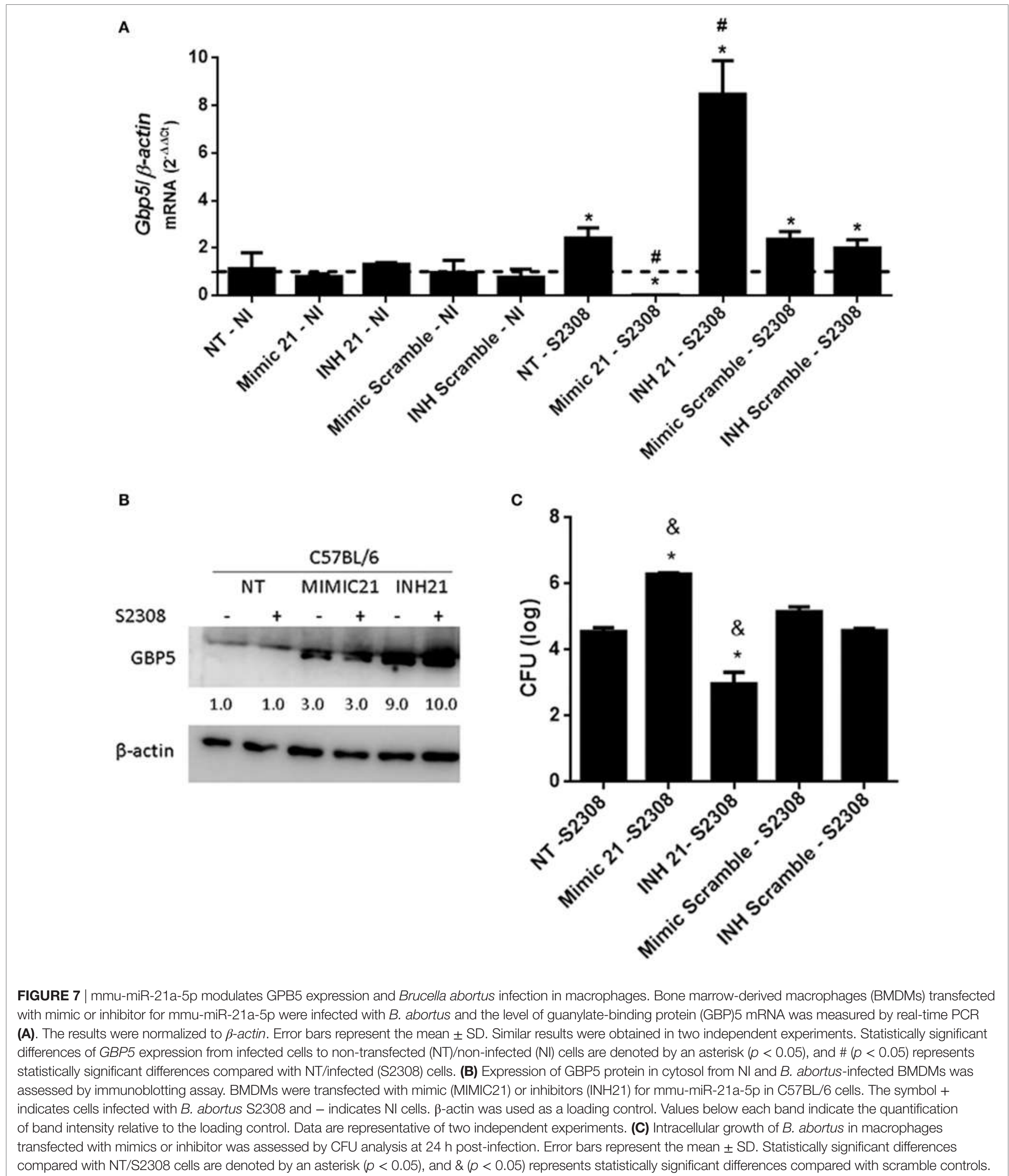

FIGURE 7 | mmu-miR-21a-5p modulates GPB5 expression and *Brucella abortus* infection in macrophages. Bone marrow-derived macrophages (BMDMs) transfected with mimic or inhibitor for mmu-miR-21a-5p were infected with *B. abortus* and the level of guanylate-binding protein (GBP)5 mRNA was measured by real-time PCR **(A)**. The results were normalized to *β-actin*. Error bars represent the mean ± SD. Similar results were obtained in two independent experiments. Statistically significant differences of *GBP5* expression from infected cells to non-transfected (NT)/non-infected (NI) cells are denoted by an asterisk ($p < 0.05$), and # ($p < 0.05$) represents statistically significant differences compared with NT/infected (S2308) cells. **(B)** Expression of GBP5 protein in cytosol from NI and *B. abortus*-infected BMDMs was assessed by immunoblotting assay. BMDMs were transfected with mimic (MIMIC21) or inhibitors (INH21) for mmu-miR-21a-5p in C57BL/6 cells. The symbol + indicates cells infected with *B. abortus* S2308 and – indicates NI cells. β-actin was used as a loading control. Values below each band indicate the quantification of band intensity relative to the loading control. Data are representative of two independent experiments. **(C)** Intracellular growth of *B. abortus* in macrophages transfected with mimics or inhibitor was assessed by CFU analysis at 24 h post-infection. Error bars represent the mean ± SD. Statistically significant differences compared with NT/S2308 cells are denoted by an asterisk ($p < 0.05$), and & ($p < 0.05$) represents statistically significant differences compared with scramble controls.

used by bacteria to evade immune response and survive inside phagocytes, and recently it was characterized novel virulence-related sRNA in *B. melitensis* (34). However, the host triggers intracellular signaling pathways to induce an effective immune response (35). In this battle between host and pathogen, many regulators are induced, including miRNAs. During

B. melitensis infection in Raw264.7 cells, 57 miRNAs were differentially expressed and they potentially play important regulatory roles in the *Brucella*–host interactions (24). Among these 57, 3 miRNAs (miR-145a-3p, miR-146b-5p, and miR-151a-3p) were also identified as differentially expressed in our study. However, they showed a different profile. miR-145a-3p and miR-146b-5p were upregulated in *B. melitensis*-infected Raw264.7 cells and downregulated in *B. abortus*-infected BMDMs. In addition, miR-151a-3p was downregulated in *B. melitensis*-infected Raw264.7 cells and upregulated in *B. abortus*-infected BMDMs. These differences could be related to the virulence of the *Brucella* species studied or the source of host cells used. Macrophages sense *B. abortus* and induce upregulation of *IL-12*, *IL-1β*, *TNF-α*, and *IL-6* genes as early as 30 min after infection. This acute inflammatory response against *B. abortus* has been receiving attention by our group since this bacterium is rapidly recognized by several innate immunity receptors such as TLRs (25) and inflammasomes (36). Therefore, we decided to identify miRNAs that could be related to the initial phase of immune response against *B. abortus* using high-throughput sequencing of small RNAs. RNAseq indicated that 69 miRNAs were differentially expressed between infected and control macrophages, 38 upregulated and 31 downregulated. According to the expression levels and fold-change comparing *Brucella*-infected versus NI libraries, we selected four miRNAs that were upregulated (mmu-miR-151-3p, mmu-miR-155-5p, mmu-miR-181a-5p, and mmu-miR-328-3p) and five miRNAs that were downregulated (mmu-miR-21a-5p, mmu-miR-98-5p, mmu-miR-145a-5p, mmu-miR-146b-5p, and mmu-miR-374b-5p) for validation and further analysis. By qPCR, we validated the differential expression of four upregulated and five downregulated miRNAs in *Brucella*-infected macrophages or spleen cells thus confirming the profile observed in the RNAseq analysis.

Previously, we (25) and others (37) have shown the critical role of MyD88 adaptor molecule in triggering innate immune responses against *B. abortus* and *B. melitensis*, respectively. Since MyD88 pathway is important for control of *Brucella* infection, we evaluated the role of this adaptor molecule in the differential expression of miRNAs. We observed a dependence of MyD88 in upregulation of mmu-miR-181a-5p, while it was not observed differences of upregulation of mmu-miR-328-3p in the absence of MyD88. On the other hand, we observed a dependence of MyD88 for the downregulation of mmu-miR-21a-5p, mmu-miR-98-5p, and mmu-miR-146b-5p during *B. abortus* infection in macrophages. Furthermore, analysis of MyD88 regulation of miRNAs expression *ex vivo* in mouse spleens revealed that among upregulated miRNAs, only mmu-miR-181a-5p shows the same profile in macrophages and *ex vivo*. However, among all downregulated miRNAs tested, the profile observed was similar *in vitro* and *ex vivo* highlighting the importance of MyD88 in regulating miRNA expression. Even though we did not study the involvement of individual TLRs in regulating the miRNAs tested here, we hypothesize that TLR9 may play a role since we have previously demonstrated this is the most critical TLR involved in *B. abortus* infection (38). Recently, Jentho et al. (39) characterized miRNA regulation during *Legionella pneumophila* infection and demonstrated that miR-125a-3p expression is downregulated in a MyD88-dependent manner thus affecting *Legionella*–host cell interactions. These results demonstrate the importance of MyD88 in regulating miRNA expression and the host response during bacterial infections.

We carried out further functional analysis using mmu-miR-181a-5p and mmu-miR-21a-5p. Recently, Luo et al. (29) have identified that *B. suis* upregulates miR-146a, miR-181a, miR-181b, and miR-301a-3p leading to reduced TNF-α expression in Raw264.7 cells. In addition, according to Dan et al. (40), stability of *TNF-α* mRNA was influenced by miR-181a-5p. Therefore, we tested the effect of miR-181a-5p mimic or inhibitor in *TNF-α* expression during *Brucella*-infected BMDMs. miR-181a-5p mimic diminished *TNF-α* expression whereas miRNA inhibitor increased *TNF-α* transcripts. These results corroborate the ability of miR-181a-5p to downregulate TNF-α in *Brucella*-infected BMDMs. As for mmu-miR-21a-5p, one defined target is PDCD4, which is a suppressor of the anti-inflammatory cytokine IL-10. Cohen and Prince (41) showed that during bacterial pneumonia type III IFNλ promotes inflammation by inhibiting miR-21, upregulating PDCD4, and consequently diminishing IL-10 production. PDCD4 influences IL-10 mRNA stability leading to a reduced production of this anti-inflammatory cytokine (42). Therefore, we decided to test the effect of miR-21a-5p mimic or inhibitor in *IL-10* expression on *Brucella*-infected macrophages. Herein, we show that when miR-21a-5p mimic was used higher levels of *IL-10* mRNA were detected in infected cells compared with the controls. By contrast, when the cells were transfected with miR-21a-5p inhibitor, we observed a strong reduction in *IL-10* levels. This result suggests the role of miR-21a-5p in controlling *IL-10* expression *via* PDCD4 during *B. abortus* infection. Previously, we and others demonstrated that IL-10 modulates the pro-inflammatory immune response to *B. abortus* and the lack of IL-10 increases resistance to *Brucella* infection (8, 9).

Recently, we have determined that GBPs encoded by genes on mouse chromosome 3 (GBP1, GBP2, GBP3, GBP5, and GBP7) are critical for *B. abortus* control in macrophages and in mice (33). Therefore, we decided to search for GBP genes as potential targets for miR-181a-5p or miR-21a-5p regulation. We found that GBP2, GBP4, GBP5, and GBP8 are putative targets for miR-21a-5p but not for miR-181a-5p. We then selected GBP5 as a target to evaluate the potential regulatory role of miR-21a-5p. When miR-21a-5p mimic was transfected in macrophages, we observed reduced *GBP5* expression. By contrast, the miR-21a-5p inhibitor increased *GBP5* transcripts. As for GBP5 protein, miR-21a-5p inhibitor enhanced GBP5 levels in infected and uninfected macrophages compared with miR-21a-5p mimic treated cells. Unexpectedly, we observed a small increase in GBP5 in the Western blot when cells were transfected with miR-21a-5p mimic. It is possible that the transfection reagent induced a background level of GBP5 that was detected by the polyclonal antibody. These results show that *GBP5* expression is regulated by miR-21a-5p. In addition, pretreatment of BMDMs with miR-21a-5p inhibitor significantly decreased the intracellular *Brucella* numbers upon bacterial infection. By contrast, miR-21a-5p mimic pretreatment increased bacterial load in macrophages.

In summary, the findings present here provide evidences that miR-181a-5p regulates *TNF-α* and miR-21a-5p influences *IL-10* expression during *B. abortus* infection (**Figure 8**). In addition,

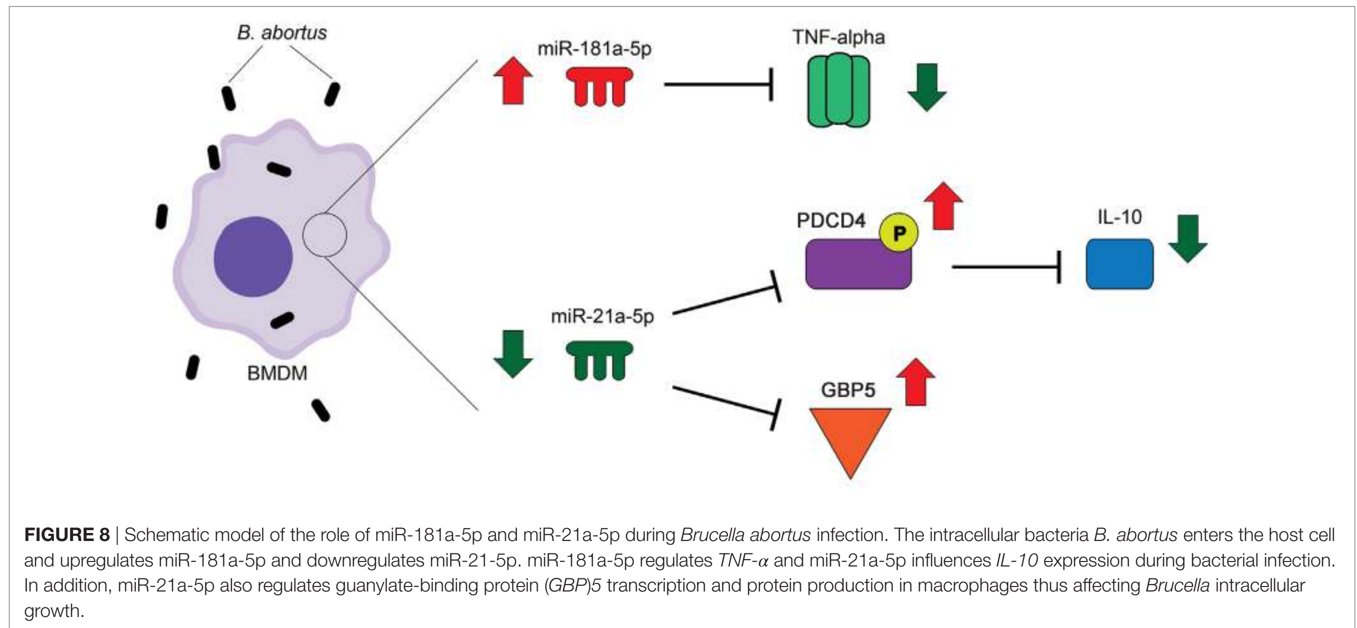

FIGURE 8 | Schematic model of the role of miR-181a-5p and miR-21a-5p during *Brucella abortus* infection. The intracellular bacteria *B. abortus* enters the host cell and upregulates miR-181a-5p and downregulates miR-21-5p. miR-181a-5p regulates *TNF-α* and miR-21a-5p influences *IL-10* expression during bacterial infection. In addition, miR-21a-5p also regulates guanylate-binding protein (*GBP*)*5* transcription and protein production in macrophages thus affecting *Brucella* intracellular growth.

miR-21a-5p also regulates *GBP5* transcription and protein production in macrophages thus affecting *Brucella* intracellular growth. Herein, we hypothesize that in initial phase of infection host cells can downregulate miR-21a-5p expression to reduce *IL-10* and increase *GBP5* expression thus resulting in improved control of *Brucella* replication.

ETHICS STATEMENT

This study was carried out in strict accordance with the Brazilian laws 6638 and 9605 in Animal Experimentation. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Minas Gerais (Permit Number: CETEA no. 128/2014).

AUTHOR CONTRIBUTIONS

PC, LA, JM, and SO designed the project and experiments. PC, LA, MG, EG, and AG carried out most of the experiments. PC, JM, and SO wrote the manuscript. PC, LA, and AG carried out statistical analysis and prepared figures. SO submitted this paper. All the authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01331/full#supplementary-material.

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Correlation Between the Hepatic Expression of Human MicroRNA hsa-miR-125a-5p and the Progression of Fibrosis in Patients With Overt and Occult HBV Infection

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Aims: To evaluate the correlation between the hepatic expression pattern of hsa-miR-125a-5p and HBV-DNA and the progression of fibrosis in patients with overt or occult HBV infection.

Methods: We enrolled all the HBsAg-positive treatment naive patients (overt HBV group) and all the HBsAg-negative patients with hepatocellular carcinoma and with a positive HBV-DNA in their hepatic tissue (occult HBV group), who underwent a diagnostic liver biopsy between April 2007 and April 2015. Tissue concentrations of HBV-DNA and hsa-miR-125a-5p were then analyzed by real-time quantitative PCR. Necroinflammatory activity and fibrosis were evaluated according to the Ishak score.

Results: During the study period, we enrolled 64 patients with overt and 10 patients with occult HBV infection. In the overt HBV group, 35 of 64 (54.7%) showed a mild fibrosis (staging 0–2), 17 (26.6%) a moderate fibrosis (staging 3–4), while the remaining 12 (18.7%) had a cirrhosis. All patients in the occult HBV group were cirrhotic. Patients with more advanced fibrosis stage showed a higher mean age when compared with those with mild ($p < 0.00001$) or moderate fibrosis ($p < 0.00001$) and were more frequently male than patients with staging 0–2 ($p = 0.04$). Similarly, patients with occult B infection were older than HBsAg-positive patients. Liver concentrations of miR-125a-5p were significantly higher in patients with cirrhosis (9.75 ± 4.42 AU) when compared with patients with mild (1.39 ± 0.94, $p = 0.0002$) or moderate fibrosis (2.43 ± 2.18, $p = 0.0006$) and were moderately higher in occult than in overt HBV infection ($p = 0.09$). Moreover, we found an inverse correlation, although not statistically significant, between the tissue HBV-DNA levels and the staging of fibrosis.

Conclusion: This study suggests a correlation between the tissue expression of hsa-miR-125a-5p and the progression of liver damage in a group of patients with occult or overt HBV infection. If confirmed, these data suggest the hsa-miR-125a-5p may be a novel biomarker of hepatic damage.

Keywords: HBV infection, microRNA, chronic hepatitis, liver fibrosis, occult B infection

INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at post-transcriptional level by inducing the degradation of target mRNAs or inhibiting their translation in protein (1). They are involved in a large variety of physiological processes playing crucial roles in cell differentiation and development (2). In addition, several studies indicate that miRNAs are important regulators of virus–host interactions (3–5).

HBV represents a leading cause of cirrhosis and hepatocellular carcinoma (HCC) all over the world. World Health Organization estimates that 257 million persons, or 3.5% of the world population, were living in 2015 with chronic HBV infection, which was responsible of more than 900,000 deaths each year (6). The severity of chronic hepatitis B (CHB) is variable, with a clinical presentation ranging from a healthy HBV carriage to the more severe expressions of the disease and with a clinical course ranging from a benign indolent progression over decades to a rapid evolution to liver cirrhosis and HCC (7, 8). Moreover, after HBsAg seroclearance, HBV-DNA can persist inside the hepatocytes, causing a condition known as occult B infection, characterized by HBsAg negativity but persistence of HBV DNA in the liver. The occult HBV infection may be associated with the progression of liver damage and the development of HCC in patients with liver diseases due to different etiologies (9, 10).

Despite the efforts of the scientific community, the interactions between the virus and the host and the mechanisms at the base of liver damage are still largely elusive, which represents a significant barrier to the treatment and the eradication of HBV infection. At this regard, many research groups have investigated the relationship between the expression profile of several miRNAs and HBV replication (11). In particular, it has been shown that hsa-miR-125a-5p, a miRNA expressed in the human liver (12), is able to target a viral sequence within the overlapping polymerase and surface antigen coding regions (13), inhibiting the expression of HBsAg *in vitro*. Moreover, miRNAs are involved in the progression of liver fibrosis at multiple levels, by regulating the activation of hepatic stellate cells, the production of TGF-β, and the expression of matrix metalloproteinases (14).

In this study, we aimed to correlate the hepatic expression pattern of hsa-miR-125a-5p with the concentrations of HBV-DNA in liver tissue and the progression of fibrosis in patients with overt or occult HBV infection.

PATIENTS AND METHODS

Patients

We enrolled in this cross-sectional study all the consecutive HBsAg-positive patients who underwent a diagnostic liver biopsy (overt HBV group) between April 2012 and April 2015 in one of the three liver units participating in the study. The three units involved, two in Naples and one in Caserta, have cooperated in several investigations with the same clinical approach and the same laboratory methods (15, 16). Moreover, of the 68 HBsAg-negative patients with HCC who underwent liver biopsy in the same period, those with a positive HBV-DNA in non-HCC liver tissue were enrolled (occult HBV group).

Exclusion criteria for HBsAg-positive patients (overt HBV group) were HIV, HCV, or HDV coinfection and previous treatment with nucleos(t)ide analogs and/or interferon therapy. For HBsAg-negative patients (occult HBV group), HIV coinfection and previous treatment with interferon-free regimens were regarded as exclusion criteria.

We collected at the enrollment the demographic characteristics of each subject (age, gender, geographical origin). All patients underwent complete physical examination, full liver function tests, assessment of triglycerides, cholesterol, blood cell counts, α-fetoprotein, viral markers (HBV, HCV, hepatitis delta virus-HDV, human immunodeficiency virus-HIV), and liver ultrasound scan.

Tissue Specimen Collection and Histological Analysis

Liver biopsy was performed for all patients and was advised by the physicians in care, and informed consent was signed by the patient. Liver specimens were fixed in formalin, embedded in paraffin and stained with Masson's trichrome stain. Liver biopsies were examined by a pathologist who, unaware of the clinical and laboratory data, used the Ishak scoring system to grade the fibrosis (17).

For each patient, a fragments of nearly 3 mg were cut away from the two extremities of the liver biopsies not useful for diagnosis (18) and stored at −80°C in RNAlater solution (Qiagen GmbH, Hilden, Germany) for subsequent molecular analyses. In addition, plasma sample was collected for each patient and stored at −80°C the same day the liver biopsies were performed.

Ethics Statement

All procedures applied in the study were in accordance with the international guidelines, with the standards on human experimentation of the Ethics Committee of the Azienda Ospedaliera Universitaria-Università della Campania and with the Helsinki Declaration of 1975 and revised in 1983. The Ethics Committee of the Azienda Ospedaliera Universitaria of the University of Campania approved the study (no. 214/2012 and no. 349/2013). All patients signed their informed consent for liver biopsy, the collection and storage of biological samples, and for the anonymous use of their data for research purposes.

Serological Analysis

HBV and HDV serum markers were sought using commercial immunoenzymatic assays (Abbott Laboratories, North Chicago, IL, USA, for HBsAg, anti-HBs, and anti-HBc, and DiaSorin, Saluggia, VC, Italy, for anti-HDV). The anti-HCV antibody was sought using a third generation commercial immunoenzymatic assay (Ortho Diagnostic Systems, Neckargemund, Germany). Antibodies to HIV 1 and 2 were sought using a commercial ELISA (Abbott Lab., North Chicago, IL, USA). Liver function tests were performed by the routine methods.

Quantitation of HBV DNA and miR-125a in Tissue Samples

The liver tissues stored at −80°C in RNAlater solution were homogenized by TissueLyser. For all patients in overt and occult HBV groups, the DNA extracted from liver homogenates was analyzed for the presence of the HBV genome by performing a real-time PCR with a wide range of linearity in a Light-cycler 1.5 (Roche Diagnostics, Branchburg, NJ, USA). An external standard curve was made to quantify the HBV genomes present in the samples; the standard was a PCR product cloned with the TA cloning system (Invitrogen k2000-01, Invitrogen, Carlsbad, CA, USA), and 8 μL of the appropriately diluted plasmid was used to generate the standard curve; by this method, the detection limit in plasma samples is estimated at around 40 IU/mL (18, 19). HBV DNA in the liver tissue was quantified in relation to β-globin DNA (LightCycler Control kit Fast star DNA Master Hyprobe, Roche Diagnostics, Branchburg, NJ, USA), a DNA present in all human cells and thus used as a positive control, using LightCycler quantification software (Roche Diagnostics, Branchburg, NJ, USA). The results were expressed as a number of IU/hepatic cell (18, 19).

For the patients in all groups, total RNA was extracted by mirVana™ miRNA isolation kit from liver tissues homogenized by TissueLyser; RT-PCR tests for hsa-miR-125a-5p and RNU6B (used as a reference gene) were carried out using TaqMan miRNA assays from Applied Biosystems.

Statistical Analysis

Continuous variables were summarized as median and interquartile range, and categorical variables as absolute and relative frequencies. For continuous variables, the differences were evaluated by Wilcoxon rank-sum test; categorical variables were compared by chi-square test, using exact procedures if needed. Odds ratios, with 95% confidence intervals, were estimated by a logistic regression model for evaluating the relationship between age, plasma HBV DNA, liver hsa-miR-125a-5p, and presence of cirrhosis. A p value <0.05 was considered to be statistically significant.

RESULTS

Sixty-four overt and 10 occult HBV patients were included in the study; in the overt group, the median age was 44 (IQR: 14.5) years and 75% of patients were males (**Table 1**); 56 (87.5) were Italian, 3 (4.7) patients came from Eastern Europe, and the remaining 5 (7.8) from Sub-Saharan Africa. The median ALT level was 0.84 (IQR: 1.18) × upper limit of normal (ULN) and the Prothrombin activity was 90% (IQR: 12.48). All patients had a positive plasma HBV-DNA, with a median viral load of 4.7E + 3 (IQR: 5.2E + 4) IU/mL. 10 of 64 (15.6%) showed a staging 0, 17 (26.6%) a staging 1, 8 (12.5%) a staging 2, 16 (25%) a staging 3, 1 (1.6%) a staging 4, while the remaining 12 (18.7%) had a cirrhosis (staging 6); all but one of them had a compensated liver disease. In the occult group, the median age was 67.5 (IQR: 15.5) years and 8 of 10 patients were males. The median ALT level was 1.17 (IQR: 1.48) × ULN and the Prothrombin activity was 86% (IQR: 8.25). Only three subjects had a positive plasma HBV-DNA, with a viral load ranging from 24 to 811 IU/mL. All patients had an HCV-related cirrhosis, with a decompensated disease (Child-Pugh score B) in 6 of 10 patients.

All patients were HIVAb and HDVAb negative; none of the patients in the overt HBV group was HCV-coinfected; two of them were HBeAg positive. Patients with more advanced fibrosis stage showed a higher median age when compared with those with mild (68.5, IQR: 2 vs 40, IQR: 15.5, $p < 0.00001$) or moderate fibrosis (40, IQR: 14, $p < 0.00001$) and were more frequently male than patients with staging 0–2 (91.7 vs 60.0%, $p = 0.04$). Similarly, patients with occult B infection were older than HBsAg-positive patients (67.5, IQR: 15.5 vs 44, IQR: 14.5, $p = 0.00017$). A lower median ALT level was found in patients with mild when compared with patients with moderate fibrosis (0.8, IQR: 0.96 vs 1.24, IQR: 1.8, $p = 0.02$); furthermore, a higher prothrombin activity was found in patients with mild fibrosis when compared with cirrhotic subjects (93, IQR: 12.5 vs 85, IQR: 9.4, $p = 0.02$). Finally, we found a higher concentration of HBV-DNA both in plasma (1.37E + 4 IU, IQR: 9.9E + 5 vs 3.6E + 2 IU, IQR: 1.16E + 4, $p = 0.03$) and in liver tissue (0.14 IU/cell, IQR: 0.27 vs 0.007 IU/cell, IQR: 0.046, $p = 0.01$) when we compared patients with moderate fibrosis to cirrhotic patients.

TABLE 1 | Demographic, biochemical, virological, and histological characteristics of the enrolled patients according to the etiologic group.

	Overt HBV group	Occult HBV group
No. patients	64	10
Median age (IQR)	44 (20.5)	67.5 (15.5)
Males, no. (%)	48 (75)	8 (80.0)
Geographical origin, no. (%)		
• Italy	56 (87.5)	10 (100)
• Western Europe	3 (4.7)	0 (0.0)
• Sub-Saharan Africa	5 (7.8)	0 (0.0)
AST/ULN (median, IQR)	0.7 (0.6)	1.35 (1.45)
ALT/ULN (median, IQR)	0.84 (1.18)	1.17 (1.48)
PT% (median, IQR)	90 (12.48)	86 (8.25)
HBV DNA positivity, no. (%)	64 (100)	3 (30)
Plasma HBV-DNA load, IU/mL (median, IQR)	4.7E + 3 (5.2E + 4)	24–811[a]
Fibrosis score (Ishak), no. (%) of patients with		
• 0	10 (15.6)	0 (0)
• 1	17 (26.6)	0 (0)
• 2	8 (12.5)	0 (0)
• 3	16 (25.0)	0 (0)
• 4	1 (1.6)	0 (0)
• 6	12 (18.7)	10 (100)
- Child-Pugh score A no. (%)	11 (17.2)	4 (40)
- Child-Pugh score B no. (%)	1 (1.6)	6 (60)

[a]*Range.*
IQR, interquartile range; ULN, upper limit of normal.

In overt HBV group, liver concentrations of miR-125a-5p (**Table 2**; **Figure 1**) were significantly higher in patients with fibrosis score of 5 or 6 (cirrhosis; median, IQR: 10.7, 6.9 AU) when compared with patients with mild (fibrosis score 0–2; median, IQR: 1.13, 1.04 AU, $p < 0.00001$) or moderate fibrosis (fibrosis score 3 or 4; median, IQR: 1.53, 2.55 AU, $p = 0.0002$). The 10 patients in occult HBV group, all cirrhotic, showed a high liver concentrations of miR-125a-5p (median, IQR: 5.47, 2.43 AU). Patients with higher liver HBV-DNA concentrations showed a slightly lower miRNA expression ($p = 0.24$ including all patients; $p = 0.35$ excluding the occult HBV group); similarly, no correlation was found between the miRNA expression and the plasmatic viral load ($p = 0.11$ including all patients; $p = 0.13$ including only the HBsAg-positive patients, data not shown).

To confirm the association between liver hsa-miR-125a-5p and the presence of an advanced liver disease and to avoid a possible confounding effect of other factors such as age and the plasma HBV-DNA level, a multivariate logistic regression analysis was performed. Multivariate analysis identified liver hsa-miR-125a-5p as an independent predictor of fibrosis >5 (OR: 2.08, CI 95%: 1.11–3.85, $p = 0.02$) (**Table 3**).

DISCUSSION

HBV represents one of the most important issues for global health. The spectrum of clinical conditions caused by HBV infection is wide, ranging from asymptomatic carriers to liver cirrhosis and hepatocellular cancer, and several authors have tried to investigate the pathogenesis of liver damage induced by this virus.

It is well known that miRNAs can regulate almost every biological process in all cell types, including hepatocytes and other liver cells. Not surprisingly, the role of miRNAs in liver fibrogenesis and carcinogenesis has emerged in the recent years as one of the most interesting issue in the field of hepatology. Many studies have highlighted the expression pattern of several miRNAs, both in plasma and in liver, in patients with chronic HBV infection (20, 21). In a recently published paper, Singh and coworkers (22) investigated the expression profile of several miRNAs by microarrays in liver biopsy samples from 65 patients with different stages of HBV infection and 7 healthy controls. They found in immune tolerant patients higher levels of miR-199a-5p, miR-221-3p, and Let-7a-3p involved in the regulation of innate immune response. Conversely, in the advanced fibrosis group they demonstrated an upregulation of miR-1 and miR-10b-5p, and a downregulation of miR-20b-5p and miR-455-3p, implicated in immune response and cellular senescence.

miR-125a-5p is present in all animals with bilateral symmetry; in mammalians, it is expressed in most tissues (23) where negatively regulates cell proliferation (24). Indeed, it is downregulated in several types of tumors (25, 26), including hepatocellular cancer (27, 28). However, its role in liver is not limited to the tumor suppressor activity; a few studies have investigated its role in liver fibrosis progression. In 2012, Park et al. (29) showed that TGF-β, a profibrogenic cytokine, could induce an upregulation of hsa-miR-125a-5p in HBV transfected hepatocytes. A more recent study by Li et al. (30) demonstrated an upregulation of this miRNA in a murine model of carbon tetrachloride-induced liver fibrosis; furthermore, they showed how the downregulation of miR-125a-5p could prevent the activation of hepatic stellate cells *in vitro*. The same group demonstrated a strong correlation between serum concentrations of miR-125a-5p and staging of fibrosis in 91 patients with CHB (31). They also found a significant positive correlation between the expression levels of the miRNA and the serum HBV-DNA.

In a previous study, we analyzed a little cohort of 27 HBsAg/anti-HBe-positive patients and correlated the liver concentrations of miR-125a-5p with the clinical, virological, and histological characteristics of the enrolled patients (19, 32). Liver miRNA expression was identified as an independent predictor of higher necroinflammatory activity (HAI > 6) and more advanced liver fibrosis (staging > 2). Furthermore, more elevated miRNA expression was found in patients with higher serum and liver HBV-DNA levels. In this study, on a larger HBV population, 64 consecutive patients with overt and 10 with occult HBV infection were enrolled and the expression levels of liver hsa-miR-125a-5p were determined, along with clinical, biochemical, and histological parameters. As expected, patients with more advanced liver disease were

TABLE 2 | Characteristics of HBV patients stratified according to liver fibrosis.

	Overt HBV group			Occult HBV group	*p* Value			
	Staging 0–2	Staging 3–4	Staging 5–6		Staging 0–2 vs 3–4	Staging 0–2 vs 5–6	Staging 3–4 vs 5–6	Occult vs overt HBV group
No. patients	35	17	12	10				
Age (median, IQR)	40 (15.5)	40 (14)	68.5 (2)	67.5 (15.5)	0.36	**<0.00001**	**<0.00001**	**0.00017**
Males, no. (%)	21 (60)	14 (82.3)	11 (91.7)	8 (80.0)	0.11	**0.04**	0.47	0.73
AST/ULN (median, IQR)	0.7 (0.57)	0.76 (1.05)	0.7 (1)	1.35 (1.46)	0.09	0.38	0.28	**0.01**
ALT/ULN (median, IQR)	0.8 (0.96)	1.24 (1.8)	0.5 (1.31)	1.18 (1.47)	**0.02**	0.32	0.08	0.14
PT% (median, IQR)	93 (12.5)	91.5 (13.3)	85 (9.4)	86 (8.25)	0.29	**0.02**	0.05	0.22
Plasma HBV-DNA UI/ml (median, IQR)	4.7E + 3 (4.7E + 4)	1.37E + 4 (9.9E + 5)	3.6E + 2 (1.16E + 4)	//	0.18	0.16	**0.03**	**//**
Liver HBV-DNA IU/cell (median, IQR)	0.03 (0.1)	0.14 (0.27)	0.007 (0.046)	0.015 (0.34)	0.14	**0.04**	**0.01**	0.37
miR-125a-5p AU (median, IQR)	1.13 (1.04)	1.53 (2.55)	10.7 (6.9)	5.47 (2.43)	0.11	**<0.00001**	**0.0002**	**0.006**

IQR, interquartile range; ULN, upper limit of normal; cp, copies; AU, arbitrary units.
P values <0.05 are displayed in bold font.

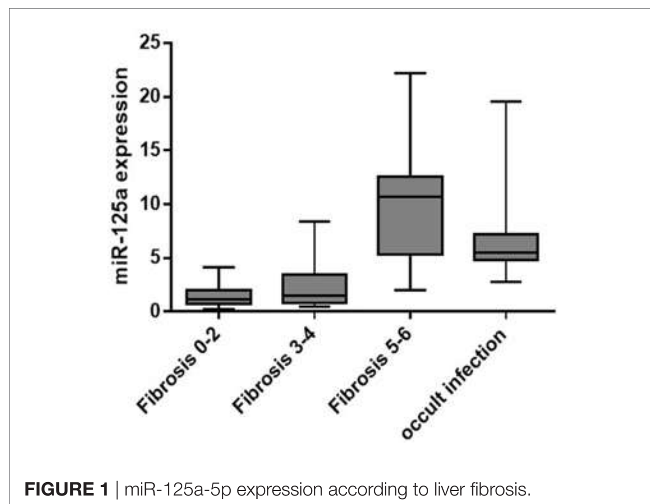

FIGURE 1 | miR-125a-5p expression according to liver fibrosis.

TABLE 3 | Multiple logistic regression analysis for independent predictors of staging >5.

	OR	95% CI		*p* Value
		Lower limit	Upper limit	
Age	1.37	0.94	1.96	0.10
miR-125a-5p	2.08	1.11	3.85	**0.02**
Plasma HBV-DNA	0.49	0.16	1.51	0.21

OR, odds ratio; CI, confidence interval.
P values <0.05 are displayed in bold font.

older than patients with mild or moderate fibrosis. Furthermore, they were more frequently male; these data can be explained with a higher incidence in male gender of HCC, which represents one of the main indications for liver biopsy in cirrhotic subjects. Finally, as expected, patients with initial fibrosis (staging 0–2) showed a higher prothrombin activity than cirrhotic subjects and a lower ALT level than patients with more advanced liver damage (staging 3–4). Regarding the virological characteristics, we found a lower plasma and liver viral load in cirrhotic subjects than in patients with less advanced disease; this could be related to the clinical and demographic features of patients with mild or moderate fibrosis (i.e., younger and more frequently HBeAg-positive patients).

In the HBV-infected patients, we demonstrated a significant correlation between the miRNA concentrations and the progression of liver fibrosis. In fact, the liver has-miR-125a-5p concentrations were higher in the patients with higher fibrosis score. However, we did not find a correlation with the plasma and tissue HBV viral load. This is may be due to the inclusion in this study of patients with advanced stages of fibrosis, not included in our previous study, infection by different HBV strains inducing a lower miR-125a expression response (all patients in the former study were infected with a genotype D, while five patients in this paper have a genotype E and two a genotype A), or different serological characteristics (e.g., HBeAg-positive patients, not included previously).

The study has some limitations. First, the cross-sectional design does not allow to clearly evaluate the impact of single factors on the progression of liver damage, as a prospective study could do. Moreover, the lack of control groups with different etiologies and virological characteristics makes it difficult to understand the real correlation between the miR-125a expression and the liver fibrosis. However, the multivariate analysis that identified the miRNA concentrations as independent predictor of advanced liver damage (regardless of the viral load) suggest a role of miR-125a-5p in the process of fibrogenesis, not correlated to the inhibitory effect on HBV replication.

CONCLUSION

This study demonstrates a correlation between the tissue expression of hsa-miR-125a-5p and the progression of liver damage in a group of patients with occult or overt HBV infection. However, further studies are needed to investigate the role of this miRNA in pathogenesis of HBV infection, to assess novel biomarkers of hepatic damage. Moreover, we need more data to understand the role of this miRNA in the process of fibrogenesis induced by etiologies other than HBV.

ETHICS STATEMENT

All procedures applied in the study were in accordance with the international guidelines, with the standards on human experimentation of the Ethics Committee of the Azienda Ospedaliera Universitaria-Università della Campania and with the Helsinki Declaration of 1975 and revised in 1983. The Ethics Committee of the Azienda Ospedaliera Universitaria of the University of Campania approved the study (no. 214/2012 and no. 349/2013). All patients signed their informed consent for liver biopsy, the collection and storage of biological samples, and for the anonymous use of their data for research purposes.

AUTHOR CONTRIBUTIONS

Concept and design: NC, AR, and ES. Production and analysis of data: MS, CM, MP, NM, and NP. Patients inclusion, collection of samples, analysis of data: LO, GS, NF, LA, and VM. Writing of article: NC, LO, and AR.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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MicroRNA-Related Polymorphisms in Infectious Diseases—Tiny Changes With a Huge Impact on Viral Infections and Potential Clinical Applications

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MicroRNAs (miRNAs) are single-stranded sequences of non-coding RNA with approximately 22 nucleotides that act posttranscriptionally on gene expression. miRNAs are important gene regulators in physiological contexts, but they also impact the pathogenesis of various diseases. The role of miRNAs in viral infections has been explored by different authors in both population-based as well as in functional studies. However, the effect of miRNA polymorphisms on the susceptibility to viral infections and on the clinical course of these diseases is still an emerging topic. Thus, this review will compile and organize the findings described in studies that evaluated the effects of genetic variations on miRNA genes and on their binding sites, in the context of human viral diseases. In addition to discussing the basic aspects of miRNAs biology, we will cover the studies that investigated miRNA polymorphisms in infections caused by hepatitis B virus, hepatitis C virus, human immunodeficiency virus, Epstein–Barr virus, and human papillomavirus. Finally, emerging topics concerning the importance of miRNA genetic variants will be presented, focusing on the context of viral infectious diseases.

Keywords: microRNA, miR, polymorphism, hepatitis B virus, hepatitis C virus, human immunodeficiency virus, Epstein–Barr virus, human papillomavirus

INTRODUCTION

Viruses are found abundantly in the most diverse environments on earth (1). Some of the animal viruses are responsible for causing human infections. Viral diseases weaken humans individually and have important impacts on the environment, on the social organization and public health systems of populations worldwide. Historically, viruses are responsible for epidemics and outbreaks that impact all nations, being especially a burden in developing countries. Moreover, some viral diseases, such as the acquired immunodeficiency syndrome [AIDS, caused by human immunodeficiency virus (HIV)], affect the entire world, assuming a pandemic characteristic.

Many advances have been made in the combat against viral diseases. Vaccination and antiviral drugs development are examples of medical technologies effectively used against viruses. However, the number of people affected by viral diseases around the world is still alarming. The HIV pandemic

alone affects about 37 million people worldwide (2). Our knowledge about the pathogenesis of many viruses is still incipient. Similarly, the natural human defenses against pathogens or the immunogenetic aspects that determine, individually or in terms of a whole population, the degree of susceptibility or resistance to viral infections can still be greatly explored.

Within the context of the host genetics, this review will discuss the impact of microRNA (miRNA)-related polymorphisms on infections caused by hepatitis B virus (HBV), hepatitis C virus (HCV), HIV, Epstein–Barr virus (EBV), and human papillomavirus (HPV). Taking into account the interaction of miRNAs with the epigenetic machinery (3, 4), this review will be relevant to readers interested in epigenetics, genetic polymorphisms, and/or viral diseases.

From this point onward the word "microRNA" will be abbreviated to "miRNA" when we are referring to miRNAs in a general way. However, some clarifications regarding the terminologies used in this review for specific miRNAs are important. miRNAs are named according to the species of which they were derived, indicating it before the prefix "miR," followed by the identification number of each miRNA (for example, hsa-miR-101 for *Homo sapiens* and mmu-miR-101 for mouse). The prefix "miR" is used to identify mature miRNAs and the prefix "mir" is used to identify precursor hairpins (5, 6). In this review, most cited miRNAs are human-derived mature miRNAs. Thus, the miRNAs quotation was standardized as follows: miR-101, miR-102, miR-103, for example. The few cases of miRNAs encoded by viral genes will be adequately indicated. Besides, in this review, the quotation of the polymorphisms was standardized according to the Single Nucleotide Polymorphism Database (dbSNP) of NCBI (https://www.ncbi.nlm.nih.gov/snp/), based on the reference SNP cluster (rs#) of each polymorphism. Importantly, some authors refer to the forward strand alleles of a given polymorphism while other authors, who studied the same polymorphism, refer to the reverse strand alleles. Although we have standardized the quotations of the SNPs according to the dbSNP, we respect the quotations of the alleles according to the original cited article. Thus, the reader should be aware of this aspect.

GENERAL ASPECTS OF miRNAs

MicroRNAs are small non-coding single-stranded RNA molecules of 19–25 nucleotides in length, well known by its important role in posttranscriptional regulation of gene expression (7). They are present in almost all eukaryotes, including humans, and regulate diverse biological processes in both physiological and pathological conditions (8–10). miRNAs were described to interfere in processes as distinct as cell proliferation and differentiation, apoptosis, or even in viral infections (11, 12). In such infections, the main focus of this review, miRNAs stand up as relevant mediators of the host response, and studies have demonstrated that these molecules can contribute to intracellular defense against the infection, to individual resistance to certain viruses, as well as control the survival, amplification, and modulation of cellular tropism of viruses. On the other hand, also viruses can produce miRNAs. Actually, they use the host cell machinery to generate their own miRNAs (10, 13, 14), which can, for example, to induce viral latency and decrease inflammatory responses, as well as to prevent cell apoptosis, contributing to the oncoviruses-related malignant transformation (15).

To understand how polymorphisms can influence the gene expression regulation by miRNAs, and even alter a given biological process, it is important to remember how miRNAs are generated. These molecules can be codified by independent genes or can be inserted in exons or introns from other genes. Briefly, in humans, miRNA biogenesis begins when they are transcribed by the RNA polymerase II as a primary transcript (pri-miRNA), consisting of a molecule encompassing 500–3,000 bases (see **Figure 1**). In the nucleus, the pri-miRNA is cleaved into pre-miRNA (60–70 nucleotides long) by a complex formed by the Drosha enzyme and its cofactor DGCR8 (DiGeorge syndrome critical region 8 protein) (7). After translocation from nucleus to cytoplasm, a process mediated by the molecule exportin-5 (Exp-5, a nuclear transport factor), pre-miRNAs are cleaved in a mature miRNA (19–25 bases long) by the Dicer/TRBP (trans-activation response RNA-binding protein) complex. Next, the mature single-stranded miRNA and the Argonaut protein (AGO) constitute a multicomponent complex called RNA-induced silencing complex, which allows the binding to complementary sequences in the 3′ untranslated region (3′UTR) of a target mRNA, leading to translational repression or degradation of the mRNA (7, 16–18). The key binding point for miRNA–mRNA interaction is the seed region, located within nucleotides 2–8 from the 5′ end of the mature miRNA sequence (19, 20). In general, a partial complementarity of the mRNA 3′UTR to the miRNA seed sequence leads to translational inhibition, while a perfect complementarity results in mRNA degradation. A slightly distinct process occurs when the miRNA precursor is located in mRNA introns (see **Figure 1**). In this case, the pre-miRNA will be spliced out and then exported from the nucleus to the cytoplasm, bypassing the Drosha/DGCR8 complex, and then will follow the remaining aforementioned pathway (10).

Polymorphisms in miRNA genes can influence gene transcription, alter the processing of pri- or pre-miRNA, and affect miRNA–mRNA interactions. Moreover, such miRNA–mRNA interactions can also be either facilitated or hindered by polymorphisms located in the 3′UTR of the target mRNAs, by the generation or loss of miRNA-binding sites, for example (25). The effect of gene regulation by miRNAs is quite complex, since a certain miRNA can target several mRNAs, and conversely, a single mRNA can bind to distinct miRNAs, being the final effect determined by the joint action of, potentially, several miRNAs (26). Despite best known by their capacity to impair translational processes, decreasing the rates of protein expression, miRNAs, in some cases, can bind to 5′ untranslated regions, to exons, or even to DNA elements, leading to increased transcription or translation (27–29).

An important emerging research topic concerns the effects of polymorphisms in miRNAs and its target-sites in viral-associated diseases. Recent case–control and functional studies have pointed out to a role of such polymorphisms in susceptibility to viral infection, as well as in chronicity of the disease versus viral clearance, and even in viral-associated cancer development (30–33). Given the increasing interest in such processes and phenomena,

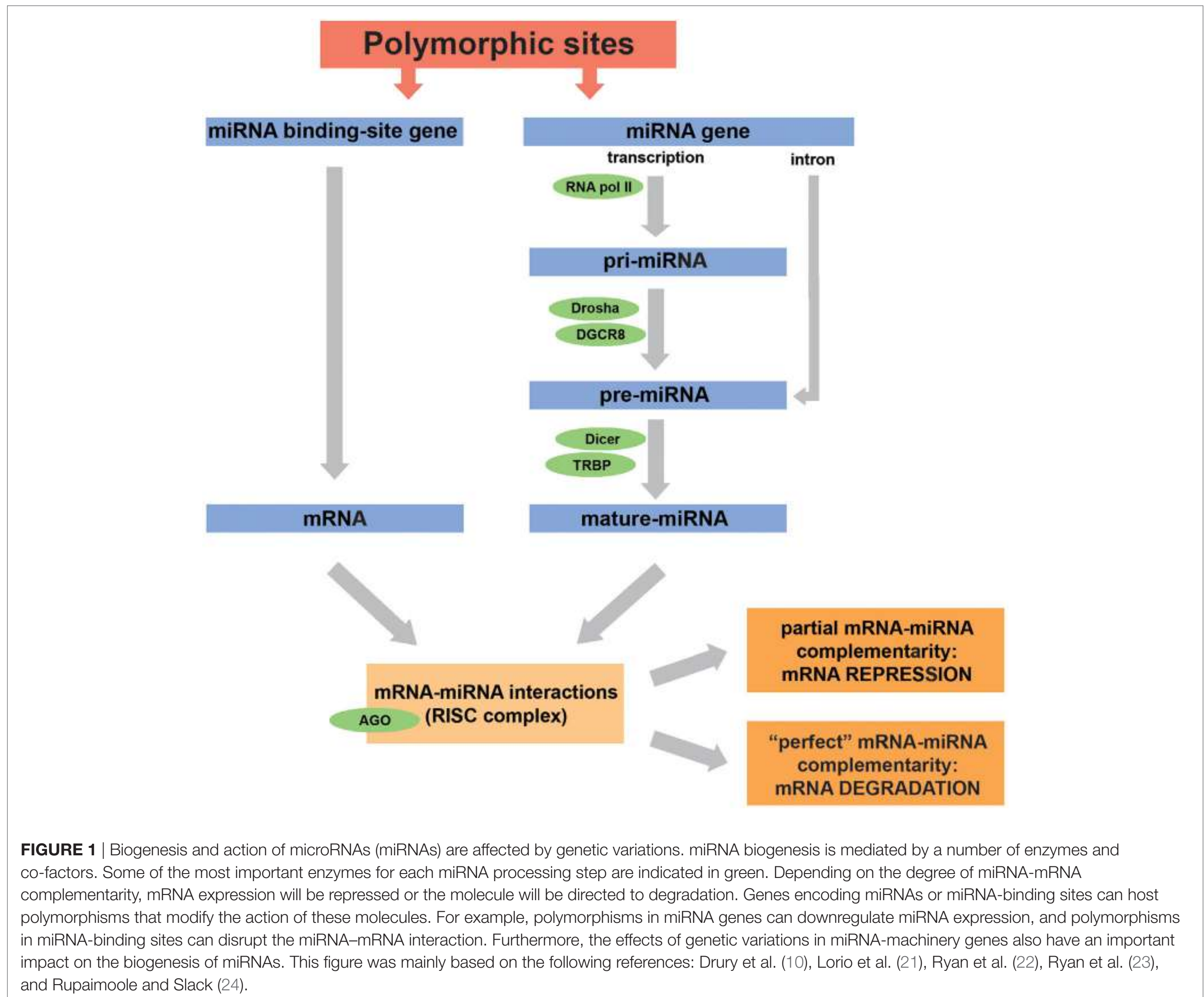

FIGURE 1 | Biogenesis and action of microRNAs (miRNAs) are affected by genetic variations. miRNA biogenesis is mediated by a number of enzymes and co-factors. Some of the most important enzymes for each miRNA processing step are indicated in green. Depending on the degree of miRNA-mRNA complementarity, mRNA expression will be repressed or the molecule will be directed to degradation. Genes encoding miRNAs or miRNA-binding sites can host polymorphisms that modify the action of these molecules. For example, polymorphisms in miRNA genes can downregulate miRNA expression, and polymorphisms in miRNA-binding sites can disrupt the miRNA–mRNA interaction. Furthermore, the effects of genetic variations in miRNA-machinery genes also have an important impact on the biogenesis of miRNAs. This figure was mainly based on the following references: Drury et al. (10), Lorio et al. (21), Ryan et al. (22), Ryan et al. (23), and Rupaimoole and Slack (24).

in addition to the potential clinical use of miRNAs as molecular biomarkers and therapeutic targets, we will present a review of the existing literature on these topics.

VIRAL INFECTIONS AND POLYMORPHIC VARIANTS THAT AFFECT miRNAs

HBV Infection, HBV-Associated Diseases, and miRNAs

It was estimated that around 30% of the world's population is a HBV carrier or has been infected with the virus in the past (34). HBV infection is classically responsible by triggering several types of liver damage, including cirrhosis and hepatocellular carcinoma (HCC) (34). Africa and Asia concentrate the largest number of countries with high prevalence of chronic HBV infection (34), and particularly in China, HBV infection is an endemic problem (35). Although several advances in the fight against HBV have been made, a large part of the Chinese population still suffers from HBV-associated diseases (36). Therefore, it was not a surprise, when the literature regarding the influence of miRNA-related polymorphisms on HBV-associated diseases was reviewed, that a large number of studies were performed with individuals from China.

