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The role of epigenetics in infectious diseases

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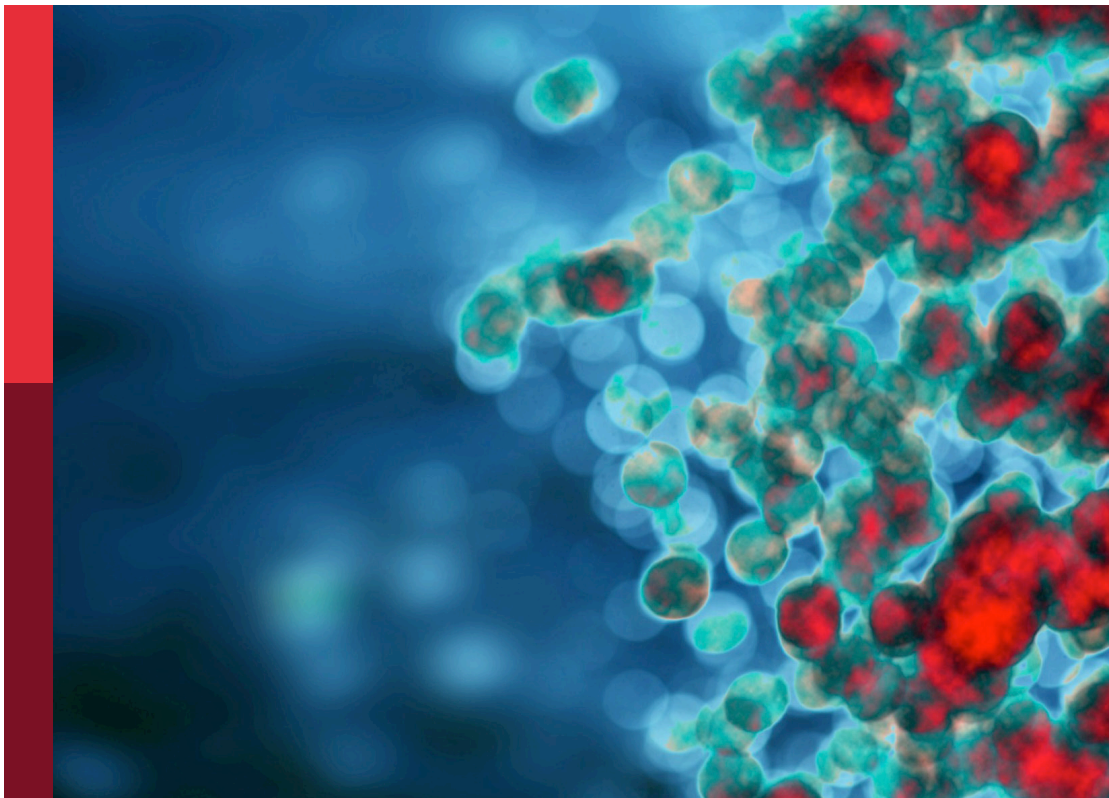

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The role of epigenetics in infectious diseases

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Editorial: Epigenetics of infectious diseases

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Editorial on the Research Topic
The role of epigenetics in infectious diseases

Host genetics contribute to variations in the acquisition, disease manifestation, and outcomes of pathogenic infections across individuals. One of the factors influencing the variability in infection and pathogenesis is epigenetics. Epigenetic factors such as DNA methylation, histone modifications, and non-coding RNAs (ncRNAs) are instrumental in host-pathogen interactions. These factors regulate pathogen and host genes without altering their genetic sequences. For instance, viruses may modify host-specific histones to make the host genome more accessible for viral replication. In contrast, the host may silence the integrated viral genome through DNA methylation, inhibiting viral replication. Thus, the series of contributions gathered in this Research Topic explores intriguing epigenetic factors that influence host-pathogen interactions. This research will drive our understanding of the role of epigenetics in immunology, pathogenesis, and possible clinical intervention of infectious diseases.

Pathogen-associated molecular patterns (PAMP) from bacteria, fungi, viruses, and protozoa may alter the epigenetic landscape of host immune cells involved in pathogen recognition. Ramendra et al. (2021) demonstrate that the potent fungal PAMP, 1,3-β- d – glucan (BDG), alters monocyte chromatin accessibility and epigenetic landscape. The changes in histone modifications in monocytes match the accessibility of the chromatin on a global scale. The changes in the epigenetic landscape prompts glutathione synthesis and metabolism, which promotes the acute functional response of monocytes to infections. The hepatitis B viral HBx protein can dysregulate host microRNA (miRNA) profiles which, in turn, modulates its viral load and enhances persistence. Sartorius et al. (2021) reviews the literature on the influence HBx has on the host and viral epigenome. The authors further attempt to link HBx dysregulated epigenetic pathways in hepatitis B virus-induced hepatocellular carcinomas.

Although our understanding of epigenetic mechanisms in infectious diseases has recently improved, its therapeutic use is still in the developmental stages. Much research has been undertaken to develop epigenetic modifying drugs for various cancers. Repurposing drugs for other diseases is an effective tool to speed up drug discovery. The histone deacetylase inhibitors (HDACi), belinostat and vorinostat, have been FDA-approved to treat various cancers. Barman et al. (2021) findings suggest that these two HDACi are effective against the *Theileria annulate* parasite in an *in vitro* model. These parasite-specific HDACi induces apoptosis in the parasite-infected cells *via* the caspase-dependent pathway while having low host cytotoxicity.

Singh et al. (2021) also repurpose an anti-cancer drug regulating DNA methylation against infectious disease, in this case, HIV. DNA methylation regulates the expression of the anti-viral restriction factor BST-2. The authors findings suggest that individuals with higher DNA methylation levels near the transcription start site of *BST2* had lower *BST2* expression and worse HIV disease outcomes. A significant negative correlation between *BST2*-methylation and *BST2* expression exists in HIV patients. Higher *BST2* expression and lower DNA methylation inhibits HIV replication in an *in vitro* HIV replication model. Treatment with a DNA-demethylating drug 5-Aza-2 – deoxycytidine increases *BST2* expression, which was associated with a lower HIV viral load.

Arumugam et al. (2021) delves further into the role of DNA methylation in the context of HIV. The authors provide a comprehensive discussion on the effect DNA methylation has on both viral and host genes. The authors also provide a detailed list of HIV-associated host genes with evidence of methylation in other disease models that should be further studied in the context of HIV. In addition, the potential use of DNA methylation as both a biomarker and therapeutic strategy against HIV is critically explored in this review.

DNA methylation not only plays a role in the pathogenesis of viral infection but also regulates the host's immune response to bacterial infections. The changes in host methylation profiles may be brought about, in part, by the bacteria. The review by Qin et al. (2021) provides an in-depth discussion on factors regulating DNA methylation and recent insights into the regulation of host DNA methylation during bacterial infection.

NcRNAs, which include miRNAs, long non-coding RNAs (lncRNAs), and circular RNA (circRNA), are significant regulators of genes involved in the immune response. The expression of ncRNAs can differ in different physiological or disease states. Bacteria, viruses, or fungi can significantly change the pathogen and host's ncRNA profiles in sepsis. Thus, over the past decade, more attention has been given to understanding the role of ncRNAs in disease etiology. Ghafouri-Fard et al. (2021) provide an extensive list of lncRNAs, miRNAs, and circRNAs involved in the initiation and progression of sepsis. In sepsis, these ncRNA generally interact to regulate inflammatory signaling pathways such as NF-kB, PI3K/AKT, and JAK/STAT pathways. Sepsis often leads to multi-organ failure; however, septic cardiomyopathy may be reversible. Recent research has focused on preventing and reducing mitochondrial dysfunction, which is involved in the pathogenesis of septic cardiomyopathy. Liu and Chong (2021) summarize recent studies on the role of lncRNA in the mitochondrial dysfunction of septic cardiomyopathy.

Tamgue et al. (2021) discusses the function of ncRNAs in the etiology and control of major human tropical diseases, including tuberculosis, HIV/AIDS, and malaria, and neglected tropical diseases including leishmaniasis, African trypanosomiasis, and leprosy. The authors highlight several ncRNAs involved at different stages of these diseases. The authors describe several ncRNAs that have potential as biomarkers for disease diagnosis. They further identify and discuss knowledge gaps that warrant further investigation, such as potentially targeting ncRNAs for adjunctive therapy and vaccine development.

Exposure of the cornea to pathogens results in an inflammatory cascade, eventually leading to keratitis. Verma et al. (2021) summarize the clinical perspective of infectious keratitis, the role of epigenetics in infectious keratitis, and the potential of epigenetic modifiers in treating infectious keratitis.

Epigenetic factors may explain the heterogeneity of COVID-19 disease severity. Kgatle et al. (2021) highlight the role epigenetics play in regulating viral entry points and immunoregulatory genes during SARS-CoV-2 infection and the potential of epigenetic drug treatments against COVID-19. Roy et al. (2021) further elaborate by providing their opinion on how ncRNAs regulate macrophage plasticity during the pathogenesis of COVID-19 disease. The authors consider a pool of miRNAs and LncRNAs that regulate the expression of the SARS-CoV-2 receptor *ACE2* as potential direct targets for therapeutic manipulation. They also reason that macrophage overactivation in the lung and uncontrolled systemic inflammatory responses can be lowered by existing drugs such as the p38MAPK specific inhibitor simvastatinin and the toll-like receptor (TLR) antagonist Tocilizumab.

Evidence from animal models and *in vitro* studies suggests that chronic and severe infections alter the epigenetic landscape of immune cells, often leading to long-lasting immune suppression. Infection-induced epigenetic changes cause exhaustion, tolerance, and anergy in the immune cells making the surviving host susceptible to secondary infections. Epigenetic drugs can directly reverse drug-induced immune suppression. Abhimanyu et al. (2021) discuss studies demonstrating the reversal of infection-induced epigenetic-mediated immune suppression and postulate how these approaches could become clinically relevant to decrease post-infectious morbidity and mortality.

This Research Topic brings together contributions highlighting the importance of epigenetic processes involved in the pathogenesis of infectious diseases. Findings from novel research studies found in the Research Topic provide evidence

of the dynamic interaction between the host epigenome and pathogen. Furthermore, the use of epigenome-modifying drugs are shown to be effective against pathogens in *in vitro* settings. The review articles and opinion pieces found in the Research Topic help to drive forward our understanding of the role of epigenetics in immunology, pathogenesis, and possible clinical intervention of infectious diseases. These articles also provide suggestions on what future research regarding the epigenetics of infectious diseases should hold. Thus, we hope that this Research Topic sparks new ideas in researchers who want to further explore both basic and translational aspects of epigenetic mechanisms in infectious diseases.

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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REVIEW
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The Epigenetic Modulation of Cancer and Immune Pathways in Hepatitis B Virus-Associated Hepatocellular Carcinoma: The Influence of HBx and miRNA Dysregulation

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Hepatitis B virus (HBV)-associated hepatocellular carcinoma (HBV-HCC) pathogenesis is fueled by persistent HBV infection that stealthily maintains a delicate balance between viral replication and evasion of the host immune system. HBV is remarkably adept at using a combination of both its own, as well as host machinery to ensure its own replication and survival. A key tool in its arsenal, is the HBx protein which can manipulate the epigenetic landscape to decrease its own viral load and enhance persistence, as well as manage host genome epigenetic responses to the presence of viral infection. The HBx protein can initiate epigenetic modifications to dysregulate miRNA expression which, in turn, can regulate downstream epigenetic changes in HBV-HCC pathogenesis. We attempt to link the HBx and miRNA induced epigenetic modulations that influence both the HBV and host genome expression in HBV-HCC pathogenesis. In particular, the review investigates the interplay between CHB infection, the silencing role of miRNA, epigenetic change, immune system expression and HBV-HCC pathogenesis. The review demonstrates exactly how HBx-dysregulated miRNA in HBV-HCC pathogenesis influence and are influenced by epigenetic changes to modulate both viral and host genome expression. In particular, the review identifies a specific subset of HBx induced epigenetic miRNA pathways in

HBV-HCC pathogenesis demonstrating the complex interplay between HBV infection, epigenetic change, disease and immune response. The wide-ranging influence of epigenetic change and miRNA modulation offers considerable potential as a therapeutic option in HBV-HCC.

Keywords: hepatitis B virus-associated hepatocellular carcinoma, epigenetic modulation, HBx, microRNA, immunology

INTRODUCTION

In the Globocan 2018 report, 841 080 new liver cancer cases were diagnosed with hepatocellular carcinoma (HCC) (1). Chronic hepatitis B virus (HBV) infection, which has been etiologically implicated in 43% to 80% of total HCC incidence (1–3) remains a primary risk factor and high HBsAg seroprevalence (>5%) levels currently persist in Western Pacific, Africa, East Mediterranean, and East Asia (2). The downstream effect of widespread HBV infection could precipitate 5 million deaths by 2030 from HBV-HCC (3). HBV-HCC pathogenesis is fueled by persistent HBV infection that stealthily ensures a careful balance between replication and the need to remain under the "radar" of the host immune system. HBV is remarkably adept at using a combination of both its own, as well as host machinery to manage viral load and enhance persistence (4, 5). A key tool in this regard, is the manipulation of the epigenetic landscape to modulate the silencing role microRNA (miRNA). The role of HBV dysregulated miRNA at each stage of the HBV-HCC continuum has been well documented from the onset of HBV infection to fibrosis/cirrhosis and the onset of HBV-HCC (6). The regulatory role of HBV dysregulated miRNA has also been extended to specifically examine the interplay between HCC and immune pathways (7).

This review examines the epigenetic role of HBx dysregulated miRNA in HBV-HCC pathogenesis. HBx is a 17 kDa transactivating protein expressed from the *X* open reading frame of HBV, with little sequence homology to any known genes, hence the name "X". HBx can modulate several hepatocyte signaling cascades and factors associated with mechanisms that induce cellular transformation. Unlike mammalian hepadnaviruses (HBVs), avian HBVs do not express HBx and do not develop HCC leading to the postulation that the HBx protein has oncogenic potential (8, 9). HBx, can initiate a wide range of epigenetic changes implicated in hepatocarcinogenesis including DNA methylation, histone modifications, chromatin remodeling and microRNA (miRNA) dysregulation (10). One of these epigenetic factors, namely, miRNA are themselves influenced by epigenetic modulation often forming feedback loops that modulate epigenetic change (11, 12).

We attempt to link the epigenetic role of HBx dysregulated miRNA in HBV-HCC pathogenesis in both the HCC and immune pathways. The review commences by describing how the HBx viral protein can modulate both the host, as well as HBV genome expression by influencing the epigenetic landscape. Next a comprehensive list of HBx dysregulated miRNA are presented in **Table 1**, which are influenced by upstream epigenetic changes, and/or their downstream epigenetic targets. This table also identifies host gene targets in both the HBV-HCC cancer and immune pathways. Four comprehensive examples of epigenetically modified miRNA are then outlined in the next section to demonstrate the complex interplay between viral infection, HBV-HCC pathogenesis and immune system response. The review then examines the potential therapeutic role of miRNA that has yet to be deployed outside of the laboratory setting.

HBV-HCC PATHOGENESIS: ROLE OF MIRNA

Persistent HBV infection remains a global risk factor that can promote the development of fibrosis/cirrhosis and ultimately the onset of HBV-HCC (120). A significant component of HBV-HCC pathogenesis involves the integration of HBV DNA into the host genome that results in the oncogenic disruption of cellular genes (121). HBV DNA integration can cause host cell deletions, *cis*/*trans*-activation, translocations, the increased production of fusion transcripts, aberrant epigenetic changes and generalized genomic instability (122). These changes take place in the form of chronic inflammation and tissue damage that result in the continuous destruction of well differentiated hepatocytes and organized extracellular matrix (ECM). Over time the depletion of hepatocytes and well organized ECM results in their replacement with undifferentiated liver stem cells and poorly organized fibrotic tissue (120) that display changing patterns of apoptosis, regeneration, senescence and survival (123). HBV-HCC pathogenesis involves the deregulation of many cellular signaling pathways including the Wingless-related integration site/Beta-Catenin (WNT/β-CAT), in the Retinoblastoma-Tumor Protein 53 (RB1-TP53) suppressor networks, the Phosphoinositide 3-kinase/mitogen-activated protein kinase (PI3K/MAPK), the Janus kinase/signal transducer (JAK/STAT) pathways and the insulin receptor substrate-1/insulin growth factor (IRS1/IGF) pathways (124–126).

MicroRNA (miRNA) are a subsidiary subset of epigenetic factors that act as post-transcriptional gene silencers in the HBV-HCC pathways. miRNA collectively attempt to repress target mRNA expression in order to ensure homeostasis and their fluctuating role is explained by the inherently stochastic nature of gene transcription and environmental fluctuations (127). In the HBV-HCC continuum, from asymptomatic HBV infection leading to HCC, an increasing number of miRNA are

TABLE 1 | HBx-dysregulated epi-miRNA and their targets in HBV-HCC.

HBx-epi-regulator	miRNA	Epi target	HBV-HCC gene target	Immune gene target	Epi-Ref	HCC Reference
HMT/EZH2 LIN28/ tet1/EED/ SUZ12	Let-7c	SUZ12/EED/ EZH1/2	*CNKD1/PRICKLE/SFRP5/B-CAT/STAT3/RAS/HMGA2/ MYC/ IL-6/IL-10/TLR-4/COL1A2/NGF/BCL-XL/BCL-2/ MCL-1z*	*MYC/STAT3/IFN-b/RAS/TLR4 BCL-XL/SMAD2/ SMAD4/NFZ APC2/WNT1/HMGA2/PLZF/IFN/ IL-4/IL-17/LIN28B/IGF2BP1*	(13–15)	(16–19)
DNMT	miR-1	HDAC4	*EDN1/PI3K/AKT/METFOXP1*	*E2F5/HSP60/HSP70/KCNJ2/GJA1*	(20–22)	(20, 21, 23)
HMT/ EZH2/ EED/C-MYC/ SUZ12	miR-101	DNMT3A/ EZH2/EED/ SUZ12/	*GSTP1/FOS/MCL-1/RASSF1A/PRDM2/CNKD1/ PRICKLE/SFRP5/B-CAT/AP1/DUSP1/MCL-1/ROCK2/ ATG4D/MTOR/SOX9/COX2/RAB5A/STMN1/DNMT3A/ FOS/RAP1B/VEGF*	*ICOS (naïve T-cells)/MCL-1*	(13, 14, 24)	(25) (14, 24, 26–28)
DNMT/ PPARα	miR-122		*CTNNB1/CCNG1 modulated p53/GLD2/NDRG3/ GALNT10/CCNG1/PTTG1/PBF/ADAM10/CCNG1/ Igf1R/ADAM 17/BCL-W/NDRG3*	*SOCS3/IFN/IP-10/BCL-W*	(29, 30)	(31–34)
DNMT1	miR-124	EZH2/BMI1	*STAT3/PIK3CA/ROCK2/STAT3/Cyclin D*/CDK6, VIM, SMYD3, E2F6, IQGAP1	*STAT3/TRAF6/CYCLIND3/BM11*	(14, 35, 36)	(37)
	miR-125a	SIRT7	*MMP11/VEGF-A/ERBB2/HBsAg*	*NF-α/BCL-2/KLF13/BMF*	(38, 39)	(40–42)
EZH1/2/ HMT/ SUZI2/ EED/p53	miR-125b	SIRT7/SUZI2 SUV39H1/ EED/EZH1/ EZH2/	*SMAD2/4/Sirtuin7/SUV39H1/LIN28 B/PIGF/BCL-2/ MCL-1/CNKD1/PRICKLE/SRFRP5/B-CAT*/PIGF/MMP2/ MMP9	*PRDM1/IRF4/TNF_/BCL-2/MCL-1/LIN28/IRF4/ KLF13/BMF/BCL-2/SMAD2/SMAD4 APC2/ WNT1//KLF13/TRP5 3INPI/LIN28A/IRF4/ BLIMP1 IRF4/BMF*	(13, 36, 43, 44)	(45, 46)
DNMT3	miR-132	p300	*AKT/GSK3/WNT-BCAT*	*p300/IRAK4/FOXO3/SOX4/*	(47, 48)	(48)
HMT/EED/ SUZ12/ EZH2	miR-139-5p	EED/SUZ12/ EZH1/2	*ZEB1/2/CNKD1/PRICKLE/SFRP5/B-CAT*	*IL-4/IFN-γ*	(13, 14)	(45, 49, 50)
	miR-140	DNMT1	*NF-kB/TGFβRI/FIF9/Pin1*		(51)	
	miR-145	HDAC2	*MAP3K/CUL5/ADAM17*	*IFN-b/TIRAP/TRAF6*	(52–54)	(55–57)
DNMT1/ p53C-MYC	miR-148a	DNMT1	*HPIP/AKT/ERK/FOXO4/ATF5/ERBB3/BCL-2/IRS-1/ MTOR/MET/ACVR1/SNAIL/IGF-IR/MIG6/CAND1/ CDC25B*	*CaMKIIα/KIT/MET/SIPI/BACH/PTEN/BIM/ GADD45*	(58, 59)	(45, 60–62)
	miR-152	DNMT1/ DNMT3A	*GSTP/CDH1/KIT*	*CaMKII/KIT*	(22, 47, 63)	(42, 63–65)
HDAC-I/ EZH2	miR-155	PRC2/Phf19/ p300/CBP	*PTEN/SOX6/ZHX2/SOCS1*	*AID/Blimp-1/PRDM1/IFN/SHIP1/SOCSI/ BMAL1/PU.1/BACH1/CSFIR/CEBP/ETS1/Th2 induction3/SOCS1/C/EBP/AID/FOXP3*	(43, 66, 67)	(66, 68, 69)
HDAC-I SAHA/ C-MYC	miR-17-92 family	DNMT	*E2F1, Cyclin G1/PTEN/p21/p27/p57/cccDNA*	*TNFSF9/CCL-5/IKBKE/c-MAF/AMLI/TP53INPI c-MAF/IFN/CD69/PTEN/TGFBR11/p27/p21/ E2F/PHLPP2/BIM/CREB1*	(70, 71)	(72–74)
HMT	miR-199a/b		*CHC*	*CD19+*	(75)	(76)
DNMT/p53	miR-200a	HDAC4	*ZEB1/2/HNF-3β Rho/ROCK/ASB4*		(77, 78)	(45, 79)
EED/ SUZ12/ EZH1/2	miR-200b	EED/SUZ12/ EZH1/2	*CNKD1/PRICKLE/SFRP5/B-CAT*		(13, 78)	(45, 79)
EZH2/ BMI1	miR-203		*RAP1A*	*SMAD1/BCL11B/RARB/PRKCA/PRKCB1/ FMRP*	(80)	(81)
DNMT	miR-205		*ACSL4/E2F1/ZEB1/2*		(82)	(45, 82, 83)
DNMT	miR-221	HDAC6	*ERα/DDIT4/BMF/p27 p57/PTEN/p21/SOCS3*	*PTEN/SOCS3/p57/KIT/p27*	(84, 85)	(86–88)
DNMT	Mir-222		*P27kip 1/PTEN/PPP2R2A/p57/p21*	*p27 kip 1/PTEN/KIT*	(85)	(55, 88–90)
HAT/ HDAC1/3/ EP300/ p50/p65	miR-224		*PAK4/MMP9 inhibitor-5/SMAD4*	*AP15/SMAD4*	(91, 92)	(93–95)

(Continued)

TABLE 1 | Continued

HBx-epi-regulator	miRNA	Epi target	HBV-HCC gene target	Immune gene target	Epi-Ref	HCC Reference
	miR-26a	EZH2	*IL-6/IFN_/ER_/Cyclin D2/Cyclin E2/c-JUN/CDK4/6*	*IFN-b CDK4/6/MALT1*	(96–98)	(99–101)
	miR-29c	DNMT3B/ SIRT1	*BCL-2/MCL-1/TNFA1P3*	*TCL-1/MCL-1/IFN-*	(102–104)	(105, 106)
H3K4ac	miR-29a/b	DNMT1/ DNMT3A/B/ SETDB1/ H3K9/SIRT1	*PTEN/PI3K/AKT/MMP-2*	*IFNARI/IFN/T-Bet/EOMES/PTEN/MCL-1/IFN-/ SLFN4/CDC42/HBP1/TCL1*	(22, 102, 103, 107, 108)	(55, 109)
HBx/P53/ DNMT1/ 3A/3B	miR-34a	SIRTI	*CCL22/MAP4K4/SIRT1/CCND1/CDK4/6/MET/C-JUN/ CDK2*	*IFN-b/FOXP1/CDK2/4/6/CCL22/FOXPN*	(110)	(111–115)
HDAC	miR-373	HDAC/SIRT1	*SNAIL-1/CDH1*	*MTOR/SIRT1/RELA*	(116)	(116)
	miR-548a	HDAC4	*HBXIP, IFN-λ1*	*IFN-λ1*	(117)	(118, 119)

Column 1 indicates upstream epigenetic proteins that can influence miRNA expression in column 2 (blue miRNA- downregulated and red miRNA-upregulated). Column 3 is downstream epigenetic targets of dysregulated miRNA. Columns 4 and 5 are specific HBV-HCC and immune gene targets. Column 6 refers to epigenetic references only (column 1 and 3) and column 7 to HBV-HCC and immune gene targets.

dysregulated due to the need to respond to viral infection, epigenetic changes (35), inflammation (128), fibrosis (129), cirrhosis (123) and finally, the onset of HCC. One of the most documented HBV tools to modulate both its own, as well as host genome expression is the HBx protein which has been shown to dysregulate multiple miRNA species in key HCC cancer and immune pathways (7).

HBX INDUCED EPIGENETIC CHANGES IN HBV-HCC PATHOGENESIS

HBV DNA and its proteins influence own and host genome expression by employing a range of epigenetic modifications in HBV-HCC pathogenesis, as well as modulating signal transduction, transactivation and transcription to regulate immune response, cell cycle, apoptosis and DNA repair (130). In particular, the HBx protein influences DNA methylation (131), histone modifications (132), chromatin remodeling (133) and miRNA dysregulation (6) which is the central focus of this review (**Figure 1**).

Regulation of cccDNA Activity

The HBV covalently closed circular DNA (cccDNA), a stable mini-chromosome that is classified as nuclear episomal DNA, serves as the template for viral RNA transcription. In order to enhance the efficiency of HBV replication, this virus co-opts host transcription machinery like HNF1/2/3, C/EBP, CREB and CRTC1 to trigger cccDNA transcription (4). The HBx protein appears to be co-opted to influence its own transcription by triggering histone modifications including histone acetylation and deacetylation (HAT/HDAC), histone methylation and demethylation (HMT/HDMT), DNA methylation (DNMT) and ubiquitination (5). An example in this respect, is when cccDNA transcription is increased the HBx protein co-opts DDB1 and Cul4 to create a ubiquitination complex to repress SMC5/6 which maintains the structure of chromosomes. The repression of SMC5/6, therefore, promotes DNA relaxation to increase the transcription of cccDNA (131, 134) (**Figure 1A**). The HBx protein can also increase cccDNA transcription by recruiting CBP/p300 (pCAF) to transactivate HATs (H3K9ac) that leads to hyperacetylation (131, 135) (**Figure 1B**).

DNA Methylation

DNA methylation, the addition of a methyl group by the enzymes DNMT1/2/3A/3B, can silence target gene promoters or enhance target gene expression by silencing transcription regulators in both the HBV and host genome (4, 5). In percentage terms, common host genes targeted for DNA methylation in HBV-HCC include WT1 (54%), SOCS1 (43-65%), SEMA3B (83%), RASSF1A (59-75%), p300 (65%), p27 (48%), p21 (63%), p16 (16-83%), GSTP1 (41-76%), E-cadherin (33-67%), E2F1 (70%), CPS1 (80%) and APC (53-81%) (10). In many cases these epigenetic changes silence tumor suppressor or activate oncogenic proteins and the HBx protein influences DNMT expression to subdue the host immune response, as well as manage its own expression to promote a balance between survival and replication (4). The HBx protein, for example, often promotes DNMT1/3A/3B expression to silence tumor suppressor genes like SOCS1/RASSF1A in HBV-HCC pathogenesis (4, 10) (**Figure 1C**). It can also use this machinery to suppress cccDNA expression in order to evade the host immune system (136).

Histone Modifications

Histone modifications are post translation modifications to histone proteins that include HAT/HDAC, HMT/HDMT, phosphorylation and ubiquitylation. These post translational modifications primarily occur at the N-terminal of the histone tails and have a fundamental impact on chromatin remodeling which essentially alters the structure of host and HBV DNA in

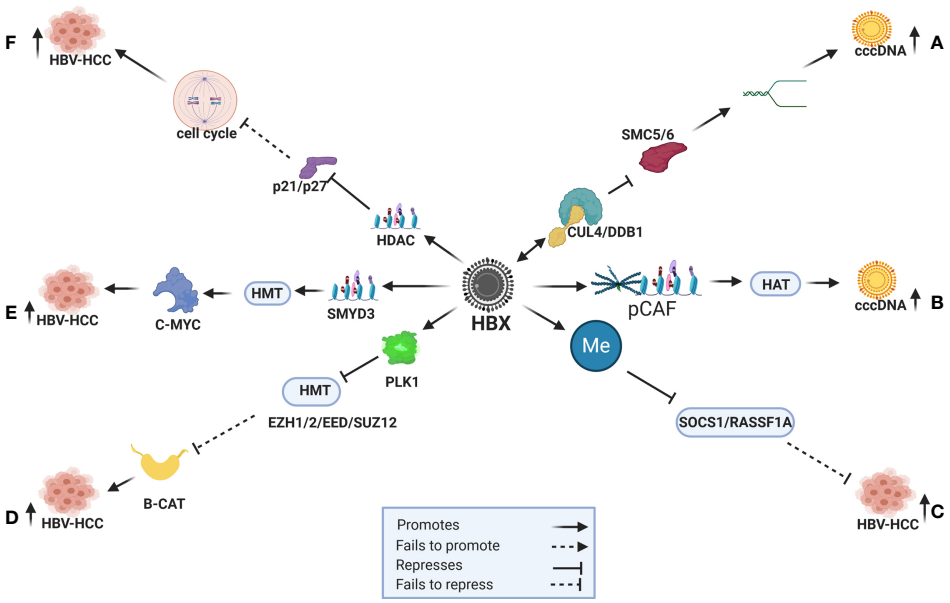

FIGURE 1 | HBx induced epigenetic expression in HBV-HCC. **(A)** HBx recruits the CUL4/DDB1 ubiquitination complex to repress SMC5/6 and promote DNA relaxation and cccDNA transcription. **(B)** HBx recruits CBP/p300 (pCAF) to transactivate HAT (H3K9ac) to promote cccDNA expression. **(C)** HBx promotes DNMT to repress SOCS1/RASSF1A tumor suppressors that fail to modulate HCC pathogenesis. **(D)** HBx promotes PLK1 to block HMTs (EZH1/2/EED/SUZ12) that then fail to modulate B-CATENIN induced hepatocarcinogenesis. **(E)** HBx initiates HMTs (SMYD3) to promote C-MYC induced HCC. **(F)** HBx promotes HDAC to silence cell cycle controls like p21/p26 and promote HCC.

HBV-HCC pathogenesis. For example, the HBx protein can promote HAT by recruiting trans-activator proteins like CBP/p300 complex to induce H3K9ac (135) Conversely, the HBx protein can induce HDAC to promote cell proliferation in HBV-HCC by repressing tumor suppressors like p21/p27 that are important regulators of cell cycle control (132) (see **Figure 1F**) and promote epithelial-mesenchymal transition (EMT) by repressing CDH1 (137). The HBx protein can also use HDAC to modulate the viral genome expression by recruiting HDAC1/2 to cccDNA to suppress its expression (4, 5). In addition, HBx also induces HMT by upregulation of the SMYD3 gene, which encodes a histone H3-K4-specific methyltransferase to trigger oncogene expression (138). The HBx protein can also induce histone methylation transferases and it has been demonstrated that HBx induced upregulation of SMYD3, that encodes for histone H3-K4-specific methyltransferase (HMT), is linked to the upregulation of the oncogene *C-MYC* in HCC (138, 139) (**Figure 1E**). Conversely, the HBx protein can reverse the repressive effect of histone methylation (H3K9me3) on cccDNA by initiating a histone demethylation (HDMT) agent (140).

Polycomb Proteins

The Polycomb repressive complex (PcG) proteins, namely, Polycomb repressive complex 1 (PRC1) and 2 (PRC2) form part of the histone modification machinery that epigenetically regulate chromatin remodeling. PRC2 participates in histone methylation (H3K27me3) and, following histone H2AK119 mono-ubiquitination by PRC1, collaboratively represses target gene transcription (141). In the HBV-HCC continuum, the HBx protein can co-opt the proteins of these two complexes to influence epigenetic changes. For example, HBx upregulates the proto-oncogene PLK1, an enzyme that can block the repressive effect of the PRC2 complex (SUZ12/EED/EZH1/EZH2) to down-regulate WNT antagonists (CNDK1/PRICKLE/SFRP5). The repression of these WNT antagonists leads to increased β-catenin transcription and hepatocarcinogenesis (13, 142) (**Figure 1D**).

HBx DYSREGULATED miRNA IN HBV-HCC AND EPIGENETIC CHANGE

In the HBV-HCC continuum, the HBx protein can influence epigenetic changes like DNA methylation, histone modifications and other non-coding RNA that dysregulate a specific subset of miRNA (called epi-miRNA) that forms the central focus of this review (**Table 1**). In addition, the HBx protein can dysregulate host genes that modulate miRNA biosynthesis, transcription and translation (47). Simultaneously, miRNA expression modulates downstream epigenetic modulation by targeting epigenetic modifiers suggesting epigenetic feedback loops that directly influence both miRNA and their downstream epigenetic targets (4, 10, 45). In the HBV-HCC continuum, HBx-dysregulated miRNA, therefore, are epigenetic regulators that are themselves epigenetically modulated. In **Table 1** we show the HBV-HCC specific gene targets of miRNA identified in the literature, as well as their specific immune targets in HCC.

It should be borne in mind that these are dynamic pathways and that the proposed regulatory effect of miRNA is also dynamic.

The HBx protein can upregulate or downregulate specific miRNA expression using specific epigenetic regulation (**Table 1**). These mechanisms, for instance, include DNA methylation of miR-1/-122/-124/-132/-/148/-200/-205 genes to downregulate miRNA expression (24, 48), histone acetylation or HDAC inhibitors to upregulate miR-224/-29/-155/-17-92 and histone methylation to downregulate Let-7c/miR-101/-125b/-139-5p (45). The HBx protein can also target upstream transcription factors essential for miRNA expression like p53 suppression of miR-23a/-34/-125b/-148a/-192/-200 (143) and C-MYC upregulation of miR-15a/-16/-26a/-101/-148a/-363 (144). This protein also targets p50/65 upregulation of miR-143/-224 (93) and NF-$_K$B upregulation of miR-143/-146a, as well as dysregulating miRNA expression by repressing miRNA biosynthesis machinery like DROSHA (145, 146).

HBx-DYSREGULATED miRNA IN HBV-HCC PATHWAYS

In the HBV-HCC continuum, upregulated miRNA often reduce tumor suppressor expression in the four key HCC cancer pathways, namely, the P13K/MAPK, WNT/β-Catenin, TP53 and JAK/STAT pathways (125). In this section we seek to demonstrate the complexity of the interlocking roles of viral infection, selected epigenetic changes of miRNA in HBV-HCC pathogenesis and the resulting modulation of the host immune system. We illustrate some of the proven epigenetic pathways in HBV-HCC pathogenesis by using four well researched miRNA (miR-29a/b, miR-155, miR-148/152 and miR-101). Two of these miRNA are downregulated (blue) and two are upregulated (red) (see **Figures 2**–**5**). These four miRNA are all HBx epigenetically dysregulated in various HBV-HCC pathways, as well as exercise diverse roles in both the innate and adaptive immune pathways. In many cases, the same miRNA plays a significant regulatory role in many other cancers like those of the breast, lung and colon (147). It is important to highlight that the illustrated hypothetical pathways in **Figures 2**–**5** occur in a dynamic context and that the degree of influence of any single path is non constant. It is also important to keep in mind that the HBx protein can modulate miRNA *via* non epigenetic pathways (e.g. C-MYC/p53) and that in HBV-HCC pathogenesis multiple other factors (e.g. somatic mutations) also influence miRNA expression (45).

HBx-Dysregulated Epi-miR-101

HBV-HCC Pathogenesis

In HBV-HCC and many other cancers, the tumor suppressor miR-101 is regarded as a key miRNA in epigenetic systems (147) (see **Figure 2**). This HBx-dysregulated miRNA involves both upstream and downstream epigenetic systems and has been

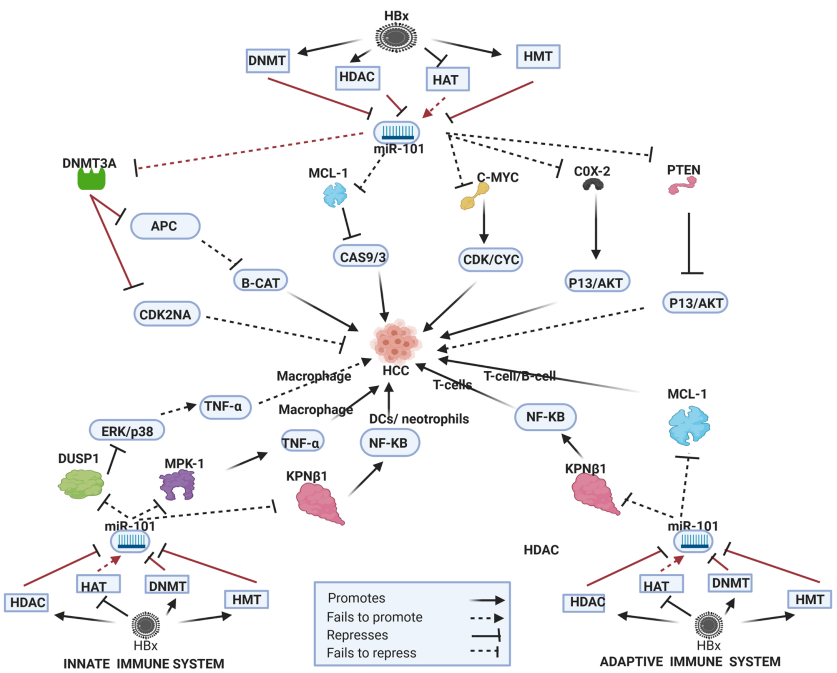

FIGURE 2 | Epi-miR-101 in HBV-HCC and immune pathways. HDAC-Histone deacetylation, HAT-histone acetylation, HMT-Histone methylation, DNMT-DNA methylation (pathways in dark red involve identified direct upstream or downstream epigenetic proteins/enzymes). HBx protein can downregulate or initiate HDAC/HAT/DNMT/HMT to repress miR-101 modulation in various HBV-HCC pathways including WNT-B-CATENIN, TP53, and P13K/AKT to influence HCC pathogenesis. HBx epigenetically downregulated miR-101 also regulates macrophage and DC expression in the innate immune system, as well as T-cell and B-cell expression in the adaptive immune system.

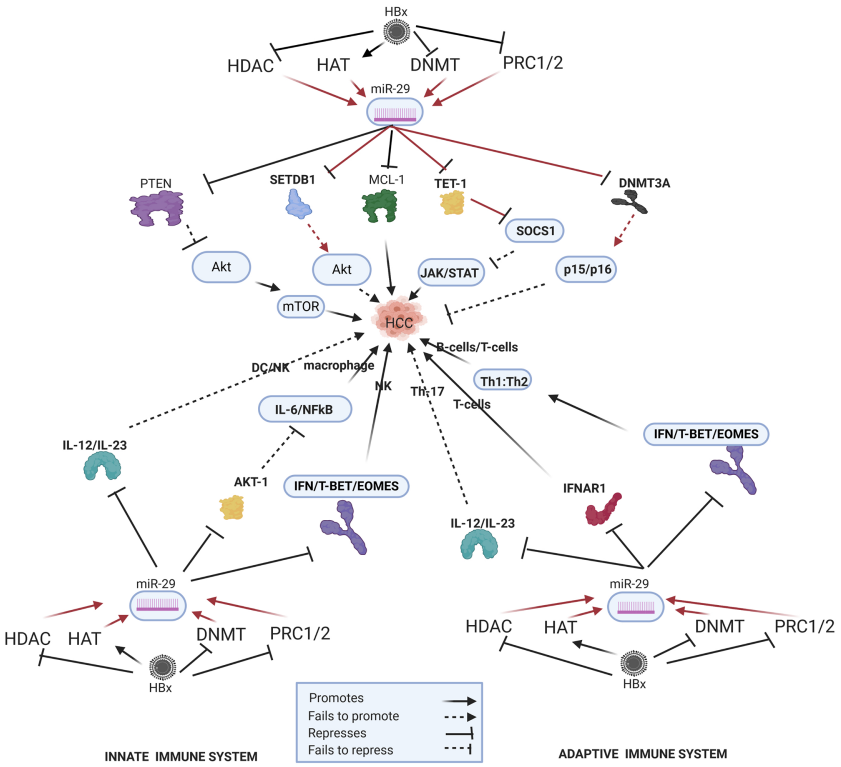

FIGURE 3 | Epi-miR-29 in HBV-HCC and immune pathways. HDAC-Histone deacetylation, HAT-histone acetylation, DNMT-DNA methylation, PRC1/2-Polycomb Repressive Complex, SETDB1-SET Domain Bifurcated Histone Lysine Methyltransferase, TET1-Ten-eleven translocation methylcytosine dioxygenase 1 (pathways in dark red involve identified direct upstream or downstream epigenetic proteins/enzymes). HBx protein can downregulate or initiate HDAC/HAT/DNMT/HMT to upregulate miR-29 modulation in various HBV-HCC pathways including AKT/MTOR, TP53, and JAK/STAT to influence HCC pathogenesis. HBx epigenetically upregulated miR-29 also regulates macrophage and DC/NK expression in the innate immune system, as well as T-cell and B-cell expression in the adaptive immune system.

widely reported as downregulated in HBV-HCC (147). The HBx protein can recruit the Polycomb protein EZH2 (148) to downregulate miR-101 but a feedback loop exists because this miRNA also targets EZH2 (149). In HBV-HCC pathogenesis a demonstrated EZH2 downregulated miR-101 pathway promotes HCC progression as a result of failing to modulate COX-2 activated AKT signaling (14, 150). This downregulated miRNA also fails to block the oncogenic MYCN, a member of the MYC family of proteins that are widely cited in many cancers including HCC. The MYC family can directly promote proliferation by promoting CDK/CYC expression (14, 149, 150) as well as promote angiogenesis *via* promoting VEGFA expression (151). In HBV-HCC pathogenesis, a direct epigenetic target of this miRNA is DNMT3A which targets a range of tumor suppressors. It has been demonstrated that HBx-downregulated miR-101 fails to modulate DNMT3A expression in HBV-HCC and this can contribute to the silencing of tumor suppressor genes like SF1/PRDM2/GSTP1/RUNX3/APC/CDKN2A/STMN1 (24, 149). The silencing of cell cycle inhibitors like CDKN2A in the TP53 pathway, for example promotes cell proliferation in HCC while silencing of the APC tumor suppressor promotes β-catenin expression and the development of EMT (152, 153) HBx (EZH2) downregulated miR-101 can also fail to modulate MCL-1, a key anti-apoptotic member of the BCL-2 family, thus promoting survival in HCC cells as a result of suppressing caspase driven apoptosis (152, 154). This downregulated miRNA also fails to repress the proto-oncogene c-FOS which regulates transcription activity and results in increased invasiveness in HCC pathogenesis (26). EZH2 downregulated miR-101 also fails to modulate *CCDC88A* that codes for the oncogenic protein GIRDIN which regulates many signal transduction pathways such as AKT/PKB, GAI/S, EGFR and is linked to increased migration and invasiveness in HCC (155). Conversely, downregulated miR-101 also targets the tumor suppressor PTEN in the P13/MAPK pathway that contributes to the activation of this pathway and HBV-HCC pathogenesis (156).

Innate Immune System

Downregulated miR-101 can downregulate the activation of LPS-stimulated macrophages by failing to modulate MKP-1 which de-activates p38 and JUN induction of pro-inflammatory cytokines like TNF-α (157, 158). In another pathway in the innate immune system, LPS-stimulated macrophages are reduced by HBx repressed miR-101 as a result of its failure to modulate DUSP1 which, in turn downregulates ERK1/2/p38/JNK promotion of pro-inflammatory cytokines like TGF-β (159). Reduced DUSP1

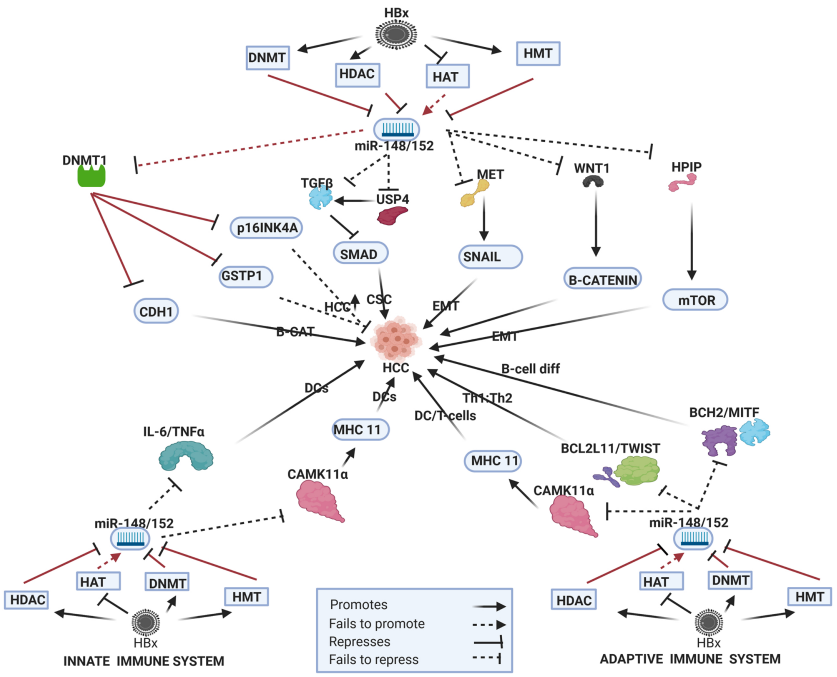

FIGURE 4 | Epi-miR-148/152 in HBV-HCC and immune pathways. HDAC-Histone deacetylation, HAT-histone acetylation, DNMT-DNA methylation, HMT-Histone methylation (pathways in dark red involve identified direct upstream or downstream epigenetic proteins/enzymes). HBx protein can downregulate or initiate HDAC/HAT/DNMT/HMT to repress miR-148/152 modulation in various HBV-HCC pathways including WNT-B-CATENIN, TP53, and AKT/mTOR to influence HCC pathogenesis. HBx epigenetically downregulated miR-148/152 also regulates DC expression in the innate immune system, as well as T-cell and B-cell expression in the adaptive immune system.

is often noted in HCC, however, and it has been demonstrated that it plays a protective role in HCC by lowering the ERK cascade and thus repressing cell proliferation (160). TGF- β plays a major role in the regulation of inflammatory processes that influence immune cell development (159). Alternatively, miR-101 can play a role macrophage expression *via* activating the NF-$_K$B signally as a result of the upregulation of the KPNB1 transport protein (161–163) which is commonly reported in many cancers including HCC (164). In the innate immune system the activation of dendritic cells (DCs) and neutrophils is also directly influenced by NF-$_K$B signaling suggesting that miR-101 can potentially play a wider role in the activation of leucocytes (165).

Adaptive Immune System

The differential expression of miR-101 has been implicated in modulating T-cell activation. A direct target of miR-101 is the Inducible T-cell co-stimulator (ICOS) mRNA that acts in concert with interaction between T cell receptor(TCR) and MHC class 1 and 2 peptides (166, 167). T-cell function is highly dependent on miR-101 modulation of ICOS mRNA and a reduction in miR-101 mediated regulation can increase ICOS expression on naïve T-cells increases, causing an effector T-cell-like phenotype and that results in autoimmunity (166). HBx-induced stimulation of EZH2 suppresses miR-101 in HBV-HCC (14, 24) and this effect could lead to ICOS upregulation that influences T-cell activation (166). HBx-downregulated miR-101 in HBV-HCC results in a failure to modulate the anti-apoptotic protein MCL-1 thus promoting cell survival (152). Acting alongside this relationship, MCL-1 expression influences an increase in CD8+ T-cell activity (168). A further hypothetical pathway, supported by various studies indicates that miR-101 can initiate T-cell expression, T-cell differentiation and memory T-cells *via* activating NF-$_K$B signaling by promoting the expression of the KPNB1 transport protein. KPNB1 is often reported as upregulated in many cancers including HCC (161, 162, 169).

B-Cells

HBx-downregulated miR-101 can also influence B-cell development *via* the regulation of MCL-1 which is regarded as a crucial input to B-cell synthesis (170).

HBx-Dysregulated miRNA-29 Family

HBV-HCC Pathogenesis

HBx upregulated miR-29a/b plays a key epigenetic role in modulating aberrant DNA methylation which is often a key feature of HBV-HCC pathogenesis (171) (**Figure 3**). HBx-upregulated miR-29 can repress DNMT3A/3B in HBV-HCC (102) acting as a feedback mechanism to modulate DNA methylation. This can influence a range of downstream effects because DNMT3A/3B often targets cell cycle controls in HBV-HCC including CDKN2A, p16(^{INK4A}) and p15(^{INK4B}) (171). By repressing DNMT3A/3B, HBx-upregulated expression of

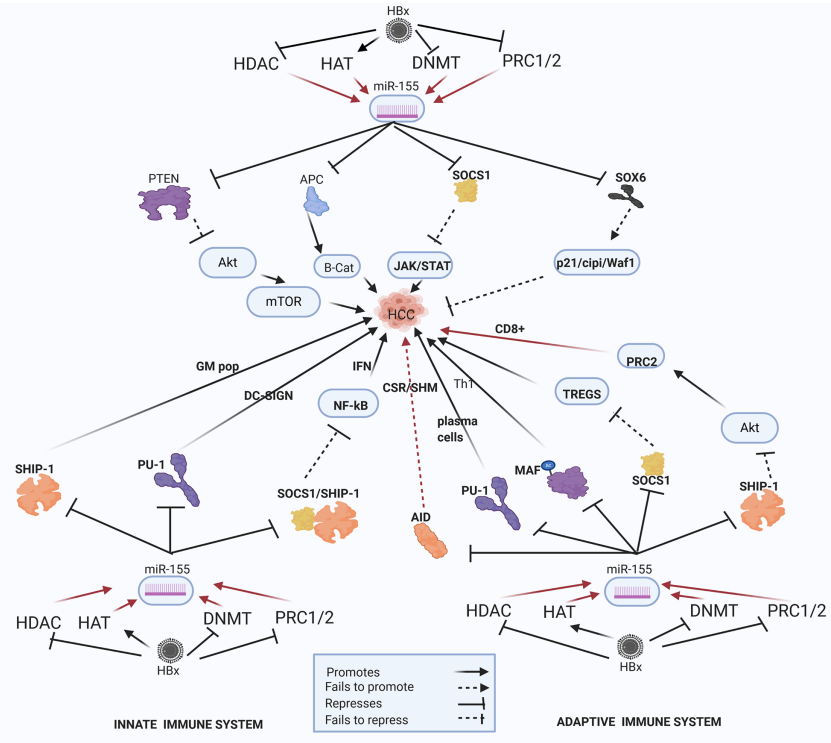

FIGURE 5 | Epi-miR-155 and HBV-HCC and immune pathways. HDAC-Histone deacetylation, HAT-histone acetylation, DNMT-DNA methylation, PRC1/2-Polycomb Repressive Complex 1 and 2 (pathways in dark red involve identified direct upstream or downstream epigenetic proteins/enzymes). HMT-Histone methylation. HBx protein can downregulate or initiate HDAC/HAT/DNMT/HMT to upregulate miR-155 modulation in various HBV-HCC pathways including AKT/MTOR, TP53, and JAK/STAT to influence HCC pathogenesis. HBx epigenetically upregulated miR-155 also regulates germinal matrix (GM) population and NF-kB expression in the innate immune system, as well as T-cell and B-cell expression in the adaptive immune system.

miR-29 acts in support of host cell cycle controls. DNMT3A/3B also silences many tumor suppressors in HBV-HCC pathways like RASSF1/PRDM2/GSTP1/RUNX3/APC therefore, miR-29 induced DNMT3A/3B repression contributes to a secondary support system for tumor suppressor expression to slow HCC progression (172). HBx-upregulated miR-29 family members could increase cell survival by targeting BCL-2 proteins (MCL-1) that retard apoptosis by contributing to the down regulation Caspase 9/3 driven apoptosis in the TP53 cancer pathway (105, 173, 174). The upregulation of the histone methyltransferase (HMT) SETDB1/SIRT1 is a common feature in HCC. HBx-upregulated miR-29, therefore, acts as a tumor suppressor to modulate the histone methylation transferase SETDB1/SIRT1 leading to a reduction in hepatocarcinogenesis possibly because of a reduction in AKT signaling and/or an increase in pro-apoptotic expression (107, 175). The upregulation of miR-29b has been demonstrated to increase HCC carcinogenesis by repressing SOCS1 expression in the JAK/STAT pathway *via* directly targeting the TET1 DNA demethylation enzyme (176, 177).

Innate Immune System

HBx-upregulated miR-29 upregulates LPS induced macrophage activation by repressing AKT1 suppression of a pro-inflammatory response resulting in increased IL-6, IL-1β and NF-$_K$B signaling (178, 179). In addition, the upregulation of miR-29 can repress Il-12/Il-23 activation of mature DCs (180) and can influence NK production *via* targeting TBX21/EOMES promotion of IFN-γ (181).

Adaptive Immune System

The miR-29a/b cluster plays a crucial role in the thymic production of T-cells, T-cell differentiation and B-cell oncogenic transformation (182, 183) and miR-29 has been cited as a repressor of the immune system because it directly targets IFN-λ in IFN producing immune cells (184). It has also been demonstrated that miR-29 targets IFNAR1 to promote T-cell production (185). In the presence of infection, type 1 IFN signaling and T-BET/EOMES expression modulate Th1:Th2 differentiation. In turn, miR-29a/b directly targets type 1 IFN/T-BET/EOMES thus playing an important role in Th1:Th2 differentiation. Upregulated miR-29a/b blocks type 1 IFN/T-BET/EOMES to promote Th2 expression and reduce Th1 expression. In the HBV-HCC continuum, miR-29a/b is upregulated by the HBx protein suggesting a viral intervention to promote modulate Th1:Th2 differentiation (182). A similar role is played by miR-29a/b when this miRNA is downregulated by intracellular bacteria and fails to modulate type 1 IFN

resulting in an imbalance of the production of CD8+ T-cell (182, 183). The upregulation of miR-29 therefore tilts the Th1:Th2 ratio in favor of Th2 expression that also influences B-cell production (184). In addition, the upregulation of miR-29 can repress Il-12/Il-23 activation of Th17 cells (180).

HBx Epigenetically Dysregulated miR-148/152 Family

HBV-HCC Pathogenesis

HBx downregulation of miR-148/152 has a downstream influence on multiple HBV-HCC pathways (**Figure 4**). HBx-downregulated miR-152 influences DNMT1 to silence both the CDH1 and GSTP1 tumor suppressors to promote carcinogenesis. The downregulation of CDH1 increases B-CATENIN in the WNT/B-CATENIN pathway and the downregulation of GSTP1 increases cell proliferation (63). Interestingly HBx activation of DNMT1 also represses cell cycle controls like $p16^{INK4A}$ to promote cell proliferation (186) thus further widening the downstream epigenetic change in HBV-HCC pathways. HBx- downregulated miR-148a fails to suppress HPIP induced upregulation of the mTOR pathway contributing to EMT and increased hepatocarcinogenesis (60). The progression of EMT is also less modulated as a result of this downregulated miRNA failing to repress expression in the MET/SNAIL/EMT pathway contributed to progression of HBV-HCC (61). This downregulated family also fails to regulate the production of B-catenin as a result of the reduced repression of WNT1 signaling (187). HBx-downregulated miR-148a increases SMAD pathway expression by failing to directly regulate TGF-B signaling and by failing to repress USP4 induced TGF-B signaling (188, 189).

Innate and Adaptive Immune Pathways

The miR-148/152 family play an especially important role modulating the adaptive immune system. In the adaptive immune system this family targets many genes that influence B and T lymphocyte function. This HBx-downregulated miRNA in HBV-HCC targets BCL2L11/TWIST to influence T-cell differentiation and BACH2/MITF to influence B-cell differentiation (190, 191). This family also plays a role in B-cell tolerance and elevated levels of miR-148a which have been noted in autoimmune disorders. In particular, miR-148a targets GADD45, BIM and PTEN that suppress B-cell tolerance (192). This family also targets CAMK11α to suppress MHC class II levels in antigen stimulated DCs that promote T-cell activation. It can thus be hypothesized that if the HBx protein epigenetically downregulates miR-148/152 then this would result in reduced suppression of MHC class II levels in DC activation of T-cells (193). This family, therefore, plays an important role in the innate system as a result of its ability to modulate antigen presenting (APC) DCs which are regarded as the most important class of APC in the innate immune system. DCs, therefore, link the innate and adaptive immune systems (194). This family also modulates IL-6, TNF-α and IFN-β to repress TLR induced DC activation and it can be hypothesized that downregulated miR-148/152 would fail to repress IL-6/TNF-α/IFN-β induced DC expression (194).

HBx Epigenetically Dysregulated miR-155

HBV-HCC Pathogenesis

HBx-upregulated miR-155 is a key epi-miRNA that targets both the HBV-HCC immune and cancer pathways (**Figure 5**). HBx dysregulation of miR-155 can occur due to histone modifications like histone deacetylase inhibitors (HDAC-I) or the repression of polycomb proteins (EZH2) can contribute to upregulated miR-155 expression in HBV-HCC pathways (43, 66, 67). This well researched miRNA is cited as an epigenetic modulator in many cancers including those of the breast, lung and colon (195–197), as well as playing multiple different roles in both the innate and adaptive immune system response (7). In HBV-HCC pathogenesis, upregulated miR-155 typically represses PTEN modulation of AKT/MTOR signaling in the P13K/MAPK pathway that promotes epithelial to mesenchymal transition (EMT) (68, 198). In addition, this miRNA can promote β-catenin expression in the WNT/β-Catenin pathway by repressing the APC/GSK3 destruction complex to thus promoting the transcription of oncogenic proteins like C-MYC (45, 199). This miRNA also represses the SOCS1 tumor suppressor in the JAK/STAT pathway to induce the transcription of CCND1 and c-MYC thus promoting HCC cell proliferation (200, 201). In the TP53 pathway, this key HBx epigenetically upregulated miRNA can repress SOX6 to negate its promotion of p21/Waf1/cip1 modulation of cell cycle controls directly promoting HCC proliferation (6, 69). In a strategy to possibly evade immune system response, this HBx upregulated miR-155 can also subdue HBV replication by blocking the CCAAT/enhancer-binding protein (C/EBP) protein that binds and activates the HBV Enhancer 11/core promoter (199).

Innate Immune System

Epigenetically upregulated miR-155 is a key modulator of pro- and anti-inflammatory responses in the innate immune system (202, 203). It is a particularly important miRNA in the modulation of $NF\text{-}_KB$ driven induced myelopoiesis as a result of targeting IRAK1/TRAF6 and SHIP1/SOCS1 respectively (204–206) and also targets CSFR to influence myeloid differentiation (207).

Macrophages

SHIP1, an important regulator of the innate system, is a primary target of miR-155 and its repression influences an increase in granulocyte/monocyte cell populations and a reduction in lymphocyte numbers (208, 209) and reduced levels of SHIP1 appears to induce myeloproliferative disorders (208). Interestingly, SHIP-1 is classified as a tumor suppressor in HBV-HCC and reduced levels of SHIP-1 are associated with a poorer prognosis (210). Upregulated mIR-155 in viral infection can induce type 1 IFN induced macrophages *via* by activating the TLR4/MyD88/JNK/$NF\text{-}_KB$ dependent pathway. In order to upregulate TLR4 signaling, upregulated miR-155 can suppress both SHIP1 and SOCSI to block their regulation of downstream TLR signaling directly contributing to increased inflammatory signaling and macrophage activation (208). Furthermore, SOCS1 which regulates type I IFN signaling, is targeted by miR-155 in

macrophages (211, 212) and the loss of function of SOCS-1 is a common feature in HCC clearly supporting a hypothesis that HBx-upregulated miR-155 promotes the progression of HBV-HCC (200, 201). Finally, it has been demonstrated that AKT signaling can repress miR-155 in macrophages thus indicating a negative feedback loop to fine-tune TLR signaling (213).

Dendritic Cells (DC)

Upregulated-miR-155 modulates the TLR/IL-1 (interleukin-1) inflammation signaling pathway to regulate human monocyte-derived DCs in order to ensure excess damage does not occur (214). TLR/TNF/IFN upregulated miR-155 *via* AP1/BIC plays a significant homeostatic role in monocytepoiesis by repressing PU.1 which activates DC-SIGN, a C-type lectin receptor to increase pathogen cell surface uptake on DCs (207, 215). Decreased DC-SIGN expression in HCC is related to poor prognosis and PU.I has been identified as a metastasis suppressor possibly relating to the impairment of the antigen presenting capabilities of APCs (216).

Adaptive Immune System: B-Cells

In the adaptive immune system the epigenetically modulated miR-155 can influence B-cell expression by triggering downstream epigenetic changes. Epigenetically upregulated miR-155 (HDAC-I/EZH2) can repress the expression of an important epigenetic regulator like activation induced cytidine de-aminase (AID) which acts as a HDAC inhibitor that binds to specific immunoglobin genes in the nucleus to induce CSR/SHM/antibody diversification (43). The repression of AID by upregulated miR-155 thus leads to a reduction in CSR/SHM/Plasma B cell diversification thus contributing to reduced ability to synthesize pathogen specific antibodies (217, 218). Interestingly, different studies indicate AID is upregulated in both HBV and HCV induced hepatocarcinogenesis (219, 220). This upregulated miRNA also influences B-cell synthesis by targeting Ship-1 which plays an important role in the regulation of immune cell activation in both the innate and adaptive pathways (221). Epigenetically upregulated miR-155 targets PU-1, a critical transcription factor, to block GC B-cell to Plasma cell transition thereby modulating germinal cell B-cell differentiation into memory cells or plasma cells (222).

T-Cells

Epigenetically upregulated miR-155 can target Ship-1 to promote histone building capacity (Phf19) to promote PRC2 expression that promotes histone modifications to repress T-cell senescence and promote CD8+ T-cell expression (67). This miRNA can also modulate IFNγ expression by repressing SHIP1to play a critical role in the reciprocal regulation of CD4+ and CD8+ leukopoiesis (223). MiR-155 also has a role in the generation of exhausted dysfunctional T cells and Fosl2 antagonism of miR-155 can reduce T cell exhaustion during chronic viral infection (224). This upregulated miRNA modulates T helper cell differentiation and the germinal center reaction to synthesize T-cell dependent antibody response. In order to do this, upregulated miR-155 can repress SOCSI to maintain $Foxp3^{+}$ regulatory T-cell (Treg) generation in order to regulate an autoimmune response (225, 226). It can also enhance Treg and Th17 cells differentiation and IL-17A production by targeting SOCS1 (206). A supporting meta study also confirms that the elevated expression of Tregs can be associated with HCC pathogenesis and Treg upregulation is a feature of the HCC tumor microenvironment (227). Conversely, Tregs can also target miR-155 to provide a negative feedback loop to control Treg expression (228). In the Th1/2 differentiation stage upregulated miR-155 can promote Th1 differentiation as a result of targeting C-MAF (229, 230) and an elevated Th17 to Th1 ratio has been associated with tumor progression in HBV-HCC (231). MiR-155 in Th17 cells can also trigger autoimmune inflammation through a signaling network by targeting the Ets1/IL-23/IL-23R pathway (205).

CLINICAL THERAPEUTIC OPTIONS

The five-year survival rate of advanced HCC remains dismally low and the treatment of advanced stages is limited by a paucity of targeted options despite the fact that HCC cancer pathways and their targeted genes have been well documented. Since the introduction of the multi-kinase inhibitor sorafenib, very little progress has been made in treatment of advanced HCC (232). Novel targeted therapies developed for a range of cancers include the development of immune checkpoint inhibitors like anti-CTLA4 or anti-PD-1/PD-L1 antibodies has introduced new opportunities in clinical oncology (233, 234). Chronic CHB, inflammation and the development of cirrhosis are all hallmarks of HBV-HCC pathogenesis. The question remains as to how miRNA-regulated epigenetic expression can prompt an appropriate immune response.

Our review demonstrates that multiple miRNA can influence epigenetic changes in multiple pathways in HBV-HCC pathogenesis by regulating histone modifications, DNA Methylation and chromatin modelling. For example, we show that HBx- downregulated miR-101 fails to modulate DNMT3A silencing of multiple tumor suppressors in HBV-HCC (24) while simultaneously modulating the expression of macrophages (157), DCs (165), T-cells (166) and B-cells (170). The question remains, however, as to the *in vivo* clinical potential of deploying miR-101 replacement therapy in HBV-HCC. Current trials using HDAC inhibitors to inhibit cell cycle in HCC have been disappointing (235) and to date there has been no attempt to modulate HDAC expression using miRNA in HCC. In a breast cancer study, for instance, HDAC inhibitors reduced tumorigenesis and apoptosis *via* microRNA miR-125a-5p *in vivo* and *in vitro* (236). Epigenetic targeting of EZH2, a histone-lysine-N-methyltransferase and DNMT1 inhibitor reactivated transcriptionally repressed chemokine genes and augmented T cell response in HCC (237). Our review shows that 29 dysregulated miRNA in HBV-HCC (**Table 1**) are both regulated and regulate epigenetic changes offering numerous hypotheses to be tested *in vitro*. In the case of our four HBV-HCC pathways, miR-101 influences PRC2 and DNMT3A silencing, miR-148/152influenced DNMT1/3A silencing,

miR-155 repressed PRC2 silencing and miR-29a/b repressed DNMT1/3A silencing in parallel with affecting immune expression in both the innate and adaptive immune systems.

The use of miRNA-led therapeutics is still a work in progress and most likely these therapeutic options would be used as an ancillary form of treatment in support of current options. In theory miRNA-led therapeutics attempt to repress or restore oncogenic and tumor suppressor expression respectively. Currently, miRNA replacement therapy has started investigating whether this could be an adjuvant therapy in support of chemotherapy and radiation (238, 239). This approach relies on the use of synthetic miRNA or miRNA inhibitors to upregulate or downregulate miRNA expression respectively (240). However, this approach has yet to capture the synergistic response generated by multiple miRNA, nor its dynamic homeostatic shifts and this is the puzzle yet to be solved.

CONCLUSION

This review attempts to link the epigenetic modifications that influence HBV and host genome expression in HBV-HCC pathogenesis in both the hepatocyte and immune pathways. We examine the interplay between CHB infection, the silencing role of miRNA, epigenetic change, immune system expression and HBV-HCC pathogenesis. In particular, we demonstrate how HBx dysregulated miRNA in HBV-HCC pathogenesis influence and are influenced by epigenetic changes to modulate both the HBV and host genome expression. The paper provides useful insights and potential hypotheses of the complex interplay between host gene targets in the principal cancer and immune pathways as a result of HBx dysregulated miRNA, epigenetic change, HBV-HCC pathogenesis and immune response.

This review paper tries to provide a platform for a wide range of evidence-based hypotheses rather than an (exactly) correct snapshot of the role of miRNA in HBV-HCC pathways. Even though HBV-HCC is a specific sub-type of HCC, there are multiple different classes and stages in which the hypothesized figures would operate to greater or lesser degree. This review should contribute to the point of view that our understanding of miRNA-based pathogenesis is far superior to our current ability to translate this knowledge to improve clinical outcomes.

AUTHOR CONTRIBUTIONS

KS conceptualized review article, performed literature review, wrote up first draft including figures and tables. Rewrote drafts 2-4 after fellow author comment. PA assisted re conceptualization of article, reviewed article. CW reviewed article, added text comments. AC reviewed article, added text comments XL reviewed article, added text comments. AK reviewed article, added text comments. All authors contributed to the article and approved the submitted version.

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Epigenetic Regulation of *BST-2* Expression Levels and the Effect on HIV-1 Pathogenesis

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HIV-1 must overcome host antiviral restriction factors for efficient replication. We hypothesized that elevated levels of bone marrow stromal cell antigen 2 (*BST-2*), a potent host restriction factor that interferes with HIV-1 particle release in some human cells and is antagonized by the viral protein Vpu, may associate with viral control. Using cryopreserved samples, from HIV-1 seronegative and seropositive Black women, we measured *in vitro* expression levels of *BST-2* mRNA using a real-time PCR assay and protein levels were validated by Western blotting. The expression level of *BST-2* showed an association with viral control within two independent cohorts of Black HIV infected females (r=-0.53, p=0.015, [n =21]; and r=-0.62, p=0.0006, [n=28]). DNA methylation was identified as a mechanism regulating *BST-2* levels, where increased *BST-2* methylation results in lower expression levels and associates with worse HIV disease outcome. We further demonstrate the ability to regulate *BST-2* levels using a DNA hypomethylation drug. Our results suggest *BST-2* as a factor for potential therapeutic intervention against HIV and other diseases known to involve *BST-2*.

Keywords: *BST-2*, HIV-1, DNA methylation, epigenetic regulation, expression

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INTRODUCTION

To establish infection and replicate efficiently, HIV-1 must overcome host antiviral restriction factors. Host restriction factors that inhibit HIV-1 replication are an important component of the innate immune system that forms the first line of defense before adaptive immune responses are mobilized and established (1–4). *BST-2* (also termed *Tetherin/CD317/HM1.24*) was discovered as an anti-HIV host factor responsible for the prevention of virus release (5). Subsequently, additional mechanisms of HIV inhibition by *BST-2* have been reported (6) and studies have associated BST-2

expression levels with HIV viral control (6–8). Downregulation of BST-2 expression correlated with Vpu expression and elevated BST-2 induced a requirement for Vpu to facilitate HIV particle release in some cells (5, 9). Vpu promotes intracellular down-regulation of BST-2 (10, 11). However, BST-2 is an interferon-induced protein, which gets activated upon HIV infection (7, 12). Factors regulating the expression levels of the *BST-2* gene have not been fully resolved.

The "tethering" effect mediated by *BST-2* on HIV has subsequently been shown to restrict the replication of a diverse array of other enveloped viruses including other retroviruses, rhabdoviruses, alphaviruses, arenaviruses, filoviruses, herpesviruses, paramyxoviruses, orthomyxoviruses, orthohepadnaviruses and flaviviruses (12–21). In addition, BST-2 expression levels are elevated in several cancers such as head and neck, breast, cervical, lung, endometrial, myelomas, and glioblastomas (22–29) as well as lupus erythematosus (SLE) an autoimmune disease (30), suggesting that BST-2 could be an immunotherapeutic target for several diseases. If *BST-2* is directly affecting these conditions, then identifying the factors regulating *BST-2* expression could develop strategies against an array of diseases. DNA methylation has been linked with the regulation of *BST-2* expression particularly in cancer cells (24), and in lupus (30). A few human genes implicated in HIV control are known to be regulated by DNA methylation. Hypermethylation of FOXP3, EPB41L3, IL-2, CCR5 and HLA-A at gene regulatory sites, are associated with reduced gene expression and worse HIV outcome, whereas reduced methylation corresponds with increased expression of these genes and improved disease outcome (31–36). This highlights the potential importance of this epigenetic mode of gene regulation in HIV disease pathogenesis.

In this study, we found that increased BST-2 levels associated with HIV control. We further show DNA methylation as one of the regulatory mechanisms responsible for *BST-2* expression variation within HIV infected individuals. Furthermore, *BST-2* methylation levels correlate with HIV outcomes in both *ex vivo* and *in vitro* experiments, and experimental manipulation of *BST-2* methylation altered its expression levels. Together, these data suggest that manipulation of *BST-2* expression levels could be used as a therapeutic target for viral control.

MATERIALS AND METHODS

Study Design

A chronic HIV infection cohort, Sinikithemba (SK; n=21) (37), was compared to the HIV negative arm of the acute infection cohort from the Females Rising through Education, Support, and Health (FRESH; n=65) study (38, 39) in a cross sectional analysis. We further studied a longitudinal HIV acute infection cohort, CAPRISA 002 (n=55) (40, 41), from pre-infection to >36 months of follow-up post HIV infection. All the samples used in this study were from South African females of Black ancestry. The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal.

Sample Processing, Viral Load Quantification and CD4 Cell Enumeration

Peripheral blood mononuclear cells (PBMCs) were isolated within 6 hours of blood collection, and frozen in liquid nitrogen until use. Viral load was determined using the automated COBAS AMPLICOR HIV-1 Monitor Test v1.5 (Roche Diagnostics, Mannheim, Germany). $CD4^+$ T cells were enumerated using the Multitest kit (CD4/CD3/CD8/CD45) on a four-parameter FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Real Time PCR Quantitation

RNA was extracted from 2 x 10^6 PBMCs using the TRIzol LS Reagent (Invitrogen, Carlsbad, CA, USA). RNA from each sample was reversed transcribed using the iScript cDNA synthesis kit (Bio-Rad, California, United States of America). PCR primer and cycling conditions for *BST-2* and *GAPDH* (housekeeping gene) are available on request. *GAPDH* was used as reference gene (42). PCR-product amplification specificity was confirmed *via* melting curve analysis and agarose gel electrophoresis.

Western Blotting

Cell lysates were boiled for 10 minutes in 4X Laemmli sample buffer (Bio-Rad), then separated by SDS-PAGE on 4 to 15% gels (Bio-Rad, California, United States of America) and transferred onto nitrocellulose membrane following standard methods. The membrane was then incubated with the primary antibody (rabbit monoclonal anti-BST-2 [cat. no. ab243229, Abcam, Cambridge, United Kingdom], and mouse polyclonal anti-alpha-tubulin [cat. no. ab7291, Abcam, Cambridge, United Kingdom]), diluted in 5% bovine serum albumin (BSA) (Roche, Basel, Switzerland) in tris-buffered saline and Tween 20 at a 1:100 or 1:5000 dilution overnight, followed by three washes in tris-buffered saline (TBS) and Tween 20 (TBST) for 10 minutes. The membrane was incubated with the secondary antibody (anti-rabbit, or anti-mouse) at a 1:20,000 dilution in 5% BSA in TBST for 1 hour on a rocker, followed by three washes in TBST for 10 minutes. Antibody-antigen complexes were detected *via* enhanced chemiluminescence reagents (SuperSignal West Dura extended-duration substrate, Thermo Scientific, Pierce Protein Research, United States of America). Proteins were visualized using the ChemiDoc XRS+ system with Image Lab software (Bio-Rad, California, United States of America).

In Vitro HIV Infection

HIV-1 replication *in vitro* was assessed on PBMCs from 22 donors selected from the 65 healthy HIV uninfected individuals from the FRESH cohort. Individuals with the highest (n =11) and lowest (n=11) *BST-2* mRNA levels were included. PBMCs (2×10^6/mL) were stimulated for 48 hours in R10 buffer [Roswell Park Memorial Institute (RPMI)-1640 medium (Grand Island, NY, USA) supplemented with 10% fetal calf serum (Hyclone Inc., Logan, UT, USA), gentamicin (Gibco-Brl, Gaithersburg, MD, USA) (100 mg/mL)] containing 5 mg/mL phytohemagglutinin (PHA) (Roche, Basel, Switzerland) and

5 mg/mL interleukin-2 (IL-2) (Roche, Basel, Switzerland). Following stimulation with PHA/IL-2, cells were washed with R10 buffer and then infected with HIV IIIB (NIH AIDS Reagent Repository) at 0.1 multiplicity of infection (MOI) by spinoculation (2 hours, 300 x *g* at 37°C). Infection was performed in a 24-well plate. Virus was subsequently removed by washing the cells, followed by cell culture for 7 days. *BST-2* mRNA expression levels and DNA methylation were analyzed by real-time PCR and pyrosequencing. Supernatants from days 2, 4 and 7 were harvested and analyzed by p24 antigen capture enzyme-linked immunosorbent assay (ELISA [Biomérieux, Marcy-l'Étoile, France]).

DNA Methylation by Sequencing

Primer design for the detection of methylation within the *BST-2* promoter region was performed using MethPrimer online software, default settings (43) (Forward meth primer 1 GGTTAGTTTTTGTTGTAGGAGATGG; Reverse meth primer 1 AACTATTACAAAATACCCATAAAAAAC; Forward meth primer 2 TTGATGGTGAAGATAATTAAGGGTATT; Reverse meth primer 2 AAAAACTACTAATCAAAACACTTC CTAAAA). Sodium bisulphite conversion was performed on genomic DNA extracted from PBMCs using the EZ DNA methylation™ kit (Zymo Research, Irvine, USA). Using the *BST-2* specific primers on the bisulphite converted DNA, a PCR was run using the following conditions (95°C for 15 minutes, 45 cycles of 95°C for 30 seconds, 60°C for 45 seconds, 72°C for 30 seconds and one cycle of 72°C for 10 minutes). The level of methylation at specific sites within the *BST-2* promoter was measured using pyrosequencing (Roche, Basel, Switzerland).

5'-aza-2-deoxycytidine Treatment

Treatment of cells with the DNA hypomethylation drug, 5'-aza-2-deoxycytidine (5'-Aza-CdR), was performed as previously described (32). Briefly, PBMCs from healthy donors (n=40) were treated with 10 μM 5'-Aza-CdR (Sigma, St. Louis, United States America) or with dimethyl sulfoxide (DMSO; treatment control) for 24 hours at 37°C. *BST-2* mRNA levels from 5'-Aza-CdR treated cells were compared to DMSO treated and then plotted against the untreated mRNA levels.

Statistical Analysis

Statistical analyses were conducted using Instat Graphpad Prism V.5 and SAS version 9.4. All expression data was $\log_{10}$ transformed to ensure normality (44, 45). Gene expression levels between HIV negative and HV infected donors were compared using an unpaired t-test. *BST-2* mRNA expression levels for HIV positive donors prior to infection and at three months post-infection were compared using paired t-test. Furthermore, we calculated the Pearson correlation coefficient to measure the strength of an association between *BST-2* mRNA expression levels and methylation at each time-point.

Univariable linear mixed model with autoregressive order one covariance structure were fitted to determine if there was an association between *BST-2* gene expression and viral load. In this model, we included a random effect for the participant or subject.

RESULTS

BST-2 mRNA Expression Level Associates With HIV Viral Control

We investigated the effect of HIV infection on *BST-2* mRNA expression levels in PBMCs *ex vivo*. We found significantly higher levels of *BST-2* in HIV negative individuals (n=32, FRESH cohort, black dots) compared to HIV infected late stage antiretroviral (ARV)-naïve individuals (n=21, SK cohort, red dots; $p < 0.0001$; **Figure 1A**). To validate these findings for consistency of mRNA expression with protein levels, we randomly selected donors, based on sample availability, from 5 HIV negative donors and 4 HIV infected donors, which formed subsets of the FRESH and SK cohorts, respectively. Western blot assays showed consistent BST-2 protein expression levels relative to mRNA expression levels, with protein expression higher in HIV- compared to HIV+ donors (**Figure 1B**). We next explored the relationship between *BST-2* mRNA expression levels and HIV-1 viral load. A negative correlation was observed in both SK (r=-0.53, p=0.015; **Figure 1C**), and CAPRISA 002 cohorts (n=28, r=-0.62, p=0.0006; **Figure 1D**), all individuals analysed cross-sectionally were past 36 months post infection in both cohorts.

BST-2 mRNA levels and viral load were also tested longitudinally at three timepoints (3, 12 and >36 months) in the CAPRISA 002 cohort. The results of the generalized estimating equation (GEE) model revealed consistent results to the cross-sectional data where higher mRNA levels associated with decreased viral load (Effect = -0.022; Standard error = 0.009; p=0.0003).

Effect of *BST-2* DNA Methylation on *BST-2* Expression and HIV Disease

The inverse effect of DNA methylation on *BST-2* expression has been shown previously in the context of cancer and autoimmune studies (24, 30). Here, we examined the effect DNA methylation on *BST-2* expression levels within an HIV setting. Nine CpG sites located within 200 bp of the transcription start site were evaluated for methylation levels (**Figure 2A**) in HIV positive and negative individuals (SK *vs.* FRESH cohort respectively). All sites showed significantly higher methylation levels within the HIV infected group (**Figures 2B–J**), suggesting that increased *BST-2* methylation levels in chronic HIV infection results in decreased expression level of the gene as observed in **Figure 1**.

The average methylation across the nine CpG sites was compared to *BST-2* mRNA expression levels in samples with four different HIV serostatus or disease stages, i.e. pre-infection, 3, 12- and >36-months' post-infection using n=27 matched ARV-naïve samples, based on sample availability. An inverse correlation was observed at all time points; pre-infection (r=-0.52, p=0.0056; **Figure 3A**), 3 months (r=-0.50, p=0.0097; **Figure 3B**), 12 months (r=-0.44, p=0.02; **Figure 3C**) and >36 months (r=-0.46, p=0.0178; **Figure 3D**). These data strongly point to methylation as a major contributor in regulation of *BST-2* expression levels.

Comparison of *BST-2* DNA methylation pattern with mRNA expression levels indicate distinctions at the four timepoints.

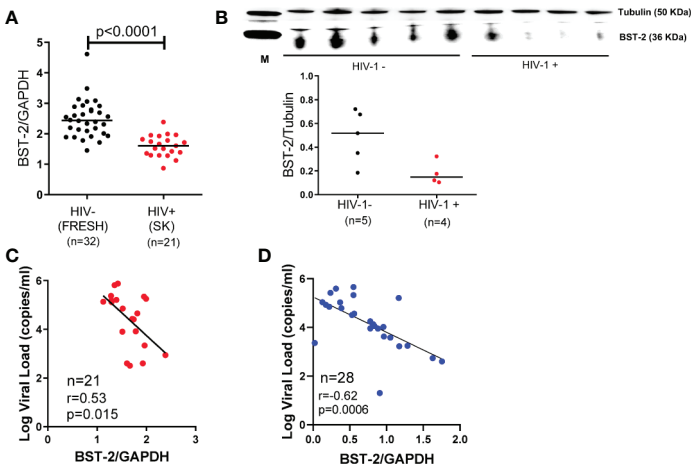

FIGURE 1 | *BST-2* mRNA and protein expression levels within HIV-negative and positive individuals. **(A)** Comparison of *BST-2* mRNA expression levels measured in HIV negative and positive donors from the FRESH, (black dots), and SK, (red dots) cohorts, respectively. Significantly elevated *BST-2* levels are found within HIV negative donors *vs.* positives (p<0.0001). These represent unmatched donors from two separate cohorts. The HIV positive donors are ARV naïve chronically infected. **(B)** Protein levels of BST-2 were measured on 5 HIV negative donors and 4 HIV infected donors from the FRESH and SK cohorts, respectively. BST-2 protein levels were assessed using a Western blot assay. The levels of HIV infected donors are lower than the HIV negative. **(C)** *BST-2* mRNA expression levels were correlated with log viral load within the SK cohort. Higher mRNA levels correlated with lower log viral load levels (r=-0.53, p=0.0150). **(D)** A negative correlation was also observed when examining the effect of *BST-2* mRNA expression levels and viral load using the CAPRISA 002 cohort at the >36 month time point (r=-0.62, p=0.0006).

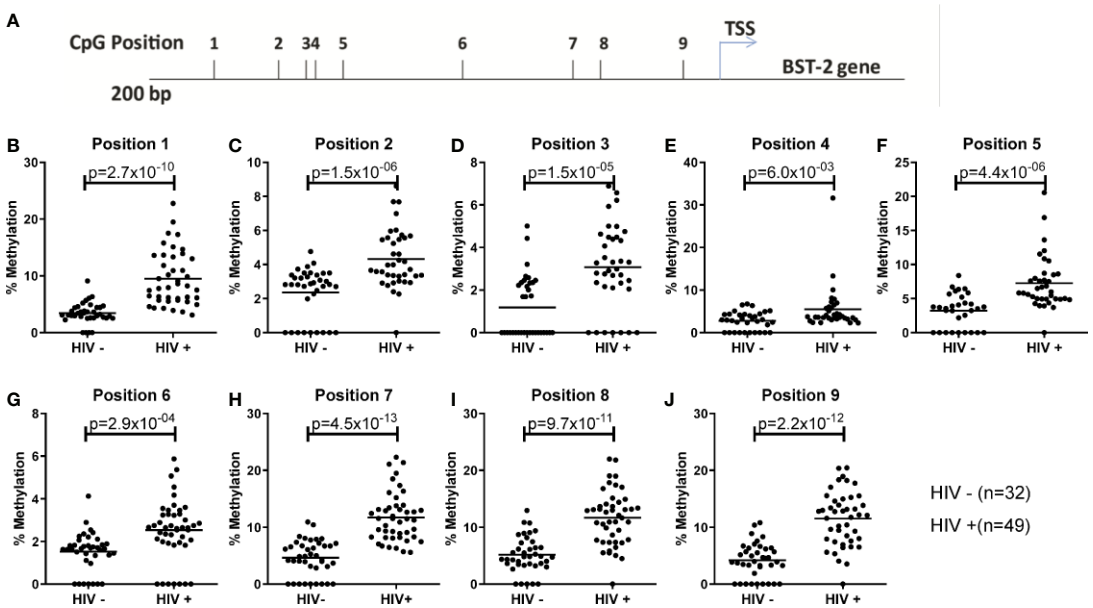

FIGURE 2 | Examining DNA methylation levels across unmatched HIV uninfected and infected donors. **(A)** Location of nine CpG sites within the *BST-2* promoter region 200bp upstream of the TSS. **(B-J)** Using HIV negative (FRESH) and HIV positive (SK) cohorts the percentage methylation, using pyrosequencing of bisulfite converted DNA, was calculated for each of the nine sites.

