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Community series in epigenetics of the immune component of inflammation, volume II

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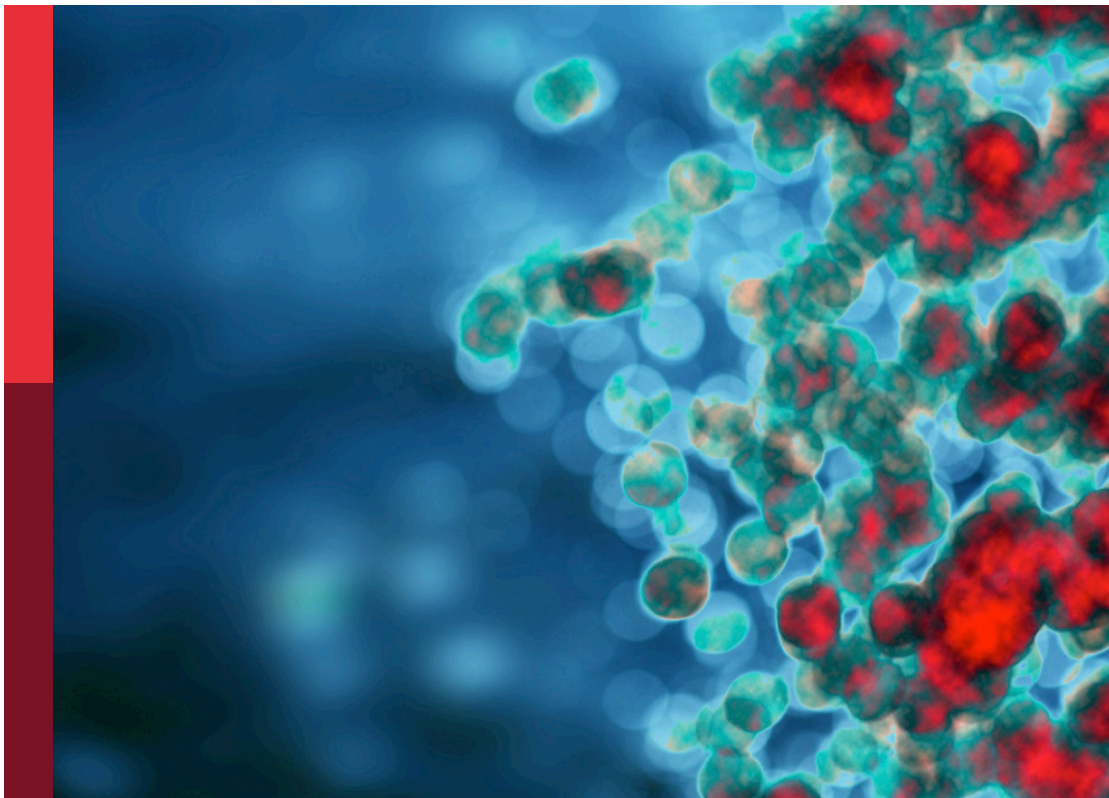

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Community series in epigenetics of the immune component of inflammation, volume II

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Editorial: Community series in epigenetics of the immune component of inflammation-volume II

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Editorial on the Research Topic
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Epigenetics is also known as pseudogenetics or postgenetics, and explores heritable changes in gene expression or cell phenotype through certain mechanisms, without changes in the DNA sequence in biology and specific genetics (1, 2). Inflammation is a basic pathological process that occurs in living tissues with a vascular system in response to the stimulation of various damage factors (3–6). It is well-known that inflammation is mediated by a variety of immune components (including complements, cytokines, chemokines, transcriptional factors, pattern recognition receptors, etc.) secreted or expressed by immune/non-immune cells (2). Mounting evidence supports that epigenetic modifications are associated with the occurrence, development, and resolution of inflammation via remodeling immune/non-immune cells and the microenvironment (5, 6), thus promoting or repressing the progression of many inflammatory diseases such as diabetes (7–9), rheumatoid arthritis (RA) (10, 11), asthma (12, 13), fatty liver diseases (14, 15), and cancer (16–18). Mechanically, inflammation can induce changes in the epigenetic landscape in an inflammatory microenvironment (6, 19), and epigenetic modifications can in turn maintain and promote the development of inflammation by regulating the expression of various immune components (20, 21). With studies on the development of epigenetic modifications in inflammation and with rapid research progress on mechanisms and drug discovery, some star targets (lysine-specific demethylases (22, 23), BRD4 (16, 17, 24), EZH2-EED protein-protein interaction (25, 26), and HDACs (27)) have been used in the diagnosis or treatment of inflammatory diseases *in cellulo* and *in vivo*. Therefore, investigating the functions of epigenetic immune components in inflammatory diseases not only helps reveal the molecular mechanism of a variety of inflammatory diseases, but also develop novel theranostical strategies against these diseases.