In order to give a comprehensive idea of the studies approaching miRNA polymorphisms, minimizing the potential problems of comparing ethnically distinct populations, we will initially focus on studies performed in China; all other studies being gathered in the next section. Nevertheless, even considering only those studies, and centering in human populations with a relatively homogeneous ethnic origin, conflicting data arouses. In a research performed by Xu et al. (37), the GG genotype of miR-146a G/C SNP (rs2910164) was associated with increased risk of HCC in males. Their study compared 479 HCC patients with 504 controls. Of note, 88.9% of the HCC patients were infected with HBV. Moreover, through *in vitro* assays, the same authors

described how miR-146a rs2910164 would be linked to HCC. Briefly, the G allele increases miR-146a maturation, potentially contributing to HCC-related cell proliferation (37). A number of authors reported no impact of miR-146a rs2910164 on HBV-associated HCC (38–43). On the other hand, according to Cong et al. (44), the GG genotype and G allele of miR-146a rs2910164 increase the risk of HCC among HBV-infected individuals. In a recent meta-analysis including eleven studies performed in Chinese populations, the miR-146a rs2910164 was linked to an increased risk of HBV-associated HCC (45). Besides HCC development, other HBV-associated diseases are potentially influenced by this polymorphism. For example, Jiang et al. (46) investigated the miR-146a rs2910164 in patients with acute-on-chronic hepatitis B liver failure and in individuals with chronic HBV infection. Individuals carrying the GG genotype had reduced susceptibility to the disease, lower levels of TNF-α, and higher survival rate (46).

Xiang et al. (38) genotyped the miR-499a C/T SNP (rs3746444) in chronic HBV-infected individuals, HCC patients (HBV-infected and non-infected), and controls. They identified the CC genotype as a risk factor for the development of HBV-associated HCC (38). Posteriorly, and in conflict with the data from the previously cited work, a small case–control study found, in a dominant model, that AG + GG genotypes of miR-499a rs3746444 were associated with a reduced risk of HCC when HBV-infected patients were analyzed (47). In addition, another study with a small sample size reported an increased risk of HBV-associated HCC linked to the A allele of miR-499a rs3746444 (42). Ma et al. (48) investigated the miR-499 rs3746444 and the miR-423 A/C/T (rs6505162) SNP in 984 HCC patients and compared the genotype frequencies with a similar number of controls. Of note, among the HCC group, 760 individuals were infected with HBV. MiR-423 rs6505162 had no effect on HCC risk, independently of the HBV infection status. However, miR-499a TC + CC (in a dominant model) increased the risk of HBV-associated HCC, when compared to the TT genotype (48). Finally, a meta-analysis including case–control studies reinforced the involvement of miR-499a rs3746444 in the susceptibility to HCC among HBV-infected individuals (49). Nevertheless, it is important to consider that several authors did not found a statistically significant link between miR-499a rs3746444 and HBV-associated HCC (39, 41, 43, 50, 51). This fact evidences the need for new investigations aiming to establish with more robustness the impact of this SNP on HBV-associated HCC and reinforces the fact that, in multifactorial diseases, multiple variants of susceptibility can be identified, each of them with a small contribution.

In a study evaluating the miR-196a2 C/T SNP (rs11614913), Qi et al. (52) genotyped 199 chronic HBV-infected individuals without HCC, 361 chronic HBV-infected individuals with HCC, and 391 healthy controls. An increased risk of HBV-associated HCC was found in males carrying the C allele and the CC genotype. Regarding HCC progression, no statistically significant influence of miR-196a2 rs11614913 on tumor number, size, growth phase, stage, and lymph node metastasis was found. However, when stratified by sex, in male patients with lymphatic metastasis, a higher frequency of the T allele was observed (52).

The potential role of miR-196a2 rs11614913 on the risk of HBV-associated HCC was investigated by a number of authors. In a study performed by Hao et al. (39), CT and TT genotypes of miR-196a2 rs11614913 were considered risk factors for HCC development in HBV-infected individuals. In addition, the influence of miR-196a2 rs11614913 on HCC risk was investigated by Li et al. (43) in a small case–control sample (266 individuals in each group). 110 individuals from the HCC group and 32 individuals from the control group were HBV infected. Looking at these individuals, it cames out that CT + TT genotypes increase the risk of HCC development (43), a finding in line with the study performed by Hao et al. (39). However, conflicting results were also published. Kou et al. (41) evaluated this same miRNA variant site in 532 controls and 271 HCC patients. Approximately, 58% of the patients were HBV infected, and CT and TT genotypes presented a reduced risk of HCC (41). Furthermore, Zhang et al. (40) evaluated the miR-196a2 rs11614913 in a relatively large sample of the Chinese population. Their study included a control group (~1,000 individuals) and a group of HCC patients (~1,000, including 771 HBV-associated HCC patients). In brief, CT + TT genotypes and the T allele were linked to a lower chance of HBV-associated HCC development (40). Supporting this result, a small case–control study described CT and TT genotypes as well as the T allele of miR-196a2 rs11614913 as markers of reduced risk of HBV-associated HCC (53). Recently, the miR-196a2 rs11614913 was associated with a decreased risk of HBV-associated HCC in a meta-analysis including eleven studies carried out with Chinese populations (45). A previous meta-analysis (51), approaching a total of 2,693 HCC cases and 3,594 controls, had already associated the T allele and the TT genotype with reduced risk of HCC. Interestingly, this finding had been observed only considering the total pool of individuals, but not when stratifying the populations according to ethnicity (51). Actually, there are studies in Chinese populations where no statistically significant association between the miR-196a2 rs11614913 and risk of HBV-associated HCC were observed [see Yan et al. (54), for example], although these results seem to have been "diluted" with the inclusion of new studies in the more recent meta-analysis.

Another important point to be discussed refers to the interactions between viruses and host genetic factors. To highlight this point let's take the study from Han et al. (30), which, using quantitative PCR, explored the interaction of miR-196a2 rs11614913 and miR-34b/c T/C SNP (rs4938723) with HBV mutations in a sample of 3,325 individuals (1,021 of them with HBV-associated HCC). Among several results, the most interesting finding was that the effects caused by miRNA SNPs on HBV-associated HCC susceptibility can be strongly influenced by HBV mutations (30). Thus, host genetic polymorphisms may be relevant in the presence of an infection associated to a specific HBV genotype, but less important in the presence of HBVs showing different genetic features. In this sense, conflicting findings in studies evaluating the same particular host polymorphism in the context of HBV-associated diseases may be due not only to differences in the ethnic background of the studied population, but can also result from the HBV genetic variants circulating in this given population.

Some SNPs were studied in a particular context or population and few (or no) further studies were performed to confirm or refute these initial results. Wang et al. (55) investigated the

influence of miR-608 C/G SNP (rs4919510) and miR-149 C/T SNP (rs2292832) on the risk of HCC development. No link between miR-608 rs4919510 and HBV-associated HCC was reported. On the other hand, in men, the TT genotype of miR-149 rs2292832 was associated with an increased chance of HBV-associated HCC development when compared to the wild-type genotype (55). Differently, but also evaluating the miR-149 rs2292832, Liu et al. (56) found that the TC + CC genotypes, when compared with TT genotype, increased the risk of HCC in HBV-infected individuals. No link between miR-149 rs2292832 and HBV-associated HCC was reported in other studies (43, 50).

Wang et al. (57) investigated the miR-646 G/T SNP (rs6513497) in HCC patients and controls. Among the 771 HCC patients enrolled in the study, 81.1% were infected with HBV. Among males, the GT genotype and G allele were considered as protective factors against HBV-associated HCC (57). In this same direction, miR-378a C/T SNP (rs1076064) was also described as a protective factor of HBV-associated HCC. Specifically, AG + GG genotypes were associated with a decreased risk of HCC and higher HCC survival rate (58). Of note, these results were attributed, at least partially, to the effects that miR-378a rs1076064 exerts on miR-378 transcription (58). Although the results regarding miR-646 rs6513497 and miR-378a rs1076064 are quite interesting, the lack of confirmatory cohorts hinders further conclusions.

As the miR-122 expression is reduced in tissue samples of HBV-associated HCC (59), Liu et al. (60) evaluated the role of miR-122 A/C SNP (rs4309483) and miR-122 C/T SNP (rs4503880) on the risk of HCC. Their study included 1,300 HBV-infected patients with HCC, 1,344 HBV-infected patients without HCC, and 1,344 patients showing HBV clearance. The expression of pri-miR-122 and mature-miR-122 was measured in 29 HCC patients, comparing the levels in tumoral liver tissue and in adjacent tumor-free regions. In short, based on genotypes and gene expression, the authors concluded that miR-122 rs4309483 increases the risk of HBV-associated HCC (60). On the other hand, the same authors reported this SNP also acts as a protective factor against chronic HBV infection (60). This can be interpreted as follows: miR-122 rs4309483 hampers chronic HBV infection, but if the infection is established, this same SNP facilitates carcinogenesis.

Liu et al. (61) focused their investigation on the MCM7 C/T SNP (rs999885). Importantly, *MCM7* gene is the location of the miR-106b, miR-93 and miR-25 cluster (miR-106b-25) (61). They evaluated the influence of MCM7 rs999885 on the clinical outcome of HBV infection. In addition, the expression of miR-106b-25 was measured both in the HCC tissue and in adjacent tumor-free liver regions of 25 HBV-infected patients. AG/GG genotypes were associated to a higher expression of miR-106b-25 and a higher risk of HBV-associated HCC. Interestingly, these same genotypes were linked to lower risk of chronic HBV infection (61). The impact of MCM7 rs999885 of miR-106b-25 cluster on the outcome of HBV-associated HCC was also studied by Qi et al. (62). In summary, these authors observed that the AG/GG genotypes and G allele of MCM7 rs999885 were linked to a better HCC prognostic (62).

Zhou et al. (63) studied the GAGA ins/del polymorphism (rs17875871) of the 3′UTR of *IFNAR1* gene in a sample of HCC individuals and controls ($n = 420$ in each group). This polymorphism potentially affects the miR-1231-binding site. The deletion allele was associated with an increased risk of HCC, especially in the presence of HBV (63). The influence of polymorphisms in genes that affect the biogenesis/binding of miRNAs was also subject of study of Liu et al. (64). Specifically, polymorphisms in *DICER1*, *RAN*, *PIWIL1* genes (C/T rs1057035, A/C/G rs3803012, and C/T rs10773771, respectively) were genotyped in HBV-infected individuals with different clinical outcomes. Of note, DICER1 rs1057035 affects the miR-574-3p binding, RAN rs3803012 impacts the miR-199a-3p binding, and PIWIL1 rs10773771 influences the miR-1264 binding. The impact of the SNPs on the binding of these specific miRNAs was also tested *in vitro*. In brief, the authors found evidence that CT/CC genotypes of both DICER1 rs1057035 and PIWIL1 rs10773771 decreased the risk of HBV-associated HCC. Differently, RAN rs3803012 AG/GG genotypes were a risk factor of HBV persistent infection (64).

Xiong et al. (65) studied the KRAS G/T SNP (rs712), a genetic variation with implications to the binding of miR-let-7 and miR-181. According to these authors, the TT genotype increases the risk of HBV-associated HCC. This influence occurs possibly by a modified expression of *KRAS* due to the rs712-induced changes in the miR-let-7-binding site (65). Li et al. (66) explored the effect of five SNPs in miRNA-binding sites (located at *RAD52* gene) on the risk of HBV-associated HCC. The SNPs analyzed were: RAD52 A/G (rs1051669), RAD52 A/T (rs10774474), RAD52 A/T (rs11571378), RAD52 G/T (rs7963551), and RAD52 C/T (rs6489769). The C allele of RAD52 rs7963551 reduced the risk of HCC development. Of note, this SNP may affect the binding of miR-let-7. The authors also showed that CC or AC genotypes of RAD52 rs7963551 were associated with an increased RAD52 expression. Due to the role of RAD52 in DNA repair, changes in its expression or regulation caused by polymorphisms affecting the miRNA-binding sites may have a significant impact on the risk of HBV-associated HCC (66).

Zhang et al. (67) investigated the impact of PD1 A/G SNP (rs10204525) on the binding of miRNAs in the context of susceptibility to HBV-associated diseases. In summary, their results suggest that the *PD-1* regulation by miR-4717 is modified in response to PD1 rs10204525 genotypes. For example, *in vitro* experiments showed miR-4717 decreased *PD-1* expression in lymphocytes isolated from patients showing chronic HBV infection and GG genotype of PD1 rs10204525. In addition, this phenomenon was found in association with increased levels of TNF-α and IFN-γ. Together, these events may have an important impact on the HBV infection clinical course (67).

The influence of variations in genes of the miRNA machinery on chronic HBV infection was investigated by Shang et al. (68). Such study specifically addressed the following SNPs: DGCR8 A/G (rs3757), AGO1 A/G (rs636832), and GEMIN4 C/T (rs7813). The A allele of AGO1 rs636832 decreased the risk of chronic HBV infection. Moreover, compared to the AA genotype, AG + GG increased the risk of chronic HBV infection, suggesting the AA genotype as a protective factor to the disease. No statistically significant associations were reported in relation to the other analyzed SNPs (68).

In summary, it is evident that polymorphisms can interfere with the maturation and/or in the action of miRNAs, modifying

the risk of HBV-associated diseases. Therefore, it is important not only focus on genes that actually encode miRNAs or their binding sites, but also on those miRNA maturation/action modifier genes.

The interaction of a miR-122-binding site TTCA ins/del polymorphism (rs3783553, located at the *IL-1A* gene) and HBV mutations was investigated, in the context of HBV-associated HCC, by Du et al. (69). Interestingly, the TTCA insertion allele was linked to an increased frequency of the HBV C7A mutation. In general, rs3783553 did not modify the risk of HBV-associated HCC, but its interaction with HBV preS deletion reduced the risk of HCC development (69). According to the authors, host genetic polymorphisms influence the risk of HCC more subtly than the influence exerted by the genetic features of HBV. However, there is a strong interaction between viral and host genetic factors defining the course of HBV infection (69). Similar to Du et al. (69), Han et al. (70) evaluated the risk of HBV-associated HCC diseases taking into consideration virus–host interactions, meaning miR-218-2 A/G SNP (rs11134527) and HBV mutations. Briefly, miR-218-2 rs11134527 modified the risk of HCC, cirrhosis development, inflammation, and HBV clearance. Moreover, and again similar to the findings of Du et al. (69), the host genetic variation was associated with HBV preS deletion in men (70). However, in the study performed by Han et al. (70), the interaction of miR-218-2 rs11134527 with HBV preS deletion was linked to an increased risk of HCC. Finally, the T1674C/G HBV mutation reduced the increased risk of HCC linked to miR-218-2 rs11134527 (70). The results of these two studies exemplify the complex relationships between viral and host genetic factors. Besides, it is necessary to study the influence of gene–gene and gene–environment interactions to better understand the effect of miRNA SNPs on HBV-associated HCC (51).

Considering all articles mentioned above, we note that only a few miRNA SNPs have been studied in depth. This is the case of miR-146a G/C SNP (rs2910164) and miR-196a2 C/T SNP (rs11614913). The influence of these genetic variants on HBV-associated diseases is relatively well studied, at least in Chinese populations. However, even in these cases, conflicting results arouse. In order to synthesize the information described in this topic, the main interactions between miRNA SNPs and HVB-associated diseases were compiled in **Table 1**. In addition to data from studies performed with populations from China, **Table 1** also shows information obtained from studies performed in other populations. These studies will be discussed in the next topic.

HBV: More Studies With Diverse Human Populations

The potential role of miR-196a2 C/T SNP (rs11614913) on HBV-associated diseases was addressed by different authors in distinct human populations comprising ethnic backgrounds other than Chinese (focus of the previous topic). Data from a small case–control study performed by Akkiz et al. (73) in a Turkish population, pointed the C allele and the CC genotype as potential markers to identify individuals at high risk for developing HBV-associated HCC who could benefit from more frequent HCC preventive examinations. However, conflicting results regarding the effects of such variant were published later. Kim et al. (74) studied in a Korean population the impact of miR-196a2 rs11614913 and miR-196a2 A/C SNP (rs12304647) on the clinical outcome of HBV infection. In addition to 404 patients with HBV spontaneous recovery, the study included 313 HBV-infected patients with chronic hepatitis, 305 HBV-infected patients with liver cirrhosis, and 417 HBV-patients with HCC, in a total of 1,035 HBV-infected individuals. Briefly, among HBV-infected patients with chronic hepatitis or cirrhosis, the CC genotype of miR-196a2 rs12304647 was linked to a reduced risk of HCC, although no statistically significant influence of the miR-196a2 rs11614913 on HCC development was observed (74).

In a case–control study, Riazalhosseini et al. (75) genotyped three polymorphisms in three Malaysian ethnical groups (Malays, Chinese, and Indians): miR-196a2 C/T SNP (rs11614913), miR-196a2 A/C SNP (rs12304647), and miR-146a C/G SNP (rs2910164). The authors evaluated the influence of these SNPs on the development of HBV-associated cirrhosis and HCC, comparing 103 chronic HBV-infected patients with liver cirrhosis or with cirrhosis and HCC to 423 chronic HBV-infected patients without such conditions. No statistically significant influence of miR-196a2 rs11614913 and miR-146a rs2910164 on the HBV-associated diseases was observed. However, when compared to CC genotype, AA + AC genotype of miR-196a2 rs12304647 was linked to a reduced risk of cirrhosis/HCC (75).

Kim et al. (71) investigated in a case–control study with a Korean population the role of miR-196a2 C/T SNP (rs11614913), miR-149 C/T SNP (rs2292832), miR-146a C/G SNP (rs2910164), and miR-499a C/T SNP (rs3746444) on the risk of HCC development. Among 159 HCC patients, 127 were HBV infected. In relation to miR-149 rs2292832, CT genotype and CT + CC in a dominant model reduced the risk of HCC in HBV-infected and non-infected individuals. Considering miR-499a rs3746444, an AG + GG model also reduced the risk of HBV-associated HCC. No influence on HBV-associated HCC was observed for miR-146a rs2910164 and miR-196a2 rs11614913 in this study (71), although a meta-analysis (72) suggested that the miR-146a rs2910164 C allele decreases the risk of HCC in populations with an Asian ethnic background and also in Caucasians. No effect of miR-499a rs3746444 was observed in this same meta-analysis (72).

The influence of miR-149 C/T SNP (rs2292832) and miR-101-1 C/G/T SNP (rs7536540) on the risk of HCC in Thai population was evaluated by Pratedrat et al. (81), in a study including 95 healthy controls, 90 chronic HBV-infected individuals, and 104 HCC patients. However, no statistically significant association was found (81). In addition to miR-101-1 rs7536540, the influence of the following variants on clinical outcome of HBV infection was investigated in Korean individuals (77): miR-101-2 C/T SNP (rs17803780), miR-101-2 C/T SNP (rs12375841), and miR-338 C/T SNP (rs62073058). In brief, miR-101-1 rs7536540 had an impact on the risk of liver cirrhosis and HCC, and miR-101-2 rs12375841 and the haplotype ht2 (T-C) of miR-101-2 influenced the HBV clearance (77).

The role of three variants of the miRs-371-372-373 cluster (C/T SNP rs28461391, A/C rs3859501, and C/T rs12983273) on the risk of HCC and HBV clearance was investigated by Kwak et al. (76) in a sample of 1,439 Korean individuals. The miRs-371-373 rs3859501 and the ht2 (C-A-C) haplotype were linked to a reduced

TABLE 1 | Main microRNA (miRNA)-related polymorphisms showing statistically significant influence on hepatitis B virus (HBV) infection and HBV-related diseases.

miRNA or miRNA-binding site[a]	Polymorphism[b]	Influence on	Population	Reference
miR-146a	C/G rs2910164	Susceptibility to HBV infection	Chinese	Cong et al. (44)
		HBV-associated hepatocellular carcinoma (HCC)	Chinese	Zhou et al. (29); Cong et al. (44)
			Meta-analysis	Tian et al. (45)
		Acute-on-chronic hepatitis B liver failure	Chinese	Jiang et al. (46)
		Susceptibility to HBV infection; HBV clearance	Saudi Arabian	Al-Qahtani et al. (33)
miR-149	C/T rs2292832	HBV-associated HCC	Chinese	Wang et al. (55); Liu et al. (56)
			Korean	Kim et al. (71)
		Susceptibility to HBV infection; HBV clearance; HBV persistence; HBV-associated cirrhosis/HCC	Saudi Arabian	Al-Qahtani et al. (33)
miR-499	C/T rs3746444	HBV-associated HCC	Korean	Kim et al. (71)
			Chinese	Xiang et al. (38); Li et al. (42); Zou and Zhao (47); Ma et al. (48)
			Meta-analysis	Yu et al. (49)
miR-196a2	C/T rs11614913	HBV-associated HCC	Chinese	Hao et al. (39); Zhang et al. (40); Kou et al. (41); Li et al. (43); Qi et al. (52); Zhou et al. (53)
			Meta-analysis	Tian et al. (45); Zhu et al. (51); Xu et al. (72)
			Turkish	Akkiz et al. (73)
		Gene-gene interaction; HCC-related HBV mutations	Chinese	Han et al. (30)
		Susceptibility to HBV infection; HBV clearance; HBV-associated cirrhosis/HCC	Saudi Arabian	Al-Qahtani et al. (33)
miR-196a2	A/C rs12304647	HBV-associated HCC	Korean	Kim et al. (74)
		HBV-associated cirrhosis/HCC	Malaysian	Riazalhosseini et al. (75)
miR-34b/c	C/T rs4938723	Gene-gene interaction; HCC-related HBV mutations	Chinese	Han et al. (30)
miR-423	A/C/T rs6505162	HBV clearance; HBV-associated cirrhosis/HCC	Saudi Arabian	Al-Qahtani et al. (33)
miR-26a1	C/T rs7372209	HBV-associated cirrhosis/HCC	Saudi Arabian	Al-Qahtani et al. (33)
miR-608	C/G rs4919510	HBV-associated cirrhosis/HCC	Saudi Arabian	Al-Qahtani et al. (33)
miR-492	C/G rs2289030	HBV clearance	Saudi Arabian	Al-Qahtani et al. (33)
miR-30a	A/G rs1358379	Susceptibility to HBV infection; HBV clearance; HBV persistence; HBV-associated cirrhosis/HCC	Saudi Arabian	Al-Qahtani et al. (33)
miR-122	A/C rs4309483	Chronic HBV infection; HBV-associated HCC	Chinese	Liu et al. (60)
miR-122-binding site	ins/del rs3783553	HCC-related HBV mutations	Chinese	Du et al. (69)
miR-371-372-373 cluster	A/C rs3859501	HBV-associated HCC	Korean	Kwak et al. (76)
miR-106b-25 cluster	C/T rs999885	HBV-associated HCC	Chinese	Liu et al. (61); Qi et al. (62)
		Chronic HBV infection	Chinese	Liu et al. (61)
miR-101-1	C/G/T rs7536540	HBV-associated cirrhosis/HCC	Korean	Bae et al. (77)
miR-101-2	C/T rs12375841	HBV clearance	Korean	Bae et al. (77)
miR-1231-binding site	ins/del rs17875871	HBV-associated HCC	Chinese	Zhou et al. (63)
miR-219-1	A/G rs107822	HBV clearance	Korean	Cheong et al. (78)
miR-219-1	C/T rs421446	HBV clearance	Korean	Cheong et al. (78)
miR-219-1	C/T rs213210	HBV clearance	Korean	Cheong et al. (78)
miR-574-3p-binding site	C/T rs1057035	HBV-associated HCC	Chinese	Liu et al. (64)
miR-1264-binding site	C/T rs10773771	HBV-associated HCC	Chinese	Liu et al. (64)
miR-199a-3p-binding site	A/C/G rs3803012	HBV-associated HCC; HBV persistance	Chinese	Liu et al. (64)
miR-378	C/T rs1076064	HBV-associated HCC	Chinese	An et al. (58)
miR-604	C/T rs2368392	HBV-associated HCC; HBV persistance	Korean	Cheong et al. (79)
miR-218	A/G rs11134527	Gene-gene interaction; HCC-related HBV mutations; HBV-associated cirrhosis/HCC; HBV clearance	Chinese	Han et al. (70)
miR-646	G/T rs6513497	HBV-associated HCC	Chinese	Wang et al. (57)
miR-let-7-binding site	G/T rs7963551	HBV-associated HCC	Chinese	Li et al. (66)
miR-let-7-binding site	G/T rs712	HBV-associated HCC	Chinese	Xiong et al. (65)
miR-4717-binding site	A/G rs10204525	Chronic HBV infection	Chinese	Zhang et al. (67)
miR-323b	A/C/T rs56103835	HBV persistence	Korean	Yu et al. (80)

[a]*Seed or regulatory region.*

[b]*Polymorphism quotations were standardized according to the Single Nucleotide Polymorphism Database (dbSNP) of NCBI (https://www.ncbi.nlm.nih.gov/snp/), based on the reference SNP cluster (rs#) of each polymorphism.*

risk of HBV-associated HCC. However, no statistically significant influence of those SNPs was observed concerning HBV clearance (76). Another study from Korea evaluated the impact of three distinct variants of miR-219a1 (C/T rs421446, A/G rs107822, and C/T rs213210) on HBV clinical outcome (78). In brief, all SNPs evaluated and the ht1 (C-A-C) and ht2 (T-G-T) haplotypes showed some influence on HBV clearance. Conversely, no statistically significant influence of those SNPs on HBV-associated HCC was reported. These results indicate that miR-219a1 has an important influence specifically on HBV clearance. However, the mechanisms by which miR-219a1 acts on HBV infection and how its SNPs can affect those mechanisms are still unclear and may be subject to functional studies (78). Posteriorly, the T allele of miR-604 C/T SNP (rs2368392) was linked to HBV chronic infection in Korean patients (79), although, unexpectedly, in patients chronically infected with HBV this allele reduced the risk of HCC occurrence (79). In other words, this SNP seems to play a role in the maintenance of the infection, but it does not necessarily contribute to the mechanisms of hepatocarcinogenesis.

Still considering Korean patients, Yu et al. (80) evaluated the miR-323b A/C/T SNP (rs56103835) on HBV replication and clinical course of infection. In that study, miR-323b rs56103835 was associated with persistent infection and was hypothesized as a factor which facilitates chronic HBV infection. In line with this interpretation, this SNP promoted HBV replication *in vitro* (80). In association, these findings support an important role for miR-323b rs56103835 in HBV chronic infection, once miR-323b can be considered an HBV suppressor (80). Of note, some points in the study of Yu et al. (80) (statistical analysis and interpretation of results) were target of criticism (82) which should be taken into account when interpreting the results mentioned above.

Recently, Al-Qahtani et al. (33) investigated the role of a number of miRNA SNPs on HBV-associated liver diseases in Saudi Arabia, including 1,352 HBV-infected patients and 600 healthy HBV uninfected controls. The genotyped variants were: miR-499a C/T SNP (rs3746444), miR-423 A/C/T SNP (rs6505162), miR-26a1 SNP C/T (rs7372209), miR-608 C/G SNP (rs4919510), miR-604 C/T SNP (rs2368392), miR-492 C/G SNP (rs2289030), miR-149 C/T SNP (rs2292832), miR-146a C/G SNP (rs2910164), miR-196a2 C/T SNP (rs11614913), and miR-30a A/G SNP (rs1358379). Briefly, the authors evidenced that the polymorphisms of miR-149 rs2292832, miR-146a rs2910164, miR-196a2 rs11614913, and miR-30a rs1358379 were significantly more frequent in patients than in the control group (33). As a remark, Cong et al. (44) have already described that miRNA-146a rs2910164 may be involved in immune regulation during HBV infection in a Chinese population. Moreover, in this same study miR-30a rs1358379, miR-149 rs2292832, miR-146a rs2910164, miR-423 rs6505162, miR-492 rs2289030, and miR-196a2 rs11614913 were associated to HBV clearance (33). HBV persistence was impacted by miR-149 rs2292832 and miR-30a rs1358379. Finally, miR-196a2 rs11614913, miR-30a rs1358379, miR-26a1 rs7372209, miR-608 rs4919510, miR-149 rs2292832, and miR-423 rs6505162 impact the development of HBV-associated cirrhosis, or HBV-associated HCC. No statistically significant associations were reported concerning miR-604 rs2368392 or miR-499a rs3746444 on HBV-associated diseases (33). Of particular interest, the finding regarding miR-499a rs3746444 corroborates the previously mentioned study of Xu et al. (72).

Behelgardi et al. (83) studied the influence of IL-16 T/C (rs1131445), an SNP located in a miRNA-binding site in 3′UTR of the *IL-16* gene, and the risk of HBV chronic infection in an Iranian population. After adjustment for covariates, including age and gender, the TC genotype was associated with an increased risk of HBV chronic infection. IL-16 is a pro-inflammatory cytokine that activates T cells, monocytes, dendritic cells, and macrophages, as well as stimulates other pro-inflammatory cytokines, such as IL-1β, IL-6, and IL-15. Thus, polymorphisms that modulate the production of IL-16 could be important regulators of susceptibility to viral infections (83).

Hepatitis C Virus

The susceptibility to HCV infection as well as the progression of HCV-related diseases result from the interaction of host and viral genetic characteristics, and are mediated by environmental and different physio-metabolic factors (84). Focusing on the host genetics, the importance of miR-146a G/C SNP (rs2910164) (44, 53) and miR-196a2 C/T SNP (rs11614913) (39, 41, 53, 54) on HCV-associated disease was investigated in Chinese individuals. Despite these efforts, no statistically significant association was found between the SNPs and HCV-associated diseases (39, 41, 44, 53, 54). Furthermore, no statistically significant association between miR-196a2 rs11614913 and HCV-related HCC was reported in an investigation encompassing the Turkish population (73). Although disappointing at a first glance, these data are quite relevant. Knowing which SNPs (and genes) have little or no clinical importance on a particular disease helps to refine our choices and redirect new studies into variants and pathways relevant to the field.

MiR-122 is abundantly expressed in hepatic cells (85–87) and markedly influences the clinical course of HCV infection (86, 88). Some attempts to explain this influence have focused on genetic variants that affect miR-122 expression. For instance, Urban et al. (89) evaluated the relationships between the IFNL4/IL28B C/T SNP (rs12979860) and miR-122 expression in liver samples of HCV-infected patients from the United States presenting distinct ancestry (Asian, African American, Caucasian, and Hispanic). They observed a reduced miR-122 expression in samples of patients showing poor response to the treatment. However, this finding was independent of the IFNL4/IL28B rs12979860 genotype. On the other hand, this SNP may also influence the course of HCV infection independently, once carriers of CT or TT genotypes showed higher levels of interferon-stimulated genes compared to those levels linked to CC genotype (89). Evaluating the same SNP in a small sample of HCV-infected patients, Estrabaud et al. (86) observed an increased miR-122 expression in the liver of CC genotype carriers. In this same context, Spaniel et al. (59) reported a reduced miR-122 expression in non-tumor liver samples of HCV-infected individuals, a finding also linked to another SNP of *IFNL4/IL28B* gene: G/T rs8099917. In this study, the TG genotype was associated with a lower expression of miR-122 in non-tumor liver samples of HCV-infected Japanese individuals (59). Moreover, evaluation HCV-infected patients,

Su et al. (90) found an association between TT genotype of IFNL4/IL28B rs8099917 and high levels of serum miR-122. In agreement with results from population studies, an *in vitro* assay suggested that both IFNL4/IL28B rs12979860 and IFNL4/IL28B rs8099917 modulate the course of HCV infection, although how exactly this modulation happens is still not understood (87). However, as often is the case, there are conflicting data from studies that do not corroborate these associations (88, 91, 92).

The IFNL3/IL28B A/C SNP (rs4803217) affects the binding of HCV-induced miRNAs (miR-208b and miR-499a-5p) with the *IFNL3* mRNA (93, 94). According to McFarland et al. (93), this phenomenon has important implications for the HCV pathogenesis. Specifically, the G allele of IFNL3/IL28B rs4803217 impairs the activity of both these miRNAs, promoting a high expression of *IFLN3*. As a consequence, the G allele contributes to HCV clearance while the T allele favors (or is neutral) the infection process (93, 94). Based on the study of McFarland et al. (93), Tiang (94) highlighted that miR-208b and miR-499a-5p are potential targets for therapy against HCV infection. Posteriorly, the functional effects of IFNL3/IL28B rs4803217 on miR-208b and miR-499a-5p were challenged by other investigations (95, 96), since it was suggested that the influence of IFNL3/IL28B rs4803217 on HCV infection is promoted by miRNA-independent mechanisms (96). Thus, more studies regarding the role of IFNL3/IL28B rs4803217 polymorphism and miR-208b and miR-499a-5p on HCV infection are welcome.

Hepatitis C virus uses several strategies to evade the immune system, including miRNAs engagement (84). The disruption of miRNAs that promote HCV infection (for example, those that help HCV to evade the immune system) is a potential therapy for HCV-associated diseases (93). The understanding of how polymorphisms affect this phenomenon can help us in the development of new drugs based on this mechanism (**Figure 2**). Following these ideas, and based on a study investigating miRNA-101-1 and miRNA-221 expression and their respective SNPs (miR-101-1 C/G/T rs7536540 and miR-221 A/G rs17084733) in an Egyptian population, Shaker et al. (97) proposed the use of miR-101-1 and miR-221 as biomarkers of HCV-associated HCC. However, before applied to the clinical practice, these findings must be validated in different populations in studies with large sample sizes.

Finally, there is some evidence showing that TGFBR1 A/G SNP (rs868) (located at miR-let-7 and miR-98-binding sites) could have an impact on clinical parameters of HCV infection, especially on HCV RNA loads and hepatic inflammation (98). However, in the current scenario, the interaction between this TGFBR1 SNP (rs868) and HCV infection is poorly understood.

HIV Infection

According to Corbeau (99), different human miRNAs have a close relationship with HIV, both by interacting with HIV RNA as well as with mRNAs of cellular proteins essential for HIV replication. These interactions impact HIV replication, latency, pathogenesis, and also affect the host antiviral immune response. Therefore, the manipulation of these miRNAs expression can be approached as a potential therapeutic tool to mitigate the impact of HIV infection (99). Hariharan et al. (100) suggested that polymorphisms in miRNAs targeting HIV genes may influence the infection progression. However, although the relationship between HIV and miRNAs has already been studied and debated, the effects of miRNA SNPs on HIV-miRNAs interaction have been poorly explored.

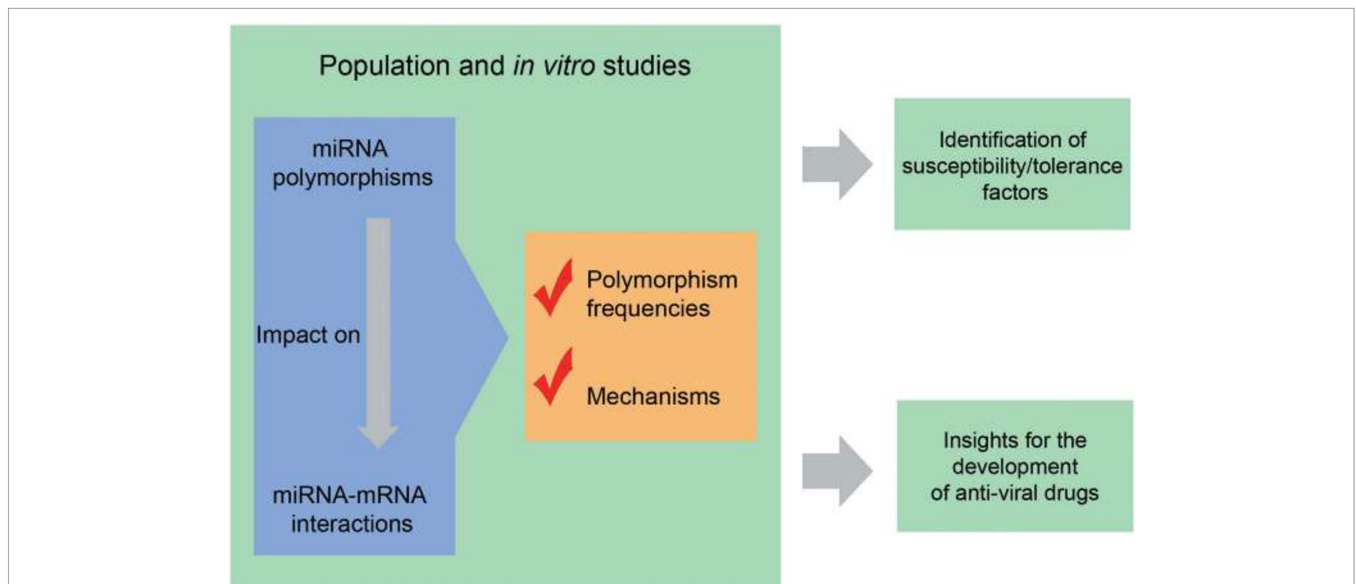

FIGURE 2 | Why to study polymorphisms in the context of viral diseases? Population or *in vitro* studies help to understand which and how polymorphisms impact on microRNA (miRNA)–mRNA interactions. Knowing the population distribution of polymorphisms and relationships of susceptibility/tolerance in the context of viral diseases, make it possible to identify individuals and populations with increased or decreased susceptibility to viral infections, allowing the development of strategies for infection control. In addition, the comprehension of the mechanisms by which miRNA-related polymorphisms influence the outcomes of viral diseases provides insights for the development of new antivirals. See text for references.

One of the few genes evaluated in the context of miRNA and HIV is the *Human Leukocyte Antigen-C* gene (*HLA-C*). The HLA-C ins/del variant (rs67384697) already evaluated in Europeans (101) and in Chinese populations (102), disrupts the binding site of miR-148, impacting the control of HIV infection. The deletion allele was associated to an HIV controller phenotype (low viral loads and high CD4 T^+ cell counts) and the insertion alleles were associated to an HIV noncontroller phenotype (high viral loads and low CD4 T^+ cell counts) (101, 102). The HIV controller phenotype was also associated to the CC genotype of HLA-C C/T SNP (rs9264942) (102), although it is worth to note that HLA-C rs67384697 and rs9264942 are in linkage disequilibrium (101, 102). Finally, miR-148a A/G SNP (rs735316) seems to influence the progression of HIV infection by interfering with the expression of HLA-C on the cell surface (103).

Peckham-Gregory et al. (104) evaluated 25 miRNA SNPs in patients with AIDS-associated non-Hodgkin lymphoma (AIDS-NHL) and HIV-infected controls. The authors included in their analyses SNPs located at miRNA coding regions, at miRNA biogenesis genes, and near/within miRNA-binding sites. Among the different results of this study, it worth to highlight: (I) The DDX20 C/T SNP (rs197412) affected miRNA biogenesis and this SNP C allele was associated with an increased risk of AIDS-NHL; (II) The T allele of miR-196a2 C/T SNP (rs11614913) (located at miR-196a2 coding region) was linked to a decreased risk of central nervous system (CNS) AIDS-NHL; (III) The T allele of HIF1A-AS2 C/T SNP (rs2057482) was associated with an increased risk of systemic AIDS-NHL, and (IV) the same allele decreased the risk of CNS AIDS-NHL (104). Of particular interest, HIF1A-AS2 rs2057482 is a variant that creates a binding-site to miR-196a2 (104).

Several CYP2B6 SNPs were evaluated concerning their potential influence in the metabolization of the anti-HIV drug Efavirenz in different contexts (105–109). Among the main findings, the CC genotype of CYP2B6 C/T SNP (rs1042389) was associated to low Efavirenz plasma concentration in Black HIV + individuals from South Africa (110). It is believed that this SNP modifies the expression of *CYP2B6* mRNA since it affects the binding-site of different miRNAs (110, 111). However, the association found in this study was quite weak and the clinical significance of this variant is controversial. Also in South Africa, Maharaj et al. (112) genotyped the miR-27a A/C/G/T SNP (rs895819) in HIV-negative and HIV-positive pregnant women subdivided according to a normotensive or a preeclamptic status. Although the TC/CC genotype of miR-27a rs895819 was associated to increased body mass index (BMI) in the group of HIV-positive women with preeclampsia, it was not associated with preeclampsia susceptibility (112). As miR-27a is an inhibitor of adipogenesis (113, 114) it is believed that miR-27a rs895819 can disrupt this miR-27a action, and then contribute to an increased BMI (112). The association between miR-27a rs895819 and BMI described by Maharaj et al. (112) is quite interesting and deserves to be replicated in other populations with different genetic backgrounds. Posteriorly, the same group described a potential impact of miR-146a C/G SNP (rs2910164) on HIV-positive South African women with preeclampsia (115). Specifically, GC/CC genotypes were associated with a reduced susceptibility to severe preeclampsia in HIV-positive pregnant women on HAART (Highly Active Antiretroviral Therapy). In addition, the miR-146a rs2910164 seems to have an influence on IL-2 levels of pregnant women (115). These results suggest an influence on the progression of HIV-related diseases. However, the patients studied by Maharaj et al. (112, 115) represent a very particular group of women, and before assuming that miR-27a rs895819 or miR-146a rs2910164 have an important influence on the clinical status of HIV-infected individuals from different genetic backgrounds, these SNPs must be studied in distinct populations (infected and non-infected by HIV) in studies recruiting men and women with different health status.

Finally, the A allele of TREX1 A/G SNP (rs3135945), a variant from a gene which encodes a restriction factor against HIV-1, was associated with higher susceptibility to HIV infection in a Caucasian cohort evaluated by Pontillo et al. (116). Since this SNP does not induce aminoacid sequence change, the authors hypothesized that a miRNA-mediated mechanism could explain how TREX1 rs3135945 impacts on HIV infection (116).

Epstein–Barr Virus

Epstein–Barr virus belongs to the herpesvirus family and is one of the most common viruses in humans, infecting more than 90% of the people worldwide. EBV is well known to cause the infectious mononucleosis (117). However, this virus is also associated with the development of several human tumors (118). EBV infection is a relevant susceptibility factor to nasopharyngeal carcinoma (NPC), and the few data available about the role of polymorphisms in miRNAs and binding target-sites in EBV infection came from studies focused in this type of cancer (32, 119, 120). Actually, the interest in NPC-associated EBV miRNAs emerged from the identification of EBV-encoded viral miRNA in lymphoid malignancies. Given that only a few viral latent proteins are expressed in NPC, researchers have hypothesized that EBV may contribute to cancer development through the viral miRNAs (120). The role of EBV miRNAs is still little known, but studies are pointing to important roles in both viral and cellular gene expression modulation (10, 121, 122).

An interesting case–control association study related to the current topic showed the influence of SNPs within mature-miRNA sequences in NPC susceptibility, assessing a southern China population (32). Further, these preliminary results were validated in a sample from eastern China. Eight SNPs were evaluated in the referred study, including miR-499 rs3746444 C/T, miR-608 rs4919510 C/G, miR-3152 rs13299349 A/G, miR-4293 rs12220909 C/G, miR-4513 rs2168518 C/T, miR-4520a rs8078913 C/T, miR-5579 rs11237828 C/T, and miR-5689 rs9295535 C/T. Among them, only the miR-608 rs4919510 SNP was associated with NPC risk. The presence of the G allele was reported as a susceptibility factor in both Chinese samples, in the two merged populations, and especially in individuals with EBV infection, where the risk effect was more prominent in comparison with individuals not infected. Aiming to evaluate the effects of the miR-608 rs4919510 SNP on NPC tumorigenesis, CNE-1 and CNE-2 cells (both NPC cell lines) were transfected with constructs containing G or C alleles and a soft-agar colony formation assay was performed. In agreement with population-based results,

functional analyses indicated that G allele of miR-608 rs4919510 SNP induced more colonies compared to C allele in CNE-2 cells (32). Another investigation also based on population-derived data and functional experiments had previously linked the miR-608 rs4910510 G allele with NPC locoregional recurrence (123). Based on this body of evidence, it is possible to assume that the G allele of miR-608 rs4919510 SNP significantly interacts with EBV, resulting in an increased NPC susceptibility (32). Some of the miR-608 target genes (immune system related genes, or genes associated to DNA repair, metastasis-related, cell death-related, among many others), can have their expression rates altered by the miR-608 rs4919510 SNP (32, 123). Furthermore, EBV can influence host gene transcription (32, 124, 125). In line with this view, Qiu et al. (32) suggested that miR-608 target genes could be directly activated by EBV and the influence of miR-608 rs4919510 SNP on gene transcription could be modified by EBV infection. These complex interaction networks would result in an increased NPC risk, as previously mentioned. Although the impact of miR-608 rs4919510 SNP on host gene expression and the interactions between EVB and host genes are plausible and supported by different data (32, 123–125), it must be characterized in more detail. Finally, the same authors also proposed the use of miR-608 rs4919510 SNP as a marker of NPC risk in a Chinese population (32). Although the above-mentioned data support this suggestion, it is important to replicate these findings in different populations before miR-608 rs4919510 SNP be used as a marker of NPC risk. Further functional studies fully characterizing the effects of this variant on gene regulation are also welcome to help us understand the role of this SNP (32).

Host–EBV interactions have also been investigated revealing that EBV gene regulation can be influenced by host transcriptional regulators. In addition, it was shown that EBV-encoded miRNAs can induce cell transformation in the host (126–128). In fact, EBV-encoded miRNAs have been involved in the regulation of both EBV and human gene expression in NPC. In a study from Lung et al. (120), two nucleotide variations in the primary transcript of miR-BART22 were identified as responsible for its increased biogenesis *in vitro*. This miRNA is coded by EBV and is highly expressed in NPC. Moreover, miR-BART22 modulates the EBV-encoded LMP2A protein expression, which is an oncoprotein recognized by cytotoxic T cells in the host (120). MiR-BART22-induced LMP2A down-modulation may promote EBV-infected cells evasion of the immune system (120). Based on these findings, it is possible to assume that miR-BART22 contributes to EBV pathogenesis. Thus, it makes sense the suggestion that polymorphisms in the miR-BART22 transcript could affect its maturation in NPC, contributing to a higher miR-BART22 expression, which in turn would induce a decreased LMP2A expression facilitating cancer development through the evasion of host immune response (120). Although this is not a case–control association study, it highlights and reinforces the importance of studies about polymorphisms in miRNAs and their binding target-sites in the context of the EBV infection. However, we consider that the most interesting in the study performed by Lung et al. (120) is that it supports the expression control of oncogenic and immunogenic viral proteins by EBV-derived miRNAs. Based on this information, polymorphisms affecting this control potentially play a pivotal role in the NPC development. In this sense, these polymorphisms may be used as models for the study of NPC, once understanding the mechanisms by which polymorphisms in EBV miRNAs interfere with the expression of proteins that modulate tumor biology may provide important insights for the development of NPC therapies. However, to achieve this goal, it is essential to perform functional studies focused on the understanding of the effect of EVB miRNAs and their polymorphisms in pathological and physiological contexts. Since EBV miRNAs modulate the expression of cancer-related proteins (120, 121, 128), they potentially also influence basic cellular physiological mechanisms, such as cell growth, differentiation, and signaling.

A recent characterization of the mRNA and miRNA transcriptome in NPC models (in cell lines that actually harbor EBV), provides a general view about miRNA–mRNA regulation and polymorphisms that can interfere in such regulation (127). This approach represents an interesting starting point for planning new studies about polymorphisms within miRNAs or miRNA target-sites potentially related to EBV infection.

Finally, we call attention to the need for conducting studies involving the characterization of EBV miRNAs, once information on this subject is still scarce. From the characterization of these miRNAs, it will be possible to deepen the investigations of polymorphisms found in sequences of EBV miRNAs. Although NPC is a disease of great relevance, it is also essential do not neglect other EBV-related diseases.

Human Papillomavirus

Studies encompassing miRNAs and HPV infection are incipient. Actually, a search in article databases returned only 12 articles focusing on genetic polymorphisms related to miRNAs and HPV, being the vast majority on HPV-related cancer development, progression, and prognosis. In this sense, miRNA-related polymorphisms that could modulate immune response and viral restriction, as well as cell cycle, proliferation and death, related to HPV infection often will be evaluated considering tumoral clinical outcomes.

Analyzing an Italian cohort of patients with penile squamous cell carcinomas (PSCCs), Peta et al. (129) investigated the association of a common functional miR-146a C/G SNP (rs2910164) with risk to cancer development. The frequencies of miR-146a rs2910164 genotypes in PSCCs patients, as well as its expression levels, were not different from the distribution observed in the general population, although miR-146a targets various genes that control of immune response, inflammation, cell proliferation, differentiation, and metastasis formation. Considering their potential effects, miRNA expression might act as a two-edged sword, being its upregulation related to immunosuppressive effects and its downregulation associated to cell proliferation and metastases development (129, 130). The authors found an inverse correlation regarding the expression levels of miR-146a and the expression of epidermal growth factor receptor (EGFR), a well-established target of miR-146a. The activation of EGFR pathways is known to increase keratinocytes proliferation and migration, and to be related to HPV-mediated cell immortalization and transformation (131). In fact, miR-146a expression levels were lower in high-risk HPV-positive than in HPV-negative patients,

although that difference was not statistically significant. This lower expression was also observed in HPV-positive carcinoma cell lines when compared to cultures from healthy cells. Thus, the authors suggested that HPV-16 E6 downregulates miR-146a expression, leading to an overexpression of EGFR, increasing, this way, the risk of cancer development (129).

Revathidevi et al. (132) studied the effect of a deletion in the *APOBEC3* gene cluster in an attempt to associate HPV infection and cancer development in a South Indian population. This polymorphism corresponds to a deletion of a 29.5-kb fragment, removing sequences from the fifth exon of *APOBEC3A* to the eighth exon of *APOBEC3B*. The polymorphic transcript encompasses the coding sequence of APOBEC3A and the 3′UTR *APOBEC3B*. The APOBEC3A/3B deletion polymorphism has been associated to poor HPV restriction and carcinogenesis promotion (133). Since APOBEC3A/3B deletion involves 3′UTR alterations, Revathidevi et al. (132) hypothesized that miRNA-mediated posttranscriptional regulation could be important to the *APOBEC3A/3B* overexpression. Nevertheless, no association between APOBEC3A/3B deletion polymorphism and the cancer development was observed, independently of the cancer type (they evaluated breast, cervical, and oral cancer samples), contrasting with previous studies that found associations of this polymorphism with cancer development (134, 135). Interestingly, the expression of an *APOBEC3B* miRNA that could regulate *APOBEC3A/3B* fusion transcript (miR-34b-3p) was downregulated in cervical tumor samples, suggesting that this miRNA may lead to a loss of miRNA repression and a consequently increased expression level of the APOBEC3A/3B protein.

In a Chinese population, Wu and Zhang (136) studied the association of miR-124 C/G (rs531564) with susceptibility to HPV infection and cervical cancer. The authors found that the miR-124 rs531564 G allele and the CG genotype were associated to a reduced risk of HPV infection, compared to the C allele and the CC genotype. Additionally, the miR-124 rs531564 G allele was described as associated to a reduced susceptibility to cervical cancer, corroborating other studies (137, 138). The authors hypothesize that the miR-124 rs531564 G allele promotes the expression of a mature form of this miRNA, leading to a lower risk of HPV infection and a subsequent reduced risk to cervical cancer.

Zhou et al. (139), studying another Chinese population, described the influence of miR-218 on HPV-related cervical cancer. Two polymorphisms were investigated: one at the primary-miR-218 (pri-miR-218) A/G SNP (rs11134527), and the second located at the 3′UTR of *LAMB3* (laminin-5 β3) gene (rs2566, C/T), a known target of miR-218, which is suppressed by the HPV-16 E6 protein. In fact, the expression of LAMB3 is augmented in the presence of the HPV-16 E6 oncoprotein and this effect is regulated through miR-218 (140). Laminin-5 plays an important role on the development of cervical lesions, and has been indicated as a marker of invasiveness (141). The authors evidenced an association of the pri-miR-218 rs11134527 variant homozygote GG genotype with a decreased susceptibility of cervical cancer development, as compared to the AA genotype. Regarding the LAMB3 rs2566 polymorphism, Zhou et al. (139) showed that the presence of the T allele, in a dominant model, was significantly associated to a higher risk of cervical cancer. Moreover, when these susceptibility variants were present together, the risk of cervical cancer was significantly higher, in a dose-dependent manner (139).