At the pre-infection time point, *BST-2* expression levels are relatively low, with modest methylation of the gene. Three months after HIV infection, *BST-2* expression levels increase with a concomitant decrease in methylation, perhaps as a result of the immune response in acute infection, including IFN-I production, which is known to enhance *BST-2* production (3) (**Figure 4**). Methylation begins to increase at 12 months' post infection, and by 36 months post infection, the mean expression

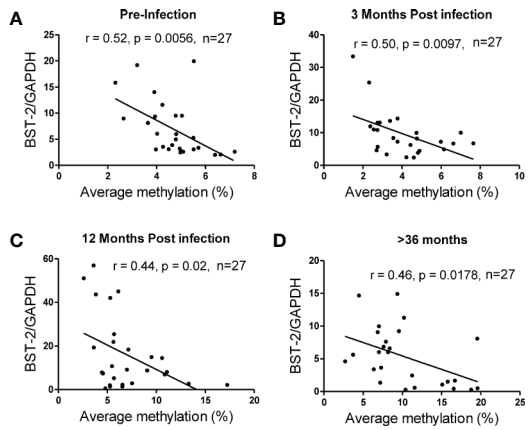

FIGURE 3 | Correlation of DNA methylation and *BST-2* mRNA expression levels across HIV disease. Average methylation was calculated as the average methylation level across nine sites within 200bp upstream of the transcription start site. A strong negative correlation was observed at each of the time points examined for a set of n=27 matched samples at varying time points across disease progression. **(A)** pre-infection (r=-0.52, p=0.0056), **(B)** 3 months' post infection (r=-0.50, p=0.0097), **(C)** 12 months' post infection (r=-0.44, p=0.02) and **(D)** >36 months (r=-0.46, p=0.0178).

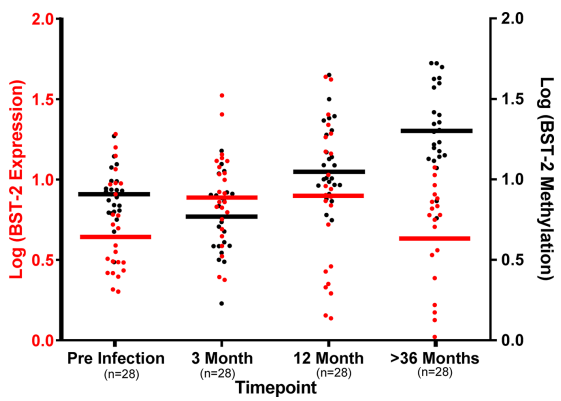

FIGURE 4 | DNA methylation levels dictate *BST-2* mRNA levels during HIV disease. Baseline levels of 27 matched samples at varying time points across disease progression show at the pre-infection higher methylation (Black) and low *BST-2* expression (Red), while at acute infection (3-month post infection) methylation and expression levels are at similar level, due to IFN induction. The *BST-2* expression and methylation levels invert at 12 months' post infection. The most dramatic difference is observed at the >36 months timepoint, these individuals are at a chronic phase of infection, at this time point we observe the lowest expression and highest methylation.

of *BST-2* dips to pre-infection levels while methylation is considerably higher than that at pre-infection timepoint (**Figure 4**). Overall, these results suggest that DNA methylation is not the sole contributor to BST-2 expression variation.

In Vitro HIV Infection and 5'-Aza-CdR Treatment

We next examined the impact of *BST-2* mRNA expression levels on HIV replication *in vitro*. HIV replication was assessed by the amount of p24 released into tissue culture supernatant following infection of PBMCs from HIV negative individuals either having the highest (n=11) or lowest (n=11) *BST-2* mRNA expression levels screened from a cohort of 65 HIV negative donors. p24 measurements, taken at days 2, 4 and 7, showed that individuals with higher *BST-2* expression (dotted line, **Figure 5A**) significantly associated with lower viral replication, at days 4 and 7 post infection, compared to lower *BST-2* expression individuals (solid line, **Figure 5A**; ANOVA, p<0.001). Further, a negative correlation between HIV replication and *BST-2* mRNA expression levels was observed at day 7 (r=-0.63, p=0.0019, **Figure 5B**). These data support a model in which higher *BST-2* levels diminish HIV replication.

Next, we tested whether DNA methylation correlated with *BST-2* mRNA expression levels in an *in vitro* HIV infection assay. Individuals with high *BST-2* expression levels (red dotted line, **Figure 5C**) possessed low methylation levels (blue dotted line) measured at days 0, 2, 4 and 7 days post HIV infection. Conversely, low *BST-2* mRNA expression donors (red solid line, **Figure 5C**) associated with high methylation levels (blue solid line) throughout the time course. Further, the overall difference between the methylation levels within donors either possessing high or low *BST-2* expression levels was significant (**Figure 5C**; ANOVA, p<0.001). Thus, *BST-2* mRNA expression levels associate with the level of *BST-2* DNA methylation, even within an *in vitro* time course of HIV infection.

5'-Aza-CdR induces hypomethylation due to its ability to inhibit DNA methyltransferases, the enzymes responsible for methylation. As manipulation of *BST-2* expression could be considered as a therapeutic intervention in HIV disease, we tested whether 5'-Aza-CdR enhanced *BST-2* expression differentially among donors as a function of the intrinsic expression level of *BST-2*. *BST-2* levels were measured from HIV negative healthy donor PBMCs (n=40) treated with either 5'-Aza-CdR or DMSO (to measure baseline potential for stimulation in each subject). *BST-2* ratios of 5'-Aza-CdR/DMSO treated mRNA levels were then plotted against the *BST-2* levels measured in corresponding untreated PBMCs (**Figure 5D**). A negative correlation between levels of *BST-2* mRNA expression in untreated and Aza-CdR treated PBMC (R=-0.46, p=0.0027; **Figure 5D**) was observed. Donors with the lowest intrinsic (i.e. untreated) *BST-2* mRNA expression levels had the greatest increase in mRNA expression following 5'-Aza-CdR treatment. These data point directly to DNA methylation as a primary factor in regulating *BST-2* gene expression. Increasing *BST-2* gene expression by demethylation may therefore enhance resistance to HIV, given the observation that higher *BST-2* expression associates with HIV control.

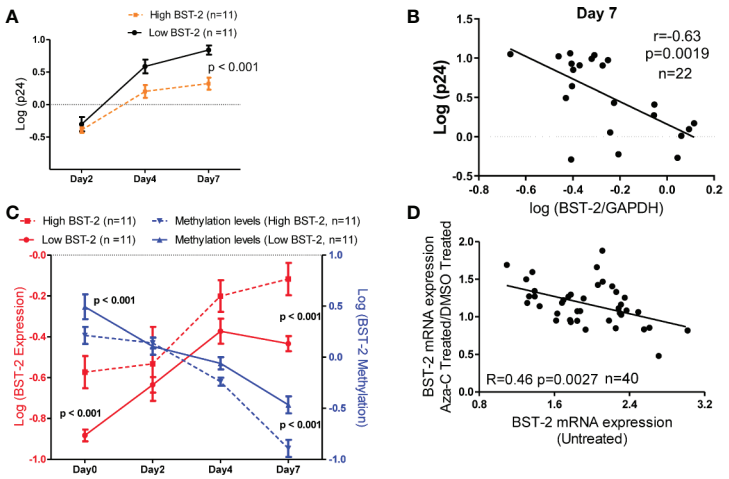

FIGURE 5 | *BST-2* mRNA expression and methylation levels correlate in an *in vitro* viral replication assay, and treatment with a DNA hypo-methylation drug increases *BST-2* mRNA expression levels. **(A)** Individuals were pre-selected based on *BST-2* expression levels for a HIV replication assay. PBMCs from HIV negative donors (n=22) were infected with HIV IIIB viral strain, the amount of virus present was determined by measuring the p24 antigen using an ELISA assay. Measurements of p24 for both high and low *BST-2* donors were taken at days 2, 4 and 7. Donors with higher *BST-2* levels (dotted line) had lower level of p24, while donors with lower *BST-2* levels (solid line) had significantly higher p24 levels ($p<0.001$). **(B)** A negative correlation was observed when comparing the HIV replication levels against the BST-2 mRNA levels at day 7 from the *in vitro* HIV infection assay ($r=-0.63$, $p=0.0019$). **(C)** mRNA and DNA were used to measure *BST-2* expression (red) and methylation levels (blue), respectively, from high and low *BST-2* donors (n=22) at four time points during the viral replication assay, days 0, 2, 4 and 7. Within high *BST-2* donors, we find high expression (red dotted line) associated with lower methylation (blue dotted line) and vice versa for low *BST-2* levels, where low expression (red solid line) associated with higher methylation (blue solid line). **(D)** PBMCs from HIV negative donors (n=40) were split into three subsets; the first subset was treated with a DNA methyltransferase inhibitor that causes hypomethylation (5'-Aza-CdR), while the second subset was treated with DMSO. Both subsets were incubated for 24 hours. *BST-2* mRNA expression from 5'-Aza-CdR and DMSO treated cells were compared and plotted as a fold change against *BST-2* mRNA from an untreated time point (third subset), a significant correlation was observed ($R=-0.46$, $p=0.0027$).

DISCUSSION

Here we show that expression levels of *BST-2*, a potent antiviral cellular protein are negatively associated with viral loads in an antiretroviral-naive cohort of women followed longitudinally from acute HIV-1 infection. Similar results were obtained from an ART-naive chronically infected cohort of participants with unknown time of infection. *BST-2* levels are lower in chronically infected HIV individuals compared to uninfected persons, however in longitudinally followed matched samples, *BST-2* levels first increase significantly over baseline and then decline slowly. We showed *BST-2* expression and DNA methylation levels within the gene promoter region are negatively correlated. These findings are consistent in HIV infected subjects in studies performed *ex vivo* and *in vitro*. Moreover, we pharmacologically altered *BST-2* expression levels by manipulating methylation levels with 5'-Aza-CdR, leading to an increase in *BST-2* mRNA expression, especially within cells with lower intrinsic *BST-2* levels.

BST-2 levels have been shown to inhibit the production of HIV-1 particles by hindering the release of virion progeny (5, 46). However, HIV-1 has developed the ability to counteract this mechanism through the accessory viral protein, Vpu. BST-2 is trafficked from the viral budding sites on the cell surface by a Vpu-mediated mechanism, which thereafter sequesters the host protein to a perinuclear compartment (47). Vpu-null or defective viruses are most prone to BST-2-mediated inhibition. Previous studies have demonstrated that BST-2 surface levels are elevated during acute infection and then progressively decrease throughout the stages of infection, even after initiation of ART (7, 8). In line with these findings, we observed an elevation in mRNA expression of *BST-2* during acute infection both *ex vivo* and *in vitro*, with a subsequent decrease observed by 36 months post HIV-infection. The plasticity of *BST-2* methylation observed suggests that methylation levels are a strong regulator of *BST-2* expression even within a disease setting, although the mechanism regulating the methylation levels requires investigation.

Due to sample availability, we used bulk PBMCs to measure *BST-2* mRNA expression, rather than CD4+ T cells specifically. A previous study measuring cell surface BST-2 showed no differences in expression patterns between individual cells types, PBMCs, mononuclear leukocytes, including CD4-positive, CD8-positive T lymphocytes, B cells, across stages of HIV infection (7). Although the level of mRNA expression does not always reflect protein expression levels, our Western blot assay in a small number of participants suggested a fair correlation. Sample limitations prevented us from examining the correlation between BST-2 mRNA levels and cellular surface expression, however, previous studies have demonstrated the correlation between these subsets (24, 48, 49). These studies have shown that BST-2 mRNA and protein levels correlate in mice, monkeys and humans. Furthermore, the studies also show specific tissues and cell types have strong correlations. The effect is observed across diseases (cancer, SIV, and Mouse mammary tumor virus) and healthy human controls (24, 48, 49).

It is plausible that other human HIV restriction factors could be regulated through DNA methylation. Each factor contributing toward the overall HIV disease outcome. Whole genome methylation analysis on a pair of monozygotic twins with discordant HIV status found several distinct differential methylation regions in the HIV infected twins (50, 51). Furthermore genome-wide methylation analysis of 85 unrelated individuals with varying HIV statuses showed differential genome-wide patterns which was associated with their ability to control HIV replication (52). Future studies should focus on larger cohorts of monozygotic twins or consider longitudinal studies such that the changes in DNA methylation profiles may be followed up at the different time points of HIV infection.

DNA methylation is just one of the mechanisms contributing to the variation in *BST-2* expression levels. Another mechanism identified is a proposed regulatory variant, rs12609479, located in the *BST-2* promoter region, which associated with decreased risk of acquiring HIV-1. The rs12609479-A allele associated with increased BST-2 expression and decreased risk of acquiring HIV-1 (53, 54). The 9 CpG sites that were examined in this study did not contain any polymorphisms and rs12609479 was not located in a CpG site. Despite rs12609479 not being affected by methylation, previous studies have shown diverse changes with respect to minor allele frequency across various ethnic groups (55, 56). Future studies are required to fully understand all the contributing factors responsible for BST-2 expression variation including methylation status across various ethnic groups. Despite these limitations, we found a reproducible association of BST-2 mRNA expression levels with HIV control. Our results were further validated with *in vitro* data.

In conclusion, we reproducibly demonstrate *BST-2* expression levels associate with HIV viral control within a high disease burden setting. DNA methylation was shown to regulate *BST-2* levels and observed to associate with HIV disease. The use of the demethylating drug 5'-Aza-CdR *in vitro* resulted in increased *BST-2* expression levels among donors with low baseline expression levels. Thus, HIV control through higher *BST-2* expression levels, as determined in part by decreased methylation, may suggest strategic mechanisms for HIV cure therapy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Biomedical Research Ethics Committee of the University of KwaZulu-Natal. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RS, VR, MC and TN conceptualized the study. RS, VR, TN, NG, KM, KD, BW, and SK assisted with the cohort setup and proposal design. RS and VR performed the laboratory work. RS, VR, VN, and NY-Z performed the data analysis. RS, VR, MC and TN wrote the paper. All authors contributed to the article and approved the submitted version.

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REVIEW
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Reversing Post-Infectious Epigenetic-Mediated Immune Suppression

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The immune response must balance the pro-inflammatory, cell-mediated cytotoxicity with the anti-inflammatory and wound repair response. Epigenetic mechanisms mediate this balance and limit host immunity from inducing exuberant collateral damage to host tissue after severe and chronic infections. However, following treatment for these infections, including sepsis, pneumonia, hepatitis B, hepatitis C, HIV, tuberculosis (TB) or schistosomiasis, detrimental epigenetic scars persist, and result in long-lasting immune suppression. This is hypothesized to be one of the contributing mechanisms explaining why survivors of infection have increased all-cause mortality and increased rates of unrelated secondary infections. The mechanisms that induce epigenetic-mediated immune suppression have been demonstrated *in-vitro* and in animal models. Modulation of the AMP-activated protein kinase (AMPK)-mammalian target of rapamycin (mTOR), nuclear factor of activated T cells (NFAT) or nuclear receptor (NR4A) pathways is able to block or reverse the development of detrimental epigenetic scars. Similarly, drugs that directly modify epigenetic enzymes, such as those that inhibit histone deacetylases (HDAC) inhibitors, DNA hypomethylating agents or modifiers of the Nucleosome Remodeling and DNA methylation (NuRD) complex or Polycomb Repressive Complex (PRC) have demonstrated capacity to restore host immunity in the setting of cancer-, LCMV- or murine sepsis-induced epigenetic-mediated immune suppression. A third clinically feasible strategy for reversing detrimental epigenetic scars includes bioengineering approaches to either directly reverse the detrimental epigenetic marks or to modify the epigenetic enzymes or transcription factors that induce detrimental epigenetic scars. Each of these approaches, alone or in combination, have ablated or reversed detrimental epigenetic marks in *in-vitro* or in animal models; translational studies are now required to evaluate clinical applicability.

Keywords: tolerance, immune exhaustion, epigenetics, chronic infections, bioengineering

INTRODUCTION

Epigenetic mechanisms guide gene expression to maintain homeostasis by balancing the nature of expressed and non-expressed genes. This balance can be perturbed either by pathogen- induced epigenetic changes, such as through Rv1998 antigen secreted by *Mycobacterium tuberculosis* (*Mtb*) (1) or by chronic and severe stimulation of the immune system as in case of LCMV (2), HCV (3), sepsis (4), Schistosomiasis (5) and TB (6). Long-lasting immune suppression that follows severe or chronic infections increases the risk for secondary infections. This was recognized in 1909 when German researchers noted that TB recurrence occurred after measles (7). In the 1950s, clinicians reported an increased risk for histoplasmosis reactivation among patients recovering from TB (8). Similarly, after surviving sepsis, host immunity remains in a suppressed state that increases the risk for secondary bacterial infections and doubles mortality risk (9, 10). Survivors of pneumonia have increased risk of death with the severity of pneumonia correlated with mortality risk (11). TB survivors also have increased risk of mortality, not only from secondary infections and recurrent TB, but from increased risk of cardiovascular disease and cancer (12, 13). Although epigenetic immune suppression is needed acutely to temper exuberant immunity (14), these immunosuppressive epigenetic marks are long-lived and are thought to be a major contributing factor for increased secondary infections long after resolution of the first insult (12, 15–17). Proper epidemiological studies matched with translational studies need to be conducted, but epigenetic-mediated post-infectious myeloid and lymphoid immune suppression is a suspected explanation for why individuals retain increased mortality risks even after successful treatment for pneumonia, TB or sepsis (11, 13, 15).

After chronic and severe infections, $CD4^+$ T cells are characterized as being anergic and $CD8^+$ cells as being exhausted (18, 19). Functionally, anergic $CD4^+$ T cells fail to recognize and respond to foreign antigen, as measured by decreased antigen-induced cellular proliferation and cytokine production (18). Similarly, $CD8^+$ T cell immune exhaustion is defined by decreased antigen-induced proliferation, cytokine production and an increase in immune checkpoint inhibitors (19). Myeloid cell immune tolerance is defined by a decreased responsiveness, usually measured by decreased phagocytic capacity, killing capacity and cytokine production, e.g., TNF, IL-6 and IL-1β (10, 20–22). Animal models have demonstrated that post-infectious immune suppression is epigenetically mediated and that the detrimental epigenetic marks induced by chronic infections overlap with those induced by cancer (5, 6, 23–30). There are many studies demonstrating how cancer induced epigenetic-mediated immune suppression can be reversed. Herein, we review the growing literature of *in-vitro* and animal model studies demonstrating how to block or reverse infection induced epigenetic-mediated immune suppression and postulate how these approaches could become clinically relevant to decrease post-infectious morbidity and mortality.

EPIGENETIC MECHANISMS AND GENE EXPRESSION

Epigenetic mechanisms are one major means of regulating gene expression. This regulation comes from nucleosomal scaffolding of the negatively charged DNA around positively charged proteins, called histones, present as two functional copies apiece of the type H2A, H2B, H3 and H4. Each nucleosome is further condensed in a higher-order structure, the chromatin. Both nucleosome and chromatin can guide accessibility of molecular factors to the DNA, thus resulting in differential gene expression (31). Cells can either circumvent or reinforce these barriers, depending on the context, by dynamically modifying DNA and histones at specific nucleotide or amino acid residues, transiently creating regions of the genome differentially accessible to gene expression machinery. Histones are modified on their free N-terminal tails, or their globular domains that physically interact with the DNA, through chemical modifications including acetylation, methylation, phosphorylation, ubiquitylation, acylation, hydroxylation, glycation, serotonylation, glycosylation, sumoylation and ADP-ribosylation (32). DNA is methylated at cytosine and adenine residues. Epigenetic marks other than acetylation and methylation are not as well studied and are less understood. The gene expression implications of certain epigenetic marks are well established. DNA methylation directly interferes with the binding of DNA and transcription factors, or it can attract proteins that bind specifically to modify DNA, thereby blocking other transcription factors from binding the site (33). Acetylation of histones H3 and H4 relaxes the nucleosome compactness and leads to partial de-condensation of chromatin locally, making the DNA more accessible. Such accessibility is referred to as "permissive" and the loss of accessibility and increased compactness referred to as "restrictive" (34). Histone modifications can be either permissive or restrictive. For example, trimethylation of histone 3 at lysine 4 (H3K4me1, H3K4me3) promotes open chromatin, while trimethylation of histone 3 at lysine 27 (H3K27me3) and at lysine 9 (H3K9me3) promotes restrictive heterochromatin (35).

SIGNALING PATHWAYS THAT INDUCE T CELL IMMUNE EXHAUSTION

Broadly, T-cell activation involves tightly controlled signaling pathways and cascades, that when perturbed lead to transcription factor (TF) imbalances that then drive epigenetic-mediated gene expression inhibition. Activation of the T-cell receptor (TCR) by MHC-antigen complexes assembles the "TCR signalosome" that results in downstream phosphorylation events and activation of secondary signaling molecules (18, 36–40). Downstream of the TCR, key events include phosphorylation of tyrosine kinases and phospholipase C (PLC)1 (41–43). Activated PLCγ1 cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3), the former being

critical for proper activation of activator protein-1 (AP-1) complex, a heterodimer of c-Fos and c-Jun (**Figure 1**) (44–47). IP3 plays a central role in calcium signaling by releasing intracellular calcium stores from the endoplasmic reticulum (ER) (48) and thereby dephosphorylating NFAT proteins which translocate to the nucleus, and bind with their transcriptional partners, such as AP-1, to activate distinct ranscriptional programs (**Figure 1**) (49). NFAT/AP-1 transcriptional complexes bind to the promoters of various cytokine genes, including *Il2*, leading to their active transcription. In the absence of co-stimulation, impaired AP-1 activation results in NFAT homodimerization inducing transcriptional and epigenetic changes that yield anergic and exhausted T cells (**Figure 1**) (18, 44, 50, 51).

Activation of the TCR and CD28 co-stimulatory induces a flux of intracellular Ca^{2+} and activation of the PI3K-AMPK-mTOR signaling pathway. Activated mTOR engages several downstream effector pathways, including promoting metabolism by activating gene expression of the TFs hypoxia-inducible factor 1α (HIF1α), MYC and sterol regulatory element-binding protein (SREBP). Upregulation of inhibitory receptor signaling recruits Src homology 2 domain-containing tyrosine phosphatase 2 (SHP2) phosphatase which interferes with CD28 costimulatory signaling by blocking PKC-θ (52) and PLCγ1 (53). Increased programmed death protein 1 (PD-1) inhibits AKT and mTOR pathways (54), activates Basic leucine transcription factor (BATF) to repress T-cell proliferation and cytokine secretion in HIV-specific CD8+ T cells (55), and inhibits IL-2 production to limit T-cell proliferation (52). NFAT1 in the absence of AP-1 interaction promotes the expression of *Pdcd1* (PD-1 encoding gene) (56). Inactivation of the AKT/mTOR pathway promotes FOXO1 retention in the nucleus to enable continued inhibitory receptor *Pdcd1* gene transactivation (57). PD-1 signaling through SHP2 activates AMPK, which is an inhibitor of mTOR signaling (already abrogated by inactivation of PI3K and AKT), leading to downregulation of HIF-1α and MYC, which in turn governs the transcription of the glycolytic enzymes such as GLUT1, thereby decreasing cellular metabolism.

TRANSCRIPTION FACTORS DRIVING T CELL IMMUNE EXHAUSTION

NFAT homodimers play a critical role in induction of the T cell anergy transcriptional program (44). NFAT1 homodimers bind to specific NFAT binding sites on T cell anergy-associated gene promoters. For example, NFAT homodimer consensus binding sites are present in the promoter of *Grail*, a T cell anergy-associated gene (50). Expression of the early growth response gene 2 (Egr2) and Egr3 is NFAT-dependent, and these TFs are associated with regulation of gene expression of the Casitas B-lineage lymphoma b (Cbl-b) E3 ubiquitin ligase in anergic T cells (58). Downstream of NFAT signaling, the TF Ikaros, binds to the *IL2* gene locus, and recruits the NuRD complex, including histone deacetylases (HDACs), thereby facilitating epigenetic remodeling, specifically histone deacetylation, of the *Il2* promoter, thus effectively silencing gene expression (59, 60).

Highlighting the critical importance of a balanced NFAT response, a bioengineered constitutively active form of NFAT, termed CA-RIT-NFAT1, closes chromatin conformation inducing epigenetic-mediated immune exhaustion, including decreased microbial killing capacity in $CD8^+$ T cells (56, 61). Constitutively active NFAT1 leads to the enrichment of genes belonging to the nuclear receptor (NR) family of genes, specifically members of the NR4A family. In particular, NR4A2 (NURR1) and NR4A3 (NOR1) exhibit high enrichment upon CA-RIT-NFAT1 expression. NR4A family member genes exhibit greater chromatin accessibility in exhausted tumor infiltrating lymphocytes (62). Using a chimeric antigen receptor T cell (CAR T cell) model, a NR4A triple knockout reversed detrimental chromatin accessibility, and promoted tumor regression and prolonged survival of tumor-bearing mice, thus illustrating the epigenetic and functional relevance of the NR4A family in T cell exhaustion. NR4A is also important for PD-1 and TIM3 expression, markers of T cell exhaustion (62).

NFAT homodimers also induce the thymocyte selection-associated high mobility group box (TOX) proteins which mediate the expression of inhibitory receptors such as PD-1 and TIM3, leading to the T cell exhaustion phenotype. Increased TOX expression occurs in chronic infection models such as LCMV and chronic hepatitis C (HCV) infection (51, 63). Removal of the nuclear localization sequence (NLS) and part of the DNA-binding domain from TOX *via* deletion of exon 5 resulted in decreased PD-1 expression and impaired generation of the T cell exhaustion phenotype. In addition, TOX exon 5 deletion resulted in differential expression of genes associated with T cell exhaustion such as *Id3, Helios (Ikzf2), Nr4a1, Nr4a2, Pdcd1*, and *Klrg1*. Conversely, over-expressing TOX in healthy T cells increases PD-1 expression, demonstrating a role in inducing the T cell exhaustion phenotype (63). Deletion of TOX exon 5 leads to decreased chromatin accessibility of the *Pdcd1* gene locus, which encodes PD-1, and increased chromatin accessibility to *Tnf* (63). Knocking out *Tox* in $CD8^+$ CAR tumor infiltrating lymphocytes (TILs) increase cytolytic activity further supporting the notion that TOX specifically attenuates $CD8^+$ T cell effector function (**Figure 1**).

These studies provide evidence for the importance of the NFAT, TOX and NR4A TFs in driving epigenetic-mediated immune exhaustion, and also suggest strategies to alter their activation could be therapeutically pivotal (**Figure 1**). For example, Cyclosporin A (CsA), a calcineurin inhibitor, inhibits NFAT activation, thereby inhibiting TOX, NR4A1, NR4A2 and NR4A3 and the subsequent detrimental chromatin conformation changes that leads to immune exhaustion (51). Tacrolimus (binding to FK506) inhibits calcineurin by a different mechanism, but similarly decreases NFAT and TOX, blocking the chromatin confirmation changes that upregulate PD-1 and LAG3, thereby preserving capacity to produce TNF and IFN (64–66). Discussed in more detail below, bioengineered upregulation of c-Jun rescues NFAT-AP-1 imbalance, thereby restoring immune function. Therefore, while tacrolimus and CsA are considered immune suppressants, they can prevent detrimental

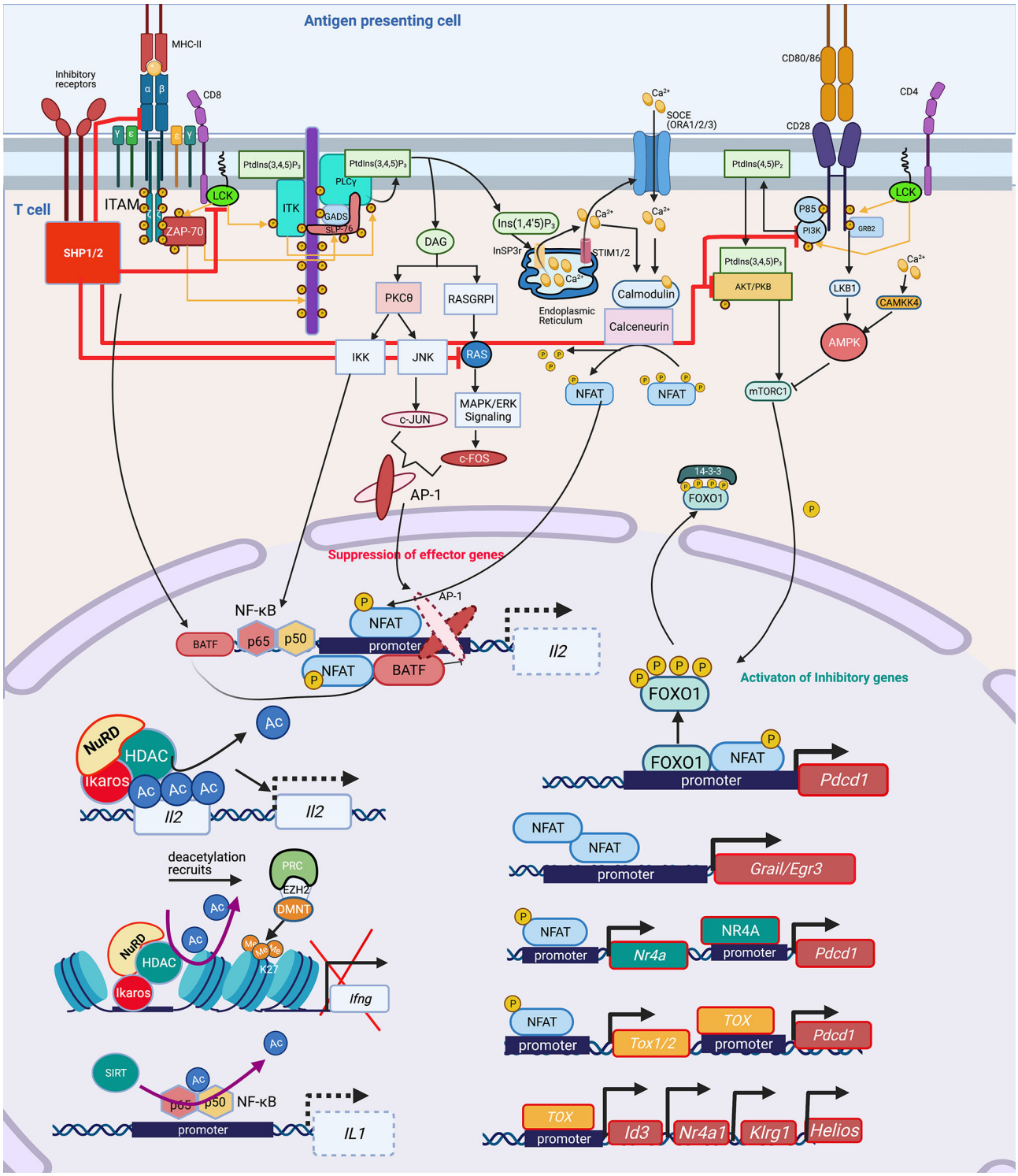

FIGURE 1 | Continued

FIGURE 1 | Signaling cascade and transcription factors that mediate epigenetic changes that inhibit host immunity. In T cells, protein kinase Lck and ZAP-70 initiate a signaling cascade that result in activation of PLCγ1 and production of InsP3 (IP3), a second messenger, binding to the InSP3 receptor on the ER leading to release of Ca^{2+} from the ER. The reduction of Ca^{2+} activates STIM, which recruits SOCE such as ORAI in the plasma membrane. Opening of ORAI channels in the plasma membrane results in sustained Ca^{2+} influx and activation of several Ca^{2+} regulated enzymes such as serine/threonine phosphatase calcineurin, which dephosphorylates NFAT enabling its translocation to the nucleus where it binds to promoters of effector genes including *Il2*. NFAT requires AP-1 generated through another second messenger DAG activation of PKCΦ and RAS/MAPK/ERK1 pathways. Lck also mediates activation of PI3K activating AKT and mTOR, which govern the phosphorylation of FOXO1. Phosphorylated FOXO1 is transported out of the nucleus and exists in complex with 14-3-3 in the cytoplasm. In exhausted T cells, either through activation of inhibitory receptors such as PD-1, CTL4, a dephosphorylating protein SHP1/2 is activated, which dephosphorylates Lck and ZAP70, suppressing the subsequent signaling cascades. SHP2 inhibits among others RAS, AKT, PI3K and even the TCR-MHCII microcluster, thus weakening or abrogating the effector signals at multiple levels (red inhibition arrows). This leads to widespread change in the cellular TF landscape. SHP1/2 activate BATF3, a TF, due to non-availability of AP-1 to partner with NFAT. Partnerless NFAT, alone leads to transcription of inhibitory genes and receptors including *pdcd1*, which is also transcribed by increased nuclear retention of unphosphorylated FOXO1 in the nucleus, in absence of a PI3K/AKT/mTOR activation. Unpartnered NFAT transcribes, *Nr4A* and *TOX1/2*, which further contribute to inhibitory signaling by increasing transcription of *Pdcd1*. NFAT homodimer transcribes inhibitory genes *Grail3/Erg3*. TOX leads to transcription of genes such as *Id3*, *Nr4a1*, *Klrg1*, *Helios*. Many of these genes and TF lead to epigenetic modifications, which further contribute to exhausted phenotype. TF, Ikaros (Helios family) can directly bind to the *Il2* promoter and recruit NuRD, which has HDAC and deacetylates *Il2* leading to its transcriptional repression. Deacetylation is usually followed by recruitment of PRC, which through EZH2 can further add to closing of chromatin by adding methylation marks at H3K27, as seen at the *Ifng* locus. Another, NAD: NADH+ dependent deacetylase, SIRT can directly deacetylate NF-κB to decreases *IL1* transcription. Lck, LCK proto-oncogene, Src family tyrosine kinase; ZAP-70, zeta chain of T cell receptor associated protein kinase 70; PLC, Phospholipase C; IP3/$InsP_3$, inositol 1,4,5-trisphosphate; ER, Endoplasmic reticulum; STIM, stromal interaction molecule 1; SOCE, Store-operated calcium entry; ORAI, ORAI calcium release-activated calcium modulator; NFAT, Nuclear factor of activated T-cells; IL, Interleukin; AP-1, Activator protein1; DAG, Di-Acyl Glycerol; PKCΦ, Protein kinase C; MAPK, Mitogen-Activated Protein Kinase; FOXO1, Forkhead box protein O1; 14-3-3, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta (encoded by *YWHAQ*); PD-1/Pdcd, programmed cell death 1; CTL4, Cytotoxic T-Lymphocyte Associated Protein 4; SHP, Src homology 2 domain-containing tyrosine phosphatase 2; PI3K, phosphatidylinositol 3-kinase; AKT, Protein kinase B; mTOR, mammalian target of rapamycin; MHC, Major Histocompatibility complex; TF, Transcription factors; BATF, Basic Leucine Zipper ATF-Like Transcription Factor; Nr4A, Nuclear Receptor Subfamily 4 Group A Member 1; TOX, Thymocyte Selection Associated High Mobility Group Box; Erg, ETS transcription factor ERG; Id3, Inhibitor Of DNA Binding 3; Klrg1, Killer Cell Lectin Like Receptor G1; NuRD, Nucleosome and DNA Remodeling complex; PRC, Polycomb Repressive Complex; EZH2, Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit; H3K27me3, H3 lysine 27 trimethylation; Ac, Acetylation; Me, Methylation. Created with BioRender.com.

chromatin conformation that leads to immune exhaustion, thereby preserving host immunity. Studies are needed to evaluate if these agents could be of benefit in humans following pneumonia, sepsis or TB.

SIGNALING PATHWAYS THAT INDUCE MYELOID IMMUNE TOLERANCE

For myeloid cells, the best studied model for immune tolerance is LPS challenge or sepsis. The exact myeloid tolerance mechanism(s) has not yet been elucidated for other infections. Thus, we discuss LPS/sepsis as a central theme for the signaling pathways inducing myeloid tolerance. Following overstimulation, myeloid cells, including monocytes and macrophages, develop tolerance, a state of cell refractoriness defined by an inability to mount an inflammatory response to a secondary stimulation (67). Pathogen- or danger-associated molecular patterns (PAMPs/DAMPs) such as LPS are sensed *via* pattern recognition receptors (PRR), e.g., toll-like receptors (TLR). LPS is recognized by TLR4, mediating signaling through two distinct adaptor pathways, myeloid differentiation factor 88 (MyD88) and TIR-domain containing adapter-inducing interferon (TRIF). The MyD88 pathway employs interleukin-1 receptor-associated kinase (IRAK)1 and 4 kinases and TNF receptor-associated factor (TRAF) 6 to activate NFκB and AP-1 signaling, promoting transcription of pro-inflammatory cytokines. Activation of TRIF pathway leads to janus kinase (JAK)/signal transducer and activator of transcription (STAT)1 and type I interferon activation and increases the expression of interferon-inducible genes such as *TNFA, IFNB, IL1B, IL6*, and *COX2* (68, 69). PI3K interacts with MYD88 and also influences TLR4 signaling (70). LPS-induced myeloid tolerance involves downregulation of TLR4 expression, decreased recruitment of MyD88 or TRIF to TLR4, decreased activation of IRAK1/4 and diminished canonical NFκB signaling (p65/p50 heterodimer) *via* formation of inactive p50 homodimers (67, 71), decreased AP-1, reduced expression of *TNFA, IL1B, IL6 and IL12B*, and increased expression of *IL10* and *TGFB1* (**Figure 2**).

LPS-tolerized myeloid cells are also characterized by negative regulatory molecules IRAK-M, A20, SH2 domain-containing inositol phosphatase 1 (SHIP1) (72), Pellino-3 (73), suppression of tumorigenicity 2 (ST2) (74), suppression of cytokine signaling (SOCS)1 and SOCS3 that inhibit TLR signaling (67, 69) (**Figure 2**). PI3K pathway, activated in LPS tolerance, also contributes to production of anti-inflammatory cytokines such as sIL-1RA (75) and its inhibition with wortmannin mitigates tolerance and increases TNF production (76). NFκB upregulates HDACs that remove histone acetyl marks and recruit the NuRD complex with the net result of a "repressome" such that euchromatin marks (e.g., histone acetylation) are removed and heterochromatin marks (e.g., DNA methylation and H3K9 and H3K27me) are induced (**Figure 2**) (20, 77–79). MyD88 activation, *via* non-coding RNAs, also contributes to decreased chromatin accessibility changes thereby inducing tolerance (80, 81). Acutely, tolerance is beneficial as studies have demonstrated that inhibiting post-sepsis epigenetic-mediated immune suppression too early exacerbates immune pathology (14).

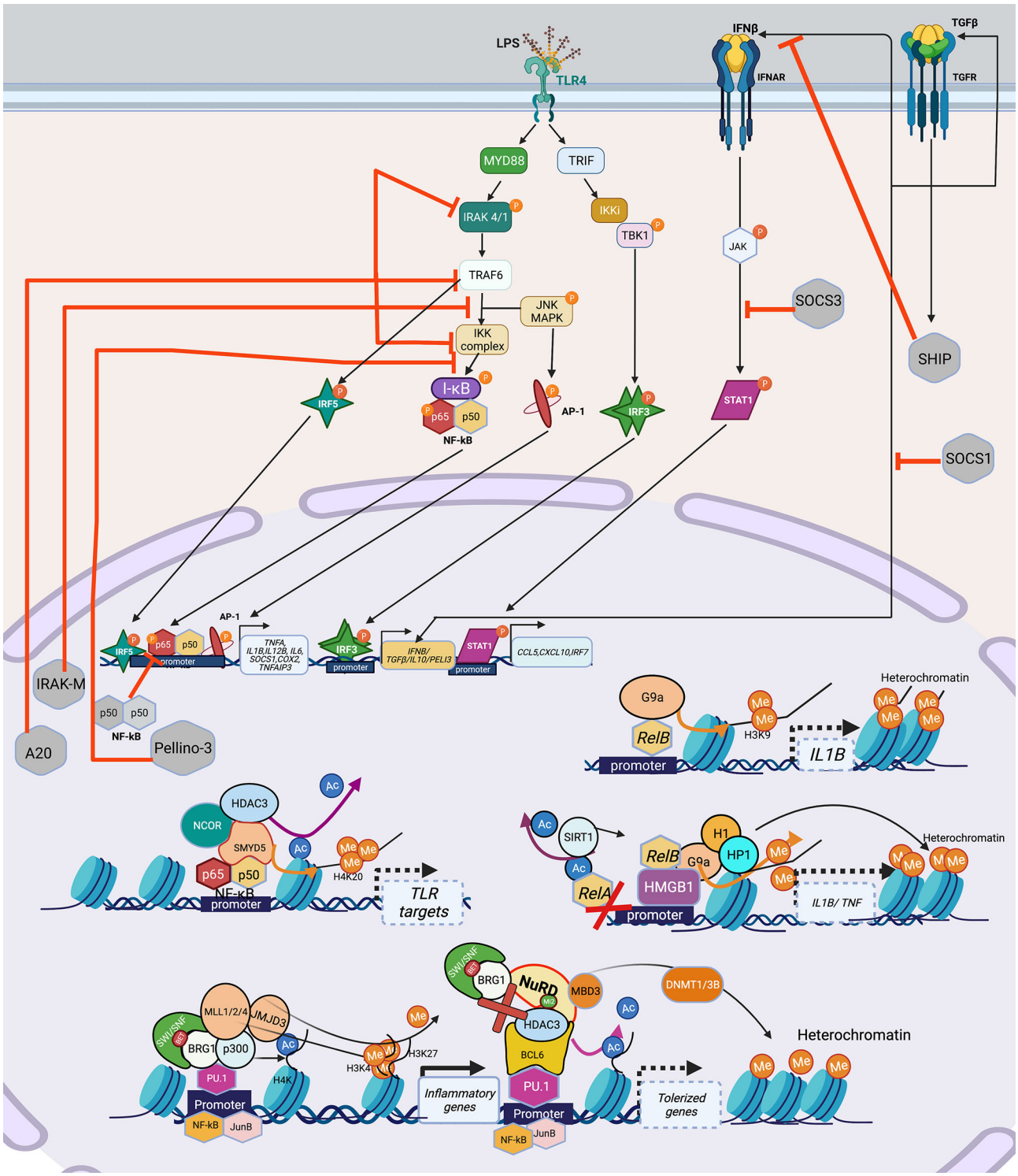

FIGURE 2 | Continued

FIGURE 2 | Signaling cascade and transcription factors that mediate epigenetic changes that inhibit host immunity in myeloid cells. TLR4 recognizes LPS, and engages the MyD88-TRIF pathway to induce the TFs: NF-κB, AP-1, IRF3, IRF5 which leads to the induction of pro-inflammatory genes such as *TNFA, IL1B, IL6, COX2* etc. IRF3 induces the production of IFNβ and TGFβ, which adds to the IFN signaling and induces STAT1 leading to transcription of *CCL5, CXCL10 and IRF7*. Overwhelming LPS stimulation as seen in sepsis, leads to lesser production and engagement of TLR4 and its pathway components, with over-inflammation leading to production of inhibitory molecules such as IRAK-M, A20, Pellino-3, SHIP, which inhibit various parts of the LPS-TLR4 signaling cascade, leading to a tolerized phenotype. Epigenetically, multiple mechanisms have been shown to lead to and maintenance of the tolerized phenotype. Guided by TFs such as NF-κB and its isoform RelB, which can recruit HDACs (SIRT included) either alone or in a repressome complex, usually with a chromatin modifier such as SWI/SNF results in deacetylation of histones, followed by addition of repressive methylation (H3K9, H3K3) by DNMT such as SMYD5 (in the NCOR-HDAC3 repressome), or KMT such as G9a bound to HMGB1 (can recruit H1 and HP1) to close the chromatin and suppress gene expression. Lineage TFs such as PU.1 provide good example of this assembly of the SWI/SNF complex containing BRG1 which can recruit HAT (p300) to acetylate H4K, HMT (MLL1/2/3) to add permissive H3K4 and demethylase such as JMJD3 to remove repressive H3K27 to activate inflammatory genes upon LPS stimulation. The same PU.1 when bound to co-repressor BCL6 can induce tolerance by losing the SWI/SNF complex and recruitment of NuRD, which recruits HDAC3 to remove acetylation and induce *de novo* methylation *via* DNMT1/3B to close the chromatin and thus shutting down inflammatory gene transcription in tolerance. Created with BioRender.com. TLR4, Toll-like receptor 4; LPS, Bacterial Lipopolysaccharide; MyD88, myeloid differentiation factor 88; TRIF, TIR-domain containing adapter-inducing interferon; TF, Transcription factor; STAT1, signal transducer and activator of transcription; IRAK, interleukin-1 receptor-associated kinase; TRAF6, TNF Receptor Associated Factor 6; SHIP; SH2 domain-containing inositol phosphatase 1; IKK, Ikappa B Kinase; TBK, TANK-binding kinase 1; MAPK, Mitogen-Activated Protein Kinase; I-KB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; IRF, Interferon regulatory factors; SWI/SNF, SWItch/Sucrose Non-Fermentable; BRG1, Brahma-related *gene*-1; HDAC, Histone deacetylase; H1, *H1*.1 Linker Histone; HP1,Heterochromatin protein-1; HMGB1, High Mobility Group Box 1; DNMT, DNA methyltransferase; MLL, mixed lineage leukemia (lysine methyl transferase); JMJD, Jumonji domain containing protein; BCL6, B-cell lymphoma 6; MBD3, Methyl-CpG Binding Domain Protein 3.

TRANSCRIPTION FACTORS DRIVING MYELOID IMMUNE TOLERANCE

Similar to the situation with immune exhaustion, myeloid cell immune tolerance is mediated by TFs that recruit co-activator and corepressor complexes that modify chromatin accessibility through post-translational modifications (**Figure 2**). The myeloid lineage defining transcription factor PU.1 facilitates chromatin opening with an increase in H3K4me3 at promoters and H3K4me1 at enhancers. However, in resting macrophages, corepressors such as B-cell lymphoma 6 (BCL-6) associate with PU.1 and recruit HDACs and histone demethylase resulting in repression of many LPS-inducible genes (82). In an analysis of LPS-induced tolerant and non-tolerant genes, NF-κB and MAPK were downregulated in tolerant macrophages (83). NFκB family TF isoform, RelB, mediates epigenetic silencing *via* facilitating the direct deposition of repressive histone marks by the H3 lysine methyltransferase (KMT) G9a at the *IL1B* promoter (78). Similarly, binding of high-mobility group box-1 protein (HMGB1) and histone H1 linker at the promoters of *TNF* and *IL1B* genes leads to transcription silencing by promoting assembly of RelB, which results in deposition of H3K9me2 mediated by the KMT G9a. Depletion of HMGB1 by siRNA results in dissociation of RelB from the promoter and partially restores *TNF* transcription (84).

Tolerized myeloid cells exhibit decreased chromatin accessibility due to decreased TLR-induced recruitment of the BRG1-containing SWI/SNF nucleosome remodeling complex and changes in histone acetylation and methylation (83). The NCoR-Hdac3-p50 repressome contains histone deacetylase and SET histone methyltransferases (SMYD5) that result in H3K9/14 deacetylation (77) and H4K20 methylation (85) respectively, both contributing to heterochromatin and repression of tolerizeable genes, thereby inhibiting the expression of genes downstream of TLR4 activation. Genetic disruption of the NcoR-Hdac3 interaction abolishes TLR4 tolerance (83). Interestingly, IFN-γ prevents tolerance by preserving expression of the receptor-interacting protein 140 (RIP140) coactivator and promoting TLR-induced chromatin accessibility upon secondary TLR challenge (86). In contrast, non-tolerized genes maintain an open chromatin state and exhibit more H4 acetylation and maintain H3K4me3 after re-stimulation (83). Interestingly, the NuRD complex acts antagonistically, and in a SWI/SNF-BRG1 dependent manner in LPS stimulated macrophages showing that these complexes exhibit concerted action to guide gene expression in myeloid cells (87) (**Figure 2**).

In summary, PU.1 facilitates myeloid gene transcription, while tolerance is associated with binding of co-repressor BCL-6 to PU.1, disruption of the NFκB active heterodimer and epigenetic silencing *via* HMGB1, RelB, NCoR-HDAC3-p50 repressome (**Figure 2**), increased SMYD5 and G9a methyltransferase and decreased chromatin accessibility due to reduced recruitment of BRG1-NRC. BET inhibitors (that bind to the bromodomain in the BRG1-NRC) such as IBET151, rescue tolerance in a preventative way when administered along with LPS, and not post LPS exposure (88).

Thus, HDAC inhibitors and G9a inhibitors if given after the resolution of acute infection, could potentially mitigate aspects of long-lived myeloid cell tolerance, while BET inhibitors act in a more preventative way (88).

METABOLIC MECHANISMS LEADING TO IMMUNE EXHAUSTION AND TOLERANCE

Upon immune activation *via* mTOR and NFAT signaling, shifts in cellular metabolism increase glycolysis, the tricarboxylic acid cycle (TCA, also known as the Krebs cycle) and electron transport chain (ETC), not only to meet high energy demands for proliferation and effector function, but also to produce the intermediate metabolites that fuel the biosynthesis of effector protein functions (10, 89–91). While initially beneficial, in severe or chronic infection, these metabolic shifts contribute to epigenetic changes that induce immune suppression (35, 89,

90, 92–95). In both lymphoid and myeloid cells, these metabolic shifts are mediated by the PI3K-Akt-mTOR pathway (96) and if the infection persists, the associated signaling cascades are downregulated and epigenetic mechanisms suppress host immunity, with loss of accessible chromatin that allows for expression of cytokines such as *Tnf* and *Ifng* and gain of chromatin accessible regions in inhibitory loci such as *Pdcd1* (97, 98), thereby placing cells into an immune suppressed state (97). The described metabolic shifts induce epigenetic changes due to alteration in metabolic precursors required for epigenetic marks. At least three overlapping metabolic-epigenetic rheostats (**Figure 3**) have been identified that regulate host immunity (99–101).

Histone acetylation, an epigenetic mark characteristic of euchromatin is regulated by the availability of nicotinamide adenine dinucleotide (NAD^+). High NAD^+ levels and NAD^+/NADH ratios induce the NAD-dependent deacetylase sirtuins (SIRTs) which deacetylate both histone and non-histone proteins. High dose LPS exposure, *via* upregulation of IDO1-induced *de novo* synthesis of NAD^+, activates SIRT1, leading to histone deacetylation and gene silencing of proinflammatory genes such as *Tnf* and *Il1b* (102, 103). SIRT1 deacetylates p65 lysine of RelA (NF-κB) and nucleosomal H4K16 to terminate NF-κB dependent transcription and remains bound to assembled RelB and recruited transcriptional repressor complex (including heterochromatin linker H1) generating tolerance (102). NAD levels are regulated by CD38 and IDO1. CD38 levels correlate with T cell exhaustion (104, 105) and are elevated in patients with Cytomegalovirus (CMV) (106), Epstein-Barr-virus (EBV) (107), mycobacteria (108) and HIV, and are associated with poor prognosis (109). CD38, an extracellular and intracellular NADase, converts NAD^+ molecules to a single cyclic ADP ribose thereby drastically shifting NAD^+/NADH ratios, and activating sirtuin-mediated epigenetic mediated immune suppression (104). IDO, another mediator of immune suppression, is the rate limiting enzyme step in *de novo* NAD synthesis, converting tryptophan to kyneurine. IDO, elevated in sepsis and TB, inhibits host immunity by decreasing nuclear NAD^+ concentrations, and initiating sirtuin activation (103, 110). Sirtuins regulate post-infectious immune suppression in both lymphoid and myeloid cells (102, 111), with inhibitors of CD38, IDO1 or SIRT1 able to restore host immunity and prevent mortality in animal models (104, 110, 112).

The second metabolic-epigenetic immune rheostat mechanism is guided by the balance of α-ketoglutarate (α-KG) and succinate. DNA methyltransferases (DNMT), lysine demethylase (KDM), jumonji domain-containing protein D3 (JMJD3) and Ten-eleven translocase (TET), require α-KG (also known as known as 2-oxoglutarate-2OG) as a co-substrate (113). Therefore, these epigenetic enzymes are known as α-KG or 2OG dependent dioxygenases (α-KG-DD) (113). Succinate, the end product of these chemical reactions, acts as a negative feedback loop to inhibit their function (114). In addition to succinate, other late-stage TCA metabolites including fumarate, malate, itaconate and 2-hydroxyglutarate (2HG) inhibit the α-KG-DD epigenetic enzymes (115–120). The importance of TCA metabolites in epigenetic regulation was first described in cancer where mutations in IDH, succinate dehydrogenase (SDH) or fumarate hydratase were found to induce global epigenetic disturbances (121, 122). These mutations lead to TCA metabolite imbalances that drive global DNA and histone hyper-methylation and immune tolerance (123–126). While originally described in cancer, studies in wild type mouse and human healthy T cells demonstrate that, upon immune activation, 2HG is increased *via* Von Hippel-Lindau (VHL)-HIF1α (120). Initially, 2HG increases T cell IL-2 production, but when 2HG elevations persist, there are global increases in the inhibitory epigenetic mark H3K27me3 with suppression of T cell cytotoxic function (120). Dimethyl fumarate (DMF), an immune suppressive therapy for multiple sclerosis, induces DNA methylation and heterochromatin in monocytes and T cells to suppress exuberant immunity (127, 128).The timing and duration of TCA metabolite shifts need to be further explored as short term shifts induce immune beneficial immunity, while others are immune suppressive (118, 120, 129, 130).

The known metabolic-epigenetic immune rheostats are overlapping and redundant, as demonstrated by the third known metabolic-epigenetic rheostat. SDH is unique in that it is both part of the TCA cycle and the ETC. Upon immune activation, the increase in glycolysis fuels the ETC and when persistent, electrons leak out of the inner mitochondrial space (**Figure 3**), increasing reactive oxygen species (ROS) in the mitochondrial matrix. This increase in ROS triggers phosphatase of activated cells 1 (PAC1, encoded by *DUSP2*, dual specificity protein phosphatase 2) and nuclear factor erythroid 2-related factor (NRF2, encoded by *NFE2L2*) to activate the NuRD complex (131, 132). The NuRD induces histone deacetylation and DNA hypermethylation (*via* MBD2/3) and is instrumental in limiting exuberant immune pathology in macrophages after sepsis and preventing T cell autoimmunity (133). Mice with tumor-induced immune exhaustion demonstrate elevated mitochondrial ROS that correlates with detrimental epigenetic marks (DNA hypermethylation and closed chromatin conformation) and immune suppression (134). Inhibiting mitochondrial ROS accumulation ablates immune suppression (92, 135), however to date, the direct link *via* the NuRD has not been demonstrated.

The immune inhibitory effects due to itaconate further demonstrate the overlap of mechanisms by which metabolism acts as an immune rheostat. The direct epigenetic effect of itaconate has not yet been described. Itaconate is produced by diverting cis-aconitate in the TCA cycle by the enzyme cis-aconitate decarboxylase. Itaconate inhibits SDH, leading to succinate accumulation (**Figure 3**). Therefore, it is presumed but not proven that itaconate induces epigenetic changes akin to succinate. Itaconate acts as a negative feedback to limit exuberant immune pathology, inducing NRF2 nuclear translocation and downregulation of IL1β and IL-6 (93). Upon *Mtb* infection, mice with knockdown of *Irg1* (gene that codes for CAD, the enzyme

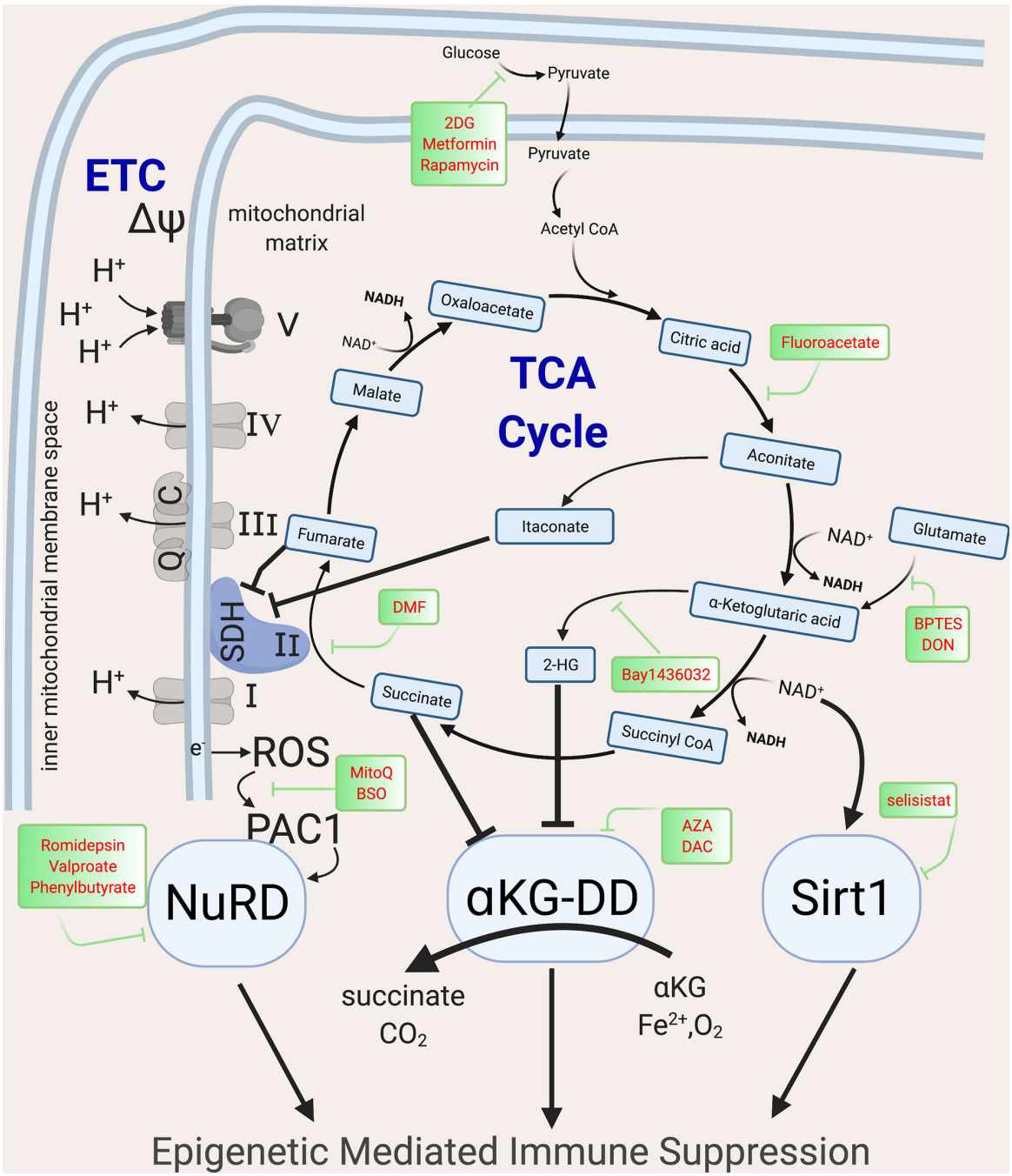

FIGURE 3 | Metabolic intermediates of the TCA cycle guide epigenetic changes that inhibit host immunity. The TCA cycle metabolites act as co-factor for major epigenetic enzymes that shape the epigenomic landscape post infection *via* three overlapping and redundant major metabolic-epigenetic rheostats (RST). RST1: NAD+: NADH-SIRT: Dependent on the level of NAD+ in the cell, Sirtuins, which are histone deacetylases, can remove acetyl groups and lead to immune suppression; RST2: Succinate-αKG-αKGDD: Dependent on the levels of α–ketoglutarate and succinate (which along with fumarate, malate and Itaconate acts as inhibitors of KGDDs), leads to the activation/inhibition of a family of enzyme dioxygenases which regulate the DNA methylation levels by methylating (via DNMTs) and demethylate (via KDM, JMJD and TET) the DNA; RST3: ROS-PAC1-NuRD: guided by the activation of the ETC, which leads to electron leak and induction of ROS and activation of NuRD, which is multiprotein complex guiding DNA methylation and chromatin accessibility. The main enzymes of the TCA cycle and the ETC are shown, along with the drugs that can be used to block specific enzymes and help with reversing epigenetic mediated Immune suppression. NAD, Nicotinamide adenine dinucleotide; SIRT, Sirtuins; αKGDD, α–ketoglutarate-dependent-dioxygenases; DNMT, DNA methyl transferase; KDM, Histone demethylase; JMJD, Jumonji domain containing protein; ROS, reactive oxygen species; PAC1, Phosphatase of activated cells 1; NuRD, Nucleosome Remodeling and DNA methylation complex; TCA, Tri-carboxylic acid cycle; ETC, Electron transport chain. Created with BioRender.com.

that converts citrate to itaconate) have increased proinflammatory cytokine production (IL1β, IL-6, IFN-γ, IL12) and fatal exuberant pulmonary infiltration of innate immune cells (94, 95).

As noted earlier, most epigenetic marks depend upon metabolic precursors which, when altered, influence the epigenetic landscape. For example, S-adenosylmethionine (SAM)-mediated one-carbon metabolism supplies methyl groups for histone and DNMT. Chronic antigenic stimulation, as occurs in chronic LCMV infection, induces cellular metabolism from glycolysis and glutaminolysis, decreasing amino acid metabolic pathways feeding into the one-carbon metabolism such that reduced threonine, reduces SAM, leading to decreased H3K4me3 levels and impaired cytokine production (136, 137). SAM supplementation increased H4 arginine 31 methylation of STAT1 by Arginine methyl transferase (PRMT1), which is inhibited by HBV and is functionally essential for STAT1 function, improved antiviral effects of IFN-α in HBV infection (138) and HCV (139).

Current knowledge of the metabolic-epigenetic immune rheostat axes indicates functional roles in inhibiting acute exuberant immune pathology at the cost of long-lasting epigenetic marks and long-lasting immune suppression. To date, mechanistic studies have identified inhibitors of glycolysis, glutaminolysis and mTOR as well as transient glucose (140) restriction as possible means to block metabolic-epigenetic immune suppression. However, studies are needed to evaluate the clinical applicability of these mechanisms in severe and chronic infections.

EPIGENETIC DRUGS TO RESTORE IMMUNE RESPONSE

Epigenetic drugs have been developed predominantly for cancer therapeutics, however some, such as valproic acid and hydralazine are routinely used as antiepileptics and antihypertensives. Currently approved or in-development epigenetic modifying drugs include DNA hypomethylating agents (HMAs), HDAC inhibitors, lysine methyltransferase inhibitors (targeting EZH2, G9a, DOTL), and BET bromodomain (BRD) inhibitors. Several studies, most in cancer but a growing number in infectious diseases, have demonstrated that epigenetic drugs can reverse epigenetic-mediated immune suppression. Animal models, especially those for sepsis and chronic LCMV, have documented the mechanisms by which epigenetic drugs are able to restore host immunity.

Infection with clone 13 LCMV, the prototypical model for inducing $CD8^+$ T cell immune exhaustion induces global DNA methylation changes associated with immune exhaustion (2, 25, 141). Applying either a conditional knock of *DNMT3a* or the hypomethylating drug decitabine was able to restore $CD8^+$ T cell effector function (25). Humans with sepsis upregulate DNMT1, DNMT3a and DNMT3b, resulting in global DNA methylation differences, 82.6% of which are suppressive hypermethylated marks (142). In a cecal ligation model of murine sepsis, decitabine restored immune function and decreased mortality (142). Similarly, application of the hypomethylating drug azacytidine or decitabine to cancer cell lines increased interferon responsiveness and antigen presentation (143–145).

DNA methylation changes occur in parallel to other epigenetic modifications to inhibit host immunity. Co-immunoprecipitation studies demonstrated that DNMT associates with EZH2, the catalytic unit of the PRC (146). EZH2 methylates H3K27, inhibiting gene expression (146) and the combination of EZH2 inhibition (RNAi EZH2) and azacytidine can restore gene expression (146). EZH2 acts as an anchor point for multiple epigenetic mechanisms to suppress gene expression (**Table 1**). In ovarian cancer cell lines, inhibition of EZH2 using DZNep (inhibitor of SAM dependent enzymes), EPZ6438 and GSK126 (selective inhibitor of EZH2) and DNMT (azacytadine) synergistically increase IFN-γ responsiveness, and CXCL9 and CXCL10 expression, while shrinking tumor size (165). In melanoma, prostate cancer, hepatocellular cancer and colon cancer, EZH2-induced epigenetic marks inhibit Th1 polarization and IFN-γ-JAK-STAT signaling, with EZH2 knockdown or pharmacologic inhibition using DZNep or GSK126, restoring IFN-γ-induced gene expression (157–160). *In-vitro*, EZH2 regulates both Th1 and Th2 polarization, and inhibiting EZH2 genetically or by using EZH2 inhibitor DZNep, results in reduction of the suppressive histone mark H3K27me3, thereby augmenting both Th1 and Th2 polarization and effector cytokine production (166). In exhausted CD8 T cells, GSK126, a specific EZH2 inhibitor, restores CD8 cell cytotoxicity (164). In a CLP model of sepsis, H3K27me3, the repressive epigenetic mark induced by EZH2, persists to inhibit IL12 immunity at least 6 weeks after the initial septic insult (155). In clinical studies of sepsis, EZH2 expression increases proportional to disease severity and correlates with poor clinical outcomes (156). In TB, EZH2 is expressed early (161), with EZH2 inhibition decreasing *Ifng* and *Tnf* H3K27me2 and increasing TNF and IFN-γ production (162).

The PRC, which includes the HMT EZH2, interacts with DNMTs and also the NuRD complex. Therefore, these three suppressive epigenetic marks, H3K27 deacetylation, H3K27 methylation, and DNA methylation, often occur together, resulting in heterochromatin, thereby silencing gene expression (167). Chronic LCMV infection results in global decreased histone acetylation that limits both LCMV-specific and non-specific CD8 T cell effector function (147). Chronic LCMV infection also non-specifically decreases effector responses to influenza peptides, and decreases *salmonella* and *listeria* killing capacity (147, 168). Valproic acid, an HDAC inhibitor, was able to restore LCMV-specific and non-specific CD8 T cell effector function, including non-specific *listeria* killing capacity (147). Several studies indicate that HDAC inhibitors can restore host immunity when applied to chronic infections. Entinostat (MS-275), an inhibitor of HDAC1 and HDAC3, increases host anti-tumor immunity (152). Considering the long-term increased mortality that persists following a bout of sepsis, it would seem prudent to conduct a clinical trial to evaluate the efficacy of an

TABLE 1 | Drugs targeting epigenetic enzymes restore Immunity and Reverse Epigenetic-mediated Immune suppression.

Epigenetic post translational modification	Target enzyme/ Action	Drugs	Drug Mechanisms of Action	Evidence for Causing disease	Evidence for improving Infection outcome	Evidence for improving outcome Cancer
Histone Acetylation	Histone Deacetylases (HDAC)	Valproic acid; Sodium or phenyl butyrate; Trichostatin-A (TSA)	Reduces histone deacetylation.	Histone deacetylation limits acute immunity during chronic viral infection (147). *Mtb* suppress critical immune genes, such as IL12, by upregulating HDAC1, leading to deacetylation of histone H3 (148).	Valproic acid, restores CD8 T cell effector function and listeria killing capacity in LCMV (147). TSA and sodium butyrate restore host immunity, cytokine production restoring *Mtb* killing capacity (148). Sodium or phenyl butyrate, restores IFN-γ downstream responsiveness (149, 150), as well as inflammasome and IL1 pathway gene expression (151).	Entinostat Increases host anti-tumor immunity (152).
	(HDAC1, 3)	Entinostat (MS-275)	Entinostat, preferentially reduces histone deacetylation HDAC1 and HDAC3.			
	Sirtuins (SIRT), NAD+ dependent deacetylators of proteins, including histones	EX-527	Elective Sirt1 inhibitor prevents histone deacetylation	LPS induced immune tolerance characterized by deacetylation and silencing of TNF, IL1β and NFκB by Sirt in a NAD dependent manner (20, 102). In T cells, *Mtb*-induced upregulation of SIRT2 deacetylates NFκB to suppress immunity and *Mtb* killing capacity in mouse models (153). Increased SIRT1 confers chemoresistance (154).	When used in the acute phase of sepsis, it increases morbidity. When used in the immune hyporesponsive phase of sepsis, it is able to reduce post-sepsis mortality (112). SIRT2 inhibition improves both myeloid and lymphoid immunity and *Mtb* killing capacity in mouse models (153).	EX-527, increases chemosensitivity in cancer (154).
DNA Methylation	DNA methyl Transferase (DNMT)3A	Decitabine; Azacytidine	Demethylation/ Hypomethylation	Upregulated *DNMT1*, *DNMT3a* and *DNMT3b*, resulting in global DNA hypermethylation methylation in sepsis (142).	Knock out *DNMT3a*, or Decabitine Restore CD8 + T cell effector function (25). Decabitine restore immune function and decreases mortality in sepsis (142).	Azacytidine, increases interferon responsiveness and antigen presentation in cancer (143–145).
Histone Methylation	EZH2/ increases the methylation	3-deazaneplanocin (DZNeP).	DZNep (Inhibitor of SAM dependent enzymes), decreases methylation	H3K27me3, the repressive epigenetic mark induced by EZH2, persists to inhibit IL12 immunity at least 6 weeks after the initial septic insult (155). EZH2 expression increases proportional to disease severity and correlates with poor clinical outcomes in sepsis (156). In multiple cancers EZH2 induced epigenetic mark inhibit IFN-γ-JAK-STAT signaling (157–160). In TB, EZH2 is expressed early (161), with EZH2 inhibition decreasing *Ifng* and *Tnf* H3K27me2 and increasing TNF and IFN-γ production (162).	Inhibition of EZH2 with DZNep improved acute septic morbidity and mortality, lessen cytokine levels and bacterial burden in mice (163). EZH2 inhibition in TB decreases *Ifng* and *Tnf* H3K27me2 resulting in increased TNF and IFN-γ production (162).	GSK126, restores CD8 cell cytotoxicity (164). Combination of EZH2 and DNMT inhibitors synergistically increased IFN-γ responsiveness and CXCL9 and CXCL10 expression and shrinks tumor size during immunotherapy in ovarian cancer cell lines (165). EZH2 knockdown or pharmacologic inhibition restoring IFN-γ-induced gene expression in various cancers (157–160).
		GSK126	GSK126 (Selective inhibitor blocking EZH2), decrease methylation			

HDAC inhibitor in reversing long-term sepsis-induced immune suppression (9).

Sirtuins, a class of HDACs, recognize the NAD^+: NADH ratio and then deacetylate and silence NFκB, TNF and IL1b after LPS-induced immune tolerance (20, 102). EX-527, a Sirt1 inhibitor restored myeloid cell IL1B and TNF production when administered after sepsis. Reinforcing the importance of timing, administering EX-527 early during sepsis increases mortality, however if given later during the immune hyporesponsive phase of sepsis, it reduces post-sepsis mortality in mice (112).

TB is the archetypical chronic infection. Macrophages infected with *Mtb* upregulate HDAC and undergo deacetylation of critical immune genes, such as IL12. Inhibition of HDAC restores immune function including cytokine production and *Mtb* killing capacity (148). In T cells, *Mtb*-induced upregulation of SIRT2 deacetylates NFκB (p65) with SIRT2 inhibition improving both myeloid and lymphoid immunity, and *Mtb* killing capacity in mouse models (153). Sodium or phenyl butyrate, an HDAC inhibitor, restores IFN-γ downstream responsiveness (149, 150) as well as inflammasome and IL1 pathway gene expression (151). In a clinical trial that did not evaluate epigenetic or immunologic outcomes, the combination of Vitamin D_3 and phenylbutyrate did not change time to sputum conversion but did ameliorate TB disease severity (151). Like sepsis, survivors of TB retain detrimental epigenetic scars (6, 22) and have increased all-cause mortality (12, 13). Large clinical trials should evaluate if reversing these detrimental epigenetic marks are able to reverse the post-infectious morbidity and mortality risk due to TB.

BIOENGINEERING APPROACHES TO REVERSE EPIGENETIC-MEDIATED IMMUNE EXHAUSTION & SUPPRESSION

Systemic means to reverse immune suppression, such as immune checkpoint inhibitor blockade (e.g., anti-PD-1 and anti-LAG-3), have short and long-term toxicities (169). Newer technology such as the CRISPR/Cas9 system holds promise as a precise and controlled bioengineering tool (170–175) to reverse immune suppression. Typically, the CRISPR/Cas9 system includes a guide RNA (gRNA) complexed with the Cas9 protein to specifically edit a unique genomic address (170). For example, *in vitro* gene editing of the *PD-1* and *LAG-3* genes using CRISPR-Cas9 in CAR-T cells has improved their anti-tumor function (169, 176).

A catalytically inactive version of the Cas9 protein called dead, or deactivated, Cas9 (dCas9) (177) repurposes the CRISPR-Cas9 platform for precision edited of the epigenome or gene expression machinery (173–175, 177). A diverse spectrum of epigenetic effectors has been tethered to dCas9 to deliver epigenetic payloads to specific sites across the genome, giving rise to a continually expanding epigenome editing toolkit (173, 178). The Krüppel-associated box (KRAB) is a repressive domain that is a component of several zinc-finger transcription factors (179). A fusion protein between the KRAB domain and dCas9 (dCas9-KRAB) has been shown to promote highly specific gene silencing when targeted to mammalian genes (180) and to distal regulatory elements such as enhancers (181). A version of dCas9-KRAB with a linker for activation of T cells (LAT-dCas9-KRAB) was recently shown to silence the *PD-1* gene when targeted to its transcription start site (176).

Targeting the transcriptional start sites and promoters with dCas9 coupled with the *de novo* methyltransferases DNMT3A and its homolog DNMT3L (dCas9-DNMT3A/3L) has been described to produce widespread DNA methylation of CpG islands at the targeted loci for up to 1200 bp (182). In addition, tethering the catalytic domain of the DNA demethylase TET1 to dCas9 (dCas9-TET1) to promoters previously silenced by engineered transcriptional repressors can generate a stable, long-term reactivation of the silenced gene by demethylation of targeted CpG islands (183). Previous work has also shown that a fusion protein consisting of the catalytic core of the human acetyltransferase p300 and dCas9 (dCas9-p300) can achieve robust genetic transcriptional activation by targeting either promoters, proximal enhancers or distal enhancers (184).

The epigenome editing tools dCas9-TET1 and dCas9-p300 were recently employed to elucidate the epigenetic landscape of the *Foxp3* locus, an important transcription factor in T cells. Demethylation of the enhancer region of the *Foxp3* locus was achieved in mouse primary T cells, although without strong *Foxp3* gene expression. In contrast, targeting dCas9-p300 to the *Foxp3* promoter stabilized Foxp3 expression under both normal and inflammatory culture conditions *in vitro* (185). This technical approach provides new opportunities to revert anomalous post-infectious epigenetic modifications in other immunologically relevant genes using dCas9-based epigenome editing.

Robust targeted transcriptional activation has also been achieved by using CRISPR activation (CRISPRa) tools. dCas9 fused to an engineered tripartie activation domain consisting of VP64, p65 and Rta, (dCas9-VPR) has proven to be a potent synthetic CRISPR/Cas9-based transcriptional activator. For example, dCas9-VPR is able to induce gene activation of some target genes up to 320-fold compared to the original, conventional dCas9-VP64 activator (186).

Another method to increase transcriptional activation is by recruiting several copies of the regulatory proteins at once to the target gene. This can be achieved by fusing dCas9 to the SunTag, an array of a repeated short peptide sequence with strong affinity for a single-chain variable fragment (scFv) antibody fused to the activation domain. The SunTag can recruit up to 24 copies of the antibody-fused protein and has been used to recruit multiple copies of the transcriptional activation domain VP64, increasing gene expression of the targeted locus (187).

RNA aptamers that interact with transcriptional activation domains have been inserted into gRNAs, and these systems have been used to recruit transcriptional regulatory domains *via* dCas9 (188, 189). For example, the synergistic activation mediator consists of an MS2 bacteriophage coat protein-binding aptamer that is placed in the gRNA loops, which enables a fusion between MS2 p65 and Heat Shock Factor 1

(HSF1) to be successfully recruited to targeted genomic loci (188). Recently the SAM system was used to increase the expression of key endogenous genes related to immunological exhaustion in the context of boosting anticancer immunotherapy. Multiplexed gene activation of *Cd70*, *Cd80*, *Cd86*, *Ifnα4*, *Ifnβ1*, and *Ifnγ* was achieved in mice using a CRISPRa gRNA library improving immunogenicity of the transduced cells and leading to tumor rejection *in vivo* (190).

Finally, Proteolysis Targeting Chimeras (PROTACs) are small molecules which induce the targeted degradation of a protein by linking it to an E3 ubiquitin ligase. The ubiquitinated protein is then recognized and degraded by the 26S proteasome (191). Recently, Si et al. demonstrated that a hematopoietic progenitor kinase has a key role in T cell exhaustion and could be targeted by employing PROTACs and CRISPR/Cas9 technology. Increased gene expression of the *MAP4K1* gene has been correlated with increased T cell exhaustion due to dysregulation of the NFκB signaling pathway. Knocking out this gene using a CRISPR/Cas9 system in CAR-T cells improved their persistence and functionality *in vivo*. Similarly, developing a small molecule PROTAC that selectively degrades the HPK1 protein encoded by the *MAP4K1* gene in CAR-T cells improves their efficacy as well (192).

A major challenge in reducing T cell exhaustion is the enduring epigenetic changes that differ from their normal state (25). The CRISPR/dCas9-based protein fusions to epigenetic writers and erasers are a potential tool to robustly and precisely modulate the epigenome of exhausted T cells, reverting them to their pre-infected functional state.

As previously discussed, T cell function requires balanced AP-1 and NFAT heterodimerization. CAR T cells experience tonic activation that induces characteristic features of exhaustion (193). By manipulating HA-28z CAR T cells to over-express c-Jun, AP-1-NFAT balance was restored, increasing IL-2 production (193). Recent studies have shown that the HDAC SIRT1 functions to deacetylate c-Jun, inactivating it, and thus effectively preventing the formation of the NFAT/AP-1 complexes required to induce *Il-2* expression in activated T cells. In this way SIRT1 acts as an epigenetic promoter of immune exhaustion (18, 194). In a follow-up CAR T study, the incorporation of a titratable FK506 binding protein 12 (FKBP) destabilizing domain (DD) emphasized the importance of timing and rest (195). Simply put, this engineered CAR T cell model demonstrated that interrupting tonic T cell activation, either through the titratable FK506 DD or through dasatinib, a tyrosine kinase inhibitor, could block epigenetic-mediated immune exhaustion.

CONCLUSION

Increasing evidence from *in-vitro* studies and animal models has demonstrated the signaling pathways, TFs, metabolic intermediates and epigenetic enzymes that remodel chromatin in order to suppress gene expression and limit exuberant immune pathology. Although acutely, this suppression helps regulate an overly exuberant immune response, it makes individuals more susceptible to secondary infections and cancers leading to increased long-term morbidity and mortality. Other fields have harnessed drugs to manipulate epigenetic enzymes, metabolic pathways, TFs and signaling pathways to improve clinical outcomes. Similar studies need to evaluate which strategy limits off-target adverse effects in order to restore host immunity. For example, theoretically, upstream moderation of the three-metabolic-epigenetic-immune rheostats might better block detrimental epigenetic marks than a specific epigenetic modifying drug. Considering the significant long-term mortality that exists after pneumonia, sepsis and TB, translational studies using emerging immunologic approaches and bioengineering tools are needed to evaluate if modulating these pathways improve clinical outcomes.

AUTHOR CONTRIBUTIONS

All authors made a contribution to the acquisition of the information for the work, critically revised the manuscript for important intellectual content, and gave final approval of the current version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REVIEW
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The Role of Host Cell DNA Methylation in the Immune Response to Bacterial Infection

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Host cells undergo complex transcriptional reprogramming upon infection. Epigenetic changes play a key role in the immune response to bacteria, among which DNA modifications that include methylation have received much attention in recent years. The extent of DNA methylation is well known to regulate gene expression. Whilst historically DNA methylation was considered to be a stable epigenetic modification, accumulating evidence indicates that DNA methylation patterns can be altered rapidly upon exposure of cells to changing environments and pathogens. Furthermore, the action of proteins regulating DNA methylation, particularly DNA methyltransferases and ten-eleven translocation methylcytosine dioxygenases, may be modulated, at least in part, by bacteria. This review discusses the principles of DNA methylation, and recent insights about the regulation of host DNA methylation during bacterial infection.

Keywords: DNA methylation, immune response, bacteria, infection, mechanism, review

INTRODUCTION

DNA methylation refers to the addition of a methyl group to the DNA cytosine residues at the fifth carbon position (5mC), which is a common epigenetic mark in many eukaryotes and often found in the sequence context CpG (i.e., regions in the DNA where a cytosine nucleotide is followed by a guanine nucleotide along the 5' to 3' direction) (1). The methylation process is promoted by the DNA methyltransferases (DNMTs), of which DNMT3A and DNMT3B mediate *de novo* DNA methylation, establishing a pattern of methylation that is then sustained by the maintenance methyltransferase, DNMT1 (2). DNMT2 is not involved in DNA methylation, but rather mediates methylation of RNA (3), and therefore is further not discussed in this review. The process of DNA methylation can be reversed passively through cell division or actively catalyzed by ten-eleven translocation (TET) methylcytosine dioxygenases family proteins, and a subsequent nucleotide excision and repair process, called DNA demethylation (4). There are three members in the TET family, namely TET1, TET2 and TET3, all sharing a conserved catalytic domain in their C terminus (5). DNA methylation is generally associated with transcriptional silencing, although this paradigm has been challenged by recent studies showing that DNA methylation can both positively and negatively regulate gene expression depending on the position where it occurred (6).

Both innate and adaptive immune responses contribute to protection of the host against bacterial pathogens (7). The innate immune system functions as the first line of defense against invading pathogens and is composed of innate immune cells (including basophils, dendritic cells, eosinophils, Langerhans cells, mast cells, monocytes, macrophages, neutrophils and natural killer cells) and some stromal cells, such as epithelial cells that sense bacteria by their surface or endosomal pathogen recognition receptors (PRRs). Toll-like receptors (TLRs), RIG-I-like receptors, NOD-like receptors and C-type lectin receptors are among the large array of PPRs that are able to detect pathogens by recognizing microbial components known as pathogen-associated molecular patterns, among which lipopolysaccharide (LPS), flagellin and lipoteichoic acid (8, 9). Upon recognition of bacteria or bacterial components, innate immune cells initiate intracellular signaling cascades to induce functional changes and to elicit the production of immune effectors, such as cytokines, chemokines and antimicrobial peptides, that directly or indirectly contribute to host antibacterial defense and inflammatory responses. When bacterial pathogens evade host innate immunity, adaptive immune responses can contribute to defense mechanisms. T and B cells are dominant players in adaptive immunity, activated through presentation of bacterial antigens by antigen-presenting cells. Innate and adaptive immune responses do not act independently, but coordinated actions of these two systems are required for efficient elimination of bacterial invaders. Furthermore, in order to prevent collateral damage both innate and adaptive immune responses need to be tightly regulated at different levels (10). Modification of DNA methylation in host cells, induced by infectious agents, has been implicated in the induction and regulation of the immune response to bacteria.

DNA methylation has been considered to be relatively stable when compared with other epigenetic modifications, such as those involving histones, but recent findings have documented that DNA methylation can occur faster than previously thought, particularly when cells are exposed to changing environments, including contact with pathogens during infection (11). Importantly, accumulating evidence indicates that pathogens can alter DNA methylation and/or regulate the expression and function of DNA methylation modifiers such as TETs and DNMTs, resulting in altered expression of important host genes involved in immune responses (11). These alterations in DNA methylation or its related factors can either contribute to protective host immunity to eliminate pathogens or benefit pathogens to evade immune responses for persistence within the host. This review summarizes current understanding of the effects of DNA methylation on host immune responses and pathogen elimination during infection.

DNA METHYLATION

Two families of proteins directly contribute to the DNA methylation pathway: the DNMTs promote and maintain DNA methylation, while the TETs catalyze demethylation *via* multiple steps (**Figure 1**). DNA methylation is established by the *de novo* methyltransferases DNMT3A and DNMT3A with the help of catalytically inactive DNMT3L in mammals, whilst the maintenance of DNA methylation is mediated by DNMT1 and its obligate partner ubiquitin-like plant homeodomain and RING finger domain 1 (UHRF1), which preferentially recognizes hemimethylated CpGs during cell division (12).