Several authors have studied the effect of miRNA-related polymorphisms on the development of oral squamous cell carcinoma (OSCC) and its variations [oropharynx (SCCOP) and oral cavity (SCCOC)] (142–147). These cancer types respond by the majority of head and neck malignant tumors worldwide and are highly associated to HPV infection. Song et al. (143) described the effect of four miRNA SNPs [miR-146 G/C (rs2910164), miR-149 C/T (rs2292832), miR-196 C/T (rs11614913), and miR-499 C/T (rs3746444)] in HPV-16 seropositivity and OSCC in a population from the United States. No statistically significant associations of these polymorphisms were observed. In fact, also an absence of association was observed between miR-146 rs2910164 and miR-196 rs11614913 with OSCC overall survival rates in a European cohort (144). However, Song et al. (143) described that, according to HPV-16 seropositivity, miRNA SNPs profiles could play a role in OSCC. Compared with individuals both miR-146 rs2910164 GG genotype and HPV-16 negative, those both GG genotype and HPV-16 positive presented an augmented risk of OSCC, and the susceptibility was even higher when the C allele was present. Similar results were obtained for the associations between miR-149 rs2292832 (CC genotype), miR-196 rs11614913 (C allele presence), and miR-499 rs3746444 (C allele presence) SNPs and risk of HPV-16-associated OSCC (143). Specifically to SCCOP, Guan et al. (142) described in the same population that compared to miR-146 rs2910164 CG/CC and miR-196 rs11614913 CC genotypes, individuals carrying both miR-146 rs2910164 GG and miR-196 rs11614913 CT/TT genotypes were significantly associated to a better overall, disease-specific, and disease-free survival in HPV-positive tumors (142). In line with these data, Song et al. (143) found that individuals with the combined miR-146 rs2910164 CG and CC genotypes had a higher risk of SCCOP than individuals with the GG genotype, and individuals with the miR-499 rs3746444 combined CT and CC genotypes had a higher risk of SCCOP than individuals with the TT genotype (143).

The same research group also studied the effect of polymorphisms located in putative miRNA-binding sites in the 3′UTR of genes related to DNA repair pathways in SCCOP recurrence in HPV-16-positive tumors (147). The authors found that only BRCA1 C/T (rs12516) and RAD51 A/G (rs7180135) SNPs were associated with SCCOP incidence. Patients with the variant genotypes of BRCA1 rs12516 (CT/TT) and RAD51 rs7180135 (AG/GG) SNPs presented a significantly lower susceptibility of disease recurrence as compared to patients with the corresponding common homozygous genotypes. Moreover, BRCA1 rs12516 CC genotype had a significantly higher BRCA1 protein expression, compared to CT/TT variant genotypes and RAD51 rs7180135 AA genotype had a borderline significant association to a higher expression of RAD51 protein, compared to RAD51 rs7180135 AG/GG variant genotypes. BRCA1 rs12516 SNP has been described as a potential binding site of several miRNAs, two of them (miR-118 and miR-639) have already been associated to cancer risk, while RAD51 rs7180135 SNP was described as a potential binding site of miR-197. Other miRNAs were

also described to target *RAD51* gene (miRNA-182, miR-155, miR-103, and miR-107), showing the importance of further studies to determine how this gene expression could be regulated.

Yuan et al. (146) and Zhang et al. (145) described in the same population that ins/del polymorphisms in 3′UTR of *E2F1* and *IL-1*α genes, respectively, are associated to OSCC HPV related (145, 146). Both genes have been described as important on the control of cell death and proliferation and variations on 3′UTR are supposed to impact on miRNA targeting. The authors showed that the E2F1 rs3213180 ins/del and ins/ins and the IL-1α 3′UTR (rs3783553) del/del genotypes, jointly to HPV seropositivity, are associated to a higher susceptibility to HPV-related OSCC. The E2F1 rs3213180 is the only miRNA-binding site described at E2F1 3′UTR that may affect E2F1 expression levels (148), while IL-1α rs3783553 interfere on miR-122-binding site, regulating IL-1α expression levels (149). In fact, Zhang et al. (145) described a significantly increased expression of IL-1α in patients with del/del genotype as compared to ins/ins and ins/del genotypes (145).

Another crucial regulatory gene is the *Cyclin-dependent kinase 6* (*CDK6*), which is associated with cell cycle and tumorigenesis. Various miRNAs are reported to be involved in CDK6-mediated tumorigenesis, such as miR-145, miR-320, and miR-29. In a Chinese population, Ye et al. (150) studied for the first time the effect of five genetic variations in the 3′UTR of *CDK6* gene (rs8179 G/A, rs4272 A/G, rs42033 A/T, rs42035 T/C, and rs42377 G/A) on susceptibility to precancerous cervical lesions. The authors found that the rs8179 A and rs42033 T alleles were associated to a lower risk to develop precancerous cervical lesions and had an antagonistic interaction with the HPV infection. This lower susceptibility to cervical lesions was also observed to rs8179 GA, compared to AA genotype and rs42033 AT, compared to AA genotype, after adjustments for HPV infection and others clinical and demographic characteristics. Strong linkage disequilibrium values were observed between rs8179, rs4272, rs42033 and rs42377 and the haplotype AGTA was significantly associated to a reduced risk to precancerous cervical lesions when compared to GAAG haplotype (150).

To our knowledge, only one study described polymorphisms in HPV related to miRNA-binding sites. Mandal et al. (151) showed that polymorphisms located at a short non-coding region (NCR2), commonly present between HPV E5 and L2 open reading frames, could lead to a loss of human miRNA sites. Through *in silico* analysis, the authors identified binding sites at the NCR2

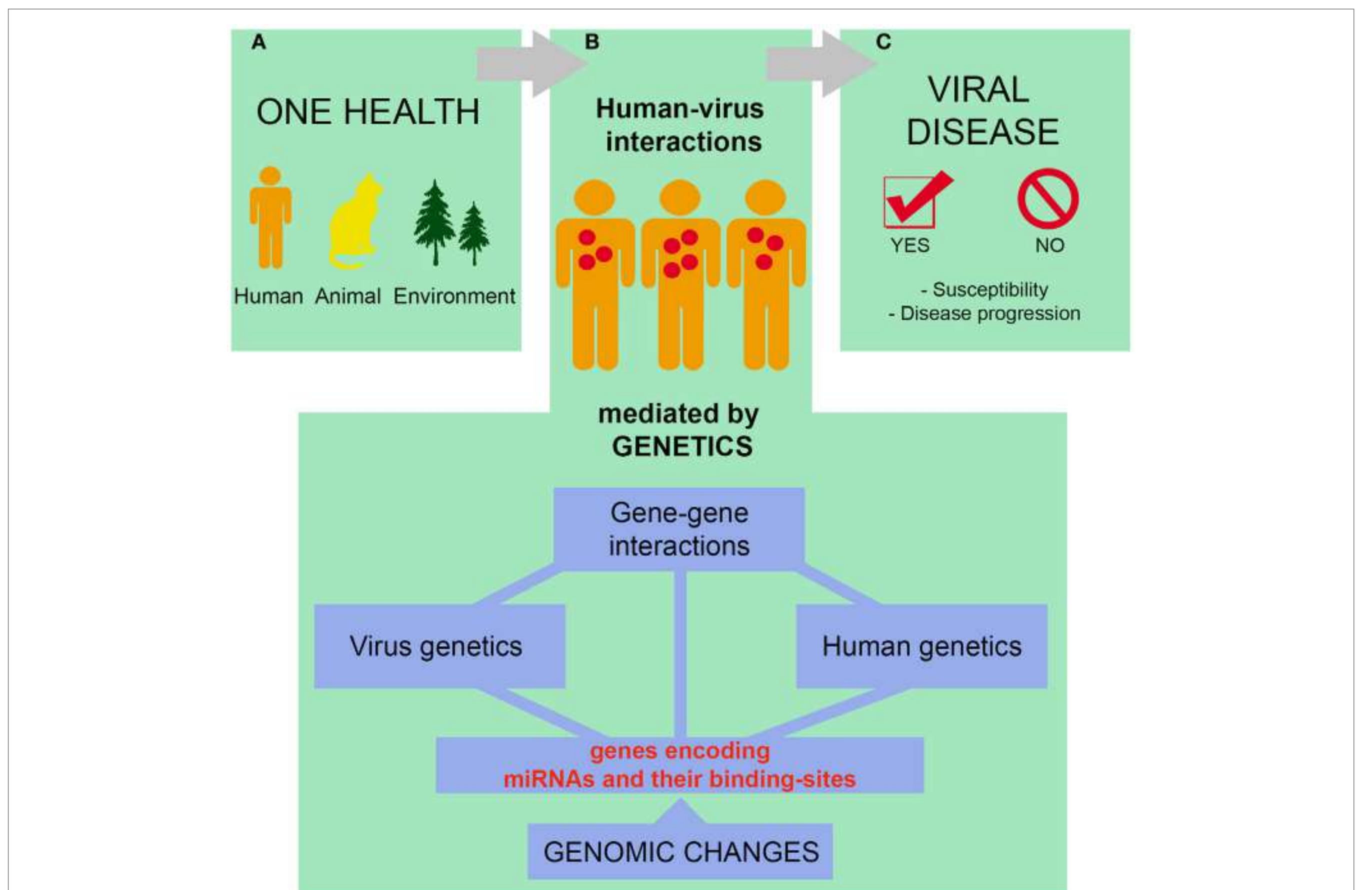

FIGURE 3 | Susceptibility to infections and progression of viral diseases are complex processes that must be thought within the context of One Health. Human, non-human, and environmental factors define whether a given individual will come into contact with a particular virus and the consequences of such interaction **(A)**. Of note, human–virus interactions are mediated by both host and viral genetic factors, including microRNAs (miRNA) and miRNA-related polymorphisms **(B)**. The complex interactions mentioned in **(A,B)** influence the susceptibility to infections as well as the progression of viral diseases **(C)**. See text for references.

region in HPV-16 corresponding to 14 human miRNAs (miR-3148, miR-3174, miR-3613-3p, miR-3916, miR-495, miR-548a-5p, miR-548b-5p, miR-548c-5p, miR-548d-5p, miR-548h-5p, miR-548i-5p, miR-548j-5p, miR-548w-5p, and miR-548y-5p). Moreover, the authors revealed the occurrence of an SNP (T4228C) in the NCR2 of a variant isolate, which could lead to loss of 9 miRNA-binding sites in the corresponding transcripts (151).

EMERGING TOPICS

Currently, the prevention and control of infectious diseases should be approached as a global strategy, in which human, animal, and environmental factors are integrated (152–154), allied to the evaluation of the genomic characteristics of the pathogens (155, 156). Among the human factors that should be taken into consideration, the investigation of genetic features that alter the susceptibility to infection and progression of viral diseases is essential for the prevention and clinical management of infectious diseases in specific human populations (157–159) (**Figure 3**).

SNPs are the most common form of genetic variation in the human genome (160). For many years human SNPs have been highly studied worldwide. However, it is necessary to focus those studies also on viral genetic variants and try to understand how these variants affect the action of miRNAs, for example. For instance, some evidence indicates that SNPs have a relevant impact on the biogenesis and action of Kaposi's sarcoma-associated herpesvirus (KSHV) miRNAs (161). KSHV infection is highly linked to Kaposi's sarcoma development (162). According to a set of *in vitro* analyzes based on clinical observations, Han et al. (161) have shown that different SNPs in KSHV miRNAs alter the expression level of these miRNAs, as well as modify their processing and silencing activities. These changes may alter KSHV pathogenesis, potentially impacting Kaposi's sarcoma development (161). Of note, a number of polymorphisms (SNPs, deletions, and insertions) in KSHV miRNAs have already been observed in pri-miRNAs, pre-miRNAs, and mature miRNAs (163–165). Looking at KSHV-related diseases, these polymorphisms affect miRNAs maturation and some of them may also affect the risk of Kaposi's sarcoma development in patients with AIDS (164), although as a whole the effects of these polymorphisms on KSHV pathogenesis are poorly understood. Furthermore, it has been shown that polymorphisms in KSHV miRNAs influence the risk for the development and the pathogenesis of multicentric Castleman disease and KSHV-associated inflammatory cytokine syndrome, diseases also linked to KSHV infection (165).

The number of KSHV miRNAs described in the literature is increasing (166–168). Similarly, the effects of them on immune response, KSHV pathogenesis and Kaposi's sarcoma development have already been described (166–172). The implications of KSHV miRNA SNPs on the miRNA processing are also being characterized (164, 173). However, there is much to be explored about the SNPs located in KSHV miRNAs. Once these SNPs are well characterized, we will better understand their effects on Kaposi's sarcoma development and other KSHV-related diseases.

Another interesting example of viral miRNA variant involves the human T cell leukemia virus-type 1 (HTLV-1); Host miR-28-3p is an inhibitor of HTLV-1 replication and infection (31). The Thr-to-Cys (AAT-to-AAC) polymorphism in ATK-1 HTLV strain (subtype 1A) disrupts the miR-28-3p target site. This disruption affects the anti-HTLV action of miRNA-28-3p. However,

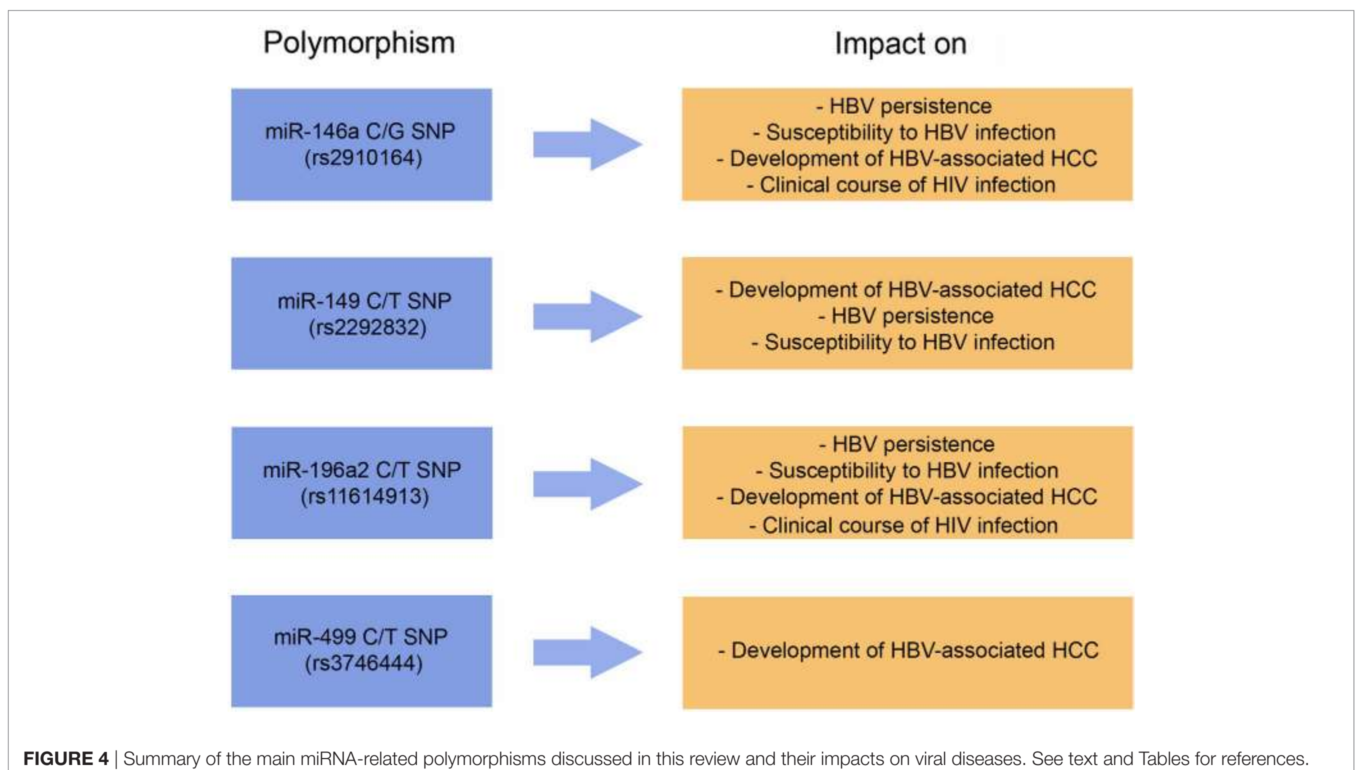

FIGURE 4 | Summary of the main miRNA-related polymorphisms discussed in this review and their impacts on viral diseases. See text and Tables for references.

miR-28-3p target site is highly conserved in the HTLV-1 subtypes B and C, and therefore, this miRNA has a therapeutic potential in strategies to control HTLV-1 infection (31).

The role of miRNA SNPs in viral infections other than those previously approached in the present review has been poorly explored, although some studies can be cited. For example, Misra et al. (174) evaluated the influence of the following host miRNAs SNPs on human cytomegalovirus (HCMV) infection: miR-146a C/G (rs2910164), miR-196a2 C/T (rs11614913), miR-499a C/T (rs3746444), and miR-149 C/T (rs2292832). In brief, with exception of miR-149 rs2292832, mutant genotypes of the other three SNPs were linked to increased risk of symptomatic HCMV infection. Multifactor Dimensionality Reduction analysis (applied to access SNP–SNP interactions) indicated an association between increased risk of symptomatic HCMV infection with the four interaction models tested (174). This result indicates that miRNA SNPs play a relevant role in the pathogenesis of HCMV. However, to the best of our knowledge, no other study focusing on the role of miRNA SNPs in HCMV infection was performed, making this a blank spot to further studies.

Finally, we should highlight the triad (I) exosomes, (II) miRNAs, and (III) viral infections. Exosomes are extracellular nanovesicles originated from multivesicular bodies. These vesicles have drawn attention from the scientific community due to their ability to transport protein, lipid, and genetic components between different cells in a highly regulated manner (175). Moreover, a large body of evidence showed that host and viral miRNAs are one of the major types of components transported by exosomes (176, 177). Exosomes have a relevant immunomodulatory action (176, 178) and have been shown to strongly interact with different viruses, such as HIV (179, 180), Ebola (181), HBV (182), and others (183). Taking into account the therapeutic potentials of the exosomes-mediated miRNA delivery pathway supported by recent findings (184, 185), in the near future, it will be possible to modulate the exosomes-mediated miRNA trafficking aiming to mitigate viral infections. In addition, a therapeutic control of exosomes-mediated miRNA delivery could be used to induce, or avoid, similar effects to those triggered by miRNA SNPs. However, in order to these therapeutics become a reality, one must to disclosure the influences of the miRNAs transported

TABLE 2 | Main microRNAs (miRNA)-related polymorphisms showing statistically significant influence on Epstein–Barr virus (EBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), and Human Papillomavirus (HPV) infections.

Virus	miRNA or miRNA-binding site[a]	Polymorphism[b]	Influence on	Population	Reference
EBV	miR-608	C/G rs4919510	EBV-related nasopharyngeal carcinoma (NPC); NPC risk	Chinese	Qiu et al. (32)
HCV	miR-208b- and miR-499a-5p-binding sites	A/C rs4803217	HCV clearance	*In vitro* experiment	McFarland et al. (93)
	miR-let-7- and miR-98-binding sites	A/G rs868	HCV loads; hepatic inflammation	Polish	Sajjad et al. (98)
HIV	miR-148-binding site	ins/del rs67384697	HIV loads	European descendant	Kulkarni et al. (101)
			HIV loads; CD4 T^+ cell counts	Chinese	Blais et al. (102)
	miR-148-binding site	C/T rs9264942	HIV loads; CD4 T^+ cell counts	Chinese	Blais et al. (102)
	miR-148a	A/G rs735316	Progression of HIV infection	European descendant	Kulkarni et al. (103)
	miRNA biogenesis	C/T rs197412	AIDS-associated non-Hodgkin lymphoma risk	American	Peckham-Gregory et al. (104)
	miR-196a2	C/T rs11614913	Central nervous system (CNS) AIDS-associated non-Hodgkin lymphoma risk	American	Peckham-Gregory et al. (104)
	miR-196a2-binding site	C/T rs2057482	CNS and systemic AIDS-associated non-Hodgkin lymphoma risk	American	Peckham-Gregory et al. (104)
	miR-27a	A/C/G/T rs895819	HIV/AIDS-associated nutritional status	African descendant	Maharaj et al. (112)
	miR-146a	C/G rs2910164	HIV-related diseases (particularly preeclampsia)	African descendant	Maharaj et al. (115)
HPV	miR-146a	C/G rs2910164	HPV-related cancer	Chinese	Guan et al. (142); Song et al. (143)
	miR-149	C/T rs2292832	HPV-related cancer	Chinese	Song et al. (143)
	miR-196a	C/T rs11614913	HPV-related cancer	Chinese	Guan et al. (142); Song et al. (143)
	miR-499	C/T rs3746444	HPV-related cancer	Chinese	Song et al. (143)
	miRNA-binding sites	C/T rs12516 and A/G rs7180135	HPV-related cancer	Chinese	Zhu et al. (147)
	miRNA-binding sites	ins/del rs3213180	HPV-related cancer	Chinese	Yuan et al. (146)
	miR-122-binding site	ins/del rs3783553	HPV-related cancer	Chinese	Zhang et al. (145)
	miR-218	A/G rs11134527	HPV-related cancer	Chinese	Zhou et al. (139)
	miR-218-binding site	C/T rs2566	HPV-related cancer	Chinese	Zhou et al. (139)
	miRNA-binding sites	A/G rs8179 and A/T rs42033	HPV-related cancer	Chinese	Ye et al. (150)
	miR-124	C/G rs531564	HPV infection and cervical cancer	Chinese	Wu and Zhang (136)

[a] *Seed or regulatory region.*
[b] *Polymorphism quotations were standardized according to the Single Nucleotide Polymorphism Database (dbSNP) of NCBI (https://www.ncbi.nlm.nih.gov/snp/), based on the reference SNP cluster (rs#) of each polymorphism.*

by the exosomes in viral diseases, and to decipher how miRNA SNPs modulate the pathogenesis of different viruses.

CONCLUSION AND PERSPECTIVES

Based on the articles discussed in this review, we present in **Figure 4** the miRNA SNPs that were studied with greater robustness in the context of the viral infections, as well as the influences of these selected SNPs on viral diseases. In summary, development of HBV-associated HCC is influenced by the following polymorphisms: miR-146a G/C SNP (rs2910164), miR-149 C/T SNP (rs2292832), miR-196a2 C/T SNP (rs11614913), and miR-499 C/T SNP (rs3746444). In addition, in **Table 2** the main findings of the studies addressing HCV, HIV, EBV, and HPV infections are presented. The study of genetic variants located in miRNA genes or in genes of miRNA-binding sites is incipient. As research continues, new SNPs will be described and new influences of miRNA SNPs on viral diseases will be reported. It is also possible that future investigations will redirect the discussions about the biological or clinical significance of the variants presented in this review. On the other hand, part of these results can be strengthened with the completion of new studies. Of note, the majority of the studies mentioned in this review are punctual and specific of particular populations. Studies investigating miRNA SNPs in Asian populations highly outnumber the studies performed with non-Asian populations. Thus, it is essential to investigate the frequencies of miRNA SNPs in worldwide populations in order to gather better data about susceptibility and progression of viral diseases in different ethnic/genetic backgrounds. The identification of SNPs that influence characteristics of susceptibility or clinical outcome in different populations will be essential for the understanding of the biological significance of such genetic factors. Thus, new meta-analyses will be essential to establish with robustness the effects of those SNPs on infectious diseases.

We also draw attention to the fact that most of the studies within the scope of this review investigated human miRNA SNPs. It is necessary to explore the importance of viral miRNAs and their variants on the clinical course of infectious diseases. Also of great importance, the prevalence of viruses of different genotypes is variable around the world, which may or may not complicate what this review is dealing with. Thus, the evaluation of viral genetic characteristics is significant in population-based studies focused on miRNA SNPs. In this sense, when virus genotype is available, this information must be considered during the interpretation of the studies here mentioned.

Finally, directing further investigations to the SNPs discussed here may provide important insights for the development of new therapies against infectious diseases based on inhibitors or stimulators of the action of miRNAs. As discussed earlier, knowing how SNPs alter biogenesis, processing or the action of miRNAs may also be useful for the development of antiviral therapies or for the treatment of complications caused by viral infections. Technologies focused on the delivery of miRNAs in an accurate manner, as engineered exosomes, will also contribute to the success of these therapies. We believe that we are close to experiencing a boom of the miRNA-based therapies.

AUTHOR CONTRIBUTIONS

JE, FZ, and RG reviewed the studies and wrote the manuscript. JC wrote and reviewed the manuscript.

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Toll-Like Receptor-1 Single-Nucleotide Polymorphism 1805T/G Is Associated With Predisposition to Multibacillary Tuberculosis

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Tuberculosis (TB), caused by mycobacterial species of the *Mycobacterium tuberculosis* complex, is a serious global health issue. Brazil is among the 22 countries with the highest number of TB cases, and the state of Amazonas has the highest incidence of TB cases in the country. Toll-like receptors (TLRs) are important pattern recognition receptors of the innate immunity and play a key role in orchestrating an effective immune response. We investigated whether the single-nucleotide polymorphisms (SNPs) 1805T/G *TLR1*, 2258G/A *TLR2*, 896A/G and 1196C/T of *TLR4*, 745T/C *TLR6*, and −1237A/G and −1486A/G of *TLR9* are associated with the predisposition to TB and/or bacillary load. The SNPs genotyping was performed by nucleotide sequencing in 263 TB patients and 232 healthy controls residing in the state of Amazonas. Alleles and genotypes frequencies were similar between patients and healthy individuals for most of the investigated SNPs. Stratification of the TB patients according to their bacillary load showed that the genotype 1805TT *TLR1* (rs5743618) was prevalent among paucibacillary patients [odds ratio (OR) = 0.38; 95% confidence interval (CI) = 0.19–0.76; $p = 0.009$] while the genotype 1805TG was common among multibacillary patients (OR = 3.72; CI = 1.65–8.4; $p = 0.004$). Comparison of demographic characteristics of patients to controls showed that TB is strongly associated with smoking (OR = 6.55; 95% CI = 3.2–13.6; $p < 0.0001$); alcohol use disorder (OR = 7.14; 95% CI = 3.7–13.9; $p < 0.0001$); and male gender (OR = 3.66; 95% CI = 2.52–5.3; $p < 0.0001$). Multivariate logistic regression demonstrated that alcoholism (OR = 2.93; 95% CI = 1.05–8.16; $p = 0.03$) and the 1805G allele (OR = 2.75; 95% CI = 1.33–5.7; $p = 0.006$) are predictive variables for multibacillary TB. Altogether, we suggest that the *TLR1* 1805G allele may be a relevant immunogenetic factor for the epidemiology of TB together with environmental, sociodemographic, and behavioral factors.

Keywords: tuberculosis, immunogenetics, single-nucleotide polymorphism, toll-like receptors, Brazil

INTRODUCTION

Tuberculosis (TB) is a contagious disease caused by mycobacteria of the *Mycobacterium tuberculosis* complex. The most common clinical form is pulmonary TB. TB causes a serious public health burden worldwide and its control remains a major challenge for the scientific community and TB control programs globally. Nearly 10 million people worldwide suffered from TB each year (1). Brazil is one of the 30 countries considered a priority for TB control as more than 69 thousand new cases occur annually. The state of Amazonas has the highest incidence of TB in the country with 67.2/100 thousand inhabitants (1, 2).

Multiple socioeconomic, behavioral, medical, and genetic factors affect TB infection (3, 4). Many single-nucleotide polymorphisms (SNPs) in immune response genes are associated with TB (5–7). The most reported are *HLA-DRB1*, *VDR*, *TIRAP*, and *NRAMP1* (6, 8). Other genes such as toll-like receptors (TLRs), *TLR1*, 2, 4, 6, and 9 that are involved in the initial recognition of pathogen-associated molecular patterns to trigger the activation of the effector mechanisms of innate immunity and subsequent targeting of the specific adaptive immune response to *Mycobacterium tuberculosis* have also been associated with susceptibility or resistance to TB (6, 9, 10).

In this study, we investigated whether the SNPs rs5743618 (1805T/G) of *TLR1*, rs5743708 (2258G/A) of *TLR2*, rs4986790 (896A/G) and rs4986791 (1196C/T) of *TLR4*, rs5743810 (745C/T) of *TLR6*, and the rs5743836 (−1237A/G) and rs187084 (−1486A/G) of *TLR9* are associated with TB and with the bacterial load. We identified that alcoholism and the *TLR1* 1805G allele may be predictive variables for multibacillary TB.

MATERIALS AND METHODS

Study Population

This is a case-control study investigating the association of demographic and immunogenetics factors with susceptibility or resistance to TB. Patients with TB and healthy controls, aged 18–65 years, were recruited in Manaus, the capital city of Amazonas state, Brazil. The patients with TB were recruited at the Policlínica de Referência em Pneumologia Sanitária Cardoso Fontes, a referral center of the state for diagnosis and treatment of TB. Patients with a diagnosis of pulmonary and pleural TB were selected according to guidelines of the Brazilian Ministry of Health. Data collected included social-demographic characteristics and the presence of the following clinical symptoms: cough, expectoration, chest pain, dyspnea, hemoptysis, malaise, fever, chills, night sweats, loss of appetite, weight loss, and fatigue. The controls consisted of health professionals and healthy contacts without consanguinity to the patients with TB and without any previous history of the disease. TB patients with associated comorbidities or lack of information on the patient's chart or subsequent diagnosis of non-tuberculous mycobacteria were excluded. TB patients were stratified into paucibacillary (including pleural TB) and multibacillary. The bacillary load was determined by bacilloscopy, as recommended by Brazilian Ministry of Health (11). This study protocol was approved by the Institutional Review Board of the National Institute of Amazonian Research (INPA) under the number CAAE: 05925212.8.0000.0006.

Identification of *M. tuberculosis*

Sputum samples were submitted to microscopy (12) and solid culture in according to PKO method (initials in tribute to Petroff, Kudoh, and Ogawa) (13). A smear of culture, stained by Kinyoun method, was carried out to confirm if the culture was composed of acid-fast bacilli. Later, the colony aspect (not pigmented and rough morphology) was evaluated. The DNA extraction from pure culture was made as described elsewhere (14). So, the molecular identification of *M. tuberculosis* complex was performed by PCR, amplification of 123 bp fragment from IS*6110* insertion sequence, as described (15). If, the PCR for IS*6110* was negative, the differential diagnosis of mycobacteria was performed as established (12, 16). This study also included samples (32/263) with diagnosis confirmed for TB by the molecular test, GeneXpert MTB/RIF [*M. tuberculosis*/rifampin (RIF) resistance] assay (Cepheid, Sunnyvale, CA, USA).

Genotyping of the Different TLR Polymorphisms

DNA from whole blood samples was extracted using a QIAamp DNA Blood kit (QIAGEN) in the automated QIAcube platform following the manufacturer's instructions. The primers were designed to flank the different polymorphisms studied using the Primer-3 version 2.3.7 embedded in Geneious software version 6.1.7 (17) and Primer-BLAST (Table S1 in Supplementary Material). PCR conditions for the five evaluated genes were optimized separately to use only one cycling amplification program of an initial cycle of 94°C for 2 min followed by 33 cycles of 94°C for 2 min, 63°C for 30 s and 72°C for 60 s, and a final cycle of 72°C for 17 min. The PCR mix in a final volume of 25 µL contains 1× Buffer; 1.5 mM $MgCl_2$; 0.2 mM dNTPs; 0.4 µM of sense and antisense primers; enzyme GoTaq Hot Start 1 U/µL; and approximate 100 ng of DNA. PCR products were precipitated with polyethylene glycol (PEG) (PEG 8000 at 20% P/V, 2.5 M NaCl) based on the protocol originally described by Lis and Schleif (18), with slight modifications subsequently by Lis (19) and Paithankar and Prasad (20). The purified amplicons were adjusted to the concentration range of 5–20 ng/µL, proportional to their size. The allelic discrimination was performed by direct capillary sequencing. Sequencing reaction was performed with the BigDye® Terminator Kit (3.1) (Life technologies) using the protocol suggested by Platt et al. (21). Either sense or antisense primers used in PCR of each gene was used. The sequencing product was purified with Ethanol/EDTA/Sodium Acetate, according to the recommendations of Life Technologies and submitted to capillary electrophoresis in the ABI 3130 Genetic Analyzer (Applied Biosystems) and the resulting electropherograms were edited and analyzed using the Sequencing Analysis Program (Life Technologies, version 5.3.1) and Geneious.

Statistical Analysis

Parametric Student's *t*-test was used for means age comparison. Two-tailed Fisher's exact test was applied for association of TB with BCG vaccine, sex, smoking, and alcoholism. The frequency of the alleles and genotypes between groups was compared with two-tailed Fisher's exact test. Variables with a *p* value were considered significant. Genetic models also were applied to

elucidate the association of the SNPs with susceptibility to TB and bacillary load. Akaike information criterion (AIC) was considered in the evaluation of applied genetic models. The free software R, version 3.1.2 and the embedded function to study SNP association—SNPassoc[1] were used. The multivariate logistic regression analysis with stepwise approach was applied in the choice of variables predictive of multibacillary TB. The analyzed variables were age, sex, BCG, alcoholism, and the presence of the 1805G allele. Variables with a p value <0.05 were considered and the variables p >0.1 were excluded from the regression analysis. For each regression model proposed, the discrimination capacity of each model was analyzed as described previously by Boechat et al. (22, 23). The model with the largest area under the curve (AUC) was considered. Statistical analyses were performed using MedCalc Statistical Software version for Windows, version 15.2 (MedCalc Software, Ostend, Belgium[2]).

RESULTS

Baseline Characteristics of All the Participants of the Study

This study included 263 patients with TB and 232 healthy controls. The baseline characteristics of the study population are shown in **Table 1**. The age distribution between TB patients and controls was similar. TB patients had a mean of 33.4 ± 11.9 years of age and were predominantly male (65.7%). By contrast, the control group was predominantly female (65.9%). BCG vaccine adherence was higher among the controls, 92.0% compared to 78.7% among the TB patients. Smoking and alcoholism were significantly associated with a greater predisposition to TB. The clinical characteristics of the patients with TB are shown in **Table 2**. Pulmonary TB (88%) was the most prevalent and the majority of the patients reported more than three clinical symptoms.

Comparison of the Genotypes and Alleles Frequencies of the Polymorphisms of the Different TLRs Between TB Patients and Healthy Controls

The genotypes and alleles frequencies of the different SNPs studied are shown in **Table 3**. Differences in the number of samples for each SNP are due to the rigorous adoption of only high-quality sequences analyzed. The Hardy–Weinberg equilibrium (HWE) was observed for the different SNPs in both cases and healthy controls except for both SNPs of *TLR4*. Further analysis of the SNPs of *TLR4* was abandoned due the deviations from the HWE. The frequencies of the genotypes and alleles of the other SNPs were similar between patients and controls except for the SNPs of *TLR9*. Notably, *TLR2* SNP 2258G/A was not observed in our population of study. *TLR9* −1237A/G and −1486A/G SNPs showed minor allele frequency (MAF) for patient populations and controls of 15.3 and 17.4% for the first SNP and 38.5 and 33.3% for the second, respectively. The genotype −1237AG was associated with TB protection [odds ratio (OR): 0.62; confidence interval (CI): 0.39–0.97; $p = 0.04$].

Association of *TLR1* SNP 1805T/G and Multibacillary TB

Patients with TB were stratified into paucibacillary and multibacillary TB. Comparison of genotypes and allele frequencies of all the SNPs between multibacillary and paucibacillary TB revealed that the genotype 1805TT *TLR1* (rs5743618) was prevalent among paucibacillary patients (OR = 0.38; 95% CI = 0.19–0.76; $p = 0.009$) while the genotype 1805TG was common among multibacillary patients (OR = 3.72; CI = 1.65–8.4; $p = 0.004$). The 1805G allele (OR = 1.71; CI = 0.97–3.01; $p = 0.05$) showed statistical trend for association. Further analysis applying four genetic models as shown in **Table 4** showed that the codominant model with the genotype (1805TG) was associated with the multibacillary TB form (OR = 3.69; CI = 1.62–8.42; $p = 0.002$). The same genotype was associated with the multibacillary form in the overdominant model (OR = 3.73; CI = 1.65–8.41; $p = 0.0004$). In the dominant model, the genotypes (1805TG + 1805GG) also showed association with the multibacillary TB (OR = 2.63;

[1] https://cran.r-project.org/web/packages/SNPassoc/index.html (Accessed: March 09, 2016).

[2] https://www.medcalc.org/ (Accessed: January 18, 2016).

TABLE 1 | Baseline characteristics of patients with TB and the healthy controls.

General characteristics	TB patients (n = 263)	Controls (n = 232)	OR	95% CI	p-Value
Age	33.4 ± 11.9	33.5 ± 11.9	–	–	0.92
Male (%)	172 (65.4)	79 (34.1)	3.66	2.52–5.3	<0.0001
Vaccine BCG (%)	207 (78.7)	213 (92)	0.26	0.13–0.5	<0.0001
Smoking (%)	55 (20.9)	9 (3.9)	6.55	3.2–13.6	<0.0001
Alcohol use disorder (%)	69 (26.2)	11 (4.7)	7.14	3.7–13.9	<0.0001

TB, tuberculosis.

TABLE 2 | Clinical characteristics of the patients with TB.

Characteristics	Patients (n = 263) n (%)
Clinical form of TB	
Pulmonary	231 (88.0)
Pleural	32 (12.0)
Family history of TB	136 (51.7)
Bacillary load[a]	
Multibacillary	176 (76.2)
Paucibacillary	55 (23.8)
Clinical symptoms	
Coughing with 1 symptom	19 (7.2)
Coughing with 2 symptoms	23 (8.8)
Coughing with 3 symptoms	35 (13.3)
Coughing with 4 or more symptoms	172 (65.4)
No information	14 (5.3)

All patients with pleural TB were grouped as paucibacillary.
[a]Bacillary loads for 32 patients were not available but were confirmed for Mycobacterium tuberculosis by Xpert MTB/RIF.
TB, tuberculosis.
Clinical symptoms in addition to the cough: expectoration, chest pain, dyspnea, hemoptysis, malaise, fever, chills, night sweats, loss of appetite, weight loss, and fatigue.

TABLE 3 | Genotypes and allele frequencies of the TLRs polymorphisms in healthy controls and patients with TB.

	Controls *n* (%)	Patients with TB *n* (%)	OR	CI 95%	*p*-Value
Genotypes					
***TLR1* 1805T/G (rs5743618)**					
T/T	116 (55.3)	146 (58.0)	1.11	0.7–1.61	
T/G	74 (35.2)	86 (34.1)	0.64	0.64–1.37	0.77
G/G	20 (9.5)	20 (7.9)	0.81	0.42–1.56	
Alleles					
T	306 (73.0)	378 (75.0)	1.11	0.83–1.5	0.48
G	114 (27.0)	126 (25.0)	0.89	0.66–1.37	
***TLR2* 2258G/A (rs5743708)**					
G/G	168 (100)	196 (100)	–	–	
G/A	0	0	–	–	–
A/A	0	0	–	–	
Alleles					
G	336(100)	392 (100)	–	–	–
A	0	0	–	–	
***TLR4* 896A/G (rs4986790)**					
A/A	199 (95.7)	221 (92.9)	0.5	(0.2–1.3)	0.41
A/G	8 (3.8)	16 (6.7)	1.8	(0.7–4.3)	
G/G	1(0.5)	1 (0.4)	–	–	
Alleles					
A	406 (97.6)	458 (96.2)	0.62	(0.29–1.37)	0.20
G	10 (2.4)	18 (3.8)	1.6	(0.73–3.5)	
***TLR4* 1196C/T (rs4986791)**					
C/C	207 (99.5)	226 (95)	0.75	(0.3–1.88)	0.17
C/T	1 (0.5)	11 (4.6)	1.39	(0.52–3.65)	
T/T	0	1 (0.4)			
Alleles					
C	415 (99.8)	463 (97.3)	0.79	(0.33–1.86)	0.003
T	1 (0.2)	13 (2.7)	1.27	(0.54–3.0)	
***TLR6* 745T/C (rs5743810)**					
T/T	120 (69)	176 (73)	1.2	(0.7–1.8)	
T/C	50 (29)	58 (24)	0.7	(0.5–1.2)	0.47
C/C	4 (2)	8 (3)	1.45	(0.43–4.9)	
Alleles					
T	290 (83)	410 (84.7)	1.1	(0.76–1.61)	0.6
C	58 (17)	74 (15.3)	0.9	(0.9–1.3)	
***TLR9* −1237A/G (rs5743836)**					
A/A	127 (66)	141 (73)	1.38	(0.89–2.14)	
A/G	63 (33)	45 (23)	0.62	(0.39–0.97)	0.04
G/G	2 (1)	7 (4)	3.75	(0.73–17.4)	
Alleles					
A	317 (82.6)	327 (84.7)	1.17	(0.79–1.71)	0.42
G	67 (17.4)	59 (15.3)	0.85	(0.58–1.25)	
***TLR9* −1486A/G (rs187084)**					
A/A	84 (44)	67 (34.9)	0.65	(0.43–0.98)	
A/G	88 (46)	102 (53.1)	1.33	(0.89–2)	0.21
G/G	20 (10)	23 (12)	1.17	(0.61–2.21)	
Alleles					
A	256 (66.7)	236 (61.5)	0.79	(0.59–1.07)	0.13
G	128 (33.3)	148 (38.5)	1.25	(0.93–1.68)	

Total of controls (C) and patients (P) analyzed for each SNP: TLR1 1805T/G 210 (C) 252 (P); TLR2 2258G/A 168 (C) 196 (P); TLR4 896A/G 208 (C) 238 (P); TLR4 1196A/G 208 (C) 238 (P); TLR6 745T/C 174 (C) 242 (P); TLR9 −1234 A/G 192 (C) 193 (P); TLR9 −1486 A/G 192 (C) 192 (P).
CI, confidence interval; OR, odds ratio; TLRs, toll-like receptors; TB, tuberculosis; SNP, single-nucleotide polymorphism.

TABLE 4 | Different genetic models of association of *TLR1* SNP with clinical forms of TB.

Model	PB *n* (%)	MB *n* (%)	OR	95% CI	*p*-Value	AIC
Codominant						
T/T	38 (74.5)	90 (52.6)	1.00	–	0.002	233.2
T/G	8 (15.7)	70 (40.9)	3.69	1.62–8.42		
G/G	5 (9.8)	11 (6.5)	0.93	0.30–2.86		
Dominant						
T/T	38 (74.5)	90 (52.6)	1.00	–	0.004	235.2
T/G—G/G	13 (25.5)	81 (47.4)	2.63	1.31–5.29		
Recessive						
T/T—T/G	46 (90.2)	160 (93.6)	1.00	–	0.429	242.7
G/G	5 (9.8)	11 (6.4)	0.63	0.21–1.91		
Overdominant						
T/T—G/G	43 (84.3)	101 (59.1)	1.00	–	0.0004	231.2
T/G	8 (15.7)	70 (40.9)	3.73	1.65–8.41		
Log-additive						
0, 1, 2	51 (23.0)	171 (77.0)	1.68	0.96–2.92	0.057	239.7

Of note, the genotypes of 30 TB patients out of 252 sequenced for TLR1 were excluded since only results of GeneXpert MTB/RIF without bacilloscopy were available. PB, paucibacilary; MB, multibacilary; CI, confidence interval; OR, odds ratio; AIC, Akaike information criterion; TLRs, toll-like receptors; SNP, single-nucleotide polymorphism; TB, tuberculosis.

TABLE 5 | Multivariate logistic regression for multibacillary TB.

Variables	OR	95% CI	*p*-Value
Age	0.98	0.96–1.01	0.29
Male	0.98	0.5–1.95	0.96
Smoking	1.18	0.42–3.35	0.74
BCG	0.71	0.3–1.67	0.43
Alcoholism	2.93	1.05–8.16	0.03
Citizenship	1.12	0.58–2.2	0.72
1805G	2.75	1.33–5.7	0.006

CI, confidence interval; OR, odds ratio; TB, tuberculosis.

CI = 1.31–5.29; p = 0.004). The overdominant model better explains the association to multibacillary TB according to AICs results.

Alcoholism and the *TLR1* SNP Guide Predisposition Multibacillary TB

Multivariate logistic regression analysis was applied to adjust baseline characteristics, using a logistic regression model for multibacillary TB shown in **Table 5**, including gender, age, smoking status, alcoholism, and citizenship. There was no influence for gender in the analysis (p = 0.96). Logistic regression showed the variables alcoholism (OR = 2.93; CI = 1.05–8.16; p = 0.03) and allele 1805G (OR = 2.75; CI = 1.33–5.7; p = 0.006) were associated with the multibacillary TB (**Table 5**).

Logistic regression with a stepwise approach only showed alcoholism (OR = 3.04; CI = 1.21–7.67; p = 0.01) and allele 1805G (OR = 2.59; CI = 1.28–5.26; p = 0.008) were associated with the multibacillary TB. The two variables were subsequently challenged in two regression models. Model 1 that included only the variable alcoholism, obtained an AUC of 0.58. In the model 2

that included both the variables alcoholism and SNP 1805T/G, the AUC increased to 0.64 suggesting that both alcoholism and SNP 1805T/G act together in the establishment of multibacillary TB.

DISCUSSION

Tuberculosis continues to be a health problem worldwide and with the emergence of HIV there is a resurgence of TB cases among AIDS patients and becomes barriers to the roll back of TB. Enormous headway has been achieved in the understanding of the immunopathology of TB. Understanding the puzzle of the host genetics contribution to the resistance or susceptibility to MTB infection may help in the strategy of designing new vaccines to TB or in the combination of chemotherapy with immunotherapy.

None of the SNPs investigated in this study were associated with a greater predisposition to TB. Only the *TLR9* −1237AG genotype was associated with TB protection. Stratification of the patients into multibacillary and paucibacillary TB did not show any association with the bacillary load. Few studies evaluated the importance of SNPs −1237A/G and −1486A/G of *TLR9* in TB, and results are conflicting. Two cited no association with TB (24, 25) while two reported association with higher risk for TB (26, 27). The predisposition to TB is known to be complex and is determined by many genetic variations in a multiple of genes contributing to smaller inputs in the development of the disease (5, 10).

We observed the 1805TG genotype is associated for the first time to the best of our knowledge with multibacillary TB. Interestingly, it has been shown that *TLR1* with this variation is hyporesponsive to its agonists and impedes its migration to the surface of cells expressing it (28–30). Among the demographic and behavioral factors investigated, only alcoholism contributed to the multibacillary form.

A previous study reported that *TLR1* regulates polypeptide-induced signaling and responds to extracts of *M. tuberculosis*, suggesting that *TLR1* may substantially regulate the immune response against the bacillus and impact on the outcome of TB. Authors also demonstrated that subjects with *TLR1* SNP1805G/G genotype produce low concentrations of IL-6, an important cytokine in the inflammatory response. Heterozygous individuals produce intermediate levels suggesting that the G and T allele are codominant. The association of heterozygous individuals to multibacillary TB in this study may in part be explained by the influence of the G allele that correlates to low level of IL-6 to inefficiently control the multiplication of the bacillus (29). However, the effect of the hypo-responsiveness of this variation on the risk of TB and the bacillary load is not clearly understood due to the complex immune response to TB and the immune escape developed by *M. tuberculosis* (31, 32).

In this study, we observed that males, smokers, and alcoholism were more common among the TB patients. Furthermore, family history of TB was reported in more than 50% of the patients reinforcing the importance of intra-domiciliary TB transmission or the component of genetic factors in the predisposition to TB. Previous studies have also observed similar features and reinforced that smoking, alcohol, and other drug abuse, along with socioeconomic factors, increase the risk for TB development (33).

There is biological evidence to explain the association of smoking with TB. Chronic exposure to cigarette components alters the normal functioning of mucociliary clearance. This facilitates the access of the bacilli to the pulmonary alveoli, giving rise to an infectious focus. Macrophages resident in the alveola of smokers also present reduced phagocytic action and decreased level of pro-inflammatory cytokines. Nicotine, one of many toxic constituents found in cigarettes, acts directly on acetylcholine receptors in macrophages and leads to a lower secretion of TNF-alpha, generating a favorable environment for bacillary survival in the macrophages and contributes to immune evasion (34, 35).

Variations in the frequency of SNPs suggest distinct evolutionary histories among populations in recent human evolution due to selective pressure exerted by diseases, such as TB and Malaria (36). This study observed MAF for SNP 1805T/G of *TLR1* of 25.0 and 27.0% in TB patients and controls, respectively. Previous studies of other infectious diseases in Brazil showed frequencies varying between 11.0 and 40.0% (37–40). Frequency of the MAF in other populations such as Spain, Turkey, and Nepal are 30.0, 43.0, and 6.0%, respectively (28, 41, 42). The 1805G mutant allele is rare in Vietnamese population (28).

TLR2 SNP 2258G/A was not observed in our study. This SNP is also rare in four populations investigated by the HapMap project and in a population from Colombia (43). The *TLR4* 896A/G and 1196C/T SNPs are co-segregated and the frequency of haplotype of the mutant alleles ranges from 0 to 5.0% among different populations worldwide. However, this frequency is 10–20 times higher among African populations and it is suggested that may be a result of selective pressure due to malaria infection (44, 45). Our study observed an MAF of 3.8 and 2.4% for 896A/G, and 2.7 and 0.2% for the 1196C/T SNP among patients and controls, respectively. Other studies in Brazil showed higher MAF varying from 4.6 to 7.0% (37, 38, 46). However, one study of case-controls of malaria from the Amazonas observed similar MAF to ours indicating the frequency is a reflex of the local population (40).

The MAF of *TLR6* 745C/T varies from 0 to 46.0% among 11 populations investigated by the HapMap. In this study, the MAF is 15.3 and 17% in TB patients and controls, respectively. Another study in Brazil reported an MAF of 18.0 and 10.0% in individuals of symptomatic and asymptomatic malaria, respectively (37). The MAF of *TLR9* −1237A/G and −1486A/G SNPs are 15.3 and 17.4% for the first SNP and 38.5 and 33.3% for the second in patients and controls, respectively. Similar high MAF for these SNPs were observed in other studies in the Amazonas (37, 40).

Our study has few limitations. Our sample size is small, and male is prevalent among the TB cases. However, this should not have a great influence on the study as the frequencies of the alleles of males and females are similar among the controls. Moreover, new study with a higher sample size is needed to confirm the results observed in this study.

The predisposition to TB and its bacillary load involves numerous factors that are determined mainly by the human intrinsic factors and the virulence of the mycobacterial strain. Further studies are necessary to evaluate the combined effect of these factors in TB. In addition, more studies are needed to understand the functional effect of this *TLR1* variant to elucidate its importance in the regulation of the immune response and in the immunopathogenesis of TB.

ETHICS STATEMENT

This study protocol was approved by the Institutional Review Board of the Instituto Nacional de Pesquisas da Amazônia (INPA) under the number CAAE: 05925212.8.0000.0006. All patients gave written informed consent.

AUTHOR CONTRIBUTIONS

RB-N, FN, AS, AB, RR, and JP conceived and designed the study. RB-N, VA, MO, and AS were responsible for acquisition and quality of the clinical and laboratory data. RB-N, VA, AS, and MO performed the identification of *Mycobacterium tuberculosis*. RB-N, FN, and GS performed the genotyping of the different TR polymorphisms. RB-N, FN, and AB conducted data mining and statistical analysis. RB-N, FN, MO, AS, AB, and RR wrote the first version of the paper. All authors were involved in the interpretation of the data and participated in the final writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01455/full#supplementary-material.

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Immune Activation, Proinflammatory Cytokines, and Conventional Risks for Cardiovascular Disease in HIV Patients: A Case-Control Study in Bahia, Brazil

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Background: Cardiovascular events (CVE) are an increasing cause of morbi-mortality for HIV patients. The antiretroviral therapy (ART), persistent immune activation, and life style are factors that can increase CVE for such patients. We performed a case-control study to evaluate the role of coinfections and immune markers associated with CVE.

Methods: We included patients under ART, with undetectable plasma viral load ≥12 months. Patients presenting any condition of risk for CVE were considered cases, and those without CVE risk conditions were controls. History of viral infections (Epstein–Barr virus, hepatitis C virus, hepatitis B virus, and cytomegalovirus), exposure to antiretroviral drugs, time since HIV diagnosis/under ART, and life style (demographics, weight, smoking, alcohol, and illicit drug use) were assessed. CD4/CD8 nadir and current counts, nadir and current CD4/CD8 ratio, immune activation markers (CD4CD38HLADR, CD8CD38HLADR), and serum levels of eight cytokines [IL-2, IL-4, IL-6, IL-10, tumoral necrosis factor-alpha (TNF-α), interferon gamma, macrophage inflammatory proteins 1 alpha, and interferon-inducing protein (IP-10)] were measured.

Results: Two-thirds of patients were males. Cases (N = 106) were older (52.8 vs 49.5 years, p = 0.002), had higher levels of creatinine (0.97 vs 0.87 mg/dL, p = 0.002) and IL-6 (0.67 vs 0.52 pg/mL, p = 0.04) than controls (N = 114). There was no difference between groups regarding frequency of CD4CD39HLADR+ or CD8CD38HLADR+ cells. We found a significant correlation (all patients) between increased frequency of CD4CD38HLADR+ cells and levels of IP-10 (r = 0.171, p = 0.02) and TNF-α (r = 0.187, p = 0.01). Levels of IL-6 (r = 0.235, p = 0.02), TNF-α (r = 0.267, p = 0.01), and IP-10 (r = 0.205, p = 0.04) were correlated with CD4CD38HLADR+ cells, in controls. Higher frequency of CD4CD38HLADR+ cells was also correlated with levels of IP-10 (r = 0.271, p = 0.04) in patients presenting with arterial hypertension. Frequency of CD4CD38HLADR+ cells was negatively correlated with levels of IL-2 (r = –0.639,

$p = 0.01$) and IL-6 ($r = -0.0561$, $p = 0.03$) in patients with hypercholesterolemia. No association was detected between viral infections or smoking/alcohol use and immune activation markers.