Although DNA methylation is reported to be stable, DNA demethylation has been widely observed during development and activation of mammalian cells. Possible mechanisms underlying DNA demethylation have been reviewed by other researchers (13–16); we here only briefly introduce the broadly recognized passive and active routes. Passive demethylation occurs in the absence of the DNA methylation maintenance

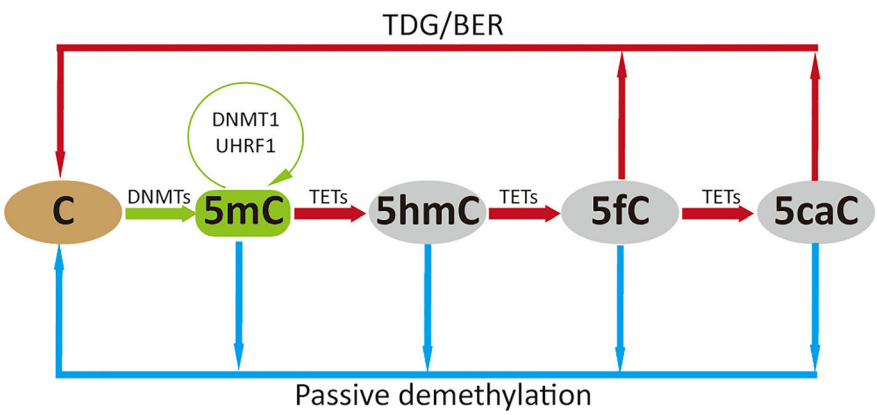

FIGURE 1 | DNA methylation cycle. DNMTs catalyze the addition of a methyl group to the fifth carbon position of cytosine to generate methylated cytosine (5mC), which is maintained by DNMT1 (green arrow); 5mC is oxidized to 5-hydroxymethylcytosine (5hmC), which can be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by TETs. The higher oxidized cytosine bases 5fC and 5caC can then be converted back to their unmodified state directly by thymine DNA glycosylase (TDG) and subsequently base excision repair (BER) processing; these oxidative steps contribute to active demethylation (red arrow). Passive demethylation removes 5mC from all forms of methylcytosine due to absence or reduction in DNMT levels and function (blue arrow).

machinery (DNMT1/UHRF1) during DNA replication, which leads to dilution of 5mC, or removal of 5mC due to absence or reduction in DNMT levels and function (17). Active demethylation is mostly dependent on the oxidation of 5mC by TETs, that oxidize 5mC to 5-hydroxymethylcytosine (5hmC), which can be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). These oxidized cytosine bases (5hmC/5fC/5caC) may facilitate DNA demethylation by impairing the binding and/or activity of enzymes regulating the maintenance methylation machinery (DNMT1/UHRF1) which impairs remethylation during DNA replication (13). The higher oxidized cytosine bases (5fC/5caC) can be efficiently excised by thymine DNA glycosylase (TDG), followed by the base-excision-repair (BER) pathway, which accounts for the major DNA demethylation mechanism. Interestingly, TETs might not decrease methylation levels, but specifically prevent aberrant methylation spreading into CpG islands (CGIs) (18), and DNMTs might also contribute to active DNA demethylation in conditions of low methyl group sources (19).

REGULATION OF DNMTs

DNMT proteins are recruited to certain locations in the genome where they catalyze the transfer of methyl groups from S-adenosyl-L-methionine (SAM) to the C5 of cytosine to establish 5mC. During this process, the activity of DNMTs can be regulated at the following levels (**Figure 2**).

First, by the Abundance of DNMTs

The expression and stability of DNMTs can be regulated by transcriptional regulation and post-translational modifications (PTMs), respectively. Numerous pathways have been shown to induce or inhibit expression of DNMTs, and the extent of their expression can be further regulated by multiple epigenetic regulatory mechanisms (20). Proteolytic degradation of DNMT proteins can be promoted or inhibited by PTMs. Acetylation and ubiquitination of DNMT1 either protect from or promote proteolytic degradation (21, 22). Phosphorylation of Ser143 stabilizes DNMT1 (23), whilst methylation of Lys142 and Lys1096 promotes its proteolytic degradation (24, 25).

Second, Through the Function/Activity of DNMTs

DNA methylation by DNMTs is dependent on their catalytic activity, which is largely regulated by PTMs or isoform variation of DNMTs. SUMOylation of DNMT1 increases the catalytic activity of this enzyme on genomic DNA (26); SUMOylation of DNMT3A, however, abolishes its capacity to interact with histone deacetylases (HDACs) (27). DNMT1 is an auto-inhibitory protein that is activated upon binding to unmethylated cytosines (28, 29). The same auto-inhibitory characteristic was also found for DNMT3A, the activation of which is induced by histone H3 (30); this is might be the reason why the histone H3 N-terminal tail with an unmethylated Lys4 (H3K4) is required for *de novo* DNA methylation (31). In addition, the activity of DNMTs can be affected by isoform variation (32, 33), and other regulatory proteins, such as the microprocessor component DROSHA that interacts with DNMT1 to ensure its full methyltransferase activity (34).

Third, Through Recruitment of DNMTs to the Genome

To successfully perform DNA methylation, DNMTs are first recruited to the targeted DNA motif, and this recruitment is affected by both the features of the target DNA motif and factors that influence DNMT recruitment to the genome. DNMTs can be specifically recruited to DNA marked with unmethylated H3K4 *via* interacting with the ADD domain of DNMTs (35), while methylated H3K4 repulses the binding of *de novo*

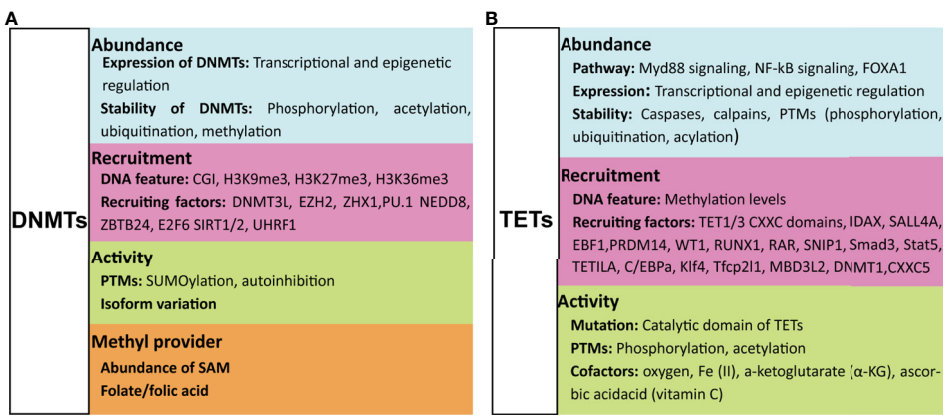

FIGURE 2 | Factors that regulate the function of DNMTs and TETs. The function of DNMTs can be influenced at four levels: their abundance, their recruitment to DNA, their catalytic activity, and the methyl group source **(A)**. The function of TETs is regulated at three levels: their abundance, their recruitment to DNA and their catalytic activity **(B)**. For details see text. DNMTs, DNA methyltransferases; TETs, ten-eleven translocation methylcytosine dioxygenases; PTMs, post-translational modifications; CGI, CpG islands; SAM, S-adenosyl-L-methionine.

methyltransferases resulting in maintaining the hypomethylated state of CGIs (36). CGIs marked by H3K27me3 are more susceptible to *de novo* DNA methylation during differentiation and in disease states such as cancer (37, 38). Gene body enriched with H3K9me3 or H3K36 tri-methylation (H3K36me3) is also reported to be favorable for DNMT3B recruitment, leading to hypermethylation at these regions that functionally relate to gene transcription initiation, proper splicing and compact chromatin at active genes (37, 39, 40). The affinity of DNMT3A and DNMT3B for DNA can be further enhanced by DNMT3L through the formation of heterotetrametric complexes with either DNMT3A or DNMT3B, resulting in more efficient DNA methylation (41, 42). A large class of proteins, including polycomb group protein enhancer of zeste homolog 2 (EZH2) (43), Zinc-fingers and homeoboxes 1 (ZHX1) (44), ubiquitin-like protein modifier NEDD8 (45), zinc-finger protein ZBTB24, transcription factor E2F6 and PU.1, and Sirtuins 1 and 2 (SIRT1/2), were reported to recruit DNMTs to genes targeted for DNA methylation mediated gene silencing (46–49). The binding of DNMT1 to hemimethylated cytosines is selectively promoted by UHRF1 (50), but this binding is prevented by a DNA aptamer named Apt. #9 that competes with the hemiDNA for binding to DNMT1 (51). Besides protein molecules discussed above, some RNAs were also reported to affect the recruitment of DNMTs (52–54).

Fourth, the Methyl Group Donors Determine the Direction of the DNA Methylation Pathway

SAM is the major source of methyl groups for DNA methylation. The addition of folate/folic acid to provide methyl groups was reported to maintain DNA methylation and/or prevent the loss of global DNA methylation in health and disease (55, 56). However, factors that lead to less SAM decreases the transfer of methyl groups to DNA and RNA (57). In the absence of SAM, DNMT3a and DNMT3b can exhibit DNA dehydryoxymethylase activity, by directly converting 5hmC and 5caC, but not 5fC, to unmodified cytosines (58, 59). In some cases, DNMT1 is able to mediate oxidation of cytosine with formaldehyde, forming 5hmC (60), which further can participate in the DNA methylation cycle.

REGULATION OF TETs

The presence and catalytic activity of TETs are necessary for DNA demethylation, but their function is affected by multiple regulatory mechanisms that (amongst others) modulate substrate accessibility, enzymatic activity, expression levels and genomic targeting of TETs. Factors that are of importance for the regulation of activity of TETs are the following.

First, the Abundance of TETs Can Be Regulated at Transcriptional and Post-Transcriptional Levels

The expression of TETs can be induced by multiple signaling pathways, such as hydrogen sulfide (61), Myd88 signaling (62), NF-kB signaling (63) and Forkhead box A1 (FOXA1) (64), and frequently regulated at transcriptional level. IDAX (also known as CXXC4) and lysine demethylase KDM2A (65) negatively regulate whilst transcription factors Oct4 and CEBPα positively regulate TET2 protein expression (66–68). TET3 can be negatively regulated by nuclear receptor TLX (69). More recently, TETs were shown to be regulated by epigenetic modifications involving long non-coding RNA's or microRNA's (70–73). The abundance of TETs can also be regulated at protein level. TETs can be directly cleaved by caspases (68) and calpains (74) or degraded through PTMs. For instance, all three TET proteins can be monoubiquitinated by the VprBP-DDB1-CUL4-ROC1 E3 ubiquitin ligase (CRL4VprBP) (75), whilst MAPK-mediated phosphorylation at Serine-99 of TET2 stabilizes this enzyme (76, 77). Moreover, the 14-3-3 proteins bind phosphorylated TET2 and protect Serine-99 phosphorylation (78). Other modifications like (de) acetylation of TETs have also been reported; for example, acetylation of TET2 by p300 stabilizes this enzyme by inhibiting ubiquitination (79), whilst deacetylation of TET2 by the deacetylase SIRT1 promotes its ubiquitination degradation as well as enhances its catalytic activity (80, 81).

Second, the Binding of TETs to Genomic DNA Sequences Can Be Modulated

Similar to DNMTs, TET proteins also need to be recruited to the genome for implementing their functions. TET1 and TET3 can be recruited to genomic target sites through direct binding of their respective CXXC domains to DNA (82). This binding process can be influenced by several proteins. For instance, Lin28A recruits TET1 to common genomic loci to regulate DNA methylation and gene expression (83), thyroid hormone receptors stabilize the association of TET3 to chromatin depending on the catalytic activity of TET3 (84). In contrast to TET1 and TET3, TET2 is recruited to genomic DNA by a distinct CXXC domain-independent mechanism since TET2 does not have any discernable domains that bind directly to DNA. Indeed, numerous proteins have been discovered that promote or inhibit binding of TET2 to DNA. IDAX/CXXC4, originally encoded within an ancestral *TET2* gene but separated from *TET2* during evolution, recruits TET2 to DNA sequences containing unmethylated CpG dinucleotides located at promoters and CGIs in genomic DNA (68, 85). Other molecules such as Wilms tumor protein 1 (WT1) (86), early B-cell factor 1 (EBF1) (87), PRDM14 (88), RUNX1 (89), retinoic acid receptor (RAR) (90), SNIP1 (91), Smad3 and Stat5 (61), TET2 interacting long noncoding RNA (TETILA) (92) and transcription factors C/EBPa, Klf4, and Tfcp2l1 (93) can interact with TETs and enhance the recruitment of TETs to target loci. In addition, some proteins like Methyl-CpG binding domain protein 3-like 2 (MBD3L2) (94), DNMT1 (79), CXXC5 (95) and SALL4A (96) can further strengthen or stabilize the binding between TETs and methylated DNA targets. Besides factors modifying the recruitment of TETs, the character of target DNA sequences can also affect the binding of TETs. For example, low-methylated regions (LMRs) of CpG-poor distal regulatory regions that are occupied with DNA-binding factors are favorable for TET binding, thereby maintaining low methylation levels in these regions (97).

Third, Dioxygenase Activity of TETs Is Tightly Regulated

The dioxygenase activity of TETs is largely dependent on their catalytic domain and any mutation or modification within this region is likely to lead to a change in their function. Enzymatic reactions mediated by TETs highly rely on the cofactors oxygen, Fe (II), and a-ketoglutarate (α-KG) (98). Therefore, any modification in the production or activity of these cofactors is expected to lead to a functional change of TETs. Mutations in the genes encoding the metabolic enzymes isocitrate dehydrogenases 1 and 2 (IDH1/2), succinate dehydrogenase, and fumarate hydratase, result in aberrant accumulation of metabolites such as 2-hydroxyglutarate (2-HG), succinate and fumarate, respectively, which act as competitors of α-KG to broadly inhibit the α-KG-dependent enzymatic activity of TETs (99–101). Hypoxia, such as frequently occurs in tumor tissues, leads to loss of TET activity (102). On the other hand, addition of ascorbic acid (vitamin C), which is needed to reduce the oxidized iron species, enhances the catalytic activity of TETs (103–105). Additionally, TETs activity has also suggested to be affected by PTMs. Acetylation enhances TET2 function (79) and phosphorylation of TET3 at the highly conserved Serine-1310 and -1379 residues within its catalytic domain by cyclin-dependent kinase 5 (cdk5) is required for its dioxygenase activity (106). Moreover, the phosphorylation of TETs can be suppressed *via* O-GlcNAcylation by the glycosyltransferase OGT (107).

DNA METHYLATION AND GENE EXPRESSION

DNA Methylation, DNA Demethylation and Gene Expression

DNA methylation plays a critical role in the regulation of many cellular processes, including X chromosome inactivation, genomic imprinting, stem cell differentiation, chromosomal conformation, chromatin structure, developmental stages and transcriptional activation/repression of genes (108). DNA methylation in the genome is not uniformly distributed: both promoter and CGIs typically are hypomethylated, whereas the extent of methylation in gene bodies is higher than that in intergenic regions (2). While early studies suggested that DNA methylation represses gene expression, a growing body of evidence has indicated that DNA methylation has a dual role, both inhibitory and permissive, depending on the genomic region at which DNA methylation occurs (2). DNA methylation of CpGs at promoters and enhancers that usually remain unmethylated is mainly coupled with transcriptional silencing (108, 109), but DNA methylation at the gene body has been associated with enhanced gene transcription or elongation (39, 110). DNA methylation can also indirectly regulate gene expression by altering the chromatin accessibility for transcription factors or by recruiting repressive proteins with methyl-binding domains (111). For instance, DNA methylation changes the accessibility of B cell enhancers for transcription factors E2A and PU.1 and blocks the binding of transcription factor erythroblastosis 1 (ETS1) at Ets binding site during B cells development (112, 113). In addition, DNA methylation closely cooperates with other regulatory machineries to modify gene expression, especially with histone modifications, which can partially be mediated through methylcytosine-binding proteins, such as MECP2 or MBD2, that are capable of recruiting histone deacetylases or transcriptional repressors to methylated regions (111, 114). DNA demethylation, on the other hand, is normally positively correlated with gene transcription (13). However, the precise relationship between DNA (de)methylation and gene expression is complex and requires further investigation. For instance, it is reported that microbe-induced changes in the expression of some genes can occur prior to modification of DNA methylation at their sites (11, 115) and that elevated DNA methylation outside of gene promoters has been shown to facilitate gene transcription to a larger extent than promoter DNA methylation (116, 117).

DNMT Related Gene Expression

DNMTs can repress gene expression by increasing DNA methylation at promoters and enhancers, resulting in reduced binding of transcriptional factors to these positions or inducing changes in the chromatin structure to make it less accessible for transcription (2, 111). For instance, DNMT3B mediated DNA methylation at the promoter regions of NF-κB responsive genes decreases NF-κB recruitment to the promoters, suppressing the expression of downstream genes (33). H3k6me3 selectively recruits DNMT3B to gene bodies of actively transcribed genes, thereby promoting DNA methylation and gene expression (37, 39, 110, 118). DNMTs can regulate gene expression not only *via* directly modifying DNA methylation, but also through mechanisms that are unrelated to DNA methylation but achieved by cooperating with other regulatory machineries. All three DNMTs (DNMT1, 3A and 3B) have been reported to repress gene transcription through interacting with HDACs independent of their catalytic activity (27, 119). DNMT3A-mediated DNA methylation increases HDAC9 transcription by repressing the inhibitory histone mark H3K27me3 at its distal promoter (116). DNMTs work together with polycomb group proteins for repression of their common target loci (43). The tricarboxylic acid cycle metabolites succinate and fumarate determine the catalytic activity of DNMTs; in turn, DNMT3B has been reported to modulate mitochondrial metabolism for maintaining articular cartilage homeostasis (120).

TET Related Gene Expression

TETs regulate gene expression directly by demethylation, dependent on their catalytic activity, or indirectly through interaction with other regulatory mechanisms, mostly independent of their catalytic activity. All three TETs contribute to dynamic demethylation during development, activation and oncologic transformation, linked with wide transcription reprogramming in cells during these processes (5, 121). In recent years, more and more DNA methylation independent functions of TETs have been discovered, indicating that TETs closely work together with other epigenetic regulatory mechanisms in the setting of infection. TET2 and TET3 have been shown to inhibit proinflammatory cytokine expression by recruiting HDAC1/2 to

the promoters of cytokine encoding genes during bacterial and viral infection, respectively (122–124). TET2 also mediated transcriptional repression by facilitating the recruitment of the polycomb Repressive Complex 2 to CpG dinucleotide-rich gene promoters (125). TET1 can be incorporated in the SIN3A co-repressor complex, resulting in transcriptional effects independent of 5hmC (126), and this might be the underlying mechanisms of TET1 mediated inhibition of *IL1B* transcription (127). The same mechanism applies to TET3 regulated inhibition of type I interferon production during viral infection or poly(I:C) stimulation (124). TET2 and TET3 facilitate OGT-dependent histone O-GlcNAcylation by interacting with the enzyme O-linked b-N-acetylglucosamine (O-GlcNAc) transferase (OGT) (128, 129). Beyond oxidation of methylated cytosine in DNA, TET2 has also been reported to promote mRNA oxidation during infection derived sepsis, thereby destabilizing target mRNA (130); TET2 can suppress expression of endogenous retroviruses through a similar mechanism (131).

MODIFICATION OF DNA METHYLATION ASSOCIATED WITH INFECTION

The host response to an infection involves transcriptional changes in different types of immune cells, which can affect their function to either promote host defense against invading pathogens or benefit pathogen persistence. The transcriptional reprogramming during infection is highly regulated and epigenetic regulatory mechanisms are involved herein (132, 133) (**Figure 3**). Until recently, the extent of DNA methylation was thought to be stable and resistant to environmental stimulation. However, it is now well recognized that DNA methylation can be altered in a brief time frame in response to inflammation or infection and that these modifications in DNA methylation can influence immune cell responsiveness (11). Two possible mechanisms underlie infection induced alterations in DNA methylation: infection can directly alter DNA methylation by inducing or repressing DNA methylation enzymes (DNMTs and TETs), and/or indirectly through inflammatory mediators induced by the infection (134). Modification of host DNA methylation associated with bacterial infection and the consequent effects on immune responses were summarized in **Table 1** and detailed below.

Gut Microbiota and Intestinal Pathogens

Commensal bacteria contribute to the maintenance of intestinal symbiosis by shaping host gene expression *via* epigenetic modification (187). Gut microbiota-dependent and -independent processes act together to form the postnatal development of the transcriptome and DNA methylation

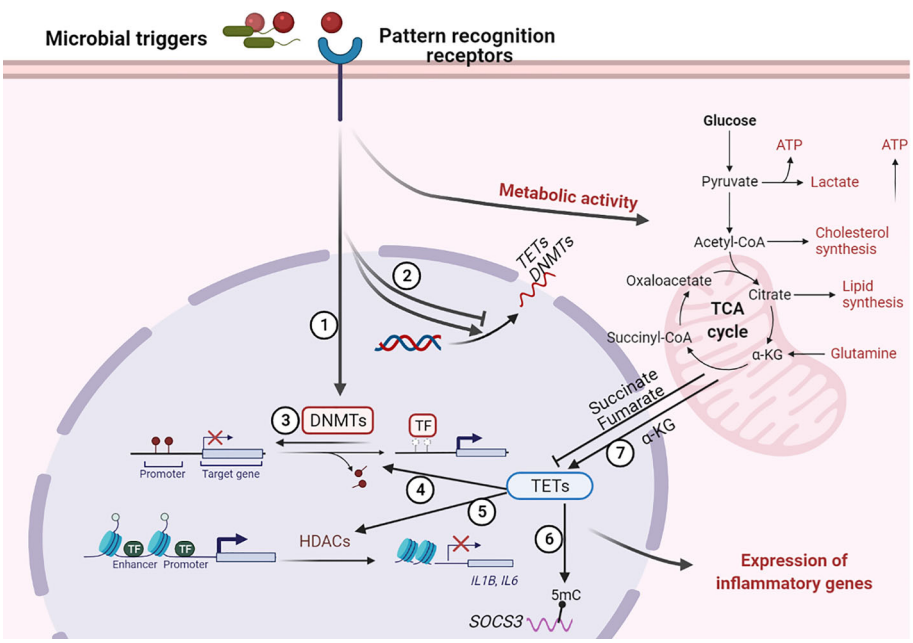

FIGURE 3 | Regulation of host DNA methylation of immune responses during infection. Figure representing a general overview of how infection can affect DNA methylation. Note: not all infection modify DNA methylation; an overview of changes induced by specific pathogens is provided in the table. ① Infection induces DNA (de)methylation at target genes; ② Infection alters the transcription of DNA methylation modifiers TETs and DNMTs; ③ Loss of DNMTs promotes infection induced DNA demethylation at target genes; ④ TET proteins promote infection induced DNA demethylation at target genes; ⑤ TET proteins recruit HDACs for histone modification at *IL1B* and *IL6* promoters; ⑥ TET proteins oxidize 5-methylcytosine (5-mC) on SOCS3 messenger RNA (mRNA); ⑦ Infection alter metabolic products that regulate the activity of TET proteins. "arrow" symbol represents promotion, "bar-headed arrow" symbol represents inhibition. DNMTs, DNA methyltransferases; TETs, ten-eleven translocation methylcytosine dioxygenases; HDACs, Histone deacetylases; TFs, transcription factors; IL, interleukin; SOCS3, Suppressor of cytokine signaling 3; ATP, Adenosine triphosphate; α-KG, a-ketoglutarate.

TABLE 1 | Modification of DNA methylation induced by bacteria and its effects on immune responses.

Bacteria	Effect on DNA methylation	Impact on immune response	References
Gut microbiota	Altered DNA methylation in IECs	Changed expression of genes related to immunity and metabolism in IECs	(135–137)
	Hypermethylation of *TLR4* in IECs	Suppressed response to LPS and commensal microbiota, maintaining intestinal homeostasis	(138, 139)
	Demethylation in IECs mediated by TET2/TET3	Maintained intestinal homeostasis and inhibition of acute inflammation in experimental colitis	(137, 140)
Polymicrobial	Altered DNA methylation in whole blood leukocytes	Changed gene expression in whole blood leukocytes of septic patients	(141, 142)
	Altered DNA methylation in monocytes	Increased IL-10 and IL-6 levels and organ dysfunction in septic patients	(143)
	Altered expression of DNMTs and TETs	Increased disease severity in septic patients or experimental septic mice	(130, 144, 145)
Helicobacter pylori	Aberrant DNA methylation in gastric mucosae caused by infection induced inflammation	Increased risk of gastric cancer	(134, 146–149)
	Aberrant DNMT activity in gastric tissues	Increased susceptibility to infection	(55, 150)
Mycobacterium tuberculosis	Altered DNA methylation in dendritic cells and macrophages *in vitro* and *in vivo*	Altered transcription of genes involved in immune response	(11, 151, 152)
	Aberrant DNA methylation in monocytes	Increased disease severity	(153–155)
	Demethylation at the promoter region of *Nlrp3* in macrophages	Increased NLRP3 inflammasome activation and downstream release of IL-1β and IL-18	(156)
	Aberrant methylation at the *TLR2* promoter in human blood leukocytes	Negatively regulated *TLR2* expression; increased bacterial burden and disease severity	(154)
Escherichia coli	Aberrant DNA methylation by altered DNMT activity in T cells	Dysregulation of immune responses to bacterial infection induced lung injury	(157, 158)
	Increased DNMT1 activity in uroepithelial cells	Downregulation of *CDKN2A* (tumor suppressor gene) and increased risk of bladder cancer consequently	(159, 160)
	Decreased DNMT3A activity in porcine mammary epithelial cells	Enhanced immune response	(161)
	Downregulation of *TET1* in THP1 macrophages	Reduced NF-κB signaling pathway and inhibition of macrophage M1 polarization	(162)
Salmonella	Altered DNA methylation in chicken cecum and blood leukocytes	Changed expression of immune and metabolic genes	(163, 164)
	Enhanced DNA methylation at the promoters of *TLR4*, *TLR21* and *TLR2-1* in chicken blood leukocytes	Reduced MyD88 signaling and increased susceptibility to *Salmonella enterica*	(165, 166)
Pseudomonas aeruginosa	Altered DNA methylation at *NODAL* in bronchial epithelial cells	Changed airway homeostasis	(167)
	Aberrant function of DNMT3B	Increased susceptibility to infection	(168, 169)
Methicillin-resistant Staphylococcus aureus	Reduced *DNMT3A* in macrophage and neutrophils	Reduced IL-10 production and increased inflammatory responses in patients; Increased susceptibility and mortality in murine models	(170)
	Modified DNA methylation signatures in circulating immune cells	Increased disease severity in patients	(171)
Campylobacter rectus	Hypermethylation of *Igf2* in mouse placenta	Down-regulation of *Igf2* and aberrant placental growth	(172)
Porphyromonas gingivalis	Decreased *DNMT1* expression in gingival epithelial cells	Increased antibacterial responses by promoting β-defensin 2 and CC chemokine ligand 20 expression	(173)
Anaplasma phagocytophilum	DNA hypermethylation in neutrophils potentially by promoting *DNMT3A* expression	Reduced neutrophil antibacterial functions	(174)
Bacterial products	**Effect on DNA methylation**	**Impact on immune response**	**References**
LPS	Aberrant DNA methylation at *TLR*s, inflammatory cytokines (*IL6*, *TNF*)	Dysregulation of cellular responses to LPS stimulation	(175–178)
	Increased DNMT1 activity in macrophages	Enhanced inflammatory responses by hypermethylation of anti-inflammatory factors such as KLF4, miR-145 and SOCS3	(178–180)
	Downregulation of *TET1* in macrophages	Inhibition of NF-κB signaling and decreased inflammatory responses	(162)
	Increased *Tet2* expression in myeloid cells	Decreased IL-6 production and reduced inflammation *in vivo*	(63, 122)
***Staphylococcal* enterotoxin B**	Modified DNA methylation of some genes with important roles in immunity in nasal polyp explants	Potentially altered immune responses related to T-cell maturation/activation	(181)

(Continued)

TABLE 1 | Continued

Bacteria	Effect on DNA methylation	Impact on immune response	References
Peptidoglycan and lipoteichoic acid	Suppressed DNMT activity and hypomethylation of global DNA	Enhanced inflammatory responses	(182)
Rv2966c from *Mycobacterium tuberculosis*; Mhy1, Mhy2, and Mhy3 produced by *Mycoplasma hyorhinis*	Hypermethylation of host genes by acting as DNA methyltransferase	Interference with host immune response	(183–185, 212)
Extracellular vesicles secreted by *P. aeruginosa*	Modified DNA methylation at enhancers of immune-related genes in human lung macrophages	Abnormal innate immune response	(203)
Bacterial metabolite folate	Increased DNMT activity with altered DNA methylation in host cells	Unknown	(186)

IECs, intestinal epithelial cells; TLR, Toll-like receptors; LPS, lipopolysaccharide; DNMT, DNA methyltransferase; TET, ten-eleven translocation methylcytosine; KLF4, Krüppel-like factor 4; SOCS3, Suppressor of cytokine signaling 3; IL, interleukin; TNF, tumor necrosis factor.

signatures of intestinal epithelial cells (IECs) early after birth. The formation of microbiota related "functional" methylation sites might impact long-term gene expression signatures in IECs (135, 136). Furthermore, some intestinal genes, related to innate immunity, phagocytosis, endothelial homeostasis and tissue metabolism are influenced by microbiota through DNA methylation (136). For instance, exposure of colonic epithelial cells to commensal bacteria results in Toll-like receptor (*TLR*)*4* gene hypermethylation and transcriptional downregulation, thereby suppressing responsiveness to LPS (138, 139). More importantly, TET2/3 in IECs contribute to enhanced demethylation induced by microbiota under homeostasis and during acute inflammation (137). Besides IECs, the development and function of immune cells at nonmucosal sites, such as the bone marrow, peripheral lymph nodes and spleen, are also suggested to be regulated by microbiota *via* DNA methylation (188). On the other hand, TET2 deficiency in hematopoietic cells can lead to a microbiota-dependent impairment of gut barrier (140).

Many intestinal pathogenic bacteria have been suggested to cause aberrant DNA methylation in host cells. In this context. *Helicobacter (H.) pylori* is one of the most investigated enteric pathogens. *H. pylori* is able to change DNA methylation directly. High levels of aberrant DNA methylation in *H. pylori*–infected gastric mucosae have been associated with gastric cancer risk (146). Indeed, several tumor suppressing genes were found downregulated in gastric mucosae through *H. pylori*–infection induced hypermethylation. DNA methylation at the promoter region of trefoil factors, which regulate mucosal repair and suppress tumor formation in the stomach, was found increased early after *H. pylori* infection and throughout gastric tumor progression (189). Similarly, hypermethylation of DNA repair protein O6-methylguanine DNA methyltransferase (MGMT) and reduced levels of MGMT were common in the gastric epithelium of *H. pylori* infected patients, increasing mutagenesis in *H. pylori*-infected gastric mucosa (190). Other important genes like *CX32* and *CX43* were also repressed by *H. pylori* induced hypermethylation (191). DNA hypermethylation in the context of *H. pylori* infection was partially reversible after eradication of this bacterium or administration of a DNA demethylating agent, 5-aza-2-deoxycytidine, resulting in decreased the incidence of gastric cancers induced by *H. pylori* infection (190, 192). Single nucleotide polymorphisms in *DNMT1* were reported to be genotypic markers for predicting genetic susceptibility to *H. pylori* infection (150), whilst the addition of folic acid to promote the activity of DNMTs was able to counteract *H. pylori* induced DNA demethylation (55), suggesting a direct role for methylation related factors herein. More recent evidence suggests that *H. pylori* induced inflammatory responses rather than the bacteria itself cause aberrant DNA methylation in the gastric mucosa (147). DNA hypermethylation induced by *H. pylori* infection was associated with down-regulation of genes involved in cell cycle progression control and DNA repair, thereby increasing the risk for gastric cancer (148). Mechanisms implicated in DNA hypermethylation during *H. pylori* infection include inflammation associated with the infection (134, 149) and altered expression or activity of DNA methylation related enzymes (62); as an example, IL-1β is able to induce *TET2* expression in macrophages *via* IL-1R-Myd88 signaling (62).

Polymicrobial Infection and Sepsis

Sepsis is defined as life-threatening organ dysfunction resulting from a dysregulated host response to infection (193) and one of the leading causes of death globally (194). Sepsis is associated with changes in DNA methylation patterns in blood leukocytes of critically ill patients, and the majority of the differentially methylated region-associated genes were differentially expressed (141). Functional analysis showed that these sepsis related alterations in DNA methylation involved inflammatory pathways participating in both the innate and adaptive immune response, as well as in cell adhesion and cell junctions (141, 195). Likewise, the altered DNA methylation profiles in monocytes of septic patients correlated with increased IL-10 and IL-6 levels, as well as with organ dysfunction (143). Analysis of the CpG methylation status in blood cells of neonates with sepsis showed differential methylation of several CpGs located in functionally important genes including a group of *PCDHB* genes that play vital roles in leukocyte cell adhesion and the Wnt signaling pathway when compared to health (142). Another investigation indicated that the DNA methylation pattern of CpG sites in the promoter region of the calcitonin-related polypeptide α (*CALCA*) gene might be used as an epigenetic

biomarker for bacterial sepsis in preterm newborns (196). Sepsis associated DNA methylation signatures in either specific genes or at genome-wide level have potential as diagnostic tools for predicting sepsis outcome or distinguishing sepsis subtypes. For instance, methylation of the NF-κB binding site in the Aquaporin5 (*AQP5*) promoter diminishes the binding of NF-κB and increased the expression of *AQP5* in blood cells of septic patients is associated with substantially greater 30-day mortality (197). Similarly, DNA methylation signatures in critically ill adults can distinguish septic and nonseptic patients, and can associate with clinical traits including severity of illness, need for vasopressors, and length of stay (141). These changes in DNA methylation likely at least in part are caused by sepsis-induced changes in the levels of enzymes mediating DNA methylation, as indicated by decreased DNMT1 and increased TET2 mRNA levels in blood leukocytes of sepsis patients (144). However, *de novo* DNMT mRNAs (*DNMT3A* and *DNMT3B*) in extracellular vesicles in blood were much higher than in healthy controls and strongly correlated with disease severity; DNMT mRNA levels were higher in septic shock patients than in sepsis patients without shock (145). In sepsis models, the inhibition of DNA methyltransferases by Decitabine attenuated NF-κB activation, downregulated inflammatory cytokine levels, inhibited the progression of sepsis and improved survival in mice with severe sepsis induced by cecal ligation and puncture (198). The presence of TET2 impaired survival in mice with sepsis by promoting emergency myelopoiesis and a cytokine storm through oxidation of 5-mC in Socs3 mRNA resulting in destabilization of this mRNA (130). Collectively, DNA methylation could be a potential diagnostic tool or biomarker for sepsis, and manipulation of DNA methylation enzymes might be a novel strategy in the treatment of sepsis.

Specific Pathogens

Mycobacterium tuberculosis

Mycobacterium tuberculosis (MTB) infection has been reported to change DNA methylation at global level and at specific target CpGs both *in vivo* and *in vitro*. An *in vitro* study showed that MTB infection can lead to rapid changes in DNA methylation in non-proliferating cells, in parallel with the transcriptional response (11). Altered DNA methylation in macrophages was predominantly found at non-CpG dinucleotide sites during MTB infection (151), and the mycobacterial protein Rv2966c might be responsible for this type of DNA methylation change (183). Macrophages isolated from MTB infected patients also showed altered DNA methylation profiles of the promoter sequences of many cytokines and their receptors (152). For instance, demethylation at the promoter region of *NLRP3* by MTB infection activates the NLRP3 inflammasome and increases IL-1β and IL-18 release (156). Peripheral blood mononuclear cells from TB patients are characterized by DNA hyper-methylation of genes critical to mycobacterial immunity resulting in decreased mycobacteria-specific and non-specific immune responsiveness (153). Aberrant methylation of certain CpG sites over the *TLR2* promoter negatively regulated *TLR2* expression in NK cells/monocytes of patients with active pulmonary TB and correlated with the bacterial burden and disease severity (154); likewise, increased DNA methylation in monocytes from tuberculosis patients was suggested to reflect disease severity (155). Collectively, these results suggest that DNA methylation profiles of leukocyte subsets might be used as clinically prognostic tools for TB.

Escherichia coli

Escherichia (E.) coli is a Gram-negative and common causative pathogen in gastroenteritis, urinary tract infection, neonatal meningitis, hemorrhagic colitis, peritonitis and pneumonia. Several studies have documented modifications of DNA methylation in host cells during *E.coli* infection. DNA methylation within the promoters of a core set of $CD4^+$ T-cell pathway genes attenuated neonatal immune responses to pneumonia-induced injury (157). Yet, DNMT inhibition by 5-aza-2-deoxycytidine (DAC) augmented the number and function of regulatory T cells thereby accelerating the repair of experimental lung injury (158), suggesting that the altered DNA methylation might be caused by the changes in the abundance or activity of regulatory enzymes during *E.coli* infection. Moreover, *E. coli* induced alterations in DNA methylation are frequently accompanied by changes in the expression of genes encoding proteins that are required for controlling bacterial infection. Uropathogenic *E. coli* infection induces *de novo* methyltransferase activity and *DNMT1* expression causing increased methylation of *CDKN2A* exon 1 and downregulation of this tumor suppressor gene in uroepithelial cells, which may increase the risk of bladder cancer (159, 160). However, downregulation of *de novo* methyltransferase *DNMT3A* by *E. coli* was accompanied by hypomethylation of some immune response genes in porcine mammary epithelial cells (161). Additionally, knockdown of *TET1* in THP1 macrophages downregulated the activity of the NF-κB signaling pathway activated by *E. coli*, thus inhibiting macrophage M1 polarization (162). Avian pathogenic *E. coli* infection led to changes of DNA methylation at gene body regions in the spleen, which negatively correlated with the expression of genes involved in the host inflammatory response and other networks and pathways related to injury/survival (199).

Salmonella

Salmonella is the most frequently detected causative agent in foodborne outbreaks worldwide. *Salmonella (S.) typhimurium* and *S. enteritidis* are the most common serotypes associated with foodborne diseases (200). The domestic chicken is an important host of *S. enterica*, and some studies showed that *S. enterica* infection alters DNA methylation in immune and metabolism related genes in chicken cecum and blood leukocytes (163, 164). Furthermore, enhanced DNA methylation levels at the promoters of *Tlr4*, *Tlr21* and *Tlr2-1* of blood leukocytes is related to reduced expression of genes in the MyD88 signaling pathway and increased susceptibility to *S. enterica* infection (165, 166). Notably, although *Salmonella* is an important pathogen in humans, knowledge of its capacity to modify DNA methylation in human cells is lacking.

Pseudomonas aeruginosa

P. aeruginosa is one of the main causative pathogens in hospital-acquired pneumonia and chronic airway infection associated

with cystic fibrosis (201). Bronchial epithelial cells (BECs) are activated by and required for host defense against *P. aeruginosa* infection (202). Recently *P. aeruginosa* was shown to inhibit *NODAL* expression in BECs through methylation modification of its promoter. Nodal is vital for regulating proliferation of BECs and BEC-induced differentiation of T helper (Th) cells from Th1 to Th2 and Th17, thus regulating the immunological balance of the airway microenvironment (167). DNA methylation in human lung macrophages can be modified by *P. aeruginosa* secreted extracellular vesicles; DNA methylation modifications particularly occurred at distal DNA regulatory elements, including enhancer regions and DNase hypersensitive sites, and some CpGs associated with cytokines such as *CSF3* displayed strong negative correlations between DNA methylation and gene expression (203). DNA methylation enzymes are important for regulating host immune responses against this bacterium infection, as indicated by the association between genetic variants of *DNMT3B* and *P. aeruginosa* infection in children (168). We recently identified a role for DNMT3B in bronchial epithelial cells during *P. aeruginosa* pneumonia (169). DNMT3B deficient human bronchial epithelial cells produced more CXCL1 and related chemokines than control cells when stimulated with *P. aeruginosa*. Mechanistically, DNMT3B deficiency reduced DNA methylation at exon 1 of *CXCL1* and increased NF-κB p65 binding to the *CXCL1* promoter. These *in vitro* findings were corroborated by studies in mice with bronchial epithelial Dntm3b deficiency infected with viable *P. aeruginosa via* the airways, which showed increased *Cxcl1* expression in bronchial epithelium and CXCL1 protein release together with enhanced neutrophil recruitment and accelerated bacterial clearance. Additional studies using purified flagellin (an important virulent factor expressed by *Pseudomonas*) and a flagellin-deficient *P. aeruginosa* strain demonstrated that bronchial epithelial DNMT3b impaired host defense during *Pseudomonas* induced pneumonia at least in part by diminishing mucosal responses to flagellin (169). In separate investigations we showed that the DNA methylation eraser TET2 maintains epithelium barrier function during acute *P. aeruginosa* infection in mice (204).

Burkholderia pseudomallei

B. pseudomallei is an intracellular Gram-negative pathogen causing melioidosis, a common cause of sepsis in Southeast Asia and Australia. *B. pseudomallei* induced changes in DNA methylation of human macrophage-like U937 cells *in vitro*, particularly in the vicinity of genes involved in inflammatory responses, intracellular signaling and apoptosis (205).

Methicillin-Resistant Staphylococcus aureus (MRSA)

MRSA infection significantly decreased *DNMT3A* in blood leukocytes *in vivo* and in macrophage and neutrophils *in vitro*. *DNMT3A* knockdown increased *S. aureus* induced IL-10 production by macrophages *in vitro* and pretreatment with DAC increased mortality in a *S. aureus* murine sepsis model. However, a *DNMT3A* polymorphism increased the capacity to resolve MRSA bacteremia, potentially by reducing IL-10 production though a DNA methylation dependent mechanism (170). Indeed, persistent and resolving MRSA bacteremia were associated with different DNA methylation signatures in circulating immune cells of patients, particularly in neutrophils, and this distinct DNA methylation patterns were able to predict persistent MRSA bacteremia (171).

Campylobacter rectus

Placental and fetal infection with *C. rectus* in mice caused hypermethylation in the promoter region of *Igf2* in the placenta, resulting in down-regulation of *Igf2*, which affects the growth of the fetus by controlling both the placental supply of, and the genetic demand for, maternal nutrients to the fetus (172).

Porphyromonas gingivalis

P. gingivalis, the major pathogen in chronic periodontits, modifies *DNMT1* expression and changes methylation at the promoter region of several genes implicated in the innate immune response against bacteria and during tissue remodeling, whilst the DNMTs inhibitor DAC restores the expression of these genes in infected gingival epithelial cells (173).

Anaplasma phagocytophilum

A. phagocytophilum is a Gram-negative bacterium with a strong tropism for neutrophils that causes human granulocytic anaplasmosis, a zoonosis transmitted by ticks. *A. phagocytophilum* infection induces genome-wide hypermethylation in neutrophils potentially by promoting *DNMT3A* expression (174). Furthermore, inhibition of DNMTs by 5-azacytidine resulted in a partially recovery of neutrophil antibacterial functions and decreased bacterial growth (174).

Bacterial Products

DNA methylation of immune cells can affect their responsiveness to microbial products, as illustrated by strong correlations between DNA methylation in human peripheral blood mononuclear cells and IL-6 production elicited by various TLR agonists (206). LPS is one of the major virulence factors of Gram-negative bacteria and the most used molecule for studying mechanisms underlying cellular immune responses. Recent evidence has indicated that changes in DNA methylation regulate LPS-induced immune responses and that modifying DNMT activity influences cellular responses to LPS (175). One way by which DNA methylation might influence LPS responsiveness is by affecting the expression of TLR4, the LPS receptor, as has been documented in intestinal epithelial cells (207). However, the most frequently reported mechanisms by which DNA methylation regulates LPS induced responses are associated with the function of DNA methylation modifiers. Increasing the methyl donor for DNA methylation by adding the *S*-adenosylmethionine (SAM) precursor methionine attenuated LPS-induced inflammatory responses in macrophages, whilst the DNMTs inhibitor DAC partially suppressed inflammatory responses induced by LPS in macrophages and other cell types (208, 209). Furthermore, DAC reduced lung inflammation and injury by inhibiting M1 macrophage activation *in vivo* (210). DNMTs were altered in bovine endometrial cells and microglia upon LPS stimulation and the expression of some inflammatory cytokines such as IL-1β, IL-6 and IL-8 were negatively regulated by

methylation at their promoters (176, 177). Similarly, DNMT3B was reported to inhibit pro-inflammatory cytokine production by hypermethylation at their promoters or by downregulation of PPARγ expression (33, 211). Conversely, DNMTs mediated hypermethylation at promoters of anti-inflammatory factors, such as *SOCS1, KLF4* and miR-145 – and as a consequence thereof – their downregulation, exacerbates inflammatory responses either *in vivo* or *in vitro* (178–180). The role of TET proteins in LPS induced activation of immune cells was intensively studied, revealing both inhibitory and stimulatory functions. TET1 is able to interfere with the NF-κB signaling pathway and knockdown of *TET1* resulted in decreased production of proinflammatory markers by LPS/IFN-γ-induced M1 macrophages (162). TET2 functions downstream of the NF-κB signaling pathway by recruiting HDACs to the *IL6* promoter resulting in reduced *IL6* expression in macrophages and attenuation of inflammatory responses in murine endotoxemia model (63, 122). Besides LPS, there are few other bacterial compounds reported to affect DNA methylation in host cells. *Staphylococcus aureus* enterotoxin B altered the DNA methylation pattern in nasal polyp explants, most notably in *IKBKB* and *STAT5B*, genes encoding proteins with important roles in immunity (181). Likewise, peptidoglycan and lipoteichoic acid from this bacterium are able to suppress DNMT activity, resulting in enhanced inflammatory responses in bovine mammary epithelial cells (182). While the majority of bacterial compounds alter host DNA methylation by modifying the expression and activity of DNA methylation enzymes, mycobacterial protein Rv2966c by itself acts as a DNA methyltransferase that binds to host specific DNA sequences and methylates cytosines predominantly in a non-CpG context (183). Likewise, the swine pneumonia pathogen *Mycoplasma hyorhinis* produces Mhy1, Mhy2 and Mhy3, which can serve as mammalian DNMTs able to modify host DNA methylation (184, 185, 212). Besides bacterial components, bacterial metabolites might also affect host cell DNA methylation after uptake by these cells. For instance, folate produced by the commensal bacteria *Bifidobacterium* and *Lactobacillus* contributes to the generation of SAM resulting in increased DNMT activity and altered DNA methylation in host cells (186).

CONCLUSION AND PERSPECTIVES

Bacterial infection can alter the DNA methylation pattern of host cells, which may represent a strategy of pathogens to modify host gene expression to avoid clearance and facilitate colonization (213, 214). Changes in DNA methylation may also contribute to short-term memory in innate immune cells (215). Most of our current understanding of DNA methylation is derived from research fields outside infection immunity, in particular cancer and developmental immunology. Whilst awareness of the crucial role of DNA methylation and the proteins involved herein in regulating host immune defense against bacterial infection has increased, much remains to be learned about the mechanisms by which bacterial infection alters host DNA methylation and how this interferes with immune responses. Additionally, compared to a broad spectrum of bacteria that can modify host DNA methylation, thus far only few bacterial components or products have been reported to alter host DNA methylation, through mechanisms that are incompletely understood. Therefore, further research is warranted to reveal which bacterial effectors and mechanisms are involved in modification of host DNA methylation in bacterial infection. Expanding our knowledge of the role of variations in the methylation of DNA in host immune cells may not only enhance our understanding of host defense and the pathogenesis of bacterial infection, but also may provide clues for the development of novel therapeutics.

AUTHOR CONTRIBUTIONS

WQ and TP wrote the first draft of the article, with subsequent input from BC. All authors contributed to the article and approved the submitted version.

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Macrophage Activation Syndrome and COVID 19: Impact of MAPK Driven Immune-Epigenetic Programming by SARS-Cov-2

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INTRODUCTION

The current coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has the worst affected the entire population on the earth (1, 2). This is currently a major concern for the global health care system, as declared by the World Health Organization (WHO). Ample pieces of evidences suggested the idiopathic association of the SARS-CoV-2 with many diseases in COVID-19 cases. Given the aberrant immunopathology of COVID-19, a single approach may not be sufficient to control the disease effectively. Severely infected patients displaying acute respiratory distress syndrome (ARDS) need additional modalities for their management (3). This could be due to the host's epigenetic programming of infected macrophages, which may be responsible for negative prognosis and inadequate response to the current therapeutic regimen for controlling disease manifestation.

SARS-CoV-2 enters the host cells *via* ACE-II receptor and triggers the secretion of the copious amount of IL-6;promote pulmonary fibrosis and Th2/17 programming of lungs, leading to severe lung infection in COVID-19 patients. SARS-CoV-2 interacts and tweaks all kind of cells like epithelium, macrophages, dendritic cells, and T cells and exploit them in a way that supports its replication for progression of the disease.

Out of these, uncontrolled activation of macrophages (also known as double edge component of immunity) leads to Macrophage activation syndrome which is responsible for acute respiratory distress syndrome (ARDS) and subsequent death of COVID-19 patients (4, 5). This is mainly characterized by the increased infiltration of committed $F_C^N 1^+$ macrophages and their Th2/Th17 programming leading to mortality. Once derailed, hyperactive macrophages secrete high levels of IFN-γ, IP-10 (IP-10), IL-6, IL-17, TNF-α along with TGF-β and IL-10/23, leading to the Th2/Th17 programming in the infected lungsof severe cases of COVID-19 (6).

At molecular levels, this is accompanied by the activation of inflammasome pathways which are important forTh17 programming of tissue. Activated CD14+ monocytes phagocytose dead neutrophils and promotes NETosis in the lung. This promotes Th2 bias, decreases lymphocyte/neutrophils ratio and increases the risk of COVID-19 patients for death. Given this, *in situ* reprogramming Th2/Th17 programmed macrophages towards their M1 phenotype is expected to afford protective immunity in COVID-19 cases (4) as shown in **Figure 1**.

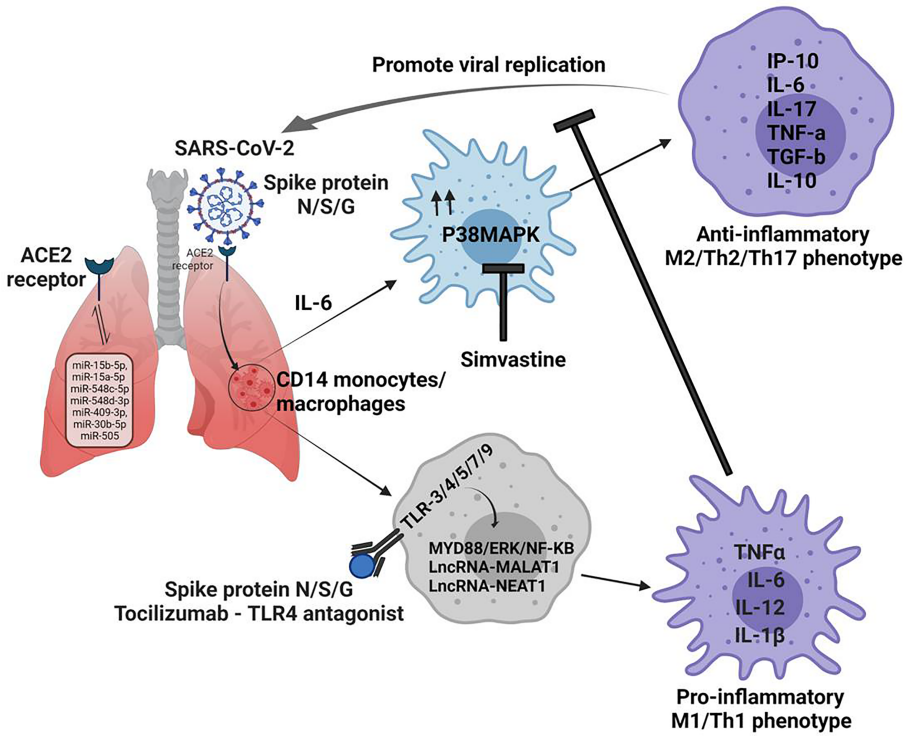

FIGURE 1 | Non-coding RNAs regulates macrophage plasticity during the pathogenesis of Covid 19 disease. 1. N/S/G spike proteins bind to ACE2 receptors on lung cells and determine the entry of the SARS-CoV-2 virus. 2. miRNA can be direct targets since they can regulate the expression of ACE2 in various organs. 3. Infection produces an copius amount of IL-6, which drives the fate of CD14 monocytes/macrophages towards M2 phenotype *via* MAPK signaling, which promotes viral replication. 4. In view of this virus eliminating inflammatory niche could be achievd by promting M1 phenotype in TLR depenedent MYD88/ERK/NF-Kb pathway. 5. This could be fosterd by application of MAPK inhibitors like simvastatin in conjuction of TLR antagonist which can help immune cells to curb SARS-CoV-2 in the host effectively.

Committed macrophages rely upon Toll-like Receptors (TLRs) and associated pathways, the guardian for Th1/2/17 effector responsesduring any infection, including SARS-CoV-2 (7). Among various TLRs on macrophages, TLR-4, 5, 3, 7,and 9 actively sense spike proteins (N, S or G) or mRNA of NSP-10, S2, and E proteins of SARS-CoV-2 and promote M1 polarization of macrophages (8). Apart from ACE-2, the spike protein of SARS-CoV-2 uses TLR-2, 4 and 5 signaling pathways also *via* MyD88 and triggers Th1 effector response through NF-κB and ERK signaling cascade (9). Given this, tweaking TLR signaling like TLR5 can restore or promote Th1 response in derailed macrophages in COVID-19 patients. Indeed, a recent report suggests that conjunction therapy with antivirals and TLR-7 agonists may benefit patients (7) who are believed to harbor Th2/17 programmed macrophage. Similarly, the application of Tocilizumab and TLR-4 antagonists is expected to promote M1 re-polarization of derailed macrophage in patients with severe disease displaying ARDS.

Several intracellular pathways like nk-kb/STAT/and p38MAPK are essential for the immune polarization of macrophages during infection and cancer. p38MAPK pathway is one of the host factors implicated in lung and heart injury in COVID-19 patients (10, 11). P38MPAK landscape is decisive for sterile inflammatory responses, desmoplastic reactions, T cell exhaustion, and epigenetic programming of severely infected COVID-19 cases. P38 MAPK controls macrophage plasticity *via* promoting ER stress, unfolded protein responses, and glucose intolerance which are associated with energy imbalance in the infected host. Since SARS-CoV-2 directly up-regulates p38 activity for promoting its replication in epithelium and macrophages (12), we presume that hyper-activation of p38MAPK may contribute to Th2 bias in these macrophages and aberrant inflammation in the lung.

SARS-CoV-2 regulates P38MAPK signaling in multiple ways to support its replication, one of the prominent mechanisms is downregulation of *ACE2* activity, which negatively regulate expression of *ICAM-1* (intercellular adhesion molecule-1), *VCAM-1* (vascular cell adhesion molecule-1) (13) and *NF-KB* activation (14) leading to Th2 bias in the host. Loss of *ACE2* function leads to enhanced concentration of intracellular Angiotensin 2, which directly activates P38MAPK (10) in the host, leading to Th17 response in the host (15). This progress to ARDS (acute respiratory distress syndrome) and myocarditis are primary reasons for death in critically infected patients (16). Several studies with severely infected patients suggested that SARS-CoV-2 promotes degradation of DUSPs (dual-specificity phosphatase) transcripts, this promotes *P38MAPK* hyperactivation (17) in the host. Besides ACE2 and DUSPs, SARS-CoV-2 also triggers TAB1 (TGF-β activated kinase 1 (MAP3K7)-bindingprotein 1) mediated P38A auto-phosphorylation and P38MAPK hyper-activation,

adding to the reason for increased MAPK activity in the infected cells. Studies with several MAPK inhibitors like SB203580 (18), Losmapimod (19) and Dilmapimod (20) have shown promising results in mitigating pathogenic inflammation in COPD patients and advocated their potential application in hashing SARS-CoV-2 burden. Therefore targeting p38MAPK could be of direct interest in controlling viral burden and M1 retuning of infected macrophages *viz-a-viz* mitigating T- cell exhaustion in patients.

Apart from activating several cytoplasmic signaling pathways, p38MAPK also activate the expression of various transcription factors. Recent studies have provided compelling evidence that activated MAPK influence the expression of differentially expressed mi/lncRNAs,which are important for sterile inflammation and M2/Th2 polarization of macrophages. Most intriguingly, the lncRNA landscape is proposed as a prognostic factor responsible for the severity of COVID-19 cases (21). Among pool of miRNAs; miR-15b-5p, miR-15a-5p, miR-548c-5p, miR-548d-3p, miR-409-3p, miR-30b-5p and miR-505 have been validated as potent targets for controlling SARS-CoV-2 infection (22). These miRNAs regulate the expression of ACE-2 in various organs, including the kidney, heart, blood vessels, and lungs which are important for COVID-19 pathophysiology (23). Other than this, several LncRNA like *WAKMAR2*, *EGOT*, *EPB41L4A-AS1*, *ENSG00000271646*, *MALAT1* and *NEAT1* are known to contribute to skewing the immune response against SARS-CoV-2 infection (24, 25).

Overexpression of *NEAT1* stabilizes the mature caspase-1 to promote interleukin-1β production and modulate inflammasome activation (26), which is associated with Th2/17 programming of immune cells like macrophages. *MALAT1* promotes Th1 effector responses and apoptosis in airway epithelial cells conditioned DCs and cardiac cells (6, 27) *via* miR-125b and p38MAPK/NF-κB pathways (7). This loop is potentially involved in the maturation and pro-inflammatory programming of CD14+/Gr-1-/iNOs+ M1 macrophages, which is essential for the adaptive immunity of the host.

MAJOR PERSPECTIVE

Lowering p38MAPK with specific inhibitors like simvastatinin conjunction with TLR antagonist and Tocilizumab is anticipated to be a prudent approach for augmenting immunity of COVID-19 infected cases. The uncontrolled systemic inflammatory response and cytokine storm is the main mechanism of ARDS caused by the excessive release of interferon, interleukins, TNF-α and chemokines. Thus, it was proposed that statins (28), which are well known for their anti-inflammatory effects,could treat MERS-CoV infection and perhaps COVID-19 patients (29) as well. However, statins in COVID-19 patients sometimes increase the risk and severity of myopathies and acute kidney injury (29). On the other hand, statin therapy increases liver enzymes, leading to severe complications in the COVID-19 patients (30). Thus, guideline-directed statin therapy in COVID-19 patients is necessary.

It was proposed that early intervention with interleukin-6 receptor blockade by Tocilizumab could effectively control the progression to hypoxemic respiratory failure or death of severe COVID-19 patients (31). There are conflicting results obtained for tocilizumab in COVID-19 patients. Several treatment lines suggest that using a monoclonal antibody against IL-6 is an attractive strategy to manage severe COVID-19 as Tocilizumab has the potential to reduce mortality and the need for mechanical ventilation (32, 33). However, a clinical trial on 243 patients revealed that tocilizumab was not effective for preventing death in moderately ill hospitalized COVID-19 patients (34). In a recent study on the hospitalized COVID-19 patients, although tocilizumab reduced the progression to the composite outcome of mechanical ventilation, however could not improve their survival (35). Besides, this targeting miRNA which modulates the expression of ACE2 receptor activities, can also be of significant value to currently explored therapeutics/interventions. This conjuction approach is expected to enhance the sensitivity of infected host cells for currently employed drugs. Taken together, above interventions would help in curbing the SARS-CoV-2 virus for the effective management of COVID-19 disease.

AUTHOR CONTRIBUTIONS

Conceptualization and conceiving of idea, HP. Writing, AJ, RR, MW, and HP. Resources, AJ, MH, HP, and US. Editing of manuscript, HP. All authors contributed to the article and approved the submitted version.

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frontiers in Immunology

REVIEW
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COVID-19 Is a Multi-Organ Aggressor: Epigenetic and Clinical Marks

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The progression of coronavirus disease 2019 (COVID-19), resulting from a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, may be influenced by both genetic and environmental factors. Several viruses hijack the host genome machinery for their own advantage and survival, and similar phenomena might occur upon SARS-CoV-2 infection. Severe cases of COVID-19 may be driven by metabolic and epigenetic driven mechanisms, including DNA methylation and histone/chromatin alterations. These epigenetic phenomena may respond to enhanced viral replication and mediate persistent long-term infection and clinical phenotypes associated with severe COVID-19 cases and fatalities. Understanding the epigenetic events involved, and their clinical significance, may provide novel insights valuable for the therapeutic control and management of the COVID-19 pandemic. This review highlights different epigenetic marks potentially associated with COVID-19 development, clinical manifestation, and progression.

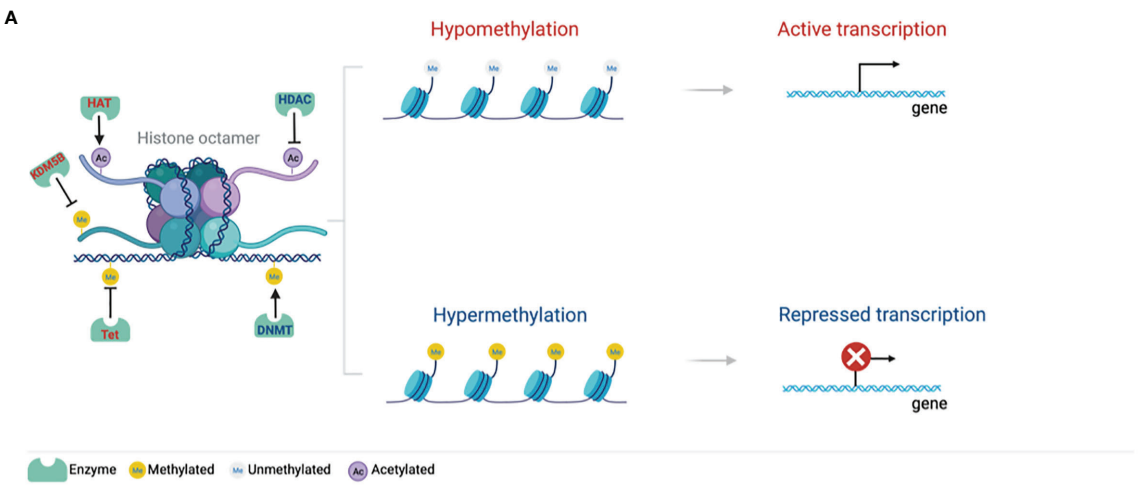
Keywords: ACE2, COVID-19, cytokine storm, epigenetics, multi-organ, pro-inflammatory cytokines, SARS-CoV-2, TMPRSS2

MAIN BACKGROUND

Epigenetics is a branch of biology arising from inheritable gene transcription alterations in response to environmental cues, such as pollutants, chemicals, radiation, diet, stress, and pathogenic organisms (1). Epigenetic phenomena do not cause any genetic alterations or mutations. However, as the new phenotypes that are somatically heritable, epigenetic tags alter gene

transcription and normal functions. Epigenetic marks are either suppressive or active and include DNA methylation, histone modification/chromatin remodelling, non-coding RNA, and RNA modification (**Figure 1**). These marks are implicated in activating or suppressing gene promoters, bodies, or transposable elements in normal processes such as ageing, genomic imprinting, and X-chromosome inactivation (2). DNA methylation is the best-studied stable epigenetic mark that occurs within CpG island promoter regions enriched with >70% of CpG (cytosine phosphate guanine) sites in the genome (3). It involves tagging or deposition of the methyl group of 5-methylcytosine to the DNA molecule through catalysis by DNA methyltransferases (DNMTs), which can be reversed by another family of enzymes called ten-eleven translocation (Tet 1-3) methyldioxygenases (4). DNMTs are regarded as writers of DNA methylation, recognised or read by methyl-CpG binding domains (MBDs) and then erased by TETs (**Figure 1**).

Eukaryotic cell DNA is packaged into chromatin wrapped around an octamer of four core histone proteins (5). Histones can be post-translationally modified by repressive or active histone marks that impact the interaction of histones with DNA or the occupancy of transcriptional machineries for gene expression (**Figure 1**). They dictate the chromatin transcriptional state of the local genomic regions *via* histone



B

WRITERS	READERS	ERASERS
DNA methylation DNMT 1-3	**DNA methylation** MBDs	**DNA methylation** TET 1-3
Hystone Acetylation HATs (p300/CBP), GNATs, MYSTs	**Hystone Acetylation** Bromodomains	**Hystone Acetylation** HDAC 1-11 Sirtuins (SIRT 1-7)
Hystone Methylation COMPASS-like proteins (SET1, MLL1-4) PRC2 GLP	**Hystone Methylation** Cromodomains PHD fingers Ankyrin repeats	**Hystone Methylation** KDM 1-6 LSD 1-2 UTX1 JMJD 1-3

FIGURE 1 | Chromatin structure. **(A)** A 147bp DNA wraps around the histone octamer with two copies of each of the histones H2A, H2B, H3, and H4. Various epigenetic mechanisms that modify chromatin, such as DNA methylation and histone modifications, are highlighted. DNA and histone methylation collaborate with different modifying enzymes and creates a tightly packed chromatin and suppress gene transcription by preventing the transcription machinery from binding DNA. Histone acetylation perturbs structural electrostatic interactions between the DNA and histones, resulting in the less compact structure of chromatin structure. This allows DNA access by transcription factors that promote gene transcription. **(B)** Writing, erasing, and reading chromatin methylation markers are highlighted. These mark various sites on the tail and globular domains of histones. Writers and erasers are methyltransferases and demethylases, respectively. These are recognised by distinct effector proteins called readers. (Created with BioRender.com) ac, Acetylation; DNMT, DNA methyltransferase; GLP, G9a-like protein; GNATs, Gcn5-related N-acetyltransferases; HATs, Histone acetyltransferases; HDACs, Histone deacetylases; JmjC, Jumonji C; KDM, Histone lysine demethylases; LSD, Lysine-specific demethylases; MBDs, Methyl-CpG binding domains; me, Methylation; MLL, Mixed-lineage leukaemia; PHD - Plant homeodomain; PRC2, Polycomb repressive complex 2; p300/CBP, p300 and cyclic AMP response element-binding protein; SET1, Suppressor of variegation 3–9, Enhancer of Zeste, Trithorax 1; SIRT, sirtuins; TET, Ten-eleven translocation; UTX1, Ubiquitously transcribed tetratricopeptide repeat, X chromosome 1.

methylation, acetylation, ubiquitination, and phosphorylation. Chromatin forms a higher-order structure classified as euchromatin and heterochromatin (6). Euchromatin is a loosely packed or open form of chromatin enriched with DNA accessible to regulatory transcription complexes and promotes active gene transcription. Excessive acetylation of histone lysine residues is a common feature of euchromatin (7). It correlates with COMPASS-like proteins as binding partners and methylation of lysine 4 of histone 3 (H3K4), H3K36, and H3K79 that mark transcriptional activation of enhancers, gene promoters, and transcribed genes in gene bodies, respectively (8–10). Lysine can be mono-(me1), di- (me2), or tri-methylation (me3), providing unique functionality to each methylation site (9, 11). A tight or closed form of chromatin is called heterochromatin, protecting the DNA from being accessible to repressive transcriptional marks that restrict gene expression. Heterochromatin is further categorized into constitutive and facultative heterochromatins that are enriched in hypoacetylated or hypomethylated histones (9). The former is a stable form of heterochromatin comprised of repetitive DNA sequences (called DNA satellites) located at the transposon elements, centromere, and telomere. It is characterised by a repressive H3K9 epigenetic mark and heterochromatin protein 1 (HP1) chromodomain binding partner (8, 9, 12–14). Facultative heterochromatin is enriched with long interspersed nucleotide elements (LINE)-type sequences, repressive H3K27me2/3 epigenetic mark and its binding partner, polycomb repressive complex 2 (PRC2)-enhancer of zeste homolog 2 (EHZ2) (15, 16).

Writers, readers, and erasures of DNA methylation and histone modifications are listed in **Figure 1**. This review will discuss the role of epigenetics in COVID-19 infection, susceptibility to infection, and clinical markers established systemically during COVID-19 and may be associated with various epigenetic alterations.

MECHANISMS OF SARS-COV-2 VIRAL INFECTION AND MULTI-ORGAN SYSTEM INVASION

ACE2 and TMPRSS2: Viral Entry and Regulation

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the aetiological agent of the current pandemic, coronavirus disease 2019 (COVID-19) (17). This pathogen is enabled by the angiotensin-converting enzyme 2 (ACE2) (18). Mechanistically, SARS-CoV-2 penetrates and enters the host cell by binding to the ACE2 receptor as the primary target. This is facilitated by proteolytic priming by the cellular transmembrane serine protease 2 (TMPRSS2) (19). In the proposed model of respiratory failure, SARS-CoV-2 downregulates ACE2 through the SARS-CoV spike (SARS-S) protein, explaining the renin-angiotensin-aldosterone systems (RAAS) dysregulation and cardiotoxicity in severe COVID-19 infection (20). Suppression of ACE2 also induces tumour necrosis factor alpha (TNF-α) converting enzyme (TACE) that antagonises ACE2 shedding of the SARS-S (19). Modulation of TACE activity by SARS-S protein was found to depend on the cytoplasmic domain of ACE2 as ACE2 mutants devoid of the carboxyl-terminal region could not induce ACE2 shedding or TNF-α production (21). Moreover, deletion of the cytoplasmic tail of ACE2 or knock-down of TACE expression significantly attenuates viral infection (21). It has been shown that Ang II induces ACE2 shedding by promoting TACE activity as a positive feedback mechanism, suggesting that SARS-CoV mediated ACE2 down-regulation will promote Ang II accumulation and HIF-1α activation, which positively activates disintegrin and metalloproteinase domain-containing protein 17 (ADAM17) activity, thus perpetuating membrane shedding of ACE2, RAAS overactivation, and inflammation (22–26). This mechanism, however, is not universal to all coronaviruses because the spike protein of HNL63-CoV (NL63-S), a coronavirus that also utilizes ACE2 and is known to cause common influenza, did not produce similar cellular responses (21).

Lung as the Primary Target for SARS-CoV-2 Infection

SARS-CoV-2 infection is primarily a respiratory infection that targets type II alveolar epithelial cells (83%) in the lungs (27, 28). Upregulation of ACE2 in various cells usually disrupts ACE2 normal function from cleaving and converting angiotensin II to angiotensin 1-7 for tissue protection (29). SARS-CoV-2-infected type II alveolar epithelial cells leads to inflammation and severe damage in the lung tissue that is clinically manifested by elevated levels of ferritin and D-dimer, and association with oxygen desaturation, chest pain, and disease progression as indicated by computed tomography (CT) pulmonary angiography (30, 31). Elevated levels of macrophage/monocyte colony-stimulating factor (M-CSF, also known as colony-stimulating factor 1 receptor), granulocyte-monocyte colony-stimulating factor (GM-CSF), and interleukin (IL)-6 have also been reported in the later stages of COVID-19 (32–34). This correlates with pneumonia and acute respiratory distress syndrome (ARDS) that may lead to organ failure as observed in severe or critical cases of COVID-19 (31, 33, 35–37).

Most recently, the study of Ferreira-Gomes et al. (38) has shown that cells isolated from bronchoalveolar lavage of intensive care unit (ICU) patients with severe COVID-19 cases were enriched with tumour growth factor-beta 1 (TGF-β1)-expressing Th17, regulatory T cells, and CD14-positive cells, immune cells that are usually recruited to fight the infection. TGF-β1 is a master regulator of immune reaction and pulmonary fibrosis in COVID-19 patients (39). Its expression was associated with SARS-CoV-2 spike protein-specific IgM, IgG (IgG1 and IgG2), and IgA (IgA1 and IgA2) antibodies that protect systemic organs and mucosal surfaces, respectively (38, 40). SARS-CoV-2 spike protein-specific antibodies were also an indication of ongoing immune reaction and damage in secondary organs from the spread of viral infection (41). In the early days of ICU admission, IgG antibodies are predominantly generated by IL-10/21 specific to SARS-CoV-2 proteins (42). As a result of clonal expansion, later these antibodies become somatically mutated, virus non-specific, and undergo switching as instructed by TGF-β1 (38, 43). Ferreira-Gomes and co-

authors have demonstrated that TGF-β1 induces chronic immune reaction by regulating antibody switching from IgG to IgA and this correlates with prolonged ICU stays of more than seven days (38).

Overall, systemic COVID-19 infection is characterised by various immunoregulatory and pro-inflammatory cytokines such as IL-1β, IL-2, IL-6, IL-7, IL-10, IL-18, D-Dimer, C-reactive protein (CRP), GM-CSF, interferon gamma-induced protein 10 (IP10), macrophage inflammatory protein 1 alpha (MIP1α), chemokine (C-C motif) ligand 2 (CCL2, also known as MCP1), interferon gamma (IFN-γ), and tumour necrosis factor alpha (TNF-α), which are mainly observed in ICU patients rather than in non-ICU patients (33, 44–49). This signifies a cytokine storm characterised by an abnormal overreaction of the body's immune system that causes a loss of communication between the infected cells and the host immune defence mechanism. Cytokine storm triggers severe inflammation and infiltration of neutrophils, macrophages, and T cells that may damage several tissues leading to multi-organ failure (50). Carveli et al. (51) demonstrated an association between COVID-19 mediated inflammation and activation of the C5 complement factor with its receptor called complement component C51 receptor (C5AR1). C5AR1 or C5a is a G-protein coupled receptor that modulates inflammatory response by activating neutrophils and monocytes to the site of damage.

Invasion of SARS-CoV-2 in Secondary Organs

ACE2 is widely expressed in a heterogeneous population of systemic cells (**Figure 2**), making it possible for SARS-CoV-2 to damage several systemic tissues leading to various clinical phenotypes that result in multi-organ dysfunction (**Figure 2**) (52–61). A high level of ACE2 in nasal epithelial cells correlates with increased viral load, especially in the early stages of SARS-CoV-2 infection (62). This may explain the accuracy of nasal and nasopharynx aspirates for SARS-CoV-2 diagnosis (62). The highest viral load was reported in the olfactory epithelium, suggesting damage in the supporting cells (61, 63–66). Although ACE2 level is low in the capillary endothelial cells of the cerebral circulation, circumstantial evidence suggests that SARS-CoV-2 may access these cells by crossing the blood-brain barrier, as demonstrated by *in vitro* studies. This may involve unknown indirect mechanisms that may be responsible for clinical manifestation (examples are anosmia, ageusia, and altered mental status) and neurological complications that have been observed in critical cases of COVID-19 infections (**Figure 2**) (67–73).

ACE2 and TMPRSS2 are also expressed in cardiomyocytes, cholangiocytes, hepatocytes, and enterocytes, suggesting potential targets for SARS-CoV-2 infection (60, 74–76). ACE2 synergises with the RAAS to regulate angiotensin to balance the normal function of the cardiovascular system (77, 78). Upon SARS-CoV-2 infection, ACE2 is suppressed and fails to counteract the vasoconstrictive and pro-inflammatory function of the RAAS to balance the system. This may lead to increased vascular permeability, tissue oedema/damage, and systemic microcirculatory dysfunction associated with cardiovascular-related disease (79). Approximately 50% of COVID-19 hospitalised patients exhibit abnormal levels of alanine transaminase (ALT) and aspartic transaminase (AST), slightly elevated level of bilirubin, higher alveolar-arterial oxygen gradient (A-aDO2)/gamma-glutamyl transferase (GGT), and hypoalbuminemia that suggests hepatic damage (80–82). Elevated levels of ALT (7590 U/L) and AST (1445 U/L) were almost doubled in severe/critical cases as relative to mild/moderate cases, and correlate with nausea, vomiting, and anorexia (83–85). In addition, a sub-group of COVID-19 patients present with darkened faces and pigmentation (86, 87). This may suggest abnormal liver function probably from failing to metabolise oestrogen, increased iron level, and melanin secretion as well as adrenocortical hypofunction associated with hepatic injury (88–91).

Zhao et al. (92), demonstrated that SARS-CoV-2 infection triggers direct cholangiocytes damage by perturbing the barrier and bile acid transporting functions of cholangiocytes *via* abnormal regulation of *solute carrier family 10-member 2* (*SLC10A2*) gene and *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene, resulting in bile acid accumulation and consequent hepatic injury aggravation. Mechanisms associated with COVID-19-related hypoxia, antiviral drugs/incorrect drug dosage, and use of herbs or traditional medicines to counteract COVID-19 effects may also participate in liver injury (93–99). Numerous studies have reported successful isolation of SARS-COV-2 from faecal/stool samples of COVID-19 patients with and without inflammatory bowel disease (IBD) (100–103). Interestingly, in some COVID-19 cases, the faecal viral load was even higher (10^7 copies/g) than in pharyngeal swabs (101, 104). This observation disputes the pharyngeal infection as the source of faecal viral RNA and supports the theory of enteric infection of SARS-CoV-2 (101, 104). An elevated level of faecal calprotectin, largely expressed by neutrophils and a reliable faecal biomarker of intestinal inflammation, has been reported in COVID-19 patients with diarrhoea as compared to patients without diarrhoea (105).

It has been demonstrated that nephrons, undifferentiated spermatogonia, testicular Sertoli, and Leydig cells express a considerable abundance of ACE2 receptor expression, making the kidney and testes further potential SARS-CoV-2 reservoirs (106, 107). Renal damage in cases with no underlying renal conditions suggested SARS-CoV-2 as the underlying cause, and this was marked by abnormal blood work and increased levels of proteins in the urine. Lengthy hospitalisation stays, acute kidney injury (AKI), and increased mortality were the most common consequences of severe or critical cases of COVID-19 (107–113). COVID-19 causes severe physiologic and neurological stress, which may release increased stress hormone and alter testosterone levels. Testes play an important role in regulating the hypothalamic-pituitary-testicular (HPT) axis, which governs the male reproductive hormonal cascade (114). HPT axis endocrinologically links testes to the brain by gonadotropins (luteinising hormone-LH and follicle-stimulating hormone-FSH) and testosterone. LH and FSH that normally activate Leydig and Sertoli cells, respectively, are altered in COVID-19 patients, and this is hypothesised to be due to imbalances in testosterone production (115–118). Levels of LH seem to increase in male patients with severe COVID-19 leading to abnormal FSH/LH ratios (115, 116).

A recent case report of semen analysis for *in vitro* fertilization procedure revealed that mild COVID-19 infection in men could result in long-term alterations in sperm morphology and sperm

FIGURE 2 | Potential underlying mechanisms of SARS-CoV2 invasion and multi-organ induced damage. Inflammation mediated by SARS-CoV-2 infection and its primary receptor ACE2 drive multi-organ failure in severe COVID-19 cases. ACE2 is widely expressed in multiple organs, and its suppression may aggravate COVID-19 severity and negatively impacts multiple organs via regulation of RAS. Moreover, this leads to severe cases of COVID-19 that are often associated with ARDS and increased mortality rate, partially mediated by the overproduction of pro-inflammatory cytokines (cytokine storm). Cytokine storm results from increased levels of inflammatory mediators, endothelial dysfunction, coagulation abnormalities, and infiltration of inflammatory cells into the organs. This may be characterised by elevated levels of interleukin-6 (IL-6), nuclear factor kappa B (NFκB), and tumour necrosis factor-alpha (TNFα) released from SARS-CoV-2-infected macrophages and monocytes. The involvement of different organs in severe patients is characterised by multi-organ failure and a broad spectrum of haematological abnormalities and neurological disorders that lengthen the hospitalisation duration and increase mortality. The most important mechanisms are related to the direct and indirect pathogenic features of SARS-CoV2 infection. (Created with BioRender.com). ACE2, Angiotensin I-converting enzyme-2; AoDO2, First alveolar-arterial oxygen gradient; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; IFN-γ, Interferon-gamma; IL-1β, Interleukin- 1β; IL-4/6/10, Interleukin- 4/6/10; TNF-α, Tumour necrosis factor-alpha; MCP-1, Monocyte chemoattractant protein-1.

DNA integrity that may ultimately lead to male infertility (119). It was previously thought that the sperm parameters would take 70 – 90 days to return to their basal state after recovering from the infection. However, this published case has shown that this can take a much longer time of >4 months (119). Although these findings are based largely on case studies and lack further validation, it is plausible to hypothesize that increased risk of infertility as a COVID-19 long-term complication, especially in young men, will be observed after the pandemic. Therefore, more studies are needed to determine the negative impact of COVID-19 in a large cohort of infected males with varying severity of disease during infection and after recovery.

THE ROLE OF EPIGENETICS IN INFECTION SUSCEPTIBILITY: X-CHROMOSOME INACTIVATION AND COVID-19

In terms of Betacoronavirus (SARS-CoV-2, severe acute respiratory syndrome coronavirus/SARS-CoV and Middle East respiratory coronavirus/MERS), men usually experience severe infections complicated with poorer clinical outcomes than women (120–124). It was observed that SARS-CoV-1 infected males had a significantly (21.9%, $p < 0.0001$) higher case fatality rate than females (13.2%) with a relative risk of 1.66

(95% confidence interval (CI): 1.35, 2.05) before age adjustment and 1.62 (95% CI: 1.21, 2.16) after adjustment (120). Peckham et al. (123), demonstrated through a meta-analysis of 3,111,714 reported global COVID-19 cases that males have almost three times the odds of requiring ICU admission (OR = 2.84; 95% CI = 2.06, 3.92) and higher odds of fatality (OR = 1.39; 95% CI = 1.31, 1.47) compared to females. X-chromosome inactivation (XCI) may explain some of the disparities in infection susceptibility (**Figure 3**). As an epigenetic hallmark of normal human development, XCI is regulated by a progressive and stepwise epigenetic phenomenon that ensures an equal dosage compensation of the X-chromosome encoded genes expression level between females and males (125, 126). XCI is regulated by the X-inactivation centre (XIC) and established by long non-coding X inactive specific transcript (Xist) RNA through several heterochromatin changes as largely demonstrated by seminal work conducted by the Brockdorff lab (127, 128). The suppression of X-linked genes through recruitment of the PRCs is a common XCI feature (129). Acquisition of histone deacetylase 3 (HDAC3) and H2A by adding a single ubiquitin group to lysine-119 (H2AK119) are the earliest repressive epigenetic marks required for efficient XCI. H3K27me3, a transcriptional silence mark that is catalysed by PRC2-EZH2 for inactive heterochromatin, is enriched and later spread at the promoters of silenced X-linked genes for long-term stable XCI maintenance (129, 130).

Notably, for counteracting invading pathogens, the X-chromosome is enriched with many immune-related genes and regulatory elements that activate host immune defence mechanisms (131). While this may increase women's susceptibility to autoimmune disease, it may also provide them with immunological and survival advantages against pathogen insults (132). Females have two copies of X-chromosome (XX), and one becomes randomly and permanently silenced during embryogenesis through XCI (125, 126). An inactivated chromosome is called a Barr body or sex chromatin (**Figure 3**). Some genes located in the silenced X-chromosome may escape XCI and remain expressed to perform their normal activities (133). Fortunately for women, these XCI skewing genes/escapees may lead to an elevated level and high immune responsiveness of such genes (134). Subsequently, this results in double and exclusive protection for women against defective X-linked genes and infections relative to men (**Figure 3**). As a result of having a single copy of X-chromosome (XY), males are at high risk of X-linked or sex-linked disorders (134), and this may explain why males tend to suffer more severe cases of COVID-19/other infections and fatal complications than females. Sex different effects in COVID-19 may be attributable to various external risk factors that are more prevalent in men *versus* women (135–140). Comorbidities such as cancer, heart failure, hypertension, diabetes, obesity and chronic obstructive pulmonary disease coupled with behavioural factors including smoking and alcohol consumption are generally increased in males than females, and these have been shown to correlate with poor clinical outcomes, increased risk of ICU admission and fatalities in COVID-19 infected patients (135–138, 140, 141). Men have been shown to have an increased level of circulating plasma ACE2 receptor, the primary receptor that enables SARS-

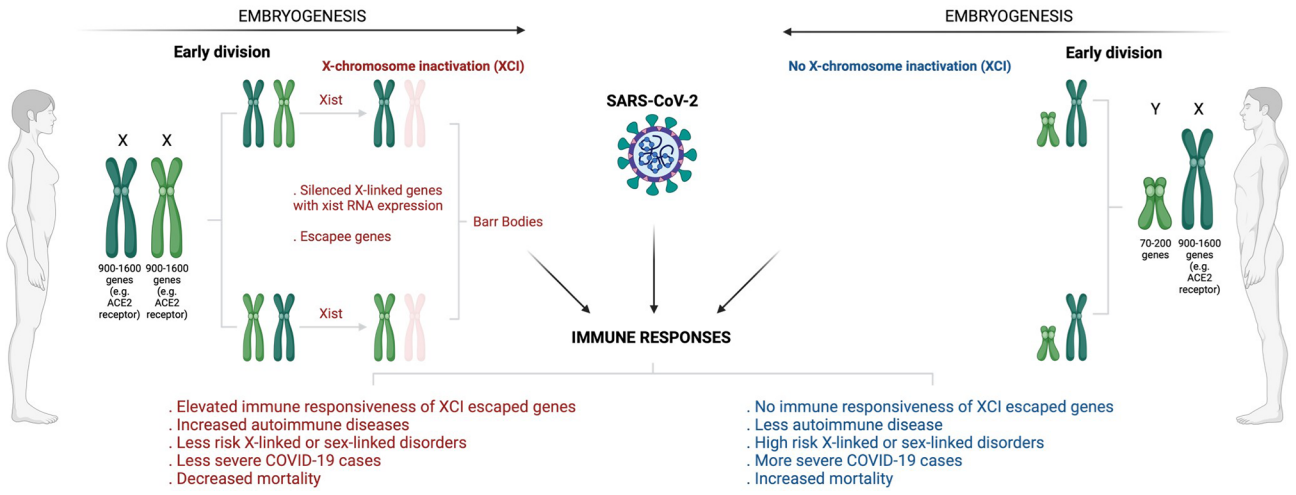


FIGURE 3 | Overview of sex-based differences in the immune response to COVID-19. The diagram shows how X-chromosome inactivation escapee genes may underlie sex bias differences in COVID-infection, severity, and mortality. Sex-bias differences in COVID-19 may be linked to ACE2, the primary receptor that enables SARS-CoV-2 infection. Having double X-chromosomes protects women against increased susceptibility to COVID-19 infection and associated severe complications as compared to men who have just a single X-chromosome. ACE2 is an X-chromosome-linked gene that escapes X-inactivation, a phenomenon that suppresses gene transcription from one of the two X chromosomes in female mammalian cells to balance expression dosage between XX females and XY males. This means that women have twice more genetic instructions to transcribe ACE2 and many more X-chromosome-linked immunoregulatory genes that protect women from increased COVID-19 susceptibility and associated severe complications. (Created with BioRender.com). ACE2, Angiotensin I-converting enzyme-2; COVID -19, Coronavirus disease 2019; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; XCI, X-chromosome inactivation; XIST, X-inactive specific transcript.