Conclusion: Our results indicate IL-6 levels are associated with increased CV risk. Activated CD4+ T cells were associated with increased levels of proinflammatory cytokines.

Keywords: HIV, cytokines, cardiovascular, immune activation, dyslipidemia

INTRODUCTION

The life expectancy of people living with HIV (PLHIV) has substantially increased over the last decades (1–3). In Brazil, at least 30% of PLHIV are older than 50 years, with a detection rate of new HIV infections increasing fast (by 14% in recent years) in this age range (4). A recent mathematical model (ATHENA Cohort) estimates that 73% of PLHIV will be older than 50 years by 2030 (5). This trend has led to an increasing proportion of patients affected by non-communicable diseases (NCDs), in countries where antiretroviral treatment is available (5–8).

The increasing incidence of NCD among PLHIV presents emergent challenges for clinical care and public health (5, 9, 10). Currently, the proportion of deaths caused by NCD in PLHIV is higher than those related to AIDS (6, 10, 11). Increases in NCD causes of death include cancer (not related to HIV), diabetes, nephropathy, liver disturbances, and especially cardiovascular disease (CVD), comparable to seronegative individuals at similar age.

Risk of CVD, a common NCD in aging HIV patients, may be linked to viral coinfections, concomitant T-cell activation, and increased levels of proinflammatory cytokines (12–16).

Inflammatory cytokines [like tumoral necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ), and IL-6] are important players in lipid metabolism. For instance, TNF-α promotes changes in lipids levels of HIV patients by affecting lipolysis, the adipose tissue lipoprotein lipase and the synthesis of hepatic fatty acids (17). Other proinflammatory cytokines can alter levels of circulating lipids. IL-4 counterbalances the proinflammatory cytokines effects, while IL-10 increases the transformation of lipoproteins into foam cells (18, 19). Although there are consistent evidences on the role of proinflammatory cytokines on lipids metabolism, the mechanism is not clear. However, increased lipids level is a typical risk factor for CVD, while chronic inflammation is considered a potential trigger for the occurrence of such health problems.

We aimed to investigate immune activation in HIV-infected patients and the relationship with cardiovascular risk factors in a well-characterized, stable population under outpatient clinic follow-up in Salvador, Brazil.

MATERIALS AND METHODS

Study Design and Population

The AIDS clinics of Federal University of Bahia Hospital (UFBA) follow up to 3,000 HIV patients a year. All of them are regularly seen, with standardized and systematic clinical follow-up. We used a non-matched, case-control study design. Cases were defined as HIV-infected patients *recently* diagnosed *(last 2 years)* with cardiovascular (CV) comorbidity (acute myocardial infarction, coronary *arterial* disease, hypertension, stroke, diabetes, and dyslipidemia) in the past 2 years, both genders, 35 years and older, on stable antiretroviral therapy (ART), and HIV RNA plasma viral load <50 copies for at least 12 months. Controls were HIV-infected patients with no diagnosis of CV disease based on medical record review of the past 2 years, 35 years and older, on stable ART, and HIV RNA plasma viral load <50 copies for at least 12 months. Patients were compared with respect to age, sex, life style-related variables [weight, smoking, and history of illicit drug use (marijuana, cocaine, crack, and intravenous cocaine)], prevalence of syphilis, hepatitis C virus (HCV), hepatitis B virus (HBV), human T-cell lymphotropic virus (HTLV), cytomegalovirus (CMV), and Epstein–Barr virus (EBV), and differences in levels of proinflamatory cytokines/chemokines, and markers of immune activation.

Cases and controls were identified through medical record review of UFBA's AIDS outpatient clinics. Medical releases were obtained from patients to gather all relevant information regarding non-infectious conditions, including cardiovascular, pulmonary, renal, lipids, bone abnormalities, central nervous system, and malignancies. Blood samples were collected to confirm coinfection status, and for evaluation of immune activation and inflammatory markers. We used the recommendations of the American Association of Clinical Endocrinologists and American College of Endocrinology to define dyslipidemia (20).

The *serum* levels of eight different cytokines were measured by a multiplex biometric immunoassay containing monoclonal antibody-conjugated microspheres for protein targets revealed by fluorescent staining for cytokine quantification, according to the manufacturer's instructions (Bio-Plex Human Cytokine Assay; Bio-Rad Inc., Hercules, CA, USA). We compared levels of cytokines [IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α, macrophage inflammatory proteins 1 (MIP-1)-α, interferon-inducing protein (IP)-10, and markers of T-cells activation (CD4, CD8, CD38, and HLADR)] for patients with and without CV comorbidity. All infections were detected by serology. *HIV, HCV, HBV, and HTLV infections were confirmed by PCR.*

Frequency of CD4CD38HLADR+ and CD8CD38HLADR+ cells were measured by flow cytometry (FacsCalibur, Becton Dickinson, *San Jose, CA*, USA). *Briefly, 20 µL of Multitest (CD3/CD4/CD8/CD38/HLA) monoclonal antibodies were added. Monoclonal antibodies were added to Trucount tubes containing reference beads (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). 50 µL of whole blood was mixed and incubated at room temperature for 20 min in dark. Red blood cells were lysed by adding 450 µL of*

fluorescence-activated cell sorter lysing solution (Becton Dickinson Immunocytometry Systems). The tubes were incubated at room temperature for 20 min, and samples were analyzed with the Multiset software (Becton Dickinson Immunocytometry Systems) within 6 h.

Statistical Analysis

Frequency distribution of the variables means and measures of dispersion (SD) for continuous variables with normal distribution were calculated. For variables with non-normal distribution, the median and interquartile range (IQR) were used. Nominal and ordinal variables were presented as proportions. Chi-square or Fisher's exact test were used to test associations between independent variables and CV outcome. The correlation between the cytokines and frequency of activated CD4/CD8+ T cells were calculated assuming non-normal distribution using Spearman's rho coefficient. If the distribution was not normal, the Kruskal–Wallis test was used. All tests comparing the groups were two-sided at a level of 0.05 ($\alpha = 0.05$). Statistical analysis was performed by using the statistical package SPSS® (version 18.0).

Ethics Considerations

The study is part of a larger project and was approved by institutional ethics in research committee, number 1035.826, April 2015. All patients provided a written informed consent, before entering the protocol. The study was conducted according to the Helsinki's declaration.

RESULTS

We included 220 patients in the study (106 cases, 114 controls). **Table 1** summarizes the main characteristics of cases and controls. Cases were older, more likely to be retired, and included a higher proportion of males than controls. On the other hand, more controls reported the use of illicit drugs than cases. *We detected no case of alcohol addiction in the study sample.* However, weight, route of HIV infection, and frequency of smoking were similar for both groups. **Table 2** shows the main clinical and laboratorial findings for included patients. Regarding the detected conventional risks for CV comorbidities (cases), the most prevalent was arterial hypertension (64.2%), followed by hypercholesterolemia (19.8%), hypertriglyceridemia (18.8%), and mixed dyslipidemia (17.9%). The frequency of other comorbidities (viral coinfections, syphilis, bone disturbances, and neoplasms) was similar for cases and controls. Almost all (>98%) patients had a positive serology for CMV and EBV. One patient tested positive for HTLV-1 and one for syphilis, both were cases.

The only difference between groups was detected in levels of IL-6, which were significantly higher in cases than in controls (**Table 3**). The frequency of CD4CD38HLDR+ or CD8CD38HLADR+ cells was similar for cases and controls.

We detected a significant correlation between frequency of CD4CD38HLDR+ cells and levels of IP-10 ($r = 0.205$, $p = 0.04$), TNF-α ($r = 0.267$, $p = 0.01$), and IL-6 ($r = 0.235$, $p = 0.02$) in control group (**Table 4**). When we looked at all patients, frequency of CD4CD38HLDR+ cells was significantly correlated with levels of IP-10 ($r = 0.171$, $p = 0.02$) and TNF-α ($r = 0.187$, $p = 0.01$), but not with IL-6 levels. We did not detect any correlation between frequency of CD8CDE38HLADR+ cells and cytokines' levels.

TABLE 1 | Demographic and laboratorial characteristics of cases and controls.

Characteristic	Cases (*N* = 106)	Controls (*N* = 114)	*p* Value
	N (%)/mean ± SD	*N* (%)/mean ± SD	
Age	52.5 ± 9.7	48.8 ± 8.5	0.02
Sex			
Male	65 (61.3)	83 (72.8)	0.04
Female	41 (38.7)	31 (27.2)	
Education			
<12 years	46 (44.2)	58 (40.5)	0.58
≥12 years	58 (55.8)	66 (59.5)	
Weight (kg)	74.1 ± 16.1	71.6 ± 12.6	0.2
Years on ART	12.5 ± 6.5	11.1 ± 6.6	0.09
Smoking			
Current	12 (11.3)	20 (17.5)	0.4
Past	25 (23.6)	26 (22.8)	
Civil status			
Steady partner	24 (22.9)	19 (16.8)	0.2
Single or widowed	82 (77.1)	94 (83.2)	
Retired	52 (49.1)	31 (27.2)	<0.01
HIV risk profile			0.85
Heterosexual	51 (52.6)	55 (53.9)	
MSM/bisexual/IV drugs	46 (47.4)	47 (46.1)	
History of illicit drug use	2 (1.9)	10 (9.4)	0.03
Nadir CD4 count	299 ± 238	333 ± 232	0.3
Current CD4 count	778 ± 350	750 ± 350	0.5
Current CD4/CD8 ratio	0.86 (0.45)	0.9 (0.49)	0.55
Platelet count	236,882 ± 73,933	217,177 ± 81,012	0.09
Creatinine (mg/dL)	0.97 ± 0.27	0.87 ± 0.16	0.002
AST (mg/dL)	32.2 ± 21.7	31.6 ± 28.1	0.8
ALT (mg/dL)	36.5 ± 34.9	35.1 ± 33.9	0.8

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ART, antiretroviral therapy.

TABLE 2 | Frequency of diagnosed cardiovascular diseases, coinfections, and other comorbidities in cases and controls.

Comorbidity	Cases	Controls	Total	*p* Value
	N (%)	*N* (%)	*N* (%)	
Hypertension	68 (64.2)	–	68 (30.1)	–
Myocardial infarction	1 (0.9)	–	1 (0.5)	–
Stroke	3 (2.7)	–	3 (1.3)	–
Diabetes	13 (12.3)	–	13 (5.9)	–
Coronary disease	2 (1.8)	–	2 (0.9)	–
Dyslipidemia	19 (17.9)	–	19 (8.6)	–
Renal dysfunction	3 (2.7)	2	5 (2.3)	0.8
AIDS-related neoplasm	0	0	0	–
Non-AIDS neoplasm	3 (2.7)	0	3 (1.4)	0.1
Osteopenia	2 (1.8)	3 (2.6)	5 (2.3)	0.5
Osteoporosis	5 (4.5)	1 (0.9)	6 (2.7)	0.09
Hepatitis C virus	6 (5.4)	7 (6.1)	13 (5.9)	0.5
Hepatitis B virus	0	3 (2.6)	3 (1.4)	0.1

In patients presenting with arterial hypertension, there was a significant correlation between frequency of CD4CD38HLADR+ cells and IP-10 ($r = 0.271$, $p = 0.04$). Among patients presenting with hypercholesterolemia, the frequency of CD4CD38HLDR+ cells were negatively correlated to IL-6 ($r = -0.561$, $p = 0.03$) and to IL-2 levels ($r = -0.630$, $p = 0.01$). However, when we grouped patients with normal serum lipids, we detected a significant correlation

between CD4CD38HLDR+ cells and levels of IP-10 ($r = 0.221$, $p = 0.01$), MIP-1-α ($r = 0.258$, $p = 0.04$), and TNF-α ($r = 0.242$, $p = 0.005$). **Table 3** details the differences between serum levels of different cytokines for cases and controls, and **Table 4** shows the detected correlations between T-cells activation markers and cytokine levels, for cases and controls. **Table 5** summarizes the correlations observed between cytokines, activated T cells, and hypertension/hypercholesterolemia.

Over 50 different antiretroviral regimens (ART) were in use by patients at study entry: the most frequent regimen (33%) was efavirenz-based ART, followed by protease/inhibitors-based regimens (25%), and nevirapine-based ones (16%).

TABLE 3 | Frequency of CD4CD38HLADR+, CD8CD38HLADR+ cells (%), and serum levels of eight cytokines (pg/mL) in cases and controls.

Cytokine/ activated T cells	Cases		Controls		
	Median	Interquartile range (IQR)	Median	IQR	p Value
IL2	0.93	(0.23–0.73)	0.93	(0.71–1.48)	0.81
IL4	12.94	(2.06–45.44)	5.79	(1.38–37.62)	0.15
IL6	0.67	(0.43–1.12)	0.52	(0.37–0.87)	0.04
IL10	0.58	(0.25–1.45)	0.56	(0.25–1.03)	0.69
Tumoral necrosis factor-alpha	7.81	(2.94–11.24)	7.26	(1.68–11.24)	0.55
Interferon-gamma	2.62	(1.49–8.40)	3.02	(1.73–8.64)	0.48
Interferon-inducing protein-10	201.6	(136.5–284.9)	202.9	(132.3–307.2)	0.74
Macrophage inflammatory proteins 1 alpha	5.34	(2.43–17.81)	5.41	(2.22–14.81)	0.61
CD4CD38HLADR+	4.23	(2.66–7.08)	4.21	(2.55–7.46)	0.92
CD8CD38HLADR+	4.56	(2.93–9.01)	4.78	(3.37–8.35)	0.71

DISCUSSION

We detected a significant correlation between T-cells activation markers, levels of cytokines and presence of specific CVD risks. The only difference between cytokines levels for cases and controls was detected for IL-6 levels, which were significantly higher in cases than in controls. However, we detected significant correlations between frequency of cellular immune activation markers and proinflammatory cytokines (IP-10 and TNF-α) for the entire population of study. Moreover, we found a significant correlation between activated CD4+ T cells and IP-10, in patients presenting with arterial hypertension. However, levels of IL-2 and IL-6 were negatively correlated with activated CD4+ T cells in patients with hypercholesterolemia.

Higher levels of IL-6 are associated with increased CVD risk, and in SMART study, increased IL-6 levels were highly predictive of death or incident CV events (21). In our study, IL-6 levels were significantly higher in cases than in controls. In addition, when we looked at all patients, we detected a significant correlation

TABLE 4 | Correlations (Spearman's rho coefficient) between frequency of CD4CD38HLADR+/CD8CD38HLADR+ cells and serum levels of eight cytokines for cases and controls.

Cytokines	CD4+CD38+HLADR+		CD4+CD38+HLADR+		CD8+CD38+HLADR+	CD8+CD38+HLADR+
	Cases	Controls	Cases	Controls	All patients	All patients
IL-2	−0.075	0.056	−0.491	0.049	0.008	−0.028
IL-4	−0.081	−0.047	−0.096	0.118	−0.062	0.007
IL-6	−0.047	0.235*	−0.041	0.127	0.104	0.039
IL-10	−0.045	−0.045	0.016	−0.074	−0.039	−0.017
Tumoral necrosis factor-alpha	0.63	0.267*	0.068	0.199	0.187*	0.151
Interferon gamma	0.019	0.015	−0.095	0.100	0.06	−0.09
Interferon-inducing protein-10	0.141	0.205*	−0.069	0.134	0.171*	0.054
Macrophage inflammatory proteins 1 alpha	−0.18	−0.006	0.144	−0.006	0.008	0.039

$p < 0.05$ for the correlation between frequency of activated T cells and cytokines' levels.

TABLE 5 | Correlations (Spearman's rho coefficient) observed between T cells activation markers and cytokines levels, according to the presence of traditional cardiovascular risk in HIV patients on stable therapy.

Cytokines	CD4CD38HLADR+		CD8CD38HLADR+	
	Cholesterol	Hypertension	Cholesterol	Hypertension
IL-2	−0.630*	−0.008	−0.227	−0.026
IL-4	0.066	−0.002	0.246	0.030
IL-6	−0.561*	0.117	−0.196	0.110
IL-10	−0.289	0.033	−0.011	0.073
Tumoral necrosis factor-alpha	0.095	0.158	0.502	0.110
Interferon gamma	−0.404	0.151	−0.241	0.016
Interferon-inducing protein-10	−0.164	0.271*	−0.323	0.130
Macrophage inflammatory proteins 1 alpha	−0.221	−0.008	−0.095	0.215

$p < 0.05$ for the correlation between frequency of activated T cells and cytokines' levels.

between frequency of CD4CD38HLADR+ cells and levels of TNF-α and IP-10. These cytokines are involved in activation of several cell types, chemotaxis and inflammatory response, and activated CD4+ T cells are likely to increase their production (22). In a recent work involving 252 HIV patients Mooney et al. detected that, despite viral suppression and immunological stability, levels of TNF-α and other inflammatory biomarkers are persistently elevated in a large proportion of HIV patients, and this fact is associated with increased cardiovascular risk, as measured by Framingham and Veterans Aging Cohort Study scores (23).

Tumoral necrosis factor-alpha and IP-10 were consistently correlated with frequency of activated CD4+ T cells for controls and pooled groups, but for IL-6, this relationship was seen only in control group. However, Il-2 and IL-6 were negatively correlated with activated CD4+ T cells in patients presenting with hypercholesterolemia. In addition, among patients without dyslipidemia, levels of IP-10 and TNF-α were significantly correlated with CD4CD38HLDR+ cells, while levels of MIP-1-α were correlated with CD8CD38HLDR+ cells in patients with normal lipids' levels. These findings suggest that IP-10 and TNF-α are involved in lipids' regulation and their levels are associated with cellular immune activation.

Tumoral necrosis factor-alpha can affect the metabolism of lipids, by modifying important functions like lipolysis, adipose tissue lipoprotein lipase, and synthesis of fatty acids by the liver (17, 19, 23). In addition, IL-6 and TNF-α stimulates the hepatic lipogenesis and were also associated with lipodystrophy in HIV patients (21, 24, 25). The detected correlation between CD4CD38HLDR+ cells and IP-10 suggests that this cytokine is involved in persistent immune activation (PIA) and inflammation. In addition, IP-10 levels were significantly correlated with CD4CD38HLDR+ cells in patients with arterial hypertension, a well-known risk factor for cardiovascular events. *The detected correlation between CD8CD38HLDR+ and MIP-1-α reinforces the link between T cell activation and inflammation, as events that can drive increased CVD risk.*

Interferon-inducing protein-10 is produced by several cell types (including monocytes, endothelial cells, and fibroblasts) in response to IFN-γ. IP-10 promotes chemo attraction (for monocytes/macrophages, T cells, NK cells, and dendritic cells), T cell adhesion to endothelial cells, antitumor activity, and inhibition of bone marrow colony formation and angiogenesis (26). Levels of IP-10 are usually elevated in patients with chronic untreated HCV infection (27). *Some previous reports indicate that IP-10 either directly or indirectly can influence hepatic lipogenesis and contributes to liver steatosis and inflammation* (28). Our results show increased levels of IP-10 in patients with higher frequency of CD4CD38HLDR+ cells, as well as in those presenting with arterial hypertension. These findings suggest IP-10 plays a role as a marker of immune activation and, perhaps, of arterial hypertension, for HIV-infected patients. TNF-α is produced by macrophages and many other cells, including T lymphocytes. It primary role is regulation of immune cells, but it is also involved in inflammation and many other activities, including regulation of lipids metabolism (21, 24, 25). The consistent correlations seen in our study between CD4CD38HLDR+ and CD8CD38HLDR+ cells and TNF-α suggest that immune activation is closely related with inflammation and CVD risks in HIV patients on stable antiretroviral treatment.

Persistent immune activation is often associated with microbial translocation, ongoing HIV replication, and viral coinfections (14, 15). However, in many of available reports the studied population was heterogeneous and included patients with detectable viremia or clinical manifestations of disease. In our study, we included only patients on stable therapy, with long-term HIV suppression, and with no current HIV-related events. We did not see any association between prevalent viral coinfections and signs of immune activation or inflammation. However, activated CD4CD38HLDR+ cells were significantly correlated with increased levels of IP-10 and TNF-α when we looked at all patients. IP-10 was also associated with hypertension, and controls showed a significant correlation between frequency of CD4CD38HLDR+ and levels of IL-6, TNF-α, and IP-10.

On the other hand, there was a consistent correlation between hypertension and frequency of CD4CD38HLDR+ cells. T cells activation was also correlated with increased production of proinflammatory cytokines, like IP-10, IL-6, and TNF-α. Our findings suggest that in treated patients activated T cells are involved in inflammation and can affect CV risk in HIV-infected patients.

The reasons for T cells activation were not clear in our study. Chronic CMV and EBV infections were seen in over 98% of our patients and would not explain the observed T cells activation. Other chronic viral infections were infrequent in the studied groups and were not associated with immune activation nor with increased levels of proinflammatory cytokines. Other causes, like microbial translocation, were not investigated, and would be *less frequent* in patients with high CD4+ cells count and suppressed viremia.

IL-6 is an already established marker for CV risk in HIV patients. Our results showed higher levels of IL-6 among cases, and a significant association between TNF-α and IP-10 for all patients. This indicates that cellular immune activation can trigger the observed increase in proinflammatory cytokines and, in consequence, increase the risk of NCD in HIV patients on stable therapy. *A long-term follow-up of patients would provide an opportunity to evaluate the NCD outcomes among those presenting signs of immune activation and inflammation.*

The case-control design of this study involves retrospective analyses of exposures in association with the outcome, limiting conclusions on causality with the observed events. However, both cases and controls are drawn from the same clinical study base, which was mostly homogeneous, on stable therapy and long-term viral suppression. These characteristics made possible to exclude potential confounding factors, like ongoing viremia, or AIDS-related comorbidities. The low prevalence of dyslipidemia in our study's population probably affected the power to detect associations between cytokines' levels and lipids. *In addition, because of the great variety of ART regimens used by patients, it was not possible to assess the potential effect of distinct antiretroviral drugs on lipids and immune activation.*

Understanding chronic micro-inflammation is very important in HIV disease. We detected a significant association between inflammatory markers, cytokines, and hypertension. Taken together, our findings indicate that activated CD4+ T cells are

linked to immune dysregulation, with increased production of proinflammatory cytokines even in patients with prolonged viral suppression. These effects can interfere on lipids metabolism and increase the risk of development of CVD in HIV-treated population.

ETHICS STATEMENT

The Project was approved by the Ethics Committee of Maternidade Climério de Oliveira (UFBA), number 1.035.826, on April 26, 2015.

AUTHOR CONTRIBUTIONS

CB-A, EL, EN, KP, RD, and CB have designed the study, supervised data collection and statistical analysis. TF and CP were responsible for collecting and testing samples. CB-A, KP, RD, and CB were responsible for writing the paper.

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The −*3279C>A* and −*924A>G polymorphisms* in the *FOXP3* Gene Are Associated With Viral Load and Liver Enzyme Levels in Patients With Chronic Viral Liver Diseases

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The transcription factor FOXP3 is an essential marker of the development and activation of regulatory T cells (Tregs), which are cells specialized in the regulation and normal tolerance of the immune response. In the context of chronic viral liver diseases, Tregs participate in the maintenance of infections by promoting histopathological control and favor the immune escape of viral agents by suppressing the antiviral response. Single nucleotide polymorphisms (SNPs) may influence the function of FOXP3 in a number of pathological conditions. The present study sought to evaluate the influence of SNPs in the *FOXP3* gene promoter region in patients with chronic viral liver diseases. Three SNPs (−*3279C>A*, −*2383C>T*, and −*924A>G*) were analyzed in groups of patients with chronic hepatitis C (CHC), active chronic hepatitis B (CHB-A), inactive chronic hepatitis B (CHB-I), and a healthy control group (CG) using real-time PCR. The frequencies of the polymorphic variants were compared between groups and correlated with liver histopathological characteristics and enzyme levels [i.e., alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase (GGT)] obtained via biopsy and from the clinical records of the participating patients, respectively. For the −*2338C>T* SNP, no significant differences were found in the frequencies of variants between groups or in the histological findings. Significant associations between the polymorphisms and the CHB-I group were not established. The −*3279C>A* SNP was associated with altered viral loads ($\log_{10}$) and GGT levels in CHC patients with advanced stages of inflammatory activity and liver fibrosis. The −*924A>G* SNP was associated with altered viral loads ($\log_{10}$) and liver enzyme levels among CHB-A patients with milder inflammation and fibrosis. However, the frequencies of the −*3279C>A* and −*924A>G* polymorphisms were not directly associated with the histopathological profiles of the analyzed patients. These polymorphic variants may influence hepatic function in patients with chronic viral liver diseases but are not directly associated with the establishment of the degree of inflammatory activity and liver fibrosis.

Keywords: immune regulation, regulatory T lymphocytes, FOXP3, SNPs, chronic viral liver diseases

INTRODUCTION

Previous studies have shown that a sustained helper T and cytotoxic T lymphocyte response is closely associated with the disease courses of hepatitis B and C infections and that these cells are crucial for successful infection control (1–3). A stronger immune response with multiple targeted epitopes is expected during the acute phase of infection; in the chronic phase, in contrast, the immune response is less effective as a result of escape mutation(s) and/or T cell exhaustion due to sustained antigenic stimulation (4–6).

However, the continued actions of these cells in liver tissue can cause damage and autoimmune reactions, especially in the context of chronic viral infection. Therefore, many regulatory mechanisms of the immune system control specific immune responses against viruses to avoid these problems (7–9). The liver itself, which is constantly exposed to antigenic stimulation, has acquired specialized mechanisms and cells that mediate immunotolerance properties and prevent excessive activation of the immune response (10–12).

In this context, there is strong evidence for the involvement of a specialized set of cells called regulatory T cells (Tregs) in the regulation of both the liver immune response and tolerance (13). During chronic hepatitis B infection, the number of Tregs has been reported to be elevated in hepatic tissue and blood in the host and is correlated with the serological status of the infection and the patient's clinical condition (14–17). In chronic hepatitis C infection, different Treg subpopulations protect the host against tissue damage, and these cells are correlated with the degree of hepatic inflammation (18).

During hepatitis B and C infection, the numerical increase in Tregs occurs due to constant tissue immunological activation (19). Viral agents utilize this robust regulatory activity (20) but also modulate it by directly influencing Treg activation, which is a known escape mechanism of the antiviral response (21). Treg activation may also influence the progression of liver disease due to the maintenance of fibrogenesis and inflammatory tissue activity (22–24).

One key marker of Tregs is the transcription factor FOXP3 (Forkhead box P3). FOXP3 is a 47-kDa protein 431 amino acids in length (25), whose function is strictly related to the development of different Treg lines and the maintenance of immunoregulation in different pathological conditions that are both autoimmune and infectious in nature (26).

Expression of the *FOXP3* gene can be triggered by distinct biological stimuli, such as the presentation of host autoantigens to T cells (27, 28), or by alternative pathways that include interactions with normal microbiota metabolites (29) and the host cytokines transforming growth factor (TGF)-β and interleukin (IL)-10 (30). The activated FOXP3 protein prevents the interaction of nuclear factor of activated T cells (NFAT) and the nuclear factor kappa B (NF-kβ) transcriptional factors with genes associated with the expression of immune response-related cytokines [e.g., IL-4 and interferon (IFN)-γ] (31, 32). However, it favors the expression of genes that confer a regulatory phenotype [i.e., CD25, CTLA-4 and glucocorticoid-induced TNFR-related protein (GITR)] (33). Thus, FOXP3 is able to activate natural Treg lines or convert non-natural lines to suppressor cells (34).

The biological significance of single nucleotide polymorphisms (SNPs) in preserving the immune response role of FOXP3, and consequently in disease susceptibility, has previously been investigated (26). The promoter region of the *FOXP3* gene may harbor relevant SNPs, as it is involved in the regulation of gene expression and Treg activation (35, 36). Among these SNPs, the *−2383C > T* (rs3761549), *−3279C > A* (rs3761548) and *−924A > G* (rs2232365) SNPs are functionally well-defined and are distinguished by the relevance of studies concerning them.

The *−2383C>T* polymorphism was functionally characterized by Inoue et al. who suggested that the **C* allele altered the binding site of the transcription factor Ying Yang 1 (YY1) to the gene (37). The CC genotype decreases the regulatory function by increasing the activity of self-reactive T cells, causing severe thyroid tissue destruction in patients with Hashimoto's disease (HD) (38). Additional published studies have also associated this polymorphism with endometriosis, regardless of the disease stage (39), psoriasis (40), food allergies (41), and systemic lupus erythematosus (SLE) (42). Only one prior study has evaluated the influence of SNP *−2383C>T* on HBV and HCV infections; the presence of the polymorphism was associated with hepatocellular carcinogenesis in Chinese patients with hepatitis B (43).

The *−3279C>A* polymorphism alters the *FOXP3* expression pathway. The **A* allele changes the E47 and c-Myb transcription factor binding sites, leading to modifications in the gene expression that predispose the individual to autoimmune disease development (44). In addition, the variant **A* interferes with Sp1 transcription factor binding to the *FOXP3* gene, affecting gene expression (45). Findings concerning the impact of the polymorphism on immunological regulation led to new research into its association with other pathologies in different populations, such as recurrent spontaneous abortion in Han Chinese individuals (46), allergic rhinitis in Hungarian individuals (47), and breast cancer tumor progression (48) and preeclampsia (49) in Indian individuals. In cancer cases in Asians, contradictory results have been reported; the **A* allele was associated with an increased risk of non-small cell lung cancer (50), while no association between the SNP and breast cancer was found, which led the authors to suggest that the polymorphism may have variable carcinogenic effects in different organs (51).

Studies have shown that the SNP *−924A>G* is located in a *FOXP3* gene region equivalent to the GATA3 transcription factor binding site, essential for differentiating the Th2 profile. The presence of the **A* allele allows the interaction between GATA3 and *FOXP3* gene, leading to a Th2 profile (46), while **G* allele may influence cell conversion (52). The **G* allele decreases the expression of the *FOXP3* gene and causes an immunological imbalance, predisposing an individual to developing autoimmune diseases, including an increased risk of developing psoriasis (40) and vitiligo (45).

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; FOXP3, Forkhead box P3; GGT, gamma-glutamyl transpeptidase; HBV, hepatitis B virus; CHB-A, active chronic hepatitis B carriers; CHB-I, inactive chronic hepatitis B carriers; CHC, chronic hepatitis C carriers; SNPs, single nucleotide polymorphisms; Tregs, regulatory T lymphocytes.

Due to the lack of associative studies concerning SNPs in the FOXP3 gene with chronic viral liver diseases, the present study breaks new ground due to the acquisition of fundamentally new knowledge concerning immunogenic factors that may interfere with host hepatic function to influence the evolution of pathologies. We believe that these data will provide a basis for future studies regarding the role of FOXP3 as a modulator of the immune response, as well as a better understanding of its function and mechanisms of action in several chronic diseases.

MATERIALS AND METHODS

Sample Characterization and Ethical Concerns

This study was performed as a cross-sectional and analytical study in the city of Belém in the state of Pará, Brazil, with the collaboration of the outpatient liver disease clinic of the Hospital of Santa Casa de Misericórdia Foundation of Pará (FSCMPA) and the João de Barros Barreto University Hospital (HUJBB). Consecutive cases of chronic hepatitis B and C carriers were identified and enrolled between 2014 and 2016.

All selected patients were clinically evaluated and subjected to complementary tests based on certain medical criteria, which included enzymatic tests (liver enzyme levels: alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT), serological tests (HBV surface antigen (HBsAg), HBV e antigen (HBeAg), anti-HBeAg, total anti-HBc and anti-HCV), virological tests (HBV DNA and *hepacivirus C* RNA), ultrasound examination, endoscopic tests and liver biopsies, which allowed the patients to be screened and classified as described below.

Forty-one active chronic hepatitis B virus carriers (CHB-A) were identified based on the presence of histological changes in the liver and persistent HBsAg for more than 6 months. Thirty-three inactive chronic hepatitis B virus carriers (CHB-I) were identified based on the absence of significant histological changes, persistently normal liver enzyme levels for at least 1 year, an HBV DNA load below 2,000 copies/mL in blood, and negative HBeAg and positive anti-HBeAg results. One hundred and one chronic hepatitis C carriers (CHC) were identified based on the positive presence of a *hepacivirus C* viral load and changes in the liver histological profile and enzyme levels.

A control group (CG) was established that consisted of 300 volunteer blood donors from the Center for Hemotherapy and Hematology of the Pará Foundation (HEMOPA). The volunteers were seronegative and had undetectable viral loads for HBV, *hepacivirus C* and other agents typically screened for at blood banks.

The inclusion and exclusion criteria in the present study were the same as those established by previous studies published by our research group (53), including an age older than 18 years, provision of consent by the participants through signing an informed consent form and the biological aspects inherent to the study.

In compliance with Resolutions 466/2012 and 347/05 of the National Health Council, which established guidelines and standards for human research in Brazil, the present study was submitted to and approved by the Research Ethics Committee of FSCMPA (protocol #772,782/2014) and HUJBB (protocol 962,537/2015).

Laboratory Data

The liver enzyme levels, serology for the investigated viral liver diseases and plasma viral loads were obtained from updated clinical records when the patients agreed to participate in the study. These data were organized into an access-restricted worksheet used only for updates and for obtaining information related to the study objectives.

Histopathological Procedures

Liver biopsy specimens were obtained only from patients with a medical indication for investigation of hepatic parenchyma alterations within the clinical care protocol. The liver biopsies were performed by medical professionals with a Tru Cut needle (UNIT comércio, importação e exportação LTDA, São Paulo, Brazil) using an ultrasound-guided approach. Each sample was examined at the Pathological Anatomy Service of the Federal University of Pará (UFPA) according to the service's routine protocols. The specimens were stained with hematoxylin and eosin (HE), chromotrope aniline blue (CAB), Gomori's reticulin and Shikata's orcein stains.

The histopathological diagnosis was based on the French METAVIR classification system (54). The activity of the peri-portal and peri-septal inflammatory infiltrates (inflammatory activity) was scored from 0 to 3 (A0-A3), with "A0-A1" indicating absent to mild inflammation and "A2-A3" indicating moderate to severe inflammation. The structural alterations of the hepatic parenchyma (i.e., the degree of fibrosis) were scored from 0 to 4 (F0-F4), with "F0-F1" indicating absent to mild liver fibrosis, "F2" indicating moderate liver fibrosis and "F3-F4" indicating advanced liver fibrosis to cirrhosis. All data regarding the histopathological profiles were obtained from medical records, which were available for data collection.

Patients evaluated by transient elastography (TE) whose results indicated histopathological changes were classified according to the methodology described above without the need for a liver biopsy. However, for these patients, the sensitivity of the testing only permitted the determination of the degree of fibrosis; the inflammatory activity was not evaluated.

DNA Extraction and Polymorphism Genotyping

For the molecular analyses, a vacuum collection system was used to collect peripheral blood samples directly into a 4-mL collection tube containing EDTA as an anticoagulant. These samples were transported to the Laboratory of Virology of the Federal University of Pará (Labvir-UFPA) and stored at −20°C until subsequent analysis.

DNA was extracted from these samples based on a forensic method consisting of cell lysis, protein precipitation, DNA precipitation and DNA hydration steps, as described in a previous publication (53).

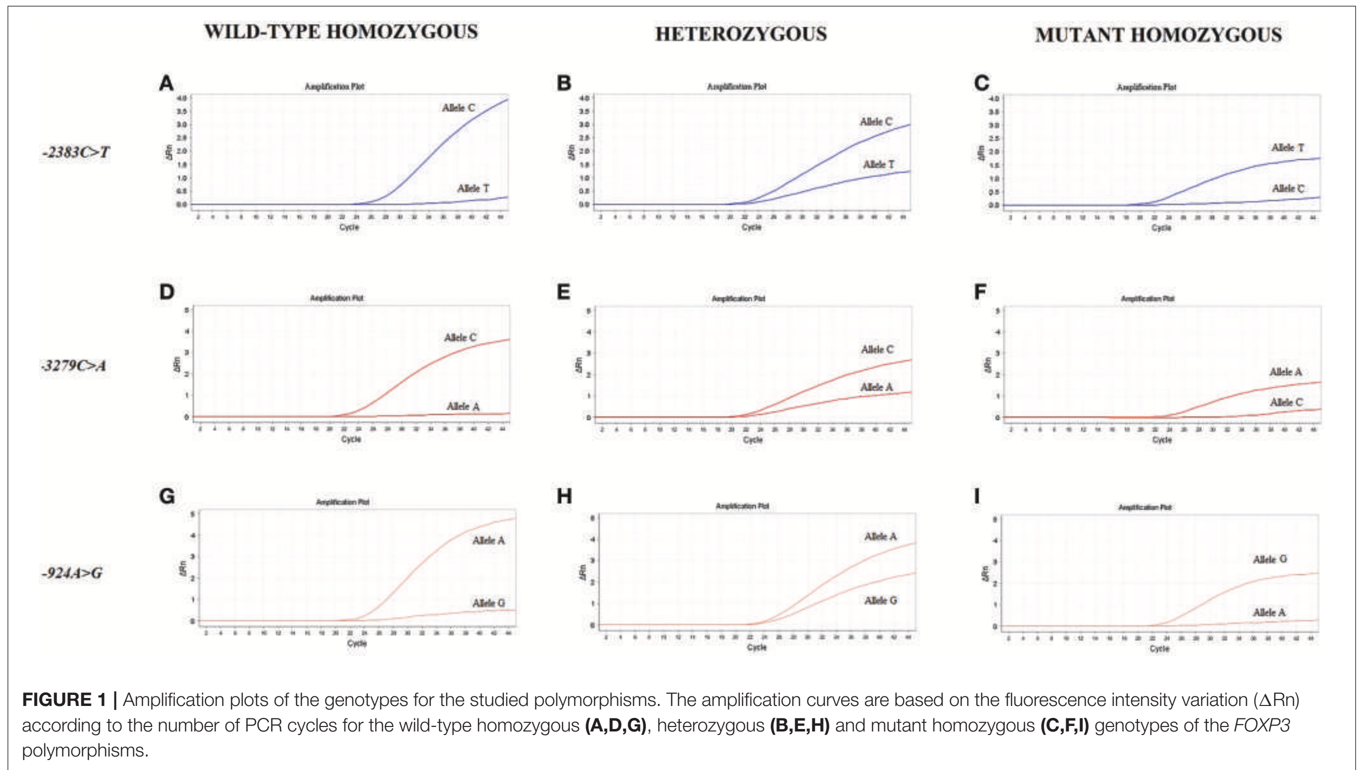

FIGURE 1 | Amplification plots of the genotypes for the studied polymorphisms. The amplification curves are based on the fluorescence intensity variation (ΔRn) according to the number of PCR cycles for the wild-type homozygous **(A,D,G)**, heterozygous **(B,E,H)** and mutant homozygous **(C,F,I)** genotypes of the *FOXP3* polymorphisms.

The *−3279C>A*, *−2383C>T*, and *−924A>G* SNPs in the *FOXP3* gene promoter region were genotyped by real-time PCR using a StepOne PLUS Sequence Detector (Applied Biosystems, Foster City, CA, USA) using primers and probes from TaqMan® SNP Genotyping Assays (***−3279***: C_27476877_10, ***−2383***: C_27058744_10, and ***−924***: C_15942641_10) (Applied Biosystems, Foster City, CA, USA).

For each reaction, 7 μL of distilled water, 10 μL of TaqMan® Universal PCR Master Mix (2X), 1 μL of TaqMan® Assay Buffer (20X) and 2 μL of extracted DNA were used in a final volume of 20 μL. The following thermal cycling program was used to amplify and detect polymorphisms: 60°C for 30 s, followed by 95°C for 10 min, 50 cycles of 92°C for 30 s and 1 cycle at 60°C for 1 min and 30 s.

Figure 1 shows the amplification plots of the different genotypes of the studied polymorphisms.

Statistical Analysis

The genotype and allele frequencies of the polymorphisms were estimated by direct counting, and these frequencies were compared between the study groups using the G and Fisher's exact tests, following the assumptions of each test. Hardy Weinberg equilibrium was estimated using the Chi-squared test. Only females were included in this analysis because the *FOXP3* gene is located on the X chromosome; the same principle was used for the determination of genotype frequencies. The statistical analyses described above were performed using BioEstat 5.0 software (55) at a significance level of 5% ($p < 0.05$).

A haplotype block was inferred from analysis of linkage disequilibrium (LD) between polymorphisms using Haploview 4.2 software (56), assuming a scheme based on the representation of the confidence interval of the normalized linkage disequilibrium coefficient (D'). The Chi-squared test was used to analyze the frequencies of the obtained haplotypes between groups.

TABLE 1 | Laboratory and histopathological data for patients with chronic hepatitis B and C virus infections.

Variables	CHC	CHB-I	CHB-A
Number of individuals	101	33	41
Sex (*F/M*)	50/51	16/17	12/29
Viral load (log^{10}) mean ± σ	5.41 ± 1.02	2.27 ± 0.78	3.74 ± 1.58
Median	5.62	2.03	3.90
ALT (IU/L) mean ± σ	77.93 ± 58.42	27.52 ± 14.83	80.05 ± 101.19
Median	58.00	31.00	44.00
AST (IU/L) mean ± σ	69.83 ± 48.62	29.82 ± 14.56	60.98 ± 54.73
Median	59.00	25.00	40.00
GGT (IU/L) mean ± σ	99.58 ± 95.18	31.79 ± 21.22	64.35 ± 28.53
Median	60.00	25.00	36.00
DEGREE OF INFLAMMATORY ACTIVITY			
Absent to mild (%)	40 (58.82)	10 (100.00)	16 (76.19)
Moderate to severe (%)	28 (41.18)	–	5 (23.81)
DEGREE OF LIVER FIBROSIS			
Absent to mild (%)	27 (34.18)	10 (100.00)	10 (34.48)
With few septa (%)	17 (21.52)	–	9 (31.04)
Advanced to cirrhosis (%)	35 (44.30)	–	10 (34.48)

σ, *Standard deviation.*

The enzymatic and virological data were compared between the polymorphism genotypes with the Mann-Whitney test using BioEstat 5.0 (55) and GraphPad Prism version 6.1 software; the latter was used specifically for estimation of the plots. For significant data, heatmap matrices were plotted using R 3.4.2 software (57) with canberra and ward.D as the distance and cluster methods, respectively, depending on the options provided by the software.

RESULTS

Sample Description

The majority of patients included in the study were hepatitis C carriers rather than CHB carriers. Male individuals predominated in the CHB-A group. The median viral load values and liver enzyme measurements were higher in CHC patients than in the other groups.

Histopathology profile showed in most cases no signs of abnormalities (with the inflammatory activity and hepatic fibrosis mostly absent to mild) with the exception of CHB-A carriers.

Eleven CHC and eight CHB-A patients were not submitted to biopsy considering the high risk procedure as the previous results of TE indicated severe alterations in the hepatic parenchyma; these patients were classified according to the degree of fibrosis.

The data on sex, viral load, and liver enzyme levels, inflammatory activity and degree of hepatic fibrosis are shown in **Table 1**.

Allele and Genotype Frequencies of *Foxp3* Gene Polymorphisms

Table 2 shows the allele and genotype frequencies of the studied polymorphisms in the *FOXP3* gene organized according to the infection status and the sex of the participants. All

TABLE 2 | Genotype and allele polymorphism frequencies in the *FOXP3* gene promoter region in patients with chronic hepatitis B and C virus infections.

Genotypic and allelic profile	CHC	CHB		CG	*p1*	*p2*	*p3*	*p4*	*p5*	*p6*
		I	A							
	n (%)	*n* (%)	*n* (%)	*n* (%)						
FEMALES#										
−2383C>T										
CC	41 (82.00)	12 (80.00)	9 (81.82)	133 (89.26)	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
CT	9 (18.00)	3 (20.00)	2 (18.18)	16 (10.74)						
TT	0	0	0	0						
**C*	0.910	0,900	0.909	0.946	0.2324	0.9731	0.9938	0.3985	1.0000	0.6185
**T*	0.090	0.100	0.091	0.054						
−3279C>A										
CC	27 (54.00)	13 (81.25)	8 (66.67)	91 (61.07)	0.4961	0.4913	0.1179	0.2500	0.3555	0.2500
AC	20 (40.00)	2 (12.50)	4 (33.33)	46 (30.87)						
AA	3 (06.00)	1 (06.25)	0	12 (08.05)						
**C*	0.740	0.875	0.833	0.765	0.6854	0.4322	0.1475	0.6152	0.7131	0.1859
**A*	0.260	0.125	0.167	0.235						
−924A>G										
AA	11 (22.00)	3 (18.75)	1 (09.09)	46 (30.87)	0.4709	0.2337	0.8526	0.1034	0.2507	0.4749
AG	27 (54.00)	8 (50.00)	9 (81.82)	73 (48.99)						
GG	12 (24.00)	5 (31.25)	1 (09.09)	30 (20.13)						
**A*	0,490	0,438	0.500	0.554	0.2975	1.0000	0.6861	0.6618	0.7827	0.2629
**G*	0.510	0.563	0.500	0.466						
MALES*										
−2383C>T										
C	48 (94.12)	13 (81.25)	24 (92,31)	138 (91.39)	0.7655	0.9967	0.1424	1.0000	0.3520	0.3692
T	3 (05.88)	3 (18.75)	2 (07.69)	13 (08,61)						
−3279C>A										
C	45 (88.24)	13 (76.47)	17 (65.39)	112 (74.17)	0.0503	0.0305	0.2535	0.4739	0.5131	1.0000
A	6 (11.76)	4 (23.53)	9 (34.61)	39 (25.83)						
−924A>G										
A	31 (60.79)	7 (41.18)	10 (65.52)	82 (54.31)	0.5144	0.0358	0.2591	0.0674	0.7553	0.3196
G	20 (39.22)	10 (58.83)	19 (34.48)	69 (45.69)						

*p1, CHC vs. GC; p2, CHC vs. CHB-A; p3, CHC vs. CHB-I; p4, CHB-A vs. GC; p5, CHB-A vs. CHB-I; p6, CHB-I vs. GC; #G Test; *Fisher's exact test.*

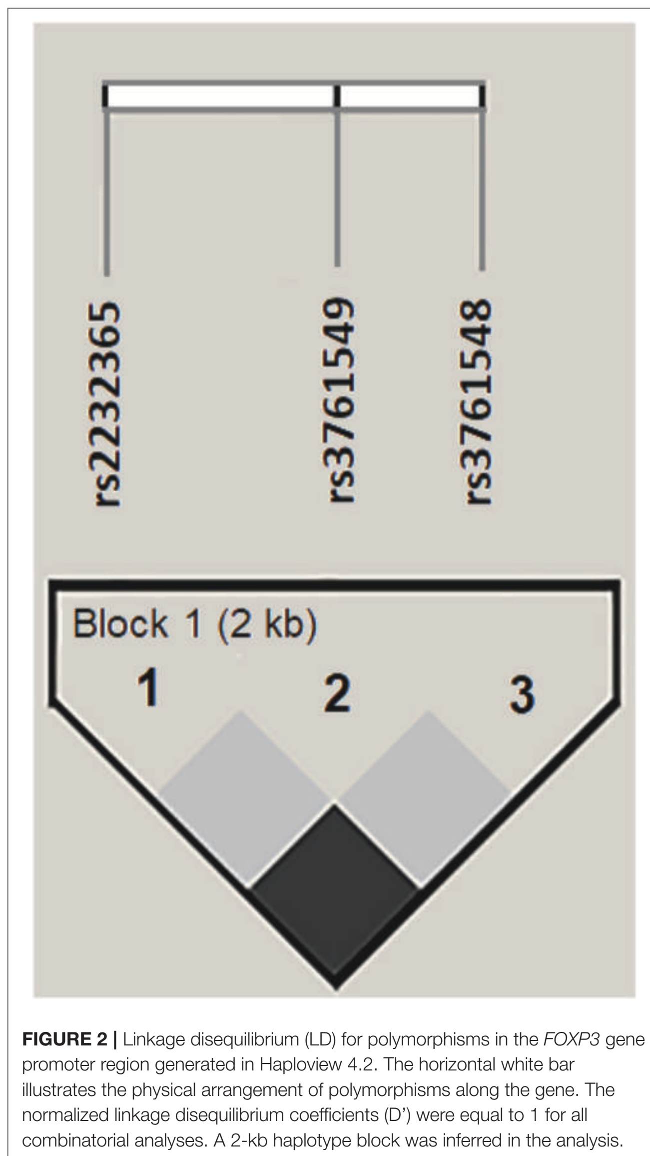

FIGURE 2 | Linkage disequilibrium (LD) for polymorphisms in the *FOXP3* gene promoter region generated in Haploview 4.2. The horizontal white bar illustrates the physical arrangement of polymorphisms along the gene. The normalized linkage disequilibrium coefficients (D') were equal to 1 for all combinatorial analyses. A 2-kb haplotype block was inferred in the analysis.

polymorphisms were in Hardy Weinberg equilibrium for the analyzed groups.

For the −*2383C>T* polymorphism, the **C* allele was the most frequent among the studied groups and both sexes; however, no significant differences were observed between the genotype and allele frequencies of the variants of this polymorphism. The frequency of the *C allelic variant of the −*3279C>A* polymorphism was significantly higher in the male patients in the CHC group than in the CHB-A group ($p = 0.0305$). A similar trend was observed between the CHC and CG groups, but the significance value in this case was borderline ($p = 0.0503$). For the −*924A>G* polymorphism, the frequency of the **A* allele was significantly higher in male patients in the CHB-A group than in the CHC group ($p = 0.0358$).

A haplotype block for the analyzed polymorphisms was inferred (**Figure 2**). It was observed that the *ACC* haplotype prevailed in all groups analyzed, and the *ATC* and *GTA* haplotypes were found only in the CHB-I group. However, no significant differences were found in the comparison of haplotype frequencies between the studied groups. The haplotypes and their respective frequencies are shown in **Table 3**.

Analysis of Polymorphisms in Terms of Liver Function and Histopathological Aspects

The distributions of the genotype frequencies of the polymorphisms based on the degrees of inflammatory activity and liver fibrosis are shown in **Table 4**.

The *CC* genotype of the −*2383C>T* polymorphism was the most frequent among the study groups when analyzing both the inflammatory activity and the degree of liver fibrosis. Similarly, for the −*3279C>A* polymorphism, the *CC* genotype was the most frequent under these same conditions. However, significant differences were not identified in the comparison of the frequencies of these polymorphisms between the different histopathological features for the analyzed groups.

For the −*924A>G* polymorphism, the *AA* genotype was the most frequent in the CHC group regardless of the degree of inflammatory activity; however, the *GG* genotype became the most frequent in this group when the liver fibrosis was absent to mild. The *GG* genotype was also the most frequent in the CHB-I group, regardless of histopathological stratification; it was also the most frequent in the CHB-A group, except in patients with liver fibrosis with few septa, for whom the *AA* genotype was the most frequent. However, no significant differences were observed in the frequencies of genotypic variants of this polymorphism according to the histopathological findings in the analyzed groups.

For SNP −*2383C>T,* no significant differences were found in the comparison of enzymatic and virological markers with the degrees of inflammatory activity and liver fibrosis (**Supplementary Figures 1–4**).

Figures 3–7 refer to comparative analyses of enzymatic markers of liver function and plasma viral loads ($\log_{10}$) between the genotypes of the −*3279C>A* and −*924A>G* polymorphisms.

The viral loads were lower in CHC patients with moderate to severe inflammation who were homozygous or heterozygous for the **A* allele of the −*3279C>A* polymorphism ($p = 0.0199$). Conversely, the GGT levels were higher for these patients ($p = 0.0454$) than for carriers of the *CC* genotype (**Figure 3A**). This result was similar to observations made in the analysis of these markers for the advanced liver fibrosis to cirrhosis stages (viral load, $p = 0.0261$; GGT, $p = 0.0405$) (**Figure 3B**).

In the heatmap matrix plotted to analyze the −*3279C>A* polymorphism in relation to the histopathological features in the CHC group (**Figure 4**), the highest viral loads in patients with the *CC* genotype with moderate to severe inflammation were predominantly clustered together with low and intermediate GGT levels. The plotted sub-clusters showed an inverse relationship; however, complementary to previously established

TABLE 3 | Haplotype frequencies of polymorphisms of the *FOXP3* gene promoter region (*−924A>G*, *−2383C>T*, and *−3279C>A*) in patients with chronic hepatitis B and C virus infections.

Haplotypes	CHC	CHB	CG	*p1*	*p2*	*p3*	*p4*	*p5*	*p6*	
		I	A							
	n (%)	*n* (%)	*n* (%)	*n* (%)						
BLOCK 1										
ACC	69 (0.550)	15 (0.400)	14 (0.383)	201 (0.548)	1.0000	1.0000	0.2015	1.0000	0.6837	0.0728
CGA	29 (0.188)	5 (0.150)	8 (0.267)	97 (0.247)						
CGC	27 (0.188)	12 (0.300)	9 (0.250)	60 (0.135)						
GTC	12 (0.074)	4 (0.100)	4 (0.100)	29 (0.070)						
ATC	–	1 (0.017)	–	–						
GTA	–	1 (0.017)	–	–						

p1, CHC vs. GC; p2, CHC vs. CHB-A; p3, CHC vs. CHB-I; p4, CHB-A vs. GC; p5, CHB-A vs. CHB-I; p6, CHB-I vs. GC.

findings, high GGT levels clustered with low viral loads in carriers of the **A* allele with moderate to severe inflammatory activity (**Figure 4A**), which was consistent with established statistical data.

A similar trend was observed for liver fibrosis. High viral load levels were grouped with low GGT levels in patients with the *CC* genotype in a cluster predominantly formed by patients with advanced fibrosis to cirrhosis (**Figure 4B**).

For the *−924A>G* polymorphism, CHB-A patients with the *GG* genotype and absent to mild inflammatory activity had lower viral loads than patients with the *AA* genotype ($p = 0.0121$), and higher GGT levels than patients with both the *AG* genotype ($p = 0.0485$) and the *AA* genotype ($p = 0.0465$) (**Figure 5**). In the liver fibrosis analysis, patients with mild fibrosis with the *GG* genotype had lower viral loads ($p = 0.0238$) but higher ALT ($p = 0.0476$), AST ($p = 0.0238$) and GGT levels ($p = 0.0357$) than patients with the *AG* genotype (**Figure 6**).

In the heatmap for the *−924A>G* polymorphism in patients in the CHB-A group with inflammatory activity, lower viral loads were clustered with high liver enzyme levels in patients with absent to mild inflammation with the *GG* genotype. Additionally, the highest viral loads tended to cluster with low and intermediate levels of liver enzymes in patients with absent to mild inflammation with the *AA* genotype. In intermediate groups, whose low levels of hepatic enzymes and viral loads were grouped, the prevalence of heterozygous genotypes is likely to be a contributing factor (**Figure 7A**).

In the heatmap matrix of liver fibrosis, the trends established for the *−924A>G* polymorphism in the CHB-A group were clearly visualized. In the group of patients with the *GG* genotype and absent to mild fibrosis, the highest levels of liver enzymes were clustered with intermediate viral loads (**Figure 7B**), similar to the obtained statistical data.

For the CHB-I patients, analyses of the liver function and virological variables did not show any significant differences in relation to the histopathological features and the genotypic profiles of the studied polymorphisms (data not shown).

DISCUSSION

Polymorphisms in the *FOXP3* gene promoter region are notable for their ability to alter gene expression of the transcription factor and consequently modify the natural course of Treg cell activation (36). Thus, conducting studies to evaluate the characteristics of these genetic variations in different contexts is important, with the goal of producing and/or substantiating knowledge of disease-related immunogenetic interactions.

The **T* variant *−2383C>T* was associated with increased FOXP3 expression in the present study; we expected that its frequency would be high in the group with viral hepatopathy. Higher *FOXP3* levels may lead to an intense regulatory response in the liver, resulting in a decline in the virus-specific immune response and viral recrudescence, which prevents collateral damage induced by excessive immune activation but may be a factor leading to the maintenance of chronic infection (15, 58, 59). However, we observed that the genotype and allele frequencies of the **C* variant were most prevalent among the analyzed groups, which also had repercussions for the sequences of the obtained haplotypes. In addition, no significant differences were identified between the genetic variants relative to the virological, histopathological and liver function characteristics, suggesting that this polymorphism might not directly influence the modulation of the immune response and/or hepatic immunotolerance via FOXP3-dependent pathways in patients with chronic viral liver diseases.

Although Chen et al. identified an association of the *CT* and *TT* genotypes with the recurrence of HBV-mediated hepatocellular carcinoma, the authors suggested a closer investigation into the incidence of tumors and its relation to the polymorphism was necessary to confirm if this relationship was due to the infection or carcinoma itself (43). Based on data from the present study, the polymorphic variants of this SNP are not determinants for the establishment of infection or its progression, since associations with aspects of chronic hepatitis B were not confirmed or established. We propose that subsequent studies evaluate the role of this polymorphism

TABLE 4 | Frequency of polymorphisms in the *FOXP3* gene promoter region according to the histopathological characteristics of patients with chronic hepatitis B and C virus infections.

Infection and genetic profile	Degree of inflammatory activity		*p1*	Degree of liver fibrosis			*p2*	*p3*	*p4*
	Absent to mild	Moderate to severe		Absent to mild	With few septa	Advanced to cirrhosis			
	n (%)	*n* (%)		*n* (%)	*n* (%)	*n* (%)			
−2383C>T									
CHC									
CC	35 (87.50)	26 (92.86)	0.6147	24 (88.89)	16 (94.12)	32 (91.43)	0.6808	0.5221	1.0000
CT	4 (10.00)	2 (07.14)		2 (07.41)	1 (05.88)	3 (08.57)			
TT	1 (02.50)	0		1 (03.71)	0	0			
CHB-I									
CC	6 (66.67)	–	–	6 (66.67)	–	–	–	–	–
CT	0	–		0	–	–			
TT	3 (33.33)	–		3 (33.33)	–	–			
CHB-A									
CC	13 (86.67)	5 (100.00)	0.7014	8 (88.89)	8 (88.89)	8 (100.00)	0.3973	1.0000	1.0000
CT	1 (06.67)	0		1 (11.11)	0	0			
TT	1 (06.67)	0		0	1 (11.11)	0			
−3279C>A									
CHC									
CC	31 (77.50)	20 (71.42)	0.6840	22 (81.48)	11 (64.70)	27 (77.14)	0.3043	0.7484	0.6046
AC	6 (15.00)	4 (14.29)		4 (14.81)	3 (17.65)	5 (14.29)			
AA	3 (07.50)	4 (14.29)		1 (03.70)	3 (17.65)	3 (08.57)			
CHB-I									
CC	7 (77.78)	–	–	7 (77.78)	–	–	–	–	–
AC	1 (11.11)	–	–	1 (11.11)	–	–			
AA	2 (22.22)	–	–	2 (22.22)	–	–			
CHB-A									
CC	10 (66.67)	4 (80.00)	0.3131	5 (50.00)	7 (87.50)	6 (66.67)	0.0943	0.5462	0.1253
AC	1 (06.67)	1 (20.00)		1 (10.00)	1 (12.50)	0			
AA	4 (26.66)	0		4 (40.00)	0	3 (33.33)			
−924A>G									
CHC									
AA	18 (45.00)	13 (46.43)	0.9920	9 (33.33)	9 (52.94)	19 (54.29)	0.4320	0.2669	0.9562
AG	9 (22.50)	6 (21.43)		8 (29.63)	3 (17.65)	7 (20.00)			
GG	13 (32.50)	9 (32.14)		10 (37.04)	5 (29.41)	9 (25.71)			
CHB-I									
AA	3 (30.00)	–	–	3 (30.00)	–	–	–	–	–
AG	1 (10.00)	–		1 (10.00)	–	–			
GG	6 (60.00)	–		6 (60.00)	–	–			
CHB-A									
AA	4 (25.00)	2 (40.00)	0.8404	1 (10.00)	5 (55.56)	2 (20.00)	0.1108	0.7890	0.3130
AG	4 (25.00)	1 (20.00)		3 (30.00)	1 (11.11)	2 (20.00)			
GG	8 (50.00)	2 (40.00)		6 (60.00)	3 (33.33)	6 (60.00)			

p1, Inflammation absent to mild vs. moderate to severe; p2, Fibrosis absent to mild vs. with few septa; p3, Fibrosis absent to mild vs. advanced to cirrhosis; p4, Fibrosis with few septa vs. advanced to cirrhosis.

in hepatocarcinogenesis, since it has been associated with susceptibility to other cancer types (60). Furthermore, in a carcinogenic microenvironment, Treg expansion favors the maintenance of anti-cancer immunity (61–63), which may explain the possible effects of this polymorphism.

In the present study, the *C allele of the *−3279C>A* polymorphism was significantly more prevalent in patients with chronic hepatitis C infection. In fact, previous studies have indicated that this polymorphism is a genetic factor that predisposes patients to susceptibility and/or progression

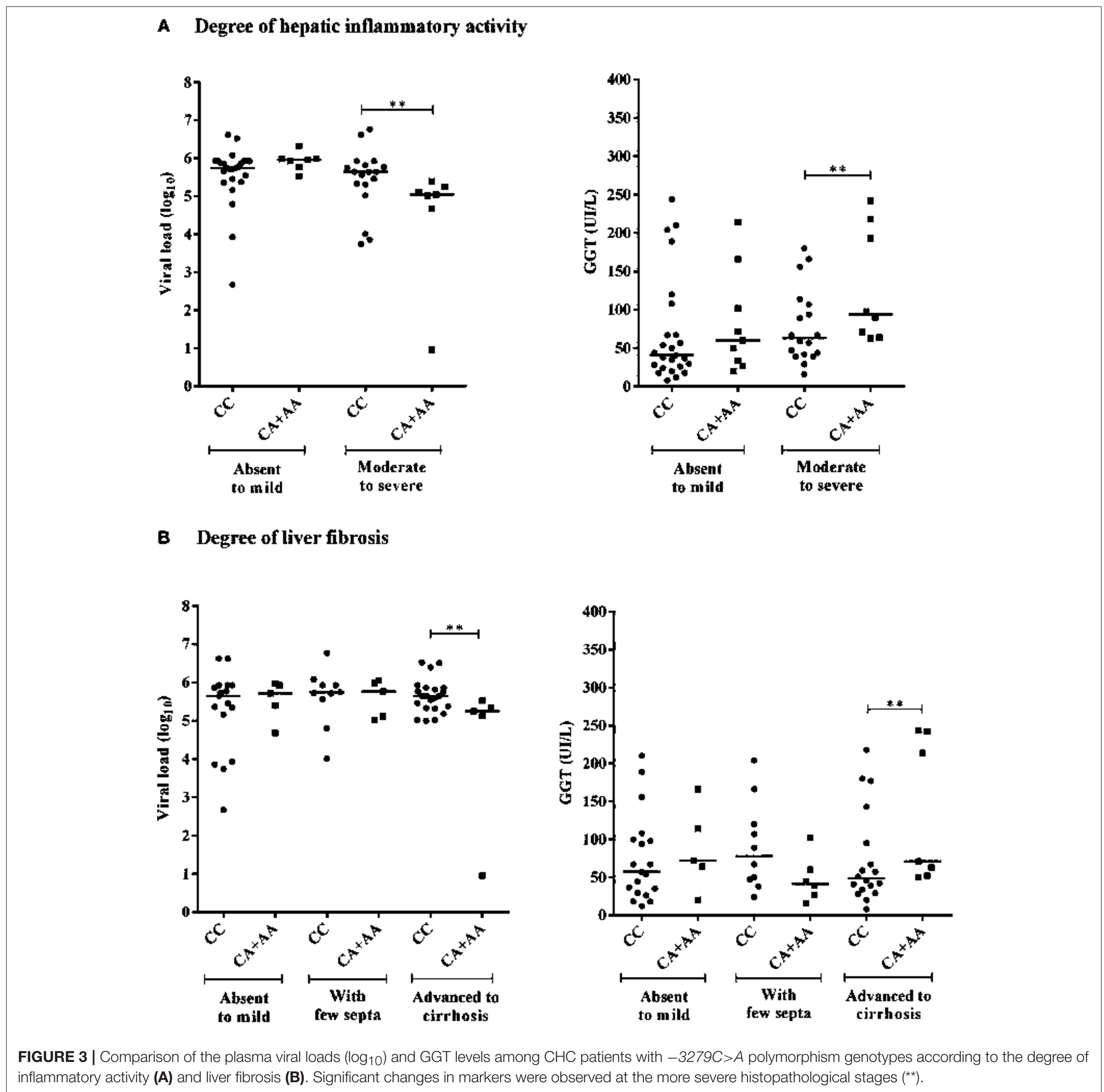

FIGURE 3 | Comparison of the plasma viral loads ($\log_{10}$) and GGT levels among CHC patients with *−3279C>A* polymorphism genotypes according to the degree of inflammatory activity **(A)** and liver fibrosis **(B)**. Significant changes in markers were observed at the more severe histopathological stages (**).

of certain pathological conditions by favoring *FOXP3* gene expression (44, 45, 48, 64–66). Therefore, if the **C* variant maintains *FOXP3* gene expression, this variant may participate in the immunopathogenesis of chronic hepatitis C infection, likely through activation of intrahepatic Tregs. This possibility may explain why these cells are abundant in lymphocytic infiltrates in the portal space and hepatic lobes (58).

This finding was in agreement with the low viral loads and high GGT levels in **A* allele carriers based strictly on the high degrees of inflammation and fibrosis represented by the clusters inferred in the heatmap matrices. If the polymorphism limits the activation and/or expansion of Tregs, a decrease in the suppressive function of these cells and an imbalance between liver damage and control of viral replication are likely to occur (67–69). Thus, in individuals with the **A* allele, these possible alterations in the regulatory response may induce maintenance of a cytolytic inflammatory response capable of leading to a decreased viral load and increased tissue damage in the hepatic microenvironment. The association with the GGT enzyme further supports the identified results, because it is

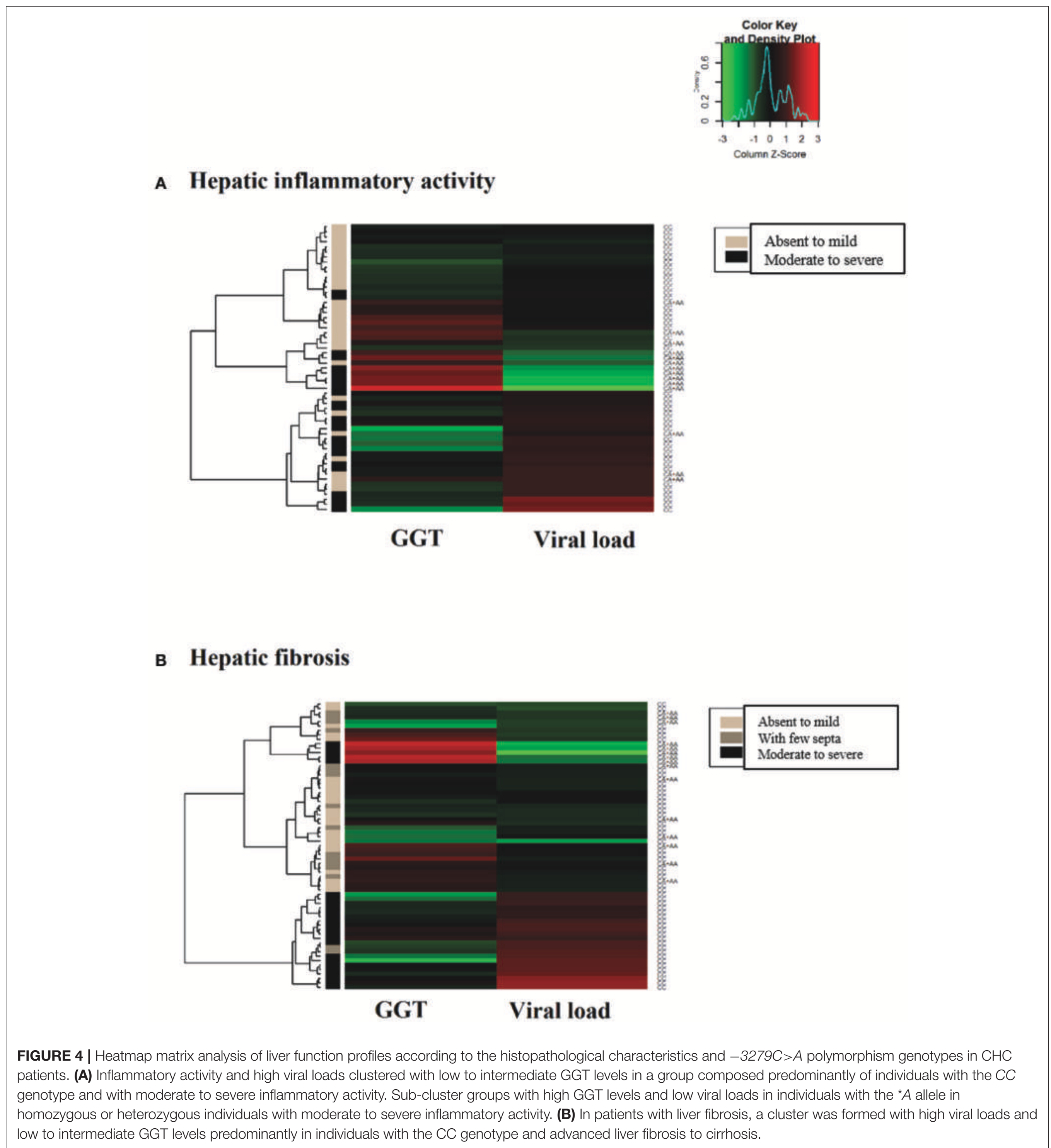

FIGURE 4 | Heatmap matrix analysis of liver function profiles according to the histopathological characteristics and *−3279C>A* polymorphism genotypes in CHC patients. **(A)** Inflammatory activity and high viral loads clustered with low to intermediate GGT levels in a group composed predominantly of individuals with the *CC* genotype and with moderate to severe inflammatory activity. Sub-cluster groups with high GGT levels and low viral loads in individuals with the **A* allele in homozygous or heterozygous individuals with moderate to severe inflammatory activity. **(B)** In patients with liver fibrosis, a cluster was formed with high viral loads and low to intermediate GGT levels predominantly in individuals with the CC genotype and advanced liver fibrosis to cirrhosis.

a marker clinically employed in the analysis of liver fibrosis, especially when there is blockage of the bile ducts and intense necroinflammatory activity (70–73).

The literature also provides data that correlate *FOXP3* expression and the presence of Treg cells with higher levels of inflammatory activity and the degree of liver fibrosis in chronic viral hepatic diseases (74–76). Thus, in patients with chronic hepatitis C infection, the *−3279C>A* polymorphism may mediate Treg activation in more advanced stages of liver inflammation and fibrosis, as shown by the relationships established in the present study. However, since the frequency of polymorphic variants was not associated with the degrees of

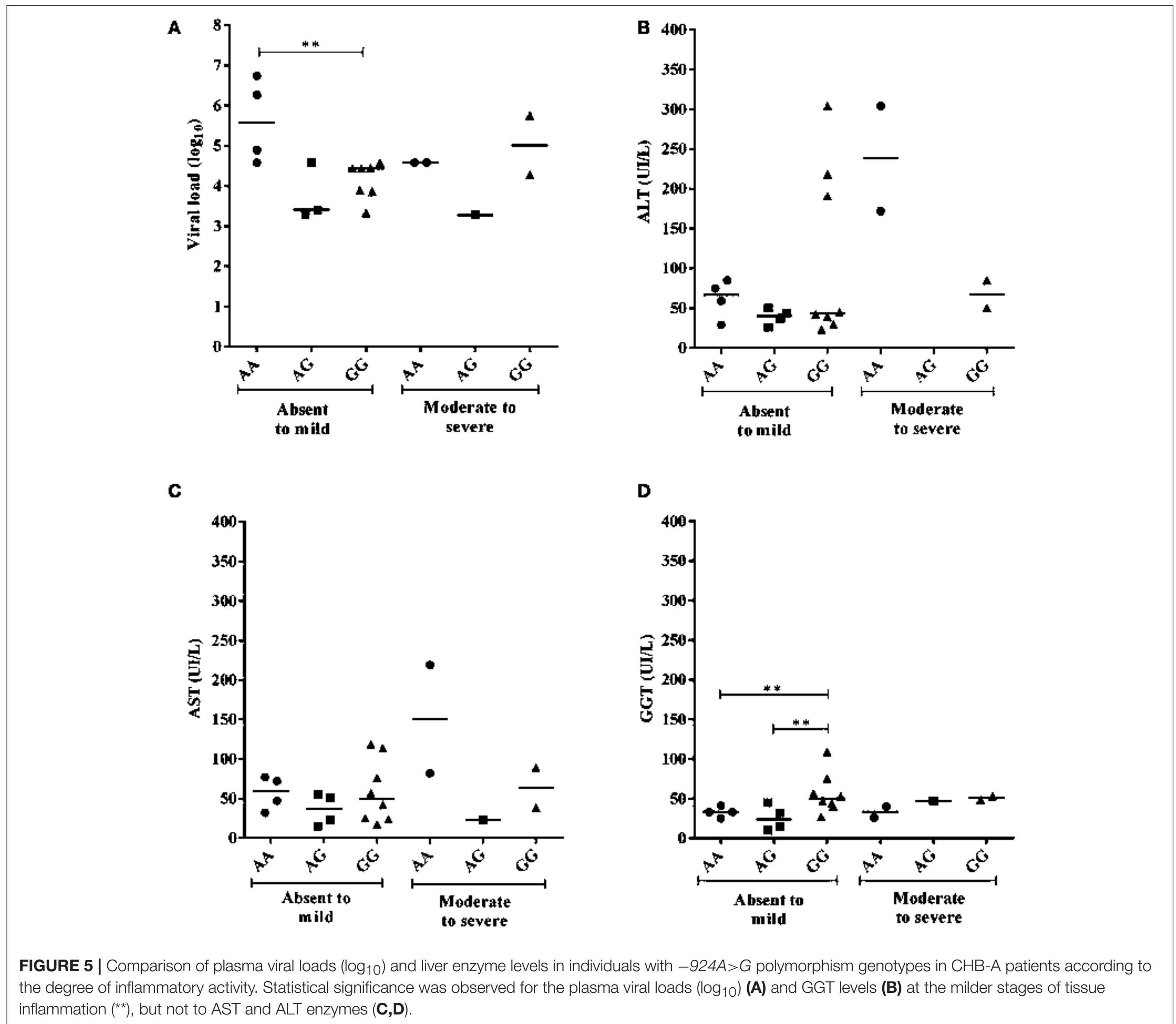

FIGURE 5 | Comparison of plasma viral loads ($\log_{10}$) and liver enzyme levels in individuals with *−924A>G* polymorphism genotypes in CHB-A patients according to the degree of inflammatory activity. Statistical significance was observed for the plasma viral loads ($\log_{10}$) **(A)** and GGT levels **(B)** at the milder stages of tissue inflammation (**), but not to AST and ALT enzymes (**C,D**).

inflammatory activity and liver fibrosis, we propose that this polymorphism may be influencing certain aspects of immune modulation during chronic hepatitis C infection but is not directly related to establishment of the histopathological scores *per se*. This proposal emphasizes the multifactorial nature of the pathological condition and the evolution of liver damage, which is not restricted to only the host-parasite relationship but also influenced by metabolic, physiological and toxicity exposure conditions (77).

For the *−924A>G* polymorphism, the frequency of the **G* allele was significantly lower in the CHB-A group than in patients with chronic hepatitis C infection. Simultaneously, patients with the *GG* genotype had lower viral loads and higher hepatic enzyme levels in the milder stages of the histopathological characteristics of the disease, as represented in the clusters plotted in the heatmaps. Changes in the balance of the Th1 and Th2 profiles generated by the allelic variants of this polymorphism have been proposed to be fundamental for the maintenance of certain pathological conditions (40, 45, 65) and may also influence chronic hepatitis B carrier status based on the data obtained in the present study.

In active hepatitis B virus infection, the Treg-Th2 conversion induced by the **A* variant most likely contributes to the development of a proinflammatory Th1 response in hepatic tissue. In fact, Th2 induction favors the persistence of infection due to stimulation of the production of anti-inflammatory cytokines, such as IL-4 and IL-10 (78), which can reduce tissue damage and decrease inflammatory activity, as proposed by the data reported in the present study.

Conversely, in the absence of Th2-specific signaling, FOXP3+ Treg cells can express high levels of IFN-γ and T-bet, which are Th1-specific transcription factors, making them susceptible to

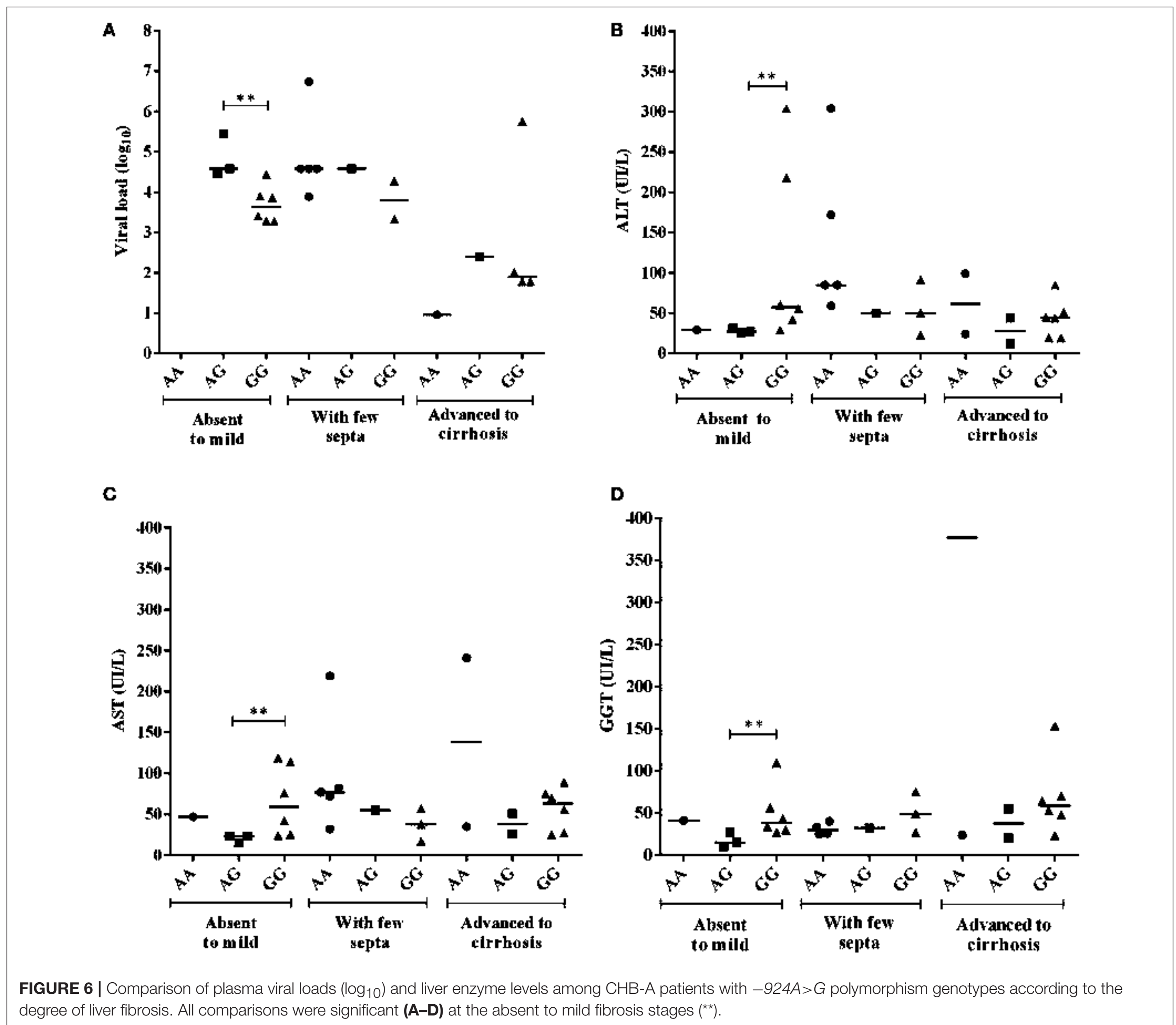

FIGURE 6 | Comparison of plasma viral loads (log_{10}) and liver enzyme levels among CHB-A patients with *−924A>G* polymorphism genotypes according to the degree of liver fibrosis. All comparisons were significant **(A–D)** at the absent to mild fibrosis stages (**).

conversion to the Th1 profile (79, 80). Reportedly, blockage of the Treg-Th2 conversion generated by the *G allele of the *−924A>G* polymorphism (76) can promote an immune balance favorable to the development of a Th1 profile, which is considered crucial for the control of viral replication by tissue inflammation (81, 82). This situation would lead to tissue damage, with a subsequent increase in liver enzyme levels in the blood of these patients.

The relationship established between the *−924A>G* polymorphism and enzymatic and virological characteristics in the early stages of inflammation and liver fibrosis indicates that the gene activation pathway for this polymorphism may be relevant in maintaining mild tissue injury during active hepatitis B infection. This possibility could complement the proposed negative correlation between the frequency and function of Treg cells in chronic hepatitis B patients with fibrosis and necroinflammatory activity scores (74, 83). However, similar to the *−3279C>A* polymorphism, the frequency of the *−924A>G* variant was not associated with the histopathological characteristic of patients with active hepatitis B. Therefore, these variants may influence the immunopathogenesis of the infection but are likely not directly related to the establishment of the degree of inflammation or tissue damage.

The results of this study highlight the importance of FOXP3 as a modulating factor of viral liver diseases, as polymorphic variations in the *FOXP3* gene can influence the pathology associated with hepatic infection. Indeed, FOXP3 is not expected to have a consistent positive association between genetic variation and a pathological condition in all study populations, which can be exacerbated by differences between ethnic populations (84). The relative risk of a variant associated with a disease is maintained among different ethnic

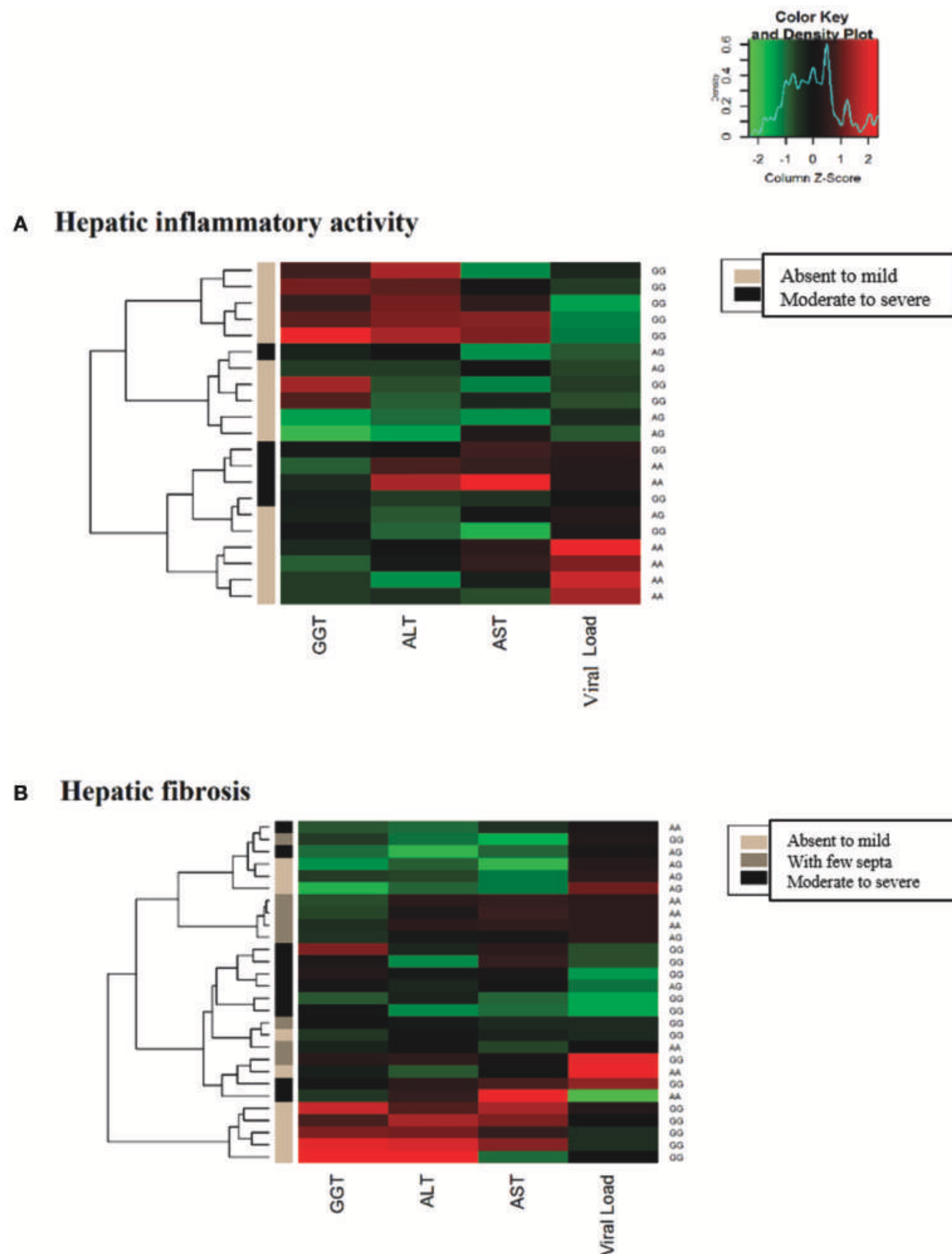

FIGURE 7 | Heatmap matrix constructed to analyze the liver function profiles according to the histopathological characteristics and *−924A>G* polymorphism genotypes in CHB-A patients. **(A)** Regarding inflammatory activity, high hepatic enzyme levels were clustered with low viral loads for patients with the *GG* genotype with mild to absent inflammatory activity. Another cluster was formed among patients with low hepatic enzyme levels and high viral loads in *AA* genotype individuals with low tissue inflammation. **(B)** Regarding liver fibrosis, elevated liver enzyme levels were clustered with intermediate viral loads for *GG* genotype patients with mild to absent fibrosis.

groups if it is a true susceptibility locus. Polymorphisms in the *FOXP3* promoter region have been associated with different pathological features despite differences in some variant frequencies among different ethnic groups. This finding supports the hypothesis that genetic variations in *FOXP3* represent true susceptibility loci and are not solely variants in linkage disequilibrium with the true locus, as has been observed in certain populations due to the structure of the ancestral haplotype (85).

CONCLUSION

Based on the data reported in this study, we conclude that the *−924A>G* and *−3279C>A* polymorphisms in the

FOXP3 gene promoter region alter viral loads and liver enzyme levels in patients with chronic viral liver diseases. The interference of these polymorphisms varies according to the histopathological stage of the hepatic tissue, although they are not direct determinants of the establishment of histopathological characteristics. In summary, the data presented here further scientific knowledge regarding the role of the *FOXP3* gene and its genetic variations in immunological regulation that determines the establishment and/or progression of pathological conditions. Further studies must be conducted to expand these analyses in the context of infectious diseases.

AUTHOR CONTRIBUTIONS

AV, SdSC, and RI conceived and designed the experiments. LP and AV wrote the paper. RM-F, AdS, and JM assisted with editing the paper; LP, SdSC, EA, and SD performed the experiments. AV, LP, AdS, and EA analyzed the data. AV, SD, and RI contributed reagents, materials, and analysis tools.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02014/full#supplementary-material

Supplementary Figure 1 | Comparison of plasma viral loads (log $_{10}$) and liver enzyme levels (ALT, AST, and GGT) among *−2383C*>T polymorphism genotype individuals in CHC patients with absent to mild and moderate to severe inflammatory activity. Reference values: ALT (8-54 IU/L), AST (16-40 IU/L), and GGT (8-63 IU/L).

Supplementary Figure 2 | Comparison of plasma viral loads (log $_{10}$) and liver enzyme levels (ALT, AST, and GGT) among *−2383C*>T polymorphism genotype individuals gene in CHC patients with absent to mild liver fibrosis, fibrosis with few septa and advanced fibrosis to cirrhosis. Reference values: ALT (8-54 IU/L), AST (16-40 IU/L), and GGT (8-63 IU/L).

Supplementary Figure 3 | Comparison of plasma viral loads (log $_{10}$) and liver enzyme levels (ALT, AST, and GGT) among *−2383C*>T polymorphism genotype individuals in CHB patients with absent to mild and moderate to severe inflammatory activity. Reference values: ALT (8-54 IU/L), AST (16-40 IU/L), and GGT (8-63 IU/L).

Supplementary Figure 4 | Comparison of plasma viral loads (log $_{10}$) and liver enzyme levels (ALT, AST and GGT) among *−2383C*>T polymorphism genotype individuals in CHB patients with absent to mild liver fibrosis, fibrosis with few septa and advanced fibrosis to cirrhosis. Reference values: ALT (8-54 IU/L), AST (16-40 IU/L) and GGT (8-63 IU/L).

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Variation in the Untranslated Genome and Susceptibility to Infections

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The clinical outcomes of infections are highly variable among individuals and are determined by complex host-pathogen interactions. Genome-wide association studies (GWAS) are powerful tools to unravel common genetic variations that are associated with disease risk and clinical outcomes. However, GWAS has only rarely revealed information on the exact genetic elements and their effects underlying an association because the majority of the hits are within non-coding regions. Some of the variants or the linked polymorphisms are now being discovered to have functional significance, such as regulatory elements in the promoter and enhancer regions or the microRNA binding sites in the 3′untranslated region of the protein-coding genes, which influence transcription, RNA stability, and translation of the protein-coding genes. However, only 3% of the entire transcriptome is protein-coding, signifying that non-coding RNAs represent most of the transcripts. Thus, a large portion of previously identified intergenic GWAS single nucleotide polymorphisms (SNPs) is in the non-coding RNAs. The non-coding RNAs form a large-scale regulatory network across the transcriptome, greatly expanding the complexity of gene regulation. Accumulating evidence also suggests that the "non-coding" genome regions actively regulate the highly dynamic three dimensional (3D) chromatin structures, which are critical for genome function. Epigenetic modulation like DNA methylation and histone modifications further affect chromatin accessibility and gene expression adding another layer of complexity to the functional interpretation of genetic variation associated with disease outcomes. We provide an overview of the current information on the influence of variation in these "untranslated" regions of the human genome on infectious diseases. The focus of this review is infectious disease-associated polymorphisms and gene regulatory mechanisms of pathophysiological relevance.

Keywords: methylation, promoter, microRNA, lncRNA, polymorphism

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INTRODUCTION

The influence of host genetic polymorphisms on inter-individual variation in disease outcomes has been investigated through candidate gene sequencing and the large scale Genome-wide association studies (GWAS). However, these studies have rarely revealed information on the exact genetic elements underlying an association because majority of the hits are in intergenic regions, some of which are now being discovered to have functional significance. This lack of functional information limits their use as biomarkers or potential targets for therapy.

Despite the high resolution and specialized genotypic platforms and the wealth of data, which allows genotype imputations (1), causal genetic variations have remained elusive. Surveys of several published GWAS indicate a multitude of disease-associated polymorphisms in enriched in non-coding regions (2), regulatory elements (3) and expression quantitative trait loci (eQTL) (3, 4), suggesting that their associations with diseases are due to their involvement in regulation of gene expression. Only about 10% of the disease associated single nucleotide polymorphisms (SNPs) in GWAS affect protein-coding sequences (2). The majority of non-coding SNPs are in the deoxyribonuclease I (DNase I) hypersensitive sites (2). These SNPs may affect binding of transcription factors to the promoter and enhancer regions and transcription of the downstream genes. It is complicated to delineate functions of enhancer region polymorphisms as they can be defined mostly by the epigenomic profile like methylation, chromatin accessibility, histone modifications and expression of enhancer RNAs (eRNAs)(2, 5–7). 3D chromatin structure has only begun to be studied since the turn of the millennium beginning with the development of the chromosome conformation capture (8). Growing literature indicates that disease associated SNPs in the non-coding genome contribute to gene regulation through 3D interactions (9, 10). A small percent of SNPs in the $3'$ untranslated region ($3'$UTR) influence post-transcriptional mRNA decay or translation by altering the microRNA (miRNA) binding sites.

Recently, another class of non-coding RNAs, termed long non-coding RNAs (lncRNAs) have been discovered. The lncRNAs regulate a number of cellular and developmental processes (11–13). The majority of the transcriptome is made up of these non-coding RNAs. Thus, it is predicted that a sizable portion of previously identified intergenic GWAS SNPs could be marking variation in function or expression of non-coding RNAs (14, 15).

Here we will review the influence of variation in the non-coding genome on transcriptional or post-transcriptional regulation of gene expression and infectious disease outcomes.

PROMOTER REGULATORY ELEMENTS

Polymorphisms in the DNA regulatory regions modulate the epigenetic alterations and transcription factor binding resulting in variations in gene expression and immune responses. Cytokines, chemokines and their receptors are the key regulators of immune response and inflammation. Promoter polymorphisms in these genes are associated with several infectious diseases. Interleukin 10 (*IL-10)* promoter polymorphism is associated with increased mortality in severe sepsis (16), susceptibility to chronic hepatitis C virus (HCV) infection, resistance to antiviral therapy (17), and predisposition to Epstein Barr virus (EBV) infection (18). *IL-8* promoter polymorphisms is associated with IL-8 release and incidence of virus bronchiolitis (19). Genomic variations in the promoters of cytokines and other innate immune genes have been linked to susceptibility to *Mycobacterium tuberculosis* (*M.tb*) infection (20). Promoter variations in the chemokine (21) and chemokine receptor genes are known to influence the course of HIV infection (22–24). Polymorphism in a TF binding site of *HLA-C* promoter associated with HIV control (25).

Some of the gene regulatory polymorphisms alter the DNA methylation pattern. A methyl group is added to the nucleotide cytosine, which is followed by a guanine to form a CpG dinucleotide (26). Short stretches of DNA with frequent CpG dinucleotides termed CpG islands are mainly located near the promoters of genes. Variation in the promoter methylation of *CCR5* (27) and human leukocyte antigen (*HLA-A)* genes have been shown to significantly impact outcomes of human immunodeficiency virus (HIV) infection (28).

SPLICING

Precise splicing of mRNAs is critical for its translation and functioning of the resulting protein. Alternative splicing is often employed by the cells to generate transcript diversity (29, 30). Splicing is orchestrated by the complex interaction between spliceosomes and intronic splicing signals. Spliceosomes are complexes of small ribonucleoproteins (snRNPs), which interact with intronic splicing signals like donor and acceptor sites, polypyrimidine tract, branch points like enhancers and silencers of splicing. Sequence variation in these splicing signals affect mRNA processing. A wide range (15–60%) of the human disease related polymorphisms are predicted to alter splicing (31). A SNP in the acceptor site of an antiviral enzyme *OAS1* associates with the level of OAS1 activity and susceptibility to viral infections (32). Intron region polymorphism in *ULK1* associate with decreased expression of the gene, compromised immune responses and associate with increase *M.tb* replication in the latently infected patients leading to the development of pulmonary TB (33). The SNPs in the splice sites of *PLCXD3* showed significant association with prion mediated sporadic Creutzfeldt-Jakob's Disease (34).

MICRORNA

MicroRNAs are small, 22 nucleotide RNAs associated with RNA-induced silencing complexes (RISC) and target specific mRNAs for degradation or inhibition of translation. The genomic variation in miRNA or the miRNA binding site in the target genes have been implicated in the differential susceptibility and clinical manifestations of infectious disease. The SNPs in host miRNA loci have been associated with susceptibility to leprosy, clearance of hepatitis B virus (HBV), human cytomegalovirus (hCMV) infection (35–39), the prion mediated spontaneous Creutzfeldt-Jakob's Disease and fatal familial insomnia (40).

A mutation in the miRNA binding site can disrupt binding to miRNA to its target thus allowing the target to be expressed at higher levels. MicroRNA binding site polymorphisms have been implicated in susceptibility or prognosis of infection. The functional effect of some of these associations have been validated. High levels of *HLA-C* mRNA and cell surface

expression associate with control HIV viremia and slower progression to acquired immunodeficiency syndrome (AIDS) (41, 42). The allele specific expression variation of *HLA-C* is partly explained by a polymorphic miR-148a binding site encoded in the 3′ UTR of *HLA-C*. The alleles with disrupted miRNA binding site escape regulation by miR-148a, are expressed at higher levels and associate with lower HIV viral loads (43). A functional SNP, within the 3′ UTR of *IFNL3* is in a binding site of HCV-induced cellular miRNAs. The allele, which allows escape of miRNA mediated downregulation associates with an increase in *IFNL3* miRNA expression and showed significant association with natural and therapy-induced HCV clearance (44).

Host miRNAs target the HIV transcripts and inhibit translation resulting in silencing of HIV gene expression in resting CD4+ T cells (45), whereas HCV requires host miR-122 for replication (46). Pathogens deregulate the host miRNA expression to their advantage, such as in Zika virus in astrocytes (47), *Mycobacterium leprae* as well as *M. tb* infection (48, 49); or directly target the host transcriptome with a miRNA encoded in their own genome, such as in Rotavirus (50).

LONG NON-CODING RNAs

The long non-coding RNAs are >200 bp transcripts without protein-coding potential. LncRNAs have been shown to enhance or repress the transcription of protein-coding genes, including immune-related genes (51–53), implicating a role for lncRNAs in disease outcomes through gene regulation. The majority of lncRNAs are found in low abundance and localized in the nucleus (54), where they participate in transcriptional regulation through diverse mechanisms (55, 56). However, some localize to the cytoplasm, where they function as competitive endogenous RNAs that can act as "sponges" for miRNAs (57). Thus, the lncRNAs form a large-scale regulatory network across the transcriptome (58). LncRNA gene variations are associated with disease outcomes, and molecular mechanisms have been delineated for a few in cancer (59) and autoimmune disease (60). A SNP in a lncRNA expressed in neutrophils was associated with doubled risk of *Pneumococcal bacteremia* in Kenyan children (46). Polymorphisms in lncRNAs are associated with tuberculosis susceptibility (61). A variant in the lncRNA gene has been shown to confer susceptibility to HBV-related carcinoma in Chinese population (62). Several viruses encode lncRNAs in their genome (63) and modulate host responses. A few known examples are arthropod-borne flaviviruses (64), Kaposi's sarcoma-associated herpesvirus (65) and HIV (66–68). Pathogens like influenza A (69), HCV (70), adenovirus (71), herpes simplex virus [HSV-1] (72), HIV (13, 73–75) and *M.tb* (76–80) alter expression of the host lncRNAs upon infection. An interferon-independent host lncRNA promotes viral replication by modulating cellular metabolism (81). The expression and functional variations within the lncRNA genes and their role in regulating immune response and inflammation is an area of intense research and likely to discover novel pathways of host-pathogen interactions.

MULTIDIMENSIONAL REGULATION AND COMPLEX GENE INTERACTIONS

The variation in gene expression levels are determined by several transcriptional, post-transcriptional and post-translational processes (**Figure 1**). A single variant or haplotype may not be the sole contributor to disease associations. This is exemplified by a few genetic associations for which molecular mechanisms have been delineated.

GWAS implicated multiple linked variations at the interferon lambda (*IFNL*) in HCV clearance (82, 83). A 3′UTR variant in *IFNL3* disrupting miRNA binding site and increasing stability of *IFNL3* mRNA was found to be protective (44). A strongly linked dinucleotide variant, which introduces a premature stop codon in another interferon gene, *IFNL4* associated with HCV clearance (84). Thus linked genotypes conferring high expression of IFNλ3 and truncated, non-functional IFNλ4 associated with HCV clearance. This paradoxical association of expression of the full length antiviral protein IFNλ4 with HCV persistence remained unexplained. Recently, it was found that the full length IFNλ4 isoform is poorly expressed due to aberrant splicing resulting in intron retention and a weak polyadenylation signal (85). The mRNA instability genotype in the *IFNL3* 3′UTR is in strong linkage disequilibrium (LD) with the full length expression genotype of *IFNL4* suggesting that the persistence of HCV is likely attributed to the impaired expression and anti-viral function of IFNλ3.

The *HLA* genes located on human chromosome 6 are extremely polymorphic, exhibit strong LD and allele-specific expression patterns. The nucleotide diversity observed within the *MHC* region, particularly within the regulatory region upstream of the initiation codon of certain class I genes, has been observed about 20-fold higher than the genome average (86). Variations within the *HLA-C* 3′UTR (43) and promoter (25) showed a strong influence on *HLA-C* mRNA and protein expression, which associates with HIV control. Both the causal variations in the 3′UTR and promoter only partially account for the allele specific gene expression variation, indicating that yet unknown variation either within the *HLA-C* gene region or elsewhere in the genome is responsible for a large part of HLA-C expression.

INTERPLAY BETWEEN GENOMIC VARIATIONS AND EPIGENOMIC MODULATIONS

Specific epigenetic changes could result in switching on or off certain genes, and determines which proteins are transcribed. In some cases, several factors have been shown to contribute to causal phenotype in addition to the genomic variation. A recent study investigated role of a SNP in 5′UTR of the gene encoding an antiviral protein, Interferon-induced transmembrane protein 3 (IFITM3), which inhibits entry of influenza virus into the host cell. The risk allele was found to disrupt a CpG site, decrease binding of a transcription factor, interferon regulatory factor (IRF3) and increase binding of CCCTC-binding factor (CTCF) leading to lower expression of

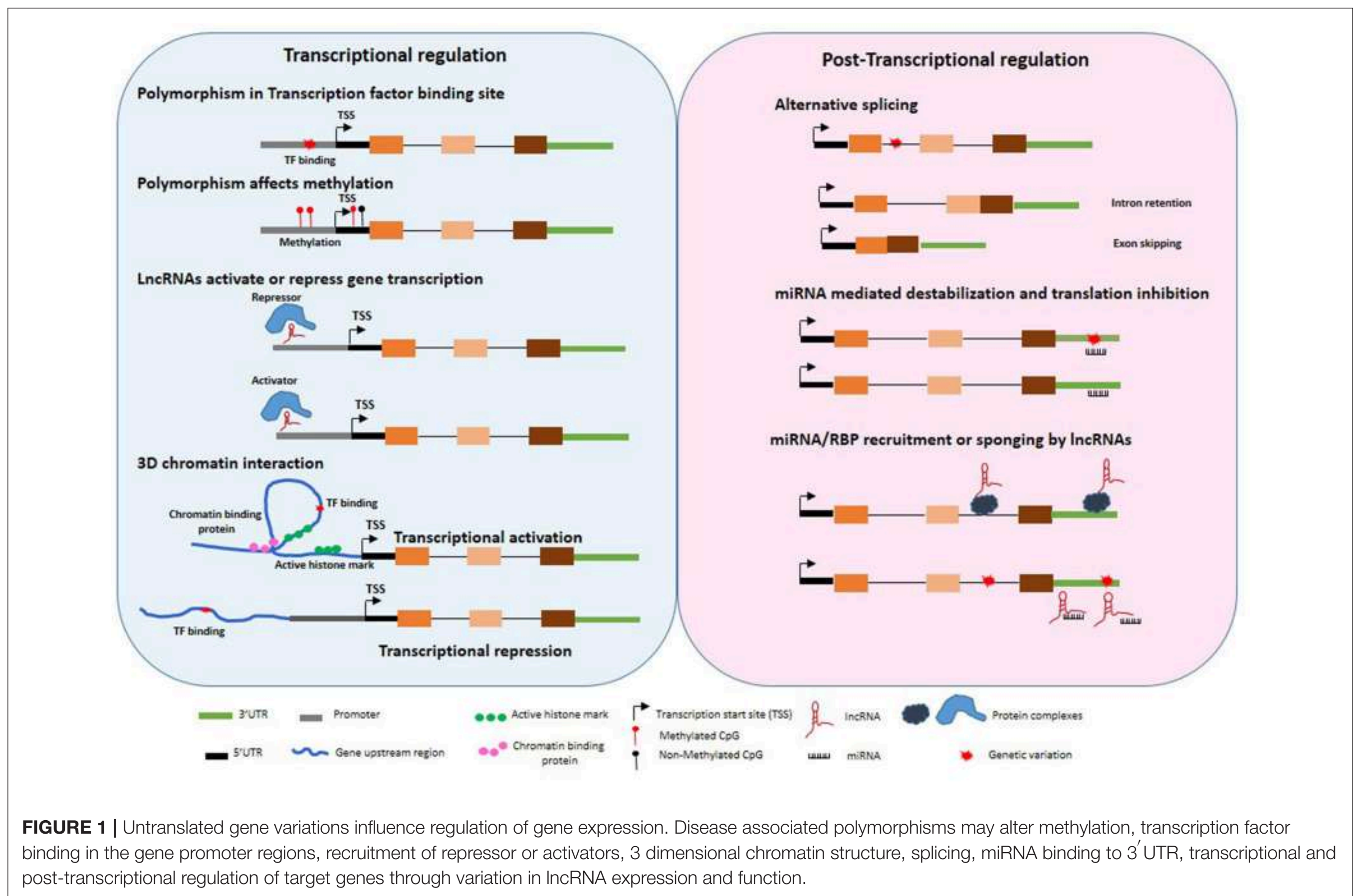

FIGURE 1 | Untranslated gene variations influence regulation of gene expression. Disease associated polymorphisms may alter methylation, transcription factor binding in the gene promoter regions, recruitment of repressor or activators, 3 dimensional chromatin structure, splicing, miRNA binding to 3′UTR, transcriptional and post-transcriptional regulation of target genes through variation in lncRNA expression and function.