CoV-2 attachment and infection (138, 139, 142). Using a high-throughput multiplex immunoassay based on a proprietary proximity extension assay (PEA) technology, Sama et al. (141) measured the ACE2 concentration in index cohort of 1485 males and 537 females with COVID-19 and heart failure, and found that the mean plasma concentration of ACE2 was higher by 5.38 in males compared with females (5.09, $P < 0.001$). This was also supported by a validation cohort that exhibited increased 5.46 ACE2 plasma concentration in males compared with 5.16 in females patients ($P < 0.001$) (141). A separate single center population-based study of 5457 Icelanders demonstrated altered serum levels of ACE2 in males, smokers and diabetes or obese patients, and this was associated with productive SARS-CoV-2 infection and severe clinical outcome (142). The expression levels of ACE2 receptor was found to be enhanced in the lungs in response to active smoking, diabetes and hypertension, explaining an increased susceptibility and severity to COVID-19 infection (138, 139, 142).

Gene expression regulation of ACE2 and other X-chromosome linked genes, including Toll-like receptors (TLRs), CD40 ligand (CD40L), and Forkhead box P3 (FOXP3)/Scurfin, expressed upon SARS-CoV-2 infection, may play a critical role in COVID-19 pathogenesis and severity. Following viral entry, SARS-CoV-2 triggers the activation of the RNA-based pathogen sensors such as TLR3, TLR4, TLR7, and retinoic acid-inducible gene-I-like receptors (RIG-I), which complex with a melanoma-differentiation associated 5 (MDA-5) to establish a frontline defence mechanism (143). This complex is epigenetically subverted to induce abnormally elevated levels of interferons (IFNs) and pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α) and interleukins (ILs), associated with critically ill and ICU admission of COVID-19 patients (131, 144).

Dai et al. (145), through integrated bioinformatics analysis revealed an upregulation of structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1) in COVID-19 patients, suggesting that it may be involved in the epigenetic control of ACE2 receptor, and thus COVID-19 pathogenesis. It is not surprising that SMCHD1 is linked to ACE2 receptor regulation, as it is an essential protein in XCI. Mouse studies have demonstrated that homozygous nonsense mutations in the *Smchd1* gene cause XCI defect that leads to female-specific embryonic lethality (146, 147). Gendrel et al. (125), demonstrated that a late step *Smchd1* gene recruitment to XCI in female XX embryonic stem cells establishes DNA methylation of CpG islands, preferably *via Dnmt3b* gene and histone mark H3K27me3 for long-term maintenance of gene silencing. An SMCHD1-dependent pathway may explain the data of Mudersbach et al. (148), demonstrating that TNF-α suppresses ACE2 mRNA and its protein expression in endothelial cells *via* hypermethylation by DNMTs, including *DNMT3b*. It has been suggested that suppression of TNF-α mediated ACE2 mRNA *via* epigenetic inhibitors may reduce SARS-CoV-2 viral replication, leading to anti-inflammatory effects associated with quicker healing and resolution of COVID-19-related complications (148). SARS-CoV-2 genome encodes mRNA Cap 2′-O-Methyltransferase (2-O-MTase), another epigenetic phenomenon that deposits a methyl group at the 2′-O position of the first nucleotide adjacent to the cap structure at the 5' end of the RNA (149–152). RNA-based viruses often use this mechanism to their advantage to escape immune surveillance. It might be tempting to speculate that drugs targeting these epigenetic marks and preventing immune evasion may also be important in fighting COVID-19 infection.

POSSIBLE EPIGENETIC DYNAMICS IN COVID-19 INFECTION

Li et al. (153) have demonstrated in a murine mouse model with the human ACE2 (hACE2) transgene that SARS-CoV-2 induces epigenetic-mediated metabolic reprogramming and alterations in both local and systemic sites of infection. These alterations are associated with systemic lethality that mirrors human COVID-19 clinical phenotypes, suggesting an epigenetic role in COVID-19 pathogenesis. Below, we discuss epigenetic marks and alterations that we hypothesize may play a role in ACE2 receptor regulation and COVID-19 pathogenesis/treatment.

Writing of DNA Methylation and Role of DNMTs

DNMT1, DNMT3A, DNMT3B, and DNMT3L are family of DNMTs that write or deposit methylation on DNA leading to hypermethylation, read by MBDs to mainly suppress gene transcription (**Figure 1**) (4, 154). DNMT1 binds to and methylates hemi-methylated CpG sites to ensure stable maintenance of DNA methylation (4). DNMT 3A and 3B are *de novo* methyltransferases that mainly lead to transcriptional repression through the establishment of non-CpG methylation, an emerging epigenetic mark that defines brain tissue-specific patterns of gene transcription (155–159). DNMT3L is catalytically inactive and serves as a cofactor for DNMT 3A and 3B (160, 161).

Although DNA methylation patterns are erased and deposited through successive normal developmental stages and cell differentiation, they also occur in the form of epigenetic memory in stem cells, and in communicable and non-communicable diseases, reviewed in (162). Most importantly, various epigenetic phenomena triggered in response to raging viral replication are usually hijacked by the same targeted virus to alter the protective immunoregulatory mechanisms for survival and propagation, reviewed in (163). For instance, during infection with hepatitis B virus (HBV), DNMTs are upregulated in response to productive viral replication mediated by the host-viral interaction as part of host immune defence mechanisms, also reviewed in (164). In the long run, the same DNA methylation machineries may start hypermethylating CpG island promoters that overlap with host-viral integration sites leading to alteration in the transcription of genes, including immunoregulators and tumour suppressors that are critical to carcinogenesis (164). COVID-19 related airborne respiratory infections such as the Middle East respiratory syndrome-CoV (MERS-CoV) and avian influenza (H5N1) have also been shown to exploit DNA methylators and

histone modifiers to suppress immunoregulators such as type 1 IFN-γ-responsive genes. These genes include *class II, major histocompatibility complex, transactivator* (*CTIIA*), *antigen peptide transporter 2* (*TAP2*), and *protein disulfide-isomerase A3* (*PDIA3*) (165). Abnormal regulation of these genes impedes the host immune system to fight infections effectively (166). This suggests that various epigenetic reprogramming phenomena may also occur during COVID-19 infection (167).

Mice transfected with hACE2 and subsequently infected with SARS-CoV-2 have been used to gain insights into epigenetic changes that drive cardiac injury in COVID-19 patients (153, 168). Li and colleagues identified 172 differentially methylated CpG sites in the hearts of SARS-CoV-2-infected mice compared with controls (153). Two genes, *paternally expressed gene 10* (*Peg10)* and *endothelin-converting enzyme 1* (*ECE1*), show high levels of differential methylation in SARS-CoV-2 mice bearing hACE2 compared with controls. For the *Peg10* gene, a hypomethylation pattern consistent with higher expression of the *Peg10* gene in hearts was seen. The loss of function of the *Peg10* gene is known to result in early embryonic death (169). *Peg10* gene also regulates cellular proliferation and viral replication through binding to the viral transcription regulators (170). SARS-CoV-2 infection was associated with increased methylation of the *ECE1* gene, the product that regulates proteolysis of endothelin precursors to form biologically active peptides (171). Loss of function of the *ECE1* gene is associated with cardiac defects, generalized oedema, and autonomic dysfunction (172). In another study, blood samples from acute SARS-CoV-2 infection *versus* healthy controls blood samples exhibited 28% of hypermethylated regions (173). Hypermethylated regions comprised of more than 5 consecutive differentially methylated CpG sites. It is not surprising that studies with SARS-CoV-1 and MERS also detected differentially methylated CpG sites, and found to be located in the promoter regions encoding genes involved in interferon and antigen presenting cells stimulation (174). This supported a recent study that identified >40 CpG sites encoding genes serving similar purposes, suggesting the role of DNA methylation influencing COVID-19 progression and target for epigenetic therapy (175).

Activation of the immunoregulatory cytoplasmic transcription factor aryl hydrocarbon receptor (AHR) may also result in hypermethylation that contributes to COVID-19 pathogenesis. The AHR has been identified as a host factor for Zika and Dengue viruses, and its inhibition was associated with significantly reduced viral replication and amelioration in the disease pathology (176–178).

It has been shown that the AHR becomes activated upon SARS-CoV-2 infection (178), and that it impacts SARS-CoV2 antiviral immunity and pathogenesis, promoting a pro-inflammatory response and participating in the severity of COVID-19 (178). Furthermore, it has been postulated that AHR activation may be the culprit behind the COVID-19-mediated cytokine storm (145, 178, 179). RNA-Seq analysis of CoV-infected cells unveils an upregulation of the AHR and its target genes, including AHRR and CYP1A1 (177). Kynurenic acid, a product of normal metabolism of L-tryptophan, and a potent endogenous AHR ligand, has also been shown to be elevated in response to COVID-19 (139, 180). This correlated with cytokine storm, age and low levels of T-cell responses, especially in males as compared to female patients, hinting for a sex-specific link to immune response and COVID-19 clinical outcome (139). Curiously, activation of the AHR has been associated with hypermethylation in acute lymphoblastic leukaemia (ALL) *in vitro*. When demethylated by methylation inhibitor zebularine, AHR-related methylation inhibition restored normal cells phenotype and prevented tumorigenesis (181). In another study, AHR activation resulted in epigenetic alteration of Foxp3 and IL-17 expression and consequently attenuated colitis (182). Recently, Jiadi et al. (183), have shown that macaques infected with SARS-CoV-2 modulation of the AHR upregulates the expression of ACE2 by binding to its promoter regions, and this is accompanied by aggressive disease. Consequently, if the AHR becomes hypermethylated, as shown in other pathologies, the level of ACE2 may also be silenced through the same methylation. This may disrupt the inhibitory mechanisms regulated by ACE inhibitors or other RAAS blockers leading to the aggressiveness of underlying cardiovascular diseases (e.g., hypertension) that have been reported in severe/critical cases of COVID-19 infection.

Interestingly, the AHR also regulates the expression of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3), that may also be epigenetically regulated (184, 185). Castro de Moura et al., revealed a strong correlation between COVID-19 clinical severity and DNA methylation of 44 CpG sites with >50% of these located in 20 promoters of annotated coding genes including *Absent in Melanoma 2* (AIM2) and *major histocompatibility class 1C (HLAC)* (175). *AIM2*, similarly to the NLRP3, is part of the inflammasome complex (186). The inflammasome is involved in caspase-1 cleavage, trigger of gasdermin D-mediated pyroptotic cell death and release of pro-inflammatory cytokines IL-1β and IL-18 in response to pathogens' insult, reviewed in (187, 188). Altered levels of IL-1β and IL-18 cytokines were observed in COVID-19, as it does in several male infertility-related disorders such as varicocele (49, 111, 189), suggesting that NLRP3 may also be activated upon COVID-19 infection. This notion is supported by the study of Su et al. (190), that demonstrated that upregulation of calcium-sensing receptor (CaSR) activates the NLRP3 pathway in testicular macrophages and impairs testosterone synthesis in a uropathogenic *Escherichia Coli* (UPEC) rat orchitis model.

Chronic infections such as HBV, hepatitis C virus (HCV), and human immunodeficiency virus (HIV) have been demonstrated to infect sperm cells and trigger oxidative stress. Subsequently, this activates histone modifications leading to long-term effects on male fertility parameters such as sperm integrity, count, motility, and morphology. During normal differentiation, sperm cells' genome undergoes successive rounds of epigenetics marks to ensure proper spermatogenesis and spermiogenesis (191). More than 85% of human mature sperm cells' DNA is bound to protamines. Protamines are sperm-specific basic nuclear proteins that take over the histones' position and function to package the sperm DNA for

compaction necessary for sperm motility (192). In the late stages of spermatogenesis, sperm cells' genome becomes dramatically reorganised and globally hyperacetylated to remove and replace histones with protamines. This phenomenon essentially erases the epigenetic modifiers laid out through histone modifications. It preserves the paternal genome by protecting it from extracellular stressors and harmful effects of the oocyte during fertilisation (192). An altered protamine ratio or histone content or distribution in sperm is a sign of aberrant chromatin packaging, associated with increased susceptibility to DNA damage or abnormal epigenetic marking that may lead to male infertility. Ma et al. (108), have detected SARS-CoV-2 in the testis' biopsies of COVID-19 patients. Immunohistochemistry analysis revealed a significant increase in spermatogenic epithelial shedding in the deceased patients with critical cases of COVID-19, which was accompanied by thinning of seminiferous tubules (193). Inflammation of the epididymis and/or testicle was associated with old age (>80yrs) and severe or critical cases of COVID-19 (P = .037) (107). More than 20% of recovered patients who previously had children through natural birth exhibited autoimmune orchitis. This was indicated by observed oligospermia, leukocytospermia, elevated sperm phagocytic CD3+/CD68+ immune responses in testes/epididymis and apoptotic cells relative to age-matched control males (193). In some cases, tocilizumab was administered in response to a progressive worsening of oxygenation, and blood biochemistry tests revealed an elevation of lactate dehydrogenase to 1213 U/l, D-dimer to 1150 ng/ml, and CRP to 23.80 mg/dl. Impaired spermatogenesis and increased apoptotic cells may be attributable to COVID-19-induced histone modifications associated with elevated CRP and fever that perturbed the optimum testicular temperature (2 – 4 ^{0}C below the average body temperature) (194). Moreover, extensive germ cell destruction, as demonstrated by the TUNEL assay, may have also been a contributing factor.

Erasing of DNA Methylation: Role of TETs

TETs are regarded as erasers of DNA methylation, reviewed in (195). They actively or passively demethylate DNA methylation by removing the 5-methylcytosine mark. TETs oxidise 5-methylcytosine to generate 5-hydroxymethylcytosine (5-hmc), 5-formylcytosine (5-fc), and 5-carboxycytosine (196–198). 5-hmc is a stable epigenetic mark that is highly abundant in the brain, liver, and stem cells, and it is crucial for neurogenesis and hepatocellular carcinoma (HCC) (199, 200). TETs are prominent regulators of immune cells. For example, Tet-2 mediates T-cell differentiation and synergises with Tet-3 to modulate the expression of Foxp3, a transcription factor responsible for T-cells development (201). Tet-deficient mice CD4-T cells exhibited impaired Th1/2/17 differentiation and cytokine production in lymphocytic choriomeningitis virus infection, supporting a critical role of Tet-2 in infections (202). In other studies, loss of Tet2/3 resulted in an antigen-driven expansion of various immune cells and rapidly developed aggressive disease phenotype (203, 204). Moreover, combined deletion of Tet2/3 in mice exhibited impaired Treg cell differentiation. This was accompanied by DNA hypermethylation of various Treg-specific demethylated regions (TSDRs) within the Foxp3 locus that resulted in aberrant Foxp3 expression (205, 206). TNFs and ILs, important cytokine storm elevated markers observed in severe or critical cases of COVID-19, are known to induce DNA demethylation *via* TETs (207–210). IL-1β and TNF-α modulate the global hydroxymethylation by activating TETs and iso-citrate dehydrogenases in the genomic DNA and specific locus in matrix metalloproteinase (MMP) promoter region in human OA chondrocytes (211).

In severe COVID-19 pneumonia cases, abnormal upregulation of T-cell proliferation, activation, and cytotoxicity was noted at the late phase of infection, suggesting an underlying perturbation resulting in the loss of an inhibitory role Tregs (212). Mohebbi et al. (210), have shown that CD4+ FoxP3+ CD25+ T cells expression level is significantly suppressed in hospitalised COVID-19 patients and led to an elevated level of IL-6. Given this evidence, it is intriguing to suggest this aberrant hyperactivation of cytotoxic cells in COVID-19 may be attributable to Tet-2/3-mediated epigenetic regulation of Tregs. Cell division occurring as a result of antigen and cytokine stimulation in response to COVID-19 infection may be the underlying mechanism for this epigenetic reprogramming. This may result in aberrant gene transcription, fatal inflammatory response, disease aggressiveness, and multi-organ disease phenotypes observed in severe and critical cases of COVID-19.

Abnormal production of the IFN and IFN-γ correlate with slowly resolved COVID-19, and enhanced viral replication was also observed, as previously reported in other studies (213–215). This may also correlate with genetic variation of heat shock protein 70 (HSP70) or A1L (HSPA1L), which has been demonstrated to result in significantly higher plasma concentrations of TNF-α and IL-6 and poor clinical outcomes after severe tissue injury from pathogens (216). Elevated levels of TNF-α and IL-6 are associated with severe cases of COVID-19 and systemic inflammation, as well as *HSPA1L* gene upregulation *via* hypomethylation of its promoter regions in response to increased SARS-CoV-2 viral replication (217). *HSPA1L* hypomethylation is catalysed by the dramatically reduced DNA methyltransferases (DNMT 1 - 3), possibly *via* TETs and postulated to enable viral cell entry and protein synthesis (217, 218).

Writing Histone Modification: Role of HATs and HMTs

Histone lysine acetylation is catalysed by conserved histone acetyltransferases (HATs) and plays a crucial role in viral infections (219, 220). It facilitates the transfer of an acetyl functional group from acetyl coenzyme A to the ε-amino group of the lysine residue at one end of the histone molecule on the chromatin. HATs alter the charge of various lysine residues within either H3 (histone acetylation at lysine 9, 14, 18, and 23, denoted as H3K9/14/18/23ac) or H4 (H4K5/8/12/16ac), reviewed in (221–223). A positive charge from lysine becomes neutralised by a negative charge from a transferred molecule, reducing the binding affinity between histones and DNA. This alters the chromatin architecture by opening the chromatin and making it accessible to the transcription factors for active gene expression (221–223). MYST writes histone

acetylation, adenoviral E1A-associated protein of 300 kDa/CREB-binding protein (p300/CBP) and general control non-derepressible 5 (GCN5)-related N-acetyltransferases (GNATs) and read by bromodomains (BRD) and extra-terminal (BET) family of proteins (221, 222).

Histone H3 and H4 form a significant component of the host immune defence mechanism against pathogen insults and other hostile environments. *In vitro* studies with retroviral infected mouse embryonic fibroblasts have shown that histones are loaded rapidly on unintegrated retroviral DNA soon after infection (219, 220). Unintegrated retroviral DNA is typically weakly expressed, but in response to interaction with loaded histones, their expression may become dramatically increased by chromatin modifiers and promote persistent infection (219, 220). Several studies have shown that histones can be released into blood circulation during an infection as damage-associated molecular patterns (DAMPs) from apoptotic and damaged cells, eliciting an inflammatory stimulus (224–226). DAMPS interact with TLRs, and trigger TLR/myeloid differentiation factor 88 (MyD88)/NLRP3 pathways leading to activation of macrophages (227). This, in turn, can cause an accumulation of neutrophil infiltration and subsequent production of neutrophil extracellular traps (NETs) and reactive oxygen species (ROS) (227). Activation of TLR/MyD88/NLRP3 pathways has been upregulated in obese patients that are at high risk of severe COVID-19 infection (228). This suggests that activating these pathways by the DAMPs and histones loaded on viral proteins may be the mechanism underlying an excessive tissue inflammation and injury that correlates with multiple organ failure and increased mortality in COVID-19 infection.

Histones can also bind to complement component 5a (C5a) and CRP, which are proteins expressed by the liver in response to systemic inflammation (229–231). CRP is a regulatory factor for angiogenesis and thrombosis associated with cardiovascular disease (CVD), which is a risk factor for COVID-19 severe cases (232). An elevated level of C5a and CRP in COVID-19 infection is an indication of excessive inflammatory response in endothelial cells and tissue damage that correlates with aggravated disease or poor prognosis. Neutrophils play an important role in the early or later stages of severe cases of influenza A virus (IAV), and COVID-19 infection cases, where circulating cell-free histones are enriched and highly pro-inflammatory (233). Hsieh et al. (233) have shown that binding of histones H4 to CRP in neutrophils models infected with IAV blocks the H4-mediated neutrophil activation and potentiates neutrophil inflammatory response during infection (233). This data suggests that H4 may be part of the host protective mechanism during excessive pro-inflammatory response. However, in response to interaction with circulating virus through molecules such as C5a and CRP, this mechanism may be hijacked by the virus for its replication advantage leading to tissue damage and fatal sequelae observed in COVID-19.

A case study of four unrelated young men who were critically ill with COVID-19 infection, and subjected to mechanical ventilation in the ICU, revealed nonsense and missense X-chromosomal TLR7 variants using whole-exome sequencing (213). This TLR7 variant mutation resulted in a unique loss of function from aberrant alteration of TLR7 mRNA expression and its downstream target genes. *Interferons regulatory factor 7* (*IRF7*), *interferon beta 1 (IFNB1*), and *interferon stimulated gene 15* (*ISG15*) are examples of genes associated with this TLR7 variant mutation. IRF7 becomes acetylated by HATs p300/CBP-associated factor (PCAF) and GCN5, and this usually impairs its binding activities leading to reduced IRF7 activity. PCAF acetylase complex and GCN5 are required for viral integration, and they have also been shown to be activated in influenza A virus to negatively regulates the viral polymerase activity (234, 235). PCAF is also known as lysine acetyltransferase 2B (KAT2B), a master regulator of TGF-β signalling pathway that triggers CVD development when altered. The SARS-CoV-2 virus induces an aberrant and excessive TGF-β-mediated chronic immune reaction creating a switching from IgM to IgA1 and IgA2 immunoglobulins (38, 236). This, in turn, causes an increased pro-inflammatory response and severe disease activity that correlates with prolonged ICU COVID-19 cases and fatalities (38, 236). It is important to investigate the possible roles of PCAF and GCN5 activities in regulating TGF-β and TLR7 signalling pathways in severe COVID-19 for novel treatments to ameliorate the severity and prevent COVID-19 fatalities.

Unlike histone acetylation, histone methylation does not modify any histone protein charge but deposits one or a set of methyl groups from S-adenosyl methionine (SAM) on the side chains of either H3 or H4 lysines or arginine (237). Histone methylation is catalysed by histone methyltransferases (HMTs) with various methylation sites (238). One of these HMTs is SET1B with H3K4me3 occupancy on open chromatin, and this recruits transcription factors for epigenetic transcriptional activation (239). This epigenetic tag has been shown to induce hypoxia, one of the emerging key drivers of COVID-19 pathogenesis and related fatalities. COVID-19 related-hypoxia manifests insufficient levels of oxygen supply in various tissues. SET1B activation is oxygen-dependent and facilitates hypoxia responses *via* site-specific histone methylation (240). In response to hypoxia, SET1B is recruited to the hypoxia-inducible transcription factor (HIF) promoter *via* HIF1α and facilitates the expression of genes involved in angiogenesis (240), one of the clinical features of COVID-19 severity. HIF-related genes will be described further in a later section of histone demethylation.

Erasing Histone Modification: Role of HDACs and LSDs/KDMs

Histone acetylation and methylation are erased by HATs and lysine demethylases (KDMs)/lysine-specific demethylases (LSDs), respectively. The former result in a more condensed, closed, and transcriptionally silenced chromatin structure that is not accessible to transcription machineries (6–10). The latter blocks the recruitment or occupancy of transcriptional factors on the chromatin sites (9). The process is called histone lysine deacetylation or demethylation, and it associates with the repression of gene transcription. HDACs are grouped into four classes, including class I (HDACs 1 - 3 & 8), class II (HDACs 4 -7, 9 & 10), class III (Sirtuin 1 - 7) class IV (HDAC 11), reviewed in (241, 242). KDMs/LSDs include KDMs/LSDs 1 – 6 with several families that act on different substrates for various cellular processes.

Histone repression marks are common phenotypical features in viral infections and other diseases, such as cancer (243). Virus-induced cancers from HBV, HPV, and EBV hijack histone acetylation marks for viral survival and propagation, and various HDACs inhibitors have been shown to circumvent these effects and alleviate the disease (244–247). Sirtuin 1 (SIRT1) is a key epigenetic regulator of CVD, metabolic and age-related disease through interaction with nuclear transcription factor-κB (NF-κB), a master regulator of inflammation activated by a signal transducer and activation of transcription 3 (STAT3) (248, 249). STAT3 becomes hyperactivated and impairs immune defence machineries that promote exacerbated inflammation and lymphocytopenia, leading to lung fibrosis and thrombosis, as demonstrated in severe COVID-19 cases (249). SIRT1 was also shown to interact with and modulate p53 activities to regulate viral replication in MERS-CoV and SARS-CoV infections (250, 251). Takahashi et al. (252), have recently shown that panobinostat, an inhibitor that counteracts HDACs effects, silenced the transcription of ACE2 receptor and ABO gene (gene encoding three blood group alleles) in cultured epithelial cell lines. This suggested a potential preventative drug against COVID-19 infection (252, 253). ACE2 is the primary host receptor for viral entry, whereas the ABO blood group system has been suspected to increase susceptibility for severe COVID-19 cases (252, 253). Related to this, Zhao et al. (254), have shown that blood group A individuals may be susceptible to COVID-19 infection, owing to the enrichment of group A antigen in respiratory cells (254).

Upregulation of HDACs by hypoxia was shown to be activated in response to a silence in hypoxia-responsive tumour suppressor genes (255, 256). These genes include HIF-1α and vascular endothelial growth factor (VEGF), and their epigenetic-mediated alteration correlates with a dramatic increase of intussusceptive angiogenic features (255). A similar clinical phenotype was observed in the lungs of deceased COVID-19 patients, exhibiting distinctive pulmonary vascular pathophysiologic features in a background of perivascular inflammation and injury, as relative to those of influenza (257). VEGF is a prominent mediator of angiogenesis and is usually involved in wound healing (257). VEGF exerts its activities through VEGFR 1–3, which are targeted and negatively regulated by epigenetics alterations (258). VEGFR3 receptor has two ligands, VEGF-C and VEGF-D, which stimulate angiogenesis. Interestingly, serum levels of VEGF-D were found to be significantly elevated in ICU COVID-19 patients as compared to non-ICU patients, a novel biomarker to trace the progression of disease (258). Current research shows that VEGF and its associated receptors undergo histone deacetylation, suggesting them as potential epigenotherapy targets. It has previously been shown that histone deacetylase 4 (HDAC4) remodels neuronal morphology by altering the transcription signature of VEGF-D (259). Activation of HDAC2 suppresses inflammatory cytokines (e.g., IL-17, **Figure 4**) in pulmonary disease, and this with the disease onset and sometimes with prognosis (260–262). On another note, Ahmad et al. demonstrated that endothelial TLR/MyD88 signalling is regulated by histone deacetylase 6 (HDAC6), contributing to alveolar remodelling architecture and pulmonary inflammation (263). Upregulation of TLR/MyD88 signalling pathway in association with elevated TNF-α and IL-6 was reported in overweight and obese individuals as compared to lean individuals (263). In referral to this observation, Cuevas and co-authors have recently published a brief communication postulating and probing for a research study that upregulation of TLR/MyD88 signalling pathway may contribute to excessive and fatal pro-inflammatory cytokine storm especially in SARS-CoV-2 vulnerable obesity individuals (228). MyD88 was shown to establish and promote CD4 T-cells responses to control viral spread to the central nervous system (CNS) in coronavirus-induced encephalomyelitis (228). Any abnormal regulation of MyD88 signalling already existing in obese individuals and other co-morbidities may impact COVID-19 disease progression leading to more fatalities (228).

The widespread methylation of genes in SARS-CoV-2 infection is associated with the downregulation of genes involved in the regulation of the tricarboxylic acid (TCA) and mitochondrion electron transport chain (153). SARS-CoV-2-induced epigenetic alterations interfere with metabolic processes that are core to generating energy for the myocardium (153). The perturbed metabolic processes restrict the energy required for uncontrolled systemic inflammatory response leading to myocardial injury. Transcriptome analysis studies conducted from patients with hypertension and DM associated with severe COVID-19 cases revealed that ACE2 expression was potentially regulated synergistically by various histone marks such as histone acetyltransferase 1 (*HAT1*), *HDAC2*, and lysine demethylase 5B (*KDM5B)* (264). *KDM5B* is a histone H3K4me2/3 demethylase that is associated with therapeutic resistance in cancer (264). Hinohara et al. (264), demonstrated that inhibition of KDM5B increases sensitivity to endocrine therapy by modulating oestrogen receptor, suggesting the therapeutic potential of this epigenetic demethylating mark. Concerning viral infections, KDM5B was shown to suppress stimulator of interferon genes (STING), a cytosolic DNA sensor that activates downstream transcription factors signal transducer and activator of transcription 6 (STAT6), and interferon regulatory factor (IRF3) through TANK-binding kinase 1 (TBK1) (265). This, in turn, protects the host cells by eliciting an antiviral response and innate immune defence against intracellular pathogens and cancer (265). SARS-CoV proteins were shown to interact with STING and activate the STING-TRAF3-TBK1 complex leading to abnormal alteration and inhibition of type 1 IFN activities that may be associated with severe disease (266). 3C-like (3CL), the main protease and regulator of viral replication for SARS-CoV-2, was shown to inhibit the activation of immune defence machinery by perturbing both RIG-I-like receptors (RLR) and cGAMP binds to stimulator of interferon genes (C-GAS-STING) pathways in human lung cells, suggesting a mechanism that will enable the virus to replicate more efficiently during infection (267). Upregulation of STING and aberrant activities usually correlate with cytokine storm in older people and those who suffer from metabolic disorders (268–271). This may explain the increased COVID-19 severe cases in patients who are older, diabetic, and hypertensive.

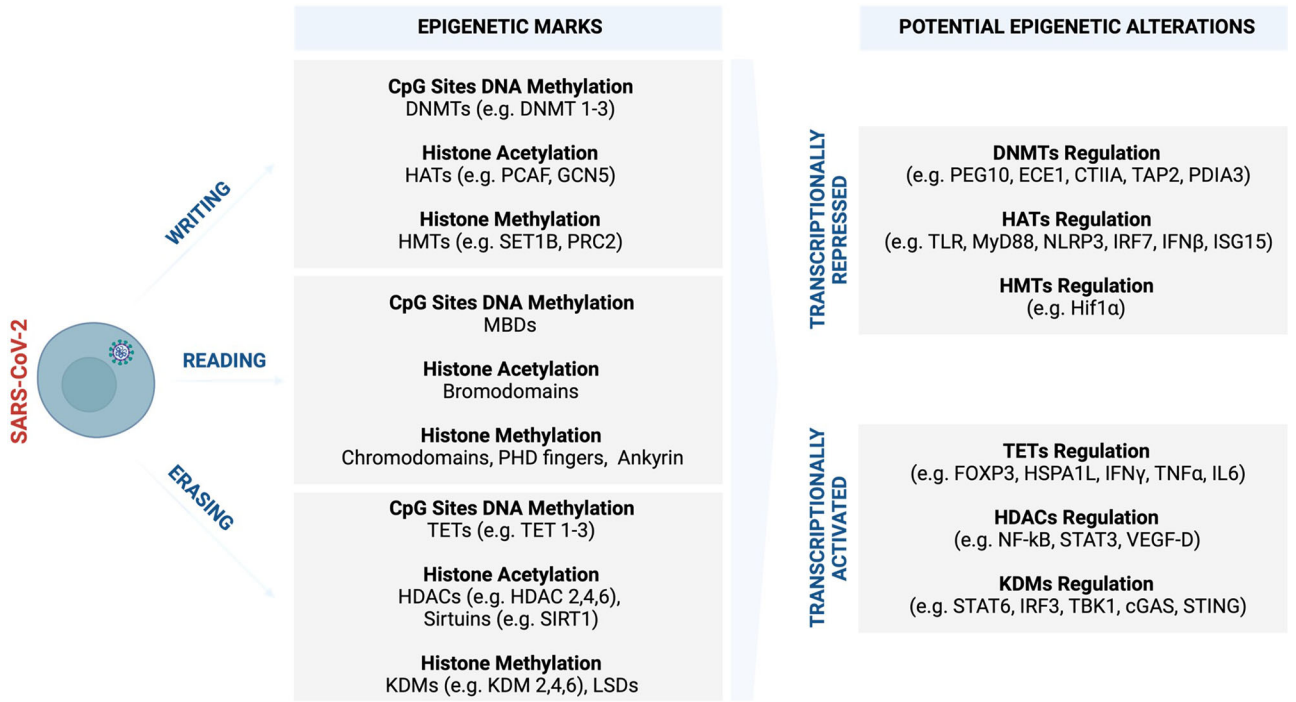


FIGURE 4 | Potential COVID-19 related epigenetic alterations and clinical implications. Like many other viruses, SARS-CoV-2 may trigger various epigenetic alterations such as global DNA methylation and histone modifications, which synergistically cooperate in influencing and driving the course of COVID-19 and sex-bias differences. In response to increased viral replication, the infected host cells may set off an epigenetic signature to antagonise the virus as part of innate immune defence machinery. Part of this epigenetic landscape may be subverted to benefit the virus and its propagation, leading to enhanced and systemic COVID-19 infection that promotes severe complications. (Created with BioRender.com). cGAS, Cyclic GMP–AMP synthase; CIITA, The major histocompatibility class (MHC) II transactivator; DNMTs, DNA methyltransferases; ECE1, endothelin Converting Enzyme 1; FOXP3, Forkhead box protein P3; GCN5, General control non-repressed 5 protein; HATs, Histone acetyltransferase; HDAC, Histone deacetylase; HIF-1α, Hypoxia-inducible factor-1α; HSPA1L, Heat shock protein family A (Hsp70) member 1-like; IFN-γ, Interferon gamma; IFN-β, Interferon-β; IL-6, Interleukin- 6; IRF2, Interferon regulatory factor 2; IRF3, Interferon regulatory factor 3; ISG15, Interferon-stimulated gene 15; JmjC, Jumonji C; KDM, Histone lysine demethylases; LSD, Lysine-specific demethylases; MBDs, Methyl-CpG binding domains; MyD88, Myeloid differentiation factor 88; MLL, Mixed-lineage leukaemia; NF-κB, Nuclear factor kappa B; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; PDIA3, Protein disulfide isomerase family A member 3; PEG10, Paternally expressed gene 10; PHD, Plant homeodomain; PRC, Polycomb repressive complex; p300/CBP, p300 and cyclic AMP response element-binding protein; STAT-3, Signal transducer and activator of transcription-3; STAT-6, Signal transducer and activator of transcription-6; SET1, Suppressor of variegation 3–9, enhancer of zeste, trithorax 1; SIRT, Sirtuins; STING, Stimulator of interferon genes; TAP2, Transporter 2, ATP Binding Cassette Subfamily B Member; TBK1, Tank binding kinase 1; TET, Ten-eleven translocation; TLR, Toll-like receptors; TNF-α, Tumour necrosis factor-alpha; UTX1, Ubiquitously transcribed tetratricopeptide repeat, X chromosome 1; VEGF-D, Vascular endothelial growth factor D.

POTENTIAL OF EPIGENETIC DRUG TREATMENT IN COVID-19 INFECTION

Given the above evidence, it is of great interest to determine the impact of various epigenetic marks in COVID-19-related severity and progression for their exploitation for future COVID-19 epigenetic therapy. Although other molecules and pathways (e.g. nuclear factor erythroid 2–related factor 2/Nrf2 and NLRP3) could also be interesting to be mentioned and included in this section (184, 185, 272), we decided to focus on the AHR due to its prominent roles in diverse diseases, including COVID-19. The AHR is a ligand-activating transcription factor that may be activated in response to infection. Its activation has been postulated many times as part of the mechanism behind the cytokine storm and poor clinical outcomes including increased fatalities associated with COVID-19 (145, 178, 179, 273, 274). While cytokines protect against viral infections, they can also be aberrantly regulated and produced excessively. This may unintentionally induce indoleamine 2,3-dioxygenase (IDO), most excessively in male COVID-19 patients, leading to abnormal accumulation of kynurenine that activates the AHR. The AhR is widely expressed in various tissues and thus transcriptionally upregulates the expression of ACE2 receptor in macaques infected with SARS-CoV-2 (273). This enhances SARS-CoV-2 infection resulting in cytopathic effects in various cells and impaired antiviral response, thereby leading to systemic tissue damage and organ failure.

Furthermore, the AHR activation has also been shown to be differentially regulated in comorbidities (e.g. smoking, age, obesity, hypertension, and diabetes) that are strongly linked to poor clinical outcomes of COVID-19 (275–278). Different epigenetic regulation of AHR (181, 181, 279–282) could explain epigenetic regulation of ACE2 receptor, differentially methylated CpG sites observed in COVID-19 and poor clinical

outcomes of COVID-19 in some individuals. For instance, activation of the AHR is also associated with reversible hypermethylation in human malignancy including acute lymphoblastic leukaemia (ALL) in *in vitro* studies (181). When demethylated by methylation inhibitor zebularine, AHR-related methylation inhibition restored normal cells phenotype and prevented tumorigenesis (181), suggesting it as a suitable and promising candidate/s for epigenetic therapy.

Likewise, various clinically approved drugs, such as dexamethasone, that are currently used/tested to ameliorate the COVID-19, have been shown to impact the activity of the AHR and to be involved in resistance to therapy, not only in infectious diseases (e.g. tuberculosis) but also in cancer (e.g. melanoma) (279, 280, 283–287). Curcumin and dexamethasone and are 2 classical examples of epigenetics reprogramming drugs and may be helpful to treat COVID-19 toxicity by counteracting the effects of molecules such as the AHR (279, 280). Of note, curcumin can modulate AHR activity (281, 288). Curcumin is a turmeric herb that exerts its potent anti-inflammatory and antioxidant properties by inducing epigenetic reprogramming *via* regulation of DNMTs, HATs, HDACs, and miRNA, reviewed in ref (282).. Various *in vitro* and *in vivo* studies in liver-related diseases have demonstrated that the use of curcumin is associated with suppressed cell growth and reduced liver injury (289, 290). It has been shown that curcumin exerts its activities by inhibiting HDAC activated by the nuclear factor kappa B (NF-κB) pathway (290). It is important to note that this pathway is known to interact with AHR and thus contributing to the regulation of COVID-19-mediated cytokine storm (274). Dexamethasone is a potent anti-oedema/fibrotic corticosteroid agent, and it was shown to accelerate AHR degradation and suppress the expression of its downstream target genes *in vitro* studies (291). Proper dosage of dexamethasone reduced the likelihood of progression of the disease, leading to shorter hospitalisation and reduced fatalities by approximately one third in COVID-19 patients requiring ventilation and by one fifth in those requiring oxygen (280). The use of dexamethasone in cholestatic rats was associated with decreased hepatic inflammation and oxidative stress (292). Investigating epigenetic reprogramming by various receptors and drugs may provide novel therapeutic opportunities to control the current pandemic.

FUTURE PERSPECTIVES AND DIRECTIONS

SARS-CoV-2 may trigger epigenetic alterations affecting the expression of ACE2 and various immunoregulatory genes that play a key role in both immune defence machinery and metabolic pathways on different cells (167, 173–175). This may promote tissue damage and augmenting multi-organ pathology in SARS-CoV-2-infected tissues. Given the evidence above, differentially methylated CpG sites of a wide variety of promoters encoding immunoregulatory genes and ACE2 gene may be the primary COVID-19 epigenetic signature that are set off in response to increased viral infections as part of host immune responses as commonly observed in viral infections. Differential epigenetic regulation associated with ACE2 receptor and AHR (153, 169, 217) may favour viral entry and regulation of ACE2 expression by modulating different epigenetic marks, including DNMTs, H3K27me, KDM5B and SIRT1. These epigenetic marks control metabolic and immunoregulatory pathways, thereby promoting immune evasion and cytokine storm, leading to severe clinical pathologies such as ARDS and widespread tissue damage associated with multi-organ failure (52–61, 175). Detection of epigenetic signatures established in COVID-19 and their dynamics during viral entry and throughout infection (e.g. from asymptomatic to mild symptomatic, severe infection and long persistent symptoms) may be valuable for timely diagnosis and to help designing therapies that may curb the severity of COVID-19 and related fatalities. Type II diabetes mellitus, hypertension and CVD are significant metabolic complications that contribute to the mortality of patients COVID-19. Discovering epigenetic markers linked to these comorbidities and how they impact the severity of COVID-19 may also be valuable for prompting treatment to prevent progression to sequelae that promote COVID-19-associated fatalities mortality.

AUTHOR CONTRIBUTIONS

MK conceived the idea and drafted the manuscript. MS, PM-A, IL, GM, TB, PK, HR, PWM, MV, JZ, and HN collected some of the literature and contributed in some sections. MS, PM-A, and SG edited and revised the manuscript. MK and PM-A made final changes, edited and finalised the manuscripts, including the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Non-Coding RNAs in the Etiology and Control of Major and Neglected Human Tropical Diseases

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Non-coding RNAs (ncRNAs) including microRNAs (miRs) and long non-coding RNAs (lncRNAs) have emerged as key regulators of gene expression in immune cells development and function. Their expression is altered in different physiological and disease conditions, hence making them attractive targets for the understanding of disease etiology and the development of adjunctive control strategies, especially within the current context of mitigated success of control measures deployed to eradicate these diseases. In this review, we summarize our current understanding of the role of ncRNAs in the etiology and control of major human tropical diseases including tuberculosis, HIV/AIDS and malaria, as well as neglected tropical diseases including leishmaniasis, African trypanosomiasis and leprosy. We highlight that several ncRNAs are involved at different stages of development of these diseases, for example miR-26-5p, miR-132-3p, miR-155-5p, miR-29-3p, miR-21-5p, miR-27b-3p, miR-99b-5p, miR-125-5p, miR-146a-5p, miR-223-3p, miR-20b-5p, miR-142-3p, miR-27a-5p, miR-144-5p, miR-889-5p and miR-582-5p in tuberculosis; miR-873, MALAT1, HEAL, LINC01426, LINC00173, NEAT1, NRON, GAS5 and lincRNA-p21 in HIV/AIDS; miR-451a, miR-let-7b and miR-106b in malaria; miR-210, miR-30A-5P, miR-294, miR-721 and lncRNA 7SL RNA in leishmaniasis; and miR-21, miR-181a, miR-146a in leprosy. We further report that several ncRNAs were investigated as diseases biomarkers and a number of them showed good potential for disease diagnosis, including miR-769-5p, miR-320a, miR-22-3p, miR-423-5p, miR-17-5p, miR-20b-5p and lncRNA LOC152742 in tuberculosis; miR-146b-5p, miR-223, miR-150, miR-16, miR-191 and lncRNA NEAT1 in HIV/AIDS; miR-451 and miR-16 in malaria; miR-361-3p, miR-193b, miR-671, lncRNA 7SL in leishmaniasis; miR-101, miR-196b, miR-27b and miR-29c in leprosy. Furthermore, some ncRNAs have emerged as potential therapeutic targets, some of which include lncRNAs NEAT1, NEAT2 and lnr6RNA, 152742 in tuberculosis; MALAT1, HEAL, SAF, lincRNA-p21, NEAT1, GAS5, NRON, LINC00173 in HIV/AIDS; miRNA-146a in malaria. Finally, miR-135 and miR-126 were proposed as potential targets for the development of therapeutic vaccine against leishmaniasis. We also identify and discuss knowledge gaps that warrant for increased

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research work. These include investigation of the role of ncRNAs in the etiology of African trypanosomiasis and the assessment of the diagnostic potential of ncRNAs for malaria, and African trypanosomiasis. The potential targeting of ncRNAs for adjunctive therapy against tuberculosis, leishmaniasis, African trypanosomiasis and leprosy, as well as their targeting in vaccine development against tuberculosis, HIV/AIDS, malaria, African trypanosomiasis and leprosy are also new avenues to explore.

Keywords: non-coding RNAs, tuberculosis, HIV/AIDS, malaria, leishmaniasis, African trypanosomiasis, leprosy

INTRODUCTION

Non-translated or non-coding RNAs (ncRNAs) are the transcripts of the genome that are not meant to be translated into proteins (1). They represent about 98% of total RNAs content within the human cells (2, 3) and were initially thought to be byproducts of transcription, therefore referred to as "Junk RNAs". However, a growing body of evidence have unveiled the role of certain ncRNAs including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) as key regulators of gene expression, that is they can alter genes expression in a reversible, transmissible, and adaptative way, without modifying the DNA sequence (4). MicroRNAs are an abundant class of highly conserved small (18-25 nucleotides long) RNA species that generally downregulate the expression of their target genes at the post-transcriptional level. Mechanically, miRNAs bind in a sequence-specific manner to complementary regions in the 3' untranslated region (3'UTR) of their target mRNAs, thereby triggering mRNA degradation or translation inhibition. In this way, a single miRNA can control the expression of several genes and a single gene expression can be controlled by several different miRNAs (5). Contrary to miRNAs, lncRNAs (at least 200 nucleotides long) are less studied, display poor sequence conservation and regulate the expression of their nearby proximal genes (Cis regulation) as well as distant genes (Trans regulation) at the chromatin, transcription and translation levels (6–8).

Several miRNAs and lncRNAs are emerging as key regulators of immune cells differentiation, activation, and function, including macrophages, dendritic cells and T lymphocytes (6). Some have been associated with specific disease conditions such as Cancer, cardiovascular, developmental (1, 9–12), neurodegenerative (13) and major infectious diseases such as tuberculosis and HIV/AIDS (14–16). There are however few or no studies addressing the role of ncRNAs in the etiology, diagnosis, treatment, or vaccine development for neglected human tropical diseases (NTDs) which are a group of less investigated infectious diseases especially common in tropical areas such as Africa and Southeast Asia where people do not have proper access to clean water and adequate means to discard their waste. In this review, we summarize most recent findings on the role of miRNAs and lncRNAs on major human tropical diseases including tuberculosis, HIV/AIDS and malaria. We also provide a first-time summary of our current understanding of the role of these ncRNAs in the etiology and control of neglected tropical diseases including leishmaniasis, African trypanosomiasis and leprosy. We also identify and discuss knowledge gaps that warrant for increased research effort.

TUBERCULOSIS

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*) which has topped HIV as the deadliest infectious agent worldwide since 2017. Developing countries are highly burdened by this disease and further threatened by the emergence of multi-drug resistant and extensively drug resistant *Mtb* strains. Accurate diagnosis and effective treatment are the key elements to interrupt TB transmission (17). Several studies have linked miRNAs and lncRNAs to the onset and progression of TB, and some of those ncRNAs were identified as biomarkers for TB diagnosis or treatment (**Table 1**).

Non-Coding RNAs in the Etiology of Tuberculosis

To fight a bacterial infection, host innate and/or adaptive immunity has to be activated. *Mycobacterium tuberculosis* like many other successful pathogens has evolved mechanisms to avoid the host immune system and ensure its intracellular survival and persistence. This is possible through the subversion of key ncRNAs that control the cellular and humoral processes enacted in host innate and adaptive immune response against *Mtb* (**Figure 1**).

The induction of a robust yet controlled inflammatory response plays a key role in the containment and eradication of the infection at an early stage. It was found that *Mtb* suppresses *inflammation via* the upregulation of miR-21-5p, miR-27b-3p, miR-99b-5p, miR-125-5p, miR-146a-5p, miR-223-3p, and the downregulation of let-7f, miR-20b-5p and miR-142-3p (18). *Mtb* HN878 infection of monocyte-derived macrophages (MDMs) induces the expression of the pro-inflammatory lncRNA-PACER (also known as lncRNA-Cox-2) which is a positive regulator of its proximal pro-inflammatory gene Ptgs-*2* (Also known as Cox-2) (25).

Macrophage and other phagocytes are the first immune cells to encounter *Mtb* in the lungs and make use of their phagocytic activity to engulf and destroy the invading *Mtb* using different cell death mechanisms. *Mtb* has developed several strategies to avoid killing within phagocytes. *Mtb* inhibits the phagosome maturation and autophagy *via* upregulation of miR-33 locus (18, 26), miR-27a-5p (18, 20), miR-144-5p and miR-889-5p. *Mtb* also

TABLE 1 | Non-coding RNAs in the etiology and control of tuberculosis.

Role in tuberculosis	Non-coding RNA	Action	Reference
Etiology	miR-26-5p	Inhibition of innate immunity	(18)
	miR-132-3p		(18)
	miR-155-5p		(18, 19)
	miR-29-3p		(18)
	miR-21-5p	Suppression of inflammation	(18)
	miR-27b-3p		(18)
	miR-99b-5p		(18)
	miR-125-5p		(18)
	miR-146a-5p		(18)
	miR-223-3p		(18)
	let-7f		(18)
	miR-20b-5p		(18)
	miR-142-3p		(18)
	miR-33 locus	Inhibition of phagosome maturation and autophagy	(18)
	miR-27a-5p		(18, 20)
	miR-144-5p		(18)
	miR-889-5p		(18)
	miR-155-5p	Apoptosis inhibition	(18)
	miR–582-5p		(18)
Diagnosis	miR-769-5p	Downregulation in TB patients	(8)
	miR-320a		
	miR-22-3p		
	miR-423-5p	Upregulation in TB patients	(21)
	miR-17-5p		
	miR-20b-5p		
	lncRNA LOC152742		(22)
Therapeutic targets	lncRNAs NEAT1	Downregulation during drug treatment, association with disease improvement	(23)
	lncRNAs NEAT2		(23)
	lnrRNA 152742		(22)
	lncRNAENST00000429730.1	Downregulation during drug treatment, associated with complete inactivation of tuberculosis lesions from sputum negative patients	(24)
	lncRNA MSTRG.93125.4		(24)

evades the host defense by Inhibiting macrophage apoptosis (27) *via* the upregulation of miR-582-5p (28). It was also observed that *Mtb* HN878 infection of MDMs induces the expression of lincRNA-p21, a positive regulator of p53-dependent cell cycle arrest and apoptosis in numerous cell types (25, 29–32).

Much research is warranted to understand the contribution of lncRNA-PACER and lincRNA-p21 to the onset and progression of TB. Also there are growing body of evidence suggesting that *Mtb*-derived ncRNAs may be delivered to the host immune cells and affect their function. The contribution of such mechanisms to host immune evasion needs in-depth investigation.

Non-Coding RNAs in the Diagnosis of Tuberculosis

Several miRNAs are decreased in the plasma of *Mycobacterium tuberculosis* infected patients compared to healthy individuals and are described as biomarkers for the diagnosis of tuberculosis (33). Amongst those miRNAs, miR-769-5p, miR-320a and miR-22-3p subsequently showed higher specificity (4, 33) at 90% sensitivity (92%), AUC (95%) and lower heterogeneity (I= 0%) in ethological-confirmation validation sets (17). Also, miR-423-5p, miR-17-5p, and miR-20b-5p were reported to be significantly increased in the serum of patients with tuberculosis and had the potential to be used to diagnose TB with an accuracy of 78.18% (21). The level of long noncoding RNA LOC152742 was found to be high in sputum and plasma of infected patients, hence could serve as novel biomarker for the diagnosis of active tuberculosis (22).

Sputum-negative pulmonary tuberculosis cases showing no clinical or microbial evidence contribute to the development and spread of active tuberculosis (34). Accurate diagnosis of sputum smear-negative cases of pulmonary TB remains very challenging. It was found that lncRNAs ENST00000429730.1 and MSTRG.93125.4 were upregulated in lung tissue samples collected from patients with sputum-negative pulmonary TB with high metabolic activity as compared to low metabolic activity according to FDG-PET/CT(Positron emission tomography with computed tomography (PET/CT) using fluorine-18-fluoro-deoxyglucose (FDG)) classification. Hence these lncRNAs might be potential biological indicators of metabolic activity in tuberculosis lesions for sputum-negative tuberculosis (24).

The emergence of multidrug-resistant strains of *Mtb* has further complicated the control and eradication of this disease. It was found that the plasma levels of miR-320a were decreased in drug-resistant TB patients as compared to pan-susceptible TB patients (33). Therefore, this miR-320a may serve as a biomarker for drug-resistant TB. Also, lncRNAs CTD-2331D11.3 and AC079779.5 were found to be increased in the Peripheral Blood Monocytic Cells (PBMCs) from patients infected with

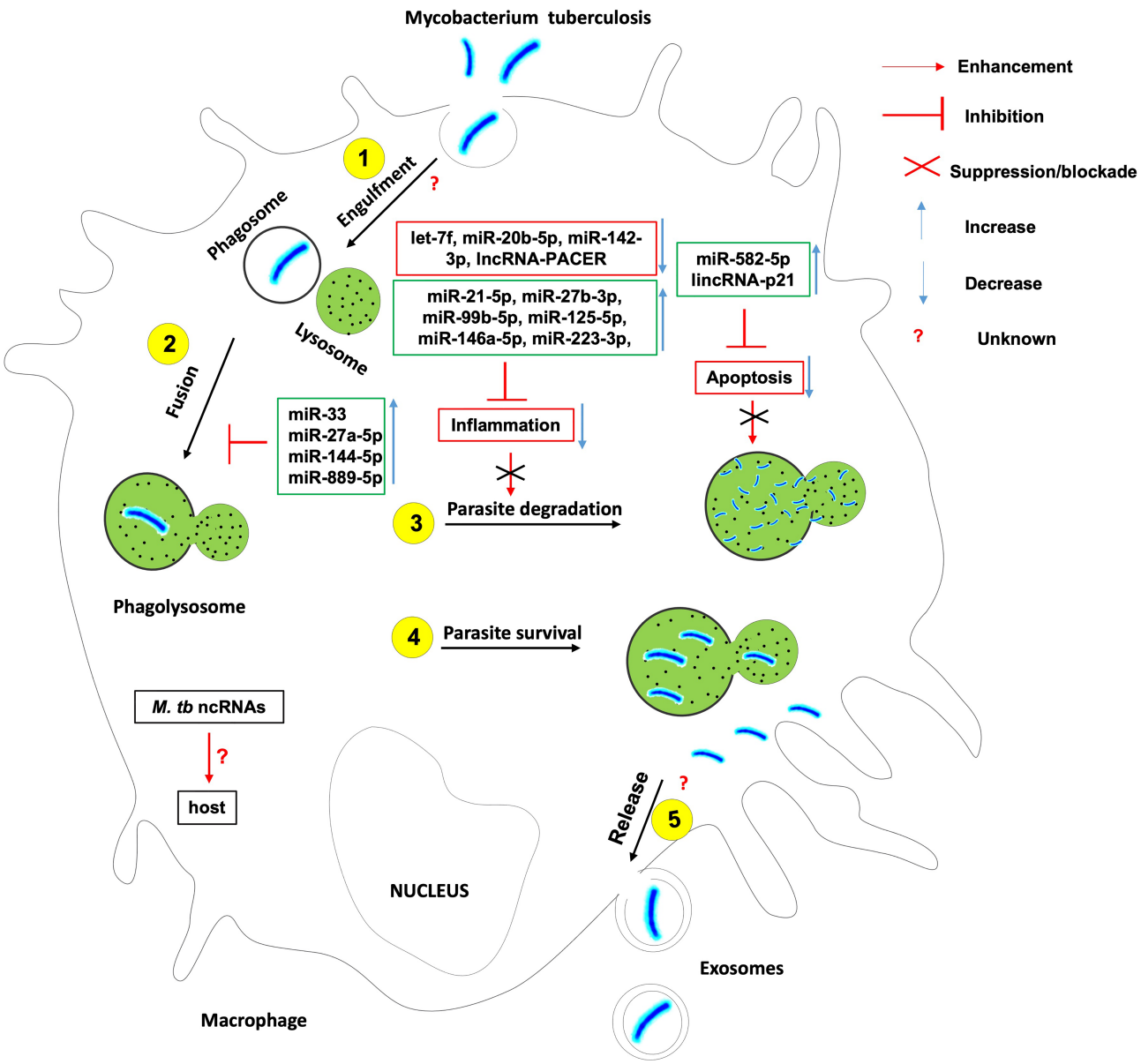

FIGURE 1 | The role of non-coding RNAs in the etiology of tuberculosis. Several ncRNAs enhance host effector killing functions against mycobacterium tuberculosis and favor the bacterium survival and persistence within the infected host. There is knowledge gap about 1- host ncRNAs that regulate the bacterium engulfment within phagosomes and release in extracellular vesicles such as exosomes; 2- the role of Mtb-derived ncRNAs on the outcome of Mtb-macrophage interaction.

Multi-drug resistant *Mtb* strains (MDR-TB) when compared to patients infected with drug-sensitive strains, indicating this lncRNAs may be potential biomarkers for multi-drug resistant TB (35).

Non-Coding RNAs as Therapeutic Biomarkers of Tuberculosis

The role of ncRNAs as potential host-directed therapeutical targets has been reviewed before (36). As a complement to that review article, recent studies have reported an increase in the expression of lncRNAs NEAT1 and NEAT2 in macrophages during *Mtb* infection. Their expression level was decreased during drug treatment, which was associated with improvement of the disease (23). The same observation was made with lnr6RNA 152742 which was upregulated in the plasma and sputum of patients and gradually downregulated in the course of the treatment (22).

Successful treatment of pulmonary tuberculosis is generally declared after absence of *Mtb* in sputum smear under microscopy and under culture. However, pulmonary TB lesions may still be harboring persisting slow growing, metabolically active but non-culturable bacilli that are less

sensitive to chemotherapy agent and may cause TB relapse (37, 38) lncRNAs ENST00000429730.1 and MSTRG.93125.4 described as indicators of metabolic activity in tuberculosis lesions for sputum-negative tuberculosis (24), hence are potential biomarkers of complete inactivation of tuberculosis lesions, thus of complete cure of tuberculosis (24).

MALARIA

Malaria is a mosquito-borne disease caused by parasites of the genus *Plasmodium*. It is transmitted through the bites of infected female *Anopheles* mosquitoes. Five parasite species cause malaria in humans: *P. knowlesi*, *P.malariae*, *P.ovale*, *P. vivax* and P. *falciparum*[1]. The two last pose the greatest threat[2]. In 2019, around 229 million cases of malaria were recorded in the world with approximately 409 000 deaths[3]. Symptoms of malaria comprise fever, shaking chills, headache, muscle aches, and tiredness. Nausea, vomiting, and diarrhea may also be involved[4]. Due to the non-specificity of its symptoms, it is difficult to distinguish malaria from other acute febrile illnesses. Non-coding RNAs, which are specific, can be of great help in resolving this problem.

Non-Coding RNAs in the Etiology of Malaria

Despite the growing recognition of the contribution of ncRNAs in the etiology of infectious diseases, only a handful studies have specifically associated ncRNAs with the onset or progression of any clinical form of malaria, be it uncomplicated or cerebral (**Figure 2**).

The clinical outcome of persons infected with Plasmodium falciparum parasites depends on many factors including parasite sequestration in tissues, host systemic inflammatory responses, and vascular dysfunction. It was found that *Plasmodium falciparum*-infected red blood cells release extracellular vesicles (EV) loaded with functional host miR-451a, miR-let-7b and miR-106b. These miRNAs-loaded EVs are internalized by endothelial cells within which they induce the production of surface receptor vascular cell adhesion protein 1 (VCAM-1) and proinflammatory cytokines such as interleukin-6 (IL-6) and interleukin-1 (IL-1). This will lead to the promotion of endothelial activation, leakage and parasite sequestration as well as vascular dysfunction and pathology during malaria infection (39). Contrary to the abovementioned microRNAs that promote malaria pathology, it was found that erythrocytes-derived miR-197-5p and miR-150-3p reduced the parasite growth, invasion and micronemal secretion *via* a mechanism involving the inhibition of the expression of apicortin, a *Plasmodium falciparum's* protein with putative microtubule-stabilizing properties (40). In addition, the resistance of sickle cell erythrocytes (carrying the HbS hemoglobin allele variant in either the heterozygous or the homozygous form) to infection by Plasmodium falciparum was associated to the expression of miR-451, miR- let-7i and miR-223 which were translocated into the parasite during its intraerythrocytic life cycle and negatively regulated the parasite growth (41). The mRNA targets of those microRNAs were however not investigated, neither was their contribution to the onset and maintenance of epigenetic marks such as DNA methylation and histone post-translational modifications on the promoter of possible target genes.

It was shown that knocking-out/down miR-155 led to reduced endothelial activation, reduced microvascular leak and preservation of blood-brain-barrier, reduced disease severity and improved survival in an experimental mouse model of cerebral malaria and an engineered human endothelialized microvessel system (42). Similarly, It was found that miR-19a-3p, miR-19b-3p, miR-540-5p, miR-142-3p and miR-223-3p were significantly upregulated in the brain of mice displaying cerebral malaria (infected with *Plasmodium berghei* ANKA) as compared to those with severe but non-cerebral malaria (infected with *Plasmodium yoelii*). These miRNA are involved in the control of TGF-β and endocytosis pathways which are known to be relevant to cerebral malaria (43). These works on gene-deficient mice models need to be repeated using humanized mice models and in-vitro human infection models for those data to have any translational value. Lastly, a mutation in the miRNA−146a was reported to be linked with increased odds for *P. falciparum* infection in first-time pregnant women, thus providing an indirect evidence of miRNA-146a protective role against P. falciparum infection (44).

Although no host lncRNA has been associated with malaria etiology to date, however, high throughput analysis of Plasmodium falciparum transcriptome have uncovered several lncRNAs including lncRNA-TARE (45, 46) and lncRNA var-AS (47, 48) that play important role in the development and virulence of the parasite. Research is warranted to investigate the role of these lncRNAs in the parasite immune evasion, host cell invasion and development within the human host.

Non-Coding RNAs in the Diagnosis of Malaria

The patients infected with *P. falciparum* manifest malaria of differing severities and clinical outcomes, such as uncomplicated malaria (UM), severe malaria, and cerebral malaria (CM). To date, few research have focused on investigating ncRNAs as biomarkers for the diagnosis of malaria. It was found that the plasma level of miR-451 and miR-16 were significantly lower in malaria patients compared to uninfected individuals, thus suggesting that plasma miR-451 and miR-16 are potentially relevant biomarkers for malaria infection (49).

Many miRNAs including miR-3135b, miR-6780b-5p, miR-1246, miR-6126, miR-3613-5p, miR-4532 and miR-6068 are upregulated in humans during the blood phase of P. falciparum infection as compared to negative controls. This upregulation was as the result of activation of host innate immunity (50). These miRNAs could be further investigated as potential blood biomarkers of the immunopathological state, thus helping in the early diagnosis of the disease. To date no lncRNA has been investigated as potential biomarker for the diagnosis of malaria.

[1] https://www.cdc.gov/malaria/about/biology/index.html
[2] https://www.who.int/news-room/fact-sheets/detail/malaria
[3] https://www.who.int/news-room/fact-sheets/detail/malaria
[4] https://www.cdc.gov/malaria/about/faqs.html

ncRNAs as Therapeutic Biomarkers of Malaria

MiRNA-146a is involved in innate immune response through a negative feedback loop comprising two key molecules downstream of the TLR machinery: the kinase associated with the interleukin -1 receptor (IRAK) -1 and the factor associated with the receptor of TNF (TRAF) -6. Recent studies have shown the potential of using miRNA-146a as a biopharmaceutical agent; The results of a current study suggest that miRNA-146a is involved in innate immunity against malaria, demonstrating its potency as a biopharmaceutical target (44).

HIV/AIDS

HIV remains a major global public health issue despite the increasing access to effective HIV prevention, diagnosis, treatment, and care, including for opportunistic infections. Approximately 38.0 million people were living with HIV at the end of 2019[5]. The Human Immunodeficiency Virus (HIV) targets the immune system and weakens people's defense against many infections and some types of cancer. As the virus destroys and impairs the function of immune cells, immunodeficiency gradually sets in the infected individual. The most advanced stage of HIV infection is Acquired Immunodeficiency Syndrome (AIDS), which can take many years to develop if not treated, depending on the individual. With the introduction of Highly Active Antiretroviral Therapy (HAART), HIV infection has become a manageable chronic health condition. There is still no cure or vaccine against HIV infection, which has been known for about forty years to date and for which research is restless (51). Many studies reported ncRNAs as novel targets for new drugs (**Table 2**). These ncRNAs influence the replication cycle of the virus.

Non-Coding RNAs in the Etiology of HIV Infection

It is known that HIV interacts with the host in order to complete its replication cycle, escape immune response and persist within infected hosts. Such interactions involve both host ncRNAs and HIV-produced ncRNAs amongst other factors (**Figure 3**).

HIV hijacks host ncRNAs to promote its replication within the host. Indeed, it was observed that at the peak of HIV-1 replication, the virus downregulates the expression of miR-29a and miR-29b in CD4(+) CD8 (–) PBMCs (67). These two miRNAs were previously reported to inhibit viral replication through direct targeting of a conserved site within the viral *nef* gene (68, 69). Also, miR-873 was shown to promote HIV-1 replication in an in-vitro infection model of Jurkat and 293T cells. miR-873 promotes the production of HIV-1 gag, pol and p24 proteins through yet undefined mechanisms (52). HIV also upregulates the expression of host miRNAs let-7c, miR-34a, and miR-124a to promote its propagation. Let-7c post-transcriptionally inhibits the expression of p21 which is a known negative regulator of viral integration and RNA expression within the infected host cell (70). miR-34a and miR-124a decrease the mRNA level of TASK1 (70), which is a mammalian potassium channel known to counteract the viral Vpu -induced release of HIV virions (71).

Besides miRNAs, HIV also hijacks numbers of lncRNAs to its advantage, including Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1), HIV-1 enhanced lncRNA (HEAL), LINC01426, lincRNA-p21 and Nuclear-Enriched Abundant Target 1 (NEAT1).

Indeed, induction of MALAT1 expression in T lymphocytes contributes to initial viral replication and to disease progression. Mechanistically, MALAT1 sequesters EZH2, the Histone H3K27 trimethylase of the polycomb repressive complex 2, hence releasing the epigenetic inhibition of the HIV-1 LTR promoter responsible for the latency (54).

HIV-1 infection-induced upregulation of lncRNA HEAL promotes the transcription of HIV-1 in both Monocytes-derived macrophages and in primary CD4+ T cells. Indeed, acting as a scaffold, HEAL recruits fused in sarcoma (FUS) RNA-binding protein to the promoter of CDK2 and HIV-1 LTR Which are known as activators of multiple proteins essential for HIV-1 transcription (55).

HIV1 infection-induced LINC01426 enhances HIV-1 replication thanks to its interaction with both the host RUNX1a transcription factor and viral Tat1 factors that mediate the lncRNA binding to the 5′ LTR of HIV-1 (56).

HIV-1 induces the complexation of the apoptosis-promoting lincRNA-p21 with the host protein human antigen R (HuR) and its subsequent degradation. This will lead to apoptosis inhibition and enhanced HIV survival within infected macrophages but not lymphocytes (62). It was observed that HIV-1 infection downregulates the expression of NEAT1 lncRNA leading to the reduction of the number of host-protective paraspeckle bodies, hence increased HIV-1 expression within CD4+ T lymphocytes (58). NEAT1 is also downregulated during viral reactivation from a resting state in CD4(+) T cells through an unknown mechanism leading to the promotion of HIV-1 transcription, and potentially HIV-1 dissemination (58, 59).

Contrary to the above-mentioned ncRNAs, some host ncRNAs were reported to repress the replication of HIV. These include miR-29a, miR-133b, miR-138, miR-149 and miR-326, NEAT1, noncoding repressor of Nuclear Factor of Activated T cells (NFAT or NRON), growth arrest-specific transcript 5 (GAS5), lincRNA-p21, 7SK and NEAT1.

Indeed, miR-29a is highly induced in HIV-1-infected Jurkat cells where it represses HIV replication through direct targeting of the HIV-1 nef 3' UTR region. However, the expression of this miR-29a is significantly downregulated at the peak of HIV-1 replication as already mentioned, thus highlighting its host-protective effect against HIV-1 infection (68, 69). In addition to miR-29a, it was found that overexpression of in-silico predicted miR-133b, miR-138, miR-149 and miR-326 decrease HIV replication in various primary T cells and T cell lines. It was further shown that miR-326 acts by direct targeting of a sequence within HIV-1 (72).

[5]https://www.who.int/news-room/fact-sheets/detail/hiv-aids

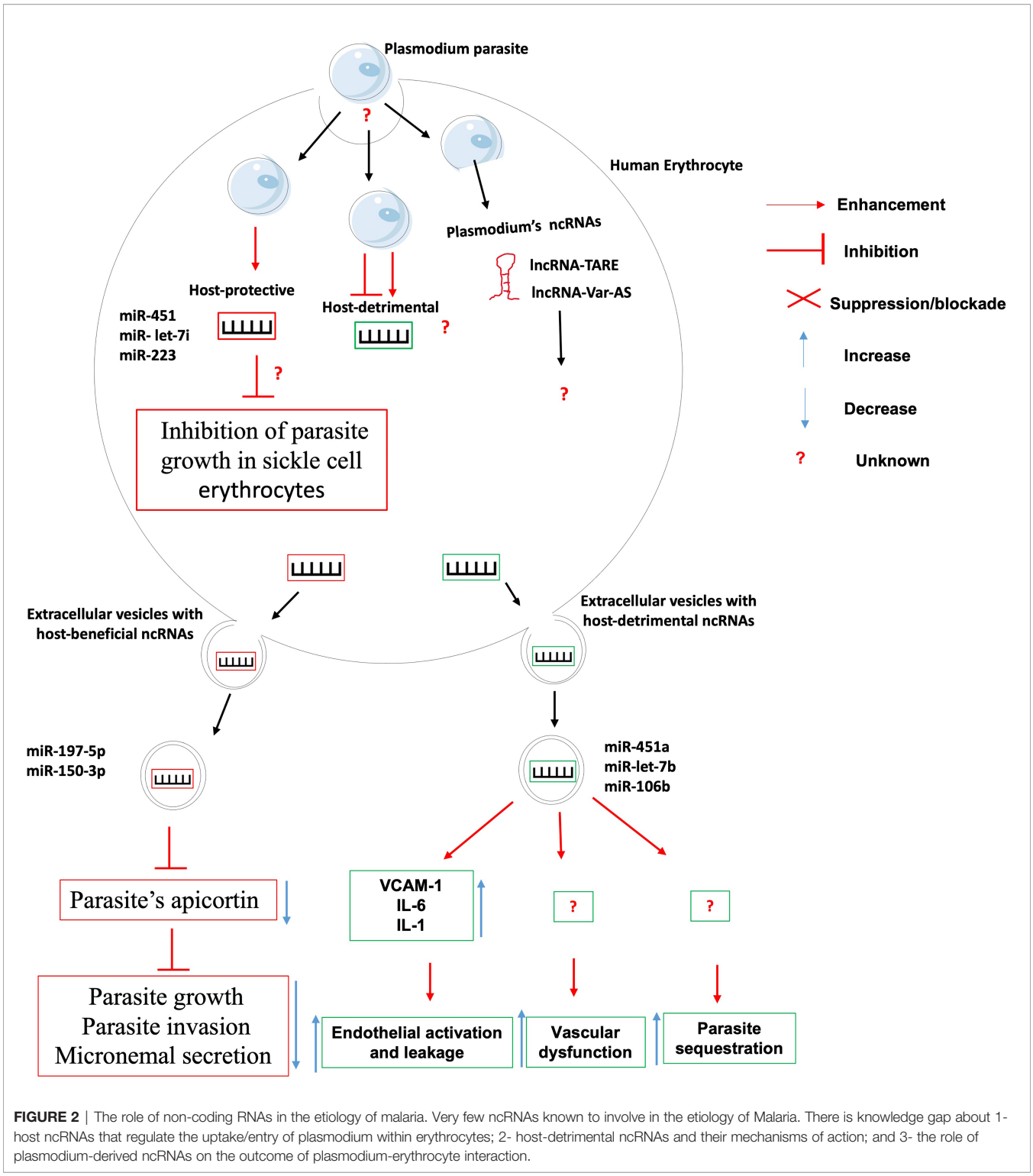

FIGURE 2 | The role of non-coding RNAs in the etiology of malaria. Very few ncRNAs known to involve in the etiology of Malaria. There is knowledge gap about 1- host ncRNAs that regulate the uptake/entry of plasmodium within erythrocytes; 2- host-detrimental ncRNAs and their mechanisms of action; and 3- the role of plasmodium-derived ncRNAs on the outcome of plasmodium-erythrocyte interaction.

As mentioned above, HIV-1 infection downregulates the expression of NEAT1 which would otherwise restrict HIV-1 replication through sequestration of unspliced viral RNA into paraspeckles (58).

The Noncoding Repressor of Nuclear Factor of Activated T cells (NFAT, or NRON) was shown to inhibit HIV-1 transcription and induce HIV-1 latency *via* induction of a proteasome-mediated HIV-1 Tat degradation, and in an NFAT-independent manner

TABLE 2 | Non-coding RNAs in the etiology and control of HIV/AIDS.

Role in HIV Infection	Non-coding RNA	Action	Reference
Etiology	miR-873	Activation of HIV transcription	(52, 53)
	MALAT1		(53, 54)
	HEAL		(53, 55)
	LINC01426		(53, 56)
	LINC00173		(57)
	NEAT1	Inhibition of HIV transcription	(53, 58, 59)
	NRON		(60, 61)
	GAS5		(52)
	lincRNA-p21		(62, 63)
Diagnosis	miR-146b-5p	Downregulation in B and T-lymphocytes	(64, 65)
	miR-223		
	miR-150		
	miR-16		
	miR-191		
	lncRNA NEAT1	Presence in the plasma	
Therapeutic targets	MALAT1	Promotion of HIV transcription,	(63)
	HEAL	Action in HIV-1 latency	(53)
	SAF	Resistance of HIV-1–infected macrophages to activation of apoptotic caspases	(66)
	lincRNA-p21	HIV-1–infected macrophages	(62)
	NEAT1	Dissemination of HIV-1	(58, 59)
	GAS5	Suppression of miR-873	(52)
	NRON	HIV-1 latency	(60, 61)
	LINC00173	Regulation of cytokine levels in T cells	(57)

(60). This findings corroborate the previous observation that NRON is highly expressed in resting CD4(+) T lymphocytes and HIV-proteins Nef and Vpu downregulate NRON expression, hence increase HIV-1 transcription *via* mechanisms that involved NFAT transcription factor but are still not fully understood (61).

The lncRNA Growth Arrest-Specific Transcript 5 (GAS5) inhibits HIV-1 replication by acting as a competing endogenous RNA (ceRNA), suppressing the effects of the host-detrimental miR-873 (52).

It is now recognized that there are ncRNAs originating from virus genomes. For instance, the antisense transcript originating from the *Nef* region in the HIV-1 genome (73, 74) which plays important role in the transcriptional control of HIV-1, notably *via* epigenetics mechanisms (75). There is currently no studies investigating the role of HIV-1-originating ncRNAs in the regulation of the outcome of host-virus interaction.

Non-Coding RNAs in the Diagnosis of HIV Infection

miR-146b-5p, miR-223, miR-150, miR-16, and miR-191 were found to be down regulated during HIV infection and plentifully expressed in B and T-lymphocytes, confirming a positive disease status (64, 65). Furthermore, some authors suggested that the presence of NEAT1 in plasma is a potential biomarker of HIV-1 infection (76).

Non-Coding RNAs as Therapeutic Targets of HIV Infection

Some ncRNAs have been associated to the treatment of HIV infection. miR-29a can be used as an indicator for the on-treatment disease evolution CD4 count or zenith HIV viral load (77). Its expression is associated to markers of HIV infection in long-term survivors, treatment-experienced patients (77). 7SK, by its pseudouridylation, can inhibit HIV transcription and escape from latency, suggesting it may be a new target for eliminating latent viral reservoirs (63, 78).

Many lncRNAs can be related to therapeutic research of HIV infection. MALAT1, as a promoter of HIV transcription, is a potential therapeutic target (54, 63). uc002yug.2, by activating latent HIV and HIV replication, can be a potential therapeutic target (56, 63). HEAL may be an attractive therapeutic target to inhibit HIV-1 latency, particularly considering that it is only upregulated in infected CD4+ and macrophages (53, 55, 63). SAF, involved in the resistance of HIV-1–infected macrophages to activation of apoptotic caspases, is a potential therapeutic target specifically intended for HIV cellular reservoirs (63, 66). lincRNA-p21 can constitute a novel therapeutic intervention strategy for HIV infection in macrophages (62, 63). NEAT1 is a feasible target for HIV treatment that involves the reactivation of latent HIV (58, 59, 63). GAS5, by suppressing miR-873, may be a novel biomarker for antiviral drugs and potential target for HIV treatment (52, 63). NRON, as an actor of HIV-1 latency, may be a novel target for reversing viral latency (60, 61, 63). TAR-gag, as an "RNA machine" of HIV genetic regulation, is a novel therapeutic target to reverse viral latency (63, 79). HIV-encoded lncRNA, as an epigenetic brake to regulate viral transcription, is a novel therapeutic target to inhibit the emergence of viral latency (51, 63, 75). LINC00173, which regulates cytokine levels in T cells, is a new therapeutic target for immunotherapy (57, 63).

LEISHMANIASIS

Leishmaniasis is a neglected tropical disease caused by infection with *Leishmania* parasites, which are spread by the bite of phlebotomine sand flies. There are different forms of leishmaniasis in people and the most common are Cutaneous Leishmaniasis (CL), which causes skin

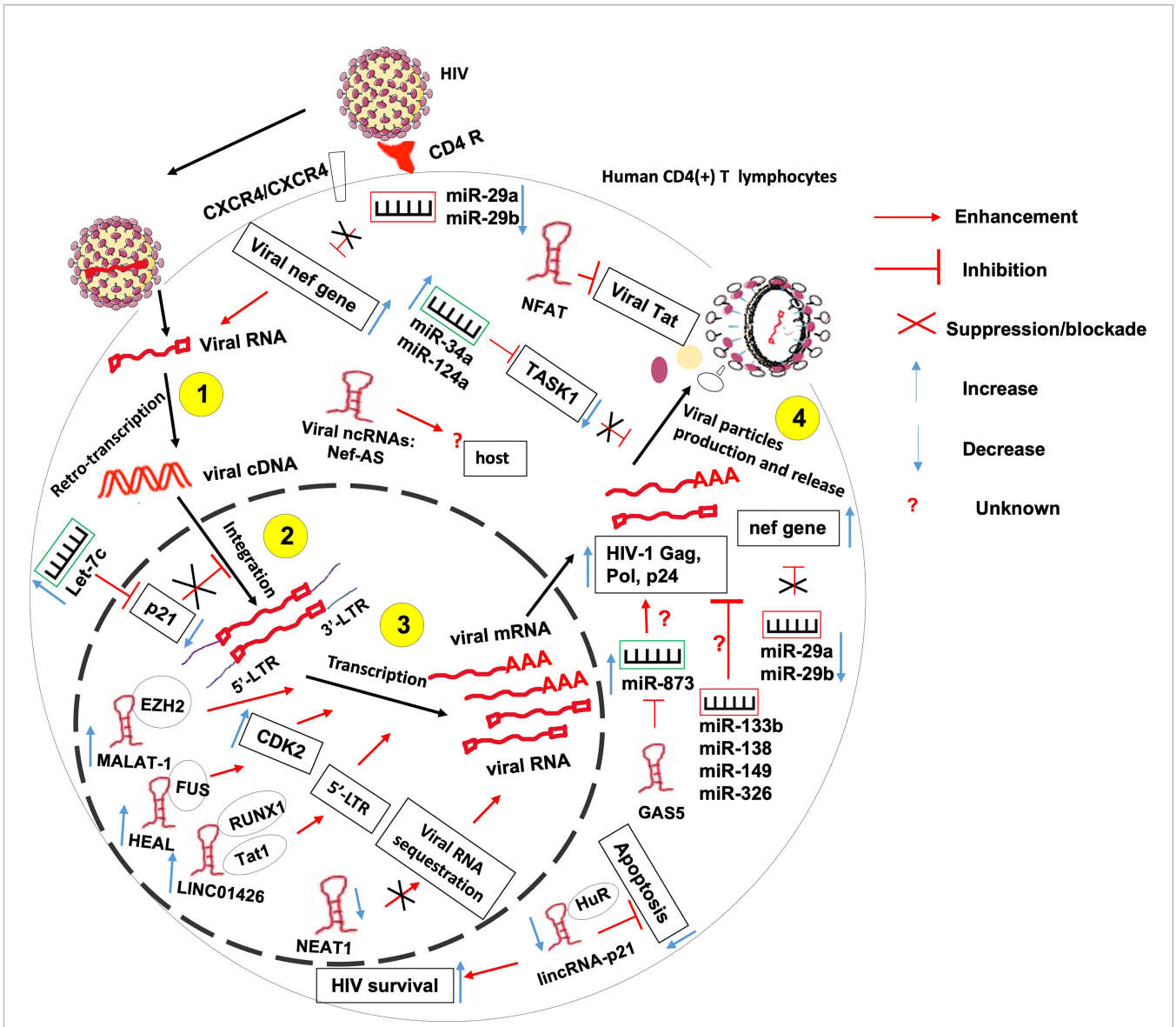

FIGURE 3 | The role of non-coding RNAs in the etiology of HIV/AIDS. ncRNAs control several steps of HIV life cycle including viral RNA retro-transcription, cDNA integration, transcription and viral particles production. More research needed to identify 1- host ncRNAs that regulate viral particles release from the host and 2- the role of HIV-derived ncRNAs on the outcome of HIV-T lymphocyte interaction.

sores, and Visceral Leishmaniasis (VL), which affects several internal organs (generally spleen, liver, and bone marrow)[6].

Leishmaniasis is prevalent on every continent except Australia and Antarctica. It is difficult to estimate the number of new cases that may vary over time. For CL, estimates of the number of new cases per year have ranged from approximately 700,000 to 1.2 million or more. For VL, the estimated number of new cases per year may have decreased to <100,000, but previous estimates ranged up to 400,000 or more cases[7]. If not treated, severe cases of visceral leishmaniasis typically are fatal.

Before considering treatment of leishmaniasis, it is essential to make sure the diagnosis is correct. It can be done by detecting Leishmania parasites (or DNA) in tissue specimens from skin lesions (CL) or bone marrow (VL). This tissue sampling is an invasive method. Conversely, the diagnosis of disease with the help of a biomarker is a non-invasive tool that has shown to have an important function in early diagnosis of infection. There is no cure for leishmaniasis and chemotherapy is threatened by limited efficacy coupled with the development of resistance and other side effects (80). Leishmania parasites elude the host defensive. Some ncRNAs have been reported as biomarkers for the diagnosis and the treatment of leishmaniasis (**Table 3**).

[6]https://www.cdc.gov/parasites/leishmaniasis/index.html

[7]https://www.cdc.gov/parasites/leishmaniasis/epi.html

TABLE 3 | Non-coding RNAs in the etiology and control of leishmaniasis.

Role in Leishmaniasis	Non-coding RNA	Action	Reference
Etiology	miR-210	Downregulation of NF-κB mediated pro-inflammatory immune responses	(81)
Therapeutic targets	miR-361-3p	Its high expression in cutaneous leishmaniasis lesions	(82)
	miR-193b	Influence in the expression of genes related to the inflammatory response observed in localized cutaneous	(83)
	miR-671	leishmaniasis	
Diagnosis	lncRNA 7SL	It makes the difference between *Leishmania* species infections	
Vaccine	miR-135	Biasing the Th2 response toward protective Th1 type	(84, 85)
development	miR-126		

Non-Coding RNAs in the Etiology of Leishmaniasis

Like many sophisticated intracellular pathogens, Leishmania has evolved mechanisms to modify the host responses to ensure their intracellular differentiation and multiplication. The parasite does so through the manipulation of the host factors including several miRNAs (**Figure 4**).

For instance, Leishmania *donovani* infection creates hypoxic conditions leading to HIF-1a-mediated induction of miR-210 in infected macrophages (81, 86). This miR-210 was shown to promote the survival of the parasite within the host by targeting the NFkb subunit p50 and subsequently downregulating the expression of pro-inflammatory cytokines TNF-α and IL-12 while upregulating the anti-inflammatory cytokine IL-10 within the infected macrophage (81). Also, L. donovani interferes with host autophagy by inducing the expression of host miR-30A-5P which in turn will downregulate the expression of the pro-autophagic BECN1 protein, hence the increased survival of the parasite within the infected macrophage (87). *Leishmania amazonensis* promotes its survival within the infected host by upregulating miR-294 and miR-721. These miRNAs target the nitric oxide synthase 2 (NOS2) mRNA at the post-transcriptional level, then leading to decreased nitric oxide production and increased arginase activity within the infected macrophage (88). miR-21 and miR-146b-5p are significantly downregulated *in monocytes-derived dendritic cells following L. donovani and L. major infection. In-silico prediction have identified* SMAD7 and TRAF6, two members of the TGF-β signaling pathway as targets for these two miRNAs (89). It was found that *L. major* infection led to the down-regulation of miR-10a in Foxp3$^+$ Treg cells. This miR-10a decreased IFNγ and enhanced the suppressive function of Treg cells (90).