IFITM3 expression, altered methylation and also affected the expression of *IFITM3* neighboring genes important in anti-viral response. CTCF is a versatile protein important in maintaining topologically associating domains of chromatin and contributing to 3D chromatin structure. CTCF has also been shown to function as an "insulator" blocking chromatin positioning and inhibiting interaction of enhancer with promoters. Increased CTCF binding associated with the IFITM3 risk allele and disruption of neighboring gene expression patterns indicative of CTCF boundary activity.

DNA methylation and histone modification are just two of the epigenetic mechanisms widely described (87). Examining certain genes, we observe the balancing act between genomics and epigenomics within infectious diseases. One such example is the *HLA-A* gene, where specific *HLA-A* alleles are observed to possess individual HIV allelic effects, based on the peptide presented (88). However, more recently it has been shown *HLA-A* alleles possess distinct allele-specific promoter methylation and mRNA expression levels (89). Furthermore, examining nearly 10,000 HIV patients across diverse ethnic backgrounds, Ramsuran *et al* showed that *HLA-A* mRNA expression levels play an important role in HIV viral control (28). The patients with high expression of *HLA-A* showed impaired HIV control. Unlike the protective effect of high HLA-C expression on HIV viral control and contrary to conventional understanding, elevated levels of *HLA-A* do not protect against HIV but rather lead to accelerated disease progression by increasing cell surface expression of a natural killer (NK) ligand, HLA-E and stronger inhibition of NK cells (**Figure 2**). HLA-E serves as a ligand for the strong inhibitory NK receptor CD94/NKG2A. A signal peptide derived from the leader sequence of *HLA-A*, *-B* and *-C* molecules stabilizes HLA-E expression on the cell surface (90). The signal peptide has a variation at position 2 (residue−21) resulting in either a methionine (-21M) or threonine (-21T). A methionine (-21M) stabilizes and increases HLA-E expression on the cell surface, while the presence of−21T results in lower HLA-E expression. High expression of HLA-A provides elevated levels of HLA-A derived signal peptide increasing HLA-E expression resulting in strong NK inhibition and impaired killing of HIV infected cells. Presence of HLA-B alleles with−21M results in strong "licensing" of NK cells and further exacerbates the inhibitory effects of high HLA-A expression on NK cell activity and results in poor HIV control. Thus, variable HLA-A expression levels in combination with either−21M or −21T HLA-B alleles lead to diverse HIV outcomes (28).

Variability within the *HLA-A* peptide binding region (exons 2 and 3) of the genomic sequence allows specific alleles to slow or accelerate HIV disease progression. However, epigenetic mechanisms alter the expression levels of these alleles, which also causes delayed or rapid HIV progression. This balance between

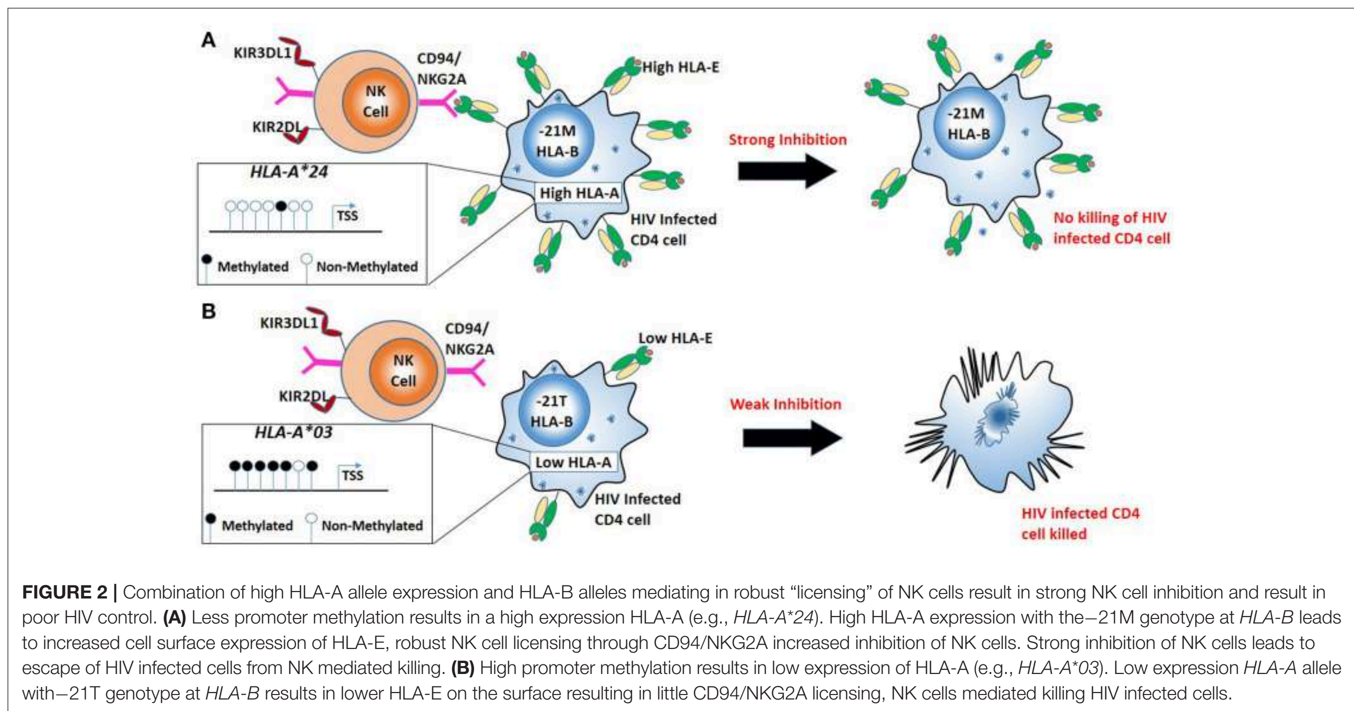

FIGURE 2 | Combination of high HLA-A allele expression and HLA-B alleles mediating in robust "licensing" of NK cells result in strong NK cell inhibition and result in poor HIV control. **(A)** Less promoter methylation results in a high expression HLA-A (e.g., *HLA-A*24*). High HLA-A expression with the−21M genotype at *HLA-B* leads to increased cell surface expression of HLA-E, robust NK cell licensing through CD94/NKG2A increased inhibition of NK cells. Strong inhibition of NK cells leads to escape of HIV infected cells from NK mediated killing. **(B)** High promoter methylation results in low expression of HLA-A (e.g., *HLA-A*03*). Low expression *HLA-A* allele with−21T genotype at *HLA-B* results in lower HLA-E on the surface resulting in little CD94/NKG2A licensing, NK cells mediated killing HIV infected cells.

genetic and epigenetic events makes this region one of the most complex multifactorial sites associating with a range of diseases.

3D CHROMATIN STRUCTURE

Over half of the significant associations in the published GWAS were reported in genome expanses lacking annotated genes in the large regions (>500 kb) flanking associated SNP. Such regions, termed "gene deserts" have been implicated in the regulation of genome function through their influence on intricate 3D genome structure. The 3D architecture of the genome facilitates spatial juxtaposition of and interaction between distant loci, which contributes to gene regulation. A SNP on chromosome 8q24 in a gene desert of 1.2Mb has been shown to affect long range (>300Kb) interactions of a cancer gene, Myc (9, 10). SNPs associated with type 2 diabetes have been implicated in 3D genome interactions leading to impaired interferon signal responses (10). Although 3D chromatin structure changes after infection have been reported in a few cases (91–95), studies on 3D genome structure are underrepresented in the field of infectious diseases.

PERSPECTIVE AND FUTURE DIRECTIONS

Gene association studies and GWAS have identified several genetic variants, which influence outcomes of complex human disease including infections. However, the majority of the disease-associated variants map to the non-coding regions of the genome. Discovery and functional validation of causal variants and the specific regulatory mechanisms are needed to advance understanding of the molecular pathways and their possible manipulation by therapy. Current literature reveals a number of efforts in determining the causal SNPs from the variants identified in the GWAS and linked SNPs. Disease-associated SNPs are further investigated to determine if they are marking coding-region variants through strong LD or are likely to alter promoter and enhancer region, splicing, miRNA binding, or lncRNA expression and function. The possible candidates are examined in a variety of *in silico* and molecular and biochemical analyses, including prediction of transcription factor or miRNA binding sites, chromatin or RNA immunoprecipitations, nucleic acid sequencing and mass-spectrometry to identify DNA, RNA or protein factors interacting with the genomic variation or the resulting RNA and protein products. Loss or gain of function studies are carried out to determine functional consequences of the RNA or protein target. Distinct regions in the genome (promoter, $3'$UTR, intron, miRNA, lncRNA) require diverse molecular and biochemical techniques. Although these focused, gene-specific studies discover novel molecular mechanisms of gene regulation, a fresh approach is needed to detangle multilayered gene regulatory pathways and complex gene interactions, which are often cell-type and context-specific (infected vs. uninfected).

Interpretation of the functional consequences of a disease associated gene variation can be complex, especially in case of the intergenic SNPs as it may affect none or multiple genes and pathways through the expression of non-coding RNAs and chromatin organization. CRISPR/Cas9 mediated editing of the SNP or the putative target region is being increasingly used to address the ambiguity. Cell line based models are more permissive for such manipulations. However, these models may not represent the complex environment of cells and tissues.

Organoid models are being developed to recapitulate the tissue environment and interaction. Multiple genetic variants co-operatively regulate a phenotype (96) Editing of a single gene will not discern this combinatorial effect. Further evolution of gene editing techniques to simultaneously target multiple regulatory regions is needed of the gene editing techniques to simultaneously target multiple regulatory regions. Gene editing techniques are even harder to implement in case of long non-coding RNAs. Manipulation of gene expression through short interfering RNAs (siRNA, shRNAs) and nucleases are limited by off-target effects. Alternatively approaches of nuclease deficient CRISPR/Cas9 (dCas9) mediated inhibition or activation of gene expression are being increasingly applied specially in the field of non-coding RNAs. Epigenetic modulations are hard to recapitulate as the causal and the target regions could be spread over a large genomic distance. The engineered systems utilizing dCas9 collectively called "epigenetic toggle switches" are being used to mimic deletion of enhances or recruitment of epigenetic repressors (97). Full implications of variations that influence 3D chromatin structure are yet to be realized in part due to difficulties in precisely capturing and mapping interactions between distinct regions of chromatin. However, the field has exploded with multiple complementary techniques like Hi-C, ChIA-PET, 3D-FISH, which help capture both the short and the long-range interactions (98). Integrated investigations of disease-associated genetic variations, context-specific gene expression, spatial organization of chromatin and epigenetic alterations in primary human cells, genomic and transcriptomic analysis at tissue and single cell level are needed to unravel the role of untranslated regions in susceptibility to infections.

AUTHOR CONTRIBUTIONS

VR and SK conceptualized the framework and all authors contributed to writing, reviewing and editing of the review.

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Association of *Chlamydia trachomatis*, *C. pneumoniae,* and *IL-6* and *IL-8* Gene Alterations With Heart Diseases

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Atherosclerosis is a progressive disease characterized by chronic inflammation of the arterial walls, associated with genetic and infectious factors. The present study investigated the involvement of *Chlamydia trachomatis* and *Chlamydia pneumoniae* infections and immunological markers (C-reactive protein, CRP, TNF-α, IL-6, IL-8, and IL-10) in the process of atherosclerosis. The evaluation included 159 patients for surgical revascularization (CAD) and 71 patients for surgical heart valve disease (HVD) at three hospitals in Belém, Brazil. The control group (CG) comprised 300 healthy individuals. Blood samples collected before surgery were used for antibodies detection (enzyme immunoassay), CRP (immunoturbidimetry) and IL-6 levels (enzyme immunoassay). Tissue fragments (atheroma plaque, heart valve and ascending aorta) were collected during surgery and subjected to qPCR for detection of bacterial DNA. Promoter region polymorphisms of each marker and relative quantification of *TNF-α*, *IL-8*, and *IL-10* gene expression were performed. Demography and social information were similar to the general population involved with both diseases. Antibody prevalence to *C. trachomatis* was 30.6, 20.3, and 36.7% (in the CAD, HVD, and CG, respectively) and to *C. pneumoniae* was 83.6, 84.5, and 80.3% (in the CAD, HVD, and CG, respectively). *C. trachomatis* cryptic plasmid DNA was detected in 7.4% of the samples. Frequency of *IL6*−174G>C polymorphism was higher in CAD and HVD than in CG regardless of previous exposure to *Chlamydia*. Previous *C. trachomatis* infection showed involvement in HVD and CAD. Significant association between disease and previous *C. pneumoniae* infection was found only among HVD. GG genotype of IL6−174G>C is apparently a risk factor for heart disease, whereas AT genotype of *IL8*−251A>T was mainly involved in valvulopathies, including patients with prior exposure to *C. pneumoniae*.

Keywords: ***Chlamydia pneumoniae***, ***C. trachomatis***, **polymorphisms, IL-6, IL-8, IL-10, TNF, CRP**

INTRODUCTION

Atherosclerosis is a multifactorial condition that is influenced by genetic factors and lifestyle-related risk behaviors (1). Atherosclerosis is a progressive disease characterized by chronic inflammation of the arterial walls, which develops from the accumulation of low density lipoproteins in the arterial wall, oxidative, hemodynamic, biochemical, and inflammatory changes, and highly specific cellular responses (2, 3).

Local or systemic infections may contribute to the atherogenic process (4, 5). In particular, bacterial components of *Chlamydia pneumoniae* have been associated with atheroma plaque genesis and the potential risk of acute myocardial infarction (6, 7). The bacterium is capable of infecting smooth muscle cells, endothelial cells, and human macrophages (8–10). This association has been demonstrated through seroepidemiological, anatomic-pathological, and experimental studies and by demonstration of the presence of the bacteria in arterial walls (6, 7). *Chlamydia* infection in the arterial intima layer causes lesions that stimulate the inflammatory process (11, 12) by inducing elevated levels of immunological markers that influence the stability or progression of atherosclerotic plaques (11–13).

Although endothelial and cardiac muscle cells may not be common targets for *Chlamydia trachomatis* infection, this bacterium can reach the circulatory system through infected monocytes and macrophages. Infection of these cells occurs *in vitro* as evidenced by the presence of intracellular inclusions characteristic of bacterial multiplication (14). Analysis of *C. trachomatis* primary rRNA has also shown the existence of viable and metabolically active microorganisms inside the inclusions (15, 16).

Chlamydia pneumoniae-infected individuals have elevated plasma C-reactive protein (CRP) levels and atheroma plaques located in the carotid artery (17). Polymorphisms in the inflammatory and immune response genes lead to changes in the expression levels of these molecules (18, 19).

The present work investigated the association of immune response gene polymorphisms (*TNF*-308A>G, *IL-6*-174G>C, *IL-8*-251A>T, *IL-10*-1082G>A, and *CRP*-717T>C) and gene expression levels (mRNA and plasma levels) in patients with coronary disease associated with previous *C. pneumoniae* and *C. trachomatis* infections.

MATERIALS AND METHODS

Study Population

A cross-sectional, case-control study was conducted with one group of 159 patients (109 men and 50 women) with coronary artery disease (CAD group) presenting with severe arterial obstruction with or without ischemia and a second group of 71 patients (30 men and 41 women) with heart valve disease (HVD group) presenting with a cardiac volume overload and high blood pressure. The patients with coronary disease had a surgical indication for myocardial revascularization, and the patients with valvulopathy had a surgical indication for valve prosthesis implantation (mitral or aortic).

The inclusion criteria included hospitalized individuals with an indication for one of the surgical procedures for the first time and the absence of antibiotic use. Exclusion criteria included individuals without an indication for surgery or indicated for repeat surgery those using antibiotics in the preoperative period. The samples were collected from November 2010 to July 2012 at the Portuguese Beneficent Hospital, the Ordem Terceira Hospital, and the Gaspar Viana Clinical Hospital Foundation, in the city of Belém, Pará state, Brazil.

A control group (CG) was formed with 300 individual blood donors (150 men and 150 women) from the Pará Hemotherapy and Hematology Foundation Center (HEMOPA) to compare the frequency of antibodies to *Chlamydia*, previous infection with the two species investigated, the frequency of immunological and inflammatory marker polymorphisms, and their gene expression levels (mRNA and plasma levels). The control group was matched with the cardiac patient groups (CAD and HVD) by sex and age and had no history and symptoms suggestive of heart disease.

The project was submitted and approved by the HEMOPA Human Research Ethics Committee (Protocol no. 0011.0.324.000–09). The subjects were informed about the project, and those who agreed to participate signed an informed consent form.

Sample Collection and Storage

A sample of 10 mL of blood was collected by intravenous puncture using a vacuum collection system containing EDTA as the anticoagulant. The samples were processed for the separation of plasma and leukocytes, both of which were stored at −20°C prior to use. Plasma was used for the detection of specific antibodies to *C. pneumoniae* and *C. trachomatis* species and for analysis of the plasma CRP and interleukin 6 (IL-6) levels. Leukocytes were used for genomic DNA extraction to analyze polymorphisms and gene expression. The leukocyte samples used for the gene expression assays were stored in RNAlater® (Invitrogen, Carlsbad, CA, USA) at −70°C.

The following samples were collected from the patients who underwent surgical procedures: (i) fragments of the ascending aorta and when possible and at medical discretion coronary atheroma plaques (endarterectomy) during the revascularization procedure and (ii) mitral and aortic valve fragments during the valve replacement procedure. The samples were stored in tubes containing RNAlater® (Invitrogen, Carlsbad, CA, USA).

Detection of Antibodies to *Chlamydia*

The plasma samples were tested by enzyme-linked immunosorbent assay (ELISA) to detect antibodies against *C. trachomatis* (NovaLisa™ *C. trachomatis* IgM and IgG) and *C. pneumoniae* (NovaLisa™ *C. pneumoniae* IgM and IgG) according to established protocols from the manufacturer (NovaTec, Dietzenbach, Offenbach, Germany).

Identification of the genetic polymorphisms *TNF*-308G>A (rs1800629), *IL6*-174G>C (rs1800795), *IL8*−251A>T (rs4073), *IL10*−1082G>A (rs1800896), and *CRP* -717T>C (rs2794521).

DNA was extracted from the peripheral blood leukocytes using the phenol-chloroform method. The procedure followed the cell lysis, protein precipitation, DNA precipitation, and DNA

hydration steps. The obtained DNA was quantified using the Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and the Qubit™ DNA Assay Kit solutions (Life Technologies, Carlsbad, CA, USA) following the protocol recommended by the manufacturer.

The *TNF* -308G>A, *IL6*−174G>C, and *CRP* -717T>C polymorphisms were assessed using polymerase chain reaction (PCR) followed by RFLP (restriction fragment length polymorphism) analysis. The reactions consisted of amplification of a 107-bp segment of the tumor necrosis factor (*TNF)* gene promoter region, a 169-bp segment of the *IL6* gene promoter region, and a 541-bp sequence of the *CRP* gene promoter region (20). For amplification of the *IL6* and *CRP* gene segments, specific primers were designed for each of the regions of interest using the Primer3 version 0.4.0 and FastPCR version 6.2 softwares based on the human reference sequences for the genes (NC_000001.11 and NC_000007.14, respectively). The reactions were conducted in an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany) in a final 50-μL volume containing 100 ng of extracted total DNA, 200 nM each dNTP, 200 nM each primer (a pair specific for each gene), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 1.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA).

The sequences of the primers used for partial amplification of the *TNF* gene were TNFA-F: 5′-AGGCAATAGGTTTTGAG GGCCAT-3′ and TNFA-R: 5′-TCCTCCCTGCTCCGATTCCG-3′. The following temperature and cycling protocol was used: initial denaturation at 95°C for 5 min, 35 cycles of 30 s at 94°C (denaturation), 30 s at 53°C (hybridization), and 1 min at 72°C (extension), and a final extension of 10 min at 72°C. Amplification of the *IL6* gene segment utilized the primer pair IL6-f: 5′-TTGTCAAGACATGCCAAGTGCT-3′ and IL6-r: 5′-GCCTCAGAGACATCTCCAGTCC-3′. The temperature and cycling conditions were as follows: initial denaturation at 95°C for 5 min; 35 cycles of 30 s at 95°C (denaturation), 45 s at 59°C (hybridization), and 45 s at 72°C (extension); and a final extension of 10 min at 72°C. For the *CRP* gene segment, the primer pair CRP-f: 5′-ACTGGACTTTTACTGTCAGGGC-3′ and CRP-r: 5′-ATTCCCATCTATGAGTGAGAACCT-3′ was used. The temperature and cycling protocol was as follows: initial denaturation at 95°C for 5 min; 40 cycles of 30 s at 95°C (denaturation), 40 s at 60°C (hybridization), and 1 min at 72°C (extension); and a final extension of 10 min at 72 °C.

The RFLP protocol used the *NCO*I, *Nla*III, and *BstU*I enzymes (Invitrogen, Carlsbad, CA, USA) to genotype *TNF* -308G>A, *IL6*−174G>C and *CRP* -717T>C, respectively. The *TNF* -308G>A genotypes were identified by the presence of the 107-bp (AA) and 87-bp + 20-bp (GG) fragments. For *IL-6*-17G>C, the genotypes were identified by fragments of 169 bp (GG) and 87 bp + 80 bp (CC). The *CRP* -717T>C genotypes were identified by the presence of 541-bp (CC) and 297-bp + 244-bp (TT) fragments. The fragments were visualized after electrophoresis (100 V, 45 min) of the amplification product on a 3% agarose gel in 1x TAE buffer (TAE 40x stock – 1.6 M Tris base, 0.8 M sodium acetate, and 10 mg/mL of EDTA) using the SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) and a UV transilluminator (Kasvi, Curitiba, Paraná, Brazil).

Polymorphisms in the *IL8* and *IL10* genes were identified by real-time PCR (qPCR) using TaqMan-SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). The polymorphisms *IL8*−251T>A (rs4073) and *IL10*−1082A>G (rs1800896) were identified using the C_11748116_10 and C_1747360_10 assays, respectively. The reactions occurred in the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's guidelines for an initial incubation of 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.

TNF, *IL8*, and *IL10* Gene Expression

Total RNA was extracted from peripheral blood leukocytes using the Total RNA Purification Kit (Norgen Biotek Corporation, Thorold, ON, Canada). The concentration of the extracted RNA (ng/μL) was obtained using the Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications, and the RNA integrity was examined using a 1% agarose gel stained with ethidium bromide. After the concentration and integrity check, the total RNA samples were normalized to 60 ng/μL for cDNA synthesis using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA was subjected to qPCR for relative quantification of the indicated markers with the Taqman® Gene Expression Assays Hs00174128_m1 (*TNF*), Hs00174103_m1 (*IL8*), and Hs00174086_m1 (*IL10*) in the StepOnePlus Sequence Detector (Applied Biosystems, Foster City, CA, USA) using the *GAPDH* gene as the internal control. The expression levels were calculated using the comparative CT method. For the relative quantification of *TNF* gene expression, 22 samples from patients with CAD, 17 samples from patients with HVD, and 28 samples from the GC were included. For *IL8* expression, 27 samples from patients with CAD, 21 samples from patients with HVD, and 32 samples from the CG were used. For the *IL-10* expression analysis, 27 samples from the patients with CAD, 20 samples from the patients with HVD, and 33 samples from the GC were tested.

Plasma CRP and IL-6 Levels

The CRP levels were measured by immunoturbidimetry using the DiaSys® PCR U-hs kit (DiaSys, Waterbury, CT, USA) on the Architect c8000/Abbott® automated system (Abbott Park, Chicago, IL, USA) in samples from 159 people in the CAD group, 71 in the HVD group, and 196 in the CG group. The IL-6 levels were measured in samples from 19 people in the CAD group, 14 in the HVD group, and 28 CG subjects. Samples were tested in duplicate using the Novex ELISA Human IL-6 kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The samples used for the IL-6 measurement were randomly selected using the BioEstat 5.3 software.

qPCR to Identify *C. pneumoniae* and *C. trachomatis*

DNA was extracted from the aorta, valve, and atheroma plaque samples obtained during surgery using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Mettmann, Germany). The presence of *C. pneumoniae* and *C. trachomatis* was investigated with qPCR using the *C. trachomatis* Q-PCR Alert Kit and the *C. pneumoniae*

Q-PCR Alert Kit (Nanogen Advanced Diagnostic, Trezzano Sul Naviglio, Lombardy, Italy). For the *C. trachomatis* detection kit, a specific amplification reaction was performed for the cryptic plasmid (~7–10 copies/cell). *C. pneumoniae* was identified by amplification of a region of the *ompA* gene. A positive control for each species, a negative control, and an internal control for the β-globin gene were included in all reaction sets. The reactions were performed in a StepOnePlus Sequence Detector (PE Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

The information obtained through the epidemiological questionnaire was inserted into a database created in Microsoft Access version 2007. The serological results among the different groups were compared using the Chi-square test (χ^2). The Hardy-Weinberg equilibrium was calculated to evaluate the genotype frequency distributions. The genotypic and allelic frequencies were calculated by direct counting, and differences between groups were evaluated using the χ^2 and G-tests.

The associations of the plasma gene expression levels of pro- and anti-inflammatory markers with the presence of antibodies to *C. trachomatis* and *C. pneumoniae* were analyzed using the non-parametric Kruskall–Wallis and Mann–Whitney tests. The statistical analysis was performed using the GraphPad Prism version 5.0 and BioEstat 5.3 softwares. Significant associations were considered to have a $p < 0.05$.

RESULTS

Demographic Information, Presence of Antibodies, and Bacterial DNA Detection

The demographic information (**Table 1**) showed that the majority of the patients were men in the CAD group (68.5%) and were women in the HVD group (57.7%). The ages ranged from 36 to 79 years (mean of 60.4 years) in the CAD group and from 14 to 80 years (mean of 45.6 years) in the HVD group. The majority of the CAD group patients were married (69.9%), and the greater frequency of the HVD patients were single (44.9%). In both groups, the most common education level was ≤5 years (79% in CAD and 68.6% in HVD), and the most common family income was ≤3 times the minimum wage (85.5 and 98.6%, respectively). The control group consisted of people aged between 24 and 68 years (mean age 40.3 years).

Antibodies (**Table 2**) for *C. trachomatis* were detected in 30.6% (48/157) of the patients in the CAD group and 20.3% (14/69) of the patients in the HVD group. In the CG, the prevalence was 36.7% (103/281), which was significantly higher than the prevalence in the HVD group ($p = 0.014$). The prevalence rates of antibodies to *C. pneumoniae* in the three groups were 83.6% (133/159), 84.5% (60/71), and 80.3% (237/295), respectively, with no significant differences between groups. All undetermined serology results were excluded from the statistical tests.

Bacterial DNA from the two species was investigated in 81 samples (atheroma plaque, valve, and aorta). The cryptic plasmid DNA of *C. trachomatis* was detected in six (7.4%) samples; four of these samples were derived from aorta fragments and two from the mitral valve (**Table 3**). No DNA was detected in the atheroma plaques. Two samples were positive for anti-*C. trachomatis* IgM. The quantification of the risk measured by the CRP level showed that three samples showed a high risk of heart disease.

TABLE 1 | Demographic and social characteristics of cardiac patients (CAD and HVD) investigated.

Demographic and social characteristics		CAD *N* = 159 *N* (%)	HVD *N* = 71 *N* (%)	CG *N* = 300 *N* (%)
Gender	Male	109 (68.5)	30 (42.3)	150 (50)
	Female	50 (31.5)	41 (57.7)	150 (50)
Age	Change (years)	36–79	14–80	24–68
	Average (years)	60.4	45.6	40.3
Marital status	Single	20 (13.1)	31 (44.9)	–
	Married	107 (69.9)	28 (40.6)	–
	Divorced/ Widowed	26 (17)	10 (14.5)	–
	No information	6	2	–
Years of education	0	10 (6.4)	7 (10)	–
	<2	16 (10.2)	3 (4.3)	–
	2–<5	78 (49.7)	34 (48.6)	–
	5	20 (12.7)	4 (5.7)	–
	<12	10 (6.4)	9 (12.9)	–
	12–16	23 (14.6)	13 (18.5)	–
	No information	2	1	–
Family income relative to minimum wage	<1 x	22 (14.5)	18 (26.1)	–
	1 to 3 x	108 (71)	50 (72.5)	–
	≥4 x	22 (14.5)	1 (1.4)	–
	No information	7	2	–

CAD, coronary artery disease group; HVD, heart valve disease group; CG, control group N, number of individuals.

Genetic Polymorphisms

The distribution of the polymorphisms of *CRP*, *TNF*, and *IL10* genes showed no significant changes irrespective of a previous infection or not with *Chlamydia*.

The genotypic distribution of the *IL-6*-174G>C polymorphisms (**Table 4**) showed a higher frequency of GG in the diseased groups (CAD and HVD) than in the controls ($p = 0.0002$). The frequency distribution according to previous exposure to the genus *Chlamydia* showed similar differences but significantly higher when compared to the controls without antibodies ($p = 0.0158$ and 0.0231 for the HVD and CAD, respectively) and with antibodies ($p = 0.0003$ and 0.0051, respectively). The presence of markers of previous exposure to *C. trachomatis* showed a significant difference between HVD and CG and between CAD and HVD ($p = 0.0199$ and 0.0122, respectively). A difference was found only between the HVD and

TABLE 2 | Frequency of antibodies to *C. trachomatis* and *C. pneumoniae* among cardiac patients (CAD and HVD) investigated.

Previous infection	Presence of antibodies (IFI)	CAD *N* = 159 *N* (%)	HVD *N* = 71 *N* (%)	CG *N* = 300 *N* (%)	*p*1	*p*2	*p*3
C. trachomatis	Positive	48 (30.6)[a]	14 (20.3)[b]	103 (36.7)[c]	0.2383	0.014	0.1516
	Negative	109 (69.4)	55 (79.7)	178 (63.3)			
C. pneumoniae	Positive	133 (83.6)	60 (84.5)	237 (80.3)[e]	0.4596	0.5240	0.9757
	Negative	26 (16.4)	11 (15.5)	58 (19.7)			

CAD, coronary artery disease group; HVD, heart valve disease group; CG, control group.
p1: CAD vs. CG; p2: HVD vs. CG; p3: CAD vs. HVD.
[a]*2 samples not included; n = 157.*
[b]*2 samples not included; n = 69.*
[c]*19 samples not included; n = 281.*
[e]*5 samples not included; n = 295.*

TABLE 3 | Serology results for *C. trachomatis* according to the patient group, the origin of the tissues investigated for the presence of the cryptic plasmid and CRP expression.

Group record	Sample	Anti-*C. trachomatis*		qPCR	CRP (mg/dL)	Risk
		IgG	IgM			
22823 CAD	Aorta	+	−	+	0.17	No
22944 CAD	Aorta	+	−	+	0.12	No
22814 CAD	Aorta	+	−	+	0.66	Yes
22990 CAD	Aorta	-	+	+	0.39	Yes
23113 HVD	Mitral V	+	−	+	0.12	No
22988 HVD	Mitral V	−	+	+	1.69	Yes

CAD, coronary artery disease group; HVD, heart valve disease group; CG, control group.
N, number of individuals.
qPCR, real-time PCR.
CRP, C-reactive protein.
Risk, risk of developing atherosclerosis based on the CRP levels.

control groups when the comparison was performed according to previous exposure to *C. pneumoniae* ($p = 0.0368$). The frequency of the *G allele was usually higher in the CAD group, sometimes significantly, than the frequency of the *C allele.

The AT genotype distribution of the *IL8*-251A>T polymorphisms (**Table 5**) was significantly different between the HVD and control groups ($p = 0.0331$), and the AT and AA genotypes differed between the diseased groups ($p = 0.0367$). A difference was also found when the presence of antibodies to *Chlamydia* species was compared to the controls without antibodies ($p = 0.0179$) and with antibodies ($p = 0.0179$). Previous exposure to *C. pneumoniae* showed involvement of the AT and TT genotypes, which significantly differed between the HVD and control groups ($p = 0.0198$) and between both diseased groups ($p = 0.0172$), but not with previous exposure to *C. trachomatis*. No differences were observed among the allele frequencies of the polymorphisms.

Plasma CRP and IL-6 Levels

The assessment of the plasma levels considered the median values described in **Figures 1–4**. The CRP values were higher in the CAD and HVD groups than in the controls (**Figure 1A**). No differences in the plasma CRP levels were found between the different genotypes in each of the investigated groups (**Figures 1B–D**). The influence of the genotypes on the plasma levels according to previous exposure to *Chlamydia* showed that the values were significantly higher among those already exposed. In the absence of previous contact with the bacterium, the difference was significant only when comparing the CAD and control groups (**Figures 1E,F**). The comparison of groups according to previous exposure to *C. trachomatis* showed significant differences between the CAD and HVD groups and between these two disease groups and the control group (**Figure 1G**). When the comparison took into account previous exposure to *C. pneumoniae*, a difference was found only between the patient groups and the control group (**Figure 1H**). The plasma CRP values among the patients in the CAD and HVD groups previously exposed to *C. trachomatis* or *C. pneumoniae* were significantly higher than the levels in the non-exposed controls (**Figures 1I,J**). CRP levels were significantly increased among the patient groups as compared with controls, regardless of a previous *Chlamydia* infection.

IL-6 plasma levels were elevated and significantly different in some specific situations, such as when comparing the patient groups (CAD and HVD) with the CG (**Figure 2A**) and when considering previous exposure to the genus *Chlamydia* and to *C. trachomatis* (**Figures 2E,G**). Previous exposure to *C. trachomatis* showed a significant difference between the HVD group and the controls (**Figure 2I**). Previous exposure to *C. pneumoniae* showed a significant difference between both the CAD and HVD groups compared to the controls (**Figure 2J**). In all comparisons, the expression level was always higher in the HVD group.

TNF, IL8, and *IL10* Gene Expression

The *IL10* mRNA expression level was not significantly different between the patient and control groups when considering the characteristics of genotype, previous exposure to the genus *Chlamydia* or the two *Chlamydia* species (data not shown).

TNF expression levels were significantly lower in the patient groups (CAD and HVD) than in the control group (**Figure 3A**) but were not correlated with the *TNF* -308G>A polymorphism genotypes (**Figures 3B–D**). The differences

TABLE 4 | Genotypic and allelic distributions of *IL-6*-174G>C markers among cardiac patients according to the presence of antibodies to *Chlamydia* and to the *C. trachomatis* and *C. pneumoniae* species.

***IL-6*-174G>C**	**Groups investigated**			***p*1**	***p*2**	***p*3**
	***N* (%) CAD (*N* = 159)**	***N* (%) HVD (*N* = 71)**	***N* (%) CG (*N* = 300)**			
GG	134 (84.28)	60 (84.51)	207 (69.00)	0.0020†	0.0002†	0.7616†
GC	19 (11.95)	07 (9.86)	85 (29.33)			
CC	06 (3.77)	04 (5.63)	08 (2.67)			
*G	0.90	0.89	0.83	0.0049†	0.0852†	0.9196†
*C	0.10	0.11	0.17			
	CAD (*N* = 138) *Chlamydia*+	**HVD (*N* = 61) *Chlamydia*+**	**CG (*N* = 51) *Chlamydia*–**			
GG	118 (85.51)	51 (83.61)	35 (68.63)	0.0158†	0.0231†	0.6778†
GC	15 (10.87)	06 (9.83)	15 (29.41)			
CC	05 (3.62)	04 (6.56)	01 (1.96)			
*G	0.91	0.88	0.83	0.0568*	0.3543*	0.5720*
*C	0.09	0.12	0.17			
	CAD (*N* = 138) *Chlamydia*+	**HVD (*N* = 61) *Chlamydia*+**	**CG (*N* = 247) *Chlamydia*+**			
GG	118 (85.51)	51 (83.61)	171 (69.23)	0.0003†	0.0051†	0.6778†
GC	15 (10.87)	06 (9.83)	69 (27.94)			
CC	05 (3.62)	04 (6.56)	07 (2.83)			
*G	0.91	0.88	0.83	0.0042*	0.1910*	0.5720*
*C	0.09	0.12	0.17			
	CAD (*N* = 48) *C. trachomatis*+	**HVD (*N* = 14) *C. trachomatis*+**	**CG (*N* = 178) *C. trachomatis*–**			
GG	43 (89.58)	9 (64.29)	122 (68.54)	0.6517†	0.0199†	0.0122†
GC	03 (6.25)	03 (21.43)	51 (28.65)			
CC	02 (4.17)	02 (14.28)	05 (2.81)			
*G	0.93	0.75	0.83	0,0255†	0.4433†	0.0350†
*C	0.07	0.25	0.17			
	CAD (*N* = 133) *C. pneumoniae*+	**HVD (*N* = 60) *C. pneumoniae*+**	**CG (*N* = 58) *C. pneumoniae*–**			
GG	113 (84.96)	51 (85.00)	41 (70.69)	0.0561†	0.0368†	0.6020†
GC	15 (11.28)	05 (8.33)	15 (25.86)			
CC	05 (3.76)	04 (6.67)	02 (3.34)			
*G	0.91	0.89	0.84	0.0554*	0.2919*	0.7095*
*C	0.09	0.11	0.16			

N, number of individuals. *Chi-square test. †G test.*
p1, CAD vs. CG; p2, HVD vs. CG; p3, CAD vs. HVD.

between the patient and control groups were evidenced when considering prior exposure to *Chlamydia* (**Figure 3E**) but remained only between the CAD and controls in persons with no prior exposure (**Figure 3F**). The *TNF* mRNA levels were significantly decreased in the HVD compared to the control group and according to prior contact with *C. trachomatis* (**Figure 3G**). However, the decrease was significant for the CAD and HVD groups according to prior contact with *C. pneumoniae* (**Figure 3H**). The decrease in *TNF* expression was significantly lower when comparing patients in the CAD and HVD groups previously exposed to *C. trachomatis* with the non-exposed controls but not to *C. pneumoniae* (**Figures 3I,J**).

IL8 gene expression was also lower among the patient groups than the controls and significantly differed between the CAD and CG groups (**Figure 4A**), between the CAD and HVD groups, and between the CAD and control groups when considering previous exposure to *Chlamydia* (**Figure 4E**). The difference

TABLE 5 | Genotypic and allelic distribution of *IL-8*-251A>T markers among cardiac patients according to the presence of antibodies to *Chlamydia* and to the *C. trachomatis* and *C. pneumoniae* species.

IL-8-251A>T	Groups investigated			*p*1	*p*2	*p*3
	***N* (%)** CAD (*N* = 159)	***N* (%)** HVD (*N* = 71)	***N* (%)** CG (*N* = 300)			
TT	59 (37.11)	27 (38.03)	96 (32.00)	0.4914*	0.0331*	0.0367*
AT	76 (47.80)	24 (33.80)	150 (50.00)			
AA	24 (15.01)	20 (28.17)	54 (18.00)			
*T	0.61	0.55	0.57	0.2707*	0.7237*	0.2618*
*A	0.39	0.45	0.43			
	CAD (*N* = 138) *Chlamydia*+	**HVD (*N* = 61) *Chlamydia*+**	**CG (*N* = 51) *Chlamydia*–**			
TT	48 (34.78)	24 (39.34)	18 (35.29)	0.9676*	0.0513*	0.0179*
AT	69 (50.00)	19 (31.15)	26 (50.98)			
AA	21 (15.22)	18 (29.51)	7 (13.73)			
*T	0.61	0.55	0.57	0.9536*	0.4539*	0.4253*
*A	0.39	0.45	0.43			
	CAD (*N* = 138) *Chlamydia*+	**HVD (*N* = 61) *Chlamydia*+**	**CG (*N* = 247) *Chlamydia*+**			
TT	48 (34.78)	24 (39.34)	77 (31.17)	0.5816*	0.0269*	0.0179*
AT	69 (50.00)	19 (31.15) (-)	123 (49.80)			
AA	21 (15.22)	18 (29.51) (+)	47 (19.03)			
*T	0.60	0.55	0.56	0.3564*	0.8980*	0.4253*
*A	0.40	0.45	0.44			
	CAD (*N* = 48) *C. trachomatis*+	**HVD (*N* = 14) *C. trachomatis*+**	**CG (*N* = 178) *C. trachomatis*-**			
TT	16 (33.3)	5 (35.71)	57 (32.02)	0.9176†	0.9494†	0.9293†
AT	23 (47.92)	7 (50.00)	91 (51.12)			
AA	9 (18.75)	2 (14.29)	30 (16.85)			
*T	0.57	0.61	0.58	0.9483*	0.9012*	0.9161*
*A	0.43	0.39	0.42			
	CAD (*N* = 133) *C. pneumoniae*+	**HVD (*N* = 60) *C. pneumoniae*+**	**CG (*N* = 58) *C. pneumoniae*-**			
TT	46 (34.59)	24 (40.00)	19 (32.76)	0.8787†	0.0198†	0.0172*
AT	66 (49.62)	18 (30.00) (-)	31 (53.45)			
AA	21 (15.79)	18 (30.00)(+)	8 (13.79)			
*T	0.60	0.55	0.60	0.9220*	0.5726*	0.4845*
*A	0.40	0.45	0.40			

*N, number of individuals. *Chi-square test. †G test.*
p1, CAD vs. CG; p2, HVD vs. CG; p3, CAD vs. HVD.

between the CAD and CG groups remained significant when previous exposure to *C. pneumoniae* was considered in all three groups (**Figure 4H**).

DISCUSSION

Cardiovascular diseases result in the death of ~17.5 million people per year[1] due to risk factors including hypertension, diabetes, smoking, excess weight, sex, age, ethnicity, genetic inheritance (21, 22), and inflammatory and rheumatic heart disease.

The patients investigated in the present study presented characteristics similar to those of other studies (21, 23, 24). The patients who underwent myocardial revascularization (CAD group) were mostly men with a mean age of 60.4 years and were married. The HVD group, which

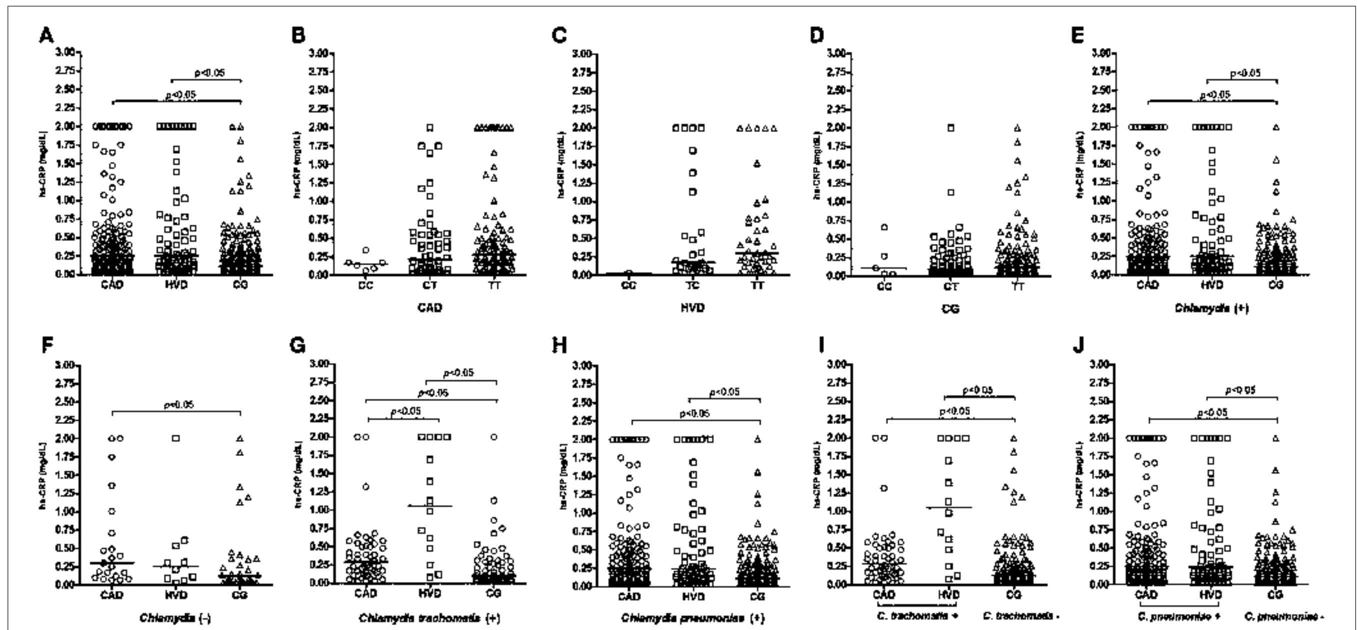

FIGURE 1 | Distribution of plasma C-reactive protein (CRP) levels **(A)** among patients receiving surgical revascularization for coronary artery disease (CAD), patients undergoing valve replacement for heart valve disease (HVD), and control subjects (CG); **(B–D)** according to the different *CRP*-717T>C genotypes; **(E)** among groups serologically positive and **(F)** negative for *Chlamydia*; **(G,I)** among groups with and without previous exposure to *C. trachomatis* and **(H,J)** to *C. pneumoniae*.

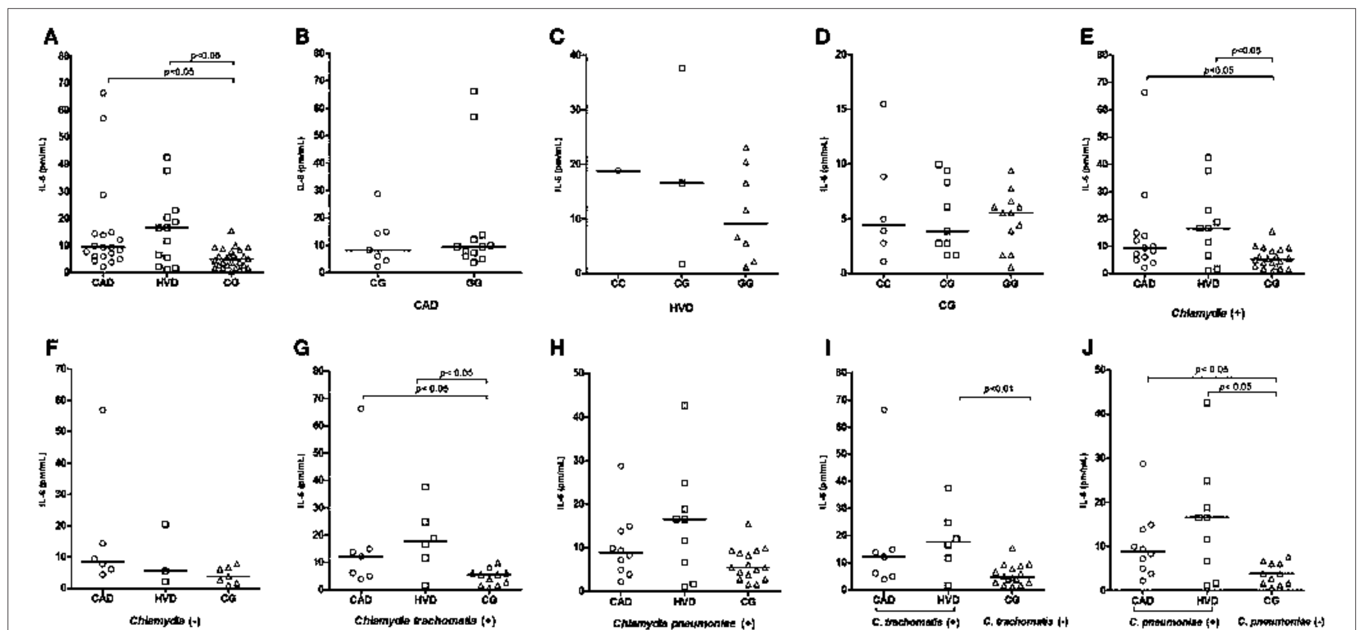

FIGURE 2 | Distribution of plasma IL-6 levels **(A)** among patients receiving surgical revascularization for coronary artery disease (CAD), patients undergoing valve replacement for heart valve disease (HVD) and control subjects (CG); **(B–D)** according to the different *IL-6*-174G>C genotypes; **(E)** among groups serologically positive and **(F)** negative for *Chlamydia*; **(G,I)** among groups with and without previous exposure to *C. trachomatis* and **(H,J)** to *C. pneumoniae*.

underwent valve replacement, was represented by a majority of women who were single with an average age of 45.6 years. Although the groups showed differences, the two groups shared a low education level and low family income. These factors do not favor adequate understanding of the protection and care associated with the prevention of heart diseases. Atherosclerosis, which is a disease of multifactorial etiology and valvulopathy caused by congenital disorders or rheumatic fever, may also develop from an infectious component (25).

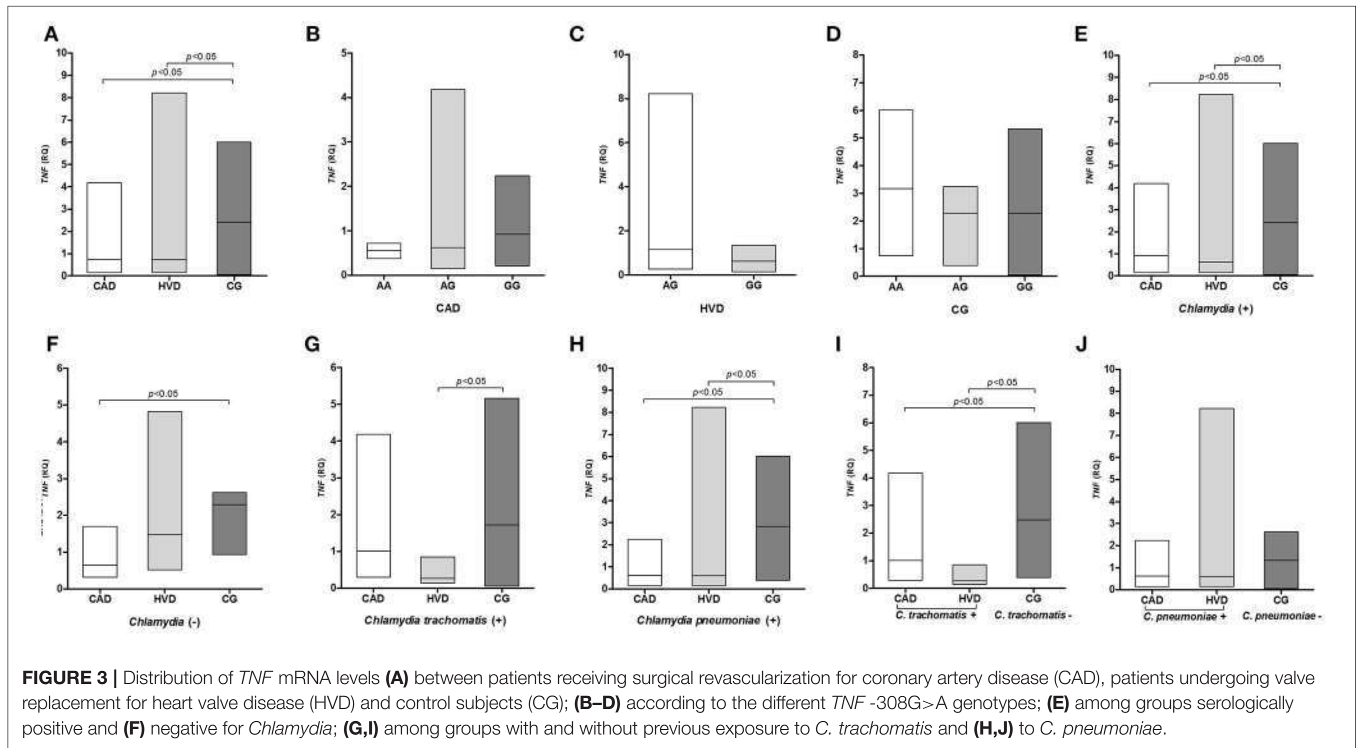

FIGURE 3 | Distribution of *TNF* mRNA levels **(A)** between patients receiving surgical revascularization for coronary artery disease (CAD), patients undergoing valve replacement for heart valve disease (HVD) and control subjects (CG); **(B–D)** according to the different *TNF* -308G>A genotypes; **(E)** among groups serologically positive and **(F)** negative for *Chlamydia*; **(G,I)** among groups with and without previous exposure to *C. trachomatis* and **(H,J)** to *C. pneumoniae*.