Leishmania promastigotes and amastigotes infection represses the expression of the lncRNA 7SL RNA, an important component of the signal recognition particle in macrophages. This will convert these cells into permissible hosts favorable to the establishment and hiding of the parasite within the macrophages' phagolysosomes (91).

It is still unknown whether and which host ncRNAs control leishmania parasites entry, engulfment within phagosomes and fusion of phagosomes with lysosomes.

An investigation on Leishmania major genome revealed that there are 1884 uniquely expressed ncRNAs in that parasite, some of which were recently shown to possess protein coding potential (92). The contribution of these parasite-derived ncRNA to the outcome of parasite-host interaction still need investigation.

Non-Coding RNAs in the Diagnosis of Leishmaniasis

The 7SL RNA gene can be used for diagnosis of human leishmaniasis (93). Diagnosis with the help of 7SL RNA is rapid, sensitive, specific, and simple (93).

Non-Coding RNAs in the Treatment of Leishmaniasis

Some authors suggested that miR-361-3p is a prognostic biomarker in cutaneous leishmaniasis lesions caused by *Leishmania braziliensis* (82). miR-193b and miR-671 were associated with a good response to treatment of Human localized cutaneous leishmaniasis caused by *Leishmania braziliensis* (83). Targeting of let-7a with Locked Nucleic Acid (LNA) Antisense Oligonucleotides (ASOs) was shown to increase L. major-infected MDMs apoptosis and necrosis, therefore, targeting let-7a was suggested as a potential therapeutic approach (94).

Non-Coding RNAs in Vaccine Development Against Leishmaniasis

miRNA21 has been recently shown to negatively associate 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 (84, 95).

Therapeutic vaccines may be developed by targeting miRNA-135 and–126 that bias the Th2 response toward protective Th1 type (84, 85).

AFRICAN TRYPANOSOMIASIS

Also known as sleeping sickness, Human African Trypanosomiasis (HAT) is a neglected tropical disease caused by microscopic parasites of the species *Trypanosoma brucei* whose vector is an insect of the genus Glossina: the tsetse fly[8]. Two subspecies of *Trypanosoma brucei* are responsible of human disease: *T. b. gambiense* in 24 countries in west and central Africa, and *T. b. rhodesiense* in 13 countries in eastern and southern Africa[9].

HAT is curable with medication but is fatal if left untreated. Diagnosis must be made as early as possible to avoid progressing to the neurological stage in order to prevent complicated and

[8] https://www.cdc.gov/parasites/sleepingsickness/index.html

[9] https://www.who.int/news-room/fact-sheets/detail/trypanosomiasis-human-african-(sleeping-sickness

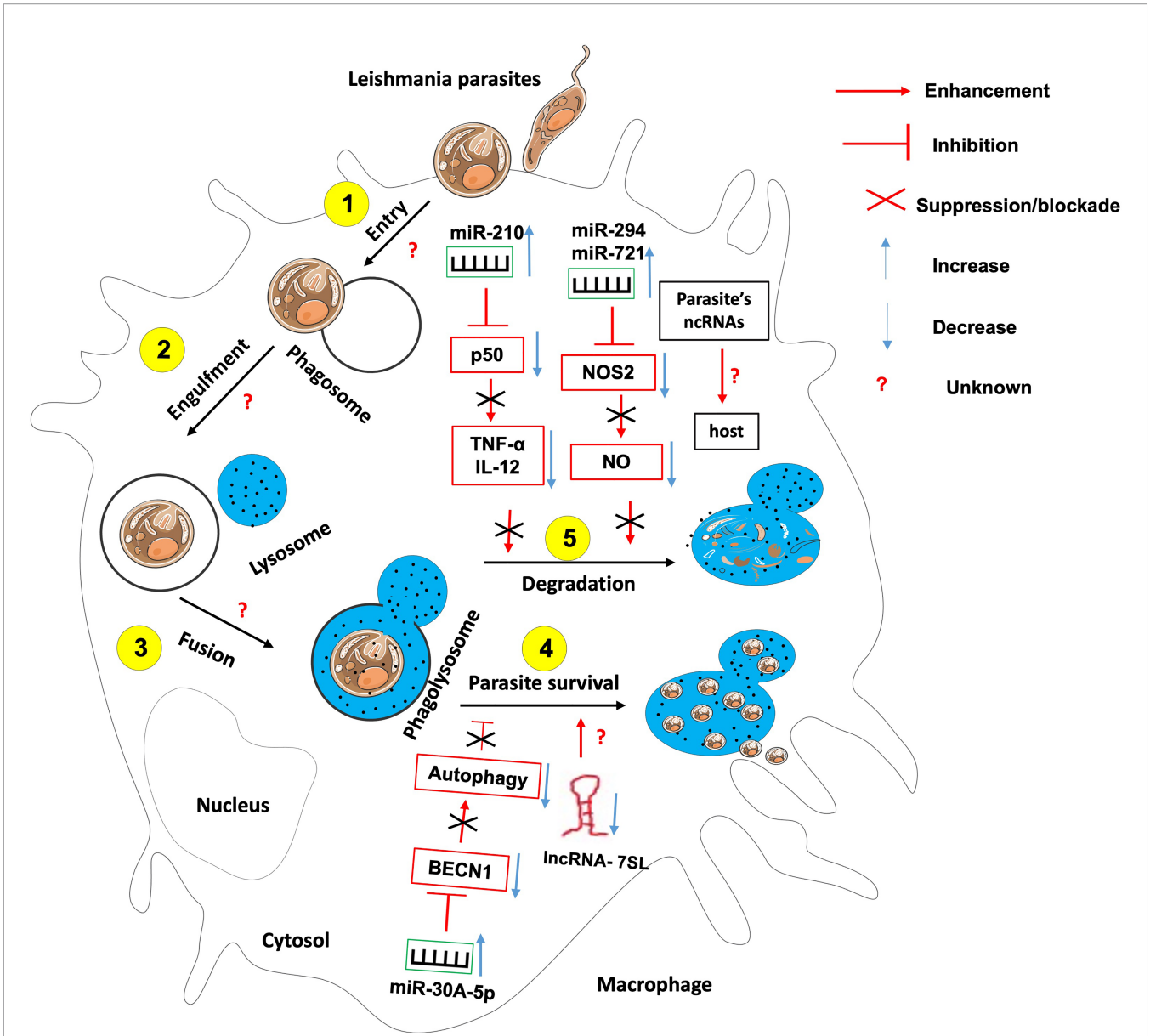

FIGURE 4 | The role of non-coding RNAs in the etiology of leishmaniasis. Most studies have identified ncRNAs that enhance host effector killing functions against leishmania parasites as well as those that favor parasite survival and persistence within the infected host. There is knowledge gap about 1- host ncRNAs that regulate the parasite entry within the host, its engulfment within phagosome and the fusion between phagosome and lysosomes; 2- the role of Leishmania-derived ncRNAs on the outcome of leishmania-macrophage interaction.

risky treatment procedures (95). Diagnosis is made clinically or by light microscopy; which are both insensitive and require certain skills[10]. The use of biomarkers such as ncRNAs could enhance sensitivity.

Non-Coding RNAs in the Etiology of African Trypanosomiasis

The alteration of nine miRNAs Including miR-193b, miR-338 (upregulated), miR-199a-3p, miR-27b and miR-126* (downregulated) has been identified in the peripheral blood of HAT patients (96). They were non-specific and some of them were previously reported changed during other infectious diseases or cancer. They might be a mirror lymphocyte activation or inflammation observed in HAT (96). However, the mechanisms of action of these microRNAs are still to be investigated.

ncRNAs as Diagnostic Biomarkers of African Trypanosomiasis

The SL-RNA was described as an attractive molecular target of the sleeping sickness (97). Later, the small RNA derived from the non-

[10]https://www.cdc.gov/parasites/sleepingsickness/diagnosis.html

coding 7SL RNA was detected at high levels in the serum of infected cattle (98). This ncRNA is highly sensitive and can be detected before the onset of parasitemia as well as during periods where there is subpatent parasitemia by microscopy (98). It can also make the difference between infections with *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma vivax*; providing the basis for the development of a cheap, non-invasive and highly effective diagnostic test for trypanosomiasis (98).

ncRNAs as Therapeutic Biomarkers of African Trypanosomiasis

The therapy of trypanosomiasis is currently based on anti-trypanosome drugs. No therapeutic field with ncRNAs has yet been investigated. Research should investigate this domain and see if ncRNAs might be useful for monitoring the treatment of this disease.

LEPROSY

Also called Hansen's disease, leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. This slow-growing, obligate intracellular bacterium is the only known bacterium that infects Schwann cells of peripheral nerves. In addition, M. leprae infects macrophages and dendritic cells (99). M leprae mainly affects the skin, the peripheral nerves, mucosa of the upper respiratory tract, and the eyes[11] [12]. According to official figures from 159 countries from the 6 WHO Regions, 208 619 new leprosy cases were globally registered in 2018[13].

Leprosy presents in many clinical forms with the two extremes being the Tuberculoid and the Lepromatous forms. In the tuberculoid forms (TT and BT), bacilli are absent or rarely found in neural branches, macrophages, or mononuclear cells of the papillary dermis. the disease is self-limited. On the other hand, in lepromatous forms (BL and LL), the bacilli are abundant and can parasitize practically all tissues, hence the disease is disseminated. These clinical forms can be recognized with the naked eye. To confirm the diagnosis, a sample of skin or nerve can be examined under the microscope and tests may also be done to differentiate it from other skin diseases[14]. These sampling techniques are invasive. Alternatively, to diagnose an infection early, biomarkers are useful and non-invasive. Some ncRNAs have been reported as biomarkers for the diagnosis of leprosy (**Table 4**).

Non-Coding RNAs in the Etiology and Occurrence of Leprosy

It is now recognized that ncRNAs play important roles in the deregulation of the immune response in the varied and polymorphic cells targeted in the leprosy skin lesions onset (99, 104, 105). Specific miRNAs regulated during infection either stimulate the immune response or facilitate immune evasion by pathogens (**Figure 5**).

For instance, miR-21 is upregulated in *M. leprae*- infected monocytes in which it downregulates the expression of genes encoding 2 vitamin D-dependent antimicrobial peptides CAMP and DEFB4A, hence used by the mycobacterium to evade the vitamin D-antimicrobial pathway (102). Also, miR-21 involves in the indirect upregulation of IL-10 and is differentially expressed in humans with progressive lepromatosis.

miR-146a expression is upregulated in hosts infected with live M. leprae. miR-146a bears a single nucleotide polymorphism associated with the risk of developing leprosy, hence the expression of this miR is dependent on the host genotype (101). Carriers of the miR-146a C allele have been shown to express high levels of mature miR-146a coupled to a reduced expression of TNF (Tumor Necrosis Factor) with a susceptibility to leprosy; suggesting that miR-146a negatively influences the secretion of TNF by controlling its level of expression.

miR-181a expression is downregulated in *M* leprae-infected T lymphocytes. This downregulation correlates with the increased expression of miR-181a target SHP2, a phosphatase involved in the inhibition of T cell receptor signaling (106). Indeed, higher miR-181a expression correlates with greater T cell sensitivity in immature T cells (107) suggesting that the downregulation of miR-181a expression in *M.* leprae-infected T lymphocytes involves in the prevailing T cell hyporesponsiveness during leprosy progression.

Recent studies have identified differentially expressed piwiRNAs (piRNAs) in leprosy skin lesions from tuberculoid tissue, lepromatous tissue, and healthy subject tissues (108). This class of small ncRNAs is closely related to miRNAs and its study will provide additional clues on the contribution of ncRNAs to the onset, development, and progression of leprosy. The contribution of M. leprae-derived ncRNAs also need to be explored.

ncRNAs as Diagnostic Biomarkers of Leprosy

Early diagnosis of leprosy is very important to control the disease and put in place preventive measures. Currently, the diagnosis of leprosy is based on clinical examination and skin biopsy. Techniques based on PCR and serological analysis have been developed but have not made it possible to diagnose leprosy with acceptable specificity and sensitivity given the different clinical forms and/or the bacterial load. However, the identification of biomarkers allows the diagnosis of leprosy with greater sensitivity and specificity. Due to the influence of the host's genetic makeup on the development of leprosy and the genetic variants associated with it. The expression profile of ncRNAs and more precisely miRNAs is a key element exploited in the development of reliable diagnostic and prognostic biomarkers. miR-101, miR-196b, miR-27b and miR-29c have been differentially expressed in different cell types: macrophages, LT, epithelial cells, dendritic cells, mast cells with establishment of an immune/inflammatory microenvironment in *M. leprea*

[11] https://www.cdc.gov/leprosy/index.html

[12] https://www.who.int/news-room/fact-sheets/detail/leprosy

[13] https://www.who.int/news-room/fact-sheets/detail/leprosy

[14] https://www.cdc.gov/leprosy/index.html

TABLE 4 | Non-coding RNAs in the etiology and control of leprosy.

Role in leprosy	Non-coding RNA	Action	Reference
Etiology	miR-181a	Rheostat of intrinsic antigen sensitivity during LT development	(100)
	miR-146a	Reduction of the TNF expression	(101)
	miR-21	Downregulation of host defense genes to evade vitamin D antimicrobial pathway	(102)
Diagnosis	miR-101	Modulation of the host immune response in leprosy	(103)
	miR-196b		
	miR-27b		
	miR-29c		

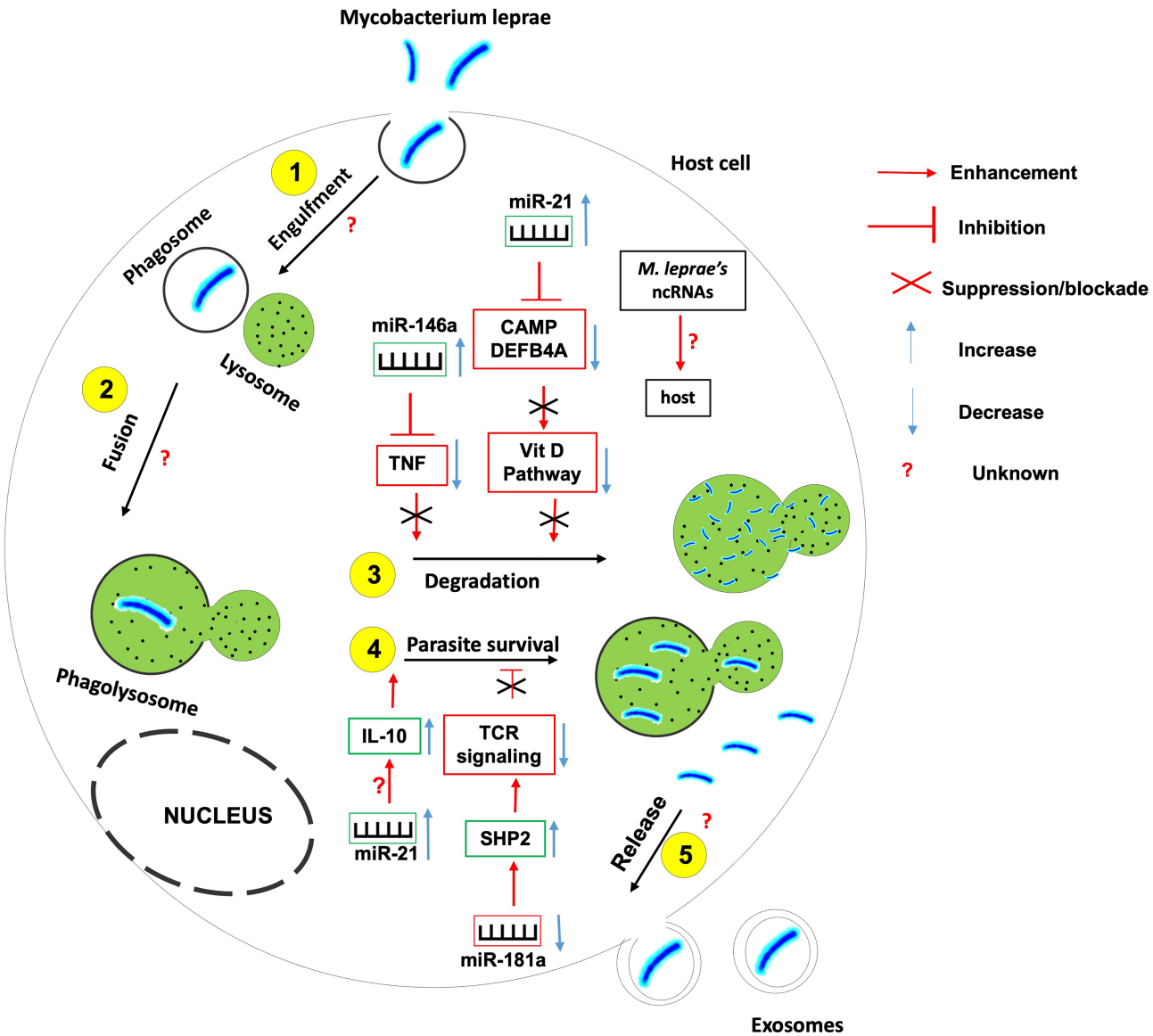

FIGURE 5 | The role of non-coding RNAs in the etiology of leprosy. Several ncRNAs are found to be instrumental in the induction or inhibition of the host effector killing functions against Mycobacterium leprae. More research needed to identify 1- host ncRNAs that regulate the parasite entry within the host, its engulfment within phagosome and the fusion between phagosome and lysosomes; 2- the role of M. leprae-derived ncRNAs on the outcome of mycobacterium-macrophage interaction.

infection. These miRNAs are linked to immune genetic targets and could modulate the host immune response in leprosy influencing its outcome. Thus, miR-101, miR-196b, miR-27b and miR-29c were identified as good discriminators in the polar forms of leprosy (LL: lepromatous leprosy and TT: tuberculoid tuberculoids) and between physiological state and pathological state (103) with high levels of sensitivity and specificity.

ncRNAs as Therapeutic Biomarkers of Leprosy

Although drug treatment has been successful, leprosy still affects people all over the world. The treatment of leprosy is based mainly on polychemotherapy, which has so far remained the only strategy for the treatment and elimination of leprosy (109). hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR-146b-5p, hsa-miR-342-3p, hsa-miR-361-3p, hsa-miR-3653 hsa-miR-484 and hsa-miR-1290 were reported deregulated in leprosy and could serve as therapeutic markers (7, 110).

Non-Coding RNAs in Vaccine Development Against Leprosy

No ncRNA-based vaccine has been developed so far against leprosy. Researchers can explore this new research avenue.

CONCLUSION

There is a growing interest in the role of host miRNAs and lncRNAs in the etiology of major human tropical diseases and the prospect of targeting these ncRNAs species as biomarkers for the early diagnosis, treatment response and vaccine development against these diseases. Although most research have shed light on the involvement of these ncRNAs in the onset and development of TB, HIV/AIDS and Malaria, but few have yet attempted to assess the potential of these ncRNAs as diagnosis biomarkers, adjunctive therapeutic targets and vaccine candidates. There is also a lack of information about the contribution of pathogen-released ncRNAs to host immune evasion and disease onset. There is a knowledge gap on the role of host miRNAs and lncRNAs in the etiology, diagnosis and vaccine development against neglected human tropical diseases. Especially, more research is warranted to understand the role of these ncRNAs in the etiology of African trypanosomiasis and the assessment of the diagnostic potential of ncRNAs for African trypanosomiasis. The potential targeting of ncRNAs for adjunctive therapy and vaccine development against leishmaniasis, African trypanosomiasis and leprosy are also new avenues to explore.

AUTHOR CONTRIBUTIONS

OT designed the writing plan and drew all the figures. CM and JAN did literature search on Leishmaniasis and HIV/AIDS. CK and USFS did literature search on Tuberculosis and Malaria. NN and FJT did literature search on Leprosy and Trypanosomiasis. OT, CM, CK, and NN wrote the draft. AM and AN proofread the manuscript. All authors contributed to the article and approved the submitted version.

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Glutathione Metabolism Is a Regulator of the Acute Inflammatory Response of Monocytes to (1→3)-β-D-Glucan

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(1→3)-β-D-Glucan (BDG) represents a potent pathogen-associated molecular pattern (PAMP) in triggering the host response to fungal and some bacterial infections. Monocytes play a key role in recognizing BDG and governing the acute host response to infections. However, the mechanisms regulating monocyte's acute response to BDG are poorly understood. We sought to investigate the response of monocytes to BDG at the epigenetic, transcriptomic, and molecular levels. Response of human monocytes to 1, 4, and 24 hours of BDG exposure was investigated using RNA-seq, ATAC-seq, H3K27ac and H3K4me1 ChIP-seq. We show that pathways including glutathione metabolism, pentose phosphate pathway, and citric acid cycle were upregulated at the epigenetic and transcriptomic levels in response to BDG exposure. Strikingly, unlike bacterial lipopolysaccharides, BDG induced intracellular glutathione synthesis. BDG exposure also induced NADP synthesis, increased NADPH/NADP ratio, and increased expression of genes involved in the pentose phosphate pathway in a GSH-dependent manner. By inhibiting GSH synthesis with L-buthionine sulfoximine (BSO) before BDG exposure we show that the GSH pathway promotes cell survival and regulates monocyte's effector functions including NO production, phagocytosis, and cytokine production. In summary, our work demonstrates that BDG induces glutathione synthesis and metabolism in monocytes, which is a major promoter of the acute functional response of monocytes to infections.

Keywords: B-glucan, glutathione, immunometabolism, innate immunity, host response

Abbreviations: UT, untreated; BDG, (1→3)-β-D-Glucan; BSO, L-buthionine sulfoximine; GSH, reduced glutathione.

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INTRODUCTION

(1→3)-β-D-Glucan (BDG) represents one of the most abundant components of the fungal and some bacterial cell walls and is a potent pathogen-associated molecular pattern (PAMP) in triggering the host response to infections (1). For example, invasive fungal infections (IFIs) represent a rising cause of human disease with increased use of immunosuppressive therapies, broad-spectrum antibiotics, and invasive medical devices. Such infections are a common complication for a wide spectrum of immunocompromised patients including people living with HIV, cancer, solid organ transplant, systemic lupus erythematosus, and other predisposing conditions like diabetes and pregnancy (2). BDG assays are now widely used as diagnostic tools for identifying mycosis in clinical settings (3–5). Recent studies have shown that circulating BDG levels are linked to immune activation and inflammation in people living with chronic conditions that induce epithelial gut damage and subsequent microbial translocation such as CMV and HIV infections (6–9). A growing body of literature has highlighted the capacity of BDG to induce long-term epigenetic reprogramming in innate immune cells, termed trained immunity, which leads to an adjusted response to a subsequent challenge and to some metabolic changes, including a switch from oxidative phosphorylation to aerobic glycolysis (10, 11). Similarly, BDG has been shown to be able to reverse LPS-induced tolerization in monocytes/macrophages and confer protection against infectious diseases including leishmaniasis and tuberculosis (12–14).

Circulating monocytes serve as an integral part of the host response to infections by detecting PAMPs, phagocytosing/presenting antigens, and producing pro-inflammatory cytokines/chemokines to help recruit a larger wave of diverse leukocytes to the site of infection. In humans, monocytes can recognize BDG using the C-type lectin receptor Dectin-1 and complement receptor 3 (CD11b/CD18 heterodimer) (15). Dectin-1 deficiency has been shown to be associated with increased susceptibility and significantly impaired immune response to *Candidiasis* by monocytes/macrophages in both mice and humans (16, 17). While the acute recognition and response of monocytes to BDG is widely recognized as a critical component of the host's response to various infections, the mechanistic pathways implicated in this response remain to be fully understood.

Herein, we exploited previously published epigenetic and transcriptomic data on monocytes exposed to BDG (12) to unveil novel components of the host response to BDG. We confirmed that BDG induces increased expression of genes involved in glucose metabolism, including pentose phosphate and cholesterol metabolism. We expand such knowledge by revealing for the first time that BDG stimulation induces epigenetic and transcriptomic changes in monocytes associated with increased glutathione synthesis and metabolism. Interestingly, intracellular glutathione levels were crucial in the regulation of several of the monocyte's antifungal functions including resilience to oxidative stress, immunometabolism, nitric oxide production, phagocytosis, and cytokine production.

MATERIALS AND METHODS

Monocytes from Healthy Donors

All primary cells for *in vitro* experiments were obtained from healthy donors who gave written informed consent (Chronic Viral Illness Service, at McGill University Health Centre (MUHC) Montreal, QC, Canada) and approved by the REB (2019–5170) of MUHC. Peripheral blood mononuclear cells (PBMC) were isolated by leukapheresis and stored in liquid nitrogen. Cells were rapidly thawed and rested for 1 hour at 37°C. Monocytes were purified from PBMC using a negative selection Human Monocyte Isolation Kit (StemCell Easy Sep). Successful isolation of monocytes was confirmed with flow cytometry (BD Fortessa) using VivaFix Viability Assay (BioRad), a cocktail of antibodies from BioLegend: anti-CD3 PE (300456), anti-CD14 BV650 (301835), anti-CD19 PerCP-Cy5.5 (302229), and anti-CD56 APC (318309). The gating strategy for the live single-cell CD3- CD19- CD56- CD14+ population is presented in **Supplementary Figure 1**. The purity of the isolated monocytes can be found in (**Supplementary Table 1**).

Our monocyte isolation protocol is similar to *Novakovic* et al. where they have enriched monocytes from healthy volunteers to generate the functional genomics datasets reanalyzed herein (12). Briefly, they isolated peripheral blood mononuclear cells (PBMC) using centrifugation in Ficoll-Paque (GE Healthcare), followed by an additional Percoll gradient to remove T cells. Monocytes were then purified using negative selection in an LD column magnet separator (Miltenyi Biotech) and monocytes purity was assessed using flow cytometry.

Cell Culture

Monocytes were cultured in complete media consisting of Dulbecco's Modified Eagle Medium containing high glucose and sodium pyruvate (ThermoFisher Scientific) with 10% heat-inactivated fetal bovine serum (Wisent BioProducts) and 100U/mL penicillin/streptomycin (Corning). NGS datasets produced by *Novakovic* et al. were produced using 5ug/mL of (1→3)-β-D-glucan from heat killed *Candida albicans* for 24 hours, as previously described (12). Cells in our *in vitro* experiments were stimulated with 5μg/mL of (1→3)-B-D-glucan from *Alcaligenes faecalis* (Sigma-Aldrich), 100ng/mL of lipopolysaccharide (LPS) from *Escherichia coli* O127:B8 (Sigma-Aldrich), 120μM L-buthionine sulfoximine (Sigma-Aldrich), and/or 1mg/mL of reduced glutathione (Sigma-Aldrich) for 24 hours unless otherwise stated in figure legends.

ATAC-Seq Peak Finding Analysis

The following published ATAC-seq datasets were analyzed: untreated, BDG 1h, BDG 4h, and BDG 24h stimulated monocytes (GEO: GSE85246) (12). Sequence reads were downloaded using fastq-dump from the SRA Toolkit with the setting –split-files and mapped to the human hg19 reference genome assembly with Bowtie 2.4.0 (18). The files containing mapped reads were converted from SAM to BAM format using samtools 0.1.18 (19) and then Tag directories were generated for further analysis using the Homer toolkit (20). To identify accessible genome regions, we processed the mapped reads

with MACS 1.4.1 (21) with a p-value cutoff of 1E-8. Significant peaks that were less than 100bp apart were merged using MergePeaks and subsequently annotated using AnnotatePeaks from the Homer toolkit (20). 55,971 regions were identified to have accessible chromatin in one or more of the four conditions and read densities were queried at each location (+/- 100bp of the peak center) in all four conditions using the AnnotatePeaks function (20). 34,572 differentially accessible regions (DARs) between conditions were then identified using a fold-change threshold of ≥ |2| when compared to the untreated condition (**Supplementary Table 2A**). These regions were clustered based on their dynamics relative to the untreated condition and the heatmap (**Figure 1A**) was generated using Java TreeView (22). Sequence read density profiles (bigwigs) were generated from BAM files using bamCoverage from the deepTools 3.1.1 toolkit (23).

Transcription Factor Binding Motifs Analyses

Known and *de novo* transcription factor binding motifs were identified in the ATAC-seq DAR clusters using the findMotifsGenome tool from Homer using the settings: -size 200 -mask. The motifs found to be enriched in one of the clusters were then queried to find their relative frequency within each cluster *vs* randomly generated sets of background sequences using the Homer toolkit (20). Potential transcriptional regulatory networks were identified by querying the list of dysregulated genes from RNA-seq against a database of TF cistromes using TRRUST (24).

ATAC-Seq Gene Ontology Analyses

Genomic coordinates for each DAR cluster ATAC-seq peaks were submitted to the GREAT 2.0.2 tool to determine if specific biological functions were enriched with differentially accessible regions at different times (25). We used 500kb as the maximum absolute distance to the nearest transcriptional start site and q-values less than 1E-10 as statistically significant. Representative Gene Ontology (GO) biological process categories were selected to remove redundancy and reported alongside the ATAC-seq DAR clusters; the full list of enriched GO categories can be found in (**Supplementary Table 2B**).

ChIP-Seq Peak Finding Analyses

H3K27ac and H3K4me1 ChIP-seq datasets for untreated, BDG 1h, BDG 4h, and BDG 24h stimulated monocytes were downloaded using fastq-dump from the SRA toolkit (GEO: GSE85246) (12). Sequence reads were aligned to the human hg19 reference genome using Bowtie 2.4.0 (18). The files containing mapped reads were converted from SAM to BAM format using samtools 0.1.18 (19) and formatted into Tag directories for further analysis using the Homer toolkit (20). Bigwigs were generated from BAM files using bamCoverage from the deepTools 3.1.1 toolkit (23).

ATAC-Seq and ChIP-Seq Density Profiles

AnnotatePeaks from the Homer toolkit (20) was used to query the read density at the genomic coordinates of the DAR clusters in the ATAC-seq, H3K27ac ChIP-seq, and H3K4me1 ChIP-seq datasets ±1kb centered around the ATAC-seq peak. Data was visualized in Microsoft Excel 2010 comparing ATAC-seq and ChIP-seq signals in each cluster after varying durations of BDG exposure.

RNA-Seq Analyses

Biological duplicates of RNA-seq on human monocytes untreated or stimulated with BDG for 1 hour, 4 hours, or 24 hours were downloaded using the SRA toolkit using –fastq-dump (GEO: GSE85246). The quality of sequence reads was confirmed using FastQC (Babraham Bioinformatics) and low-quality reads and bases were trimmed using Trimmomatic v.0.3 (26). Reads were mapped to the human hg19 assembly using HISAT2 (27). Resulting SAM files were then converted to BAM format using samtools 0.1.18 (19). Gene expression was quantified by counting the number of uniquely mapped reads to exons with featureCounts (28). Normalization and differential gene expression analysis was conducted using the edgeR Bioconductor package (29). Genes with at least three counts per million reads in at least two samples were retained for pairwise differential gene expression analyses comparing BDG exposed and unexposed monocytes. Genes with differential expression > |2| and FDR (p-value corrected using Benjamini-Hochberg method) $<1x10^{-3}$ were considered significant and clustered based on differential expression compared to untreated monocytes (**Supplementary Table 3A**). Clusters were visualized using Java TreeView (22). Bigwigs were made using genomeCoverageBed from bedtools 2.17.0 and wigToBigWig from the UCSC toolkit after scaling per 10 million reads mapping onto exons (30, 31). Gene ontology enrichment analyses was conducted on differentially expressed gene clusters by submitting gene sets to EnrichR (**Supplementary Table 3B**) (32).

RNA Extraction, cDNA Synthesis, and Real-Time qPCR

RNA was extracted using EasyPure RNA Extraction Kit (Transgen Biotech) and cDNA was synthesized using the TransScript One-Step gDNA Removal and cDNA Synthesis kit (Transgen Biotech). RT-qPCR was performed with the Luna Universal qPCR Master Mix (New England BioLabs) using the primers listed in (**Supplementary Table 4**). Data was analyzed using CFX Maestro Software for Real-Time PCR (BioRad) and normalized to *ACTB* gene expression.

Monocyte Function

Cell viability was assessed using Trypan blue and counted using a hemocytometer. Intracellular reduced and oxidized glutathione levels were measured and normalized per 1 million cells as previously described (33). NADP and NADPH levels were measured using an NADP/NADPH Assay (Abcam) and normalized by cell count. Nitric oxide production was assessed by a Griess assay on the culture supernatant (34). Phagocytic capacity of monocytes was assessed by exposing the monocytes to the pHrodo Green *E. coli* BioParticles Conjugate for Phagocytosis (ThermoFisher Scientific) for 30 minutes and the endpoint signal was measured using a spectrophotometer at OD 495nm. IL-6, IL-8,

and IL10 levels were measured in culture supernatant using Quantikine Human ELISA kits (R&D Systems).

RESULTS

Epigenetic Landscape of Monocytes Exposed to BDG

To investigate the functional changes driven by acute exposure of human monocytes to BDG, we have reanalyzed available functional genomics datasets (12). We first assessed acute changes in chromatin accessibility before and after exposure to BDG for 1, 4, and 24 hours. As expected, BDG induced significant changes in chromatin accessibility with a total of 34,572 differentially accessible regions (DARs) before and after different durations of BDG exposure. We grouped these regions based on (1) early, (2) intermediate, and (3) late chromatin opening, as well as (4) early, (5) intermediate, and (6) late chromatin closing (**Figure 1A** and **Supplementary Table 2A**). 21,870 regions had increased accessibility after BDG exposure (groups A-C) whereas 12,702 regions had decreased accessibility (groups D-F). GO analysis was then conducted to identify biological processes associated with these groups (**Figure 1B** and **Supplementary Table 2B**). DAR groups associated with increased chromatin accessibility (A-C) were proximal to genes involved in myeloid leukocyte activation, metabolic processes, antigen receptor signaling, organelle assembly, vesicle-mediated transport, and antigen presentation. Regions with decreased accessibility were associated with apoptotic processes, plasma membrane invagination, protein metabolism, cell adhesion, response to LPS, and cytokine signaling.

We next evaluated the levels of H3K27ac, a mark of active cis-regulatory regions, and H3K4me1, mark of active and primed enhancers, in monocytes before and after 1, 4, and 24 hours of BDG exposure. Groups A-C were associated with significantly increased H3K27ac and H3K4me1 signals around the ATAC-seq peak and groups D-F were associated with significantly decreased signals (**Figure 1C** and **Supplementary Figure 2**). Globally, the timing of these changes in histone modifications matched changes observed in ATAC-seq signal.

Transcriptomic Changes in Monocytes Exposed to BDG

To further characterize the acute response of monocytes to BDG we assessed transcriptomic changes using RNA-seq. Principal component analysis clearly demonstrates that duration of BDG exposure (PC1) has a major effect on monocyte gene expression (**Figure 2A**). Moreover, monocytes exposed to BDG for 4 hours have a different transcriptomic program compared to resting monocytes and monocytes exposed to BDG for 1 hour and 24 hours (PC2). After 24 hours of BDG exposure, we identified 3,852 genes with significantly dysregulated expression compared to resting monocytes ($FC \geq |2|$ and $FDR \leq 0.001$) (**Figure 2B**). Genes that were significantly dysregulated after BDG exposure were clustered as (1) early and stable upregulation, (2) transient upregulation at 4 hours, (3) intermediate and stable upregulation, (4) late upregulation, (5) early and stable downregulation, (6) transient downregulation at 4 hours, (7) intermediate and stable downregulation, and (8) late downregulation (**Figure 2C** and **Supplementary Table 3A**). There is a high degree of correlation between the ATAC-seq groups and RNA-seq clusters in both direction and timing of differential chromatin accessibility and gene expression (**Figure 2D**), showing that the BDG-induced changes in chromatin accessibility are associated with transcriptional changes of proximal genes. Gene ontology enrichment analyses were performed to identify biological processes enriched in these RNA-seq clusters (**Figure 2E** and **Supplementary Table 3B**). As previously described, the classical proinflammatory categories (i.e., TLR, MAPK, LPS, and IFNγ signaling) were enriched in the downregulated clusters (12, 35). Genes linked to cytokine signaling and transcriptional regulation were enriched in both up and down regulated gene sets. Interestingly, the categories enriched in the upregulated clusters are related to phagosome maturation, RNA and translation, but importantly multiple metabolism pathways including citric acid cycle, cholesterol metabolism, pentose phosphate pathway, electron transport chain, and glutathione metabolism.

Transcriptional Regulation of Monocytes Exposed to BDG

As the "transcriptional regulation" GO category was enriched in both up and downregulated genes clusters (**Figure 2E**), we further used the ATAC-seq and RNA-seq datasets to gain insight in the transcriptional regulation of the monocytes' response to BDG. Using Homer (20), we search for enrichment of various transcription factors (TFs) binding motifs in the ATAC-seq groups (**Figure 3A**). As expected, the motif of pioneer TFs (PU.1, CEBP, and AP-1) as well as architectural TF CTCF were enriched in all clusters. EGR and MITF binding motifs, previously shown to be enriched after BDG exposure in this dataset (12), were enriched in all ATAC-seq groups with increased DARs. The E-box and KLF motifs, were enriched in groups linked to increased but not decreased chromatin accessibility. The NRF motif, associated with the antioxidant response in myeloid cells (36), was enriched in all the groups with the highest levels of enrichment in clusters with increased chromatin accessibility (A-C) and cluster F. We then extracted the differentially expressed TF expression using the same clustering from **Figure 2C** (**Figure 3B** and **Supplementary Table 3C**). CTCF, CEBP, EGR, E-box, AP-1, MITF, and NRF motifs had enrichment in the ATAC-seq groups and the expression of genes encoding TF of these families were significantly upregulated (i.e. *CTCF, CEBPB, EGR2, CLOCK, JUN, MITF and NFE2L1-2*, respectively), while IRF, NFκB and RUNX family members were downregulated. Using TRRUST (24) we queried differentially up and down regulated genes in response to BDG exposure against a database of TF cistromes based on ChIP-chip and ChIP-seq datasets (**Figure 3C** and **Supplementary Table 5A**). Interestingly, the NRF2 cistrome was significantly enriched amongst the genes upregulated after BDG exposure. Gene ontology of genes in the NRF2 cistrome that were also upregulated by BDG exposure were enriched for the biological processes: response to oxidative stress, cell-cell

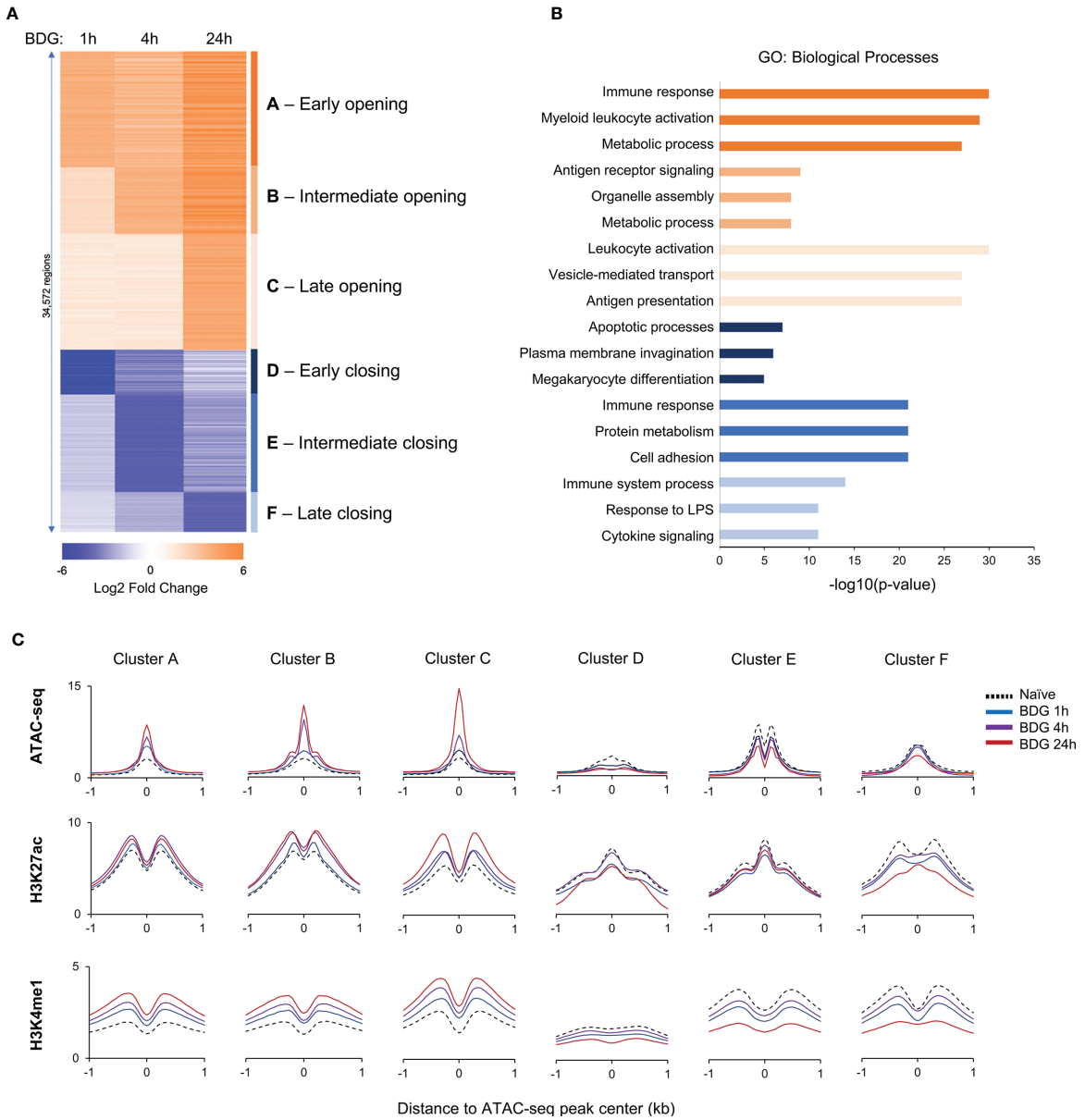

FIGURE 1 | Changes in the epigenetic landscape of monocytes after BDG exposure. **(A)** Heatmap of differentially accessible regions (DARs) after BDG exposure identified using ATAC-seq; fold change ≥ |2|. Regions were clustered (groups A-F) based on increase or decrease of chromatin accessibility after 1h, 4h, and 24h of BDG exposure. **(B)** Gene ontology enrichment analysis for each DAR group defined in panel A; the nearest gene to each DAR with a maximum distance of 500kb were used for the analysis. The top three categories are shown; see **Supplementary Table 2B** for complete list. **(C)** Density profiles of ATAC-seq, H3K27ac and H3K4me1 ChIP-seq at the DAR clusters. Signal is measured in 50bp bins (± 1kb) centered on the ATAC-seq peaks.

adhesion, cytokine signaling, and glutathione metabolism (**Figure 3D** and **Supplementary Table 5B**).

BDG Induces Glutathione Synthesis and Metabolism in Human Monocytes

Given that glutathione metabolism was enriched in the gene ontology of upregulated genes and that glutathione synthesis/metabolism are regulated by NRF2, a TF whose gene expression is increased after BDG exposure and motif is enriched in all the ATAC-seq groups, we sought to investigate the effect of BDG on this pathway in human monocytes (**Figure 4A**). Strikingly, the expression of the genes encoding the enzymes catalyzing GSH synthesis and metabolism *GCLC*, *GSS*, and *GSR* were increased after 24 hours of BDG exposure as assessed by RT-qPCR and RNA-seq (**Figures 4B, C**). At the epigenetic level, 24

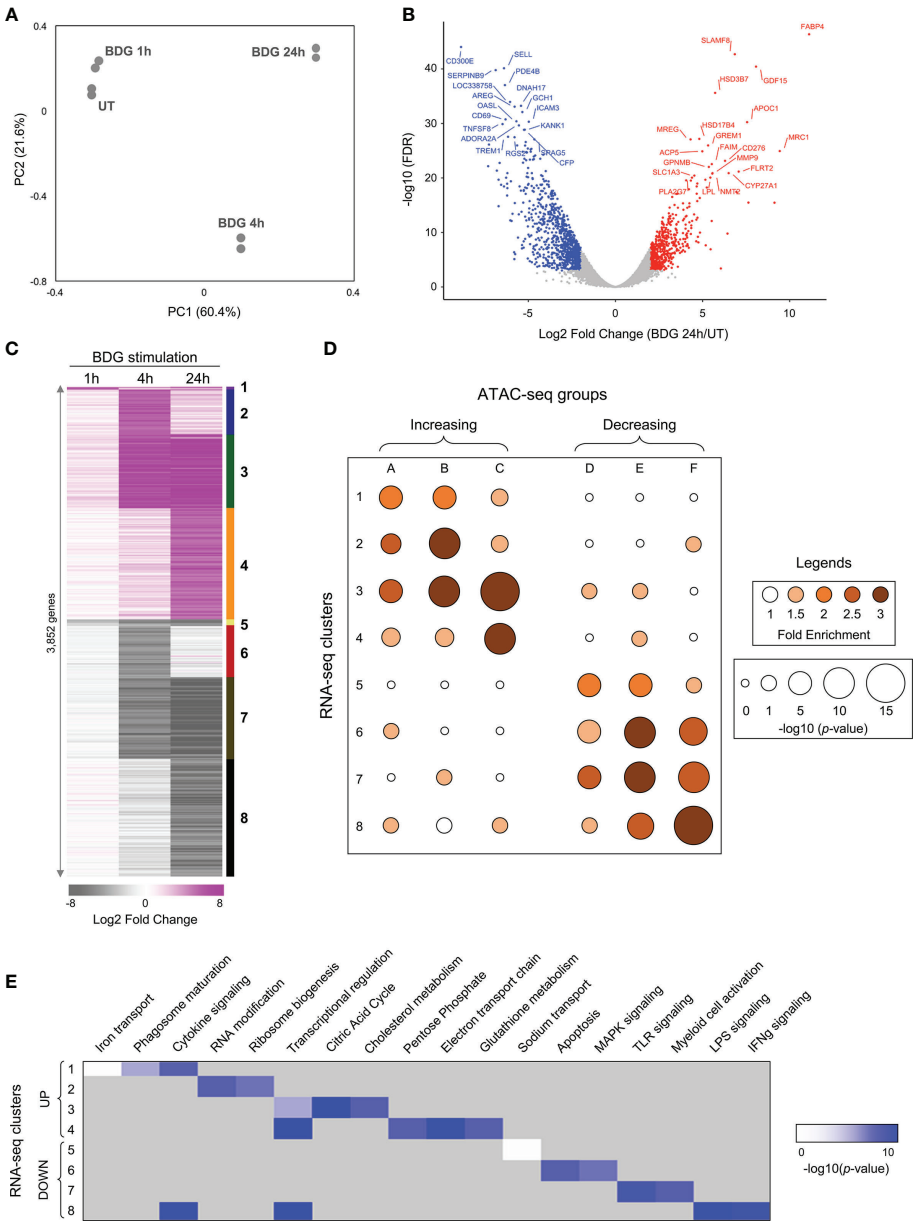

FIGURE 2 | Differential gene expression of monocytes after exposure to BDG. **(A)** Principal component analysis of RNA-seq data from untreated monocytes (UT) or monocytes exposed to BDG for 1h, 4h, and 24h. **(B)** Volcano plot of differentially expressed genes after 24h of BDG exposure compared to resting monocytes. False discovery rates (FDR) are calculated using Fisher's exact test with correction using the Benjamini and Hochberg method. **(C)** Heatmap identifying groups of genes that are differentially expressed after 1, 4, and 24 hours of BDG exposure. Genes are grouped (1–8) based on a cutoff of fold-change > 2 (upregulated) or < 0.5 (downregulated) and FDR < 0.001. **(D)** Bubble histogram showing the association between groups of differentially accessible regions and clusters of differentially expressed genes after BDG exposure. The color gradient reflects the ratio in the number of regions in each cluster associated with differentially expressed gene groups compared to control sets of randomly selected genes. Bubble size indicates the strength of the –log10 Fisher's exact test p-value for the association between DARs and differentially expressed genes compared to randomly selected control genes. **(E)** GO category enrichment analysis for groups of differentially expressed genes (white to blue gradient represents degree of enrichment, grey – not enriched).

hours of BDG exposure was not associated with strong changes in ATAC-seq signal but H3K27ac was increased at the *GCLC* and *GSR* loci. To verify if the increased expression of *GCLC*, *GSS*, and *GSR* had a functional impact on the activity of the GSH pathway, we measured total intracellular glutathione levels and ratio of reduced (GSH) to oxidized (GSSG) glutathione after 24 hours of BDG exposure (**Figure 4D**). In contrast to LPS stimulation, known to increase intracellular ROS and thus increase the intracellular GSH/GSSG ratio without changing total glutathione levels (**Supplementary**

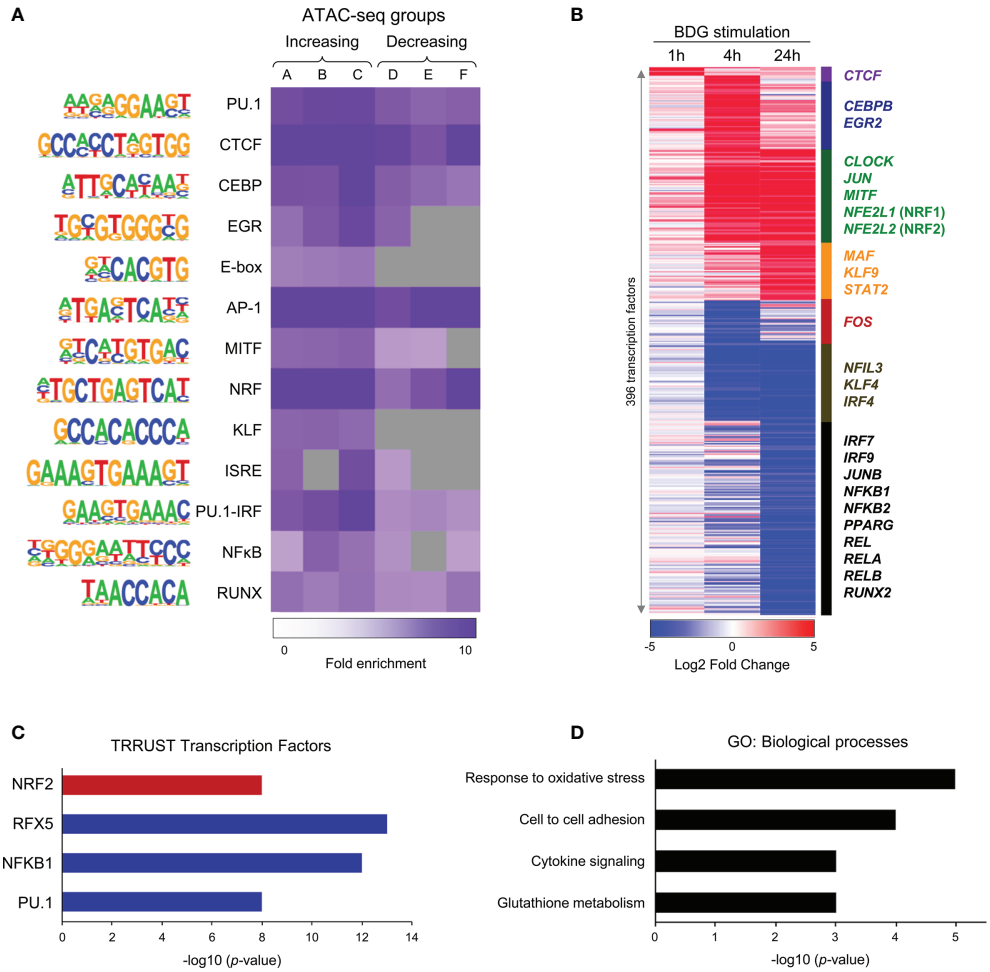

FIGURE 3 | Changes in motif enrichment and transcription factor expression in monocytes after BDG exposure. **(A)** Enriched motifs using Homer *de novo* motif analysis on the DAR clusters identified in **Figure 1A**. Color gradient indicates ratio of motif enrichment amongst regions in that cluster compared to randomly selected regions across the human genome with similar GC-content. Grey boxes indicate fold enrichment less than 1. **(B)** Heatmap of 342 differentially expressed transcription factors (TFs) after BDG exposure in the groups defined in **Figure 2C**. **(C)** TRRUST query of genes significantly dysregulated by 24h BDG exposure *vs* random sets of genes against a database of all known cistromes. Red represents cistromes enriched in genes significantly upregulated by BDG exposure, blue represents cistromes enriched amongst genes significantly downregulated by BDG exposure ($p < 10^{-7}$). **(D)** Biological process gene ontology enrichment analysis of genes significantly upregulated by BDG exposure and enriched in the NRF2 cistrome ($p < 0.01$).

Figure 3) (37), BDG exposure increases both the GSH/GSSG ratio and the total amount of intracellular GSH (**Figure 4D**).

Intracellular GSH Is a Regulator of BDG-Induced NADP Synthesis and Pentose Phosphate Pathway Metabolism

To determine the role of the GSH induction by BDG on monocyte function, we evaluated the impact of blocking GSH synthesis using L-buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase (GCLC) (38). Pretreating culture medium with 120μM of BSO was sufficient to inhibit glutathione synthesis after 48 hours (**Figures 5A, B**). Conversely, as the plasma membrane has transport proteins which can move glutathione between the intra and extracellular environments (39), adding GSH to the culture media increased intracellular levels of GSH (**Figures 5A, B**). Interestingly, while GSH supplementation or BSO pre-treatment alone did not have any effect on monocyte survival, BSO treatment followed by BDG exposure caused significant cell death (**Figure 5C**), indicating a protective effect of the elevated GSH pathway activity during BDG exposure.

The pentose phosphate pathway (PPP) was enriched amongst genes significantly upregulated after BDG exposure and one of its products, NADPH, is required for glutathione metabolism (**Figures 6A**). Hence, we sought to investigate the relationship between BDG exposure, glutathione metabolism, and the PPP. Total intracellular levels of NADP+NADPH and NADPH/NADP ratio were increased after BDG exposure (**Figures 6B, C**). Such elevation was abrogated with BSO pre-treatment and

replicable with adding GSH to culture media. At the transcriptome level, genes encoding enzymes involved in the pentose phosphate pathway, *NADK*, *NADK2*, *G6PD*, *PGLS*, *PGD*, and *TALDO1* were up-regulated with BDG exposure in a GSH-dependent manner (**Figures 6D–I** and **Supplementary Figure 4**). To some extent, glutathione exposure alone was sufficient to induce increased expression of these genes *in vitro*.

Intracellular GSH Is a Regulator of BDG-Induced NO Secretion, Phagocytosis, and Cytokine Production

As metabolism of myeloid cells is closely linked to their effector functions and intracellular GSH levels regulated BDG-induced metabolic changes, we wanted to investigate if GSH could also regulate monocyte's effector functions in response to BDG. While BDG did not induce increased expression of *NOS2*, we observed that BDG induced production of nitric oxide (NO) (**Figures 7A, B**). Inhibiting glutathione synthesis with BSO increased NO production while exposing monocytes to GSH did not induce any NO. Phagocytic capacity of monocytes was assessed using pH-sensitive rhodopsin labelled *E. coli* that fluoresces in the acidic environment of the phagolysosome. BDG induced increased phagocytic capacity of human monocytes in a GSH-dependent manner (**Figure 7C**). BDG also induced the upregulation of *IL6*, *CXCL8*, and *IL10* gene expression as well as production of IL-6, IL-8, and IL-10 in a GSH-dependent manner, GSH supplementation alone induced a similar response (**Figures 7D–I**).

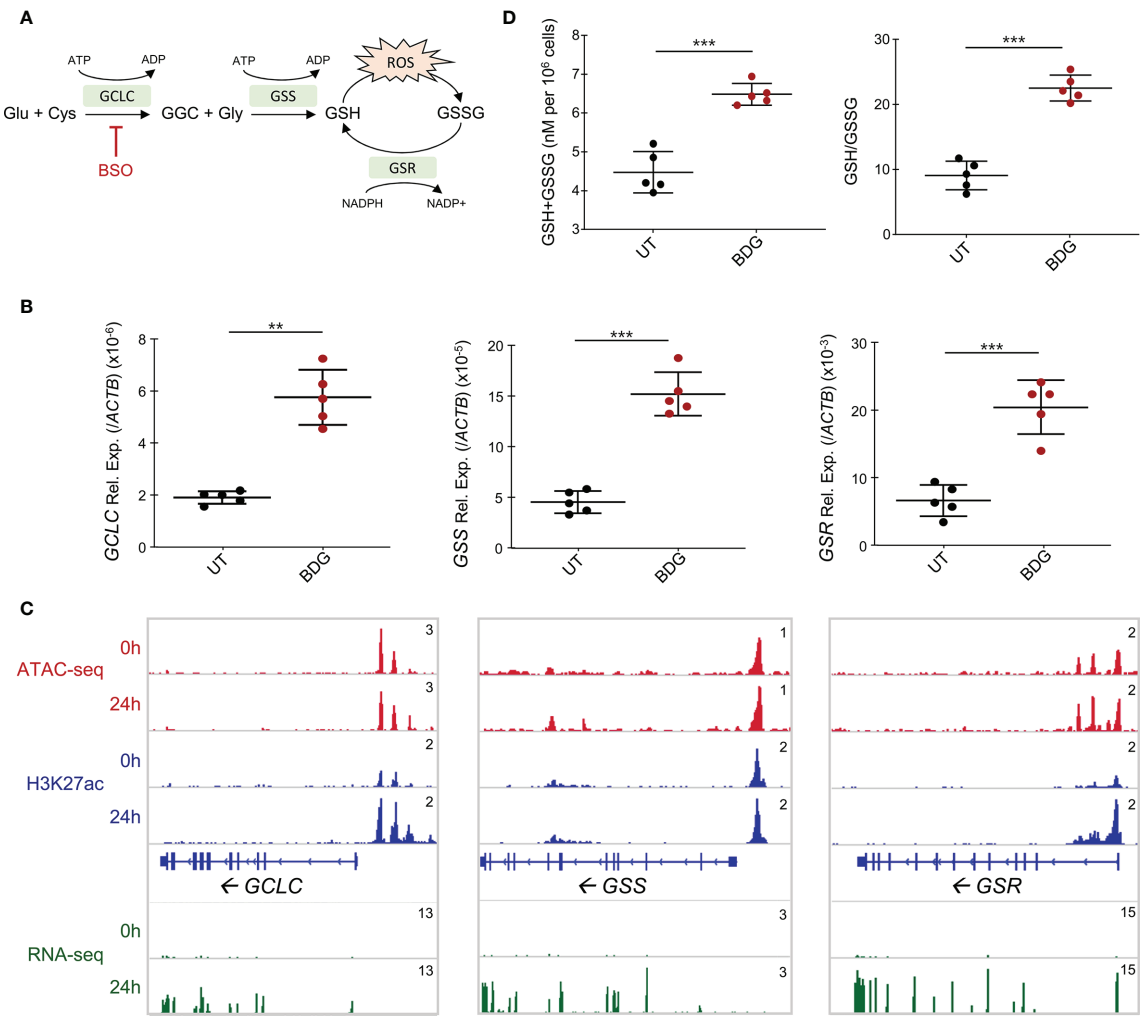

FIGURE 4 | Glutathione synthesis and metabolism are induced by BDG stimulation. **(A)** Schema of glutathione metabolic pathway with genes that are significantly upregulated from the RNA-seq in green. **(B)** Expression of *GCLC*, *GSS*, and *GSR* were evaluated with RT-qPCR. Data are presented as median ± standard deviation of n = 5 biologically independent experiments. **(C)** ATAC-seq, ChIP-seq, and RNA-seq read density profiles at the *GCLC, GSS*, and *GSR* loci. **(D)** Monocytes were stimulated with BDG for 24 hours and assessed for total intracellular glutathione levels and GSH/GSSG using calorimetry. Data are presented as median ± standard deviation of n = 5 biologically independent experiments. *P*-values were calculated using paired student's *t*-test. $^{**}P < 0.01$; $^{***}P < 0.001$.

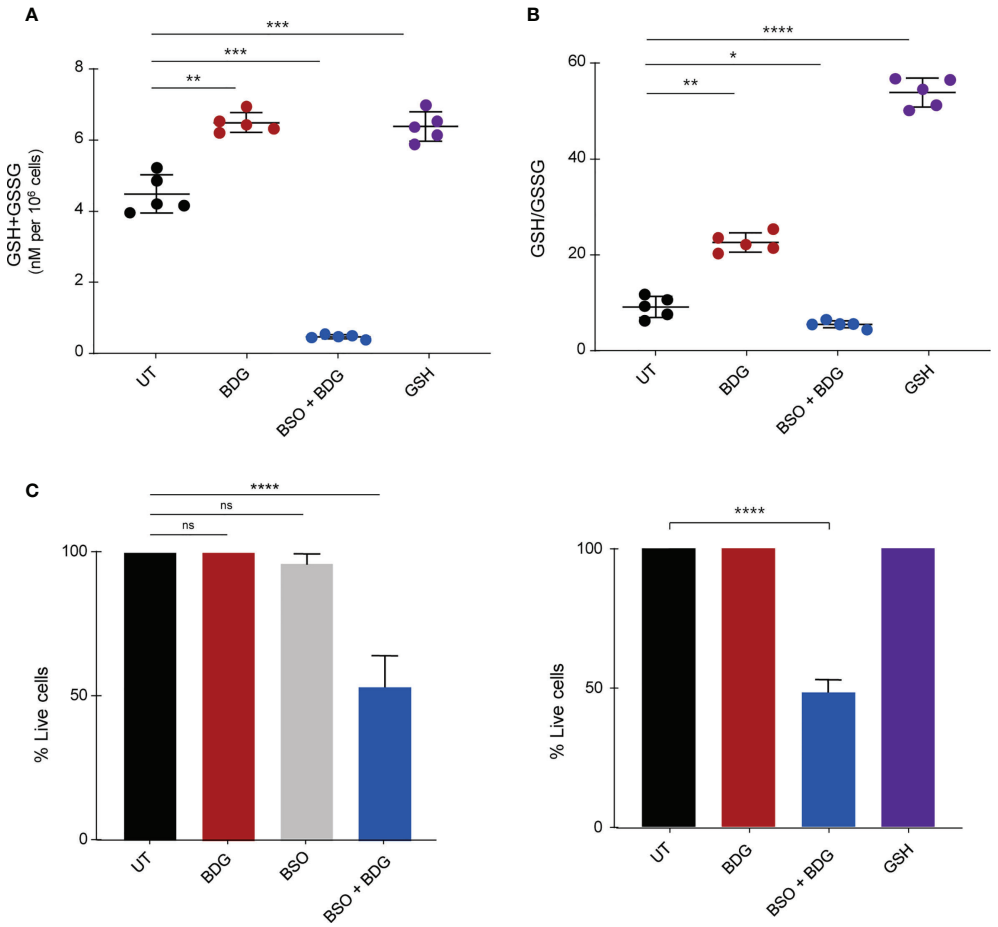

FIGURE 5 | Intracellular glutathione contributes to monocyte survival after BDG exposure. Monocytes were stimulated with BDG for 24 hours, pre-treated with BSO for 24 hours and then stimulated with BDG for 24 hours, or stimulated with GSH for 24 hours and then assessed for **(A)** Intracellular GSH+GSSG (total intracellular glutathione concentration), **(B)** GSH/GSSG (reduced to oxidized glutathione ratio), and **(C)** cell survival. Data are presented as median ± standard deviation of n≥3 biologically independent experiments. *P*-values were calculated using paired one-way ANOVA with multiple comparisons. ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

DISCUSSION

The objective of this study was to better understand the acute inflammatory response of monocytes to the PAMP BDG. Using functional genomics data (ATAC-seq, ChIP-seq, and RNA-seq) (12), we identified that BDG exposure induces changes in the immunometabolic and effector programs of human monocytes. It was previously demonstrated that BDG induces the expression of genes involved in glycolysis, cholesterol synthesis and the pentose phosphate pathways (11) and metabolomics confirmed increased activity of these pathways (40). Herein, we identified that BDG induces glutathione synthesis and metabolism at the epigenetic and transcriptomic levels. *In vitro*, we validated this increased gene expression *via* RT-qPCR and observed that BDG increases the global intracellular concentration of glutathione as well as the level of the oxidized glutathione (GSSG) form. While inhibiting glutathione synthesis with BSO had no effect on survival on its own, BSO pre-treatment followed by BDG exposure had a significant impact on monocyte survival and function. We observed that intracellular glutathione was a regulator of BDG-induced immunometabolic changes by increasing NADP synthesis and the activity of the pentose phosphate pathway. Interestingly, BDG-induced effector functions of monocytes such as NO production, phagocytosis, and cytokine production were also regulated by intracellular levels of glutathione. These results further elucidate the acute response of monocytes to BDG and establish a novel role for glutathione metabolism in monocyte biology.

Monocytes play an important role in clearing infections by detecting PAMPs, phagocytosing/presenting antigens, and producing pro-inflammatory cytokines/chemokines to help recruit other leukocytes to the site of infection. β-glucans are β-D-glucose polysaccharides with different physicochemical properties and found in the cell wall of multiple organisms including fungi, bacteria, yeast, algae, and some cereals. The pathogen-associated β-glucans are insoluble chains of D-glucose linked by 1→3 glycosidic bonds, with some 1→6 branching in yeast

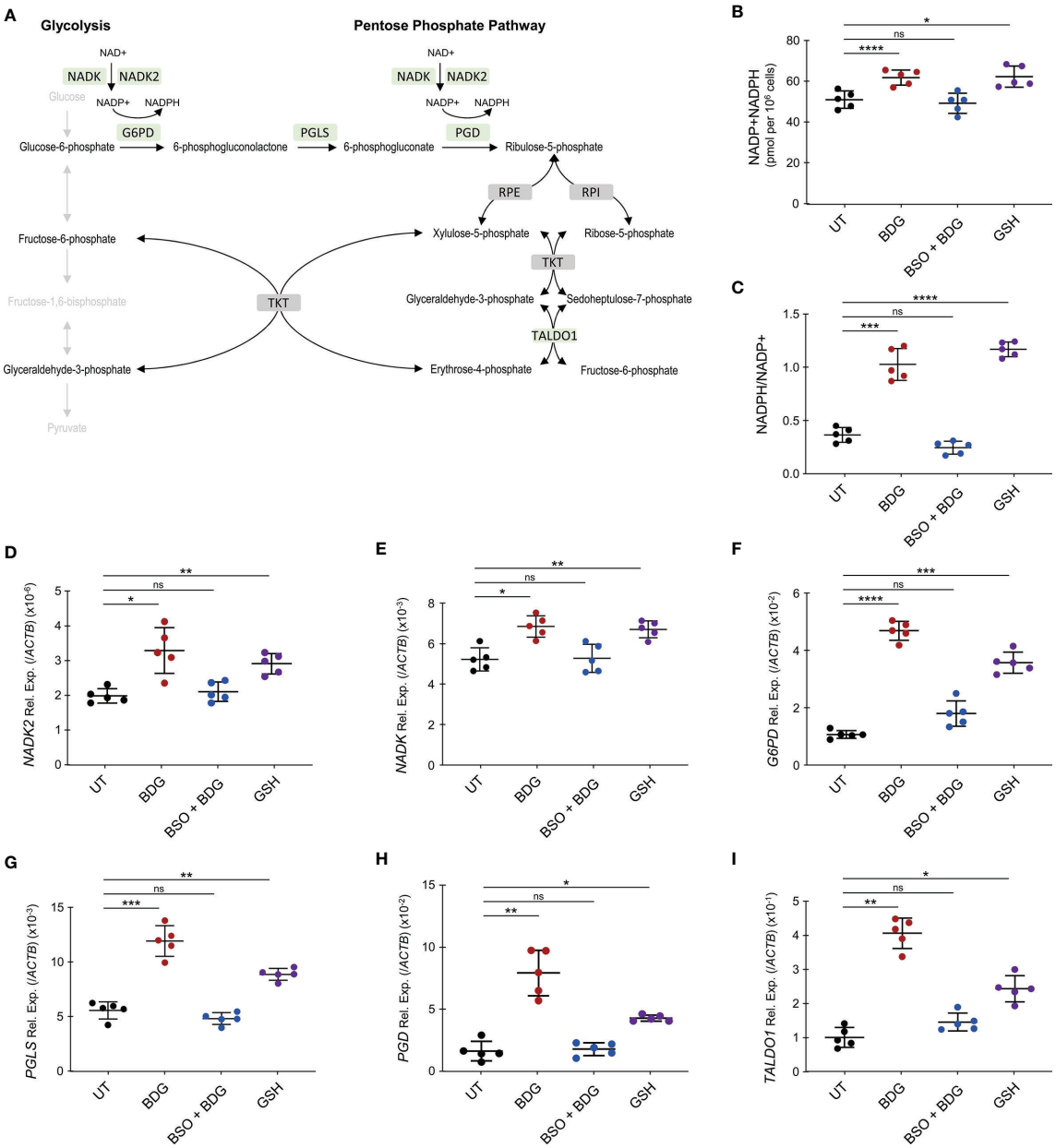

FIGURE 6 | BDG induces NADP synthesis and upregulation of the pentose phosphate pathway (PPP) in a glutathione dependent manner. **(A)** Schema representing PPP with genes that are significantly upregulated after BDG exposure as assessed by RNA-seq in green and genes that are not differentially expressed in grey. **(B–I)** Monocytes were stimulated with BDG for 24 hours, pre-treated with BSO for 24 hours and then stimulated with BDG for 24 hours, or stimulated with GSH for 24 hours and **(B)** total NADP+NADPH levels as well as **(C)** NADPH/NADP+ were measured using calorimetry. The relative expression of **(D)** *NADK*, **(E)** *NADK2*, **(F)** *G6PD*, **(G)** *PGLS*, **(H)** *PGD*, and **(I)** *TALDO1* were quantified using RT-qPCR. Data are presented as median ± standard deviation of n = 5 biologically independent experiments. *P*-values were calculated using paired one-way ANOVA with multiple comparisons. ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

and fungi. Recognition of BDG by innate immune cells is required for effective recruitment of other leukocytes and subsequent clearance of the infection (41). Mice deficient for Dectin-1, the myeloid cell receptor for BDG, rendered them susceptible to *Candida* infection. In fact, these mice had substantially increased fungal burdens and enhanced fungal dissemination due to impaired phagocytosis, antigen presentation, and cytokine/ROS production of their monocytes (16). In this study, we have used BDG from the gram negative bacterium *Alcaligenes faecalis* for our *in vitro* experiments while the RNA-seq datasets were generated using heat killed *Candida albicans* (7). Despite their different origin and that fungi BDG harbor 1→6 branching, we believe that these two reagents can be used interchangeably as we have validated the transcriptional regulation of genes induced by fungi BDG using RT-

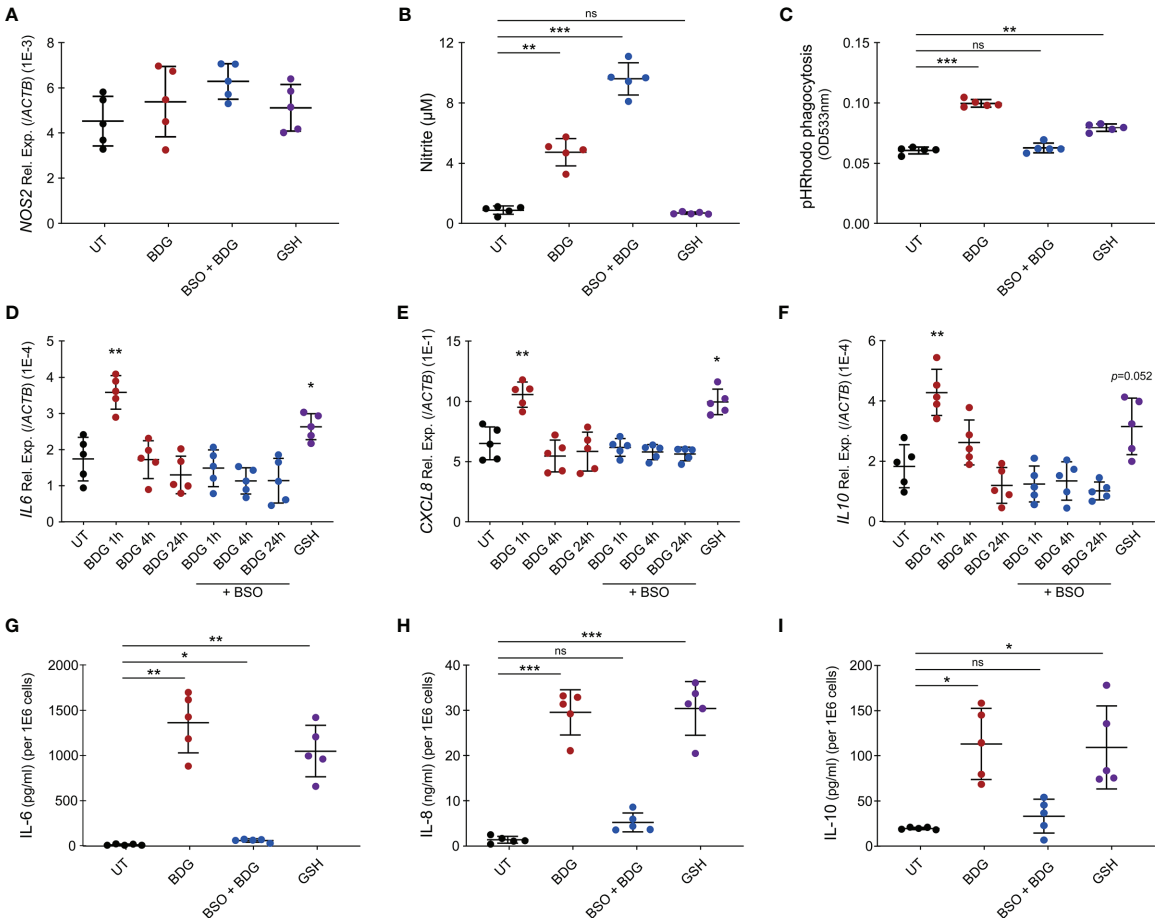

FIGURE 7 | Intracellular glutathione levels modulate monocyte's inflammatory response to BDG stimulation. **(A–C)** Human monocytes were untreated, stimulated with BDG for 24h, pre-treated with BSO for 24h then stimulated with BDG for 24h, or stimulated with reduced glutathione (GSH) for 24h and assessed for **(A)** *NOS2* expression, **(B)** Nitrite production, and **(C)** phagocytic capacity. **(D–F)** Human monocytes were untreated, stimulated with BDG for 1h, 4h, or 24h, pre-treated with BSO for 24h then stimulated with BDG for 1h, 4h, or 24h, or stimulated with GSH for 24h and then evaluated for expression of the **(D)** *IL-6*, **(E)** *CXCL8*, and **(F)** *IL-10* loci using RT-qPCR. **(G–I)** Human monocytes were untreated, stimulated with BDG for 24h, pre-treated with BSO for 24h then stimulated with BDG for 24h, or stimulated with reduced glutathione (GSH) for 24h and assessed for production of **(G)** IL-6, **(H)** IL-8, **(I)** IL-10 using ELISA. Data are presented as median ± standard deviation of n = 5 biologically independent experiments. *P*-values were calculated using paired one-way ANOVA with multiple comparisons. ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

qPCR on monocyte treated with bacterial BDG. Moreover, two recent reports show that BDG from gram negative bacterium (*Alcaligenes faecalis*), zymosan (*fungi*), and yeast-derived BDG induce a similar pro-inflammatory phenotype in primary human monocyte-derived macrophages and primary human monocytes (42, 43). From clinical applications, we know that BDG from *A. faecalis* is extremely similar to fungal BDG, as the clinical BDG test for diagnosing IFIs has false positives in patients with colonization and/or infection with this bacterium (4). Here, while we don't investigate the precise signaling pathway, we show that intracellular glutathione is required for survival and complete effector function of monocytes in response to BDG. Thus, glutathione may be an important molecule in antifungal immunity and future studies should address its role in clearing fungal infections.

Beyond mycoses, BDG has been shown to induce long-term epigenetic reprogramming, reverse LPS-induced tolerization in monocytes/macrophages, and confer protection from infectious diseases including leishmaniasis and tuberculosis (12–14). Changes in the epigenetic landscape of monocytes leading to BDG-induced trained immunity are mediated by the activation of mechanistic target of rapamycin (mTOR) and hypoxia-inducible factor 1α (HIF-1α). In fact, blocking this pathway in several studies has consistently abrogated BDG-induced trained immunity (11, 44). Interestingly, Mak et al. investigated the molecular targets downstream of intracellular GSH in T cells. As ROS are a known inhibitor of mTOR activation, they showed that intracellular GSH was able to buffer ROS leading to mTOR activation, metabolic reprogramming, and resultant inflammatory response of T cells *in vitro* and *in vivo* (45). In our study, we observed that BDG increased glutathione levels in monocytes and that intracellular GSH was able to reduce ROS production post-translationally. Hence, future studies should

investigate whether ROS buffering by intracellular GSH allows BDG-induced trained immunity in monocytes.

Previous studies have also addressed the role of glutathione in monocyte/macrophage biology. Kerstholt et al. demonstrated that *Borrelia burgdorferi* infection of human monocytes increased glutathione synthesis and GSH/GSSG ratio. Moreover, they saw that intracellular glutathione was required for production of acute pro-inflammatory cytokines in response to this bacteria (46). In RAW264.7 macrophages, BSO pre-treatment was previously shown to partially abrogate the LPS-induced pro-inflammatory response (37). Another study investigated the role of glutathione in regulating macrophage mediated killing of *Mycobacterium tuberculosis*. While LPS/IFNγ exposure induced intracellular killing of BCG in J774.1 macrophages, this response was diminished with BSO pre-treatment. To investigate whether this was due to the ability of GSH to buffer ROS, they repeated this experiment with peritoneal macrophages from iNOS knockout mice. Treatment of BCG-infected $iNOS^{-/-}$ macrophages with LPS/IFNγ, induced killing of about 75% of intracellular BCG after 72 hours. In contrast, pre-treating these macrophages with BSO before LPS/IFNγ stimulation completely abrogated this killing and resulted in BCG multiplying after 72 hours (47). Thus, there is evidence that GSH promotes intracellular killing of BCG in a ROS-independent manner. In our study, we observed that intracellular glutathione was a regulator of monocyte's effector functions including phagocytosis and cytokine production. As previously reported in the RAW264.7 macrophage cell line, we also saw that exogenous GSH was sufficient to elicit cytokine production and increased phagocytic capacity, but the mechanism is poorly understood (48). Taken together, there is converging evidence that suggests a critical role of intracellular glutathione in regulating the acute inflammatory response of monocytes/macrophages.

While there are different models to study glutathione metabolism, in this study we used BSO as it is a specific and potent chemical inhibitor of the rate limiting enzyme GCLC (38). Transient *Gclc* knockdown using siRNA has been successfully use in the mouse hepatocyte cell line FL83B to study the role of glutathione in vitamin D metabolism and other pathways (49, 50). Importantly, the authors have also demonstrated that inhibition of glutathione synthesis using BSO and *Gclc* siRNA knockdown were similarly decreasing cellular GSH levels, increasing oxidative stress and affecting the vitamin D metabolism (50). While future studies can use either *GCLC* knockdown or BSO pretreatment to further investigate the relationship between BDG response and the glutathione pathway, we have chosen chemical inhibition given efficient transient gene knockdown is technically challenging human primary monocytes. Moreover, we have further validated our findings by using exogenous GSH as a positive control. Our *in vitro* experiments on human primary monocytes show that the glutathione metabolism is involved in the regulation of the acute response to BDG at the transcriptomic and molecular levels, yet further studies are required to evaluate the functional impact of this phenomenon *in vivo*. Previous studies have attempted to create *Gclc* knockout mice, however these mice died *in utero* by gestation day 13. Although heterozygous mice were viable and fertile, they only had a 20% reduction in GSH levels making it an unideal model to study glutathione metabolism (51). While *in vivo* models of *Gclc* knockout mice have had limited success, future studies could investigate this relationship with either *Gclc* heterozygous mice or wild type mice treated on the one hand with BSO to pharmacologically block GCLC and on the other hand with exogenous glutathione.

Overall, we investigated the acute response of monocytes to a major PAMP, BDG. We showed that BDG induces glutathione synthesis and metabolism at the epigenetic, transcriptional, and molecular levels. Eliminating intracellular GSH with BSO reduced cell survival in BDG stimulated but not resting monocytes, suggesting a critical role of intracellular GSH in cell survival after an infectious challenge. Moreover, intracellular GSH buffered NO production at the post-translational level and participates in protecting monocytes from their own oxidative stress without abrogating NO production and endosome acidification. BDG was shown to be a regulator of immunometabolism and effector functions of monocytes. We now demonstrate this regulation to be dependent in part on GSH, since BSO pre-treatment abrogated BDG-induced phagocytosis and cytokine production. Overall, our findings demonstrate an important role for GSH in immunity and outline a better understanding of the acute response of monocytes to infections.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by McGill University Health Centre (MUHC) Montreal, QC, Canada, REB (#2019-5170). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RR and DL designed the study, prepared the first draft of the manuscript, and revised the final draft of the manuscript. RR, MM, JMN, LTT, SI, and JL performed the experiments and analyzed the data. J-PR, AN, and DL supervised the work. All authors contributed to the article and approved the submitted version.

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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.2021.694152/full#supplementary-material

Supplementary Figure 1 | Gating strategy to validate isolation of human monocytes from PBMC.

Supplementary Figure 2 | Longitudinal changes in epigenetic marks in monocytes after BDG exposure. **(A, B)** Clusters of differentially accessible regions identified in **Figure 1A** were queried for **(A)** H3K27ac and **(B)** H3K4me1 ChIP-seq signal in human monocytes after 0h, 1h, 4h, and 24h of BDG exposure. Data are presented as box and whisker plots with box representing median + interquartile range and whiskers representing 5th and 95th percentile marks. Red horizontal line denotes median tag count in 1kb of a specific epigenetic mark at 0h after BDG exposure. *P*-values were calculated using paired one-way ANOVA with multiple comparisons. ns, not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

Supplementary Figure 3 | Glutathione synthesis and metabolism in human monocytes before and after 24h stimulation. **(A)** Total intracellular glutathione levels in human monocytes at rest and after 24h of LPS or BDG exposure. **(B)** Intracellular GSH/GSSG ratio in human monocytes at rest and after 24h of LPS or BDG exposure. Data are presented as median ± standard deviation of n=3 biologically independent experiments. *P*-values were calculated using paired one-way ANOVA with multiple comparisons. ns, not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

Supplementary Figure 4 | ATAC-seq, ChIP-seq, and RNA-seq read density profiles at the *NADK, NADK2, G6PD, PGLS, PGD*, and *TALDO1* loci.

Supplementary Table 1 | Purity of monocyte isolations from PBMC using StemCell CD14+ monocyte negative selection kit.

Supplementary Table 2 | List of clusters of differentially accessible regions after BDG exposure and gene ontology enrichment analysis of these clusters.

Supplementary Table 3 | Groups of differential gene expression after 1h, 4h, 24h of BDG exposure and gene ontology enrichment analysis of these groups.

Supplementary Table 4 | List of primers used for RT-qPCR validations.

Supplementary Table 5 | TRUUST analysis of differentially expressed genes after 24 hours of BDG exposure in human monocytes.

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Antitheilerial Activity of the Anticancer Histone Deacetylase Inhibitors

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The apicomplexan parasite, *Theileria annulata*, is the most prevalent hemoprotozoan in livestock, causing significant economic losses worldwide. It is essential to develop new and improved therapeutics, as current control measures are compromised by the development of resistance against the only available antitheilerial drug, buparvaquone (BPQ). Histone deacetylase inhibitors (HDACi) were shown to treat cancer effectively and revealed *in vitro* antiparasitic activity against apicomplexan parasites such as *Plasmodium* and *Toxoplasma*. In this study, we investigated the antitheilerial activity of the four anti-cancer HDACi (vorinostat, romidepsin, belinostat, and panobinostat) against the schizont stage of *T. annulata* parasites. All four HDACi showed potent activity and increased hyperacetylation of the histone-4 protein. However, based on the low host cell cytotoxicity and IC_{50} values, vorinostat (0.103 μM) and belinostat (0.069 μM) were the most effective showing antiparasitic activity. The parasite-specific activities of the HDACi (vorinostat and belinostat) were evaluated by western blotting using parasite-specific antibodies and *in silico* analysis. Both vorinostat and belinostat reduced the *Theileria* infected cell viability by downregulating anti-apoptotic proteins and mitochondrial dysfunction, leading to caspase-dependent cell apoptosis. The HDACi caused irreversible and antiproliferative effects on the *Theileria* infected cell lines. Our results collectively showed that vorinostat and belinostat could be used as an alternative therapy for treating *Theileria* parasites.

Keywords: drug repurposing, HDACi, *Theileria annulata*, anticancer, molecular docking

INTRODUCTION

Bovine Theileriosis (BT), caused by *Theileria* parasites, is an economically significant parasitic disease (Brown, 1990). BT is prevalent in tropical and subtropical countries affecting millions of livestock worldwide (Nene and Morrison, 2016). In India, it is caused by the parasite *Theileria annulata* and *Theileria orientalis* (George et al., 2015a,b), mostly affecting crossbreed animals. In India, BT infections caused by *T. annulata* parasites are life-threatening, leading to the dairy industry's production and economic loss of $1,295 million/annum (Narladkar, 2018). In the last decade, because of the increase in the number of crossbreed animals, there has been a significant rise in the number of reported cases of *T. annulata* infected animals from India (Kundave et al., 2015;

Kumar et al., 2016; Larcombe et al., 2019). The single vaccine and drug buparvaquone (BPQ) are the only hope for fighting against this deadly parasite. The current schizont stage attenuated vaccine (Rakshavac-T) used in India has associated drawbacks like the infrastructure of vaccine production, its distribution, and cold chain maintenance; therefore, it is not commonly used in the field (Jeyabal et al., 2012). This leads to almost complete dependency on chemotherapy for BT treatment. In countries like Tunisia, Iran, and Sudan, BPQ resistance is reported from the field. However, in the published studies, the level of resistance or the prevalence of BPQ resistant *Theileria* parasites have not been done (Mhadhbi et al., 2010, 2015; Sharifiyazdi et al., 2012; Chatanga et al., 2019). Hence, there is an urgent need to discover new antitheilerial drugs/compounds to control the disease.

As new drug discovery takes a long time, drug repurposing is one approach that has helped researchers discover the unknown potential of the clinically approved drugs (Ashburn and Thor, 2004; Nwaka and Hudson, 2006). Identifying drugs that inhibit the parasite genes involved in transcriptional regulation, posttranslational modifications, or epigenetic regulation seems a good strategy for searching for new antiparasitic drugs (Andrews et al., 2014). In eukaryotes, HDACs have been shown to regulate multiple essential pathways, and abnormal alterations in these enzymes can lead to apoptosis or cancerous growth in cells (Gallinari et al., 2007; Li and Seto, 2016). HDAC inhibitors (HDACi) like vorinostat, romidepsin, belinostat, and panobinostat are FDA approved to treat different cancers (Grant et al., 2007; Prince and Dickinson, 2012; Thompson, 2014; Garnock-Jones, 2015). The *in vitro* antiparasitic activity of these four inhibitors has been previously investigated in protozoa parasites like *Plasmodium*, *Trypanosoma*, *Leishmania*, and *Schistosoma* (Engel et al., 2015; Chua et al., 2017). In *P. falciparum* and *P. knowlesi* parasites, all four HDACi have shown potent antiplasmodial activity. Because of differences between the human and parasitic HDACs, these enzymes seem promising targets for developing new generation antitheilerial drugs.

Keeping in mind the unavailability of backup drugs for treating BT infections, we planned to test known drugs to find their ability to target unique or unexplored pathways specific to the parasite. In this study, we tested antitheilerial activity of the four HDACi: vorinostat, romidepsin, belinostat, and panobinostat against the *T. annulata* parasites. These HDACi have never been targeted before for their antitheilerial activity and can be a new addition as an alternative therapy against *T. annulata* parasites. We have also investigated the hyperacetylation profiles of the *Theileria* infected cells after treatment with the compounds. Additionally, we have done molecular docking studies for showing the binding of HDACi to the *Theileria* specific proteins using *in silico* studies.

MATERIALS AND METHODS

Compounds

Buparvaquone (Cat No. B4725), belinostat (Cat no. A4096), and panobinostat (Cat no. 13280) were purchased from Apex Bio. Vorinostat (SAHA) was purchased from EpiGentek (M41000-2), and romidepsin (17130) was purchased from Cayman. All HDACi were prepared as 10–20 mM stock solutions in phosphate-buffered saline (PBS). BPQ was prepared as a 10 mM stock solution in 100% DMSO.

Theileria annulata Growth Inhibition Assays

Theileria annulata infected bovine cells were derived previously from the clinically infected cattle and cultured in the RPMI 1640 medium (Sigma Aldrich) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/mL Penicillin-Streptomycin at 37°C in a CO_2 incubator (George et al., 2015b; Roy et al., 2019). Antitheilerial activity of the compounds was analyzed by incubating different concentrations of compounds to *T. annulata* infected cells. Briefly, 5×10^3 *T. annulata* infected cells were seeded per well in 96 well plates in 200 μL medium at 37°C for 4 h. All four HDACi were serially diluted and added to the cells in the 96 well plate for 48 h. After 48 h, 30 μL resazurin dye (1.5 mM) was added to each well, and the fluorescence intensity of the cells was measured at 570 nm for accessing the viability of the cells based on the previously published method (Kulshrestha et al., 2013). BPQ was used as a positive control in all the assays. Each experiment was performed at least thrice independently in triplicates. The cytotoxicity profiles of the compounds were evaluated in BOMAC (Bovine macrophage cell Line) cell line using the standard protocol.

Protein Hyperacetylation Assay

Hyperacetylation assays were carried out using the protein lysate of the *T. annulata* infected bovine cells. Briefly, 1×10^5 cells were incubated for 3 h with IC_{50} concentration of test compounds (1X and 5X), and untreated cells were included as a control. BPQ treated cells were used as a negative control. *T. annulata* infected cells were then pelleted and washed thrice with 1X PBS before resuspending the cells for lysis in RIPA (Radio-Immunoprecipitation Assay) buffer. After sonication and centrifugation of the lysed cells, proteins were quantified using the BCA protein assay kit. SDS-PAGE loading dye was added to the sample, followed by denaturation (97°C, 5 min) and separation on SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane, and western blotting was done using anti-tetra acetyl histone H4 antibody (1:2,000, Sigma-Aldrich, 05-1355) and goat anti-mouse IgG secondary antibody (1:2,000) using chemiluminescent reagent (Takara). Histone H3 (1:2,000, CST, 9715S) was taken as the loading control. Membranes were imaged using the Biorad ChemiDoc Imaging system. Western blot images were processed in Image J software for protein quantification using the relative density method. Band intensities of the H3 (loading control) and anti-tetra acetyl histone H4 [the protein of interest (POI)] were quantified by taking the area of interest. Intensities were normalized by dividing the respective value with one of the samples for loading control and POI. Relative expression was calculated by dividing the normalized intensity of POI by its respective loading control.

Immunofluorescence Assay

5×10^4 *T. annulata* infected cells were incubated with IC_{50} concentration of HDAC compounds (vorinostat, romidepsin, and belinostat) with untreated cells as control. BPQ treated cells were used as the negative control. Cells were pelleted down after 3 h of incubation and washed thrice with 1X PBS. Next, the cells were fixed using 4% paraformaldehyde (37°C, 10 min) followed by 1X PBS washing and permeabilization by 0.1% Triton X-100. Permeabilized cells were incubated for 1 h with blocking buffer (2% BSA in 1X PBS) at room temperature. Cells were then incubated with anti-acetyl histone H4 (1:250, Sigma-Aldrich, 05-1355) antibody overnight at 4°C. The primary antibody was then discarded, and the slide was washed three times in PBS, followed by incubation with goat anti-mouse Cruz Fluor 555 secondary antibody (1:250, Santacruz) for 1 h at room temperature. Cells were further washed with 1X PBS, and gold antifade mountant with DAPI (1 μg/mL) was used to stain the nucleus. Images were recorded in the Airyscan microscope (Zeiss), and ZEN Blue software was used for analysis.

Western Blotting and Mitochondrial Membrane Potential

For western blot analysis, total proteins from the *T. annulata* infected cells were fractioned on 8% polyacrylamide gels before and after 48 h treatment of vorinostat and belinostat compounds. For checking the parasite-specific effect of these compounds, blotting was done for detection of *TaSP* (*Theileria annulata* surface protein) using rabbit anti-*TaSP* peptide antibody (1:3,000) and mouse anti-β-actin (1:1,000) as a loading control. The primary antibody was then discarded, and the membrane was washed thrice in PBS, followed by incubation with horseradish peroxidase-conjugated IgG secondary antibody (1:1,000; Thermo Fisher Scientific) for 1 h at room temperature. The membrane was imaged using the chemiluminescent reagent (Takara) on the Biorad ChemiDoc Imaging system.

For mitochondrial membrane potential analysis, *T. annulata* infected cells treated with and without belinostat and vorinostat drugs were incubated with a JC-1 probe. BPQ treated cells were used as a control in the experiment. After 48 h of drug treatment, cells were incubated with 2.5 μL of JC-1 dye for 20 min in the dark at 37°C. After washing, cells were resuspended in 500 μL of cell staining buffer. Data acquisition was made on the BD LSR Fortessa, followed by analysis using the Flow Jo software (Tree Star Inc., Ashland, OR). Mitochondrial depolarization was quantified by taking the ratio of red to green fluorescence emission intensity. All the fluorescence assays were carried out in two independent experiments.

Analysis of Cell Death Using Flow Cytometry

Annexin V-FITC and propidium iodide (PI) staining was done to investigate the cell death mechanism using flow cytometry. Briefly, 1×10^5 cells/well were incubated with 1X IC_{50} of test compounds (vorinostat and belinostat) with or without z-VAD-fmk (2 μM) for 48 h. Staurosporine (1 μM) with or without z-VAD-fmk (2 μM) was taken as the positive control. After 48 h, cells were washed with PBS and incubated with annexin V binding buffer (500 μL/tube) containing 5 μL annexin V and 10 μL PI for 15 min at 37°C. Data acquisition was made on the BD LSR Fortessa, followed by analysis using the Flow Jo software (Tree Star Inc., Ashland, OR) for detecting the % of apoptosis or necrosis in cells. Assays were performed in duplicate in three independent experiments.

Reverse Transcriptase-Polymerase Chain Reaction

HDACi (vorinostat and belinostat) treated and untreated cells were collected, total RNA was extracted using Trizol reagent, and 5°μg of total RNA was reverse transcribed for cDNA synthesis using a Primescript cDNA synthesis Kit (Takara) according to the manufacturer's protocol (Dandasena et al., 2018). The mRNA expression of matrix metalloproteinase 9 (MMP9) and B-cell lymphoma 2 (Bcl-2) gene was detected by real-time PCR using a BioRad CFX96 Touch System (Biorad). Relative target gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences used are as follows: MMP9: Forward-5′ CCCATTAGCACGCACGACAT-3′, Reverse 5′-TCACGTAGCCCACATAGTCCA-3′; HRPT1: Forward-5′-TGTGGCCAGCTTAATAG-3′, Reverse5′- GGCTCGTAGTGCA AATGAAG-3′, Bcl-2: Forward-5′- GATGACCGAGTACCT GAACC -3′, Reverse 5′- AGCCAGGAGAAATCAAACAGG-3′.

Homology Modeling and Molecular Docking

Since no crystal structure is available for the *TaHDAC1* putative protein, we used its amino acid sequence (TA12690) for searching its homologous proteins with available crystal structure in the Protein Data Bank (PDB). Human HDAC2 (PDB accession No. 5IWGA) was found to be a suitable template for modeling with 62.91% similarity to the TaHDAC1 protein at a resolution of 1.66 Å (**Figure 4**). Homology modeling of putative histone deacetylase of *T. annulata* (TA12690) was carried out using SWISS-MODEL Homology Modeling server.[1] Ramachandran plot, QMEAN score plot, and Local quality estimates assessed the quality of the modeled protein. The Ramachandran plot was generated using the PROCHECK program in Structure Analysis and Verification Server (SAVES) (Laskowski et al., 1996). The protein's ligand-binding site was determined using the 3DLigandSite prediction server and the previously published literature (Marks and Breslow, 2007; Wass et al., 2010). The modeled protein was saved in PDB format and docked using Schrodinger Maestro (version 12.2).

The grid generation module did the catalytic binding site's visualization and characterization. The ligands structure file was downloaded from the PubChem database [vorinostat (ID-5311), panobinostat (ID- 6918837), belinostat (ID-6918638), romidepsin (ID-5352062)] and prepared for docking to the modeled protein. The ligands were optimized using the OPLS3e force field in the Ligprep module, followed by docking into the generated receptor grid using the sitemap option in Schrodinger

[1]https://swissmodel.expasy.org/

Maestro. The ligand conformation having the lowest binding energy was considered for all the inhibitors.

Reversibility of Growth Inhibition After Treatment With Histone Deacetylase Inhibitor

Theileria infected cells were treated with IC_{50} concentration of vorinostat and belinostat compounds for 48 h to check the effect on parasite growth. After 48 h, drug pressure was removed, and parasites were grown in a traditional medium without the HDACi. The proliferation of the *T. annulata* cells was monitored by trypan blue assay for the next 12 days for assessing the effect of drug treatment.

RESULTS

Histone Deacetylase Inhibitors Showed Antitheilerial Activity Against *Theileria annulata* Parasites

For assessing the antitheilerial activity of the HDACi (vorinostat, belinostat, romidepsin, and panobinostat), *in-vitro* cultured *T. annulata* parasites were challenged with different concentrations of the compounds. BPQ was included as a control in the study. Except for panobinostat, all the other inhibitors showed potent antiparasitic activity based on the observed IC_{50} values (**Figure 1**). The values of vorinostat, belinostat, romidepsin (<0.3 μM) were at least 20 times lower than that of the panobinostat (20 μM) compound (**Table 1**). These four HDACi were previously reported to be effective ($IC_{50} \leq 0.1$ μM) against *P. falciparum* and *P. knowlesi* strains (Engel et al., 2015). A comparison was made for the effectiveness of the HDACi based on the IC_{50} values between the *Theileria* and *Plasmodium* parasites. The vorinostat, belinostat, and romidepsin values in *T. annulata* were similar to previously published data in *Plasmodium* parasites ($IC_{50} \leq 0.2$ μM) (Engel et al., 2015). However, panobinostat behaved differently, showing antiparasitic activity at significantly higher drug concentrations in *T. annulata* cells compared to *Plasmodium* parasites ($IC_{50} \leq 0.03$ μM) (Engel et al., 2015).

In vitro Cytotoxicity of Histone Deacetylase Inhibitors

Since only three HDACi (vorinostat, belinostat, and romidepsin) had potent *in-vitro* activity ($IC_{50} \leq 0.3$ μM), we decided to focus on these compounds for further studies. The *in vitro* cytotoxicity was assessed for the three HDACi against the BOMAC cells using a resazurin dye-based assay. Vorinostat and belinostat were non-toxic based on the IC_{50} values of the assay (**Table 1**). In contrast, romidepsin was equally toxic (>0.2 μM) on mammalian cells compared to *T. annulata* infected cells. The SI values of vorinostat and belinostat in *T. annulata* compared to mammalian cells (SI 140 and 195, respectively; **Table 1**) were higher than previously published data for *Plasmodium* parasites (SI 140 and 195, respectively) indicating greater selectivity for *Theileria* parasites. Our results with romidepsin were in sync with the previously published cytotoxicity results in *Plasmodium* species (Engel et al., 2015).

Histone Deacetylase Inhibitors Leads to Hyperacetylation of *Theileria annulata* Infected Cells

For checking hyperacetylation of proteins in *Theileria* infected cells, cell lysate was prepared after 3 h of treatment with HDACi (vorinostat, belinostat, and romidepsin). For quantitative assessment, *T. annulata* infected cells were treated with 1X and 5X concentrations of the IC_{50} values of the inhibitors.

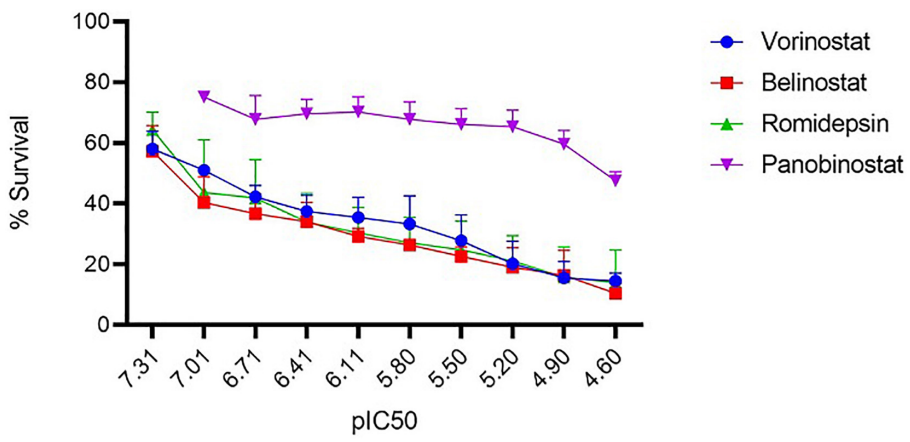

FIGURE 1 | *In vitro* antitheilerial activity of histone deacetylase (HDAC) inhibitors. Dose-response curves against the four HDACi in *T. annulata* infected cells. IC_{50} was calculated using a resazurin dye-based assay. The IC_{50} values of vorinostat, belinostat, romidepsin, and panobinostat was 0.103 (±0.005) μM, 0.069 (±0.004) μM, 0.20 (±0.006) μM, and 20.80 (±3.11) μM, respectively. IC_{50} values are represented as the negative log of IC_{50} in Molar referred to as pIC_{50} (ranged from 7.31 to 4.60 referring to IC_{50} concentration from 0.097 to 25 μM). The graph represents the mean % survival at different concentrations of HDACi. ± represents the standard deviation (SD) from the three independent experiments. All the experiments were done in triplicates.

TABLE 1 | *In vitro* antitheilerial activity of histone deacetylase (HDAC) inhibitors against *T. annulata* infected cells.

Compound	Structure	PubChem CID	*T. annulata* IC50 (μM)	Mammalian cell IC50 (μM)	SI
Vorinostat		5311	0.103 (±0.005)	>25	>140
Belinostat		6918638	0.069 (±0.004)	9.875 (±3.712)	195
Romidepsin		5352062	0.200 (±0.006)	0.296 (±0.029)	1.451
Panobinostat		6918837	20.800 (±3.110)	nd	nd
Buparvaquone		71768	0.153(±0.011)	>1.500	10.239

Nd, not determined. SI, (Mammalian cells IC_{50})/(T. annulata parasite IC_{50}); larger values = greater parasite selectivity.

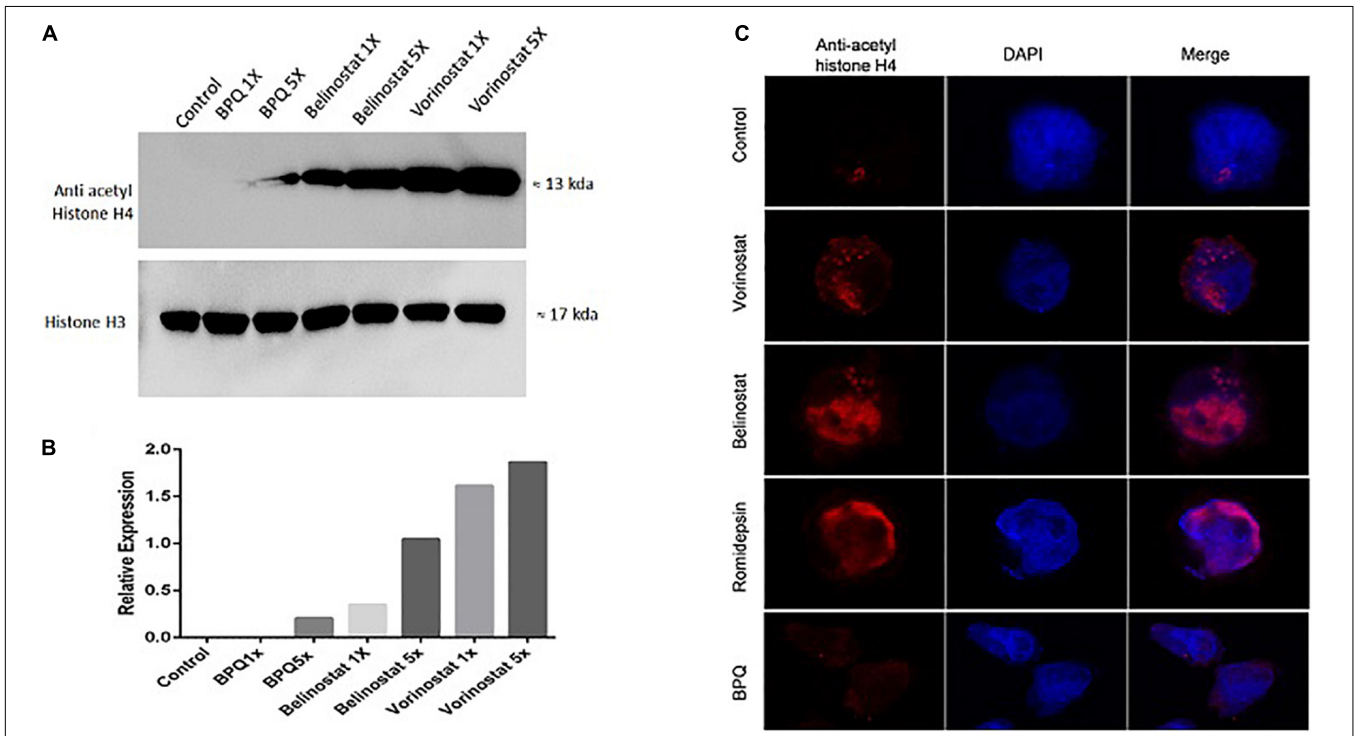

FIGURE 2 | Acetylation profiles of *T. annulata* infected cells after 3 h of treatment with histone deacetylase (HDAC) inhibitors. **(A)** Western blot analysis of the *T. annulata* infected cells treated with 1× IC_{50} and 5× IC_{50} concentrations of different HDACi (vorinostat, belinostat, and romidepsin) and controls (without treatment and with BPQ treatment) using mouse anti- acetyl histone H4 antibody. Histone H3 was taken as the loading control. **(B)** The graph shows the relative quantification of the H4 as detected in the western blot. **(C)** Immunofluorescence staining of *T. annulata* infected cells with 1× IC_{50} HDACi (vorinostat, belinostat, and romidepsin) after 3 h of treatment. BPQ was taken as control.

Vorinostat, belinostat, and romidepsin treatment clearly showed increased acetylation of H4-proteins (∼13–17 KDa) compared to untreated control and BPQ treated cells using pan acetyl histone antibody in western blot (**Figures 2A,B**). We next checked the hyperacetylation using IFA with the same antibody with cells treated with 1X concentration (IC_{50}) of the three inhibitors. The fluorescence microscopy images confirmed the increased hyperacetylation in the *T. annulata* infected cells compared to control, and BPQ treated cells (**Figure 2C**).

We also investigated whether the increase in hyperacetylation due to HDACi treatment affects the virulence of the parasite. MMP9 gene expression was analyzed in the HDACi treated and untreated samples to quantify the effect on virulence. Decreased expression of host MMP9 gene has been previously linked to attenuation or decrease in the virulence of the *T. annulata* parasites (Echebli et al., 2014). Bcl-2 gene, a well-known marker for apoptosis, was also included in the study. The quantitative SYBR green-based PCR analysis showed a twofold increase in the

MMP9 gene expression after treatment with belinostat. However, no differential expression was found in the MMP9 gene after treatment with vorinostat. Anti-apoptotic gene Bcl-2 was found to be downregulated in both the vorinostat and belinostat treated samples (**Supplementary Figure 1**).

Histone Deacetylase Inhibitors Kills the Parasite Explicitly in an Irreversible Manner and Damages the Mitochondrial Potential of *Theileria annulata* Infected Cells

Theileria annulata infected cells were incubated with belinostat (0.069 μM) and vorinostat (0.103 μM) for 48 h. After treatment, the cells were labeled with anti-*TaSP* (parasite-specific) and anti-β-actin (host-specific) antibodies, followed by western blotting. There was a significant decrease in the *TaSP* protein levels after 48 h treatment with both the compounds (**Figure 3A**). However, the intensity of the β-actin band was similar in the treated and untreated samples. BPQ treated samples used as a positive control also showed a decrease in the band intensity of the *TaSP* protein.

We also investigated whether the antiparasitic effect of HDACi is reversible after the removal of the drug pressure. Treatment of *T. annulata* infected cells with belinostat and vorinostat for 48 h resulted in the complete and irreversible suppression of the parasite growth even after drug pressure withdrawal (**Figure 3B**). There was no recovery till 12 days after drug withdrawal of the parasite.

The mitochondrial membrane potential of the *T. annulata* infected cells treated with HDACi (belinostat and vorinostat) was measured using JC1 dye to analyze their effect on the mitochondrial function. The membrane potential was measured by calculating the mean red fluorescence intensity (JC1-Aggregate) to mean green fluorescence intensity (JC1-Monomer). The flow cytometer-based analysis identified a significant decrease in the ratio of red to green fluorescence intensity in the treated cells as compared to the untreated cells (**Figures 3C,D**).

Histone Deacetylase Inhibitors Induces Caspase-Dependent Apoptosis in the *Theileria* Infected Cells

Flow cytometry analysis was performed to analyze whether belinostat and vorinostat-induced cell death is associated with apoptosis. Staurosporine (apoptosis-inducing agent) and z-VAD-fmk (pan-caspase inhibitor) were used as a control to examine the caspase-dependent apoptosis in HDACi treated *Theileria* infected cells (Belmokhtar et al., 2001). After 48 h of treatment, belinostat and vorinostat significantly promote caspase-dependent apoptosis in the infected cells (**Figures 4A,B**). Belinostat and vorinostat-induced apoptosis was completely blocked by the broad caspase inhibitor z-VAD-fmk, demonstrating that caspases were involved in the death process (**Figures 4A,B**). **Figure 4A** shows that belinostat and vorinostat significantly promote cell apoptosis, the percentages of apoptotic cells were as follows: control: 6.88 ± 0.63%, z-VAD-fmk: 7.37 ± 0.20%, staurosporine: 34.59 ± 0.13%, staurosporine + z-VAD-fmk: 9.07 ± 0.71%, belinostat: 34.26 ± 0.97, vorinostat: 49.37 ± 1.18%, belinostat + z-VAD-fmk: 15.66 ± 0.99%, vorinostat + z-VAD-fmk: 10.21 ± 0.19%.

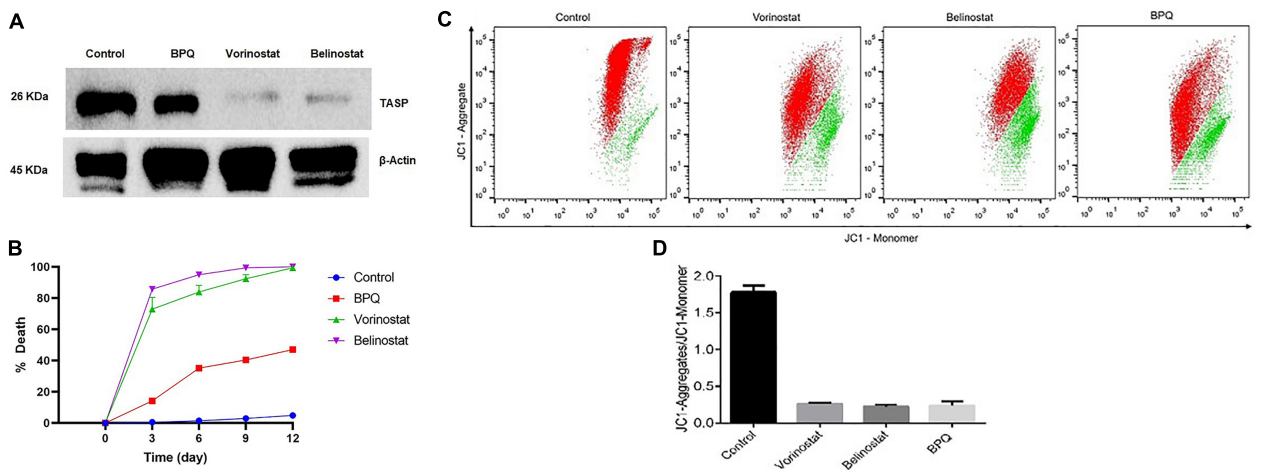

FIGURE 3 | Effect of Belinostat and Vorinostat on the parasite and its mitochondrial membrane potential: **(A)** Western blot analysis of the *T. annulata* infected cells treated with IC_{50} concentrations of vorinostat and belinostat (after 48 h) and controls [without drug treatment and buparvaquone (BPQ) treatment] using mouse anti-*TaSP* antibody and mouse anti-β-actin as a loading control. **(B)** Reversibility of growth inhibition after treatment with histone deacetylase (HDAC) inhibitor. After 48 h, HDACi and BPQ drug pressure were removed, and parasite growth was monitored for 12 days in the traditional medium. The experiment was repeated thrice. **(C)** Flow cytometry analysis using JC1 dye to analyze vorinostat and belinostat effect on the mitochondrial membrane potential of *T. annulata* infected cells. Mitochondria depolarization was quantified by taking the ratio of red to green fluorescence emission intensity. The JC1-aggregate and the JC1-monomer are represented by red and green color, respectively, in the dot-plot. **(D)** The graph shows the ratio of JC1-aggregate and the JC1-monomer from the flow cytometer analysis.

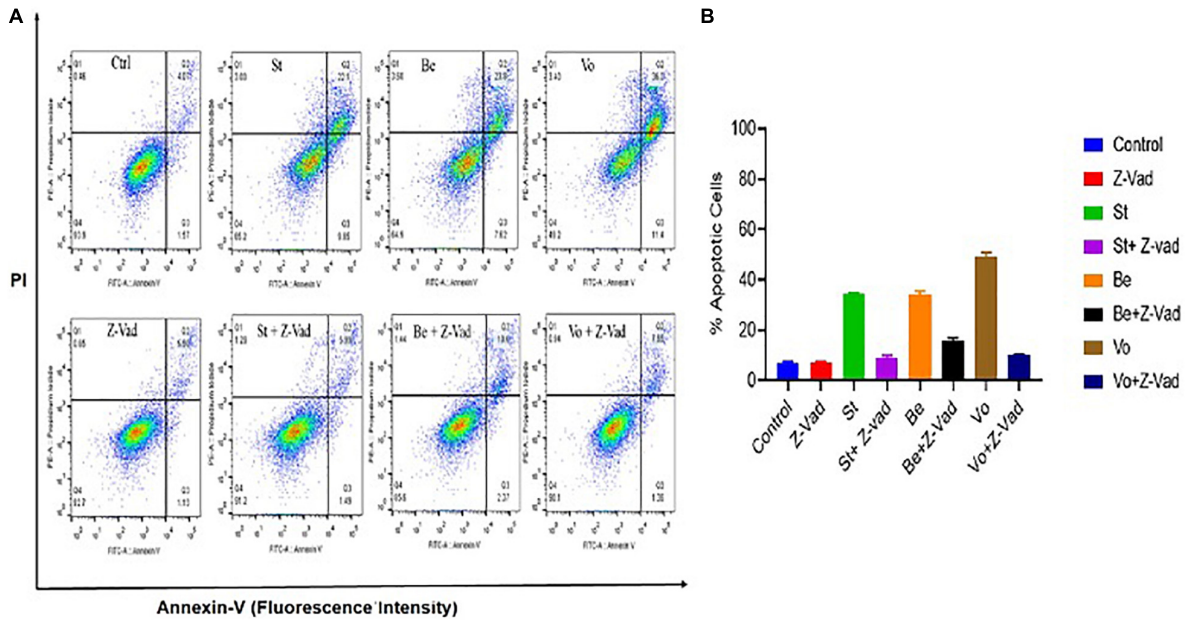

FIGURE 4 | Histone deacetylase inhibitors (HDACi) treatment induces caspase-dependent apoptosis in the *Theileria* infected cells. **(A)** The percentage of apoptosis was quantified by flow cytometry analysis using annexin V and PI staining. **(B)** Quantitative analysis of the apoptotic cells based on an average of three independent experiments (mean ± SD).

In silico Studies Predict TaHDAC1 to Be the Target of Histone Deacetylase Inhibitors

Vorinostat and belinostat hinder HDAC enzyme activity leading to hyperacetylation of proteins and parasite death in *P. falciparum* and *P. knowlesi* parasites (Sumanadasa et al., 2012; Chua et al., 2017). In *Plasmodium*, five different HDAC enzymes are reported, which can have a role in the acetylation and deacetylation of histones. We found homologs of all the five plasmodial genes in *T. annulata* genome (**Supplementary Table 1**). The antiplasmodial activity of the four HDACi used in this study was previously linked to inhibition of *PfHDAC1* (PlasmoDB—gene ID PF3D7_0925700) and PkHDAC1 gene in *P. falciparum* and *P. knowlesi*, respectively (Engel et al., 2015). We searched for the homolog of the *PfHDAC1* and *PkHDAC1* genes in the *T. annulata* database (PiroplasmaDB). The homology analysis identified the *Ta12690* gene (*TaHDAC1*, putative) as the *Plasmodium* species closest match. As the crystal structure of both *Plasmodium* and *Theileria* HDAC is not available, we used TaHDAC1 as a template and found human HDAC2 (PDB No. 5IWGA) to be very similar to the *Theileria* protein (**Supplementary Figure 2**). Using the crystal structure of the human HDAC2, we draw a three-dimensional homology structural model of *TaHDAC1* to examine the predicted binding mode of these ligands in the *Theileria*. The model's quality assessment was done based on the QMEAN score (−0.89) and GMQE (0.70) values; our structure was found to be within the allowed limits of modeling. The local quality estimates for the 3D model showed two regions with a score below 0.6, but the ligand binding/active site have scored above 0.6. The Ramachandran plot showed 91.4 and 8.6% of residues from the model located in the most favored or allowed regions (**Supplementary Figure 3**). We next docked the ligands to find the possible binding sites in the TaHDAC1. Based on the 3DLigandSite prediction tool, the expected binding of ligands was near the residues His136, His137, Asp172, Val173, His174, Asp260, Gly296, Gly297, Gly298, and Try299 of TaHDAC1. The docking of TaHDAC1 revealed hydroxamate binding of the ligands (vorinostat and belinostat) to the zinc ion in the active site (**Figure 5**). The vorinostat and belinostat made hydrogen bonds (His136, His174, Try299, and Gly145) and pi-pi (Phe200 and His174, Phe146) interactions near the active site residues in *TaHDAC1* (**Figure 5**). The docked ligands showed a high docking score of −5.233 and −8.202, respectively. Since panobinostat was previously reported to be the most potent inhibitor of the *P. falciparum* and *P. knowlesi* parasites, we compared differences in its binding to *Plasmodium* and *Theileria* HDAC1. Although panobinostat showed binding to zinc ion in the catalytic site, there was no interaction with the active site residues in *TaHDAC1* (data not shown). Thus binding of hydroxamic acid-based compounds (vorinostat and belinostat) in the active site pocket might inhibit *TaHDAC1* activity resulting in hyperacetylation of the proteins and ultimately parasite death.

DISCUSSION

Theileria annulata is the most common hemoprotozoan parasite infection in livestock, causing high mortality and production losses. The disease control efforts are badly affected due to BPQ resistance, the only available drug used for treating the parasites (Mhadhbi et al., 2010, 2015; Sharifiyazdi et al., 2012;

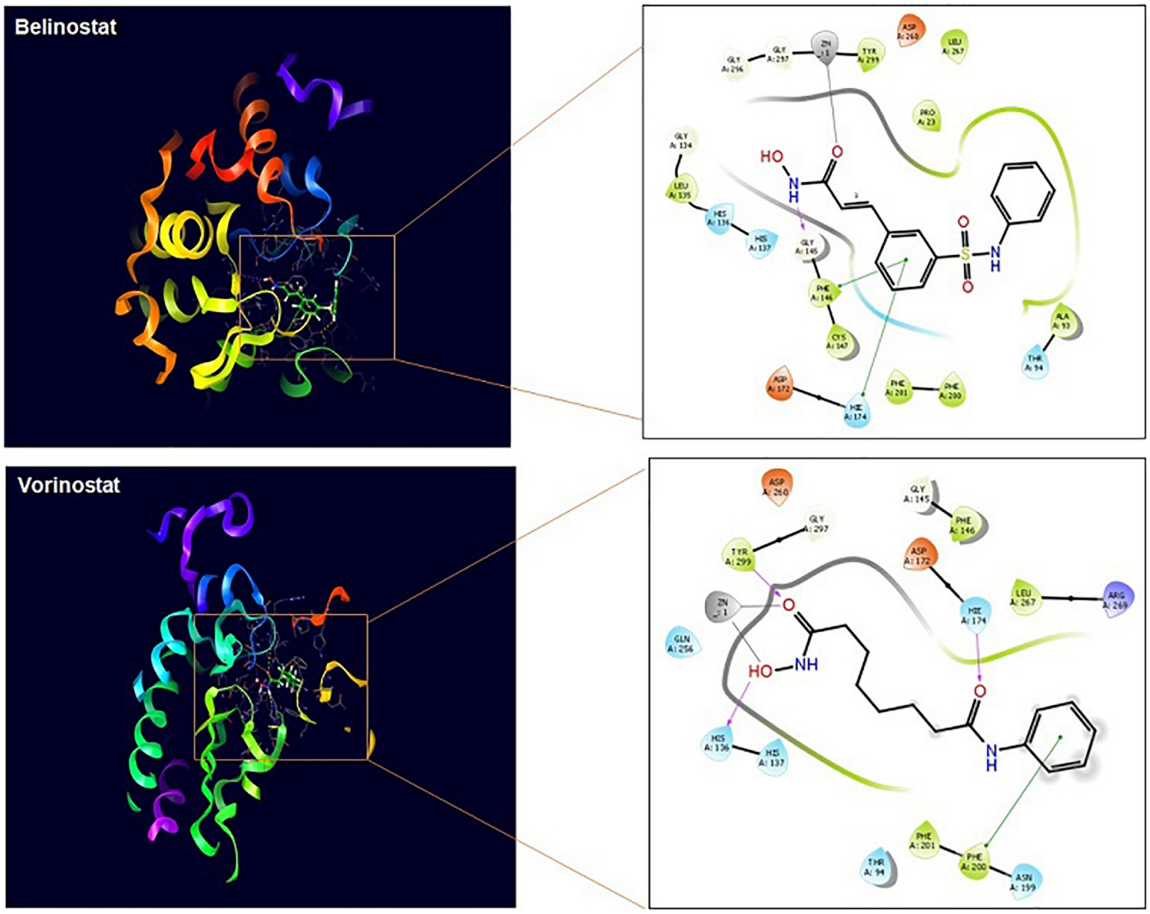

FIGURE 5 | TaHDAC1 homology model structure with docked ligands. The first figure shows the secondary structure representation of the homology model of TaHDAC1. The second part of the figure shows the 2D interaction between the inhibitor and the modeled protein amino acid residue. The docking poses are shown for ligands (vorinostat) and (belinostat) in *TaHDAC1*. Critical interactions with zinc atom (gray line), $\pi - \pi$ interactions (green line), and hydrogen bonds (pink line) are shown.

Chatanga et al., 2019). It is essential to find new therapeutic options by identifying new targets or by repurposing drugs for combatting the deadly parasite. Drug repurposing has emerged as a very effective tool to bypass the traditional method of drug discovery. Some of the common repurposed drugs include thalidomide and metformin for cancer and antibacterials such as azithromycin, tetracyclines, sulfonamides, and clindamycin for parasitic diseases (Nzila et al., 2011; Zhang et al., 2020). This study utilized the repurposing strategy by targeting epigenetic regulatory enzymes to find new treatment options against *T. annulata* parasites.

Four HDACi (vorinostat, belinostat, panobinostat, and romidepsin) that have been clinically approved for treating various cancer forms were evaluated for their antitheilerial activity. These HDACi are also well-studied in other protozoan parasites like *Plasmodium, Trypanosoma, Leishmania*, and *Schizostoma*. In *Plasmodium*, these compounds are shown to be a potential target for the treatment of *P. falciparum*, *P. knowlesi*, *P. berghei,* and *P. vivax* parasites (Andrews et al., 2008; Agbor-Enoh et al., 2009; Chaal et al., 2010; Marfurt et al., 2011; Chua et al., 2017). Vorinostat, belinostat, and panobinostat are hydroxamic acids like compounds shown to inhibit the pan-HDACs, while romidepsin is a cyclic peptide inhibiting class 1 HDACs (Mottamal et al., 2015). With an IC_{50} of <0.3 μM, belinostat, vorinostat, and romidepsin showed potent activity against the clinical isolates of the *T. annulata* parasites. Although effective in killing the parasites, the panobinostat had a significantly higher IC_{50} (20 μM) than other HDACi. Romidepsin showed high host cell cytotoxicity, which was in line with other previously reported studies where despite its effectiveness in killing *Plasmodium* and *Trypanosoma* parasites, it was not considered as a promising target (Engel et al., 2015). In previous reports, belinostat, vorinostat, and panobinostat have been shown to have potent and selective activity against the *Plasmodium* parasites, with panobinostat being the most effective (Engel et al., 2015). It was surprising that panobinostat was the least effective against the *Theileria* parasites, which might be due to changes in the gene sequence or the different life cycles of the two parasites. Vorinostat has also been recently reported to be equally effective in killing

Toxoplasma gondi parasites (Araujo-Silva et al., 2021). The compounds (belinostat and vorinostat) completely and irreversibly halted *T. annulata* proliferation even after removing the drug pressure.

Furthermore, we also revealed that treatment with belinostat and vorinostat downregulates anti-apoptotic proteins and mitochondrial dysfunction, leading to cell apoptosis. Our flow cytometry data based on the annexin V and PI labeling showed that belinostat and vorinostat inhibit the growth of the *Theileria* infected cells mainly by inducing apoptosis while necrosis was observed in a minimal number of cells. We also confirmed that apoptosis induced by the two HDACi was completely blocked by incubation with the caspase inhibitor, z-VAD-fmk, suggesting caspase-dependent cell death. Belinostat and vorinostat have previously been reported to induce similar cell death mechanisms in different cancer cells (Petruccelli et al., 2011; Sarfstein et al., 2011; Ong et al., 2016; Tuncer, 2021). Our data indicate belinostat and vorinostat to be promising leads for developing future parasite selective therapy based on the low host cell cytotoxicity and potent antiparasitic activity.

The HDACi are known to regulate gene expression by hyperacetylation of the histone proteins (H3 and H4), which is used as a marker in *P. falciparum* for confirming their parasite-specific inhibitory activity (Darkin-Rattray et al., 1996; Andrews et al., 2008; Chaal et al., 2010; Chua et al., 2017). Since *Plasmodium* and *Theileria* are apicomplexan parasites, we next checked for the hyperacetylation profiles of histone-4 protein after exposure to compounds (belinostat and vorinostat) in *T. annulata* infected cells. Hyperacetylation was observed in *T. annulata* infected cells treated with belinostat and vorinostat compared to untreated cell lines. The hyperacetylation profiles were similar to the previous studies in *Plasmodium* (Chua et al., 2017). As belinostat and vorinostat are not cytotoxic to host cells, these drugs may inhibit parasite HDACs, similar to what is shown for the other apicomplexan parasites. We confirmed this parasite-specific effect after treating these two compounds by quantifying parasite-specific protein (*TaSP*) using western blotting. The activity of the *Plasmodium* HDAC1 enzyme is previously shown to be inhibited by these inhibitors. In the absence of the recombinant HDAC1 of *T. annulata*, we did *in silico* studies to identify whether these HDACi target parasite-specific enzymes. The docking studies confirmed the binding of belinostat and vorinostat in the active site of the TaHDAC1 enzyme, which is in line with what was reported for the PfHDAC1 and PkHDAC1 (Engel et al., 2015; Chua et al., 2017).

In summary, this is the first study showing the antiparasitic activity and mechanism of action of HDACi in the *T. annulata* parasites. Our data clearly shows that drugs belinostat and vorinostat have potent activity against the *Theileria* infected cells. In the future, we will also like to check the activity and pharmacokinetics of these compounds in the *in vivo* experiments. We also plan to make parasite-specific analogs of these inhibitors, which can develop alternative therapies for treating *Theileria* parasites.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

PS designed the experiments and wrote the manuscript. MB, SK, SR, VB, SS, DD, and AS did the experiments and analysis. MB, SK, SR, VB, SS, DD, and AS helped in designing the study, data analysis, and manuscript editing. VB edited the manuscript. All authors gave approval to the final version 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/fmicb.2021.759817/full#supplementary-material

Supplementary Figure 1 | qRT-PCR determination of MMP9 and Bcl-2 gene expression relative to HDACi treatment in *Theileria* infected cells. Gene expression levels were normalized to an internal control HPRT and fold change was calculated with respect to expression levels in untreated cells.

Supplementary Figure 2 | Alignment of the Putative *T. annulata* HDAC1 to the Human HDAC 2(Uniprot—Q92769, PDB structure—5IWG).

Supplementary Figure 3 | Ramachandran plot of *T. annulata* HDAC1 homology model.

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Roles of LncRNAs in Regulating Mitochondrial Dysfunction in Septic Cardiomyopathy

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Sepsis is an abnormal systemic inflammatory response of the host immune system to infection and can lead to fatal multiorgan dysfunction syndrome. Epidemiological studies have shown that approximately 10-70% of sepsis cases can lead to septic cardiomyopathy. Since the pathogenesis of septic cardiomyopathy is not clear, it is difficult for medical doctors to treat the disease. Therefore, finding effective interventions to prevent and reduce myocardial damage in septic cardiomyopathy is clinically significant. Epigenetics is the study of stable genetic phenotype inheritance that does not involve changing gene sequences. Epigenetic inheritance is affected by both gene and environmental regulation. Epigenetic studies focus on the modification and influence of chromatin structure, mainly including chromatin remodelling, DNA methylation, histone modification and noncoding RNA (ncRNA)-related mechanisms. Recently, long ncRNA (lncRNA)-related mechanisms have been the focus of epigenetic studies. LncRNAs are expected to become important targets to prevent, diagnose and treat human diseases. As the energy metabolism centre of cells, mitochondria are important targets in septic cardiomyopathy. Intervention measures to prevent and treat mitochondrial damage are of great significance for improving the prognosis of septic cardiomyopathy. LncRNAs play important roles in life activities. Recently, studies have focused on the involvement of lncRNAs in regulating mitochondrial dysfunction. However, few studies have revealed the involvement of lncRNAs in regulating mitochondrial dysfunction in septic cardiomyopathy. In this article, we briefly review recent research in this area.

Keywords: lncRNA, epigenetics, sepsis, septic cardiomyopathy, mitochondrial dysfunction

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SEPSIS AND SEPTIC CARDIOMYOPATHY

Sepsis is an abnormal systemic inflammatory response of the host immune system to infection and can lead to fatal multiorgan dysfunction syndrome (1, 2). In severe cases, sepsis is considered a cause of death (3). Millions of human beings suffer from sepsis every year, and more than one-quarter of them lose their lives (4). According to statistics, the hospitalization rate and mortality of patients with severe sepsis increase by 8.2% and 5.6%, respectively, every year (5). Parker et al. first proposed in a 1984 study that sepsis-induced cardiac dysfunction is reversible (6). Since then, research on septic cardiomyopathy has attracted increasing attention. Epidemiological studies have shown that 10-70% of sepsis cases can lead to septic cardiomyopathy (7, 8). The mortality of patients with septic

cardiomyopathy is 70%-90%, which is 2-3-fold higher than that of patients with sepsis that does not affect the heart (9, 10). Currently, there is no formal definition of septic cardiomyopathy. It is generally recognized that septic cardiomyopathy is transient cardiac dysfunction caused by sepsis and that it manifests as heart enlargement, ventricular systolic dysfunction, hypoperfusion without ventricular systolic dysfunction, poor response to fluid resuscitation and catecholamines, and so on (11–14).

It has been revealed that the specific septic cardiomyopathy pathogenesis may include an imbalance of pro- and anti-inflammatory cytokine expression, abnormal expression of Toll-like receptors and related downstream pathways, release of nitric oxide (NO) and reactive oxygen species (ROS), complement activation, abnormal calcium processing, downregulation of the adrenergic pathway, cardiomyocyte apoptosis, autonomic nervous system dysfunction, coronary microvascular disturbance, mitochondrial dysfunction, and downregulation of sarcomere and mitochondrial proteins (15–18) (**Figures 1**, **2**).

MECHANISMS OF MITOCHONDRIAL DYSFUNCTION IN SEPTIC CARDIOMYOPATHY

Recently, researchers have focused on preventing and reducing myocardial damage in septic cardiomyopathy. Among the septic cardiomyopathy pathogenesis, mitochondrial dysfunction deserves to be a focus and further studied (19, 20) (**Figure 3**). Cardiomyocytes are rich in mitochondria, especially in the areas between sarcomeres and the subsarcolemma (21). As the energy metabolism centres of cells (22), mitochondria function to generate energy through oxidative phosphorylation (OXPHOS) (23). Of the important mechanisms of septic cardiomyopathy, the specific mechanism of mitochondrial dysfunction is under debate. Studies have shown that in the pathogenesis of septic cardiomyopathy, mitochondria undergo relevant changes that lead not only to mitochondrial dysfunction but also to the mitochondrial adaptive response (24, 25).

MITOCHONDRIAL ULTRASTRUCTURAL DAMAGE AND DECREASED ATP PRODUCTION

In 1994, morphological damage of myocardial mitochondria in septic cardiomyopathy was first described in an animal model (26). Studies have shown that the ultra-microstructural abnormalities of myocardial mitochondria in septic cardiomyopathy include swelling, ridge loss, matrix clearance, rupture of internal vesicles, and damage to internal and external membranes (27, 28), which are closely related to mitochondrial dysfunction (29). Specifically, ultra-microstructural abnormalities lead to the destruction of the OXPHOS process and further reduce adenosine triphosphate (ATP) production. Mitochondria are critical for synthesizing more than 90% of the ATP required by the body (30). The role of the respiratory chain represents the basic function of mitochondria. The respiratory chain is mainly composed of complexes I, II, III and IV (31), and F0F1 ATPase (32). Fatty acid β-oxidation supplies nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), which respectively transport electrons for OXPHOS through complexes I and II. Subsequently, electrons are transported to complex III, and then to complex IV, leading to the reduction of O_2 to H_2O. Finally, ATP is generated under the effection of F0F1 ATPase in the mitochondrial inner membrane (33–35).

NO PRODUCTION AND OXIDATIVE STRESS

Sepsis is accompanied by the excessive production of NO, ROS and inflammatory cytokines (36), leading to mitochondrial

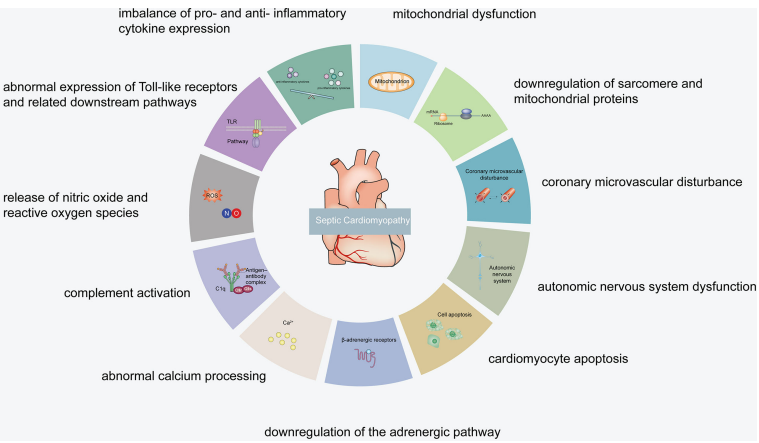

FIGURE 1 | Septic cardiomyopathy pathogenesis.

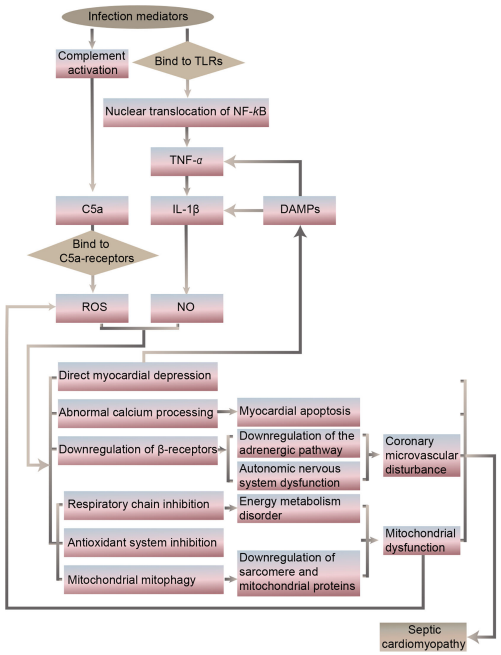

FIGURE 2 | Potential targets of protective intervention in septic cardiomyopathy.

dysfunction (37). Mitochondria produce NO through mitochondria NOS (mtNOS), which inhibits cytochrome c oxidase to regulate mitochondrial respiration (38). NO and $O_2{\cdot}^-$ produce $ONOO^-$ through diffusion-controlled reactions (39). $ONOO^-$ is a strong oxidant that can lead to direct oxidation or nitrosation damage, inhibit the OXPHOS complex and reduce O_2 consumption (40, 41). Studies have shown that knockout of inducible NOS (iNOS) can attenuate injury induced by oxidative stress, impaired OXPHOS or reduced ATP synthesis, revealing the vital role of $ONOO^-$ in regulating mitochondrial dysfunction in septic cardiomyopathy (42). The increase in ROS production, especially $O_2{\cdot}^-$, leads to excessive endogenous antioxidant capacity in the body (43). In turn, the excessive production of $O_2{\cdot}^-$ leads to further production of ROS in mitochondria, creating a vicious cycle of oxidative stress (44, 45). Excessive ROS induce protein denaturation and directly cause oxidative damage to DNA (46), which is particularly serious because mitochondrial DNA is related to the electron transport chain (ETC) (47). Furthermore, metalloproteinases and other proteases are activated, causing further functional deterioration of a variety of proteins, including antioxidant enzymes (48).

CALCIUM OVERLOAD AND CHANGES IN MITOCHONDRIAL MEMBRANE PERMEABILITY

Cytoplasmic calcium homeostasis is impaired in cardiomyocytes in septic cardiomyopathy, and Ca^{2+} enters mitochondria through unidirectional transporters (49). In addition, the rapid oscillation of Ca^{2+} between mitochondria and endoplasmic reticulum also leads to mitochondrial Ca^{2+} overload, which further initiates the opening of mitochondrial permeability transition pore (mPTP) (50). The outer mitochondrial membrane is highly permeable, substances with molecular weights less than 1500 kDa can pass through it, while the inner mitochondrial membrane allows only substances with molecular weights less than 1.5 kDa to pass through it (51). Proton pumps in the inner mitochondrial membrane pump protons from mitochondrial matrix to outer chamber, forming a potential difference between inside and outside mitochondria, which is called the mitochondrial membrane potential ($\Delta\Psi m$) (52). The mPTP opens intermittently in physiological state, and protons or positive ions in the outer chamber enter the inner chamber because of the potential difference, preventing the excessive accumulation of positive ions in the outer chamber (53). With Ca^{2+} overload, persistent oxidative stress, adenosine deficiency, increased phosphate concentration and mitochondrial depolarization occur, and then, the mPTP is in a mostly irreversibly opened state (54). The $\Delta\Psi M$ decreases rapidly, leading to ion imbalance, mitochondrial swelling and ATP depletion (55).

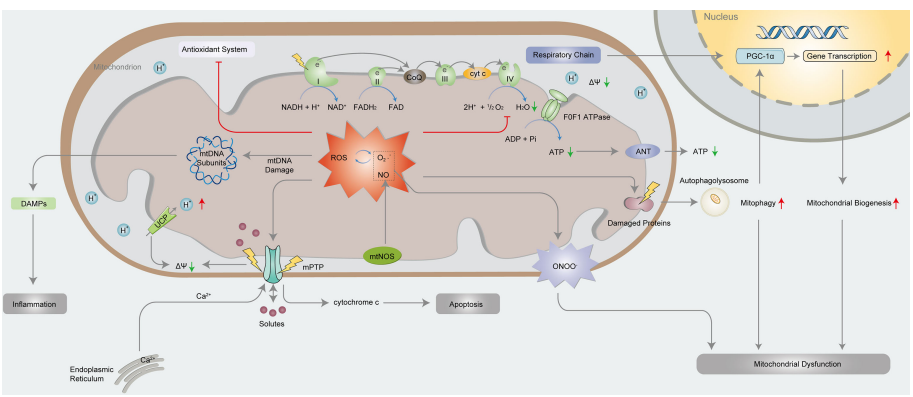

FIGURE 3 | Mechanisms of mitochondrial dysfunction in septic cardiomyopathy.

Moreover, mPTP opening leads to the release of cytochrome c into the cytoplasm, which participates in forming apoptotic bodies with APAF-1 and the precursors of caspase-9. Apoptotic bodies activate caspase-9 facilitated by deoxy-ATP (dATP), and caspase-9 then enzymatically cleaves caspase-3 to activate it, which starts the caspase-induced apoptosis cascade of reactions that ultimately leads to cell apoptosis (56, 57). In addition, electrons produced by the mitochondrial ETC can no longer be transported to oxygen molecules, resulting in the termination of OXPHOS and the inhibition of ATP synthesis (58). To maintain the $\Delta\Psi m$, mitochondria then negatively regulate F0F1 ATP synthase, leading to hydrolysis of the remaining ATP (59).

MITOCHONDRIAL BIOGENESIS AND MITOPHAGY

The levels of NO, ROS and the ratio of adenosine monophosphate (AMP)/ATP increase during septic cardiomyopathy. These changes trigger mitochondrial biogenesis (60). The main mechanism of mitochondrial biogenesis is the activation of the PGC family, especially PGC-1 α. PGC-1 α is synergistically activated, and its expression leads to the increasing expression of transcription factors, mediating the expression of nuclear proteins required for the transcription and replication of nucleus- and mitochondria-encoded OXPHOS subunits and mitochondrial DNA, transcription of OXPHOS assembly factor and mitochondrial protein import components (61). Mitochondrial biogenesis stands for the growth and division of mitochondria (62). The recovery of cardiac function in septic cardiomyopathy depends partly on mitochondrial biogenesis (63). The mechanism of mitochondrial biogenesis is debated. Some studies have shown that the clearance of damaged mitochondria in sepsis can be compensated by mitochondrial biogenesis rate, producing new mitochondria. However, other studies have shown that mitochondrial biogenesis, even as a compensatory mechanism of mitochondrial dysfunction, may lead to greater mitochondrial dysfunction by disrupting the complicated processes of gene transcription and mitochondrial dynamics. In any case, mitochondrial biogenesis in septic cardiomyopathy is insufficient to compensate for mitochondrial dysfunction (64, 65). The process opposing mitochondrial biogenesis is mitochondrial autophagy (66). Mitochondrial autophagy is a mechanism by which mitochondria eliminate dysfunctional mitochondria (67). However, it is unclear whether mitochondria clear dysfunctional mitochondria only through autophagic mechanisms and/or whether autophagy is involved in programmed cell death in septic cardiomyopathy. Recent research has not clarified the relationship between mitochondrial biogenesis and mitochondrial autophagy.

The recovery of mitochondrial function is closely related to the reversal of cardiac pump function; therefore, an increasing number of in-depth targeted intervention studies are needed to prevent or even reverse mitochondrial dysfunction. Guidelines for systematic evaluation of sepsis can improve prognosis and reduce mortality. However, there is no specific treatment for sepsis complicated with damage to some organs, including the heart. Further studies on the mechanisms of mitochondrial dysfunction in septic cardiomyopathy may supply a novel strategy to supplement the treatment options.

EPIGENETICS AND LncRNAs

Epigenetics is the study of stable genetic phenotype inheritance that does not intervene the gene sequence (68). Epigenetic modifications regulate many biological processes, including development and cell differentiation and proliferation (69). Currently, epigenetic mechanisms include the modification of DNA and proteins closely related to DNA. That is, epigenetic studies focus on the modification and influence of chromatin structure, mainly including chromatin remodelling (including advanced folding of chromatin and connections with the nuclear matrix), DNA methylation, histone modification and noncoding RNA-related mechanisms (70, 71). The reversibility of epigenetic regulation provides a targeted treatment strategy for epigenetically modified components and new ideas for innovative clinical treatment methods.

LncRNAs are endogenous RNAs with transcript lengths of more than 200 nucleotides, which do not possess the function of encoding protein. NcRNAs account for 98% of the human genome, and lncRNAs account for 80-90% of all ncRNAs (72, 73). LncRNAs are currently considered to be key epigenetic regulators (74). With increasing and in-depth research on whole-genome sequencing and function, the structure and function of lncRNAs have been found to be particularly complex (75). Although there is no consensus on the functional classification of lncRNAs, four main types are currently recognized: signals, decoys, guides and scaffolds (76). As signals or decoys, lncRNAs participate in the activation or inhibition of gene. As guides, they enlist chromatin-modifying enzymes to regulate gene expression in a cis/trans manner. As scaffolds, they enlist a variety of proteins to synthesize ribonucleoprotein complexes that regulate chromatin or histones (77). According to the classification of gene structure, lncRNAs are mainly divided into sense lncRNAs, antisense lncRNAs, intronic lncRNAs, long intergenic lncRNAs (or lincRNAs), enhancer RNAs (or erRNAs), and circular RNAs (or circRNAs) (78). LncRNAs interact with various molecules to form RNA-RNA, RNA-DNA and RNA-protein complexes, which play important roles in chromatin modification (79).

LncRNAs AND CARDIOVASCULAR DISEASES

Mutation or abnormal expression of lncRNAs is closely relevant to cardiovascular diseases (80, 81). Published research results mainly refer to MIAT, ANRIL, LIPCAR, and Braveheart. As early as 2006, scholars explored the relationship between MIAT and myocardial infarction. MIAT single-nucleotide polymorphisms can cause changes in the expression of myocardial infarction-related proteins (82). Overexpression of ANRIL can change sites of chromosome 9p21 that are closely relevant to the pathogenesis of coronary atherosclerosis (83). Further studies showed that ANRIL expression was positively related to the severity of coronary atherosclerosis (84). It was discovered that LIPCAR expression was upregulated during the early stage of heart failure and downregulated during the late stage, and therefore, changes in

LIPCAR expression can be used to predict the risk of late cardiovascular events (85). It has been confirmed that Braveheart is closely relevant to the differentiation of mouse cardiomyocytes. Studies have shown that PRC2 can inhibit the genes necessary for the differentiation and development of cardiac cells, such as the MesP1 gene, and Braveheart can interact with SUZ12 in the PRC2 complex to further control the expression of MesP1. When the expression of Braveheart is lower than normal, mouse embryonic stem cells did not differentiate into normal cardiomyocytes, which limited heart development (86).

EFFECTS OF REGULATED LncRNA EXPRESSION ON MITOCHONDRIAL FUNCTION

Mitochondria are important multifunctional organelles participating in various basic biological processes (87). The integrality of mitochondrial structure and function is significant to maintain the stability of the intracellular environment. Currently, it is generally believed that the stability of the intracellular environment depends on various mitochondrial pathways regulating energy conversion and ATP production, involving ETC and tricarboxylic acid cycle (TCA) (88). Mitochondria have genetic system independent of the nucleus, and the mitochondrial genome has a complete expression mechanism (89). However, the scale of the mitochondrial genome is small (90). The biological function of mitochondria does not solely depend on the mitochondrial genome; it also depends on nucleus-encoded proteins, which are synthesized in the cytoplasm and transported into mitochondria through specific mechanisms. In other words, mitochondrial energy metabolism and intracellular environment stability depend on the simultaneously coordinated regulation and expression of the nuclear genome and mitochondrial genome (91). Increasing evidence has shown that lncRNAs can act as messengers between nucleus and mitochondria, and participate in regulating of diverse pathways (92). However, the potential regulatory mechanisms may be very complex, and relevant research is ongoing.

LncRNAs can regulate mitochondrial function and dynamics at different levels (93). Abnormal regulation of lncRNAs leads to abnormal synthesis of ATP and ROS, thus contributing to the pathological development of many diseases. Currently, research on lncRNA regulation of mitochondrial function mainly focuses on cardiovascular diseases, neurodegenerative diseases and tumour diseases (94–96). As mentioned above, cardiomyocytes are enriched with many mitochondria, and mitochondrial dysfunction is closely relevant to the pathogenesis of cardiovascular diseases.

EFFECTS OF LncRNA REGULATION ON MITOCHONDRIAL DYSFUNCTION IN SEPTIC CARDIOMYOPATHY

As previously mentioned, various mechanisms of mitochondrial dysfunction in septic cardiomyopathy have been reported. According to the literature, recent research on lncRNAs participating in the regulation of mitochondrial dysfunction in septic cardiomyopathy has mainly focused on decreases in ATP production, mitochondrial NO production and oxidative stress. Additionally, studies have shown that lipopolysaccharide (LPS) can induce an increase in ROS, a decrease in $\Delta\Psi m$, the release of cytochrome c, and the upregulation of caspase-9 and caspase-3 in the cytoplasm, ultimately leading to cardiomyocyte apoptosis (97).

Cheng Xing Peng et al. explored the regulatory role of MIAT in septic myocardial injury. They found that MIAT knockdown significantly inhibited the production of mitochondrial ROS in LPS-treated HL-1 cells. In addition, the ratio of reduced glutathione to oxidized glutathione (GSH/GSSH) decreased with increasing malondialdehyde (MDA) content. This result suggested that MIAT aggravated myocardial damage by promoting oxidative stress. It was confirmed that MIAT acted on miR-330-5p directly to upregulate the TRAF6/NF-κB pathway, promoting inflammation and oxidative stress in LPS-induced cardiomyopathy (98).

RMRP inhibits the posttranscriptional regulatory effect of miR-1-5p on HSPA4 in LPS-induced mitochondrial damage. Overexpression of RMRP can significantly inhibit the decline in $\Delta\Psi m$, the level of intracellular ROS, and the expression of cytoplasmic cytochrome c, caspase-9 and caspase-3, thereby inhibiting cardiomyocyte apoptosis (99). Bin Shan et al. discussed H19 regulation in septic cardiomyopathy. H19 can reduce mitochondrial inner membrane damage by regulating mitochondrial membrane potential by regulating miR-93-5p/SORBS2 pathway, thereby inhibiting mitochondrial apoptosis. Inflammatory factors, involving TNF-α, IL-1β and IL-6, were markedly downregulated in LPS-induced cardiomyocytes overexpressing H19. The expression of cytochrome c in mitochondria was upregulated, while that in cytoplasm was downregulated. This result indicated that the overexpression of H19 alleviated inflammation and mitochondrial apoptosis in LPS-induced cardiomyocytes (100). Studies have also pointed out that knocking down SOX2OT can significantly enhance cardiac function, inhibit the decline in $\Delta\Psi m$, and reduce the production of mitochondrial ROS in mice with septic cardiomyopathy, while upregulating SOX2OT can reverse all of these effects. Through further research on the regulatory mechanism, it was ultimately concluded that SOX2OT aggravated mitochondrial dysfunction by downregulating the expression of SOX2, thereby affecting the prognosis of septic cardiomyopathy (101).

Studies on the involvement of lncRNAs regulating mitochondrial energy metabolism in septic cardiomyopathy are also ongoing. Dongshi Liang et al. found that the increased expression of Xist is related to the decreased level of both PGC-1α and ATP, which suggested that inhibiting the expression of Xist enhanced the production of ATP, reducing sepsis-induced myocardial injury (102).

Although the aforementioned lncRNAs have been confirmed to participate in septic cardiomyopathy by regulating mitochondrial function and apoptosis, it is still unclear whether

other lncRNAs are involved in regulating mitochondrial functions, and the specific regulatory mechanisms of participating lncRNAs are also unknown. To date, using gene chip hybridization technology, researchers at Zhejiang University identified 471 upregulated lncRNAs and 804 downregulated lncRNAs in myocardial tissues of septic mice. Ultimately, this group found that partial lncRNAs are mainly enriched in inflammation, immunity, energy metabolism and cell death, and predicted that certain lncRNAs may participate in mitochondrial dysfunction (103). All these results provide strong theoretical support for the continuing study of the involvement of lncRNAs in mitochondrial dysfunction in septic cardiomyopathy.

CONCLUSION AND PERSPECTIVE

LncRNAs will increasingly become targets for the intervention and treatment of septic cardiomyopathy, and the mechanism to target is closely related to lncRNA involvement in mitochondrial dysfunction. Finding intervention measures to prevent and treat mitochondrial damage is significant to improved treatment and prognosis of patients with septic cardiomyopathy. Although research on biomarkers for use in assessing the severity and prognosis of septic cardiomyopathy is ongoing, no clear markers with both sufficient sensitivity and specificity have been identified to date. Recent research has found that CitH3 may be recognized as a reliable blood biomarker for diagnosis and prognosis of sepsis (104). LncRNAs may be potential biomarkers for evaluating the severity and prognosis of septic cardiomyopathy, and they will also be the focus of the next phase of our research.

AUTHOR CONTRIBUTIONS

WC and SL conceived the review. SL wrote the manuscript. WC revised the manuscript. All authors contributed to the article and approved the submitted version.

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Infectious Keratitis: An Update on Role of Epigenetics

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Epigenetic mechanisms modulate gene expression and function without altering the base sequence of DNA. These reversible, heritable, and environment-influenced mechanisms generate various cell types during development and orchestrate the cellular responses to external stimuli by regulating the expression of genome. Also, the epigenetic modifications influence common pathological and physiological responses including inflammation, ischemia, neoplasia, aging and neurodegeneration etc. In recent past, the field of epigenetics has gained momentum and become an increasingly important area of biomedical research As far as eye is concerned, epigenetic mechanisms may play an important role in many complex diseases such as corneal dystrophy, cataract, glaucoma, diabetic retinopathy, ocular neoplasia, uveitis, and age-related macular degeneration. Focusing on the epigenetic mechanisms in ocular diseases may provide new understanding and insights into the pathogenesis of complex eye diseases and thus can aid in the development of novel treatments for these diseases. In the present review, we summarize the clinical perspective of infectious keratitis, role of epigenetics in infectious keratitis, therapeutic potential of epigenetic modifiers and the future perspective.

Keywords: keratitis, epigenetics, methylation, histone modifications, infectious

INTRODUCTION

While the base sequence of the gene remains same, epigenetic mechanisms alter its expression and thus its function. This can happen *via* altered methylation of DNA, post-translational modifications of histones, introduction of non-coding RNAs, remodeling of the chromatin etc. Epigenetic mechanisms are known to play an important role in several pathophysiological conditions, including those of the ocular surface. Exposure of cornea to pathogens, leading to inflammation and keratitis, has previously shown to involve epigenetic mechanisms (1, 2).

Though our understanding of epigenetic mechanisms in keratitis has advanced to some extent in recent past, the clinical implications in terms of therapeutics and treatments are yet to be explored. Some of the examples of how the mechanistic understanding of epigenetics can potentially aid drug discovery in eye diseases can be: 1) Latent infection of HSV1 (Herpes Simplex Virus 1) in corneal

cells can lead to persistent recurrence of keratitis (3). Knowing how to epigenetically reactivate the virus from its protective latent state could help in combating it *via* anti-HSV treatment. Knowing how to keep the virus in its latent state irrespective of epigenetic triggers could help in keeping the virus in a senile latent state without acute infection. 2) Fungal pathogens are known to vary their histone modifications to garner virulence and drug resistance. Down regulation of histone acetylation leads to increased inflammatory response in fungal keratitis, and histone deacetylase inhibitors could emerge as promising treatment (4). 3) In case of degenerative Keratoconus, the non-coding RNAs have potential to affect the expression of about 1000 genes (5).

Hence, understanding the epigenetic networks and interactions can possibly help in the early detection of diseases of the ocular surface and also lead to the development of novel therapeutic approaches (6). In the present review, we briefly summarize the role of epigenetics in ocular diseases followed by specifically focusing the infectious keratitis and epigenetic changes from a diagnostic and therapeutic perspective which can be possibly translated into novel therapies in the near future.

EPIGENETICS AND IT'S ROLE IN OCULAR DISEASES

'Epigenetics' refers to the heritable, reversible and environment-influenced mechanisms that affect the gene expression without altering the underlying DNA sequences (1, 7, 8). The term was initially used to refer to the complex interactions between the genome and the environment, involved in the development and differentiation of distinct cell lineages in higher organisms (9, 10). The epigenetic mechanisms that potentially mediate this dynamic interaction between the genes and the environment comprise of DNA methylation, chromatin remodeling, histone variants, post-translational modifications of histone and deployment of non-coding RNAs (11). Various factors contribute in the acquisition, maintenance and inheritance of diverse epigenetic modifications.

The modification of DNA and histone tails regulates the structure of chromatin and accessibility of DNA to transcriptional machinery. The principal epigenetic modification found in DNA is covalent attachment of methyl group by DNA methyl-transferase (DNMTs) enzymes at C5 position of cytosine residues in CpG dinucleotide sequence, which is a mark for transcriptional repression (12). On the other hand, histones can restructure the chromatin in transcriptionally permissive or restrictive states by undergoing diverse post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination etc. (13). These modifications are written, read and erased by a variety of histone modifying enzymes. The type, site, combination and the extent of histone modification adds the complexity of histone code (13–15). Besides modifications of DNA and histones, long non-coding RNAs (IncRNAs), micro RNAs (miRNAs), small inhibitory RNAs (siRNAs) and piwi interacting RNA (piRNAs) can also mediate transcriptional silencing as reviewed by Wei et al. (2017) (16). Also, the temporal and spatial regulation of transcription is regulated by ATP-dependent chromatin remodelers that re-configure the nucleosomes in response to environmental and developmental cues (17). These chromatin remodeling enzymes have been classified in subfamilies i.e. Switch/sucrose non-fermentable (SWI/SNF), imitation switch (ISWI), chromodomain helicase DNA-binding (CHD) and INO80 (18). Additionally, the replacement of canonical histones with the variants also leads to diversity of nucleosomes' structure and function. The histone variants, their chaperones/remodeler machineries and linkage to various diseases have been extensively reviewed recently (19, 20).

The human epigenome gets influenced by various factors such as diet, age, environmental factors, smoking and the infections. Evidences are growing that natural infections alter the epigenome by modulating the immune response and longitudinal disease risk. Most of the studies in infection-induced epigenetic changes have been done with respect to carcinogenic microbes and very less is known about epigenetic effects of non-carcinogenic microbial infections (21). Even less is known about the role of epigenetics in ocular infections and diseases. In this section, we briefly summarize the reported literature on involvement of different epigenetic mechanisms in ocular diseases (**Figure 1**). Some of the common eye disorders where role of epigenetic mechanism have been revealed, include retinoblastoma, diabetic retinopathy, age-related macular degeneration (AMD), glaucoma, cataract, keratoconus, corneal dystrophies, pterygium, keratitis etc. (2, 6, 22, 23), which affect different parts of the eye as shown in **Figure 1**. From the point of view of type of epigenetic mechanism involved, a large number of genes have been reported to undergo hyper- or hypo-methylation in different eye diseases such as: *MMP-2/CD24* and *TGM-2* in Pterygium; *GSTP1, OGG1*, *ERCC6* and CRYAA in cataract; *TGFBIp* in corneal dystrophies; *GSTM1, GSTM5* and *IL17RC* in *AMD, MSH6, CD44, PAX5, GATA5, TP53,VHL, GSTP1, MGMT, RB1* and *CDKN2* in retinoblastoma; *RAC1* in diabetic retinopathy; *LOXL1* in pseudo-exfoliation syndrome; *TGF-β1* in glaucoma; *RASSF1A* and telomerase reverse transcriptase gene in uveal melanoma etc. The level of various micro-RNAs has also been reported to be altered in different eye diseases such as up-regulation of hsa-miR-143-3p, hsa-miR-181a-2-3p, hsa-miR-377-5p and hsa-miR-411a in Pterygium; up-regulation of hsa-mir-494, hsa-let-7e, hsa-mir-513a-1, hsa-mir-518c, hasmiR-129, hsa-mir-198, hsa-mir-492, hsa-mir-498, hsa-mir-320, mir-503, and hsa-miR-373 in retinoblastoma; and down-regulation of hsa-mir-29b1 and 200b in diabetic retinopathy etc. Besides these, histone modifications also seem to play important role as revealed by H3K9 deacetylation in *ERCC6* in cataract; global histone acetylation in uveal melanoma etc.

These studies definitely attract our attention towards possible involvement of different epigenetic mechanism in induction, execution and promotion of various eye disorders with an opportunity to explore this area for better diagnostic and therapeutic targets. Having convinced with that, we next focus on infectious keratitis as another important eye disease of global concern and the epigenetic mechanisms involved in it.

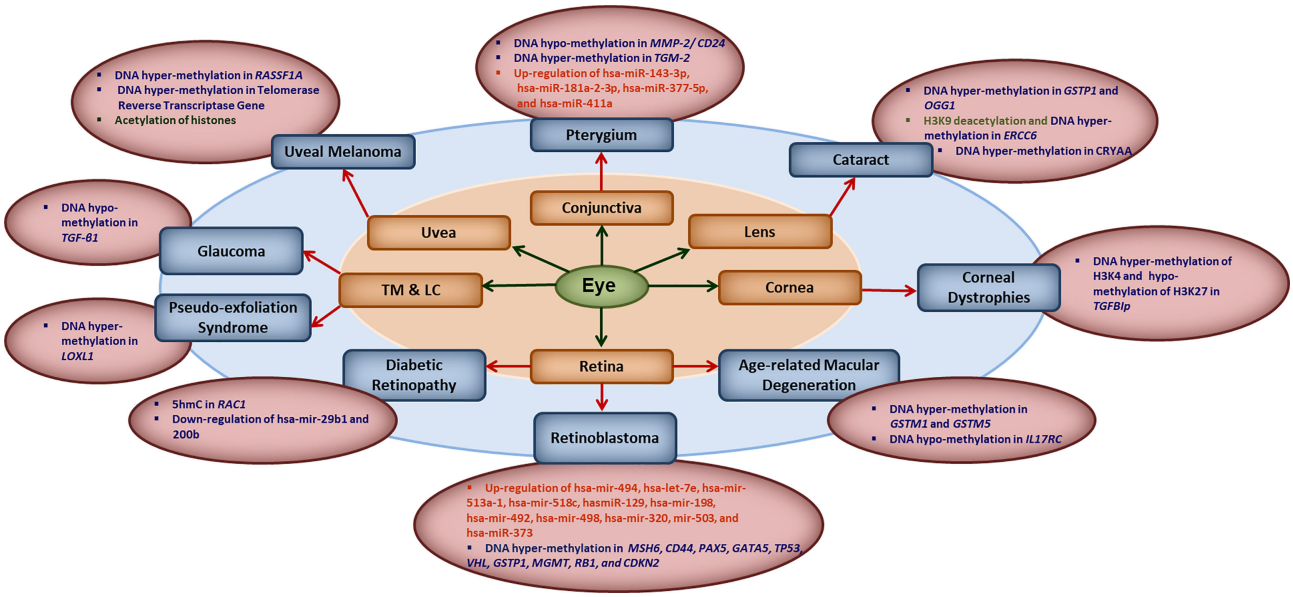

FIGURE 1 | Key epigenetic modifications in common ocular diseases: Diverse epigenetic modifications are associated with the common ocular diseases occurring in different parts of the eye. MMP2, matrix metalloproteinase 2; CD24, CD24 molecule; TGM2, transglutaminase 2; hsa-miR, human microRNA; GSTP1, pi-class glutathione-S-transferase; OGG1, 8-oxoguanine DNA glycosylase 1; ERCC6, excision repair 6 chromatin remodeling factor; CRYAA, crystalline Alpha-A; TGFBIp, transforming growth factor β- induced; GSTM1/5, glutathione S-transferase isoform mu1/mu5; IL17RC, interleukin-17 receptor C; MSH6, mutS homolog 6; CD44, cluster of differentiation 44; PAX5, paired box 5; GATA5, GATA binding protein 5; TP53, tumor protein 53; VHL, Von Hippel-Lindau gene; GSTP1, glutathione S-transferase pi-1; MGMT, methylguanine methyltransferase; RB1, retinoblastoma 1; CDKN2, cyclin-dependent kinase inhibitor 2; 5hmC, 5-hydroxymethyl cytosine; RAC1, rac family small GTPase 1; LOXL1, lysyl oxidase-like 1; TGF-β1, transforming growth factor-β1; RASSF1A, RAS association domain family 1A gene; TM, trabecular meshwork; LC, the lamina cribrosa.

INFECTIOUS KERATITIS – TYPES, CLINICAL FEATURES, AND MANAGEMENT

Keratitis refers to inflammation of cornea i.e. clear tissue in the front of eye covering pupil and iris. Depending on the causative agent, keratitis is broadly classified as non-infectious or infectious. The non-infectious keratitis results due to injury, exposure to intense sunlight, dry eyes, weak immunity etc. The infectious one on the other hand is caused by variety of microbes i.e. bacteria, viruses, fungi, parasites etc. (**Figure 2**). The cornea, which remains protected anatomically by the eyelids, a healthy tear film & its protective factors, an active lacrimal drainage system and a tenacious epithelial cover gets inflamed if any of these protective factors is breached by microbial invasion. Infectious keratitis or corneal ulceration is traditionally described as a defect in the corneal epithelium, accompanied with infiltration and inflammation. Active keratitis and its sequelae in the form of corneal perforation or scarring can cause significant morbidity and even complete vision loss (24).

- Infectious keratitis is the most common cause of corneal blindness in both developing and developed world (25). Estimated incidence of infectious keratitis is reported to be ranging from 2.5 to 799 per 100,000 population-year, depending on the study design and geographical location (26). A higher rate of infectious keratitis in under-resourced countries and a wide variation in prevalence of causative organisms and thus, the frequency of microbial keratitis has been reported from different parts of the world (26, 27)). These variations have been widely attributed to poor environmental and personal hygiene, lack of awareness and healthcare, agriculture and work-related trauma etc. But variations are also expected to exist in terms of diets and metabolites in different geographical & socio-economical regions. Thus, the gut microbiome-host immune interactions along with ocular surfaces microbiota also vary which in turn indicate the involvement of epigenetics in varied induction and promotion of infectious keratitis. Dysbiosis i.e. imbalance in gut microbiome has already been reported to be associated with bacterial keratitis (28).
- Bacterial keratitis is the commonest form of infectious keratitis globally with incidence ranging from 50 to 60% (29). The potential risk factors for bacterial keratitis include contact lenses, aqueous tear deficiencies, trauma, decreased immunologic defenses, eyelid alterations or malposition, neurotrophic keratopathy, topical corticoid medications and surgery (30). The common corneal ulcers causing bacteria are *Staphylococcus* spp., *Streptococcus* spp., Enterobacteriaceae (including *Serratia*, Klebsiella, Enterobacter and *Proteus*) and *Pseudomonas* spp. Fungal keratitis on the other hand is seen in 6-30% of cases and mainly caused by *Aspergillus* and *Fusarium species* (31). Incidence of HSV (Viral

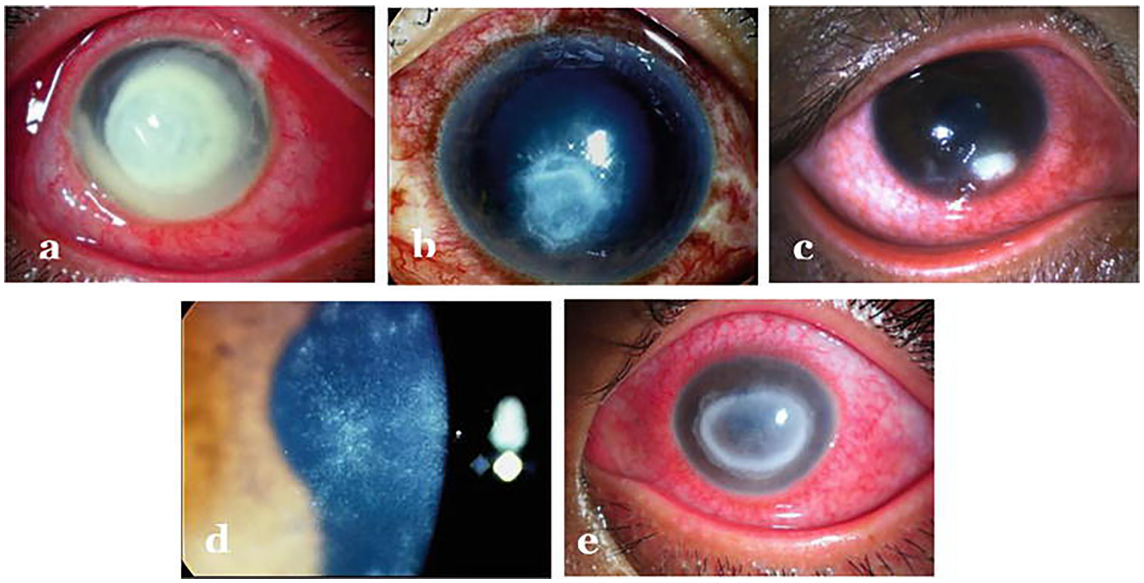

FIGURE 2 | Infectious keratitis caused by different agents: **(A)** Bacterial keratitis **(B)** Fungal keratitis **(C)** Herpes necrotizing stromal keratitis **(D)** Early *Acanthamoeba* keratitis **(E)** Late *Acanthamoeba* keratitis. Adopted from https://www.intechopen.com/chapters/69696 under Creative Commons Attribution 3.0 License.

keratitis) and *Acanthamoeba* keratitis are 15-40% and 0-5% respectively (32). Also, mixed infections comprising of infliction by more than one organism is also seen in 2-15% of patients (29).