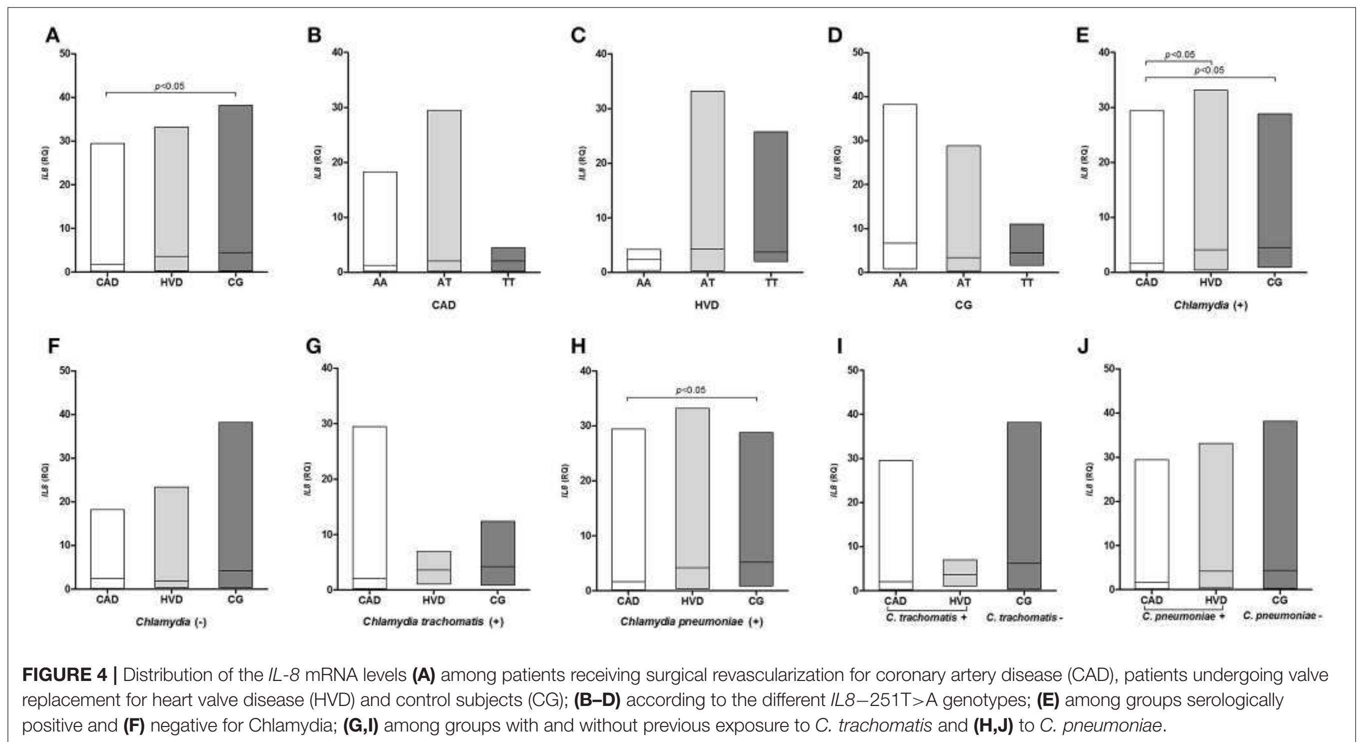

FIGURE 4 | Distribution of the *IL-8* mRNA levels **(A)** among patients receiving surgical revascularization for coronary artery disease (CAD), patients undergoing valve replacement for heart valve disease (HVD) and control subjects (CG); **(B–D)** according to the different *IL8*−251T>A genotypes; **(E)** among groups serologically positive and **(F)** negative for Chlamydia; **(G,I)** among groups with and without previous exposure to *C. trachomatis* and **(H,J)** to *C. pneumoniae*.

Chlamydia pneumoniae is a bacterium that is frequently involved in the etiology of heart disease and is widespread among adults, who have high prevalence of antibodies (26, 27). The groups involved presented prevalence rates >80%, which was common in other areas of the world (26, 28–32). Since the 1980, evidence of the presence of *C. pneumoniae* in aortic valves has been demonstrated, and the bacterium has been assigned an important role in the calcification process (33).

However, failure to detect *C. pneumoniae* is common using methods such as cell culture isolation or nucleic acid detection in cardiovascular tissues, even in populations with a high prevalence of antibodies (24–36). Biopsies of the coronary artery (37), aorta, and atheroma plaque (38) showed the presence of *C. pneumoniae* antigens by immunohistochemistry, which might be relevant in the pathogenesis of atherosclerosis.

Additionally, *C. trachomatis* is a major sexually transmitted bacterium (39) that has an extensive distribution in the Amazon region of Brazil, particularly in Pará (40–42), as has also been observed in the present study among the three groups. The occurrence of cardiopathies by *C. trachomatis* has been demonstrated in experimental studies (43–45) and in case reports (46); however, the role of this bacterium in the pathogenesis of heart disease has rarely been investigated.

Although historical studies on the etiology of atherosclerosis focused on *C. pneumoniae*, our laboratory recently demonstrated (37) the detection of bacterial antigens *in situ* in the aorta and atheroma plaque (*C. pneumoniae*) and in the heart valves (*C. trachomatis*). The present work shows for the first time the amplification of cryptic *C. trachomatis* plasmid DNA in the aorta and mitral valve samples. Two plasma samples corresponding to cases of DNA amplification showed the presence of IgM antibodies against *C. trachomatis*, indicating a recent infection in both patients and demonstrating the entrance of the bacterium in the host and the immediate invasion of tissues of the cardiovascular system. However, in one of these samples, immunohistochemistry did not detect bacterial antigens *in situ* in the aorta, atheroma plaque, or heart valve. Notably, another sample that presented antigens for both species by immunohistochemistry resulted in DNA detection only for *C. trachomatis*. Despite the differences in cellular tropism, *C. pneumoniae*, *C. trachomatis,* and *C. pecorum* have been associated with endothelial and cardiac muscle infections and have been shown to cause myocarditis in experimental and human models (8, 44, 47).

Although epithelial and muscle cells are not the preferred targets, infected monocytes and macrophages (16) eventually carry *C. trachomatis* to different regions of the human body and are found in cases of endocarditis (48), in the livers of patients with a history of high and prolonged fever (49), and in patients with myocarditis and a history of prostatitis (46). The present study is the first to demonstrate the presence of *C. trachomatis* DNA in cardiovascular tissue, which highlights the possibility that the bacterium may also cause disease. This finding indicates a need for further investigations directed toward elucidating the role of *C. trachomatis* in the pathogenesis of heart disease.

The gene polymorphisms studied were chosen according to their published frequency in the medical literature and the search for their association with different outcomes indicating exposure leading to the infection of an agent or indicating an agent infection leading to disease.

The allelic and genotypic distributions of the polymorphisms investigated in the *CRP*, *TNF,* and *IL10* genes were not associated with the risk of developing cardiovascular disease even when previous exposure to *C. trachomatis* and *C. pneumoniae* was taken into account. Although the selected polymorphisms had the highest associations with chronic diseases, including atherosclerosis, several other markers (immunological, inflammatory, and biochemical) have been identified and associated with a moderate or high risk for vascular disease (50, 51). Additionally, we should consider the multifactorial condition of diseases that do not depend only on a genetic predisposition but also depend on environmental factors (i.e., a sedentary lifestyle, smoking, and hyperlipidemia) (1).

The results of the present study and other studies showed that the *IL6*−174G>C polymorphism might represent a risk factor for cardiovascular diseases by altering the transcription levels of the gene as well as its plasma levels (52, 53). This polymorphism was initially identified among Caucasians with a G allele frequency of ∼0.60 and a C allele frequency of ∼0.40 (52). In the present study, higher frequencies of the G allele were found. The allelic distribution is heterogeneous, with the frequency of the G allele in Africans and Asians is similar to the frequency found in the present study, whereas the frequency in Europeans and Americans is lower (54). The differences may be due to the genetic contribution of different ethnic groups (Africans, Amerindians, and Europeans) in the formation of the population of Pará state (55).

Patients with the GG genotype show a two- to three-fold increased risk of developing coronary heart disease and valvulopathy compared to patients with other genotypes (53, 56); additionally, patients with this genotype have a risk of developing different pathologies, including Kaposi's sarcoma, hyperlipidemia, and growth deficiency (Crohn's disease patients) [(57), p. 56]. Heart valve lesions stimulate the inflammatory process and exacerbate the secretion of proinflammatory cytokines, including IL-6 (58). GG genotype was higher than in other studies shown above and is well correlated with the expression and consequent serum levels shown in **Figure 2** (discussed below). The major risk of allele G was not clearly detected in the present study.

The GC genotype presented a significantly higher frequency in the control population, indicating possible protection against cardiovascular disease. The presence of the C allele reduces transcriptional activation of the *IL-6* gene and may provide a more balanced cytokine secretion response (52). However, other studies noted that the C allele was associated with higher plasma IL-6 levels (59). Thus, the role of this polymorphism remains controversial (60).

The *IL8*−251A>T polymorphism showed a significantly higher frequency of the AA genotype in the HVD population than in the other groups. This genotype is related to higher transcriptional activity of the gene (61). The heterozygous genotype was found more frequently in the control population than in the patients with valvulopathies, probably because it presented a more balanced profile of IL-8 cytokine production and could act as protection against the disease (62). The role of IL-8 is associated with the inflammatory process characteristic of valvulopathies as well as the secretion of monocyte chemoattractant proteins (MCP-1), adhesion molecules, fibroblast growth factors, and neutrophil chemoattractant (62).

In the present study, the AA genotype was shown to be a risk factor for the onset of disease in the HVD group.

The GG genotype for the *IL6*−174G>C polymorphism is apparently a risk factor for the development of cardiovascular disease regardless of whether the serological result indicates previous exposure to genus *Chlamydia* or the two *Chlamydia* species investigated. The GC genotype acts as a protection factor in relation to heart disease. Importantly, previous exposure to *C. trachomatis* is a variable similar to previous exposure to *C. pneumoniae* for the risk of developing heart disease, which has not been clearly demonstrated previously. However, the significance of the *IL8*−251A>T polymorphic genotypes exists only when considering prior exposure to genus *Chlamydia* and the *C. pneumoniae* species. Due to the history of infection by this bacterium, wild genotypes for the *IL6*−174G>C and *IL8*−251A>T polymorphisms may act synergistically and accentuate the mechanisms of the immune response via specific inflammatory stimuli.

The CRP levels were higher in both patient groups than in the controls, indicating that in both cases, an acute inflammatory process was observed, reinforcing the role of CRP as a risk indicator for cardiovascular disease (unstable angina, stable angina, and acute myocardial infarction) and heart valve disease (63, 64). Several polymorphisms in the CRP gene have been shown to be associated with changes in the serum protein concentration, such as the *CRP* -717T>C polymorphism, which appears to influence the serum protein concentration and the development of cardiovascular diseases (65, 66). However, in the present study, there was no evidence of a significant association of the polymorphism with variations in the plasma CRP levels. The TT genotype raised the CRP levels higher than the controls, which was previously demonstrated (67). Despite the importance of the genetic variable, we detected no influence of the isolated form on the CRP levels (66). Previous exposure to *Chlamydia* significantly increased CRP, particularly among those who had never been infected by the bacterium, and only the CAD group showed high levels (similar to those with prior *C. pneumoniae* infection). Previous exposure to *C. trachomatis* significantly increased the levels among the valvulopathy patients. The presence of infection markers for the species can function as a transcriptional stimulus in combination with other factors to increase CRP secretion in valvulopathy patients (68).

The IL-6 levels were also higher in the patient groups, confirming the cytokine's contribution to the disease-related systemic inflammatory process (69, 70). However, no relationship was found between the IL-6 levels and the studied genotypes, which was in contrast to previous studies that found that the GG polymorphism was more frequent in the patient groups (52, 53). The previously demonstrated presence of IL-6 *in situ* is compatible with elevated levels in the two patient groups (37). Importantly, IL-6 played a role in the stimulation of acute phase proteins such as CRP (70), which was also significantly elevated in the patients in the present study.

The high prevalence of *C. pneumoniae* acts as a confounding factor when the results of IL-6 plasma levels are described. The previous exposure to both bacteria was then treated as two independent events, in order to sort out the implication of each bacterium in the inflammatory process. Previous exposure to *Chlamydia* is a variable that maintains the significance of the difference in the IL-6 levels between the patient groups. This factor maintained higher IL-6 levels in the HVD group, whereas the absence of antibodies to *Chlamydia* removed the effect on the increase in the plasma cytokine level. Both *C. trachomatis* and *C. pneumoniae* influence the IL-6 elevation, particularly in the HVD group. The immune response may be exacerbated by increased plasma IL-6 levels (71, 72) in response to microorganisms and other cytokines (73) in addition to the contribution of the effect of CRP on innate immunity and thus the systemic effects of inflammation (74). The exacerbated production of IL-6 is associated with a variety of autoimmune and inflammatory diseases and other conditions, such as atherosclerosis and acute myocardial infarction (68, 69). However, the association of the IL6−174G>C polymorphism with increased IL-6 and CRP levels has been demonstrated, and this polymorphism is considered a risk factor for the development of CAD (53, 75). Moreover, the association of the polymorphism with the persistence of antibodies to *C. pneumoniae* can lead to chronic infection (76).

TNF, *IL8*, and *IL10* gene expression was evaluated. However, no differences were found in the distribution of the IL-10 expression levels in the investigated conditions. IL-10 is an immunosuppressive cytokine that can inhibit the expression of TNF-α, IL-8, and other cytokines even at basal expression levels (77, 78). Immunosuppression was observed in the present study. Both TNF-α and IL-8 were expressed at higher levels in the control subjects than in patients despite the large concentrations detected *in situ* (37) and the lack of an influence by any genotypes of the *TNF* and *IL8* polymorphisms. Previous exposure to *Chlamydia* had a significant influence on the expression levels, but two effects were observed. The decrease in IL-8 expression was more pronounced in the CAD group following exposure to *C. pneumoniae*, whereas reduced *TNF* mRNA levels were associated with prior exposure to *C. trachomatis*.

The demographic characteristics of the patients with coronary artery disease and valvulopathies were similar to the common conditions found in the Brazilian population, and the prevalence of previous infections with *C. trachomatis* and *C. pneumoniae* did not differ from reports concerning Belém and other population groups in the Brazilian Amazon (41, 42). The presence of *C. trachomatis* cryptic plasmid DNA demonstrates the real possibility of new perspectives on the etiology of heart disease.

CONCLUSION

The wild genotypes of the *IL6* and *IL8* gene polymorphisms were present at a high frequency and with elevated CRP expression and proved to be good predictors of heart disease. The presence of bacterial antigens *in situ* (37) reinforces the suggestion that *C. trachomatis* can also reach the cardiovascular system and cause disease. The results are relevant to the understanding of genetic susceptibility, the

interaction between pro- and anti-inflammatory markers, the control of gene expression, and the role of infectious agents capable of potentiating the inflammatory reaction of heart disease.

AUTHOR CONTRIBUTIONS

NA, MI, AV, and RI designed the study. NA, MQ, MA, and IB performed the experiments. NA, MI, SL, RI, AV, and MQ analyzed and interpreted the data. NA, MQ, and RI wrote the manuscript. RI and AV oversaw the experiments and edited the manuscript. NA, MQ, SL, IB, MA, AV, MI, and RI reviewed the manuscript.

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Leishmania-Host Interactions—An Epigenetic Paradigm

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Leishmaniasis is one of the major neglected tropical diseases, for which no vaccines exist. Chemotherapy is hampered by limited efficacy coupled with development of resistance and other side effects. *Leishmania* parasites elude the host defensive mechanisms by modulating their surface proteins as well as dampening the host's immune responses. The parasites use the conventional RNA polymerases peculiarly under different environmental cues or pressures such as the host's milieu or the drugs. The mechanisms that restructure post-translational modifications are poorly understood but altered epigenetic histone modifications are believed to be instrumental in influencing the chromatin remodeling in the parasite. Interestingly, the parasite also modulates gene expression of the hosts, thereby hijacking or dampening the host immune response. Epigenetic factor such as DNA methylation of cytosine residues has been incriminated in silencing of macrophage-specific genes responsible for defense against these parasites. Although there is dearth of information regarding the epigenetic alterations-mediated pathogenesis in these parasites and the host, the unique epigenetic marks may represent targets for potential anti-leishmanial drug candidates. This review circumscribes the epigenetic changes during *Leishmania* infection, and the epigenetic modifications they enforce upon the host cells to ensure a safe haven. The non-coding micro RNAs as post-transcriptional regulators and correlates of wound healing and toll-like receptor signaling, as well as prognostic biomarkers of therapeutic failure and healing time are also explored. Finally, we highlight the recent advances on how the epigenetic perturbations may impact leishmaniasis vaccine development as biomarkers of safety and immunogenicity.

Keywords: epigenetics, DNA methylation/demethylation, histone modification, non-coding RNA, leishmaniasis, host-pathogen Interactions, therapeutics, biomarkers

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INTRODUCTION

Epigenetics encompasses any process that changes gene expression and is inherited without amending the fundamental DNA sequence (1). These variations are highly dynamic which get altered on advent of any external stress or internal cues (2, 3). Epigenetics controls several cellular processes by switching genes on or off, thereby modulating gene expression. Epigenetics is also associated with various diseased states, wherein, it is shaped by host as well as pathogen selection pressures (4, 5). Recently, there is burgeoning interest in epigenetics landscapes during an infection, particularly alterations in DNA methylome, histone marks and non-coding (nc)RNA or micro (mi)RNA profiles. The epigenetic states result in erratic gene expression profiles of host cells, which are responsible for warding off microbial infections (6, 7).

Leishmania belongs to trypanosomatid family, being among the major neglected vector-borne tropical diseases, ranging in severity from self-healing but disfiguring and stigmatizing cutaneous lesions to disseminating muco-cutaneous and fatal visceral manifestations, depending on the species and host characteristics. Globally, 0.7–1.2 million new cases of cutaneous leishmaniasis (CL) occur every year; while for visceral leishmaniasis (VL), 200,000–400,000 new cases and 20,000–40,000 deaths are reported annually, with 95% of fatal cases occurring in only six countries, namely, India, Bangladesh, Sudan, Nepal, Ethiopia, and Brazil (8). The goal of World Health Organization is to eliminate this public health problem in South-east Asia region by 2020 (9).

Leishmania parasites have a digenetic life cycle that may be zoonotic or anthroponotic, depending upon the infecting parasite species. When an infected female sandfly (*Phlebotomus* or *Lutzomyia* species) takes a blood meal, the parasites cause dermal lesions as in CL or visceralize as in VL (10). The infection is amplified in the vector's gut with successive blood meals (11). Invasion of host macrophages by *Leishmania* triggers a multitude of signaling circuits to eliminate the pathogen. However, the parasite tries to subvert these defense mechanisms to create a safe haven for its survival. *Leishmania* secretes effector molecules to modulate host immune transcriptome resulting in alterations in the host epigenome, to alter cytokine and chemokine levels, their cross talks and downstream signaling hubs. This adversely affects the recruitment and activation of immune cells, respiratory burst and antigen presentation, leading to immune evasion (12).

Though still at infancy, there is a recent surge of information on the epigenetic regulation during *Leishmania* infection. This review gives an update on *Leishmania* epigenetic landscapes and epigenome alterations imposed in the host for immune evasion (summarized in **Figure 1** and **Table 1**). Further, evolving evidence on the probable downstream effects of epigenetic regulation such as targeting epigenetic machinery to reset the waning immune response, via vaccine, or drug development and prognostic markers are also discussed.

EPIGENETIC CHANGES IN *LEISHMANIA* PARASITES

The diverse clinical manifestations of leishmaniasis may be attributed to varying genomic makeup (size, GC content, coding genes, pseudogenes, retrotransposons) of the causative *Leishmania* species (38). To promote their survival in the host environment, a huge array of epigenetic factors is speculated to interplay in these parasites (39).

DNA Modification

Glycosylated thymine, termed "Base J" or β-D-glucosylhydroxymethyluracil, is present in the telomeric repeat sequence (GGGTTA) of *Leishmania* (40). J replaces ∼1% of T in nuclear DNA and the modified T residue has been implicated in transcriptional regulation and termination. Absence of J is lethal to *Leishmania*, due to massive read through of transcriptional termination sites (41). However, repression of specific genes has now been identified as an essential role of Base J (15). A thymine base modification, 5-hydroxymethyluracil has recently been mapped to *Leishmania* genome, but its epigenetic role is yet to be elucidated (42).

Histone Alterations

Acetylation of histone H3 in telomeric divergent strand switch regions, has been reported in *L. major* promastigotes (43) resulting in chromatin state, with restriction of protein coding genes. Acetylation levels are higher in rapidly growing cells compared to stationary phase cells. Epigenetic marks such as H3K9me3, H3K14ac, H3K23ac, and H3K27ac have also been reported in promoter region of rRNA genes of *L. major*, favoring transcriptional activation of rRNA genes while H4K20me3 in the coding region is related to transcriptional silencing (44). H3K9me3 is also linked to heterochromatin formation. Histone variants such as H2A.Z and H2B.V have been identified as essential for *L. major* survival (16).

In *L. donovani*, histone acetyltransferase (HAT)4 acetylates H4K14, favoring maintenance of euchromatin state (19). H4K4 acetylation by HAT3 (18) and, HAT2-dependant H4K10 acetylation of promoters in *L. donovani* has been linked with parasite survival (17).

Epigenetic tags are differentially regulated in the promastigote and amastigote stage. Some of the histone deacetylases (HDAC) are preferentially upregulated in *L. infantum* logarithmic phase promastigotes over intracellular amastigotes, making the amastigotes better able to adapt to intraphagolysosomal environment (20). Sirtuins, NAD-dependent HDAC have been implicated in parasite survival by inhibiting apoptosis (45), and sirtinol, sirtuin inhibitor, selectively induced apoptosis in *L. infantum* axenic amastigotes but not the promastigotes (46).

Non-coding RNAs

ncRNAs, non-coding sequences of about 22 nucleotides, act as post-transcriptional regulators of RNA encoding proteins (47). A special class of nc RNAs found exclusively in amastigotes of *L. infantum* and *L. donovani*, is particularly important for intra-macrophage parasite survival (48). In *L. tarentole*, ncRNA similar to guide RNA encoded by maxicircles and minicircles has been identified (49). Significant differences in nc RNA repertoire among different *Leishmania* species and stages has been reported (50).

LEISHMANIA-INDUCED HOST EPIGENETIC ALTERATIONS

Epigenetic mechanisms regulate the interplay of host-pathogen interactions. Although information on epigenetic manipulation of hosts by *Leishmania* is scarce, the pathogen employs a number of stratagems to manipulate the host epigenome, thereby hijacking its cellular soldiers (23, 51, 52). Genetic heterogeneity among different *Leishmania species* causes altered gene expression in response to environmental conditions in the host, resulting in varied epigenetic mechanisms.

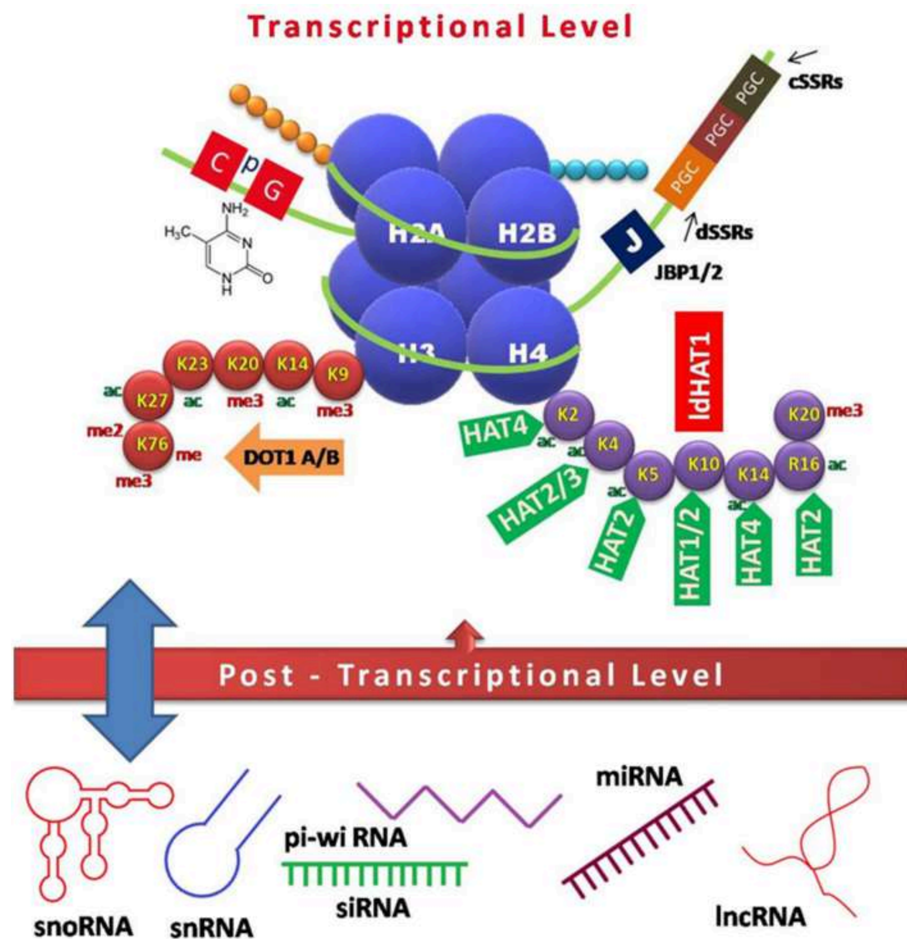

FIGURE 1 | Schematic model of epigenetic regulation during *Leishmania* infection. Interplay of various factors involved in controlling gene expression at the transcriptional (DNA and histone modifications) and post-transcriptional level (non-coding RNAs) is depicted. CpG-rich regions of repressed genes are usually methylated, which in turn recruit chromatin modifiers to keep the genes in any of the three states, i.e., repressed, expressed, or poised. Heavily expressed genes show neither DNA methylation nor acetylated histones, while repressed genes tend to have both methylated DNA and histones, which inhibit the accessibility of polymerases, and other factors required for transcription. Base J, a DNA modification is crucial for transcriptional control in *Leishmania* species. Various non-coding RNAs arising mostly from UTRs act as regulatory elements in a feedback loop (13, 14). JBP, Base J binding protein; PGC, polycistronic gene cluster; cSSRs, convergent strand switch regions; dSSRs, divergent strand switch regions; DOT, disrupter of telomere; ac, acetylation; me, methylation; me2, dimethylation; me3, trimethylation; HAT, histone acetyl transferase; sno, small nucleolar; sn, small nuclear; pi-wi, piwi interacting; si, small interfering; mi, micro; lnc, long non-coding.

DNA Modifications in Host

L. donovani has been reported to elicit epigenetic modifications in host macrophages, permanently down-modulating the innate immune defenses (23). These altered epigenetic tags comprise of cytosine methylation at CpG sites of macrophage DNA, upon infection, causing alteration in genes implicated in JAK/STAT, calcium, MAPK, notch, and mTOR signaling pathway as well as in cell adhesion involving integrin β1 and changes in host oxidative phosphorylation. *Leishmania*-driven epigenomic changes in host macrophages deactivate its innate immune defensive machinery, thereby promoting pathogen survival, and replication.

Epigenetic modification promotes self-healing in CL. Epigenetic repression of wound healing gene, Friend leukemia virus integration 1 (*FLI1*) via increased methylation of CpG islands in its promoter region, has been found to correlate with up-modulation of pro-fibrotic genes such as collagen type I alpha 1 (*Col1α1*) and alpha 2 (*Col1α2*) and, conversely, with down-regulation of matrix metalloproteinase 1 (*MMP1*) gene, resulting in resolution of lesions caused by *L. braziliensis* (53, 54). *MMP1* cleaves type I collagens to loosen keratinocytes-dermal matrix contact, favoring re-epithelialization or tissue repair. Homocystine-dependent stimulation of IL-6 has been further implicated in epigenetic DNA methylation of CpG-rich promoter of lysyloxidase (*LOX*) gene, a cross-linker of collagen and elastin, also rendering it transcriptionally inactive (55, 56). These epigenetic regulations of gene expression depend upon the infecting *Leishmania* species. Contrary to these reports, a recent study showed that increased *FLI1* promoter methylation did not translate into low *FLI1* gene expression (22).

TABLE 1 | *Leishmania* plasticity and *Leishmania*-induced host epigenetic alterations.

Epigenetic regulator	Modification	Condition	Effect	Reference
EPIGENETIC REGULATION IN *LEISHMANIA*				
Base J	↑	*L. major*	Parasite survival	(15)
H2A.Z, H2B.V	↑	*L. major*	Parasite survival	(16)
HAT2	↑	*L. donovani*	Cyclins ↑, parasite survival	(17)
HAT3	↑	*L. donovani*	Parasite survival	(18)
HAT4	↑	*L. donovani*	Cyclins ↑, parasite survival	(19)
HDAC	↑	*L. infantum* logarithmic phase promastigotes versus intracellular amastigotes	Adaptation of amastigotes to phagolysosomal milieu	(20)
Sirtuin 2	↑	Amp B^R - *L. donovani*	ROS ↓, apoptosis ↓	(21)
***LEISHMANIA*-INDUCED HOST EPIGENETIC ALTERATIONS**				
FL1 methylation	↓	*L. braziliensis* infected møs from skin lesions	FLI gene expression	(22)
IRAK2 DNA methylation at CpG sites	↑	*L. donovani* infected møs	IRAK2 mRNA ↓, NF-κB ↓, immune silencing	(23)
LARS2 related CpG site methylation	↑	*L. donovani* infected møs	LARS mRNA ↓, mTORC1 ↓, 4E-BP1 ↑, parasite proliferation	(23)
*CDC42*EP3 methylation at CpG sites	↓	*L. donovani* infected møs	CDC42EP3 mRNA ↑, Progression of infection	(23)
HDAC4	↑	*L. donovani* infected møs	Phagolysosomal formation, amastigote survival	(23)
HDAC11	↑	Imipramine treated Sb^R-*L. donovani* infected human møs	IL-12/IL-10 ratio ↑, parasite burden↓	(24)
HDAC1	↑	*L. amazonensis* infected møs	iNOS ↓, parasite survival	(25)
miRNA-294,−721	↑	*L. amazonensis* infected møs	Targets NOS-2, L-Arginine metabolism, NO ↓, parasite establishment	(26)
miRNA-210	↑	*L. major* infected møs	Activates hypoxia inducible factor-1α, parasite survival	(27), (28)
miRNA-129- 5p,−101c	↓	*L. major* infected møs	Autophagy ↑, infection↓	(28)
miRNA-25, - 26a,−140, -155, let-7a	↑	*L. major* infected human møs	Corresponding chemokine targets ↓ (CCL5, CXCL10, CXCL11, CXCL12, CCL2)	(29)
miRNA-155	↑	*L. major* infected human DCs	PU.1 (SPI1) ↑, TGF-β signaling	(30)
let7a/b	↓	*L. major* infected human DCs, møs	Pro-inflammatory cytokines IL-12↓	(30)
miRNA-193b,−671	↑	Lesions from *L. braziliensis* infected patients	CD40, TNFR, inflammatory response, faster wound healing	(31)
miRNA-361- 3p	↑	Skin lesions from *L. braziliensis* infected patients	Therapeutic failure, healing time ↑, prognostic biomarker	(31)
miRNA-30A- 3p	↑	*L. donovani* infected THP-1, HMDMs	Autophagy ↓, promotes parasite survival	(32)
miRNA122	↓	*L. donovani* infected murine hepatocytes	Serum cholesterol ↓, maintains infection	(33)
miRNA-30c	↓	DBA-treated intramacrophagic *L. donovani* amastigotes	Inhibits proliferation and virulence	(32)
miRNA-151a	↓	DBA-treated intramacrophagic *L. donovani* amastigotes	Mitochondrial dysfunction	(32)
miRNA-6540	↓	*L. donovani* infected møs	Promotes intracellular parasite survival	(34)
miRNA- 3473f	↓	*L. donovani* infected møs	Autophagy ↓, role in pathogenesis	(34)
miRNA- 6973a	↑	*L. donovani* infected møs	IL-12 ↓, Th1 → Th2, parasite survival	(34)
miRNA-3620		*L. donovani* infected møs	Iron homeostasis genes, iron in cytoplasm, parasite survival	(34)
miRNA-3620,−6385	↑	*L. donovani* infected møs	Hypoxia inducing genes ↓, macrophage effector functions ↓, parasite survival	(34)
miRNA-763,−1264,−3473f	↓	*L. donovani* infected møs	ABC transporters ↑, drug efflux ↑, resistance	(34)

(Continued)

TABLE 1 | Continued

Epigenetic regulator	Modification	Condition	Effect	Reference
miRNA-21	↑	*L. donovani* infected human DCs	SMAD7 ↓, TGF-β signaling	(30)
miRNA- 146b-5p	↑	*L. donovani* infected human DCs	TRAF6 ↑, TGF-β signaling	(30)
let7a/b	↑	*L. donovani* infected human DCs, møs	Target pro-inflammatory genes, pro-inflammatory cytokines IL-12 ↓	(30)
miRNA-511	↑	*L. donovani* infected human DCs	TLR4 activation	(30)
miRNA-488i	↑	Sb^R-*L. donovani* infected møs	MyD88 ↓, IL-10/IL-12 ↑ ratio, parasite number ↑	(35)
miRNA-34a	↓	*L. donovani* infected human møs	c-myc ↑, M2 mø activation, attenuates parasite survival	(36)
miRNA-155	↑	*L. infantum* infected J774 møs	Susceptibility to Sb ↓	(25)
miRNA-191,−374	↑	*L. infantum* infected dog PBMCs	Parasite load ↑	(37)
miRNA-150	↓	*L. infantum* infected dog PBMCs	Parasite load ↑	(37)

HDAC, Histone deacetylase; IRAK2, interleukin-1 receptor associated kinase 2; LARS, leucyl-tRNA synthetase; Amp, B^R Amphotericin B resistant; møs, macrophages; HMDMs, Human monocyte derived macrophages; DBA, dibenzalacetone; ABC, ATP-binding cassette; DCs, Dendritic cells; I, inhibitor; Sb^R, Antimony resistant; Sirtuins, Silent Information Regulator.

Histone Modifications in Host

L. amazonensis induces HDAC in infected macrophages, contributing to down regulation of inducible nitric oxide synthase (iNOS) and subsequent parasite survival (25).

ncRNA Induced Gene Silencing

Leishmania infection targets cellular miRNA repertoire and the differential miRNA expression is dependent on infecting species (37). A plethora of studies indicate miRNAs as key regulators of disease phenotype in *Leishmania*-infected cells (27, 30, 32–34). miRNA-30A-3p mediates survival of intracellular *L. donovani* and intervention targeting the miRNA resulted in significant reduction in parasite burden by restoring host autophagic machinery (32). miRNA-3620 was found to regulate iron homeostasis and hypoxia in *L. donovani* infected macrophages while miRNA-3473f was linked with autophagy inhibition (34). Drug resistance due to over expression of efflux pumps such as ABC transporters has also been linked with downregulation of miRNA-763,−1264, and−3473f (34). *L. donovani* infection causes hypoxic environment within the macrophages by activating hypoxia inducible factor-1α, that in turn up regulates miRNA-210, while down regulating NF-κB mediated pro-inflammatory immune responses, to establish a safe niche for its survival (57).

Leishmania establishes and survives in the host by manipulating its ncRNA network, which includes transcriptional arrest of the major protein coding genes in macrophages (58), downregulating 7SL RNA in SRP complex, knockdown of selected ncRNAs in their host cells by inducing degradation of a specific RNA Pol III transcription factor subunit TFIIIC110 in M2 macrophages (59). *Leishmania* surface glycoprotein, gp63 and surface glycolipid, LPG have been reported to down modulate ncRNAs in M2 macrophages, thereby promoting infection (60).

Recently, down modulation of 19 miRNAs in *L. donovani* infected macrophages has been reported (36). The miRNA gene repression correlated with upregulation of host transcription factor, c-myc upon infection, a marker of M2 macrophages, which could possibly be another virulence factor. The expression of c-myc in turn is regulated by several miRNAs, primarily miRNA-34a, which is reciprocally down modulated in *Leishmania*-infected cells.

miRNA-361-3p and−140-3p have been reported to be more expressed in skin lesions caused by *L. braziliensis* in localized cutaneous leishmaniasis (LCL) (61). While miR-193b and−671 have been correlated with faster wound healing in *L. braziliensis* infected patients (31). Autophagy in intramacrophagic *L. major* has been correlated with miRNA-101c,−129-5p and via inhibiting miRNA-210 (28).

POTENTIAL DOWNSTREAM EFFECTS OF EPIGENETIC REGULATION DURING *LEISHMANIA* INFECTION

Epigenetic Reprogramming of Innate Immune Cells

Recent reports shed light on epigenetic reprogramming in monocytes and macrophages via histone trimethylation at H3K4 for innate immune memory or trained immunity (62, 63). Natural killer (NK) cells have also been reported to differentiate into memory NK cells with distinct epigenetic profile (64). However, the epigenetic signatures of innate immune cells during *Leishmania* infection are limited (65).

Epigenetic Tuning of Cell Signaling Hubs

Epigenetic reprograming at cytokine gene loci is reported to influence its gene expression. A growing body of data suggests that differential cytokine microenvironment modulates T helper (Th) cell polarization, macrophage phenotype differentiation and cytokine-inflammasome crosstalk for optimal immune response (65). Signal transducers and activators of transcription (STAT)-4 and−6 have also been reported to play antagonistic roles in epigenetic tuning for Th cell differentiation (66). The epigenetic marks orchestrating gene regulation in Th cell differentiation

(67) and M1/M2 macrophage polarization have been extensively reviewed (68).

Differential expression of miRNAs has been reported to induce T cell differentiation during VL. While miRNA-744 suppresses TGF-β expression and subsequently Treg cell differentiation, miRNA-1272 and−155 downregulate IL-4/IL-13 signaling to mitigate Th2 response during active infection (69). Antimony-resistant *L. donovani* has been reported to activate miRNA-466 inhibitor to degrade host MyD88 and regulate IL-10/IL-12 axis and establish successful infection (35). Chemokine and chemokine receptor gene expression also contribute to immunopathogenesis of leishmaniasis (70). But the effect of *Leishmania*–induced epigenetic alterations in regulation of chemokine genes has not been much explored (29).

miRNAs are also known to be involved in activation of monocytes through toll-like receptor (TLR) signaling (31). Following infection with *L. major* and *L. donovani*, miRNA expression was down modulated through MAP kinase, JAK-STAT, and TGF-β signaling pathways (31). H3K27 has been found to suppress toll-interacting protein that negatively regulates TLR, thereby promoting TLR-mediated inflammatory cytokine production, and activation of innate immune response against the invading pathogens (65).

EXPLOITING EPIGENETICS

Leishmania have evolved stratagems to neutralize macrophage defensive arsenals, the very heart of immune system's defensive machinery, resulting in replication of parasites within phagolysosomal vacuoles of infected macrophages. Unfolding the epigenetic signatures of host-pathogen interactions would help in development of effective drug targets to modulate host immune system and ameliorate the pathogenesis of infection. Some epigenetic marks may serve as putative vaccine candidates. The epigenetic biomarkers may also complement the current diagnostic assays.

Vaccines

An essential hallmark of vaccination is to generate antigen-specific memory T cells for induction of sufficient immune response to protect against re-infection. Epigenetic modifications have been reported to contribute toward memory T cell induction (71, 72). Recombinant histone H1 has been shown to elicit protection in outbred vervet monkeys against CL (73) while histones H2A-2B-3-4 cocktail induced protective immunity against *L. donovani* challenge in hamsters (74). Sirtuins have been used as vaccine candidate against *L. donovani* infected hamsters with induction of Th1 immune response (75). Recently, miRNA-21 has been shown to negatively correlate with IL-12 production and priming of protective Th1 response, suggesting declining levels of miRNA-21 as a potential biomarker of safety and immunogenicity in anti-leishmanial vaccines (76). Therapeutic vaccines may be developed to target miRNA-135 and−126 that bias the Th2 response toward protective Th1 type (69).

Epigenomic-Therapeutics

Despite an array of chemotherapeutic arsenal, mostly targeting the parasites directly, treatment failure, and drug resistance are looming large (77). This has been partly attributed to epigenetics-driven evolution of drug resistant phenotypes to override drug pressure (78). Host-directed epigenetic reprogramming may be refractory to resistance and hence offer hope in this regard (79).

Computer-aided drug repurposing for epigenetic targets is revolutionizing drug discovery (80). DNA methylation, particularly of virulence-associated genes, suggests DNA methyl transferases as potential therapeutic targets. An inverse correlation between *FLI1* gene expression and *MMP1* in cutaneous lesions has also been observed, suggesting *MMP1* as a potential therapeutic target in severe forms of leishmaniasis (22). *FLI1* and *LOX* have also been implicated as potent drug targets in *L. braziliensis* infection (54, 56).

The enzymes effecting histone post-translational modifications, particularly those containing epigenetic reader modules, bromodomains could also be putative therapeutic targets. Sirtuins of *L. donovani* have been validated as drug targets (81). Crystal structure of *L. infantum* Sir2 has been elucidated with implications for drug design (82). Sir2 has been suggested as a resistance marker for VL (21). Phenotypic screening of compound libraries against *Leishmania* has helped in identification of bisnaphthalimidopropyl derivatives as sirtuin inhibitors (83). Imipramine has been found to mediate antileishmanial effect in antimony-resistant *Leishmania*-infected macrophages via targeting HDAC11, resulting in transcriptional inactivation of IL-10 production (24). KH-TFMDI, a novel sirtuin inhibitor, targets HDAC to promote apoptosis-like cell death in *L. amazonensis* promastigotes as well as intracellular amastigotes (84). However, none of the clinically approved HDAC inhibitors are effective against *L. amazonensis* (85).

Studies have deciphered role of miRNA-294 and−721 in *Leishmania* survival via subversion of macrophage nitric oxide production and hence these may be putative therapeutic targets (26, 32). Recent reports of *L. donovani* hijacking the host's transcription factor, c-myc and reduction of intramacrophagic parasite burden upon c-myc silencing or inhibition, with consequent miRNA upregulation, implicate c-myc as a potential therapeutic target (36). Epigenetic targets such as miRNAs screened in *L. donovani*-infected macrophages upon treatment with antileishmanial trans-dibenzalacetone, revealed an imbalance between apoptosis and autophagy (86).

Epigenetic Biomarkers

*Leishmani*a-induced changes in hosts' epigenome may help to predict the clinical outcome of infection and hence complement the existing diagnostics. The state of knowledge regarding epigenetic biomarkers in leishmaniasis is limited. A recent study showed potential of miRNA-361-3p as a prognostic biomarker in CL caused by *L. braziliensis* (61). miRNA-361-3p expression was upregulated in patients with therapeutic failure to pentavalent antimony and hence required more healing times. miRNA-193b and−671 have also been speculated to be prospective biosignatures for prognosis of LCL but require further validation (31).

CONCLUDING REMARKS

The epigenetic mechanisms work in alliance with each other to regulate life cycle of *Leishmania* parasites and ensure their survival. Pathogens are also capable of eluding cellular defensive machinery by changing the epigenetic states of host gene expression, thereby dampening their immune response. A snapshot of epigenetic imprinting of relevant genes in Th cell polarization, and memory T cell differentiation with triggering of innate immune cell populations may provide a basis for development of improved leishmaniasis vaccines.

Targeting the epigenetic marks could result in drug design with less likelihood of development of resistance, thus extending the pipeline toward disease elimination. Whether *Leishmania* parasites tailor the epigenetic mechanisms of their vector sandfly to favor their colonization remain to be elucidated. The impact of these pathogens on vector epigenetics could pave a way for development of transmission blocking vaccines. This review may assist to expand our knowledge of epigenetic influences upon host-parasite interplay, and open the doors to investigate epigenetic targets for rapid diagnostics or therapeutic interventions.

AUTHOR CONTRIBUTIONS

FA: conceptualization, reviewing the studies, writing the mini-review, and critical editing; IK: conceptualization, writing original draft, editing; HH: reviewing the draft; critical editing. All the authors are accountable for all aspects of the review and gave final approval of the version to be published.

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SYSTEMATIC REVIEW
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Evidence for Host Epigenetic Signatures Arising From Arbovirus Infections: A Systematic Review

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Background: Arbovirus infections have steadily become a major pandemic threat. This study aimed at investigating the existence of host epigenetic markers arising from the principal arboviruses infections impacting on human health. We set to systematically review all published evidence describing any epigenetic modifications associated with infections from arboviruses, including, but not limited to, microRNAs, DNA methylation, and histone modifications.

Methods: A comprehensive search was conducted using the electronic databases PubMed, Science Direct and Cochrane Library from inception to January 4th, 2018. We included reports describing original *in vivo* or *in vitro* studies investigating epigenetic changes related to arbovirus infections in either clinical subjects or human cell lines. Studies investigating epigenetic modifications related to the virus or the arthropod vector were excluded. A narrative synthesis of the findings was conducted, contextualizing comparative evidence from *in vitro* and *in vivo* studies.

Results: A total of 853 unique references were identified and screened by two independent researchers. Thirty-two studies met the inclusion criteria and were reviewed. The evidence was centered mainly on microRNA and DNA methylation signatures implicated with secondary Dengue fever. Evidence for recent epidemic threats, such as the infections by Zika or Chikungunya viruses is still scant.

Conclusions: Major epigenetic alterations found on arboviruses infections were miR-146, miR-30e and the Dicer complex. However, existing studies frequently tested distinct hypotheses resulting in a heterogeneity of methodological approaches. Whilst epigenetic signatures associated with arbovirus infections have been reported,

existing studies have largely focused on a small number of diseases, particularly dengue. Validation of epigenetic signatures have an untapped potential, but concerted investigations are certainly required to deliver robust candidates of clinical utility for diagnosis, staging and prognosis of specific arboviral diseases.

Keywords: systematic review, host epigenetics, microRNAs, histone modifications, arbovirus

INTRODUCTION

Arboviruses are virus transmitted to humans by arthropod vectors (1). They are grouped into five families: *Flaviviridae* (e.g., Dengue and Zika viruses), *Togaviridae* (e.g., Chikungunya virus), and the less commonly reported *Bunyaviridae*, *Reoviridae* and *Rhabdoviridae*.

The global distribution of arboviruses is directly linked to the areas cohabited by its vectors and most of them typically occur amongst tropical and subtropical regions (2, 3). For instance, Brazil has recently been plagued by consecutive epidemic seasons, initially with Dengue, then Zika and more recently Chikungunya, during which over 300,000 cases were reported nationwide. (1, 4–6). Ingeniously, infections of arthropod vectors do not seem to affect their fitness and are typically non-pathogenic and lifelong. By contrast, infections of the human host with these viruses, particularly *Flaviviridae* and *Togaviridae* families, are generally pathogenic, often acute and causing viremia (7). This combination largely contributes to their successful viral spread and persistence, accounting for considerable medical and economic burden to healthcare systems of endemic nations (3).

Although arboviruses undergo constant genetic evolution as they typically lack polymerases with proofreading activity and thus exhibit significant mutation frequencies, there is also strong purifying selection resulting from the requirement for their replication in two disparate hosts, and from a balanced fitness trade off they have deployed intricate mechanisms of interaction with their alternate hosts (8). The identification of such interactions may provide insights into the mechanistic basis of the infections with advanced investigative strategies aiming to tackle the current lack of solutions for intervention and therapy.

The role of environmental stimuli, such as a trauma or infection, have always been assumed but largely unexplored until recently due to the unavailability of experimental approaches. Epigenetics is a promising research field and represents a shift in paradigm as it takes into account environmental stimuli modifying gene regulation without necessarily changing the host's core DNA sequences.

In general, the most common epigenetic modifications include changes in chromatin packaging through reversible post-translational modifications in histones (e.g., acetylation, deacetylation), as well as changes in gene regulatory positions, such as gene promoter or gene enhancers, through reversible methylation/acetylation modifications of the DNA nucleotides (9). Of note, the downstream impact of epigenetic alterations may be life long, persisting through subsequent generations of cells during replication (also known as transgenerational gene regulation). Furthermore, other epigenetic events have been proposed that includes the selective neutralization of the messenger RNAs (mRNAs) used in protein translation by interference RNA (iRNA), such as micro RNA (miRNA), and small interfering RNA (siRNA) (10).

The pathogenicity and severity of clinical manifestations posed by arbovirus are likely to involve epigenetic regulation mechanisms, present at the host-pathogen interface. One example is iRNA as a host mechanism acting as an immune response against exogenous molecules, including viruses. In response, viruses are capable of neutralizing host cell defenses by producing factors known as iRNA suppressors (11).

Epigenetic changes can be triggered by several extrinsic factors and are relevant for the host homeostasis and bodily functions, including the control of diseases, which in turn can elicit specific molecular signatures. Thus, the evaluation of epigenetic changes arising from specific arbovirus infections and connected to the immune activation can provide insights into pathophysiological mechanisms prompting the course and severity of a given arbovirus condition.

To the best of our knowledge, no previous review has synthesized the host epigenetic signatures of arbovirus infections. Thus, we conducted a systematic review of original *in vivo* and *in vitro* studies investigating human epigenetic markers arising from infections with arboviruses in both clinical subjects and human cell lines.

METHODS

Search Strategy

This systematic review followed an *a priori* defined yet unpublished protocol. The electronic databases PubMed/MEDLINE, Science Direct and Cochrane Library were searched from inception to January 4th, 2018. The detailed search string used in this systematic review is provided in the supplementary online material that accompanies the online version of this article. We followed the *Preferred Reporting*

Abbreviations: mRNA, messenger RNA (mRNA); RNAi, RNA interference mechanisms; miRNA, micro RNA; miR- micro RNA marker; siRNA, small interfering RNA; DENV, dengue vírus; ADAR1, Adenosine deaminases acting on RNA; TRAF6, TNFR-associated factor 6; IFN, Interferon; LC3, microtubule-associated protein light-chain 3; TMDs, transmembrane domains; dvNS3, dengue virus non-structural protein 3; TLR, toll-like receptors; IL, interleukin; PBMCs, peripheral blood mononuclear cells; CCL5, chemokine (C-C motif) ligand 5; DHF, dengue haemorrhagic fever; SOCS1, regulation of suppressor of cytokine signaling 1 (SOCS1); GrzB, granzyme B; ZIKV, Zika virus; NK, natural killer; hNSC, human neural stem cells; JEV, Japanese encephalitis virus; KLF4, Kruppel-like-factor 4; RNF, ring finger protein 125; WNV, West Nile virus; CHIKV, Chikungunya virus; snoRNAs, small nucleolar RNAs; CCV, Crimean Congo virus; CCHF, Crimean-Congo hemorrhagic fever.

Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (12).

Eligibility Criteria

The inclusion criteria included: original studies, *in vitro* or *in vivo*, regarding epigenetic markers related to human infection by arboviruses. *in vitro* or *in vivo* studies comprised either human cell lines, or clinical subjects. Epigenetic markers consisted of changes in DNA methylation, histone post-translational modifications that affected gene expression, and iRNA suppression mechanisms. No language restrictions were applied, and reports from any country were included to avoid publication bias. Non-original publications, such as reviews, letters or comments were excluded. Studies using animal cell lines, pre-clinical animal studies, *in silico* research, as well as investigations limited to epigenetic modifications on the arbovirus or on the arthropod vector were also excluded.

Study Selection

Two investigators (GA and BD) independently screened the titles/abstracts of the unique references. After this primary screening, the full-texts of selected reports were obtained, and the same authors independently reviewed each article to determine its final inclusion in the review. Whenever a consensus could not be reached, a third author (CL or FM) made the final decision regarding inclusion.

Data Extraction

Two authors (GA and BD) independently extracted the data from the included references using a standardized form. Discrepancies were resolved through consensus. The following information was recorded for each study: author, year, virus/serotype, disease, epigenetic marker, and main results. For *in vitro* studies, we also noted the investigated cell line. For *in vivo* studies, the clinical characteristics of the sample were recorded.

Evidence Synthesis

Due to the heterogeneity of study design, participants and outcomes, we conducted a narrative synthesis of the included studies, summarizing the findings with respect to each arbovirus.

RESULTS

The search in electronic databases yielded 1,025 references. No additional references were found from manual searching the reference lists of included articles. After exclusion of duplicates, 853 unique references were selected for title/abstract screening, of which 43 were eligible for full-text review. Eleven full-text articles were excluded with reasons (see **Table S1** in the Supplementary Material that accompanies the online version of this article). Finally, 32 articles were included in this systematic review (13–44). The PRISMA flowchart of study selection for this systematic review is provided in **Figure 1**.

Characteristics of Included Studies

Overall, this systematic review included 7 *in vivo* (16, 20, 30, 34–37) and 25 *in vitro* studies (13–15, 17–19, 21–29, 31–33, 38–44). The *in vitro* studies used different human cell lines, including three with human monocytes cells lines (22, 23, 42), six with human liver cell lines (13, 15, 19, 24, 31, 40), six studied neuronal cells (26, 28, 32, 38, 41, 44), five used human-derived renal cell lines (15, 17, 25, 27, 33). Two studies were based on human fibroblasts. (14, 21), isolated studies were conducted with natural killer cells (34), epithelial cells (29), human trophoblasts (43), and human vascular endothelial cells (22).

The *in vivo* studies included a total of 171 cases and 102 controls. Sample sizes varied from 6 to 89 participants. All these studies were published in English. Description and characteristics of included studies are provided in **Table 1**.