Clinically, the patient presents with complaints of redness, pain, watering, diminished vision and intolerance to light referred to as photophobia. As far as diagnosis is concerned, distinguishing features such as - feathery borders and fixed hypopyon in fungal keratitis, diminished corneal sensations in viral keratitis and ring infiltration in *acanthamoeba* keratitis are used for clinical diagnosis (29) (**Figure 2**). Confirmatory diagnosis of infective keratitis is made conventionally on microbiological examination which includes smear examination, culture and polymerase chain reaction (PCR) evaluation (33). *Acanthamoeba* cysts have additionally been reported to be detected on confocal microscopy (34). However, culture negative keratitis poses significant problem to clinicians. Next generation sequencing (NGS) can help in diagnostic accuracy of infectious keratitis especially in culture-negative cases. Tian et al. (35) have analyzed differentially expressed genes (DEGs) in bacterial and fungal keratitis. A total of 148 DEGs were found only in bacterial keratitis and 50 DEGs only in fungal keratitis. Besides, they also identified 117 co-expressed gene pairs among bacterial keratitis DEGs and 87 pairs among fungal keratitis DEGs. Also, a total of nine biological pathways and seven KEGG pathways were screened and found that TLR4 is the representative DEG specific to bacterial keratitis, and SOD2 is the representative DEG specific to fungal keratitis, and hence can be used as promising candidate genes to distinguish between bacterial and fungal keratitis. Thus, at molecular level, genes can be quantified for identifying the causative agent for specific therapeutic outcomes. Though NGS can undoubtedly provide better insights about the ocular surface microbiome in pathophysiological circumstances, but it is not clear whether these can be effectively used to determine etiology of infection or antibiotic sensitivity. As far as management of keratitis is concerned, antimicrobial agents (36–39) besides collagen crosslinking (40–42) have been the mainstay for therapy. But, in the light of differential gene expression, specific pathways involved and eye-microbiota-immune interaction; it will be interesting to explore the epigenetic mechanisms involved so that specific epidrugs can be identified for treating infectious keratitis caused by particular type of microbe.

EPIGENETICS OF INFECTIOUS KERATITIS

Bacterial keratitis or often referred as 'corneal ulcer', is the most common form of infectious keratitis. Bacteria can induce inflammatory cascade through the interaction of pathogen associated molecular patterns (PAMP) with Toll-like receptors (TLR) expressed on corneal and conjunctival epithelial cells and subsequently activate the mitogen-activated protein kinases (MAPK) cascade and NF-κB, leading to increased production of inflammatory cytokines. Importantly, the production of inflammatory cytokines is under the control of epigenetic factors like histone acetylation/deacetylation (43). However, very little is known about epigenetic mechanisms in bacterial keratitis. Nonetheless we might be able to learn from pathogenesis of bacterial infections in other systems where the role of epigenetic factors has been investigated and can be extrapolated in the field of bacterial keratitis. In cardiomyocytes, lipopolysaccharide (LPS), a component of the bacterial cell wall, was found to increase histone

deacetylase (HDAC) activity. Since HDAC3 regulates TNF production, its inhibition decreases LPS-stimulated tumor necrosis factor (TNF) expression caused by the accumulation of nuclear factor kappa-B (NF-κB)/p65 at the TNF promoter (44, 45).

Herpetic keratitis is another common infectious corneal disease, caused by Herpes simplex virus 1 (HSV1). HSV1 infects corneal epithelial cells and sensory neurons thereby establishing latent infection, leading to recurrence of HSV1 in the cornea upon activation of virus under the influence of various stimulatory factors. Only the latency associated transcript (LAT) remains persistently expressed and lytic genes remain transcriptionally repressed, thereby maintaining the latency phase. Therefore, in order to understand and treat HSV infection, it is critical to understand the mechanism by which HSV1 is maintained in latent phase and how HSV1 is activated. The division of active and inactive genome has been shown to have epigenetic control. Histone modification for active transcription i.e. di-methylation of H3K4 and acetylation of H3K9 and H3K14 in LAT region and for inactive transcription i.e. trimethylation of H3K27 along with macro H2A histone variant have been reported to execute this. Moreover, chromatin insulators seem to separate the epigenetic domains of LAT and lytic genes. Abrogation of these insulators and CTCF (the protein that binds vertebrate insulators) binding possibly pave the way for transition from lytic to lysogenic phase (3, 46). Additionally, in neuronal cells, HDAC inhibitors (trichostatin-A) have been reported to reactivate the HSV1 infection in LAT-independent manner too (47).

Neurotrophic keratitis, also known as neurotrophic keratopathy, is a degenerative corneal disease caused by damage of trigeminal innervation. This damage to corneal innervation (from the trigeminal nucleus to the corneal nerve endings at different levels on the fifth cranial nerve) can be caused by various ocular surface disease, systemic diseases and central or peripheral nervous damages (48, 49). Though neurotrophic keratitis does not come under infectious keratitis directly but is most commonly induced by HSV (herpetic keratitis), the neurotrophic virus (50). With reactivation of latency by various stimulatory factors, virus travels back to the corneal epithelium along the axon and causes damage to corneal nerve with a severe reduction of sub-basal nerve plexus density with resultant diminished corneal sensation or corneal anesthesia (51). Thus, the epigenetic mechanisms involved in herpetic keratitis can be extrapolated to understand and manage neurotrophic keratitis too.

The pathogenesis of fungal keratitis remains poorly understood and therefore, its treatment is also yet to be explored more, especially from epigenetic perspective. However, Xiaohua Li et al. (4), have recently reported the attenuation of fungal keratitis in mice by histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA). It implies histone acetylation-deacetylation as potentially important target for understanding the fungal keratitis and expedite research in this area for better diagnosis and therapeutics. Additionally, a comprehensive human corneal miRNA expression profile and associated regulatory role in fungal keratitis has been reported (52) which again indicate the possible role of epigenetics in fungal keratitis as well.

Acanthamoeba keratitis, caused by *Acanthamoeba castellanii* remains a challenge to treat because of encystation. Even a single cyst in the tissue can cause re-infection and therefore, an effective strategy must inhibit cyst formation as well besides killing the pathogen. Epigenetic modification of the genes and proteins involved in initiation & maintenance of cyst and transition from cyst to active form can thus be a potential target for the same. Expression of encystation-mediating cyst-specific cysteine proteinase (CCSP) gene is regulated by DNA methylation (53). Similarly, silent-information regulator 2 like protein (SIR-2), which is a nicotinamide adenine dinucleotide-dependent deacetylase plays role in growth and encystation of *Acanthamoeba* (54). Though there are no direct reports available for involvement of epigenetics in *Acanthamoeba* keratitis, these reports suggest that epigenetic mechanisms play vital roles in Acanthamoeba physiology and pathology and thus, can be explored for medical purposes.

EPIGENETICS MODIFIERS AS A POTENTIAL THERAPEUTIC MOLECULE

Dysregulated epigenetics is involved in a wide range of diseases like cancer, blood disorders, neurological and neurodegenerative disorders, and respiratory disorders (55). The ability to reverse the epigenetic modifications makes them an attractive druggable target (56). The changes in epigenetic landscape can be used as diagnostic markers as well as therapeutic targets in both invasive and non-invasive samples (57). Besides pharmacokinetic effects of epigenetic-based drugs, one can also consider the pharmacodynamics effects of epigenetics. The pharmacoepigenetics, the study of the epigenetic basis for variations in drug response is a growing field which highlights that the genes encoding drug-metabolizing enzymes, nuclear receptors, drug transporters etc. are under epigenetic control and thus, can affect the pharmacodynamics of drugs (58).

The epigenetic modifiers fall into three main categories i.e. writers (ones that mark DNA and histones with chemical groups), readers (which read those marks) and erasers (which remove these marks). All three have been targeted for developing epigenetic-based drugs. Besides having precise knowledge about molecular targets and the mechanism of action involved, the demonstration of efficacy is what ultimately matters for drug. The epigenetic-based drugs are a reality now, but we need to be aware that it's a recent development and there are concerns about specificity, adversity, best schedule, ideal dosing, downstream effectors etc. Nevertheless, there are many epi-drugs which are either already approved by the U.S. Food and Drug Administration (FDA) or they are at advanced stages of approval. But most of them are for cancers. Presently, epi-drugs in three epigenetic target classes (i.e. DNMT, HDAC and EZH2 inhibitors) have been approved for the treatment of diverse malignancies (59). So far, there is no approved epigenetic-based biomarker and drug by the U.S. FDA for ocular diseases, particularly keratitis.

However, some of the recent studies have demonstrated promising therapeutic potential of epigenetics in infectious

keratitis, which develops our hope that we might have epi-drugs for ocular diseases soon as well. For example, Sivakarthik Varanasi et al. (60), have shown that 5-azacytidine (Aza; a cytosine analog), a DNA methyltransferase inhibitor, inhibits the progression of herpatic keratitis and limits the HSV-1-induced ocular inflammatory lesions by enhancing regulatory T-cell function. Similarly, attenuation of fungal keratitis in mice by histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA) has been recently reported by Xiaohua Li et al. (4),. Also, Hae-Ahm Lee et al. (61) have recently shown that histone deacetylase inhibitors MPK472 and KSK64 can be potential therapeutic targets for *Acanthamoeba* keratitis, which otherwise is difficult to treat because of cyst formation. These HDACs inhibit the encystation of A*canthamoeba* and have low cytopathic effects on human corneal epithelial cells, and therefore can be promising epidrugs for *Acanthamoeba* induced keratitis.

Using combinations of epigenetic modifiers can also be an important strategy in reducing inflammation and/or disease, for example, a single dose of combinatorial administration of as 5-Aza-2-deoxycytidine (Aza) and trichostatin A (TSA) (Aza+TSA) after the onset of acute lung injury (ALI) has been found to be an effective method to attenuate lung vascular hyper permeability and inflammatory lung injury (62).

In context of viral diseases, epigenetic modifiers in the latency period of infection can be controlled in two steps i) Shock and kill strategy-using epigenetic modifiers to revoke the expression of virus and use anti-viral drugs to decrease viral load and ii) block and lock strategy- using epigenetic modifiers permanently silencing the latent virus (63). Similar strategy could be potential used in case of ocular inflammatory disorders and infectious keratitis complications (64).

CONCLUSIONS AND FUTURE PERSPECTIVE

Disease's state represents an accelerated situation of tissue damage and aging. The role of epigenetics in maintaining normal development and function is reflected by the facts that many diseases develop when aberrant type of epigenetic footprints are introduced or are added at the wrong time or at the wrong place. DNA methylation, histone modifications and nucleosome positioning are generally used as a biomarker of tissue aging, it is not just marking time like a clock on the wall but "actually controlling the time-speed within cells" (65).

Age-associated DNA damage drives erroneous distribution of proteins at various cellular compartments. In case of epigenomic machinery it may cause unwanted genes to switch on/off associated with various diseases/degenerated state. In ocular context, epigenetic reprogramming has shown promising results in promoting optic nerve regeneration, reversal of vision loss in glaucoma, and reversal of vision loss in aging animals.

Corneal keratitis specifically neurotrophic keratitis is a condition of nerve degeneration. Manipulating epigenetic clock, thereby promoting nerve innervations could be one of the strategies to induce diseases clearance and healing. One approach will be to rewire the epigenetic memory rather than totally erasing it, by either controlling the dose, time exposure or different permutation and combinations modulating of Yamanaka factors. The reversal state can be driven by changing landscape of the tissue associated with earlier time stamp, thereby triggering local tissue regeneration (66–69). With the evolution of therapeutics, we have moved from small molecule drugs like aspirin to large molecule biologist such as insulin now moving into multicomponent system therapeutics, which may enable epigenetic reprogramming to induce targeted regeneration in the tissue of interest. Degeneration changes in a tissue associated with disease or aging are often linked with system level changes in functional gene clusters such as inflammation, fibrosis, neurodegeneration and vascular defects. Regeneration can be often looked at reversal of cell state with projections along these functional axes and changing the epigenetic state and effective time stamp and potential therapeutics using multiple factors

It is very essential to understand the epigenetic machinery and diseases specific function of its component to design and develop targeted epigenetic therapy. Importantly, it is critical to know the specific inhibitors other than the widely used pan inhibitors in clinical trials and further explore their roles in regulating specific gene expression in a more defined fashion during infection development and progression.

In the recent years, epigenetic studies advancement has provided novel insights and has significantly increased our knowledge about the interactions between pathogens, cellular factors, histones, and nonhistones modifying enzymes. As most of the epigenetics modifications are reversible, rewiring this complex machinery could be critical in determining the infection and also subsequent recovery. In case of viral keratitis, it is important to permanently maintain the virus in latency by erasing its reactivation epigenetic memory, so that the reactivation could be bypassed. Alternatively, using the epigenetic modifiers that targets the host rather than the pathogens could be helpful in tackling the complications of drug induced resistance in bacteria and viruses. In addition, addressing the role of the less studied post-translational modifications such as phosphorylation or sumoylation can shed light on new aspects of the dynamic host-pathogen interplay in case of infectious keratitis. Altogether, new therapeutic approaches are actively needed to treat infectious keratitis especially for viral infections and understanding the epigenetics of infectious keratitis and thereby repurposing drugs targeting epigenetic players could lead to major therapeutic breakthroughs in the treatment of ocular keratitis.

There are few important considerations to be taken into account, it is important to decrease the risk of epigenetic instability and abnormalities that could result due to continuous use of wide spectrum inhibitors over long-term. Therefore, it is very important to focus our research on identifying diseases specific inhibitors rather than global nonspecific inhibitors.

AUTHOR CONTRIBUTIONS

Conception, design, review of literature, writing, compiling, editing, and reviewing of the manuscript have been done by

AT and TB. Conception, design, editing, and reviewing of the manuscript and final approval have been done by SV, AS, and AV. review of literature, writing, compiling, and editing have been done by RC, JR, MA, AT, and VS. The manuscript has the final approval of all the authors. The first three authors (SV, AS, and AV) have contributed equally and share the first authorship.

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REVIEW
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Deciphering DNA Methylation in HIV Infection

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With approximately 38 million people living with HIV/AIDS globally, and a further 1.5 million new global infections per year, it is imperative that we advance our understanding of all factors contributing to HIV infection. While most studies have focused on the influence of host genetic factors on HIV pathogenesis, epigenetic factors are gaining attention. Epigenetics involves alterations in gene expression without altering the DNA sequence. DNA methylation is a critical epigenetic mechanism that influences both viral and host factors. This review has five focal points, which examines (i) fluctuations in the expression of methylation modifying factors upon HIV infection (ii) the effect of DNA methylation on HIV viral genes and (iii) host genome (iv) inferences from other infectious and non-communicable diseases, we provide a list of HIV-associated host genes that are regulated by methylation in other disease models (v) the potential of DNA methylation as an epi-therapeutic strategy and biomarker. DNA methylation has also been shown to serve as a robust therapeutic strategy and precision medicine biomarker against diseases such as cancer and autoimmune conditions. Despite new drugs being discovered for HIV, drug resistance is a problem in high disease burden settings such as Sub-Saharan Africa. Furthermore, genetic therapies that are under investigation are irreversible and may have off target effects. Alternative therapies that are nongenetic are essential. In this review, we discuss the potential role of DNA methylation as a novel therapeutic intervention against HIV.

Keywords: HIV, epigenetic regulation, DNA methylation, epigenome-wide methylation, epi-therapeutics

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INTRODUCTION

In the nuclei of eukaryotes, the chromatin is subject to intense epigenetic events resulting in either condensed repressive heterochromatin or transcriptionally permissive euchromatin (1). These epigenetic events include posttranslational modifications to histones and methylation of DNA (1). DNA methylation involves the covalent addition of methyl groups to the fifth carbon in the nitrogenous base of cytosine (5mC) bases that are usually followed by guanine bases (CpG site) in DNA (2–5). Methylation of CpG sites found in the cis-regulatory regions of genes is generally associated with silencing genes (5–7). Methylation can also occur in intergenic regions, where it

prevents the expression of potentially harmful genetic elements (4) as well as within the gene body, where a positive correlation with gene expression occurs (8–10).

DNA methylation is strongly involved in the physiological control of gene expression (4). It plays a key role in normal development (11), compaction of chromatin (12), genomic imprinting (13), X chromosome inactivation (14) and the bulk silencing of viral and transposable elements (15). However, aberrant methylation patterns are associated with a multitude of diseases [reviewed in (16–19)]. Several studies have shown that viral infections can induce aberrant methylation patterns within the host genome (20–22). On the other hand, the integrated proviral genome is also influenced by the epigenetic environment of the host (20, 23, 24). Thus virus-host interaction induces an altered epigenetic environment that affects both the virus and the infected host cell.

The human immune deficiency virus (HIV) is no exception to this phenomenon. The effect of HIV infection on DNA methylation has been characterised in HIV positive individuals (25). These effects have been associated with accelerated aging and abnormalities in gene expression, especially in immune regulating genes (25–30). Furthermore, methylation of HIV provirus by the host's methylation machinery can control HIV-1 transcription, replication, and persistence (31–35).

We review the current literature on viral and human genes affected by methylation as well as address gaps in knowledge that are yet to be explored with regards to DNA methylation and HIV. This review will focus on five aspects: (i) the fluctuations of host DNA methylation modifying factors post HIV infection, (ii) the contribution of methylation on viral genes, (iii) the contribution of human genomic methylation on HIV disease, (iv) the influence of methylation on host genes observed in other diseases and models, and (v) the potential of DNA methylation as an epi-therapeutic strategy and precision medicine biomarker.

DNA METHYLATION MODIFYING FACTORS POST HIV INFECTION

DNA methylation is not a random event. Several proteins are involved in establishing, removing, and recognising methylation marks at specific CpG sites within the eukaryotic genome (4). DNA methylation is established by a family of DNA methyltransferases (DNMTs – DNMT1, DNMT3a and DNMT3b). DNMT1 is responsible for maintaining methylation patterns following DNA replication (36), while DNMT3a and DNMT3b regulate *de novo* methylation (37). Therefore, alternations in DNMT expression usually leads to changes in DNA methylation levels within cells. Previous studies have highlighted the increase in expression of DNMTs in HIV infected $CD4^+$ T cells (38–41). HIV-1 was shown to induce the expression of DNMT1 in a non-specific tissue manner, and that overexpression of the viral genes: *nef*, *tat* and *rev*, induced *DNMT1* promoter activity (40, 42, 43). In regulatory T cells, the effect of X4-tropic HIV infection demonstrated no significant change in the expression of DNMT1 and DNMT3a, while there was a substantial increase in expression of DNMT3b (41); however, increased expression of DNMT1, DNMT3a and DNMT3b was observed in CEM*174 T cells with significantly higher expression of DNMT3b (44). Similarly, HIV-1 replication enhanced DNMT3b levels in patients receiving antiretroviral therapy (ART) (45). The expression of DNMT3b was directly correlated to patient HIV viral load, while an inverse relation was observed for DNMT1 (45). Furthermore, proteomic analysis of primary oral epithelial cells revealed significantly lower DNMT1 and DNMT3a levels in HIV patients on ART. Additionally, DNMT activity and global DNA methylation illustrated a direct correlation (46).

The effect of HIV on DNMTs has incited interest in its effect on DNA demethylase enzymes. Conversion of the methyl group from 5-methyl-cytosine are mediated by a group of ten-eleven translocation methylcytosine dioxygenase (TET) enzymes to generate 5-hydroxymethyl-cytosine. 5-hydroxymethyl-cytosine can undergo further modifications such as deamination by apolipoprotein B mRNA Editing Catalytic Polypeptide-like (APOBEC) proteins. The expression of DNMT1 and TET1 was found to be increased in HIV-1 infected individuals without ART (47). Recently, the HIV-1 Vpr, which increases HIV-1 replication in macrophages, was shown to target TET2 for degradation, exacerbating HIV-1 infection (48, 49). The status of other TET enzymes (such as TET2 and TET3) has not been explored in an HIV setting.

Interestingly, recent studies have highlighted the importance of TET2 and TET3 for regulatory T cell stability and immune homeostasis (50). The loss of TET3 gene expression may be a pivotal contributor to locus hypermethylation (51). The effect of the TET family in an HIV setting is vastly unexplored; thus, the future investigation may unearth potential mechanisms of action, as seen in non-communicable diseases (52–54). However, much interest has been given to the cytidine deamination functioning of APOBEC (especially APOBEC3G and APOBEC3F). They have been shown to extensively deaminate viral cytosine to uracil resulting in the potent inhibition of HIV-1 infections (55, 56).

Another key multifunctional epigenetic regulator associated with HIV is methyl CpG-binding protein-2 (MeCP2), which recognizes methylated CpG sites and modulates transcription and chromatin structure (57, 58). The HIV gene *tat* is known to induce miR-132 expression, which subsequently down-regulates the expression of MeCP2 (59). However, Periyasamy et al. (60) discovered that the HIV-1 tat protein downregulated miR-124, which increased MeCP2 and its phosphorylated (Ser80) analogue in microglial cells. Interestingly, phosphorylated MeCP2 (Ser80) blocks miRNA biogenesis machinery, subsequently down regulating miR-124. These contradictory observations suggest that the effect of HIV-1 on host genes desires more attention (60).

DNA methylation is also known to be recognized by methyl-CpG binding domains (MBDs) and Ubiquitin Like with PHD and Ring Finger Domains 1 (UHRF1), which recruits DNA methylation modifying enzymes to chromatin (61, 62). Evidence from Kauder et al. (31) showed that HIV latency is

regulated epigenetically *via* methylation of proviral DNA by DNMTs and its recognition by MBD2 (31). UHRF1 was also shown to facilitate latency as it was recruited to the HIV-1 5'LTR in a methylation/integration dependent fashion, where UHRF1 mediates the repression of HIV-1 gene expression (63).

CONTRIBUTION OF METHYLATION ON VIRAL GENES

Methylation of both the HIV-1 proviral genome and host genome facilitates the integration, replication, and latency of HIV-1. The integration of proviral DNA into the host chromosome is not random as it is preferentially inserted into the euchromatin or actively transcribing regions of the host (64–66). Once integrated, it becomes indistinguishable from the host genome and exploits host cellular machinery for the transcription of its genes (67). However, this also puts proviral DNA at risk for epigenetic silencing events such as DNA methylation. In most cases, presence of methylation within the viral DNA, which has been integrated into the host genome, results in the reduction of new viral particles. In contrast, when integrated viral DNA is not methylated, viral transcription and viral production proceeds as usual (**Figure 1**).

The association of proviral methylation and the transcriptional inactivation of HIV-1 was introduced as early as 1990 (68, 69). Since then, several *in vitro* studies have reported that methylation of CpG sites found within the proximal proviral promoter, located in 5' long terminal repeat (5'LTR), silences transcription of HIV-1 genes resulting in latency. This allows HIV to evade host immune responses and ART (31–35). However, *in vivo* analysis of methylation patterns in the 5'LTR with regards to latency is conflicting. High methylation patterns were found in the 5' LTR of memory CD4+ T cells isolated from aviraemic HIV positive individuals on long term ART therapy (32). However, in a subsequent study, CpG sites were poorly methylated in resting

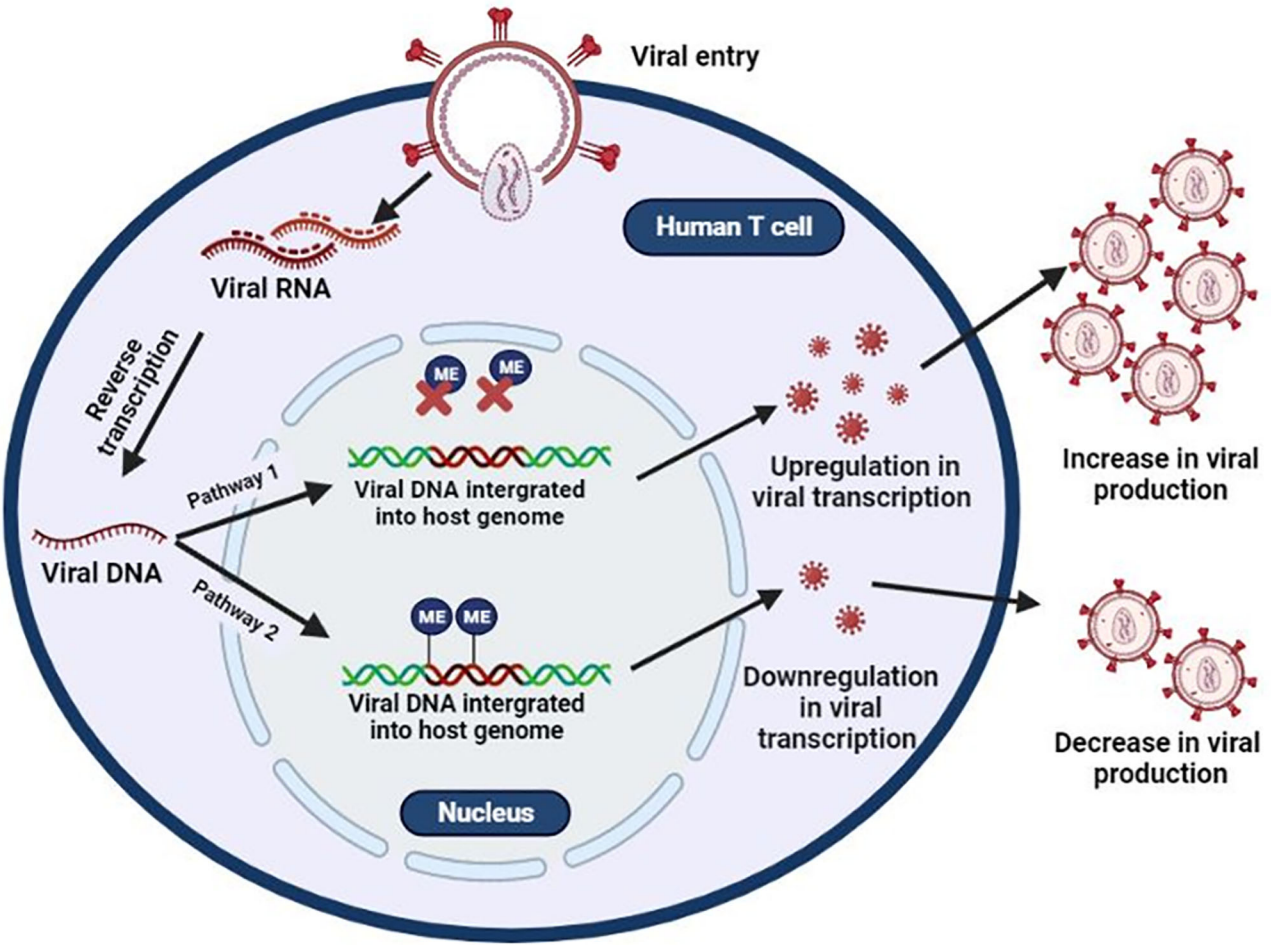

FIGURE 1 | Epigenetic silencing of HIV transcription *via* methylation of integrated provirus. HIV binds to host receptors, entering the cell. HIV viral RNA is converted to single-stranded viral DNA and integrates into the host genome. HIV uses the host's machinery to create new HIV copies (Pathway 1). However, methylation of integrated provirus results in the downregulation of HIV viral transcription, resulting in latency (Pathway 2).

CD4+ cells from HIV infected individuals (70). Trejbalova et al. (33) also observed low methylation levels in the 5'LTR of resting CD4+ cells isolated from individuals on effective ART; however, methylation levels appeared to increase with prolonged ART use (33). A comparison of methylation levels in the 5'LTR of long term non-progressors/elite controllers and virally suppressed individuals on ART found that methylation was virtually absent in individuals in the latter groups compared to the non-progressor/elite controller groups (71). These observations suggest that latency and ART-induced suppression might have different methylation patterns. The apparent difference in methylation patterns between *in vivo* and *in vitro* studies can be attributed to the pressure of "natural" selection in HIV-1-infected individuals. In contrast, under *in vitro* cell culture conditions, HIV-1 proviral genomes are not subject to the selective pressure exerted by host immune defence (72).

5'LTR methylation levels were also shown to associate with the expression of HIV-1 genes. Decreasing levels of methylation in the 5'LTR corresponded with increasing expression of HIV-1 *gag* in HIV-1 infected spermatozoa. Furthermore, gag protein was expressed in 2 cell embryos transfected with infected spermatozoa suggesting that 5'LTR methylation regulates the expression of HIV-1 *gag* in the vertical transmission from sperm to embryo (73).

Regarding methylation patterns outside the LTR, Weber et al. (72) found that CpG sites remain in a predominantly unmethylated state in the 5'LTR, 3'LTR and portions of HIV-1 *gag, env, nef* and *tat* genes. They also observed slight variations in the methylation state of the HIV-1 genome in one long term non-progressor over 11 years, although viral load and $CD4^+$ levels remained stable (72). A recent study examined methylation of intragenic regions of the proviral genome across four groups of HIV infected individuals [i.e. long term non-progressors, early combination ART (cART) treated, late cART treated and cART naïve, acutely infected] (74). As a whole, methylation of promoter regions was reduced in all four groups, while high levels of methylation were observed in the intragenic *env* region. In the ART naïve acutely infected group, a distinct increase in 5'LTR and a decrease in intragenic *env* methylation was observed (74). Taken together, these observations suggest that intragenic methylation could be a late event during infection as well as intragenic methylation was positively associated with CD4+ counts and viral loads (74).

It is important to take into consideration the high mutation rates of HIV (75). Based on levels of mononucleotide C and Gs, the frequency of CpG sites within the HIV-1 genome is much lower than one would expect. The methylation of viral CpG sites may result in the spontaneous deamination of cytosine to thymine which increases the mutation rate of HIV (76). Moreover, coding regions such as the *env* region are highly variable (75). It would be interesting if future studies would evaluate whether CpG sites within these regions are lost, retained, or gained over a period of time and whether these mutations are beneficial or harmful to the virus.

Thus far, all studies on HIV DNA methylation have focused only on CpG methylation; however, non-CpG methylation was reported in other retroviral infections (77, 78). The lack of appropriate techniques that include non-CpG methylation may be why it has not been evaluated, as most of the studies discussed used nested PCR-based methods that exclude most non-CpG methylation (79).

The variability in existing data may be due to several factors. For instance, the integrated HIV provirus is subject to its immediate chromatin environment; thus, different integration sites may influence methylation status accordingly (79). Several pitfalls arise from the amplification of HIV from bisulphite-converted DNA: (i) the high mutation rates of actively replicating HIV hinders designing PCR primers that can amplify all HIV targets, (ii) longer primers are needed for bisulphite converted DNA which can worsen the biased amplification of variable sequences, (iii) multiple rounds of amplification of multiple variants can introduce stochastic bias and variable results are obtained from different methods even when the same conditions are applied (79). There is a significant need for an approach in which HIV amplification of the provirus is reproducible across different primer sets and experiments. Furthermore, attempts to establish and measure latency are unconvincing. It has previously been shown that cell lines harbouring viruses are not genuinely latent but are instead in an incapacitated state (80). Thus, *in vitro* studies are not an accurate measurement of methylation or latency. The development of appropriate methods for specific assessment of the replication-competent HIV reservoir in clinical samples and techniques of studying DNA methylation in the context of HIV may be helpful. Furthermore, the examination of non-CpG methylation of the provirus should be undertaken (79).

THE CONTRIBUTION HUMAN GENOMIC METHYLATION ON HIV PATHOGENESIS

While DNA methylation may influence the replication of HIV and transcription of crucial HIV genes, the integration of HIV-1 DNA into the host genome is also associated with aberrant methylation of host genes. Altered DNA methylation across the host genome has been shown to contribute to HIV disease. Previous studies have identified this *via* two different mechanisms. The first mechanism is a non-hypothesis driven approach which characterizes epigenome-wide methylation patterns. The second method is a hypothesis driven approach which measures methylation of specific/candidate genes. We will discuss each approach more thoroughly in the two sections which follows.

Assessing the Epigenome-Wide Methylation Patterns of the Host

Given that methylation of specific CpG sites found in either the promoter or gene body may impact gene expression, the use of epigenome-wide characterisation of DNA methylation provides a powerful approach in identifying epigenetic variations associated with disease acquisition, severity, and predictive outcomes (81, 82). Several high-throughput methods have been

established for the genome-wide profiling of methylation at single-nucleotide resolution. These methods usually require the treatment of genomic DNA with sodium bisulphite, which deaminates unmethylated cysteine residues to uracil, leaving methylated cysteine residues unaffected (83). The most used techniques include whole genome-wide bisulphite sequencing and microarrays. Whole genome-wide bisulphite sequencing involves PCR amplification of bisulphite converted DNA coupled with next-generation sequencing, which allows for the methylation profiling of every cytosine in the genome (84). Methylation arrays such as Illumina's Infinium arrays involves amplifying bisulphite converted DNA followed by its hybridisation to arrays containing probes that distinguish methylated and unmethylated cytosine and covers CpG islands, shores, and shelves (85). Other methods include methylated DNA immunoprecipitation, comprehensive high-throughput arrays for relative methylation and reduced-representation bisulphite sequencing. Most of the studies pertaining to genome-wide methylation profiling in HIV infected hosts use either methylation arrays or whole genome-wide bisulphite sequencing.

The first large scale study to characterise altered DNA methylation patterns of the host genome associated with HIV infection was conducted on DNA extracted from whole blood collected from 261 HIV infected and 117 uninfected individuals (30). The epigenome-wide association study (EWAS) identified 20 CpG sites to be significantly associated with HIV infection. Among them, 14 CpG sites were found to be hypomethylated, and six were found hypermethylated in HIV-infected individuals. These 20 CpG sites that were significantly associated with HIV infection were found within genes involved in immune activation (30). The most significant was 2 CpG sites located in the promoter region of NOD-like receptor family CARD domain containing 5 (*NLRC5*), an important transcriptional regulator of the Human Leucocyte Antigen (HLA) class-I genes and genes related to HLA class I antigen presentation and processing, such as *TAP1 β2M, and LMP2* (86). Hypomethylation of the 2 CpG sites (cg16411857 and cg07839457) within the promoter region of the *NLRC5* inversely correlated with viral load implying that DNA methylation of *NLRC5* is associated with HIV disease outcome (30). In a recent study, similar results were observed in HIV infected and uninfected individuals who are injectable drug users during 6-month abstinence from drug injections. HIV infection was associated with 49 differentially methylated (DM) CpG sites. The top CpG sites identified were associated with immune and viral response pathways that are associated with HIV pathogenesis, with *NRLC5* being the top-ranked gene associated with HIV status (87). Strong evidence of differential methylation within the MHC region *(HLA-F, PSORS1C2, PSORS1C3* and *Notch4*) and *NLRC5* region was also observed in children with perinatally acquired HIV. HIV was also shown to stunt B cell development and maturation *via* hypermethylation of *EBF4, FOXP1* and *DLL1* in perinatally infected children (29).

While studies on adult populations found that most DM CpG sites were hypomethylated in HIV infected individuals (30, 87), 97% of DM CpG sites tend to be hypermethylated in perinatally infected children. These differences suggest childhood acquisition of HIV alters the epigenome differently than acquisition as an adult (29). Differential methylation also occurs between perinatally infected and uninfected children (44, 88). Seeing as genetic and environmental factors influence the methylome, studies comparing the epigenetic profile of the general population is less than ideal. The use of discordant monozygotic twins with perfectly matched genetic profiles and similar lifestyles eliminates potential genetic confounders when unrelated individuals are used. Thus, variations in the methylome could be accurately attributed to exogenous factors such as viral infection (89). In a study conducted on a pair of 15-year-old monozygotic twins with discordant HIV statuses, significantly higher levels of methylated differentially methylated regions (DMRs) were observed in the infected twin compared to the uninfected sibling, further suggesting that HIV infection would cause the increase of global methylation level in perinatally infected children (44, 88). DMRs were located in chromosomes 17, 19 and 22, which are known HIV integration sites as they contain actively transcribing genes (44, 90, 91). It is possible that hypermethylation of regions in these chromosomes may be a mechanism employed by the host to suppress viral propagation. Twenty-five hyper-methylated genes in the HIV infected twin were validated at the transcriptional level. The expression of 72% of genes were downregulated by more than 50% in the HIV infected twin with *IGFBP6* and *SATB2* being the most significantly reduced genes. However, information on the role of IGFBP6 and SATB2 in HIV pathogenesis is limited (44). The use of HIV discordant monozygotic twins by Zhang et al. (44, 88) was an admirable attempt to account for the influence of genetic factors; however, it failed to account for environmental effects (44, 88). Further, only a single pair of twins were used in the study and the twins were recruited seven years after the acquisition of HIV infection. Thus, methylation changes cannot be used to distinguish between cause and consequence (44, 88).

While most studies have focused on variations in global DNA methylation among uninfected and infected individuals, the disparity has also been established in individuals with variable levels of HIV-1 viral load. Oriol-Tordera et al. (92) evaluated host genome methylation patterns of chronically HIV-1 infected individuals with high (>50,000 HIV-1-RNA copies/ml) and low (<10,000 HIV-1-RNA copies/ml) viral loads. Fifty-five DMRs were found to differentiate individuals with high viral load from those with low viral loads (92). Functional analysis showed genes involved in anti-viral activity and type I interferon γ (IFNγ) signalling to be hypermethylated in HIV infected individuals with low viral loads. Of particular interest, DMRs associated with IFNγ signalling included: *PARP9*/*DTX3L*, *MX1*, *USP18*, *IFI44L* and *PLSCR1*. In contrast, genes involved in general immune activation, such as T cell activation and differentiation, were found to be hypomethylated compared to individuals with a high HIV viral load (92). Thus, the epigenetic repression of IFNγ stimulating genes may assist in achieving control of HIV.

The studies described thus far provide valuable information on the association of aberrant methylation patterns and HIV infection at an epigenome-wide level; however, the use of whole

blood, which consists of various cell types, has been used in these studies tend to be problematic. DNA methylation profiles differ strongly by cell type; therefore, variations in cell-type composition and proportions between samples can confound analysis (93). Furthermore, HIV mainly affects CD4 T cells which represents a small proportion of the tissue sampled; thus, the variation may not be detected. HIV further destroys CD4+ T cells levels; hence, measured epigenetic differences between cases and controls may only reflect differences in cell type composition and not true epigenetic differences (94). The use of homogeneous cell populations may provide a more accurate estimation of epigenome-wide methylation patterns and associated differential gene expression profiles between HIV infected and uninfected cells. $CD4^+$ T lymphocytes are significant targets of HIV, with their progressive death culminating in acquired immune deficiency syndrome (AIDS). The use of the DNMT inhibitor, 5-azacytidine (5-azaC), can reverse T cell depletion, suggesting that DNA methylation may impact T cell apoptosis during HIV infection (95). Zeng et al. (96) transfected two T-cell lines (MT-2 and Jurkat cells lines) with the T-cell-tropic HIV strain, HIV-1 pNL4-3. Whole-genome methylation analysis found 1,428 hypermethylated and 1,227 hypomethylated DMRs in HIV infected MT-2 cell line compared with the uninfected controls as well as 1,231 hypermethylated and 1,833 hypomethylated DMRs in HIV infected Jurkat cells compared to uninfected control cells (96). Hypermethylated DMRs were significantly enriched in promoter and enhancer regions, suggesting that methylation changes are prone to occur in coding and transcriptional regulatory regions during HIV-1 infection (96). Hypomethylation of DMRs in 147 transcription factor binding motifs occurred in HIV infected Jurkat cells, 94 of which overlapped with the hypomethylated DMRs in the MT-2 cell line (96). HIV infected MT-2 cell lines, and Jurkat cell lines contained 83 and 53 transcription factor binding motifs found in hypermethylated DMRs. In the MT-2 cell line, five hypermethylated transcription factor binding motifs (*WT1, HIF1A, EGR1, IRF1*, and *MEF2C*) were associated with transcription factors that have been previously associated in HIV-1 induced apoptosis (96). These results suggest that the depletion of T cells during HIV infection results from aberrant DNA methylation at the binding sites of apoptosis-related transcription factors (96). Differences in epigenome-wide methylation were observed in $CD4^+$ T cells isolated from individuals with varying degrees of control, suggesting that methylation status differs according to the progression of diseases state and control of infection. Furthermore, hypermethylation of TNF was characteristic in viremic individuals while *TRIM69* and *ITTGB2* were found to be hypomethylated in elite controllers (97). While the use of a homogenous *in vitro* models may provide more accurate methylation patterns, *in vitro* studies are not accurate representation of cells systems and are unable to account for ethnic differences.

Epigenome-wide characterisation reveals that global hypomethylation is prominent in HIV infected adults (30, 87), whereas global hypermethylation is prominent in HIV infected children compared to uninfected children (29, 88). Top hits include genes associated with anti-viral responses, immune defence, immune cell development and apoptosis (29, 30, 87, 96). However, the use of PBMCs and the comparison between unrelated, unmatched infected and uninfected individuals confounds results and thus, it is imperative to account for these factors. More studies should evaluate epigenetic events in monozygotic twins with discordant statuses, or a more desirable approach would be the longitudinal analysis of individuals pre- and post-HIV infection.

Candidate Host Gene Methylation

While EWAS characterisation provides a holistic view of methylation patterns during HIV infection, it is not feasible. Thus, many researchers opt for a targeted approach by analysing the epigenetic regulation of specific genes. The four most common techniques used to determine the methylation status of specific CpG sites includes: (i) methylation-specific restriction endonucleases (MSRE) followed by qPCR using primers surrounding the sequence of interest, (ii) pyrosequencing, (iii) methylation-specific high-resolution DNA melting analysis and (iv) quantitative methylation-specific polymerase chain reaction (98). Several studies have investigated the effect of HIV infection on specific HIV associated genes.

The surface expression of C-C chemokine receptor type 5 (CCR5) influences HIV-1 acquisition and disease progression by facilitating HIV-1 viral entry into T cells (99, 100). A common determinant of *CCR5* expression is specific polymorphisms in open reading frames and cis-regulatory regions of *CCR5* (101). One such polymorphism is a 32 base pair deletion in the open reading frame of *CCR5* (CCR5-Δ32). Individuals homozygous for the CCR5-Δ32 mutation cannot produce complete CCR5 proteins; thus, their T cells surface is devoid of the receptor, providing them with protection against HIV (102, 103). However, polymorphisms do not account for the variation in CCR5 expression between subsets of T cells and altered expression upon T cell activation (104–106). *In vivo* and *ex vivo* analysis by Gornalusse et al. (107) showed that methylation levels within the *CCR5* gene might account for these variations (107). Sorted T cells with higher methylation content within the *cis*-region of *CCR-5* correlated with low CCR5 surface levels. CpG sites in the regulatory region of *CCR5* were mostly methylated in naïve T cells, whereas hypomethylation was prevalent in memory T cells (107). *In vitro* activation of naïve T cells was associated with demethylation of *CCR5* and concomitant increase in *CCR5* expression. These results were confirmed in a cohort of individuals with primary HIV infection and two cohorts of individuals with untreated chronic infection. However, viral load suppression during ART was associated with increased methylation in *CCR5-cis* regions and low CCR5 levels during primary infection (107). Furthermore, the authors demonstrated that specific CCR5 haplotypes contain polymorphism, which may remove CpG sites, resulting in cis-regions resistant to undergoing activation-induced demethylation and are thus constitutively expressed. Therefore, CCR5 surface levels and HIV susceptibility depend on both genetic and epigenetic mechanisms (107).

Genetic variations in the HLA region are known to influence host control of HIV infection (108, 109). HLA molecules present intracellularly derived peptides to immune cells, which elicits immune response upon recognising pathogenic peptides (110). Several previously discussed EWAS have identified differential methylation within the HLA loci in HIV positive individuals (29, 86). The elevated levels of the class I HLA-A molecules are associated with higher HIV viral load and poor HIV control. In contrast, low expression of HLA-A is associated with improved control of viremia and slower progression to AIDS (111). Methylation of the HLA-A promoter results in the reduced expression of HLA-A (112). Moreover, allelic lineage-specific methylation patterns within the HLA-A promoter region are inversely related to HLA expression. Increased DNA methylation levels correlated significantly with reduced HLA-A expression levels (112). Gross et al. (26) found that an entire HLA locus had notably reduced methylation levels in HIV infected individuals compared to uninfected individuals (26). Furthermore, several differentially methylated markers were found surrounding a single nucleotide polymorphism (SNP), rs2395029, within the HLA region (26). This variant is predictive for the presence of HLA-B*5701 and is common in HIV positive non-progressors. Further examination of this locus in neutrophils and $CD4^+$ T cells found that the gene body of *HLA Complex P5 (HCP5)* was differentially methylated in neutrophils, and the methylation level of *HCP5* correlated with $CD4^+$:$CD8^+$ T cell ratio (26). Thus, methylation dynamics plays a critical role in HIV control through its regulation of the HLA system (26, 111, 112).

A specialised subset of CD4 T lymphocytes known as regulatory T cells or T_{regs} plays an essential role in suppressing hyperactive immune responses that may occur during the course of HIV infection (113). However, T_{regs} are also susceptible to HIV infection as they contain receptors that participate in viral entry (114, 115). The maintenance of T_{reg} functioning is heavily dependent on the surface expression of Forkhead Box Protein 3 (FOXP3) (116). *In vitro* transfection of T_{regs} with HIV-1 was shown to impair Treg functioning through the methylation of CpG sites found in *FOX3P* regulatory regions (41). However, *in vivo* analysis of *FOXP3* promoters from T*regs* isolated from PBMCs and colon mucosa of chronic HIV infected patients was demethylated, resulting in the increased expression of *FOX3P* (117). In both studies, *FOX3P* promoter methylation was associated with altered levels of DNA methylation regulating enzymes (41). High levels of DNMT3B were associated with the elevated methylation in the *in vitro* study while a significant reduction in DNMT1, DMAP1, METTL7B, and METTL1 was responsible for the reduced methylation in the *in vivo* study (117).

DNMTs were also shown to influence interferon-gamma (IFNγ) levels (38). INFγ, a cytokine produced by type 1 T helper cells, CD8+ cytotoxic T cells and natural killer cells, facilitates inflammation and regulates antigen presentation and macrophage differentiation upon viral infections (80). High levels of DNMTs in HIV infected T helper cells were shown to induce methylation at the SnaBI site in *INFγ* promoters resulting in low levels on *IFNγ* (38) The aberrant expression is due to methylation silencing and may play a role in the gradual loss of type 1 helper cell response seen in AIDS patients.

HIV positive women have an increased risk of developing cervical cancer and precursor lesions [cervical intraepithelial neoplasia (CIN)] (118–120). Hypermethylation and subsequent silencing of tumour suppressor genes result in gene silencing and represents an essential step for cervical cancer development (121, 122). Methylation levels of the tumour suppressor *EPB41L3* were significantly higher in HIV seropositive women with moderate grade neoplasia compared to HIV seronegative women (123). Methylation levels of microRNA-124–2 (miR-124–2), was significantly associated with HIV positive women with low, moderate and severe grade neoplasia compared to HIV negative women (124); however, no association was found between the methylation content of the tumour suppressor genes *CADM1, MAL RARB, DAPK1* and *PAX1* in HIV (124, 125). The methylation of *ASCL1, LHX8* and *ST6GALNAC5* was significantly higher in HIV seropositive women with low to moderate grade neoplasia than HIV seronegative women. However, methylation levels were comparable between HIV seropositive and HIV seronegative women with high-grade neoplasia (126).

Most recently, Singh et al. (127) found that methylation levels within the gene promoter of the host anti-viral restriction factor, *bone marrow stromal cell antigen 2* (*BST2* or *tetherin*) was associated with BST2 expression and HIV disease state. Methylation levels were significantly elevated in all nine CpG sites within HIV infected individuals compared to the uninfected group. Within the HIV positive group, CpG promoter methylation of *BST2* was further evaluated across four different time points (pre-infection, 3-months. 12-months and 36-months post-infection). An inverse correlation between *BST2* methylation and expression was observed at all time points. Furthermore, in an *in vitro* HIV replication assay, treatment with the DNA hypomethylation drug, 5'-Aza-CdR corresponded with an increased expression of *BST2* and lower viral load, suggesting that controlling regulation may be an important strategy in controlling HIV infection (127).

While DNA methylation is an epigenetic modification, candidate gene methylation may be influenced by variations in the DNA sequence. Several studies have mapped the interactions between genetic differences and variations in DNA methylation across numerous tissue and cell types (128–131). The methylation quantitative trait loci showed that up to 48% of inter-individual variation in DNA methylation was related to CpG sites that were associated with nearby single nucleotide polymorphisms (SNPs) found in *cis* regulatory regions (132, 133). SNPs located near or in CpG sites found in the promoter region of genes can either produce or remove CpG site methylation, leading to an alteration in the expression of the genes (**Figure 2A**). DNA methylation can also differ among alleles of a given gene. This is referred to allele-specific methylation (**Figure 2B**). For example, the promoter region of *HLA-A*24* (highest HLA-A expressing lineage) and *-A*03 (*lowest HLA-A expressing linage*)* contain a similar number of CpG sites; however, only one CpG site was found methylated in

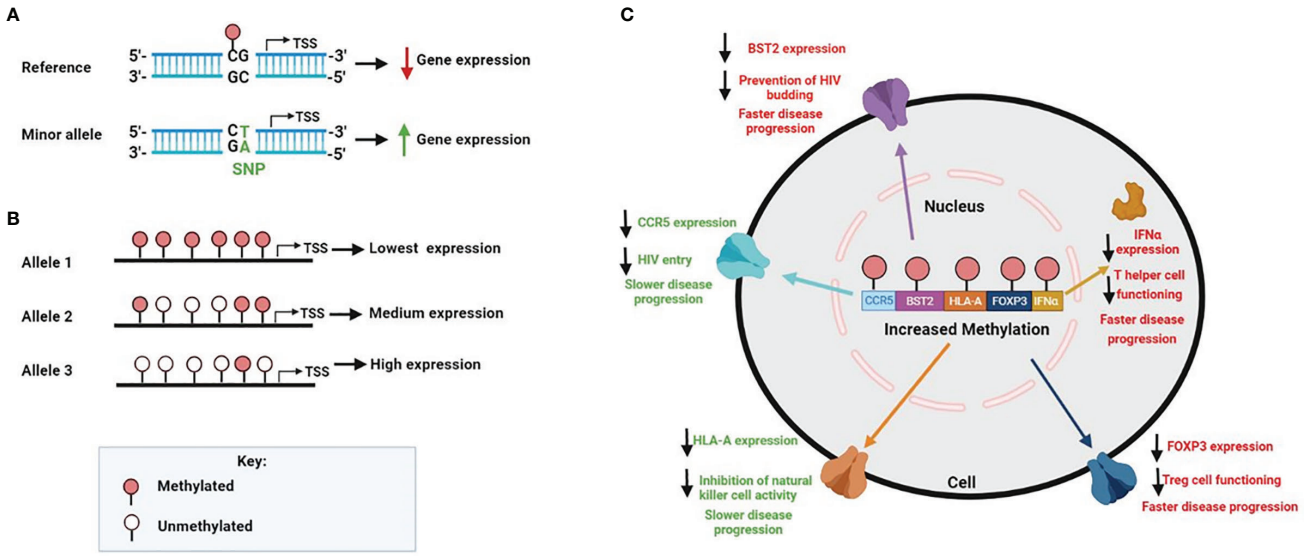

FIGURE 2 | Factors that contribute to human genomic methylation on HIV disease. **(A)** SNPs found in regulatory gene regions can create or abolish CPG sites, which in turn may affect methylation and gene expression. In the genomic sequence, if C is followed by a G, the C can be methylated; however, when the SNP is mutated from a G to a T, it removes the CpG site and methylation cannot occur **(B)** DNA methylation can also differ among alleles of a given gene. The number of methylated CpG sites on each allele can affect expression accordingly **(C)** Increased promoter methylation generally results in decreased mRNA gene expression, which is specifically observed within the HIV setting for the following genes CCR5, BST2, HLA-A, FOX3P and IFNα. Since expression variability of these genes are directly linked to HIV pathogenesis, a change in methylation levels have shown to alter HIV disease progression. Higher levels of methylation for *CCR5* and *HLA*-A results in slower HIV disease progression, however, higher levels of methylation for *BST2, FOX3P* and *IFNα* results in faster disease progression.

the promoter of the HLA-A*24 lineage, while most CpG sites were found to be methylated in the HLA-A*03 linage (112). The influence of genetic variation on promoter methylation of specific host genes in relation to HIV pathogenesis has yet to be investigated. As discussed in this section, we, however, do know that increased promoter methylation generally lowers mRNA expression of specific genes affecting HIV disease progression (**Figure 2C**).

METHYLATION CONTROLLED HOST GENES OBSERVED IN OTHER DISEASES AND MODELS

With only a few studies evaluating the influence of HIV on the methylation of specific host genes, further examination is essential (26, 38, 41, 107, 117, 123, 125, 126). Henceforth, we discuss potential host genes whose methylation status should be investigated with regard to HIV. These genes have been previously shown to associate with HIV disease and were shown to be controlled by DNA methylation in conditions other than HIV. Based on the principle that these genes have been regulated by DNA methylation for a particular disease association, we assume that they may also be regulated similarly in an HIV setting.

For instance, the co-receptor C-X-C chemokine receptor type 4 (CXCR4), like CCR5, mediates the entry of HIV into host cells. Low surface expression of CXCR4 confers with reduced viral entry, while increased expression is associated with the elevated viral entry. Therefore alternations of CXCR4 expression has a significant influence on HIV progression (134, 135). DNA methylation has been shown to regulate CXCR4 expression in pancreatic cancer (136), sporadic breast cancer (137), and primary myelofibrosis (138).

Another example is the host restriction factor, sterile alpha motif and histidine/aspartic acid domain-containing protein 1 (SAMHD1) which limits HIV reverse transcription by depleting the intracellular pool of deoxynucleotide triphosphates (139, 140). De Silva et al. (141) used $CD4^+$ T cell lines as a model to identify mechanisms that regulate *SAMHD1* gene expression. The results indicated that the *SAMHD1* promoter contains a CpG island proximal to the initiation codon of the *SAMHD1* gene, which, upon DNA methylation, leads to transcriptional repression in certain $CD4^+$ T cell lines (142). Regarding disease association, reduced levels of *SAMHD1* expression corresponded with *SAMHD1* promoter methylation in lung cancer (143) and patients with Sezary syndrome (141).

The tumour suppressor, p53 and its downstream gene, *p21*, were shown to hinder early-stage replication of HIV-1 (144). p21, a cyclin dependant kinase, promotes cell cycle arrest by downregulating G1/S transition (144, 145). p21 is also shown to regulate SAMHD1 in HIV-1 infection (145). Epigenetic alterations, including promoter DNA methylation and histone deacetylation, have long been established as crucial mechanisms of carcinogenesis (146–148). p53 promoter methylation leads to

downregulation of p53 in several cancers (149–151). Loss of p21 has been shown to occur in colorectal cancer (152). Additionally, the p21 gene is frequently methylated and is an essential factor in predicting the clinical outcome of acute lymphoblastic leukaemia patients (153). The loss of p21 expression was commonly observed in lung cancer and malignant pleural mesothelioma, and aberrant methylation was one of the mechanisms of suppression of p21 (154).

Methylation of several other host factors such as CCR2, CCL2, CXCR6, CCL5, TSG101, PD-1, PD-L1, TIM3, LAG-3, CTLA-4, TRIM22, DC-SIGN (CD209), IL-10, IL-32, IRF1, Perforin, ICAM-1, and PCSK9 could potentially play a role in HIV disease. **Table 1** provides a list of host factors associated with HIV pathogenesis which should be examined in future methylation studies. Although these disease-methylation associations have been shown in other diseases, it is yet to be proven in HIV disease. Based on the principle that these genes have been regulated by DNA methylation for a particular disease association, we assume that they may also be regulated similarly in an HIV setting. These listed genes may be potential host gene targets that may provide an alternative approach towards precision medicine or personalised therapeutic interventions against HIV and other diseases.

DNA METHYLATION: A VALUABLE TOOL FOR EPI-THERAPEUTICS AND PRECISION MEDICINE

'The Berlin patient' and 'the London patient' were the first two individuals reportedly "cured" of HIV. They both received a stem cell transplant containing the CCR5 Δ-32 mutation to treat their leukaemia which consequentially eliminated the virus from their bodies (221, 222). Such cases provided proof that HIV-1 can be eradicated in those already living with the virus. Given that this approach is not feasible for most people living with HIV, other therapeutic strategies are essential. Furthermore, recent studies have shown that early treatment with ART, is ineffective against returning the altered DNA methylation profile of HIV positive individuals during acute infection (223). Therefore, there is a need for epigenetic strategies for the treatment of HIV.

Recently, Shrivastava et al. (224) developed a zinc finger protein (ZFP-362) that specifically targeted the HIV-1 promoter region. The ZFP-362 fuses to active domains of DNMT3A and induces a long-term stable epigenetic repression of HIV-1. This suppression was found to be driven by DNA methylation (224). Like ART, this intervention may repress viral transcription and control viral replication in HIV positive individuals; however, it is ineffective against latent HIV reservoirs. Thus, efforts have mainly been focused on targeting the latent HIV-1 reservoir responsible for viral persistence and strengthening immunological defences against HIV. Many researchers are adopting the "shock and kill" approach to targeting HIV. This strategy involves the forced reversal of HIV latency (shock) followed by the robust elimination of infected cells by viral or host immune-mediated cytolysis (kill). Therefore novel approaches for the development of latency-reversing agents (LRA) are needed (225). Much interest has been given to the development of epi-LRA – agents that disrupt latency by interfering with the epigenetic silencing mechanism of the 5'LTR (226). In the instance of methylation of 5'LTR, the use of DNMT inhibitors have been considered (31).

Bouchat et al. (227) found that the DNMT inhibitor, 5-AzaC, combined with histone deacetylase inhibitors panobinostat or romidepsin, was potent in reducing HIV-1 latent reservoirs in ART-treated patients (227). The 5-AzaC analogue, 5-aza-2′ deoxycytidine (5-AzadC), alone and in combination with TNFα and prostratin, significantly increased HIV gene expression through altered methylation levels (31, 227). Both 5-AzaC and 5-AzadC, commercially known as Vidaza® and Dacogen®, respectively, have been approved by the FDA to treat myelodysplastic syndrome and in phase II clinical trials for chronic myelomonocytic leukaemia (227–229). Treatment with either 5-AzaC or 5-AzadC was shown to increase the overall survival of patients with higher-risk myelodysplastic syndromes and prolong time to leukaemia transformation and death compared to conventional care regimens (230–232). According to clinicaltrials.gov, 389 clinical trials are actively investigating 5-AzaC and 5-AzadC as interventions for various cancers and conditions. These include: ependymoma, breast cancers, lymphomas, osteosarcoma, and pancreatic cancer, as well as other conditions such as immune thrombocytopenia, sickle cell disease, myelofibrosis, and COVID-19. Therefore, the inclusion of DNMT inhibitors with ART could represent a significant step towards the elimination of the latent HIV-1 reservoir and clearance of virus from infected patients.

Other novel technologies, such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), have great potential in eradicating viral genomes from infected individuals by editing genes as well as the methylation levels associated with HIV. Ebina et al. (233) successfully excised the latently integrated provirus from the host genome and restricted transcriptionally active provirus using the CRISPR/Cas9 approach (233). CRISPR-Cas9 editing of the host genome has also been investigated as an intervention against HIV. Silencing of *CCR5* and *CXCR4* genes by CRISPR have already been shown as effective towards a functional cure for HIV-1 infection (234–236). While the conventional CRISPR approach may have revolutionised genetic therapies, it permanently switches off host genes and may have unwanted consequences such as off-target gene mutations (237, 238). Therefore, approaches that edit the epigenome rather than the genome may be a more suitable and safer strategy. CRISPR-based epigenome technologies involve the fusion of inactivated Cas9 (dCas9) with DNA methyltransferase or demethylase enzymes, allowing for manipulating methylation levels at specific CpG sites. Because this approach targets the epigenome and uses inactivated Cas9, it will enable reversible editing and prevents the formation of double-strand breaks (239–241). Therefore, this approach may be ideal in prospective studies that evaluate host gene regulation as a treatment strategy against HIV (240).

As the medical field rapidly moves towards precision medicine and theragnostic approaches, DNA methylation profiling can play

TABLE 1 | HIV-associated host genes that are regulated by methylation in other diseases or *in vitro* models.

Gene	Role in HIV-1 pathogenesis	Citation	Disease or *in vitro* models in which DNA methylation is established	Citation
Viral entry				
CXCR4	Facilitates viral entry	(134, 135)	Pancreatic cancer, Sporadic breast cancer, and primary myelofibrosis	(136–138)
CCR2	Minor HIV co-receptor which mediates viral entry	(155, 156)	Human monocytic cells	(157)
CCL2	A ligand of CCR2 which upregulates CXCR4 expression on CD4+ T cells, thus facilitating viral entry. Facilitates transmigration of HIV infected leukocytes across the blood-brain barrier	(158, 159)	Gout, Small cell lung cancer, Raw264.7 macrophages	(160–162)
CXCR6	HIV co-receptor which mediates viral entry	(163, 164)	Hepatosplenic T–cell lymphoma, and Systemic Sclerosis	(165, 166)
CCL5 (RANTES)	Ligand for CCR5. It suppresses infection of R5 strains of HIV-1 by blocking CCR5	(167, 168)	Ageing and childhood obesity-associated asthma	(169, 170)
HIV restriction factor				
SAMHD1	Restricts HIV replication	(139, 140)	Lung cancer and Sezary syndrome	(142, 143)
P53	Restricts HIV replication	(144)	Ovarian cancer, breast cancer, hepatocellular carcinoma and colon cancer	(149–151)
p21	Restricts HIV replication	(144, 145)	Colorectal cancer, lung cancer and malignant pleural mesothelioma and acute lymphoblastic leukemia	(152–154)
TSG101	Inhibits HIV budding	(171)	Cervical cancer	(172)
Immune checkpoint molecules				
PD-1	Immune checkpoint molecule expressed on exhausted T cells, inhibit productive HIV infection, thereby facilitating the establishment of latent HIV infection.	(173, 174)	Colorectal cancer, breast cancer, head and neck squamous cell carcinoma, myelodysplastic syndrome and prostate cancer	(175–179)
PD-L1	Ligand for PD-1. Immune checkpoint molecule expressed on exhausted T cells, inhibit productive HIV infection, thereby facilitating the establishment of latent HIV infection.	(180)	Colorectal cancer, Non-small-cell lung carcinoma, and acute myeloid leukaemia	(175, 181, 182)
TIM3	Suppress effector functions of activated T cells in chronic uncontrolled viral infection with HIV-1.	(183)	Colorectal cancer, breast cancer and gastric cancer	(175, 179, 184)
LAG-3	Immune checkpoint molecule, induces immune exhaustion and facilitates HIV latency	(185, 186)	Colorectal cancer, breast cancer, clear cell renal cell carcinoma, melanoma	(175, 179, 187, 188)
CTLA-4	Downregulates T cell functioning and associated with HIV disease progression	(189)	Colorectal cancer, breast cancer, rheumatoid arthritis, myasthenia gravis, head and neck squamous cell carcinomas	(175, 179, 190–192)
Other				
TRIM22	Inhibits HIV transcription and promotes HIV latency	(193)	Hepatitis B virus, Systemic lupus erythematosus	(194, 195)
DC-SIGN (CD209)	Recpetor found on dendritic cells which binds to gp120 of HIV and facilitate the dissemination of HIV	(196, 197)	Dendritic cells	(198)
IL-10	Increases post-HIV infection by inhibiting HIV-1 specific T-cell responses	(199)	Rheumathoid arthritis, Behçet's disease	(200, 201)
IL-32	Induces hostile cytokine environment that hinders HIV fusion and replication	(202, 203)	Hek293 (*in vitro*), Juvenile idiopathic arthritis, Influenza A	(204–206)
IRF1	activating the transcription of HIV genome during the early stage of HIV replication	(207, 208)	Paediatric obstructive sleep apnea	(209)
Perforin	Associated with slow HIV progression. Mediates the killing of HIV-infected cells by CD8+ T-cells	(210, 211)	CD4 and CD8 T cells, systemic lupus erythematosus, chronic fatigue syndrome, multiple sclerosis	(18, 212–214)
ICAM-1	promotes HIV-mediated syncytia formation and viral spread.	(215)	Autoimmune thyroid diseases, and primary bladder carcinoma.	(216, 217)
PCSK9	Mediates HIV-Associated Dyslipidemia		Coronary artery disease, Congenital Aortic Valve Stenosis Type 2 Diabetes and Metabolic Syndrome	(218–220)

a tremendous role in these strategies. DNA methylations can serve as biomarkers for diagnosis, prognosis, monitoring and predicting treatment response and disease outcome (242). Due to its dynamic and stable nature, it is more reliable and suitable than genetic and protein-based biomarkers. Methylation levels can be easily measured in circulating cell-free DNA, which is the preferable method in clinical settings as it is minimally invasive (243). Several DNA methylation-based *in vitro* diagnostic tests have been developed and commercialised for profiling DNA methylation (241). Tests may be specific for a disease such as Epi proColon® 2.0

CE, which detects methylated *Septin9* to diagnose colon cancer and Bladder EpiCheck®, which measures changes in methylation of 15 genes associated with bladder cancer (244, 245). The utilisation of the EpiSign assay has been well established in clinical diagnostic laboratories and uses genome-wide methylation patterns to diagnose up to 42 rare neurodevelopmental Mendelian syndromes (246, 247). Many of the commercialised clinical DNA methylation assays implement practical and cost-effective assays such as qPCR and microarrays. The use of DNA methylation-based biomarkers for precision medicine has been extensively studied with regards to cancer; however, its application has great potential in other diseases, including HIV. For instance, DNA methylation has been shown to be a potentially effective prognostic biomarker for predicting risk and type of HIV-associated lymphomas and HIV associated cognitive impairment; however, these results are yet to be translated to a clinical setting (94, 248). There is still a lot to be investigated regarding the epigenetic signature of HIV for precision medicine. Future studies should focus on using well-characterised clinical cohorts to evaluate methylation profiling as a biomarker for predicting HIV disease course, development of HIV associated comorbidities, monitoring patient response to ARVs and personalised therapy.

The Epi-therapeutic interventions, either through LRA or CRISPR technologies and DNA methylation in precision medicine and theragnostics, provides a novel and powerful approach against HIV. However, there is much-needed research to be done to translate these approaches into a clinical setting.

CONCLUSION AND FUTURE PERSPECTIVES

Since the beginning of the HIV epidemic, the impact of host genetic variations on HIV susceptibility and disease outcomes has attracted a vast amount of attention, while epigenetic changes have long been neglected. This review provided a comprehensive overview of the intricate interplay between DNA methylation and viral and host genome. Once integrated, the HIV viral genome is subject to the intense epigenetic environment of the host genome. This includes silencing of HIV transcription *via* DNA methylation. Integration of the proviral genome also induces aberrant methylation of the host genome, influencing HIV disease progression. Several host genes involved in viral entry, anti-viral responses and immune defences are altered by DNA methylation in HIV infected individuals.

However, many of the studies discussed are limited by the study designs used. Many of the studies discussed failed to account for the influence of genetic and/or environmental factors on promoter methylation. Another drawback of most studies reviewed is the type of sample that was used. The type of sample selected for a study involving DNA methylation is crucial as methylation patterns differ substantially according to cell type (93). Studies using mixed cell samples such as whole blood or PBMCs need to account for cell type composition and variation in the methylation patterns of different cells. Some studies have tried to account for account for cell type heterogeneity by transfecting homogenous T cell lines (95–97). However *in vitro* studies are not accurate representation of cells systems and are unable to account for ethnic differences. Increased susceptibility to HIV and varying responses to ARVs have been noted amongst different ethnic groups [extensively reviewed in (249)]. Disparities regarding DNA methylation have also been observed between diverse ethnic populations, including Caucasians, Hispanics, Middle Eastern, and African populations and may serve as a biomarker for underlying ethnic health disparities between human populations (250). Thus far, very little is known about the contribution of DNA methylation on ethnic differences to HIV acquisition, disease and treatment outcomes. Seeing that aberrant methylation patterns have been associated with HIV and that the rate of incidence differs amongst different ethnic groups, it is vital ethnic differences are taken into consideration when conducting studies and clinical trials therefore researchers should also take ethnicity into consideration (249, 250). The results of trials on one ethnic group may not necessarily be applicable to another ethnic, therefore researchers should also take ethnicity into consideration. We believe that the ideal model for epigenetic studies related to HIV disease are sorted PBMCs or CD4+ T cells that are isolated form a prospectively obtained longitudinal cohort consisting of different ethnic groups. Admittedly, it will be challenging to recruit and maintain such a cohort, nonetheless, more accurate and useful information can be gained from such a study design.

There is still a lot of gaps in knowledge regarding the relationship between methylation and HIV. But once we have a complete picture, the knowledge gained will contribute substantially to understanding HIV disease. Moreover, the use of epigenetic interventions such as DNMTs inhibitors as LRA, CRISPR editing, and methylation biomarkers may revolutionise our fight against HIV and the AIDS pandemic.

AUTHOR CONTRIBUTIONS

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REVIEW
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Regulatory Role of Non-Coding RNAs on Immune Responses During Sepsis

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Sepsis is resulted from a systemic inflammatory response to bacterial, viral, or fungal agents. The induced inflammatory response by these microorganisms can lead to multiple organ system failure with devastating consequences. Recent studies have shown altered expressions of several non-coding RNAs such as long non-coding RNAs (lncRNAs), microRNAs (miRNAs) and circular RNAs (circRNAs) during sepsis. These transcripts have also been found to participate in the pathogenesis of multiple organ system failure through different mechanisms. NEAT1, MALAT1, THRIL, XIST, MIAT and TUG1 are among lncRNAs that participate in the pathoetiology of sepsis-related complications. miR-21, miR-155, miR-15a-5p, miR-494-3p, miR-218, miR-122, miR-208a-5p, miR-328 and miR-218 are examples of miRNAs participating in these complications. Finally, tens of circRNAs such as circC3P1, hsa_circRNA_104484, hsa_circRNA_104670 and circVMA21 and circ-PRKCI have been found to affect pathogenesis of sepsis. In the current review, we describe the role of these three classes of noncoding RNAs in the pathoetiology of sepsis-related complications.

Keywords: lncRNA, miRNA, sepsis, expression, biomarker

INTRODUCTION

Sepsis is a systemic inflammatory response to different infections, namely bacterial, viral, or fungal agents. This condition is the principal source of mortality in intensive care units (1). These infectious microorganisms can stimulate inflammatory reactions through induction of cytokines release. These reactions lead to multiple organ system failure. Other factors that contribute in this

devastating condition during sepsis are systemic hypotension and abnormal perfusion of the microcirculatory system (2). No specific treatment modality has been suggested for prevention of multiple organ system failure during sepsis (2). Thus, identification of sepsis-related changes at cellular and biochemical levels is important. Currently, there is no effective pharmacological therapy for sepsis. Thus, early diagnosis, resuscitation and instant administration of suitable antibiotics are essential steps in decreasing the burden of this condition {Thompson, 2019 #562}.

Lipopolysaccharide (LPS) as the main constituent of the cell wall of Gram-negative bacteria has been found to stimulate apoptotic pathways in tubular epithelial cells of kidney (3). Moreover, it can prompt acute inflammatory responses through activation of NF-κB during the course of acute kidney injury (4). This molecular pathway is an important axis in mediation of immune-related organ damage.

Recent studies have shown altered expressions of several non-coding RNAs such as long non-coding RNAs (lncRNAs), microRNAs (miRNAs) and circular RNAs (circRNAs) during sepsis. These transcripts have also been found to participate in the pathogenesis of multiple organ system failure through different mechanisms. In the current review, we describe the role of these three classes of noncoding RNAs in the pathoetiology of sepsis-related complications.

LNCRNAS AND SEPSIS

LncRNAs are transcripts with sizes larger than 200 nucleotides. These transcripts regulate gene expression through modulation of chromatin configuration, regulation of splicing events, serving as decoys for other transcripts and making structures for recruitment of regulatory proteins (5). These transcripts participate in the regulation of immune reactions and pathoetiology of several immune-related disorders (6).

Experiments in animal model of acute lung injury have shown down-regulation of TUG1 and induction of apoptosis and inflammation. Up-regulation of TUG1 in these animals could ameliorate sepsis-associated lung injury, apoptosis and inflammatory reactions. TUG1 could also protect lung microvascular endothelial cells from deteriorative effects of LPS. In fact, TUG1 inhibits cell apoptosis and inflammatory reactions in LPS-stimulated microvascular endothelial cells through sponging miR-34b-5p and releasing GAB1 from its inhibitory effects. Cumulatively, TUG1 ameliorates sepsis-associated inflammation and apoptosis through miR-34b-5p/GAB1 axis (7). Another study has demonstrated down-regulation of TUG1 while up-regulation of miR-223 in the plasma samples of sepsis patients. They have also reported a negative correlation between expressions of TUG1 and miR-223 in sepsis patients. Besides, expression levels of TUG1 have been negatively correlated with respiratory infection, serum creatinine, white blood cell, C-reactive protein, APACHE II score, and SOFA score. Based on these results, TUG1 has been suggested as a biomarker for prediction of course and prognosis of sepsis (8). TUG1 has also been shown to interact with miR-27a. Over-expression of TUG1 has resulted in down-regulation of TNF-α, while up-regulation of miR-27a has enhanced expression of TNF-α in cardiomyocytes. TNF-α and miR-27a up-regulation could enhance LPS-induced apoptosis of cardiomyocytes. On the other hand, TUG1 up-regulation has exerted opposite effects (9).

MALAT1 is another lncRNA that affects immune responses of rats with LPS-induced sepsis through influencing the miR-146a/NF-κB P65 axis (10). Moreover, MALAT1 could increase apoptosis skeletal muscle cells and sepsis-associated immune responses through down-regulating BRCA1 levels *via* recruitment of EZH2 (11). The miR-150-5p/NF-κB axis is another axis that mediates the effects of MALAT1 in sepsis-associated cardiac inflammation (12). In addition, the protective effects of Ulinastatin against LPS-associated dysfunction of heart microvascular endothelial cells have been shown to be exerted through down-regulation of MALAT1 (13). Most notably, MALAT1/miR-125a axis has been shown to discriminate sepsis patients based on their severity of diseases, organ damage, levels of inflammatory responses and mortality (14). **Figure 1** depicts function of MALAT1 in sepsis-related events.

NEAT1 is another lncRNA whose participation in the pathophysiology of sepsis has been vastly investigated. This lncRNA could promote inflammatory responses and aggravate sepsis-associated hepatic damage through the Let-7a/TLR4 axis (15). Moreover, NEAT1 can accelerate progression of sepsis *via* miR-370-3p/TSP-1 axis (16). This lncRNA could also promote LPS-induced inflammatory responses in macrophages through regulation of miR-17-5p/TLR4 axis (17). NEAT1 silencing could suppress immune responses during sepsis through miR-125/MCEMP1 axis (18). **Figure 2** shows the function of NEAT1 in sepsis-related events. Several other lncRNAs have also been found to influence course of sepsis through modulation of immune responses (**Table 1**).

miRNAs AND SEPSIS

miRNAs have sizes about 22 nucleotides and regulate expression of genes through binding with different regions of target mRNAs, particularly their 3' UTR. They can either degrade target mRNA or suppress its translation. Several miRNAs have been found to influence course of sepsis. Altered expression of these small-sized transcripts has been reported in sepsis by numerous research groups. For instance, plasma levels of miR-494-3p have been shown to be decreased in sepsis patients compared with healthy controls in correlation with up-regulation of TLR6. Expression level of miR-494-3p has been decreased in LPS-induced RAW264.7 cells, parallel with up-regulation of TLR6 and TNF-α. Forced over-expression of miR-494-3p in RAW264.7 cells could reduce TNF-α level and suppress translocation of NF-κB p65 to the nucleus.

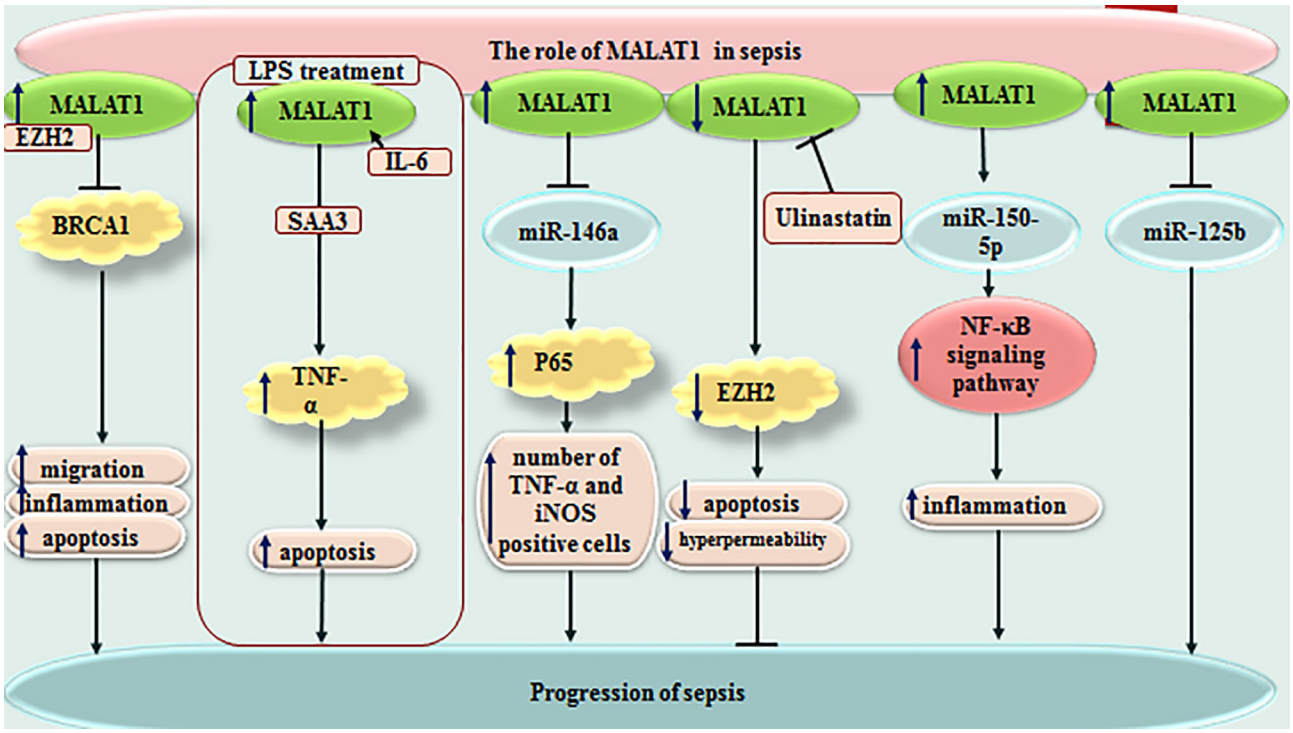

FIGURE 1 | Function of MALAT1 in sepsis-related events.

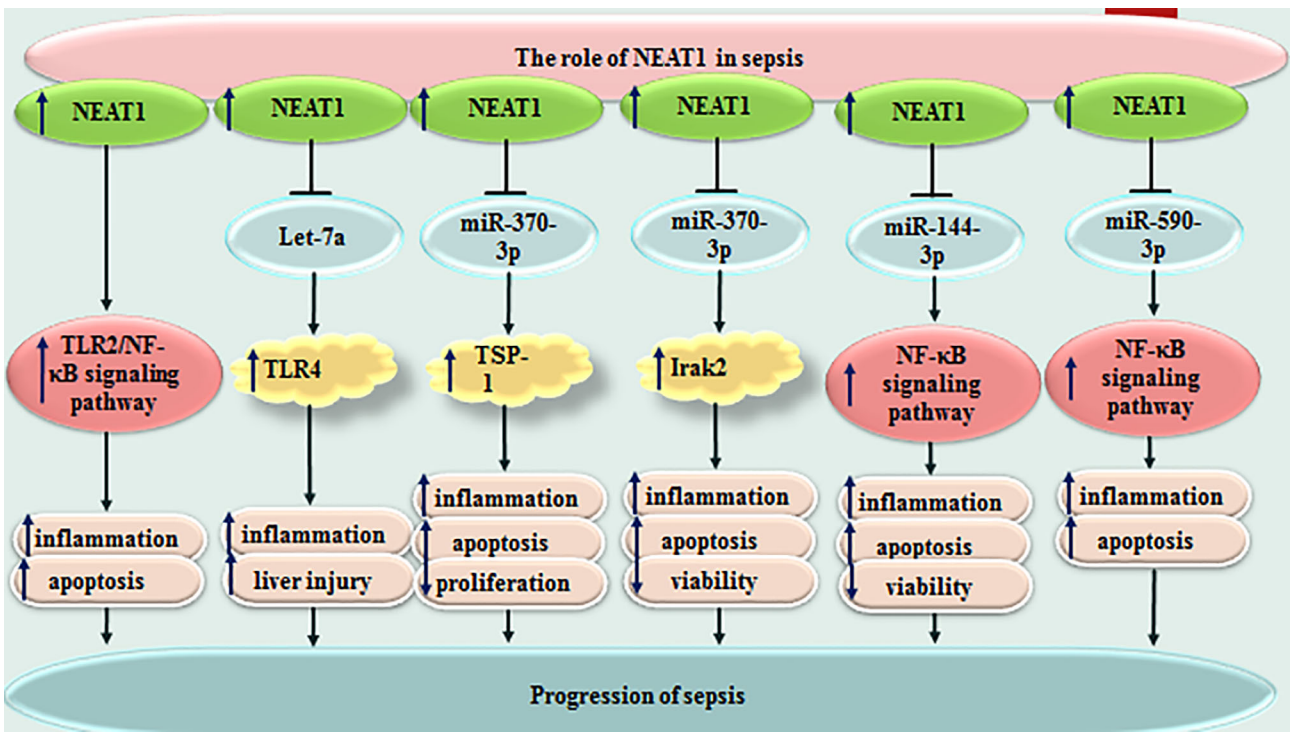

FIGURE 2 | Function of NEAT1 in sepsis-related events. Several other lncRNAs have also been found to influence course of sepsis through modulation of immune responses (**Table 1**).

TLR6 has been shown to be targeted by miR-494-3p. Taken together, miR-494-3p could attenuate sepsis-associated inflammatory responses through influencing expression of TLR6 (132). miR-218 is another miRNA which participates in the pathoetiology of sepsis. This miRNA could reduce inflammatory responses in the sepsis through decreasing expression of VOPP1 *via* JAK/STAT axis (133).

miR-122 is another important miRNA in the sepsis which has superior diagnostic power compared with CRP and total leucocytes count for distinguishing sepsis from wound infection. miR-122 has also been found to be a prognostic marker for sepsis, albeit with poor specificity and accuracy values (134).

In the mice model of sepsis, decreased levels of miR-208a-5p and increased levels of SOCS2 has been associated with enhanced activity of SOD, while reduction in LDH and MDA activities. Moreover, down-regulation of miR-208a-5p has been associated with low levels TNF-α, IL-6, NF-κB p65 and HIF-1α in this animal model. miR-208a-5p silencing could decrease the extent of mitochondria swelling, and inhibit apoptosis of cardiomyocytes in animal model of sepsis. Taken together, miR-208a-5p suppression has been suggested as a modality to attenuate sepsis-related myocardial damage. This function is mediated through NF-κB/HIF-1α axis (135).

miR-21 is another miRNA whose role in sepsis has been investigated by several groups. Down-regulation of miR-21 has been shown to inhibit inflammasome activation, ASC pyroptosome, LPS-induced pyroptosis and septic shock in one study (136). On the other hand, another study in animal models of sepsis has shown that up-regulation of miR-21 reduced inflammation and apoptosis (137). Similarly, βMSCs-derived exosomes have been shown to reduce symptoms in septic mice and improve their survival rate through up-regulation of miR-21 (138).

miR-328 is another miRNA which is dysregulated in sepsis patients as well as animal models of sepsis. Serum levels of this miRNA could properly differentiate sepsis from normal conditions. Thus, miR-328 has been suggested as a diagnostic biomarker for sepsis. Moreover, down-regulation of miR-328 could amend sepsis-related heart dysfunction and inflammatory responses in this tissue (139). miR-452 is another miRNA with diagnostic applications in sepsis. Notably, serum and urinary levels of this miRNA have been suggested as possible markers for early diagnosis of sepsis-associated acute kidney injury, since expression of this miRNA has been higher in sepsis patients with acute kidney injury compared with those without this condition (140) (**Table 2**). **Figure 3** depicts miRNAs that are down-regulated in sepsis.

CircRNAs AND SEPSIS

CircRNAs are a recently appreciated group of non-coding RNAs with enclosed circular configuration formed by covalent bonds between two ends of linear transcripts. However, some of these transcripts have been shown to produce proteins. They mostly exert regulatory functions in the transcriptome. Impact of circRNAs in the sepsis has been assessed by several groups (303). For instance, circC3P1 has been shown to attenuate production of inflammatory cytokines and decrease cell apoptosis in sepsis-associated acute lung injury *via* influencing expression of miR-21 (304).

A microarray-based has shown differential expression of 132 circRNAs between sepsis patients and healthy controls among them have been hsa_circRNA_104484 and hsa_circRNA_104670 whose up-regulation in sepsis serum exosomes has been verified been RT-PCR. Expression levels of these two circRNAs have been suggested as diagnostic biomarkers for sepsis (305).

CircVMA21 is another circRNA that has been shown to ameliorate sepsis-related acute kidney injury through modulation of oxidative stress and inflammatory responses *via* miR-9-3p/SMG1 axis (306). Circ_0114428/miR-495-3p/CRBN axis is another molecular axis which is involved in the pathoetiology of sepsis-related acute kidney injury (307). Moreover, expression levels of circPRKCI have been correlated with sepsis risk, severity of sepsis and mortality during a period of 28 days (308). **Table 3** summarizes the role of circRNAs in sepsis.