Epigenetic markers associated with host responses to arboviral infections were mostly reported for Dengue [20 studies; (13–16, 18–20, 22–25, 29–31, 34–36, 39–42), followed by Zika [4 studies (33, 38, 41, 43)], Japanese encephalitis [4 studies(26, 28, 32, 44)], Chikungunya [2 studies;(17, 21)], West Nile [1 study (19)], and Congo Crimean [1 study;(37)] viruses. Overall, the studies investigated a number of epigenetic modifications, notably miRNAs/siRNAs [25 studies,(13, 14, 16, 18–20, 22–25, 29–31, 34–36, 39, 40, 42)], iRNA suppression [3 studies (15, 25, 32)], modifications of iRNA regulators [2 studies (19)], and DNA methylation [1 study (13)]. Only a single study described interactions of viral proteins with histones (13).

Dengue Virus (DENV)

The majority of the studies included in this review investigated DENV, nevertheless these studies analyzed different serotypes and cells types. Overall, 20 studies investigated epigenetic markers associated with DENV infection (**Table 1**). Fourteen of them conducted *in vitro* studies, using a variety of human cell lines (including macrophages/monocytes, hematopoietic cells, Huh7 hepatocarcinoma cells, EAhy926 endothelial cells, HepG2 liver cancer cells and A-549 lung cancer cells). Six studies investigated markers in the blood of clinical samples. Most studies focused on a specific DENV serotype, i.e., DENV-2 (10 studies), DENV-4 (2 studies), DENV-1 (1 study). The other seven studies did not inform the DENV serotype. A description of the *in vitro* and *in vivo* studies included is summarized separately in the sections below.

Epigenetic Mechanisms Associated With DENV Infection Investigated by *in vitro* Studies.

Most DENV *in vitro* studies centered in investigating the interaction of specific DENV serotypes with human cell lines and the elicited epigenetic changes associated with viral replication or the host immune response. These studies focused mainly on iRNA-based mechanisms with eight of them investigating miRNA/siRNA expression. Of these, two used high-throughput tools to determine expression profiles, while the remaining six examined miRNAs associated with DENV infection defined *a priori*. The former assessed profiles following infection of human cell lines with DENV, determining alterations and downstream targets associated with viral replication. Diosa-Toro et al. (39) used small RNA-Seq analysis to investigate the miRNAome and its role in the infection of human macrophages by DENV. They found that only miR-3614-5p was upregulated

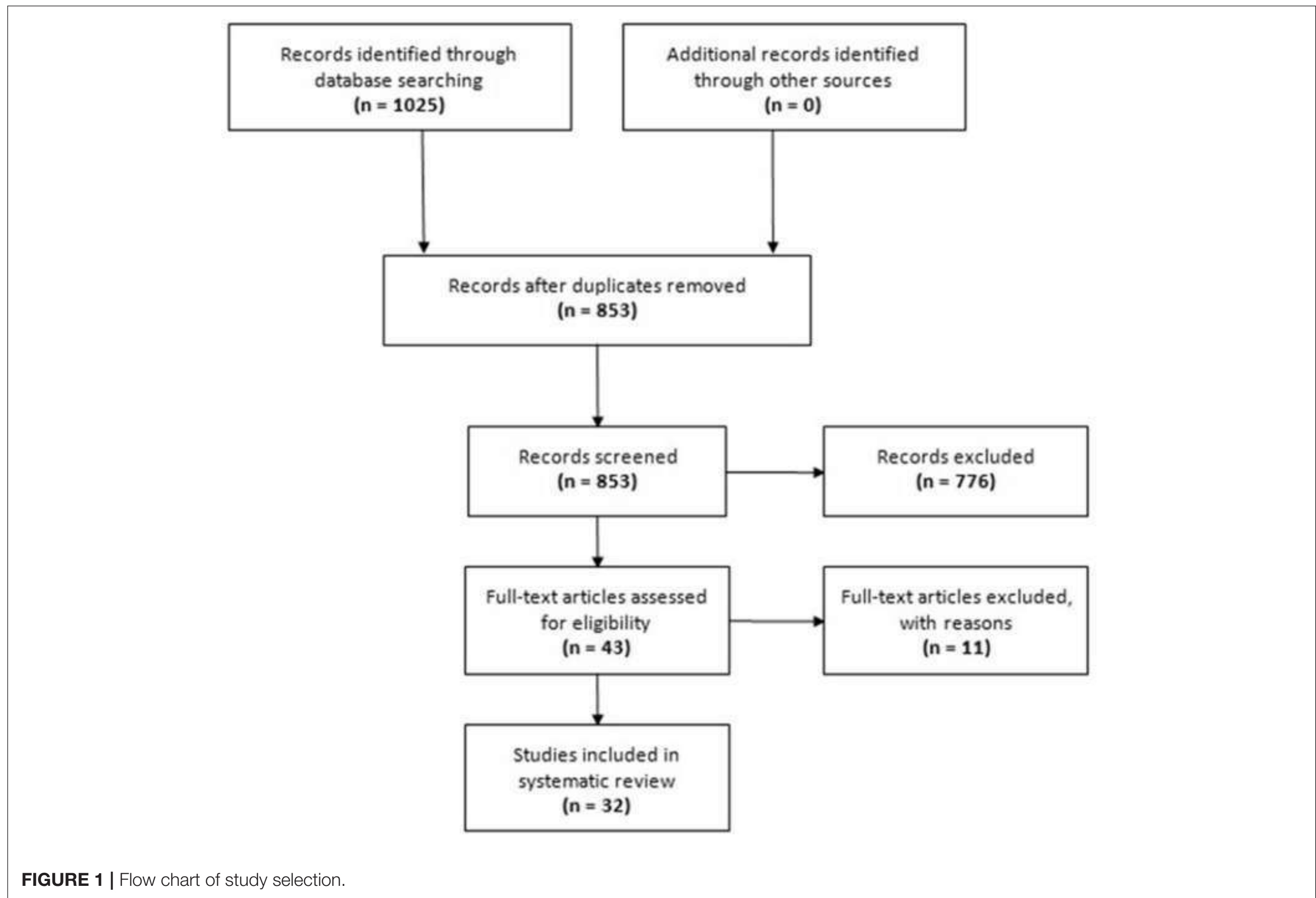

FIGURE 1 | Flow chart of study selection.

in DENV negative cells and its overexpression reduced DENV infectivity. By contrast, miR-3614-5p modified the expression of adenosine deaminases acting on RNA (ADAR1), an RNA-editing enzyme that was expected to have an antiviral role but instead it has been reported to facilitate viral replication. Their findings support a role for miR-3614 as a regulator of DENV infection. The other study by Escalera-Cueto et al. (24) used microarray and quantitative PCR to study the expression of miRNAs in human hepatoma cells (Huh-7) infected with DENV-2. Whilst investigating the longitudinal expression profiles of miRNAs, the authors observed that only Let-7c was overexpressed at different time points after infection with DENV-2, with a peak at 12 h post-infection. Let-7c overexpression seemingly correlated with the reduction of DENV replication through the targeting of the transcription factor BACH-1, whose primary downstream target is the HO-1 enzyme, a marker associated with cellular oxidative stress. As such, the downregulation of BACH-1 is implicated with the upregulation of HO-1 and these findings support the fact that Let-7c has an antiviral effect through the modulation of BACH-1 and HO-1, enhancing cellular oxidative stress. Overall, the two studies above provide evidence for epigenetic parameters associated with viral replication, with each supporting specific miRNAs and downstream targets although this may be due to specific events arising from the use of distinct cell lines and/or DENV serotypes.

Wu et al. (22) identified the role of miR-223 in the regulation of DENV-2 replication and then used three software packages (miRanda, PicTar, and Target Scan) to predict the target genes for miR-223. The analysis suggested that miR-223 targeted the microtubule destabilizer *STMN1* gene, and subsequent experiments demonstrated high levels of *STMN1* during viral replication. In short, the study suggests that miR-223 controls DENV-2 replication by negatively regulating *STMN1*. The same group also looked at the role of miR-146a in DENV replication using human monocytic cells infected with DENV-1, 2, or 3 serotypes (18). The expression levels of miR-146a measured by quantitative PCR were significantly increased after the infection by any of the DENV serotypes. These changes correlate with decreased levels of the TNFR-associated factor 6 (TRAF6) protein and secreted interferon (IFN)-β. The study goes on to suggest that miR-146a facilitates DENV replication by targeting TRAF6 and consequently reducing IFN-β levels.

Pham et al. (14) investigated the role of hematopoietic cells in modulating DENV replication by targeting miR-142, one of the most abundant hematopoietic-specific miRNAs. After infection with DENV-2, they showed that the incorporation of miR-142 onto the 3'UTR of gene target sites induced an

TABLE 1 | Characteristics and main findings of the studies investigating epigenetic markers associated with arbovirus infections in humans.

References	Virus	Study type	Epigenetic alteration	Results	Cell line
Bayer et al., (43)	ZIKV	*in vitro*	chromosome 19 microRNA cluster overexpression	C19MC miRNAs limit ZIKV infection	human throphoblasts
Bogerd et al. (19)	WNV and DENV	*in vitro*	Dicer mRNA	DENV and WNV do not express any viral miRNAs in human cells and neither DENV nor WNV induce the production of viral siRNAs in infected human somatic cells	Huh7 cells, a type of human liver cell line
Casseb et al. (29)	DENV	*in vitro*	Drosha, DGCR8, and Dicer mRNAs	Drosha, DGCR8, and Dicer mRNAs are down-regulated in human cells infected with DENV-4	epithelial cells
Chen et al. (20)	DENV	*in vivo*	miR-150 overexpression	reduced SOCS1 expression was significantly associated with up-regulated miR-150 levels	peripheral blood mononuclear cells
Colpitts et al. (13)	DENV	*in vitro*	Nuclear histone proteins H2A, H2B, H3 and H4 as target of the capsid protein of the virus	All four core histones were pulled out of the cell lysates by DEN2 C and were identified with high percent coverage and low expectation values	Huh7 cells, a type of human liver cell line
Demir et al. (37)	CCHFV	*in vivo*	downregulation of miR-146a and upregulation of miR-451 and miR-31	downregulation od miR-146aand upregulation of hsa-miR-451 and hsa-miR-31 leads to augmentation of proinflammatory cytokines	peripheral blood mononuclear cells
Devhare et al. (38)	ZIKV	*in vitro*	reduced p53 phosphorylation	ZIKV strains induce human neural stem cells (hNSC) growth inhibition and display distinct E glycoprotein localization	human neural stem cells
Diosa-Toro et al. (39)	DENV	*in vitro*	miR-3614-5p overexpression	miR-3614-5p regulated ADAR1 expression, a protein that facilitates viral replication	primary human macrophage
Escalera-Cueto et al. (24)	DENV	*in vitro*	miRNA Let-7 overexpression	Let-7c has an antiviral effect through the modulation of BACH1 and HO-1	Huh7 cells, a type of human liver cell line
Gomes et al. (30)	DENV	*in vivo*	Methylation of cytokine genes	Significant frequency of demethylation in the region of the TNF-a promoter in DENV infected patients	Total RNA was isolated from whole blood
Kakumani et al. (31)	DENV	*in vitro*	human microRNA mir-126	The chaperone and the miRNA, hsa-mir-126-5p restricts dengue virus replication in human cell lines	Huh7 cells, a type of human liver cell line
Kakumani et al. (15)	DENV	*in vitro*	RNAi suppression	Downregulation of iRNA factors as Dicer, Drosha, Ago1 and Ago2. Also, NS4B, a viral protein, as a RNAi supressor.	Huh 7 and HEK293T (human embryonic kidney cells)
Kakumani et al. (25)	DENV	*in vitro*	RNAi suppression	DENV non-structural protein 3 suppresses human miRNA machinery	human embryonic kidney (HEK) HEK293T cells
Kanokudom et al. (40)	DENV	*in vitro*	microRNA 21 overexpression	miR-21 is significantly increased upon DENV 2 infection and that expression of this miRNA promotes DENV replication	HepG2 cells, a human liver cancer cell line
Kozak et al. (41)	ZIKV	*in vitro*	miR-30e-3p, miR-30e-5p, miR-17-5p	induction of genetic pathway of autophagy including CHOP and GADD34	astrocytes
Kumari et al. (32)	JEV	*in vitro*	miR-34c-5p suppression	miR-34c-5p which is suppressed during JEV infection and overexpression of this miRNA modulates JEV induced proinflammatory cytokine production	human microglial cells
Lichinchi et al. (33)	ZIKV	*In vitro*	human m6A RNA	m6A RNA Methylation Modulates the ZIKV Life Cycle	human HEK293T cells
Liu et al. (34)	DENV	*In vivo*	miR-27a*, miR-30e, and miR-378 were down-regulated	miR-378, but not miR-27a* or miR-30e, suppressed GrzB expression in NK cells	NK cells
Ouyang et al. (35)	DENV	*In vivo*	miR-21-5p, miR-590-5p, miR-188-5p, and miR-152-3p overexpression, whereas miR-146a-5p was down-regulated	miR-21-5p and miR-146a-5p could distinguish dengue-infected patients with preferable sensitivity and specificity	Total RNA was isolated from whole blood
Pham et al. (14)	DENV	*In vitro*	miR-142 incorporation	Incorporation of miR-142 target sites into the 3UTR of DENV-2 confers endogenous attenuation of the virus in a cell-specific manner	human fibroblasts
Pu et al. (42)	DENV	*In vitro*	miR-146a overexpression	miR-146a significantly blocked DENV2-induced autophagy	A549 cells (adenocarcinoma human alveolar basal epithelial cells) and THP-1 cells (a human monocytic cell line)

(Continued)

TABLE 1 | Continued

References	Virus	Study type	Epigenetic alteration	Results	Cell line
Qi et al. (16)	DENV	*In vivo*	miR-4290,−4279,−625*, -let-7e,−1290,−33a,−378,−1246,−767-5p,−320c,−720,−491-3p,−3647,−451 and−4286, miR-106b,−20a,−30b and−3653	Upregulated miRNA subsets included: miR-4290,−4279,−625*, -let-7e,−1290,−33a,−378,−1246,−767-5p,−320c,−720,−491-3p,−3647,−451 and−4286. Downregulated miRNA subsets included: miR-106b,−20a,−30b and−3653	peripheral blood mononuclear cells
Rastogi et al. (44)	JEV	*In vitro*	48 differentially expressed microRNAs26/02/2018 34 are significantly up regulated and 14 are down regulated	miR-32-5p is up regulated in JEV infected human microglia with respect its pleiotropic to control. KLF4 (Kruppel-like-factor 4) gene was predicted as one of its target. The down-regulation of miR-432-5p and NEDD4L (neural precursor cell expressed developmentally down-regulated protein 4) has been identified as one of the potential target as well	human microglial cells
Saxena et al. (17)	CHIKV	*In vitro*	miR-744, miR-638, miR-503	miRNA expression profiling revealed regulation of 152 miRNAs post CHIKV infection	HEK293T cells
Selvamani et al. (21)	CHIKV	*In vitro*	miR-146a overexpression	CHIKV infection induced the expression of cellular miR-146a, which resulted into down-regulation of TRAF6, IRAK1, IRAK2 and increased replication of CHIKV in human synovial fibroblasts. Downregulation of TRAF6, IRAK1 and IRAK2 led to downstream decreased NF-kB activation through negative feedback loop	Human Synovial Fibroblasts
Sharma et al. (26)	JEV	*In vitro*	miR146-a overexpression	Exogenous overexpression of miR-146a led to suppression of NF-κB activation and abrogation of Jak-STAT pathway upon JEV infection which led to downregulation of interferon-stimulated genes (IFIT-1 and IFIT-2) and facilitated viral replication	human microglial cells
Slonchak et al. (27)	WNV	*In vitro*	miR-532-5p overexpression	miR-532-5p exhibits antiviral activity against West Nile virus via suppression of host genes SESTD1 and TAB3 required for virus replication	Human embryonic kidney cells (HEK293)
Tambyah et al. (36)	DENV	*In vivo*	Overexpression of a cluster of 12 miRNA (miR-450b-5p,−491-5p,−499a-3p,−512-5p,−615-5p,−624-5p,−892b,−1204,−1225-5p,−3121-3p,−4259 and−4327)	Expression patterns of 12 miRNAs (miR-450b-5p,−491-5p,−499a-3p,−512-5p,−615-5p,−624-5p,−892b,−1204,−1225-5p,−3121-3p,−4259 and−4327) were seen to be significant and specific for acute dengue cases. Expression of miR-24-1-5p, miR-512-5p and miR-4640-3p distinguished mild dengue from those exhibiting liver complications whereas miR-383 was significantly upregulated in mild dengue compared to those diagnosed as severe dengue with fluid accumulation.	Total RNA was isolated from whole blood
Wu et al. (22)	DENV	*In vitro*	miR-223 overexpression	miR-223 overexpression controls DENV-2 replication by negatively regulating microtubule destabilizer STMN1 gene	EAhy926 cells, a human vascular endothelial line
Wu et al. (18)	DENV	*In vitro*	miR-146a overexpression	miR-146a facilitates DENV replication by targeting TRAF6 and consequently reducing IFN-B levels	human monocytic cell THP-1, a model for human monocytes
Zhu et al. (28)	JEV	*In vitro*	MicroRNA-15b overexpression	miR-15b Modulates JEV– mediated Inflammation via Targeting RNF125	human astrocytoma cell and HeLa cells
Zhu et al. (23)	DENV	*In vitro*	induction of MicroRNA-30e*	miR-30e* Suppresses DENV Replication by promoting NF-kB–Dependent IFN Production	human monocyte cell line U937

endogenous attenuation of the virus in hematopoietic but not in non-hematopoietic cells.

A recent work by Kanokudom et al. (40) examined the expression of 7 miRNAs chosen from the miRbase. Following infection of human liver cancer cell line HepG2 with DENV-2, only miR-21 was differently expressed, in which its upregulation was found to promote DENV replication.

Zhu et al. (23) looked at the possible role of miR-30e* in the modulation of innate immunity associated with DENV infection. Human monocyte cell lines were infected with serotypes DENV-1, DENV-2 or DENV-3, and the expression of miR-30e* was measured by quantitative PCR. The results showed that infection with all three serotypes significantly induced miR-30e* expression. Furthermore, silencing miR-30e* was associated with

an increase in DENV replication irrespective of the serotype tested. A further investigation into downstream targets showed that miR-30e* significantly induced the expression of IFN-β protein. Finally, the simultaneous overexpression of the protein IκBα ORF (without 3′-UTR) in miR-30e*-overexpressed cells restored cellular IκBα protein levels. Altogether, this suggests that miR-30e* directly targets the 3′-UTR sequences of IκBα, thus enhancing IFN-β production and consequently suppressing DENV replication.

Recent work by Pu et al. (42) on the role of miR-146a in the autophagy pathway induced by DENV-2 infection in human lung carcinoma epithelial (A-549) cells confirmed that the overexpression of miR-146a was associated with decreased levels of TRAF6. This protein suppresses autophagossome formation and reduces the microtubule-associated protein light-chain 3 (LC3), thus constituting in a marker for authophagy. These results suggest that miR-146a is a negative regulator of DENV-induced autophagy, and TRAF6 is a key target of this microRNA mediating this process. Altogether, the modulation of DENV-associated targets can potentially suppress the excessive inflammation in host cells, thereby lessening the pathological damage caused by DENV infection.

The host iRNA response is regulated by ribonucleases that control the processing of miRNAs and siRNAs, such as Dicer, Drosha, Ago1, and Ago2 complexes (45). These enzymes could be targeted by viral components to regulate host iRNA mechanisms. Overall, five studies investigated the association of DENV infection and the function of these critical enzymes.

Kakumani et al. (15) demonstrated that the infection of human hepatoma Huh7 cells by DENV reduces the expression of the iRNA response regulators, including Dicer, Drosha, Ago1, and Ago2. Furthermore, they showed that DENV replication increased after the suppression of these iRNA regulators. The authors demonstrated that the non-strutural viral protein 4B (dvNS4B) acts as an iRNA suppressor. In fact, NS4B mediates iRNA suppression via transmembrane domains 3 and 5 (TMD3 and TMD5) irrespective of its interferon antagonistic properties, as NS4B can directly inhibit the dicing process. A follow-up study from the same group (25) showed that the DENV nonstructural protein 3 (dvNS3) is another DENV component that potentially acts as a iRNA suppressor in human cell lines. Conversely, Weiskopt et al. (46) reported that NS3 is the main target of CD8+ T cells response, which play a crucial role in the control of DENV infection, though in the context of antibody-dependent enhancement of infection, this may lead to the occurrence of severe dengue. These findings support a role for dvNS3 in favoring viral replication, either by protecting the viral genome from degradation, or by operating the host transcript levels through miRNA regulation. In summary, these studies suggest that DENV encodes suppressing proteins to regulate its replication in mammalian cell lines. Finally, another follow-up study (31) identified that the mitochondrial heat-shock protein GRP75 (which is also part of the human Dicer complex) is involved in the processing of hsa-miR-126, which restricts DENV replication in human cell lines.

Casseb et al. (29) investigated the expression levels of the genes encoding Drosha, DGCR8, and Dicer after the infection of human A-549 cells with DENV-4. DGCR8 is a double-stranded RNA-binding protein that interacts with Drosha and facilitates miRNA maturation. The mRNA levels of Dicer, Drosha, and DGCR8 were all significantly downregulated following the infection, bottoming out at 3 days post-infection and coinciding with an increase in viral replication. Given these DENV-4-mediated changes mirror events described *in vivo* in a timely manner and implicate the toll-like receptors (TLR) and interleukin (IL-1) signaling pathways, authors concluded that the changes in the gene expression pattern of iRNA regulators were directly influencing the innate immune system of the host.

Bogred et al. (19) employed human cell lines that entirely lacked Dicer function to provide better insights into the effects of miRNAs on DENV replication. The study had two principal rationales. First, if a virus is dependent on a particular miRNA, Dicer-deficient cells should be partly or entirely non-permissive with the virus replication. Similarly, had an endogenous miRNA or siRNA impaired viral replication, then the replication process would be accelerated in Dicer-deficient cells. Conversely, their findings appear to suggest that, in general, viral replication in humans is, neither inhibited, nor enhanced by the complete loss of endogenous human miRNAs, which in turn supports the fact that wild-type viruses have evolved mechanisms to evade host miRNA-mediated suppression of the viral gene expression during the replication cycles. Since DENV replication may not necessarily depend on the presence/absence of endogenous miRNAs, their study inferred that the manipulation of miRNA levels may not be as effective to tackle DENV replication as previously thought.

Lastly, a single study investigated the association of histone function with DENV infection and host response. Using the human liver cell line Huh7 infected with DENV-2 serotype, Colpitts et al. (13) used a tandem-affinity purification (TAP) assay to assess which nuclear proteins of the host cell were bound to the DENV-capsid protein (C). They found that the nuclear histone proteins H2A, H2B, H3, and H4 were in fact specific targets of DENV C. The colocalization of viral proteins with all four histones in the cytoplasm of infected cells demonstrated the formation of dimers, which interestingly were resistant to both heat and denaturation. Therefore, DENV C may either bind to histones before they enter the nucleus, or pull them into the cytoplasm after getting translocated into the nucleus and binding to the histones. In addition, DENV C binds cellular DNA, either directly or by forming a complex with core histones. This leads to disruption of histone dimerization and nucleosome formation, thus resulting in gene expression alterations, impairment of DNA transcription, increased DNA damage and, allegedly, a shift favoring the translation of viral over cellular mRNA. The authors also reported that the levels of the four histone proteins examined increased following the infection and that the presence of the DENV-2 virus was associated with changes in H2A phosphorylation over time. At 24 h post-infection, phosphorylation of H2A was significantly higher than in uninfected cells. In contract, the data revealed decreased phosphorylation at 48 h post-infection, whilst the levels increased again at both 72 and 96 h post-infection. In conclusion, this study suggests that the capsid protein of the DENV-2 virus may target

histones to disrupt normal host cell genetic machinery in favor of viral replication and perpetuation of its life cycle.

Epigenetic Mechanisms Associated With DENV Infection Investigated by *in vivo* Studies

Several studies investigated blood samples of DENV-infected patients to prospect epigenetic indicators with diagnostic and/or therapeutic potential.

Ouyang et al. (35) collected a total of 72 serum specimens (i.e., 40 patients with active DENV-1 replication and 32 healthy controls). Initially six serum samples (3 patients and 3 controls) were profiled using miRNA PCR arrays in blood. Compared to healthy controls, 41 miRNAs were found to be upregulated and a further 12 miRNAs were downregulated in the sera of DENV-1 patients. The validation analysis was performed with the remaining samples using quantitative PCR and confirmed that only serum miR-21-5p, miR-590-5p, miR-188-5p, and miR-152-3p were upregulated, while only miR-146a-5p was downregulated in DENV-1-infected patients. Receiver operating characteristic (ROC) curve and correlation analyses were performed to evaluate the potential of these miRNAs for the diagnosis of DENV infection. ROC curve suggested that only serum miR-21-5p and miR-146a-5p could reasonably discriminate DENV-infected patients from healthy controls.

Tambyah et al. (36) investigated blood samples of DENV-infected patients to shortlist miRNAs that could be associated with the pathophysiological mechanisms. Patients infected by influenza virus were used as a comparison group to determine the specificity of the differentially expressed miRNAs to DENV infection. Results showed that 12 miRNAs (miR-450b-5p, miR-491-5p, miR-499a-3p, miR-512-5p, miR-615-5p, miR-624-5p, miR-892b, miR-1204, miR-1225-5p, miR-3121-3p, miR-4259, and miR-4327) were specifically linked with acute dengue cases.

Qi et al. (16) reported results from miRNA and inflammatory cytokine profiles from the peripheral blood mononuclear cells (PBMCs) of 24 DENV-2-infected patients. Changes in the expression of cytokines were investigated using multiplex arrays with levels of the chemokine (C-C motif) ligand 5 (CCL5), IL-6, and IL-8 were increased in DENV-2-infected PBMCs in comparison to uninfected PMBCs, while levels of TNF-α, IL-10, MCP-1, and CCL4 were decreased compared to uninfected PBMCs. Their initial miRNA array analysis identified 11 upregulated and 4 downregulated miRNAs with replication analysis using quantitative PCR analyses confirming that miR-106b, miR-20a, and miR-30b were downregulated in DENV-2-infected PMBCs, while miR-4290, miR-let-7e, miR-1290, and miR-33a were found to be upregulated. Whereas follow-up *in silico* analyses suggested that miR-106b may target CCL5 mRNA, miR-let-7e were found to inhibit CCL3 and IL-6 mRNA expression. Altogether, this supports a role for miRNAs to modulate immune activation of DENV-2-infected PMBCs, highlighting that specific miRNAs target specific inflammatory mediators.

Chen et al. (20) investigated the role of miRNAs in the regulation of suppressor of cytokine signaling 1 (SOCS1) and its association with dengue haemorrhagic fever (DHF). *SOCS1* is a negative regulator of cytokine signaling and may be involved in the development of DHF during DENV infection. Quantitative PCR was used to measure *SOCS1* mRNA and its regulatory influence on miRNA levels in PBMCs obtained from the blood of patients with non-hemorrhagic dengue fever (DF) vs DHF. Patients with DF but not DHF had higher levels of *SOCS1*, as well as increased production of IFN-γ. On the other hand, DHF patients had increased production of IL-10. miR-221 and miR-572 levels were higher in patients with DF when compared to DHF. On the other hand, DF patients had decreased levels of miR-150 in comparison to DHF. With respect to *SOCS1* mRNA expression, this was inversely correlated with miR-150 expression in the PMBCs of patients with DHF, with an *in vitro* experiment replicating this association using DENV-2-infected PMBCs. Altogether, these findings support that *SOCS1* expression is reduced in PBMCs derived from patients with DHF, which in turn was significantly associated with the upregulation of miR-150 levels in a dose-dependent manner.

Liu et al. (34) studied the role of miRNAs in the regulation of granzyme B (GrzB), a serine protease found in the granules of cytotoxic lymphocytes (CTLs), natural killer cells (NK cells) and cytotoxic T cells. Software packages miRanda and TargetScan were used to predict sequences of miRNAs that potentially bind to the 3'-UTR regions of perforin (PRF1) and GrzB mRNA sequences, and as a result regulating the expression of these cytotoxic molecules in humans. They measured expression levels of the miRNAs selected by *in silico* analysis (i.e., miR-27a*, miR-30e, and miR-378) using quantitative PCR assays. All three miRNAs were reported to be downregulated, but miR-378 exhibited remarkably higher binding potential to the 3'-UTR of GrzB compared to the analogous PRF1 mRNA sequence. Therefore, this study suggests that miR-378 is a key player in the host immune response by modulating NK cell GrzB production during acute DENV infection.

A single study examined the gene methylation status of genes encoding for proteins modulating the innate immune response. Gomes et al. (30) used methylation-specific PCR assays to identify the methylation status of the IFN-γ and TNF-α gene promoters in the DNA extracted from peripheral blood from DENV infected subjects. Their comparative analysis showed that these patients had significant demethylation in the TNF-α but not in the IFN-γ promoter regions.

Zika Virus (ZIKV)

In total, four *in vitro* studies investigated epigenetic modifications associated with ZIKV infection.

Bayer et al. (43) investigated the hypothesis that trophoblastic chromosome 19 miRNA cluster (C19MC) expressed in primary human trophoblasts (PHTs) might reduce ZIKV infection in non-trophoblastic cells. PHTs are relatively resistant to infection by several viruses. The fact that the resistance to ZIKV is lost in embryonic stem cells when they are differentiated into an early trophoblast lineage suggests that term trophoblasts utilize a diverse array of antiviral pathways to protect the placenta, and consequently, the fetus, from ZIKV infection. However, at very early stages of pregnancy, when embryonic lineages are being differentiated, there is a probable period of trophoblast vulnerability that seemingly ends when the subsequent villous

placenta emerges and begins to mature. Therefore, C19MC could regulate the susceptibility of non-trophoblastic cells to ZIKV. In their work, human osteosarcoma U2OS cells were transfected with the most highly expressed members of C19MC (miR-512-3p, miR-516b, miR-517a, and miR-525-5p) and infected with ZIKV. C19MC miRNA reduced ZIKV infection, as denoted by a 50% drop in the production of viral RNA. As PHT cells can also regulate type III IFNs as a secondary pathway to achieve resistance to infection, it was demonstrated that C19MC does not exert any effect on interferon-stimulated genes, and that type III IFNs do not affect the expression levels of miRNAs in U2OS cells. Together, these findings provide evidence that the miRNAs stimulate resistance to the disease through a type III IFN-independent pathway.

Devhare et al. (38) investigated mechanisms associated with ZIKV-mediated impairment of human neural stem cells (hNSC) differentiation and progenitor cell growth, and their relationship with different sets of ZIKV strains. The hNSCs were infected with either the African, the Asian ZIKV strains, or both. Cells infected with both strains displayed distinct E glycoprotein localization and growth pattern. Their results showed an upregulation of several genes upon viral infection and a more effective growth of African ZIKV in hNSCs (though authors mentioned this may be in part due to this variant being utilized for several passages in cell culture), but not of Asian ZIKV, for which less studies were also documented. Furthermore, expression of genes associated with cell cycle arrest were enhanced in virus-infected hNSCs, consequently inducing DNA damage responses though this might be dependent on specific virus strain's mechanisms. They also demonstrated a modest increase in p53 Ser15 phosphorylation, a marker of p53 functional activation, in Asian Zika virus-infected hNSCs. However, African Zika virus-infected cells exhibited reduced p53 phosphorylation. Lastly, it was analyzed the expression levels of p53 targets p21 and PUMA and, interestingly, p21 was detected only in Asian ZIKV-infected hNSCs, potentially promoting cell cycle arrest and the capacity to limit the DNA damage. Conversely, the African strain led to a more apoptotic cell death aspect.

Kozak et al. (41) studied the profile and temporal changes of host miRNAs and transcriptome associated with ZIKV. Human embryonic astrocytes were infected with ZIKV strain PRVABC59, and changes in miRNAs expression was measured 24, 48, or 72 h post-infection using several techniques, including immuno?uorescence, quantitative PCR and microarrays. Their findings suggest that there was a trend toward an overall decrease in miRNA expression in the cells over the course of ZIKV infection. A subset of miRNAs was found to be upregulated during infection, including miR-30e-3p, miR-30e-5p, and miR-17-5p.

Changes in the expression profile of candidate target genes suggest that the unfolded protein response pathway are a major target for ZIKV-mediated regulation, since they observed at 48 h post infection an induction of genetic pathways related to authophagy, including *CHOP* and *GADD34*.

Lichinchi et al. (33) investigated the methylation status of ZIKV RNA and the role of these epigenetic modifications in the host. Authors reported that host methyltransferases and demethylases control N6-Methyladenosine (m^6A) modification in ZIKV RNA. A metagene analysis of host epigenetic modifications showed that ZIKV infection increased m^6A levels in the 5′UTR but comparatively decreased in 3'UTR target positions of the human transcriptome. Gene ontology analyses of these genes implicated a number of immune-related mediators when m^6A levels peak in the host.

Japanese Encephalitis Virus (JEV)

Two *in vitro* studies used high-throughput screening tools to assess miRNA expression profiles in human microglial cells infected with JEV.

Kumari et al. (32) employed microarrays to investigate levels of miRNAs at 6, 24, or 48 h post-infection, aiming to identify overlapping patterns of differentially expressed markers associated with JEV. Initially only 36% of miRNAs were upregulated at 6 h post-infection, peaking at 24 h with a proportion of 71% of the investigated miRNAs and remaining high at 48 h post-infection (64%). A consistent upregulation across the time series was observed for a specific group of miRNAs, including miR-3648, miR-129-5p, miR-3687, and miR-572. The same study also found downregulation of miRNAs after infection of microglial cells with JEV. At 48 h post infection miR-128, miR-132, miR-222, and miR-130b were found to be downregulated.

Authors also investigated the role of miRNAs in regulating the expression of genes associated with the host immune response. Since activation of Notch pathway during JEV infection is modulated by miR-34c-5p and this probably binds 3′UTR of Notch gene, overexpression of miR-34c-5p is expected to lead to attenuation of JEV induced TNF and IL-6 production by microglia. They observed no apparent impact on the viral replication since no significant changes in jevNS1 protein expression were detected.

Rastogi et al. (44) used microarrays to investigate changes in the expression of 526 miRNA in JEV-infected human microglial cells. They found that 48 miRNAs were differentially expressed: 34 were upregulated, while a further 14 were downregulated. Bioinformatics tools were used to identify the targets of the differentially expressed miRNA. The upregulated miRNAs were involved in pathways associated with several processes, including the maintenance of endothelial barrier and adhesion junctions, ubiquitin-mediated signaling and protein processing in the endoplasmic reticulum, and apoptosis. The downregulated miRNAs were associated with pathways mediating proinflammatory responses, cytokine and chemokine expression and neuropeptide signaling pathway. Although the miRNA-target interactions were not specifically investigated, authors speculate on four possible mechanisms associated with susceptibility of microglial cells to JEV infection that would be related to the changes in miRNA profiles. The miR-32-5p appeared to be upregulated and the Kruppel-like-factor 4 (*KLF4*) gene was predicted as one of its target. This gene has been reported to regulate endothelial barrier function in glioma cells, by suppressing the promoter activity of tight junction proteins. Thus, regulation of the blood-brain barrier might be a pathological mechanism associated with JEV infection.

Moreover, miR-29b-3p was found to be upregulated, and Tumor Necrosis Factor receptor associated factor 4 (*TRAF4*) was identified as its downstream target. *TRAF4* regulates cell survival, and since microglial cells are activated but never do they undergo apoptosis during JEV infection, this might be the result of *TRAF4* suppression, which inhibits apoptosis and promotes cell survival. The expression of miR-205-5p was suppressed after JEV infection, and its predicted target was the NFAT5 gene, which is associated with neuroinflammation. Therefore, regulation of this pathway following Japanese encephalitis may result in inflammation and BBB changes. Finally, miR-432-5p was downregulated, and its potential target gene, neural precursor cell expressed developmentally down-regulated protein 4 (*NEDD4L*), is involved in the ubiquitin-mediated proteolysis pathway and has been reported to promote and suppress the rate of virus release from infected cells. Therefore, miR-432-5p may play a critical role in regulating virus release from microglial cells during JEV infection.

Another two *in vitro* studies investigated specific miRNAs associated with JEV infection. Zhu et al. (28) used the human astrocytoma cell line U251 to assess the role of miR-15b as a regulator of JEV-induced inflammation through targeting of ring finger protein 125 (RNF125), which may regulate the T-cell receptor signaling pathway. This study showed that virus-mediated induction of miR-15b increases RIG-I expression by direct suppression of the target gene *RNF125*, resulting in the aggravation of JEV-induced production of inflammatory mediators. miR-15b is also involved in the regulation of chemokine- and cytokine-mediated inflammation pathways, including TNF-α, IL-1β, IL-6, CCL5, IL-12p70, and CCL2. Therefore, authors finally showed that these immunological mediators were significantly upregulated during JEV infection and the inhibition of endogenous miR-15b using antisense miRNA significantly decreased JEV-triggered cytokine production.

Sharma et al. (26) studied the anti-inflammatory role of miR-146a during infection with the JEV strain JaOArS982. They used quantitative PCR and luciferase assays to determine the influence of miR-146a on the *in vitro* expression of interferon-stimulated genes using human microglial cells. Infection with JEV upregulated miR-146a, which was associated with the gene expression downregulation of *TRAF6*, *IRAK1*, *IRAK2*, and *STAT1*. The exogenous overexpression of miR-146a suppressed NF-κB activation and decreased the Jak-STAT pathway activity associated with JEV infection, which in turn led to the downregulation of interferon-stimulated genes (*IFIT-1* and *IFIT-2*) and ultimately facilitated viral replication.

West Nile Virus (WNV)

Two *in vitro* studies investigated the role of epigenetic modifications in the human response to WNV infection. Both studies used high-throughput techniques to investigate iRNA-based mechanisms.

Slonchak et al. (27) studied the role of host miRNA during WNV human infection. The authors employed RNASeq analysis to determine changes in the expression of host miRNAs in human embryonic kidney 293 (HEK293) cell line. In comparison to the uninfected controls, multiple miRNAs were differentially expressed in the infected cell group at 24 and 48 hours post-infection. Only three miRNAs were significantly upregulated: miR-1271-5p, miR-532-5p, and miR-1307-3p. To understand their functions, HEK293 cells were transfected with either miRNA-specific or nonspecific miRNA mimics or inhibitors, and then infected with WNV. WNV titers appeared to be similar to controls whenever the miR-1271-5p and miR-1307-3p mimics or the inhibitors were transfected, thus indicating that these miRNAs did not influence viral replication. In contrast, WNV titers were significantly reduced in cells transfected with the miR-532-5p mimic and increased in cells transfected with the miR-532-5p inhibitor. A genome-wide computational prediction of miR-532-5p targets shortlisted 10 potential targets. In the follow-up investigation, only the for *TAB3* and *SESTD1* mRNAs were reported to be decreased following WNV infection. Altogether, this suggests that the upregulation of miR-532-5p and subsequent suppression of *SESTD1* and *TAB3* genes represent a host antiviral response during WNV infection.

Bogred et al. (19) studied the influence of endogenous miRNAs and virus-induced siRNAs on WNV replication. In a first experiment, human Huh7 cells were infected with WNV and the expression of miRNAs/siRNAs was examined using deep sequencing. They noticed that infected cells did not express detectable levels of miRNAs and/or siRNAs. In a second experiment, authors used a human cell line that did not express Dicer, and, therefore, were unable to produce miRNAs or siRNAs. No significant differences in viral replication were observed in WNV-infected cells in relation to the controls with unchanged Dicer activity. Altogether, the results show that WNV gene expression, similarly to the findings described above for DENV, is not suppressed by endogenous miRNAs which points to the fact that viruses may have evolved mechanisms in an attempt to evade or bypass human miRNA-based defenses.

Chikungunya Virus (CHIKV)

Only two *in vitro* studies investigated epigenetic modifications associated with CHIKV infection. One used high-throughput tools to screen for potential diagnostic markers or therapeutic targets (17), while the other explicitly investigated miR-146a (21).

The first study described miRNA signatures associated with CHIKV infection from three different human cell lines: human embryonic kidney cell line HEK293T, Vero-76, and primary human dermal fibroblast cells. They employed custom microarrays to identify miRNAs with altered expression patterns. A total of 59 miRNAs were found to be upregulated, while a further 33 were downregulated at 12 and 24 hours post CHIKV infection. Approximately 53 and 45% of upregulated and downregulated miRNAs, respectively, have been implicated with other viral infections, particularly HCV, HBV, HPV and HIV1. These authors also investigated changes in the expression of small nucleolar RNAs (snoRNAs), an epigenetic mechanism that guides chemical modifications of other RNAs, mainly ribosomal RNAs (47). Findings showed that 48 snoRNAs were upregulated. Of these, 16 were of the C/D type, known to be associated to ribosomal RNA acetylation.

Conversely, the second study investigated the role of miR-146a in the regulation of inflammatory responses associated with CHIKV infection in human synovial fibroblasts. CHIKV infection was associated with increased cellular expression of miR-146a and subsequent gene expression downregulation of *TRAF6*, *IRAK1,* and *IRAK*, as well as increased viral replication. Therefore, They concluded that CHIKV reduced anti-viral immune response in human fibroblasts through enhanced expression of miR-146a.

Crimean Congo Virus (CCV)

A single *in vivo* study investigated CCV-associated epigenetic factors. Demir et al profiled miRNA expression of plasma samples from eight individuals with confirmed Crimean-Congo hemorrhagic fever (CCHF) diagnosis (37). Their results identified 106 CCV-associated miRNAs that were differentially expressed in peripheral blood mononuclear cells. Main upregulated signatures were miR-144, miR-451, miR-486-5p, miR-608, miR-363, miR-31, miR-32, miR-575, miR-632, and -miR-541 in CCHF patients. Out of that, several targets were implicated with the immune cell activation, cellular adhesion and signal transduction. Authors highlighted that a similar cytokine imbalance occurs during viral hemorrhagic fevers, regardless of the etiolopathological agent, and that could be the result of disease processes mediated by shared epigenetic alterations.

Furthermore, chief downregulated miRNA leads were miR-493, miR-889, miR-655, miR-656, miR-26a-1, miR-154, miR-335, miR-1197, and miR-146a. They proposed that at least some of these candidates were related to proinflammatory cytokine-induced alterations on the endothelium, leading to vascular dysfunctions and the development of hemorrhagic damage seen in CCHF patients.

Altogether, their findings suggest a key role for miRNAs in regulating human immune response to CCV and modulating disease progress and CCHF clinical manifestations.

DISCUSSION

In general terms there are five well-characterized processes that appear to mediate epigenetic regulation, namely DNA methylation, nucleosome positioning, histone variants, histone modifications, and regulatory RNA class family. While there is a large body of evidence suggesting that epigenetic factors play a critical role in the regulation of virus infections, in many cases the detailed mechanisms have not been elucidated. Although several limiting factors contribute to our relative lack of understanding of the intricacies of viral epigenetic regulation, the inherent complexity of epigenetic regulation most likely constitutes in the single chief hurdle. To our knowledge, this is the first review that aimed to synthetize epigenetic changes associated with arboviruses infections in the human host.

Our results reveal a significant heterogeneity across studies, with authors frequently exploring varied hypotheses and distinct sets of markers, which unquestionably restricted our abilities to draw direct comparisons and undertake a substantiated meta-analysis. Our review showed that the most frequent epigenetic events expressed during arboviruses infections were related to modifications in miRNA profiles with a number of leads implicated. Aside of this, alterations in the dicer complex and dicer-like enzymes were also reported by two independent studies (19, 29). Only a single study found association with histone modifications (13) with a further investigation describing changes in the DNA methylation pattern (30). Another study reported alterations in p53 phosphorylation using an infection model of ZIKV (38).

The most frequent human miRNA alteration implicated with arbovirus infections was the miR-146. This miRNA may constitute in an interesting biomarker as it was upregulated during infections with JEV, CHKV and DENV (18, 21, 26) though it was downregulated during CCHF (37). Two of the revised studies found miR-146 levels to be in perceivable downregulation following CCHF infection (37), suggesting that its reduction following infection led to the secretion of proinflammatory mediators. Of note, CCHF is the only non-mosquito-borne arbovirus included in this review but comes from a family almost exclusive of tick-borne viruses (*Nairoviridae*), and given these particularities will most likely elicit a rather distinct pathophysiological response compared to those of mosquito-borne viruses. Irrespective of that, not all findings were consistent since a recent study found that miR-146 was downregulated during DENV infection (35). Further to this, miR-146 seems to be a key inflammatory mediator that stimulates the release of cytokines during innate immune response (48), however it seems to work through a divisive role as a proviral factor as it targets IRAK1 and TRAF6 expression leading to the inhibition of IFN production, thus hindering antiviral host defenses (49). It is possible that its upregulation observed during infections with JEV, CHKV and DENV may actually the result of increased host susceptibility to these diseases, therefore warranting further investigations to determine its clinical utility. The miR-146 has also been proposed to be a marker of sepsis, as well as a target to reduce immune hyperactivation during infection (50).

Our review also supports the involvement of other host miRNA markers as epigenetic modulators of arbovirus infections. For instance, miR-30e has been linked with NF-kB activation and IFN-β production, and its altered levels generally associated with restriction of DENV infection, via positively modulating the antiviral innate immune response (23, 51). While another study (41) showed that miR-30e is involved in an autophagic pathway in patients infected with ZIKV, others proposed that miR-30e had no actual role in regulating expression of granzyme B in NK cells during DENV infection (34), an important cytotoxic molecule to control viral replication. Despite a growing body of evidence for miR-30e, a consensus on its actual role in modulating the human immune system following viral infection has yet to be reached. Similarly, Dicer, a class III endoribonuclease, cleaves dsRNA into siRNA and miRNA. Both siRNA and miRNA facilitate the activation of RNA-induced silencing complex (RISC), which is essential for RNA interference characteristics (9). In other words, since both processing enzymes Drosh and Dice, process the majority of pri-miRNA/pre-miRNAs into duplex mature miRNAs, which is essential for rendering their biological properties, alterations in their levels as a result of an infection would not

be confined to arbovirus and would not constitute in a specific molecular signature. Indeed, another review indicated that cellular miRNAs physiologically regulate the replication of other mammalian viruses with deliberate repression of mammalian Dicer increasing the replication of HIV-1, vesicular stomatitis virus (VSV) and influenza A virus (52).

All in all, our review suggests that epigenetic mechanisms accompanying arbovirus infections may be rather specific and distinct to the ones associated with other common viral diseases, such as DNA tumor viruses where alterations in miRNA expression patterns are not so frequent (10). These viruses have predominantly been associated with DNA methylation events in the promoter positions of key disease-associated genes (10). Similarly, DNA methylation is part of the typical gene silencing process that occurs during the latent infection by the herpes simplex virus (HSV) family (10). Conversely, polyomavirus and adenovirus frequently have their viral genome integrated into the host genome with some studies reporting these viruses may also employ DNA methylation mechanisms during the course of the infection (10). A widely described virus-driven epigenetic modification is the one associated with Papilomavirus that implicates histone modifications (10).Comparatively, Hepatitis B virus possesses a mini chromosome with nucleosome and histone modifications, which appears to dysregulate a number of cellular pathways, in part by binding to genomic DNA, by changing expression patterns of miRNAs, or by connecting to sirtuin1 gene (SIRT1), an intracellular regulatory protein known to regulate epigenetic gene silencing and suppress recombination of rDNA. Overall, this supports that viruses may use different epigenetic mechanisms, and that miRNA may be functionally more relevant for arbovirus infections in humans. Despite that, the significance of miRNAs for the development and progression of arboviral infections and for the host disease response remain largely unclear.

Only a single study reported specific epigenetic alterations when stratifying patients for cardinal clinical features (36). The authors identified differentially expressed miRNAs in patients with mild vs. severe dengue, with which they supported the existence of a relevant epigenetic basis for disease progression and complications seen in some patients. Yet, a major limitation for producing sturdier assertions from the existing literature is the limited number of *in vivo* studies available. Only six studies were encountered and met inclusion criteria of our review, with five of them restricting research to DENV infection only. In these studies, authors were mainly engaged in either, identifying a pattern of epigenetic signatures during the infection (16, 35), or searching evidence for the activation of genes that encode cytokines and the innate immune system, such as TNF-alpha (30). It has been documented that the miRNAs associated with cytokine activation may lead to either side of the disease spectra depending on the pattern of their gene targets and downstream pathways. It has been shown that sustained levels of IFNs, whether or not miRNA-dependent, in DENV-infected humans correlate with protection and sub-clinical disease (53, 54), and their secretion correlates with the induction of a cytotoxic T-cell response (46). For instance, infection with DENV up-regulated the expression of miR-30e*, which a play a role controlling the infection through the expression of IFN-β. Conversely, other authors suggest that miR-146a facilitates DENV replication (18) by reducing IFN-β levels, the opposite of what is achieved with the introduction of miR-30a, which highlight the need for carrying out further evidence-based on this topic to support these views.

A clinically relevant question that our review was unable to address concerns the persistence of early response markers, such as IgM anti-Chikungunya antibodies, in chronic patients. These have been documented even after several months since the disease onset, suggesting that single-stranded arbovirus infections are seldom self-resolving processes and certainly not short-lived as many previously speculated (55, 56). Another important limitation of our review is the lack of meaningful literature in relation to specific epigenetic events and lead biomarkers with potential to discriminate the myriad of infections caused by circulating arbovirus worldwide. In Brazil, for instance, over 210 arbovirus species have already been isolated, the vast majority from the Amazon rain forest, with at least 110 new to scientist and 34 proven to cause human infections. (57). Furthermore, arbovirus are RNA entities, very mutagenic and capable of causing renewing public health threats in the form of outbreaks and pandemics. In addition to the highly publicized emergence of ZIKV and CHKV, the last decade has witnessed an expansion of previously localized endemic arbovirus, such as Oropouche (OROV), Mayaro (MAYV), Rocio (RCV), Saint Louis encephalitis (SLEV), Ross River (RRV), and both Eastern, and Western Equine Encephalitis (EEEV and WEEV) viruses from the *Flaviviridae, Togaviridae* and *Bunyaviridae* families, with each almost certainly resulting in specific molecular signatures and distinct patterns of host responses.

In general, arboviruses are zoonosis-causing agents as they are mostly kept in nature by both a non-human vertebrate and a hematophagous arthropod vector cycles. To further compound this, the saliva from blood-feeding arthropods, such as mosquitoes is enriched with active molecules that display a variety of functions leading to the modulation of the host immune system, ultimately facilitating arbovirus evasion and the establishment of the disease (58). Depending on the vector in question and intrinsic human factors, that *per se* can result in specific host-mediated immune and epigenetic responses, adding further complexity to effectively integrating *in vitro* and *in vivo* molecular analyses.

Clearly, arboviruses infections account for a massive disease burden to healthcare systems and to labor productivity, especially in tropical and developing countries (59). This systematic review shows that the major epigenetic alterations associated with arboviruses infections were microRNAs, particularly miR-146 and miR-30e, and the dicer complex. Studies fitting our inclusion criteria have been highly heterogeneous in nature, presenting varied methodological designs and testing for distinct sets of hypotheses. Further research is certainly necessary to appreciate how epigenetic findings could be validated as biomarker tools for diagnosis, staging, and prognosis of arbovirus diseases in humans. Concerted work into the mechanisms of specific epigenetic regulations is likely to support the

development of bespoken prevention or therapeutic strategies for arboviral infections.

Certainly, more thorough out epigenetic studies on the interactions of arboviral infections with common chronic diseases in humans, as well as the role of co-infections, would be extremely opportune for meaningful progress in this field. For instance, a major outbreak driven by emerging CHKV variants in Brazil between 2016 and 2017, has reminded scientists and public health authorities of the devastating impacts of arboviral infections and disease chronification on labor productivity and outcomes of underlying diseases, especially diabetes type II (59). The untapped potential of epigenetic markers may constitute the missing link to bolster a deeper understanding of pathogenesis and shared response mechanisms across disease-causing arbovirus species and between strains/serotypes of the same species. Combined with the realization of inter-individual differences in disease susceptibility, the development of effective molecular-based tools, including epigenetics, can deliver critical insights into the dynamics of arboviruses epidemics and recurrent outbreaks, potentially enabling both patient and treatment stratifications and the refinement of effective epidemiological surveillance strategies.

AUTHOR CONTRIBUTIONS

GA, CL, SV, DM, and FM designed the study protocol. GA and BD performed the literature search. GA, BD, CL, and FM identified studies for eligibility. BD and GA extracted the data. CL, GA, BD, LC, SV, DM, and FM outlined results and work execution. GA, CL, and FM drafted the initial manuscript. LC, MM, RM, SV, DM, AV, and FM critically reviewed and revised the manuscript. All authors read and approved the final manuscript as submitted.

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SUPPLEMENTARY MATERIAL

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