DISCUSSION

A vast body of literature points to the involvement of lncRNAs, miRNAs and circRNAs in the pathoetiology of sepsis-related complications. NEAT1, MALAT1, MEG3, THRIL, XIST, CRNDE, ZFAS1, HULC, MIAT and TUG1 are among lncRNAs with the strongest evidence for their participation in this process. NEAT1 as the mostly assessed lncRNA in this regard has been shown to act as a molecular sponge for let-7a, let-7b-5p, miR-370-3p, miR-124, miR-125, miR-17-5p, miR-16-5p, miR-93-5p, miR-370-3p, miR-144-3p, miR-944, miR495-3p, miR-22-3p, miR-31-5p and miR-590-3p. Through sequestering these miRNAs, NEAT1 can affect several molecular pathways in the course of sepsis. It can enhance immune responses and the related injury in target organs, thus participating in sepsis-related multiple organ damage.

Similar to lncRNAs, circRNAs influence course of sepsis mainly through acting as molecular sponges for miRNAs. circC3P1/miR-21, circVMA21/miR-9, circVMA21/miR-199a-5p, circ-PRKCI/miR-545, circPRKCI/miR-106b-5p, circDNMT3B/miR-20b-5p, circ_0114428/miR-495-3p, circ_Ttc3/miR-148a, circPRKCI/miR-454, circ-Fryl/miR-490-3p, circ_0091702/miR-182, circTLK1/miR-106a-5p, circFADS2/miR-15a-5p, circ_0091702/miR-545-3p, hsa_circ_0068,888/miR-21-5p, circPTK2/miR-181c-5p, circ-FANCA/miR-93-5p and circANKRD36/miR-330 are among circRNA/miRNA axes which are involved in the pathophysiology of sepsis-related conditions.

TABLE 1 | LncRNAs and Sepsis.

lncRNA	Expression Pattern	Clinical Samples/ Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
TUG1	↓	35 ARDS patients and 68 HCs, male C57BL/6 mice	PMVECs	↑ miR-34b-5p, GAB1 ↓	_	TUG1 reduces sepsis-induced pulmonary injury, apoptosis and inflammation in ALI.	(7)
TUG1	↓	122 patients with sepsis and 122 HCs	_	↑ miR-223	_	Low levels of TUG1 was correlated with respiratory infection. TUG1 expression was negatively associated with Scr, WBC, SOFA score, and CRP levels and 28-day deaths, but positively associated with albumin levels.	(8)
TUG1	↓	_	HUVECs	↑ miR-27a-3p, ↓ SLIT2	_	Up-regulation of TUG1 reduced apoptosis, autophagy, and inflammatory response.	(19)
TUG1	↓	70 patients with sepsis and 70 HCs	AC16	miR-27a, ↑ TNF-α	_	Up-regulation of TUG1 reduced apoptosis.	(9)
MALAT1	↑	rats with and without LPS-induced sepsis	U937	↓ miR-146a, ↑ P65	↑ NF-κB signaling pathway	Downregulation of MALAT1 decreased the number of TNF-α and iNOS positive cells.	(10)
MALAT1	↑	BALB/c male mice	HSMKMC 3500	↓ BRCA1, EZH2	_	Downregulation of MALAT1 reduced inflammatory responses, neutrophil migration, skeletal muscle cell apoptosis, and AKT-1 phosphorylation.	(11)
MALAT1	↑	_	H9c2	↓ miR-150-5p,	↑ NF-κB signaling pathway	Downregulation of MALAT1 reduced inflammatory response and downregulated NF-κB signaling pathway.	(12)
MALAT1	↑	male SD rats	CMVECs	↑ EZH2	_	MALAT1 significantly inhibited levels of EZH2 target genes, DAB2IP and Brachyury. Up-regulation of CRNDE increased permeability and apoptosis. Ulinastatin suppressed levels of MALAT1 and EZH2.	(13)
MALAT1	↑	196 patients with sepsis and 196 HCs,	_	↓ miR-125a	_	MALAT1 expression was positively correlated with APACHE II score, SOFA score, serum creatinine, CRP, TNF-α, IL-1β, IL-6, 28-day deaths, and negatively with albumin.	(14)
MALAT1	↑	sepsis mice	_	↓ miR-23a, ↑ MCEMP1	_	Downregulation of MALAT1 suppressed expression of MPO, IL-6, IL-10, TNF-α, and IL-1β, and reduced inflammation.	(20)
MALAT1	↑	male C57 mice		↑ p38	↑ p38 MAPK/ p65 NF-κB signaling pathway	Downregulation of MALAT1 reduced MPO and inflammatory responses.	(21)
MALAT1	↑	_	a lung injury inflammatory cell model	↓ miR-149, ↑ MyD88	↑ NF-κB pathway	Downregulation of MALAT1 reduced the levels of MyD88, TNF-α, IL-1β, and IL-6, and prevented the NF-κB pathway.	(22)
MALAT1	↑	CLP-induced septic mice	HUVECs, PAECs	↓ miR-150	↑ NF-κB pathway	Downregulation of MALAT1 reduced apoptosis, ER stress and inflammation.	(23)
MALAT1	↑ in ARDS group	152 patients with sepsis (41 ARDS and 111 Non-ARDS patients)	_	_	_	MALAT1 expression was association with APACHE II score, SOFA score, inflammatory factors levels, and high mortality.	(24)
MALAT1	↑	GEO dataset (GSE3140), male C57B6/L mice	HL-1	↑ IL-6, ↑ ↑ TNF-α, SAA3	_	Downregulation of MALAT1 Protected Cardiomyocytes from LPS-induced Apoptosis.	(25)
MALAT1	↑	190 patients with sepsis and 190 HCs	_	↓ miR-125b	_	MALAT1 expression was associated with Scr, WBC, CRP, PCT, TNF-α, IL-8, IL-17, APACHE II score, SOFA score, and 28-day deaths.	(26)
MALAT1	↑	120 patients with sepsis and 60 HCs	_	_	_	Expression of MALAT1 was found to be an independent risk factor for sepsis, poor prognosis and septic shock.	(27)
MALAT1	↑	female C57BL/6 mice	THP-1	↓ miR-214, ↑ TLR5	_	Downregulation of MALAT1 attenuated the burn injury and post-burn sepsis-induced inflammatory reaction.	(28)
KCNQ1OT1	↓	male SD rats	H9c2	↑ miR-192-5p, ↓ XIAP	_	Up-regulation of KCNQ1OT1 ameliorated proliferation and impeded apoptosis in sepsis-induced myocardial injury.	(29)

(Continued)

TABLE 1 | Continued

lncRNA	Expression Pattern	Clinical Samples/ Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
CYTOR	↓	male SD rats	H9c2	↑ miR-24, ↓ XIAP	_	Up-regulation of CYTOR ameliorated viability and inhibited apoptosis in sepsis-induced myocardial injury.	(30)
lncRNA-5657	↑	15 patients with sepsis-induced ARDS and 15 non-septic and non-ARDS patients, SD rats	NR8383	↑ Spns2	_	Downregulation of lncRNA-5657 7 prevented sepsis-induced lung injury and LPS-induced inflammation.	(31)
RMRP	↓	male C57BL/6 mice	HL-1	↑ miR-1-5p, ↓ HSPA4	↑ NF-κB Pathway	Up-regulation of RMRP reduced LPS-induced damage, apoptosis and mitochondrial damage and LPS-induced sepsis.	(32)
NEAT1	↑	15 patients with sepsis-induced liver injury and 15 HCs	Kupffer, Raw264.7	↓ Let-7a, ↑ TLR4	_	Downregulation of NEAT1 reduced expression of inflammatory factors in sepsis-induced liver injury.	(15)
NEAT1	↑	25 Sepsis patients and 25 HCs	RAW 264.7	↓ miR-370-3p, ↑ TSP-1	_	Downregulation of NEAT1 prevented LPS-mediated inflammation and apoptosis and ameliorated proliferation.	(16)
NEAT1	↑	male pathogen-free C57BL/6 mice	_	↓ miR-125, ↑ MCEMP1	_	Downregulation of NEAT1 suppressed inflammation and T lymphocyte apoptosis.	(18)
NEAT1	↑	68 patients with sepsis and 32 HCs	THP-1 macrophages	↓ miR-17-5p, ↑ TLR4	_	Downregulation of NEAT1 prevented LPS-induced inflammatory responses in macrophages.	(17)
NEAT1	↑	mouse with sepsis-induced lung injury	_	↓ miR-16-5p, ↑ BRD4	_	Downregulation of NEAT1 inhibited inflammation, apoptosis, pulmonary edema, MPO activity, pathological changes, promoted viability.	(33)
NEAT1	↑	male C57 mice	_	_	↑ TLR2/ NF-κB signaling pathway	Downregulation of NEAT1 reduced LPS-induced myocardial pathological injury, apoptosis, oxidative stress, inflammatory responses.	(34)
NEAT1	↑	male C57BL/6 mice	A549	_	↑ HMGB1/ RAGE signaling	Downregulation of NEAT1 increased viability attenuated LPS-induced apoptosis and suppressed inflammation.	(35)
NEAT1	↑	30 patients with sepsis and 30 HCs	HK-2	↓ let-7b-5p, TRAF6	_	Downregulation of NEAT1 increased proliferation and inhibited apoptosis and inflammation.	(36)
NEAT1	↑	_	RAW264.7	↓ miR-125a-5p, ↑ TRAF6, ↑ P-TAK1	_	Downregulation of NEAT1 decreased inflammation by promoting macrophage M2 polarization.	(37)
NEAT1	↑	_patients with sepsis	HK2	↓ miR-93-5p, ↑ TXNIP	_	Downregulation of NEAT1 inhibited apoptosis, inflammation and oxidative stress.	(38)
NEAT1	↑	_ sepsis tissues and ANCTs	AW 264.7 and HL-1	↓ miR-370-3p, ↑ Irak2	_	Downregulation of NEAT1 ameliorated viability, prevented apoptosis and the expression of inflammatory cytokines.	(39)
NEAT1	↑	_	HL-1	↓ miR-144-3p	NF-κB signaling pathway	Downregulation of NEAT1 ameliorated viability, prevented apoptosis and inflammatory response in LPS-induced myocardial cell injury.	(40)
NEAT1	↑	152 patients with sepsis and 150	_	_	_	Up-regulation of NEAT1 was positively associated with Acute Physiology and Chronic Health Evaluation II score, inflammatory responses, while negatively associated with IL-10.	(41)
NEAT1	↑	C57BL/6 mice	WI-38	↓ miR-944, ↑ TRIM37	_	Downregulation of NEAT1 inhibited inflammatory responses and apoptosis. Overexpression of TRIM37 rescued influence of downregulation of NEAT1 on cell s.	(42)
NEAT1	↑	59 patients with sepsis, 52 patients with noninfectious SIRS, and 56 HCs	PBMCs	_	_	Levels of NEAT1 could be considered as a good predictor for the diagnosis of sepsis.	(43)
NEAT1	↑	127 patients with sepsis and 50 HCs	_	↑ Th1, ↑ Th17	_	Overexpression of NEAT1 was associated with chronic health evaluation II score, CRP level, acute physiology, and SOFA score.	(44)

(Continued)

TABLE 1 | Continued

lncRNA	Expression Pattern	Clinical Samples/ Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
NEAT1	↑	male C57BL/6 mice	RAW264.7	↓ miR495-3p, ↑STAT3, ↓ miR-211	↑ PI3K/ AKT signaling	Overexpression of NEAT1 was associated with inflammatory responses.	(45)
NEAT1	↑	102 patients with sepsis and 100 HCs	_	↓ miR-125a	_	High levels of NEAT1 was associated with SOFA score, APACHE II score, 28-day deaths, and high ARDS risk.	(46)
NEAT1	↑	Septic Mice	_	↑ NF-κB	_	Downregulation of NEAT1 increased activity of nerve cells and reduced apoptosis.	(47)
NEAT1	↑	82 patients with sepsis and 82 HCs	_	↓ miR-124	_	NEAT1 showed a good predictive value for increased sepsis risk. NEAT1 expression was positively associated with disease severity, CRP, PCT, TNF-α, and IL-1β, 28-day deaths.	(48)
NEAT1	↑	18 patients with sepsis-induced AKI and 18 HCs	HK-2	↓ miR-22-3p	↑ NF-κB pathway	Downregulation of NEAT1 reduced levels of autophagy factors and inflammatory responses.	(49)
NEAT1	↑	_	RAW264.7	↓ miR-31-5p, ↑ POU2F1	_	Downregulation of NEAT1 reduced inflammatory response and apoptosis, and increased proliferation.	(50)
NEAT1	↑	22 patients with sepsis and 22 HCs,	H9c2	↓ miR-590-3p	NF-κB signaling pathway	Downregulation of NEAT1 reduced apoptosis and inflammatory responses in LPS-induced sepsis.	(51)
H19	↓	69 patients with sepsis and HCs, male BALB/c mice	_	↑ miR-874, ↓ AQP1	_	Downregulation of H19 contributed to inflammatory responses. Up-regulation of H19 ameliorated the impairment of sepsis companied myocardial dysfunction.	(52)
H19	↓	_	H9C2	↑ miR-93-5p, ↓ SORBS2	_	Up-regulation of H19 suppressed inflammatory responses in sepsis-induced myocardial injury.	(53)
H19	↓	104 patients with sepsis, and 92 HCs	_	_	_	Expression of H19 was negatively associated with 28-day deaths and inflammatory response markers.	(54)
CASC9	↓	rats	HSAECs	↑ miR-195-5p, ↓ PDK4	_	Up-regulation of CASC9 promoted viability in sepsis-induced ALI.	(55)
LUADT1	↓	60 patients with sepsis and 60 HCs	HCAECs	miR-195, ↓ Pim-1	_	Up-regulation of LUADT1 reduced apoptosis.	(56)
MIAT	↑	male SD rats	NRK-52E	↓ miR-29a	_	Up-regulation of MIAT promoted apoptosis in sepsis-related kidney injury.	(57)
MIAT	↑	male BALB/c mice	HL-1	↓ miR-330-5p, ↑ TRAF6	↑ NF-κB signaling	Downregulation of MIAT restrained inflammation and oxidative stress in Sepsis-Induced Cardiac Injury.	(58)
THRIL	↑	66 patients with sepsis and 66 HCs	HBEpCs	↓ miR-19a, ↑ TNF-α	_	Up-regulation of THRIL promoted apoptosis.	(59)
THRIL	↑	C57BL/6 mice	MPVECs	↓ miR-424, ↑ ROCK2	_	Downregulation of THRIL prevented inflammatory responses, and apoptosis in septic-induced acute lung injury.	(60)
THRIL	↑ in ARDS group	32 sepsis patients with ARDS and 77 without ARDS	_	_	_	THRIL independently predicted increased risk of ARDS. THRIL was positively associated with APACHE II score, SOFA score, CRP, PCT, TNF-α, and IL-1β levels, and mortality rates.	(61)
XIST	↓	male SD rats	HSAECs, HEK-293T	miR-16-5p	_	Up-regulation of XIST increased viability and inhibited inflammatory response and apoptosis in sepsis-induced ALI.	(62)
XIST	↓	CLP-induced AKI mice	HK-2, TCMK-1	↑ miR-155-5p, ↓ WWC1	_	Up-regulation of XIST decreased sepsis-induced AKI.	(63)
XIST	↑	30 patients and 10 HCs, male SD rats	Kupffer	↑ BRD4	_	Downregulation of XIST reduced inflammation, oxidative stress, and apoptosis in sepsis-induced acute liver injury.	(64)

(Continued)

TABLE 1 | Continued

lncRNA	Expression Pattern	Clinical Samples/ Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
XIST	↑	GEO database: GSE94717 (6 patients with sepsis-induced AKI and 6 HCs)	MPC5	↓ miR-15a-5p, ↑ CUL3	_	Up-regulation of XIST enhanced apoptosis in sepsis-induced AKI.	(65)
xist	↑	_	MCM	↓ PGC-1α, ↓ Tfam	_	Downregulation of xist inhibited apoptosis and induced proliferation.	(66)
GAS5	↓	60 patients with sepsis and 60 HCs	AC16	↓ miR-214	_	Downregulation of GAS5 restrained apoptosis of cardiomyocytes induced by LPS. GAS5 could regulate miR-214 through methylation pathway.	(67)
CRNDE	↓	male specific-pathogen-free Wistar rats	_	↑ miR-29a, ↓ SIRT1	↑ NF-κB/ PARP1 signaling	Up-regulation of CRNDE reduced apoptosis, oxidative stress and inflammatory response.	(68)
CRNDE	↑	136 patients with sepsis and 151 HCs	THP-1	↓ miR-181a-5p, ↑ TLR4	_	Up-regulation of CRNDE was correlated with poorer OS and was a significant predictor in patients with sepsis. Downregulation of CRNDE reduced sepsis-related inflammatory pathogenesis.	(69)
CRNDE	↑	male C57 mice	_	↑ p65	↑ TLR3/ NF-κB pathway	Downregulation of CRNDE reduced edema, necrosis and apoptosis in sepsis-induced AKI.	(70)
CRNDE	↑	_	HK-2	↓ miR-146a	↑ TLR4/ NF-κB signaling pathway	Up-regulation of CRNDE enhanced cell injuries, inflammatory responses and apoptosis in sepsis-induced AKI.	(71)
CRNDE	↓	rats	HK-2, HEK293	↑ miR-181a-5p, ↓ PPARα	_	Downregulation of CRNDE increased the urea nitrogen and serum creatinine, and reduced proliferation and promoted apoptosis.	(72)
CRNDE	↓	male SD rats	L02	↑ miR-126-5p, ↓ BCL2L2	_	Up-regulation of CRNDE increased viability and repressed apoptosis in sepsis-induced liver injury.	(73)
HOTAIR	↓	male SD rats	HK-2	↑ miR-34a, ↓ Bcl-2	_	Up-regulation of HOTAIR reduced apoptosis in sepsis-induced AKI.	(74)
HULC	↑	110 patients with sepsis and 100 HCs	HMEC-1, CRL-3243	↓ miR-128-3p, ↑ RAC1	_	Downregulation of HULC restrained apoptosis and inflammation, and protected HMEC-1 cells from LPS-induced injury.	(75)
HULC	↑	174 patients with sepsis and 100 HCs	_	_	_	Expression of HULC was correlated with APACHE II, SOFA score, and 28-day deaths. It was also positively associated with Scr, WBC, and CRP, but negatively correlated with albumin.	(76)
HULC	↑	56 patients with sepsis and 56 HCs	HUVECs	↓ miR-204-5p, ↑ TRPM7	_	Downregulation of HULC promoted viability and reduced apoptosis, inflammatory responses and oxidative stress.	(77)
HULC	↑	C57BL/6 mice	HMECs	↑ IL6, ↑ ICAM1, ↑ VCAM1	_	Downregulation of HULC reduced levels of pro-inflammatory factors.	(78)
TapSAKI	↑	SD rats	HK-2	↓ miR-22	↑ TLR4/ NF-κB pathway	Downregulation of TapSAKI decreased inflammatory factors and renal function indicators, so decreased kidney injury.	(79)
ITSN1-2	↑	309 patients with intensive care unit (ICU)-treated sepsis and 300 HCs	_	_	_	High levels of ITSN1-2 were correlated with elevated disease severity, inflammation, and poor prognosis in sepsis patients.	(80)
LincRNA-p21	↑	sepsis-induced ALI rat model	BEAS-2B c	_	_	Downregulation of LincRNA-p21 restrained apoptosis, inflammatory responses and oxidative stress in sepsis-induced ALI.	(81)
TCONS_ 00016233	↑	15 patients with septic AKI and non-AKI, and 15 HCs, C57BL/6J mice	HK-2	miR-22-3p, ↑ AIFM1	TLR4/ p38MAPK axis.	Downregulation of TCONS_00016233 restrained LPS-induced apoptosis. Up-regulation of TCONS_00016233 induced LPS-induced apoptosis and inflammatory responses.	(82)
UCA1	↑	C57BL/6 mice	HMECs	↑ IL6, ↑ ICAM1, ↑ VCAM1	_	Downregulation of UCA1 reduced inflammatory responses.	(78)

(Continued)

TABLE 1 | Continued

lncRNA	Expression Pattern	Clinical Samples/ Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
NR024118	↓	82 patients with sepsis without MD, 35 patients with sepsis and MD and 82 HCs	AC16	↑ IL-6	NF-κB signaling pathway	Up-regulation of NR024118 reduced the secretion of IL-6 and apoptosis, and improved LPS-induced myocardial APD duration and cell injury.	(83)
MIR155HG	↑	28 patients with sepsis and 28 without sepsis	HL-1, RAW 264.7	↓ miR-194-5p, ↑ MEF2A	_	Downregulation of MIR155HG increased viability and decreased apoptosis and inflammatory responses.	(84)
LUCAT1	↑	GEO dataset: GSE101639	H9C2	↓ miR-642a, ↑ ROCK1	_	Downregulation of LUCAT1 decreased inflammatory responses.	(85)
SOX2OT	↑	male C57B6/L mice	H9c2	↑ SOX2	_	Downregulation of SOX2OT reduced mitochondrial dysfunction in septic cardiomyopathy. Overexpression of SOX2OT aggravated mitochondrial dysfunction in septic cardiomyopathy	(86)
MEG3	↑	male C57BL/6 mice	TECs	↓ miR-18a-3P	_	Downregulation of MEG3 reduced number of pyroptotic cells, secretion of LDH, IL-1β, and IL-18, and expression of GSDMD in LPS-induced AKI.	(87)
MEG3	↑	82 patients with sepsis and 54 HCs	Human primary renal mixed epithelial cells , AC16	_	_	Patients with high levels of MEG3 showed higher mortality rate, and downregulation of it inhibited apoptosis induced by LPS.	(88)
MEG3	↑	112 patients with sepsis and 100 HCs	_	_	_	High levels of MEG3 were associated with 28-day deaths and it was found to be a predictor of higher ARDS risk.	(89)
MEG3	↑	219 patients with sepsis and 219 HCs, male C57BL/6 J mice	_	↓ miR-21	_	Lnc-MEG3 expression was positively correlated with cardiomyopathy, APACHE II score, SOFA score, Scr, TNF-α, IL-1β, IL-6, and IL-17, 28-day deaths, while negatively correlated with albumin.	(90)
MEG3	↓	male C57/BL mice	Caco2	↑ miR-129-5p, ↓ SP-D	_	Overexpression of MEG3 reduced villus length and apoptosis, inhibited intestinal injury and enhanced proliferation.	(91)
GAS5	↓	_	conditional immortalized podocyte line	↓ PTEN	↑ PI3K/ AKT pathway	Downregulation of GAS5 elevated the Podocyte Injury.	(92)
LINC00472	↑	male SD rats	THLE-3	↓ miR-373-3p, ↑ TRIM8	_	Downregulation of LINC00472 enhanced viability and suppressed apoptosis.	(93)
HOTAIR	↑	male e C57B6/L mice	HL-1	↑ p-p65, ↑ NF-κB	NF-κB pathway	Downregulation of HOTAIR restrained LPS-induced myocardial dysfunction in septic mic. HOTAIR was involved in p65 phosphorylation and NF-κB activation, leading to 15 TNF-α production.	(94)
HOTAIR	↑	male SD rats	HK-2	↓ miR-22, ↑ HMGB1	_	Downregulation of HOTAIR reduced renal function indicators (blood urea nitrogen and serum creatinine).	(95)
Hotairm1	↑	male C57BL/6 mice	MDSCs	↑ S100A9 localization	_	Downregulation of Hotairm1 restrained the suppressive functions of late sepsis Gr1+CD11b+ MDSCs. Hotairm1 Was involved in shuttling S100A9 protein to the nucleus.	(96)
NKILA	↑	_	HK2	↓ miR-140-5p, ↑ CLDN2	_	Downregulation of NKILA restrained apoptosis, autophagy and inflammation and promoted viability in sepsis-induced AKI.	(97)
HOXA-AS2	↓	44 patients with sepsis and 44 HCs, adults clean Kunming mice	HK-2	↑ miR-106b-5p	↑ Wnt/β-catenin and NF-κB pathways	Up-regulation of HOXA-AS2 increased viability and repressed apoptosis and protect cells to resist LPS-induced damage in sepsis-induced AKI.	(98)
SNHG14	↑	_	HK-2	miR-93, ↑IL-6R, ↑IRAK4	TLR4/NF-κB pathway, ↑ NF-κB	Up-regulation of SNHG14 promoted oxidative stress, inflammation, and apoptosis. TLR4/NF-κB pathway induced upregulation of SNHG14.	(99)

(Continued)

TABLE 1 | Continued

lncRNA	Expression Pattern	Clinical Samples/ Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
					and STAT3 signaling		
lncRNA-CCL2	↑	male C57BL/6 mice	_	↓ SIRT1	_	Expression of lncRNA-CCL2 was inhibited by SIRT1 through maintaining a more repressive chromatin state in lncRNA-CCL2 locus. Downregulation of SIRT1 induced inflammatory response.	(100)
DLX6-AS1	↑	patients with septic AKI	HK-2	↓ miR-223-3p, ↑ NLRP3	_	Downregulation of DLX6-AS1 suppressed LPS-induced cytotoxicity and pyroptosis. Expression of DLX6-AS1 was positively correlated with levels of creatinine in the serum of patients.	(101)
CASC2	↓	_ patients with sepsis and HCs	HK-2	↑ miR-155	↑ NF-κB signaling pathway	The levels of CASC2 were negatively correlated with the severity of AKI. CASC2 expression induced cell viability and inhibited inflammatory response, apoptosis and oxidative stress.	(102)
CASC2	↓	patients with sepsis and HCs	HPAEpiC	↑ miR-152-3p, ↓ PDK4	_	Up-regulation of CASC2 increased viability and restrained apoptosis, inflammatory and oxidative damages.	(103)
ZFAS1	↓	202 patients with sepsis and 200 HCs	_	_	_	Expression of ZFAS1 was negatively associated with APACHE II, level of CRP, TNF-α, IL-6 and positively with IL-10.	(104)
ZFAS1	↓	male SD rats	H9C2	↑ miR-34b-5p, ↓ SIRT1	_	Up-regulation of ZFAS1 decreased inflammatory responses and apoptosis.	(105)
ZFAS1	↑	male C57BL/6 mice	_	↓ miR-590-3p, SP1	AMPK/ mTOR signaling	Downregulation of ZFAS1 reduced LPS-induced pyroptosis and enhanced LPS-suppressed autophagy in sepsis-induced cardiac dysfunction.	(106)
ZFAS1	↓	22 patients with SIMI and 24 HCs, rats treated by LPS	H9C2	↑ miR-138–5p, ↓ SESN2	_	Up-regulation of ZFAS1 attenuated myocardial injury and inflammatory response.	(107)
Mirt2	↓	male SD rats	_	↑ MiR-101	↓ PI3K/ AKT Signaling Pathway	Up-regulation of Mirt2 inhibited inflammatory responses and improved cardiac function.	(108)
Mirt2	↓	40 patients with sepsis, 40 patients with sepsis-ALI, 40 HCs	HBEpCs	↓ miR-1246	_	Up-regulation of Mirt2 inhibited LPS-induced inflammatory response, apoptosis, and promoted miR-1246 expression but reduced its gene methylation.	(109)
TCONS_00016406	↓	male C57BL/6 mice	PTEC	↑ miR-687, ↓ PTEN	_	Up-regulation of lncRNA 6406 inhibited inflammatory responses, apoptosis and oxidative stress in LPS-induced AKI.	(110)
NORAD	↑ in NS patients	88 patients with late-onset NS and 86 patients with pneumonia neonates	RAW264.7	↓ miR-410-3p	_	Expression of NORAD was closely correlated with WBC, PCT, IL-6, IL-8, and TNF-α.	(111)
GAS5	↑	_	THP-1	↓ miR-23a-3p, ↑ TLR4	_	Downregulation of GAS5 inhibited inflammation and apoptosis.	(112)
lnc-ANRIL	↑	126 patients with sepsis and 125 HCs	_	↓ miR-125a	_	lnc-ANRIL showed good predictive values for sepsis risk. lnc-ANRIL was positively associated with CRP and PCT levels, disease severity scale scores, and pro-inflammatory cytokine levels, 28-day deaths in sepsis patients,	(113)
PVT1	↑	109 patients with sepsis and 100 HCs	_	_	_	PVT1 was found to be an independent risk factor for sepsis ARDS. And PVT1 expression positively associated with disease severity and 28-day deaths.	(114)
PVT1	↑	_	THP-1	_	↑ p38 MAPK signaling pathway	Downregulation of PVT1 reduced levels of IL-1β and TNF-α mRNA and inhibited the p38 MAPK signaling pathway,	(115)

(Continued)

TABLE 1 | Continued

lncRNA	Expression Pattern	Clinical Samples/ Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
PVT1	↑	sepsis model mice	HK-2	↓ miR-20a-5p, ↑ NLRP3	_	Downregulation of PVT1 inhibited pyroptosis in septic AKI.	(116)
PVT1	↑	Mice model with sepsis	_	↓ miR-29a, ↑ HMGB1	_	Downregulation of PVT1 reduced LPS-induced myocardial injury and alleviated M1 macrophage polarization.	(117)
HOTAIR	↑	C57BL/6 mice	Monocytes	↓ miR-211	_	Overexpression of HOTAIR suppressed proliferation and promoted apoptosis.	(118)
HOTAIR	↑	LPS-induced septic cardiomyopathy mice	H9C2	↑ PDCD4, Lin28	_	Downregulation of HOTAIR inhibited inflammatory responses and apoptosis.	(119)
DILC	↓	18 patients with sepsis and 18 HCs	PBMCs, THP-1	↑ IL-6	_	DILC suppressed the transcription of IL-6, DILC decreased levels of STAT3, p-STAT3, TLR4, TNF-α, CCL5, E-selectin and CXCR1.	(120)
RMRP	↑	C57BL/6 mice	HK-2	↓ miR-206, ↑ DDX5	_	Downregulation of RMRP inhibited inflammatory response and apoptosis in sepsis-induced AKI.	(121)
GAS5	↑	C57BL/6 mice	_	↓ miR-449b, ↑ HMGB1	↑ HMGB1/ NF-κB pathway	Downregulation of GAS5 inhibited pro-inflammatory reaction and alleviated myocardial injury.	(122)
TapSAKI	↑	_	HK-2	↓ miR-205, ↑ IRF3	_	Downregulation of TapSAKI alleviated LPS-induced damage.	(123)
SNHG16	↑	male SD rats	BEAS-2B	↓ miR-128-3p, ↑ HMGB3	_	Downregulation of SNHG16 reduced the apoptosis and inflammation in sepsis-induced ALI.	(124)
DANCR	↓	20 patients with sepsis-induced AKI and 20 HCs	HK-2	↑ miR-214, ↑ KLF6	_	Up-regulation of DANCR promoted viability and suppressed cell apoptosis and inflammatory responses.	(125)
CASC2	↓	_	HK2, HEK293	↑ miR-545-3p to regulate, ↓ PPARA	_	Up-regulation of CASC2 increased viability and inhibited apoptosis, migration, epithelial-mesenchymal transition and oxidative stress.	(126)
SNHG1	↓	_	H9c2	↑ miR-181a-5p, ↓ XIAP	_	Up-regulation of SNHG1 increased viability and inhibited inflammatory responses and oxidative stress.	(127)
SNHG14	↑	_patients with sepsis	HK-2	↓ miR-495-3p, ↑ HIPK1	_	SNHG14 is upregulated in patients. SNHG14 prevented proliferation and autophagy and boosted apoptosis and inflammatory responses.	(128)
Linc-KIAA1737–2	↑	_	HK-2	↓ MiR-27a-3p	_	Downregulation of Linc-KIAA1737–2 reduced apoptosis.	(129)
PlncRNA-1	↓	6 patients with septic AKI and 6 HCs	NRK-52E	↓ BCL2	_	Up-regulation of PlncRNA-1 meliorated proliferation and prevented apoptosis and autophagy.	(130)
CDKN2B-AS1	↑	sepsis patients 47 and 55 HCs	BEAS-2B	↓ miR-140-5p , ↑ TGFBR2	↑ TGFBR2/ smad3 pathway	Downregulation of CDKN2B-AS1 promoted viability reduced apoptosis and inflammation.	(131)

ARDS, acute respiratory distress syndrome; HCs, healthy controls; ALI, acute lung injury; LPS, lipopolysaccharide; SD, Sprague–Dawley; AKI, acute kidney injury; SOFA, sequential organ failure assessment; Scr, serum creatinine; WBC, white blood cell; CRP, C-reactive protein; PBMCs, peripheral blood mononuclear cells; PCT, procalcitonin; APACHE, physiology and chronic health evaluation; MPO, Myeloperoxidase; NS, Neonatal sepsis; SIMI, sepsis-induced myocardial injury.

NF-κB, PI3K/AKT, JAK/STAT and Wnt/β-catenin pathways are the most important pathways being regulated by lncRNAs, circRNAs and miRNAs in the context of sepsis. These transcripts, particularly miRNAs can be used as diagnostic or prognostic markers in sepsis. Expression levels of these regulatory transcripts might be used for diagnosis of organ specific damages during the course of sepsis.

In general, the pathophysiology of sepsis is considered as an initial hyperinflammatory phase ("cytokine storm") followed by a protracted immunosuppressive phase. Since no data is available about the differential expression of non-coding RNAs during these two distinct phases, future studies are needed to evaluate expression patterns of non-coding RNAs in these two phases. It is possible that some of the non-coding RNAs that suppress the immune response could be used as biomarkers to indicate the immunoparalysis in sepsis.

From a therapeutic point of view, several *in vitro* and *in vivo* studies have shown that up-regulation/silencing of circRNAs,

TABLE 2 | Lists the function of miRNAs in the course of sepsis.

miRNA	Pattern of Expression	Clinical Samples/Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
miR-15a-5p	↑	GEO database: GSE94717 (6 patients with sepsis-induced AKI and 6 HCs)	MPC5	↓ XIST, ↓ CUL3	_	Downregulation of miR-15a-5p reduced apoptosis in sepsis-induced AKI.	(65)
miR-494-3p	↓	_Patients with sepsis and HCs	RAW264.7	↑ TLR6	_	Upregulation of microRNA-494-3p reduced inflammation, TNF-α level, and prevented nuclear translocation of NF-κB p65.	(132)
miR-218	↓	53 Patients with sepsis and 20 HCs, septic mouse model	PBMCs	↑ VOPP1	↑ JAK/STAT pathway	Upregulation of microRNA-494-3p reduced inflammation.	(133)
miR-218	↓	male S SD rats	RAW264.7	↑ RUNX2		Up-regulation of miR-218 inhibited inflammatory response.	(141)
miR-122	↑	25 patients with sepsis and 25 patients with local wound infections as a control group	_	_	_	miR-122 showed higher AUC in comparison with CRP and TLC which had 66.6% sensitivity, 50% specificity, and 56.0% accuracy as a prognostic biomarker for sepsis.	(134)
miR-208a-5p	↑	septic mouse model	_	↓ SOCS2	↑NF-κB/ HIF-1α pathway	Downregulation of miR-208a-5p decreased reduced degree of mitochondria swelling, and inhibited apoptosis.	(135)
miR-328	↑	110 Patients with sepsis and 89 HCs, male SD rats	_	_	_	miR-328 expression was positively associated with Scr, WBC, CRP, PTC, APACHE II score, and SOFA score. miR-328 was found to be a good diagnostic value for sepsis. Downregulation of miR-328 reduced inflammatory response.	(139)
miR-452	↑	47 sepsis patients with AKI, 50 patients without AKI, and 10 HCs	BUMPT	NF-KB	_	Serum and urinary miR-452 could be a potential biomarker for early detection of septic AKI. It was upregulated in sepsis patients with AKI compared with without AKI. miR-452 had high diagnostic value for AKI.	(140)
miR-21	↓	219 Patients with sepsis and 219 HCs	_	_	_	miR-21 was found to be a good value in predicting sepsis risk. miR-21 expression was negatively correlated with APACHE II, SOFA score, and 28-day mortality risk.	(142)
miR-126	↑	208 Patients with sepsis and 210 HCs	_	_	_	miR-126 expression was positively correlated with APACHE II, serum creatinine, CRP, TNF-α, IL-6, IL-8, mortality rate, but negatively with IL-10.	(143)
mir-103	↓	196 Patients with sepsis and 196 HCs	_	_	_	mir-103 predicted high ARDS risk. Mir-103 and was negatively associated with APACHE II score, SOFA score, serum creatinine, CRP, TNF, IL- 1β, IL-6, IL-8, 28-day deaths, but positively correlated with albumin.	(144)
mir-107	↓	196 Patients with sepsis and 196 HCs	_	_	_	mir-107 predicted high ARDS risk. mir-107 and was negatively associated with APACHE II score, SOFA score, serum creatinine, CRP, TNF, IL- 1β, IL-6, IL-8, 28-day deaths, but positively correlated with albumin	
miR-92a	↑ in sepsis-induced ARDS	53 sepsis patients (36 patients with sepsis-induced ARDS)	HPMEC, A549	_	↓ Akt/mTOR signaling pathway	Downregulation of mir-92a reduced apoptosis and inflammatory response, and enhanced migration	(145)
miR-98	↓	male C57BL/6 mice	_	↑ HMGA2	↑ NF-κB pathway	Upregulation of miR-98 prevented HMGA2, NF-κB, TNF-α, IL-6, Bcl-2 and augmented IL-10, Cleaved caspase-3 and Bax expression, it reduced LVEDP, CTn-I, BNP, ALT, AST, TBIL, LDH, and PaCO2 but elevated +dp/dt max, -dp/dt max, pH and PaO2.	(146)
miR-125a	↑	150 Patients with sepsis and 150 HCs	_	_	_	miR-125a expression was positively associated with Scr, APACHE II score, SOFA score.	(147)
miR-125b	↑	150 Patients with sepsis and 150 HCs	_	_	_	miR-125b was correlated with Scr, CRP, APACHE II score, SOFA score, and chronic obstructive pulmonary disease , and 28-day deaths.	
miR-199a	↑	male C57BL/6 mice	_	↓ SIRT1	_	Downregulation of miR-199a reduced apoptosis and inflammatory response.	(148)
miR-495	↓	105 Patients with sepsis and 100 HCs, rats	_	_	_	miR-495 was negatively correlated with Scr, WBC, CRP, PCT, APACHE II score and SOFA score. CLP rats showed worse LVSP, LVEDP, ± dp/dtmax, and exhibited an increase in serum CTn-I, CK-MB, TNF-α, IL-6 and IL-1β.	(149)
miR-106a	↑	50 patients with sepsis and 30 HCs, clean Kunming mice	TCMK-1	↓ THBS2	_	Downregulation of miR-106a reduced apoptosis and inflammatory response.	(150)

(Continued)

TABLE 2 | Continued

miRNA	Pattern of Expression	Clinical Samples/Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
miR-146a	_	male C57BL/6 mice	MSCs	IL-1β	–	IL-1β stimulation resulted in packaging miR-146a into exosomes. The exosomal miR-146a was transferred to macrophages, yielded to M2 polarization, and finally led to high survival in septic mice.	(151)
miR-574	↓	CLP-treated mice	HBE	↑ C3	_	Upregulation of mir-574 increased viability, inhibited apoptosis, and reduced sepsis-induced ERS.	(152)
miR-195	_	wistar rats with sepsis		_	TGF-β1/ Smads signaling pathway,	MicroRNA-195 could promote cardiac remodeling by up-regulating the nanoantibiotics signaling pathway in sepsis rats.	(153)
miR-133a	↑	septic mouse model	RAW264.7	↓ SIRT1	_	Downregulation of miR-133a prevented inflammatory response, sepsis-induced lung, liver and kidney injuries.	(154)
miR-191-5p	↓	female Wistar rats	_	↑ OXSR1	↑ p38 MAPK/NF-κB signaling pathway	Upregulation of miR-191-5p prevented inflammatory response and apoptosis in	(155)
miR-146a	↑	180 patients with sepsis and 180 HCs	_	_	_	MiR-146a was of good value in predicting high sepsis risk and 28-day mortality risk. MiR-146a was positively associated with biochemical indices, inflammatory cytokines, overall disease severity.	(156)
miR-146b	↑	180 patients with sepsis and 180 HCs	_	_	_	miR-146b was of good value in predicting high sepsis risk and 28-day mortality risk. MiR-146a was positively associated with biochemical indices, inflammatory cytokines, and overall disease severity. .	
miR-126	↓	20 patients with sepsis and 30 patients with general infection	_	_	_	miR-126 was negatively associated with the levels of caspase-3, APACHE II score, and positively with 28-day cumulative survival rate. AUC for predicting the prognosis by miR-126 was 0.823.	(157)
miR-223	_	C57BL/6 mice	RAW264.7	_	_	Upregulation of mir-223 impelled M2 macrophage through lower activity of glycolysis Pathway. the Implementation of miR-223 over-expressed macrophages with IL-4 pre-conditioning alleviated sepsis severity.	(158)
miR-146b	↓	septic mouse model	HK-2	↑ IRAK1	↑ NF-κB pathway	Treatment with hucMSC-Ex improved survival in mice with sepsis by reducing levels of IRAK1, increasing of miR-146b level, and inhibition of NF-κB activity.	(159)
miR-1-3p	↑	male SD rats	HUVECs	↓ SERP1	_	miR-1-3p decreased proliferation, and increased apoptosis, and permeability and HUVECs membrane injury.	(160)
miR-25	↓	70 patients with sepsis and 30 patients with SIRS	_	_	_	Levels of miR-25 was negatively associated with the severity of sepsis, SOFA score, CRP and PCT level, 28-day deaths, and levels of oxidative stress indicators.	(161)
miR-370-3p	↑ in SAE	12 patients with sepsis without encephalopathy, 17 patients with SAE, 20 patients with severe uremia and 12 HCs , male C57BL/6 mice	_	_	_	miR-370-3p was associated with TNF-α and increased brain apoptosis in SAE mice.	(162)
miR-21	↑	GEO database: GSE26440 (88 children with septic shock and 26 HCs), C57BL/6 mice	_	↓ A20, ↑ NLRP3	↑ NF-κB pathway	Downregulation of miR-21 inhibited inflammasome activation, ASC pyroptosome, LPS-induced pyroptosis and septic shock.	(136)
miR-21	↓	CLP mouse model	_	↑ PDCD4, ↑ PTEN	PDCD4/NF-κB and PTEN/AKT pathways	rIPC protected kidneys from injury by miR-21. miR-21 was transported from ischemic limbs to the kidneys by exosomes.	(163)
miR-21	↓	septic mouse model	MTEC	↑ PDCD4	↑ NF-κB pathway	Upregulation of miR-21 reduced inflammation and apoptosis.	(137)
miR-21	_	septic mice	_	_	_	Hyperoside decreased miR-21 levels so reduced inflammatory responses and increased viability.	(164)
miR-21	↓	_	MSCs	↑ PDCD4	_	βMSCs-derived exosomes reduced symptoms in septic mice and improved their survival rate through miR-21 upregulation.	(138)

(Continued)

TABLE 2 | Continued

miRNA	Pattern of Expression	Clinical Samples/Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
miR-21	↑	septic C57BL/6J mice	_	↓ PGE2, ↓ IL-10	_	Downregulation of miR-21 reduced bacterial growth, systemic inflammation, organ damage, macrophage glycolysis, and increased animal survival.	(165)
miR-21-3p	↑	SD rats	TECs	↓ AKT, ↓ CDK2, ↑ FOXO1	–	miR-21-3p regulated lipid metabolism and increased cell cycle arrest and apoptosis.	(166)
miR-34	↑	male C57BL/6 mice (15 control group and 15 sepsis model group)	_	↓ KLF4	_	Plasma miR-34a was positively associated with SCr and BUN.	(167)
miR-483-5p	↑	CLP-treated mice	PMVECs	↓ PIAS1	_	Downregulation of miR-483-5p reduced inflammation and apoptosis and improved lung injury in mice with sepsis-induced ALI.	(168)
miR-181-5p	↓	CLP- treated mice	_	↑ HMGB1	_	Upregulation of miR-181-5p reduced inflammatory response, and sepsis-induced renal and hepatic dysfunction.	(169)
miR-20a	_	SD rats	_	_	_	miR-20a could deteriorated AKI via activating autophagy in sepsis rats.	(170)
hsa-miR-92a-3p	↓ in sepsis-induced coagulopathy group	116 patients with sepsis	_	_	_	AUC of hsa-mir-92a-3p was 0.660. Levels of plasma hsa-mir-92a-3p were related to plasma lipocalin-2 level, activated partial thromboplastin time, and prothrombin activity.	(171)
miR-93-5p	↓	septic mouse model	HK2	↑ KDM6B, ↓ H3K27me3	_	Extracellular vesicles containing miR-93-5p reduced inflammation, apoptosis, multiple organ injury, and vascular leakage in septic mice.	(172)
miR-223	↓	143 patients with sepsis and 44 HCs	_	_	_	Expression of miR-223 was negatively correlated with SOFA scores and positively with survival rate. Upregulation of miR-223 decreased apoptosis and increased proliferation and G1/S transition.	(173)
miR-34a	↑	male C57BL/6 mice	_	↓ SIRT1, ↓ ATG4B	_	Downregulation of miR-34a reduced inflammatory response and pyroptosis, apoptosis and enhanced autophagy.	(174)
miR-30a	↑	septic rats	_	↓ SOCS-1	↑ JAK/STAT signaling pathway	Upregulation of miR-30a promoted apoptosis and inhibited proliferation.	(175)
miR-150-5p	↓	rat septic shock model	H9C2	↑ Akt2	_	Upregulation of miR-150-5p inhibited apoptosis.	(176)
miR-140	↓	SPF male BALB/c mice	_	_	↑ WNT signaling pathway	Upregulation of miR-140 inhibited apoptosis and inflammation, skeletal muscle glycolysis and atrophy.	(177)
miR-22-3p	↓	male SD rats	HK-2	↑ HMGB1, ↑ PTEN	_	Upregulation of miR-22-3p inhibited apoptosis and inflammatory response	(178)
miR-205-5b	↑	BALB/c mice	RAW264.7	HMGB1	_	Down regulation of miR-205-5b increased HMGB1 expression in LPS-induced sepsis.	(179)
miR-526b	↓	BALB/c mice	HK2	↑ ATG7	_	Upregulation of miR-526b increased viability by inhibiting autophagy.	(180)
miR-145a	↓	septic mouse model	_	↑ Fli-1	↑ NF-κB signaling	Upregulation of miR-526b reduced levels of proinflammatory cytokines.	(181)
miR-125a	↑	150 patients with sepsis and 150 HCs	_	_	_	AUC of miR-125a: 0.749 miR-125a was positively correlated with APACHE II score and SOFA score.	(182)
miR-125b	↑	150 patients with sepsis and 150 HCs	_	_	_	AUC of miR-125b: 0.839 miR-125b was positively correlated with APACHE II score, SOFA score CRP, TNF-α, IL-6, IL-17, IL-23, and 28-day mortality risk.	
miR-122	↑	108 patients with sepsis and 20 patients with infections without sepsis as controls	_	_	_	AUC of miR-122: 0.760 miR-122 was found as independent prognostic factor for 30-day mortality.	(183)
miR-135a	↑	_patients with sepsis and HCs, BALB/c mice	_	_	↑ p38 MAPK/NF-κB pathway	Upregulation of miR-135a exacerbated inflammation and myocardial dysfunction.	(184)
miR-133a	↓	_	TCMK-1	↑ BNIP3L	↑ NF-κB pathway	Upregulation of miR-133a reduced inflammation and apoptosis.	(185)

(Continued)

TABLE 2 | Continued

miRNA	Pattern of Expression	Clinical Samples/Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
miR-223	_	male C57BL/6 mice	_	_	_	In multiple models of experimental sepsis, miR-223 showed the complex role in the pathogenesis of septic kidney injury.	(186)
miR-155	↑	44 patients with severe sepsis, 102 patients with sepsis, and 19 HCs	↑	↑	↑	AUC of miR-155: 0.782 (for predicting 30-day mortality in ALI)	(187)
miR-146a	↑	44 patients with severe sepsis, 102 patients with sepsis, and 19 HCs	↑	↑	↑	AUC of miR-146a: 0.733 (for predicting 30-day mortality in ALI), CC genotype of rs2910164 in miR-146a was correlated with worse treatment result.	
miR-194	↑	_	H9c2	↓ Slc7a5	↑ Wnt/β-catenin pathway	Upregulation of miR-194 increased apoptosis.	(188)
miR-30a	↑	male C57BL/6 mice	RAW 264.7	↓ ADAR1, ↓ SOCS3	_	Upregulation of ADAR1 (a target of miR-30a) reduced inflammation and organ damage.	(189)
miR-27b	↓	male C57BL/6 mice	BMMSCs	↑ JMJD3	↑ NF-κB signaling pathway	Upregulation of miR-27b MSC-derived exosomes reduced pro-inflammatory cytokines.	(190)
miR-155	↑	BALB/c mice	_	↓ SOCS1	↑ JAK/STAT signaling	Downregulation of miR-155 alleviated LPS-induced mortality and liver injury	(191)
miR-155	↓	C57BL/6 mice	_	↑ Arrb2	↑ JNK signaling pathway	Upregulation of miR-155 ameliorated late sepsis survival and its cardiac dysfunction, and reduced pro-inflammatory responses.	(192)
miR-155	↑	_patients with sepsis and HCs, mouse septic shock model	_	↓ CD47	_	Downregulation of microRNA-155 reduced sepsis-associated cardiovascular dysfunction and mortality.	(193)
miR-155	↑	60 patients with sepsis and 20 HCs	_	↑ Foxp3	_	Expression of miR-155 was correlated with APACHEII score, it was significantly higher in non-survival group.	(194)
miR-155	↑ in sepsis and ALI/ARDS than sepsis but no ALI/ARDS	156 patients with sepsis (41 with ALI and 32 with ARDS)	_	_	_	AUC of miR-155: 0.87, miR-155 was positively associated with IL-1β, TNF-α levels, and ALI/ARDS score, but negatively with PaO2/FiO2.	(195)
miR-29c-3p	↑	86 patients with sepsis and 85 HCs, male SD rats	_	_	_	AUC of miR-29c-3p: 0.872 miR-29c-3p expression was positively correlated with APACHE II score, SOFA score, levels of CRP and PCT. miR-29c-3p was found to be an independent factor in the occurrence of cardiac dysfunction.	(196)
miR-125b	↓	40 patients with sepsis and HCs, female and male C57BL/6 mice	_	↓ PTEN, ↑ MyD88	_	PTEN increased miR125 production through associating with the nuclear localization of Drosha-Dgcr8. Downregulation of PTEN resulted in cytokine production, MyD88 abundance and mortality.	(197)
miR-203b	↓	40 patients with sepsis and HCs, female and male C57BL/6 mice	_	↓ PTEN, ↑ MyD88	_	PTEN increased miR203b production through associating with the nuclear localization of Drosha-Dgcr8. Downregulation of PTEN resulted in cytokine production, MyD88 abundance and mortality.	
miR-146	↓	_	EA. hy926	_	↑ NF-κB signaling pathway	Upregulation of reduced levels inflammatory cytokines.	(198)
miR-140-5p	↓	male SPF rats	MLE-12	↑ TLR4, ↑ MyD88	↑ NF-κB signaling pathway	Shikonin could alleviated sepsis- induced ALI by increasing the levels of miRA-140-5p and decreasing the levels of TLR4.	(199)
miR-125b	↓	male C57BL/6 mice	HUVECs	↑ ICAM-1, ↑ VCAM-1, ↑ TRAF6	↑ NF-κB signaling pathway	Upregulation of miR-125b alleviated sepsis-induced cardiac dysfunction and ameliorated survival.	(200)
miR-494	↑	ARDS rat models	_	_	↓ Nrf2 signaling pathway	Upregulation of miR-494 increased inflammatory response, oxidative stress and ALI.	(201)

(Continued)

TABLE 2 | Continued

miRNA	Pattern of Expression	Clinical Samples/Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
miR-146a	↓	male C57BL/6 mice	H9C2, J774	↑ IRAK, ↑ TRAF6	↑ NF-κB signaling pathway	Upregulation of miR-146 reduced levels of inflammatory cytokines and sepsis-induced cardiac dysfunction	(202)
miR-223	_	221 patients with sepsis and 75 HCs, male C57Bl/6 mice	_	_	_	Levels of serum miR-223 did not differ between critically ill patients and HCs, but ICU patients with APACHE-II score had moderately decreased circulating miR-223.	(203)
miR-300	↓	septic mouse model	_	↑ NAMPT	↓ AMPK/ mTOR signaling pathway	Upregulation of miR-300 increased autophagy, cell cycle entry and reduced apoptosis and inflammatory response.	(204)
miR-126	↓	male C57BL/6 mice	_	↓ HSPA12B	_	Upregulation of HSPA12B increased levels of miR-126, upregulation of miR-126 reduced levels of dhesion molecules and improved sepsis–induced cardiac dysfunction.	(205)
miR-10a	↓	62 patients with sepsis and 20 HCs	_	↑ MAP3K7	↑ NF-κB pathway	miR-10a expression was negatively association with disease severity scores, levels of c-reactive protein, procalcitonin, and 28-day death.	(206)
miR-146a	↓	mice	_	↑ Notch1	↑ NF-κB signaling	Upregulation of miR-146a reduced inflammatory responses of macrophages and protected mice from organ damage	(207)
miR-19a	↓	CLP mice	RAW 264.7	↑ Fn14	_	Upregulation of miR-19a reduced LPS-Induced Tubular Damage, it was found to protected mice from sepsis-induced AKI.	(208)
miR-214	_	male Kunming mice	_	_	_	Upregulation of miR-214 reduced apoptosis, inflammatory response, myocardial injury, and improved cardiac function in SIMI.	(209)
miR-539-5p	↓	male C57BL/6 mice	MPVECs	↑ ROCK1	_	Upregulation of miR-539-5p reduced apoptosis, inflammatory response, sepsis-induced pulmonary injury.	(210)
miR-155	↑	60 patients with sepsis and 30 HCs	_	_	_	miR-155 was positively correlated with a higher SOFA score and a greater severity. AUC of miR-155 for 28-day survival was 0.763. miR-155 derived immunosuppression through CD39(+) Tregs.	(211)
miR-146a	↑ in sepsis group compared to shame group	male BALB/C mice	_	_	_	Up-regulation of miR-146a reduced levels of inflammatory cytokine TNF-α and mitigated inflammatory reaction and lung tissue injury in sepsis-induced ALI.	(212)
miR-7110-5p	↑	52 patients with pneumonia, 44 patients with sepsis and 21 HCs	_	_	_	The sensitivity and specificity of miR-7110-5p were 84.2 and 90.5% respectively. (sepsis vs HCs)	(213)
miR-223-3p	↑	52 patients with pneumonia, 44 patients with sepsis and 21 HCs	_	_	_	The sensitivity and specificity of miR-223-3p were 82.9 and 100% respectively. (sepsis vs HCs)	
miR-19a	↑	patients with sepsis	B cells from patients with sepsis	CD22	_	Expression of CD22 initially increased but subsequently reduced. Upregulation of miR-19a resulted in an increased BCR signaling, while overexpression of CD22 reduced the effect of miR-19a and promoted its expression.	(214)
miR-206	↑	63 patients with sepsis, 30 patients with septic shock and HCs	_	_	_	miR-206 was positively associated with SOFA sore and APACHE-II score. It was observed an activated partial thromboplastin time and notably longer prothrombin time.	(215)
miR-146a	↓	male C57BL/6 mice	RAW264.7	_	↑ NF-κB signaling	Up-regulation of miR-146a reduced apoptosis, inflammatory response, and weakened organ injury in splenic macrophages.	(216)
miR-19b-3p	↓	103 patients with sepsis and 98 HCs	HUVECs	_	_	Up-regulation of miR-19b-3p reduced inflammatory response. miR-19b-3p was found to be an independent prognostic factor for 28-day survival.	(217)
miR-129-5p	↓	CLP mice	MLE-12	↑ HMGB1	_	Up-regulation of miR-129-5p reduced apoptosis, inflammatory response, , lung wet/dry weight ratio, and myeloperoxidase activity.	(218)

(Continued)

TABLE 2 | Continued

miRNA	Pattern of Expression	Clinical Samples/Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
miR-23b	↓	30 patients with sepsis and 30 HCs	THP-1	↑ ADAM10	_	Up-regulation of miR-23b reduced apoptosis and inflammatory response.	(219)
miR-150	↓	140 patients multiple trauma and 10 HCs	MDSCs	↑ ARG1	_	Up-regulation of miR-150 reduced IL-6, TGF-β and IL-10.	(220)
miR-375	↓	_ patients with sepsis, septic mice	MDSCs	↑ miR-21	↑ JAK2/ STAT3 pathway	Up-regulation of miR-375 reduced the number of sepsis Gr1+CD11b+ MDSCs in mice.	(221)
miR-31	↑	male SD rats	CACO-2	↓ HMOX1	↑ NF-κB/ HIF-1α pathway	Downregulation of miR-31 reduced intestinal barrier function, intestinal mucosal permeability, oxidative damage and inflammation level.	(222)
miR-21 and miR-181b	↑ (in early sepsis) sustained (in late sepsis)	male BALB/c mice	MDSCs	↑ NFI-A	_	Down regulation of miR-21 and miR-181b decreased, immunosuppression, reprograming myeloid cells, late-sepsis mortality, and improved bacterial clearance.	(223)
miR-150	↓ slightly	223 critically ill patients (including 138 fulfilled sepsis criteria) and 76 HCs	_	_	_	serum levels of miR-150 were associated with hepatic or renal dysfunction. Low levels were correlated with an unfavorable prognosis of patients. serum levels of miR-150 were not suitable for predicting of sepsis.	(224)
miR-10a	↑	SD rats	_	_	↑ TGF-β1/ Smad pathway	Up-regulation of miR-10a increased ROS, TNF-α, IL-6, and MPO, and downregulation reduced sepsis-induced liver injury.	(225)
miR-145	↓	septic mice	HUVECs	↑ TGFBR2, ↑ SMAD2, ↑ DNMT1	_	Up-regulation of miR-145 reduced LPS-induced sepsis and improved the overall survival of septic mice.	(226)
miR-150	↓	17 patients with sepsis and 32 HCs	_	_	_	Levels of miR-150 were negatively correlated with the level of disease severity, TNF-α, IL-10, and IL-18.	(227)
miR-103a-3p	↑	30 patients with sepsis and 30 HCs, male C57 BL/6 mice	AML12, LO2	↓ FBXW7	_	Downregulation of miR-103a-3p reduced apoptosis, and inflammatory response.	(228)
miR-143	↑	103 patients with sepsis, 95 patients with SIRS and 16 HCs	_	_	_	miR-143 was positively correlated with SOFA score and APACHE II score in patients with sepsis. For distinguishing between sepsis and SIRS, miR-143 showed a sensitivity of 78.6% and specificity of 91.6%.	(229)
miR-145	↓	33 patients with sepsis and 22 HCs, septic mice	BEAS-2B	↑ TGFBR2	_	Up-regulation of miR-145 reduced inflammatory response and improved the overall survival of septic mice.	(230)
miR-150	↓	C57Blk/6J mice	HPAECs	↑ Ang2	_	Downregulation of miR-150 damaged adherens junctions reannealing after injury, which caused an irreversible increase in vascular permeability. Up-regulation of miR-150 reduced vascular injury and mortality.	(231)
miR-34b-3p	↓	CLP mice	RMCs	↑ UBL4A	↑ NF-κB signaling	Up-regulation of MiR-34b-3p reduced inflammatory response and AKI in sepsis mice	(232)
miR-21-3p	↑	_patients with sepsis, C57BL/6 mice	_	↓ SORBS2	_	Downregulation of miR-21-3p induced mitochondria ultrastructural damage and autophagy in LPS-treated mice. Levels of miR-21-3p increased in patients with cardiac dysfunction than without cardiac dysfunction.	(233)
miR-199a-5p	↑	C57BL/6 mice	HEK-293T	↓ SP-D	↑ NF-κB signaling	Down regulation of miR-199a-5p reduced D-lactic acid, DAO, FD-40, oxidative damage and inflammation.	(234)
miR-17	↓	mice	BMSCs, RAW264.7	↑ BDR4, ↑ EZH2, ↑ TRAIL	_	MiR-17 carried by BMSC-EVs reduced inflammation and apoptosis.	(235)
miR-125b	↑	120 patients with sepsis and 120 HCs	_	_	_	AUC of miR-125b: 0.658 MiR-125b was positively associated with APACHE II score, SOFA score, Scr, CRP, PCT, TNF-α, and IL-6 levels. miR-125b Was found to be an independent risk factor for mortality risk.	(236)

(Continued)

TABLE 2 | Continued

miRNA	Pattern of Expression	Clinical Samples/Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
miR-30e	↓	septic rats	_	↑ FOSL2	↑ JAK/STAT signaling	Up-regulation of miR-30e increased proliferation and reduced apoptosis.	(237)
miR-20b-5p	↑	SD rats	HEK-293T	↓ circDMNT3B	_	Downregulation of miR-20b-5p reduced level of d-lactic acid, FD-40, MDA, diamine oxidase, IL-10, IL-6, oxidative damage and inflammatory factors level.	(238)
miR-146b	↓	CLP mice	_	↑ Notch1	_	Up-regulation of miR-146b reduced apoptosis and inflammatory response.	(239)
miR-25	↓	SD rats	H9C2	↑ PTEN, ↑ TLR4	↑ NF-κB signaling	Up-regulation of miR-25 reduced apoptosis and enhanced survival rate.	(240)
miR-21 and miR-181b	↑	septic mice	MDSCs, Gr1 +CD11b + cells	↑ C/EBPβ, ↑ Stat3	_	Stat3 and C/EBPβ increased miR-21 and miR-181b expression by binding to their promoters during sepsis.	(241)
miR-17-5p	↓	septic mice	LPS-induced macrophages	↑ TLR4	_	Sch B increased miR-17-5p expression and reduced inflammation.	(242)
miR-200a-3p	↑	male C57BL/6J mice	HBMECs	↑ NLRP3, ↓ Keap1, ↓ Nrf2, ↓ HO-1	_	Up-regulation of miR-200a-3p induced inflammatory response in sepsis-induced brain injury.	(243)
miR-26b	↓	14 patients with sepsis and 7 patients with septic shock and 21 HCs	MEG-01	↑ SELP, ↓ Dicer1	_	Low levels of miR-26b was correlated with the severity and mortality of sepsis.	(244)
miR-96-5p	↓	_	RAW264.7	↑ NAMPT	↑ NF-κB pathway	Up-regulation of miR-96-5p reduced inflammatory response.	(245)
miR-27a	↑	septic mice	_	_	↑ NF-κB pathway	Downregulation of miR-27a reduced inflammatory response and promoted survival of septic mice.	(246)
miR-21a-3p	↑	specific pathogen-free SD rats	NRK52E	↑ Ago2, ↑ Nrp-1	_	miR-21a-3p was found to be internalized by TECs via Nrp-1 and Ago2.	(247)
miR-574-5p	↑	118 patients with sepsis	_	_	_	miR-574-5p was associated with the death of sepsis patients.	(248)
miR-181b	↓	26 patients with sepsis, 36 patients with sepsis plus sepsis/ARDS and 16 HCs, male C57BL/6 mice	THP-1, HUVECs	↑ importin-α3	↑ NF-κB signaling pathway	Up-regulation of miR-181b reduced mortality rate, inflammation response, LPS-induced EC activation, leukocyte accumulation.	(249)
miR-182-5p	↑	pneumonia mice models	_	_	_	Downregulation of miR-182-5p reduced apoptosis, inflammation response and promoted viability and proliferation.	(250)
miR-195	↑	C57BL/6 mice	endothelial cells	↓ BCL-2, ↓ Sirt1, ↓ Pim-1	_	Downregulation of miR-182-5p reduced apoptosis, and improved survival.	(251)
miR-205	↓	male SD rats	_	_	↑ HMGB1-PTEN signaling pathway	Up-regulation of miR-205 reduced apoptosis and renal injury.	(252)
miR-21-3p	↑ in AKI group	49 patients with sepsis-induced AKI and 93 sepsis patients with non-AKI	_	↑ Scr, ↑ Cys-C, ↑ KIM-1	_	Levels of miR-21-3p was positively associated with Scr, Cys-C, and KIM-1 in the AKI group.	(253)
miR-181a-2-3p	↓	GSE46955 data set, CLP mouse model	TCMK-1	↑ GJB2	_	Up-regulation of miR-181a-2-3p reduced apoptosis and inflammatory response.	(254)
miR-21	↓	female Wistar rats	HK-2	↑ PTEN, ↓ PI3K, ↓ AKT	_	Up-regulation of miR-21 suppressed apoptosis and kidney injury.	(255)
miR-146a	↓	female ICR mice	Raw264.7	↑ JMJD3, NF-κB p65	_	GSKJ4 reduced inflammatory response by increasing miR-146a levels. Transcription of miR-146a was negatively regulated by JMJD3 through epigenetic mechanism.	(256)
miR-294	_	_	RAW264.7	TREM-1	_	miR-294 reduced TNF-α and IL-6 secretion.	(257)
miR-128-3p	↑	CLP mouse model	TCMK-1	↓ NRP1	_	Up-regulation of miR-128-3p promoted apoptosis and inflammatory response and reduced viability.	(258)

(Continued)

TABLE 2 | Continued

miRNA	Pattern of Expression	Clinical Samples/Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
miR-146a	↓	_	H9C2	↓ ErbB4, ↑ TRAF6, ↑ IRAK1	_	Up-regulation of miR-146a reduced apoptosis and inflammatory response and promoted viability.	(259)
miR-511	↑ in S mice	C57BL/6J (B) mice, SPRET/Ei (S) mice,	_	Low protein expression of TNFR1 in S mice	_	miR-511 was induced by glucocorticoids. miR-511 inhibited endotoxemia and experimental hepatitis.	(260)
miR-376b	↓ in sepsis with AKI group	20 Patients with sepsis with AKI, 20 patients with sepsis without AKI and 10 HCs, male C57BL/6 mice	BUMPT	NF-κB, NFKBIZ	_	miR-376b inhibited NF-κB inhibitor ζ (NFKBIZ) expression and NF-κB inhibited miR-376b expression so they created a negative feedback loop.	(261)
miR-155	↑	female BALB/c mice	_	_	_	DXM treatment suppressed the expression of miRNA-155.	(262)
miR-133a	↑	223 patients with sepsis and 76 HCs, C57Bl/6 mice	_	_	_	High levels of miR-133a was correlated with disease severity, inflammatory response, bacterial infection, and organ failure and predicted an unfavorable outcome of patients.	(263)
miR-203	↓	clean grade Kunming mice	HEK-293T	↑ VNN1	↓ AKT signaling pathway	Up-regulation of miR-203 reduced apoptosis, inflammatory response, MDA, ALT, and AST in lung tissues, PMN and PAM levels in BALF and increased SOD activity.	(264)
miR-223	↑	187 patients with sepsis and 186 HCs	_	_	_	AUC for miR-223: 0.754, Plasma miR-223 was associated with disease severity and inflammatory factor levels. miR-223 was found to predict sepsis risk independently.	(265)
miR-146a	↓	patients with sepsis and HCs	Human primary T cells	↑ PRKCε	_	Reduced levels of miR-146a contributes to the pathogenesis of sepsis.	(266)
miR-146-a	↓	55 patients with sepsis and 60 HCs	_	_	_	AUC for miR-146-a: 0.803 Serum levels of miR-146-a was negatively correlated with C-reactive protein, pro-calcitonin, IL-6 and TNF-α.	(267)
miR-34a	↑	CLP-induced suckling rats	U937	_	↑ STAT3 pathway	Up-regulation of miR-34a promoted iNOS secretion from pulmonary macrophages.	(268)
hsa-miR-346	↓	_	RAW264.7	↑ lncRNA MALAT1, ↑ SMAD3	_	Up-regulation of hsa-miR-346 promoted proliferation.	(269)
miR-214	↓	male Kunming mice	_	↑ PTEN	↓ AKT/ mTOR pathway	Up-regulation of miR-214 reduced oxidative stress and autophagy, so ameliorated CLP-induced AKI.	(270)
miR-27a	↑	LPS induced sepsis mice model	H9C2	↓ rhTNFR: Fc, ↓ Nrf2	_	rhTNFR:Fc elevated viability and reduced apoptosis by increasing Nrf2 levels and reducing miR-27a levels.	(271)
miR-150	↓ in non-survival group	48 patients with septic shock (23 survival patients and 25 non-survival patients)	_	_	_	MiR-150 level was positively associated with cardiac index and negatively with EVLWI and PVPI.	(272)
miR-148a-3p	↑	male adult wild-type mice and myeloid-specific RBP-J-deficient mice	RAW264.7	_	Notch signaling and NF-κB pathway	Up-regulation of miR-148a-3p increased proinflammatory cytokines and decreased protective effect of EVs in LPS induced sepsis.	(273)
miR-218-5p	↑	male ICR mice	GMCs	↓ HO-1	_	miR-218-5p was reduced in honokiol-treated septic mice, so the survival rate was increased.	(274)
miR-425-5p	↓	C57BL/6 mice	hepatocytes	↑ RIP1	_	Up-regulation of miR-425-5p reduced inflammatory response and sepsis-related liver damage.	(275)
miR-122	↑ in CA group	168 patients with sepsis (CA group and CN group)	_	_	_	Serum levels of miR-122 were associated with APTT ratios, FIB and antithrombin III levels.	(275)
miR-101-3p	↑	27 patients with SIC and 15 HCs, male SD rats	H9C2	↓ DUSP1	↑ MAPK p38 and NF-κB pathways.	Downregulation of reduced apoptosis and inflammatory response.	(276)

(Continued)

TABLE 2 | Continued

miRNA	Pattern of Expression	Clinical Samples/Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
miR-124	↓	mouse model of ALI	_	↑ MAPK14	↑ MAPK signaling pathway	Up-regulation of miR-124 reduced apoptosis and inflammatory response and promoted proliferation.	(277)
miR-942-5p	↓	_	HK-2	↑ FOXO3	_	Up-regulation of miR-942-5p reduced apoptosis and inflammatory response and promoted viability.	(278)
miR-23a-5p	↑	SD rats	NR8383	_	_	_	(279)
miR-1298-5p	↑	_	BEAS-2B	↓ SOCS6, ↑ STAT3	_	Up-regulation of miR-1298-5p induced cell permeability and inflammatory response and reduced proliferation.	(280)
miR-290-5p	↓	male C57BL/6J mice	MPC5	↑ CCL-2	_	Propofol increased levels of miR-290-5p and decreased CCL-2 and inflammatory response.	(281)
miR-146a	↓	C57BL/6 mice	BMDMs	_	_	Rg6 increased IL-10 and miR-146a levels so inhibited inflammatory responses.	(282)
miR-223	_	C57BL/6 mice	MSCs	Sema3A, Stat3	_	WT-exosomes encased high miR-223 levels induced cardio-protection in sepsis.	(283)
miR-608	_	_	U937, HEK293T	ELANE	_	miR-608 played an important role in posttranscriptional regulation of ELANE expression and upregulation of miR-608 reduced inflammation.	(284)
miR-124	↓	BALB/c and C57BL/6 mice	RAW264.7	↓ α7nAChR, ↑ STAT3	_	miR-124 was found to be a critical mediator for the cholinergic anti-inflammatory effect.	(285)
miR-26b	↑ in AKI group	155 patients with sepsis (68 AKI and 87 non-AKI) and 57 patients with non-infectious SIRS	_	_	_	Urinary miR-26b levels showed an elevated mortality rate and was correlated with the severity of the disease.	(286)
miR-146a	_	Rat model of SAKI	_	_	_	DEX pretreatment could increase the expression level of miR-146a and reduce oxidative stress and inflammatory responses.	(287)
miR-29a	↑ in AKI group	74 patients with AKI and 41 without AKI	_	_	_	AUC for miR-29a: 0.82 miR-29a was found to be an independent risk factor for mortality in the septic patients.	(288)
miR-10a-5p	↑ in AKI group	74 patients with AKI and 41 without AKI	_	_	_	AUC for miR-10a-5p: 0.75 miR-10a-5p was found to be an independent risk factor for mortality in the septic patients.	
miR-155	↑	septic mice	NCM460	_	↑ NF-κB signaling	Up-regulation of miR-155 increased hyperpermeability to FITC-dextran, TNF-α and IL-6 levels, and decreased ZO-1 and Occludin expression.	(289)
miR-155	↑	male C57BL/6 mice	Raw264.7, THP-1	_	↑ PI3K/AKT signalling pathways	Curcumin inhibited inflammatory responses and miR-155 expression.	(290)
miR-497	↑ in myocardial injury group	148 patients with sepsis (58 myocardial injury group and 90 non-myocardial injury group)	_	_	_	Plasma miRNA-497 was correlated with cTnI in patients with myocardial injury.	(291)
miR-497-5p	↑	GEO database, male C57BL/6 mice	BEAS-2B	↓ IL2RB	_	Downregulation of miR-497-5p reduced apoptosis and inflammatory responses.	(292)
miR-30a	↓	_	monocytes	↑ STAT1, ↑ MD-2	_	miR-30a could inhibit STAT1-MD-2 in monocytes of sepsis.	(293)
miR-150	↓	C57BL/6 mice	HUVECs	↑ NF-κB1	_	miR-150 increased survival in patients and inhibited apoptosis and inflammatory responses.	(294)
miR-146a	_	_	THP-1	RBM4, Ago2, p38	_	Up-regulation of miR-146a inhibited p38 activation and increased Ago2-RBM4 protein interaction, so reduced inflammatory responses.	(295)
miR-146a	_	C57BL/6 mice	HEK293TN, J774.1	_	_	Up-regulation of miR-146a reduced morphine mediated hyper-inflammation.	(296)
miR-27a	↓	septic mice	_	↑ TAB3	↑ NF-κB signaling pathway	Paclitaxel pretreatment increased miR-27a levels, so decreased inflammatory responses.	(297)
miR-146a	↓ in septic patients than SIRS and HCs groups	50 patients with sepsis, 30 patients with SIRS and 20 HCs	_	_	_	AUC for miR-146a: 0.858	(298)

(Continued)

TABLE 2 | Continued

miRNA	Pattern of Expression	Clinical Samples/Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
miR-223	↓ in septic patients than SIRS and HCs groups	50 patients with sepsis, 30 patients with SIRS and 20 HCs	_	_	_	AUC for miR-223: 0.804	
miR-339-5p	↓	septic mice	RAW264.7	↑ HMGB1, ↑ IKK-β	_	Paeonol could reduce inflammatory responses by upregulating miR-339-5p expression.	(299)
miR-99b	↑	male C57BL/6 J mice	RAW264.7	↓ MFG-E8	_	Spherical nucleic acid increased migration by inhibiting miR-99b.	(300)
miR-215-5p	↓	_	H9c2	↑ LRRFIP1, ↑ ILF3	_	miR-215-5p reduced inflammatory responses.	(301)
miR-15a	↑ in sepsis and SIRS than HCs	166 patients with sepsis, 32 patients with SIRS, and 24 HCs	_	_	_	miR-15a could distinguish sepsis/SIRS from HCs.	(302)
miR-16	↑ in sepsis and SIRS than HCs	166 patients with sepsis, 32 patients with SIRS, and 24 HCs	_	_	_	miR-16 could distinguish sepsis/SIRS from HCs.	

miRNAs and Sepsis. AKI, Acute kidney injury; HCs, healthy controls; AUC, significant higher area under curve; CRP, C-reactive protein; TLC, total leucocytes count; SD, Sprague-Dawley; SOFA, sequential organ failure assessment; Scr, serum creatinine; WBC, white blood cell; PCT, procalcitonin; APACHE, physiology and chronic health evaluation; CLP, cecal ligation and puncture; ERS, endoplasmic reticulum stress; AUC, area under the ROC curve; SAE, sepsis-associated encephalopathy; BUN, blood urine nitrogen; rIPC, remote ischemic preconditioning; SPF, specific pathogen-free; GEO, Gene Expression Omnibus; SIMI, sepsis-induced myocardial injury; Tregs, regulatory T-cells; Sch B, Schisandrin B; DXM, dexamethasone; MDA, malondialdehyde; ALT, aminotransferase; AST, aspartate aminotransferase; PAM; pulmonary alveolar macrophages; PMN, polymorphonuclear neutrophils; BALF, bronchoalveolar lavage fluid; SOD, superoxide dismutase; CA, coagulation abnormal; CN, coagulation normal; APTT, serum activated partial thromboplastin time; FIB, fibrinogen; SIC, sepsis-induced cardiomyopathy; SIRS, systemic inflammatory response syndrome; DEX, dexmedetomidine; SAKI, sepsis-induced acute kidney injury).

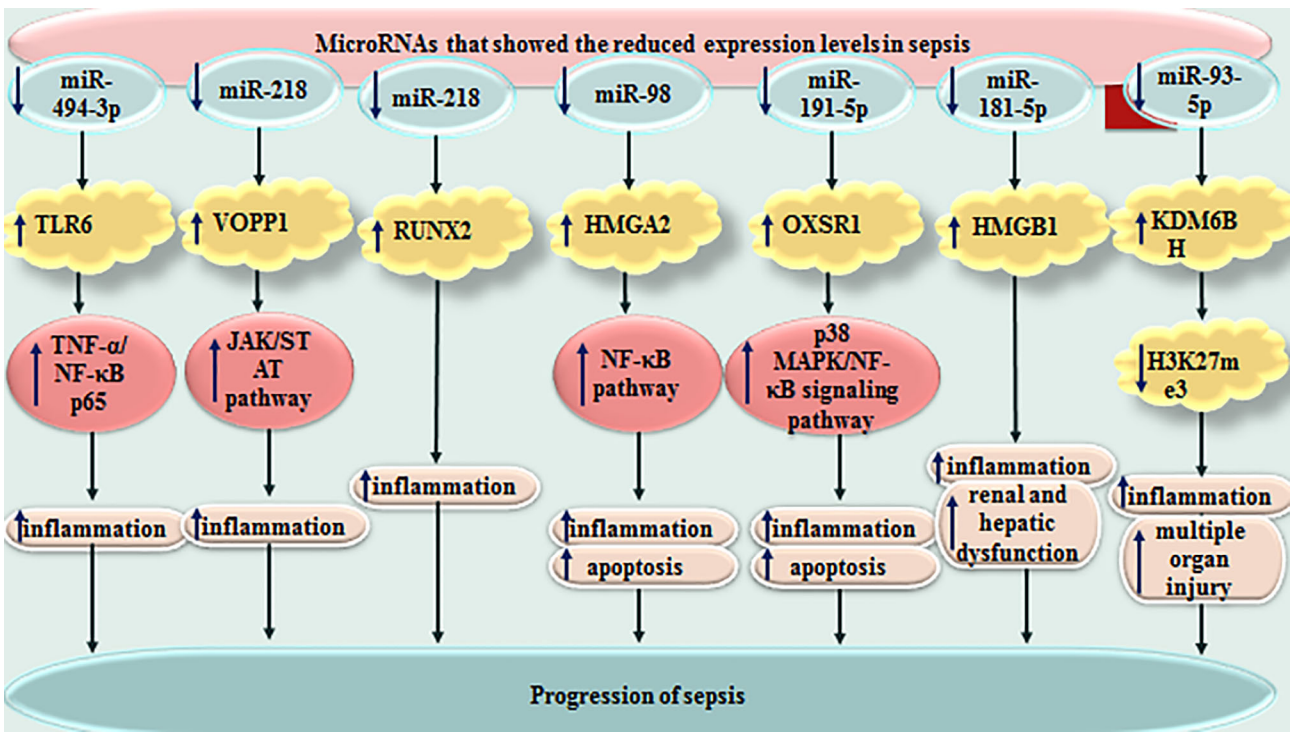

FIGURE 3 | Down-regulated miRNAs in sepsis.

TABLE 3 | CircRNAs and Sepsis.

circRNA	Pattern of Expression	Clinical Samples/ Animal Model	Assessed Cell Lines	Targets / Regulators	Signaling Pathways	Description	Reference
circC3P1	↓	male C57BL/6 mice	MPVECs	↑ miR-21	_	Upregulation of circC3P1 reduced pulmonary injury, inflammatory responses and apoptosis.	(304)
hsa_circRNA_104484	↑	25 patients with sepsis and 22 HCs	_	_	_	Hsa_circRNA_104484 showed the potential to be used as diagnostic marker for sepsis.	(305)
hsa_circRNA_104670	↑	25 patients with sepsis and 22 HCs	_	_	_	Hsa_circRNA_104670 showed the potential to be used as diagnostic marker for sepsis.	
circVMA21	↓	CLP rats	HK-2, WI-38	↑ miR-9-39, ↓ SMG1	–	CircVMA21 reduced apoptosis, inflammatory responses and oxidative stress.	(306)
circ-PRKCI	↓	121 patients with sepsis and 60 HCs	_	↑ miR-545	_	Low levels of circ-PRKCI were correlated with sepsis risk, clinical disease severity and 28-day mortality risk.	(308)
circDNMT3B	↓	male SD rats	Caco2	↑ miR-20b-5p, ↓ SOD	_	Downregulation of circDNMT3B decreased cell survival and increased apoptosis, inflammatory responses and oxidative damage.	(238)
circ_0114428	↑	_	HK2	↓ miR-495-3p, ↑ CRBN	_	Downregulation of circ_0114428 decreased apoptosis, inflammatory responses, oxidative stress, and ER stress.	(307)
circ_0001105	↓	septic rats	_	↑ YAP1	_	Up-regulation of circ_0001105 decreased apoptosis, inflammatory responses and oxidative damage .	(309)
circ_Ttc3	↓	CLP rats	_	↑ miR-148a, ↓ Rcan2	_	Up-regulation of circ_Ttc3 decreased inflammatory responses and oxidative stress in AKI rats.	(310)
circPRKCI	↓	patients with sepsis and HCs	HK2	↑ miR-545, ↓ ZEB2	NF-kB pathway	Up-regulation of circPRKCI reduced LPS-induced cell injury and inflammatory responses.	(311)
circ_0003420	↑	_patients with sepsis and HCs	Kupffer cells	↓ NPAS4	_	Up-regulation of circ_0003420 increased apoptosis, inflammatory responses and decreased proliferation.	(312)
circ-Fryl	↑ in ADSC exosomes	septic mouse model	ADSCs, LPS-induced AEC damage model	miR-490-3p, ↑ SIRT3 in ADSC exosomes	SIRT3/ AMPK signaling	Up-regulation of circ-Fryl increased autophagy and decreased apoptosis and inflammatory responses.	(313)
circ_0091702	↓	_	HK2	↑ miR-182, ↓ PDE7A	_	Up-regulation of circ_0091702 reduced LPS-induced cell injury.	(314)
circVMA21	↓	_	THP-1	↑ miR-199a-5p, ↓ NRP1	_	Up-regulation of circVMA21 reduced apoptosis, inflammatory responses and oxidative stress.	(315)
circTLK1	↑	wistar rats	HK-2, 293T	↓ miR-106a-5p, ↑ HMGB1	_	Downregulation of circTLK1 reduced apoptosis, inflammatory responses and oxidative stress.	(316)
circFADS2	↑	50 patients with sepsis and 50 HCs	HBEpCs	↓ mature miR-15a-5p	_	Up-regulation of circFADS2 reduced miR-15a-5p overexpression-induced apoptosis.	(317)
circ_0091702	↓	_	HK2	↑ miR-545-3p, ↓ THBS2.	_	Up-regulation of circ_0091702 reduced LPS-induced HK2 cell injury.	(318)
hsa_circ_0068,888	↓	_	HK-2	↑ miR-21-5p	_	Up-regulation of hsa_circ_0068,888 reduced inflammatory response and oxidative stress and increased viability.	(319)
circPTK2	↑	C57BL/6 mice	BV2 microglia	↓ miR-181c-5p, ↑ HMGB1	_	Downregulation of circPTK2 reduced apoptosis, inflammatory responses.	(320)
circ-FANCA	↑	19 patients with sepsis and 19 HCs	HK2	↓ miR-93-5p, ↑ OXSR1	_	Downregulation of circ-FANCA reduced apoptosis, inflammatory responses and oxidative stress and increased proliferation.	(321)
circANKRD36	↑	60 patients with sepsis-induced ARDS	RAW264.7	↓ miR-330, ↑ ROCK1	_	Downregulation of circANKRD36 reduced viability and migration and alleviated inflammatory responses.	(322)
circPRKCI	↓	_	HK2	↑ miR-106b-5p, ↓ GAB1	_	Up-regulation of circPRKCI reduced apoptosis, inflammatory responses and oxidative stress and increased viability.	(323)

HCs, healthy controls; AKI, acute kidney injury; ARDS, acute respiratory distress syndrome.

lncRNAs and miRNAs can ameliorate the pathologic events in the target organs, particularly heart and kidney during sepsis. Yet, this field is still in its infancy needing verification in additional animal models and cell lines. Moreover, since sepsis is an emergency situation, any therapeutic option should be verified in terms of bioavailability, efficiency and instant amelioration of pathological events.

Since the pathoetiology of sepsis-related complications is not completely understood, high throughput sequencing strategies focusing on different classes of non-coding as well coding RNAs are necessary to find the complicated networks between these transcripts in the context of sepsis.

AUTHOR CONTRIBUTIONS

SG-F wrote the draft and revised it. MT designed and supervised the study. NA, BH, and TK collected the data and designed the figures and tables. All authors contributed to the article and approved the submitted version.

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