This Volume II Research Topic continually collected excellent works on the "*Epigenetics of the Immune Component of Inflammation*," and a total of 9 articles from 77 authors were accepted, which demonstrates the great interest in this Research Topic in this field, deepens the understanding of epigenetic regulation in immune diseases and inflammation responses, and highlights the clinical significance of epigenetic regulation and inflammatory immune components in disease theranostics. This Research Topic can be roughly divided into the following three subtopics.

Genome modifications

Genome modifications mediate the progression of inflammatory diseases by modulating the expression of related inflammatory genes (28). In our Research Topic, Lagosz-Cwik et al. found that the DNA methyltransferase (DNMT) inhibitor decitabine could suppress the proliferation of gingival fibroblasts (GFs) and induce necrotic cell death via reducing genome methylation. RNA sequencing showed that decitabine raised chemokines CCL-5, -8, -13, and -20, IL-1A, -18, -33, CSF3, the matrix metalloproteinases MMP-1, -9, and -13, and intercellular adhesion molecule-1 (ICAM-1), and reduced genes mediated collagen fibril and extracellular matrix organization, which suggests that DNMT inhibitors are potential agents against periodontitis pathogenesis. However, the potential cytotoxicity of DNMT inhibitors is a non-negligible challenge for their clinical applications. Jiang et al. explored the methylation level of Homeodomain-interacting protein kinase 3 (HIPK3) in blood using a sample database including 235 RA patients, 30 osteoarthritis (OA) patients, and 30 matched healthy controls. The results revealed that all 7 CpG islands are hypomethylated in RA patients compared with OA and healthy individuals. The 33286785 CpG displays the highest predictive power (AUC=0.829) against RA, and the prediction model could be further improved by combining HIPK3 with clinical index rheumatoid factors (RF^+) and anti-citrullinated protein antibodies ($ACPA^-$). Moreover, the study also found that the methylated HIPK3 levels are negatively correlated with C-reactive protein (CRP), suggesting that the blood methylation level of HIPK3 holds potential as a clinical diagnostic biomarker and indicator for CRP in RA. Shan et al. summarized the latest application advancements of the gamma-aminobutyric acid (GABA)ergic system (mainly consisting of GABA, GABA transporter, and GABA-related receptors) in RA theranostics, which provides an insight into the potential theoretical guidance and clinical choices for RA therapy. Zheng et al. summed up the functions of serine protease granzymes (Gzms) in RA pathogenesis and showed that these enzymes are potential targets for diagnosis and therapy for RA. Natoli et al. found that the DNA methylation profiles in $CD4^+$ T-cells could discriminate the disease status of healthy controls, skin psoriasis, and psoriatic arthritis, which suggests that DNA methylation imprints may be used to determine the degree and grade of psoriasis, and thus to help carry out individualized therapeutic strategies accordingly. Sapienza et al. revealed the distinction in the DNA methylation profiles of diabetes patients who eventually developed end-stage renal disease (ESRD) and without diabetic nephropathy (DN) (29) and Wang et al. showed that this profile could be used to discriminate diabetes with ESRD and without DN. Zhang et al. described the immunoregulatory and metabolic roles and action mechanisms of genome methylation modification in the progression of metabolic-associated fatty liver disease (MAFLD), which provides references for the diagnosis and treatment of MAFLD via targeting nucleotide methylation. Xue et al. found that 8-oxoguanine DNA glycosylase1 (OGG1) inhibition or ablation enhances the antiviral activity of epithelial cells toward infection of human respiratory syncytial virus (RSV) *in vitro* and *in vivo*. Further study revealed that OGG1 recognizes 8-oxoGua in the vicinity of interferon response elements (IRF) within the *IFN-λ* promoter, and thus reduces the DNA occupancy of NF-κB/RelA and IRFs by promoting the interaction between the NF-κB homodimer p50 and p50 in guanine islets (5'-GGG-3') in the *IFN-λ* promoter, which reduces IFN-λ production, increases viral load and neutrophilia, and finally aggravates viral infection and immunopathology in mice. This finding indicates that OGG1 is a potential target for eliminating pulmonary viral infections in clinical settings.

Post-transcriptional modifications

Post-transcriptional modification refers to the various changes and alterations of RNA molecules after transcription from DNA (30). Post-transcriptional modifications including microRNA, lncRNA, tRNA, m^6A modifications, etc. mediate various inflammatory diseases by modulating multiple immune components. Wang et al. systematically summarized the dysregulation of the miRNAs miR-183/96/182 cluster (miR-183C) in many autoimmune disorders, such as systemic multiple sclerosis, ocular autoimmune diseases, and lupus erythematosus, and highlighted the potential of miR-183C as targets for diagnosis markers and therapy against these autoimmune diseases. Wang et al. described the functions and mechanisms of non-coding RNAs including microRNAs, lncRNAs and m^6A modifications in symptoms of podocytopathies, which provide a theoretical basis and target selection for the diagnosis and treatment of DN.

Post-translational modifications

Post-translational modifications are also crucial epigenetic modes involved in accurately orchestrating a variety of inflammatory processes via writing, reading, and erasing marks of specific amino acid residues within proteins (31). Wang et al. showed that histone acetylation/de-acetylation modification in podocytopathy contributes to protecting from DN progression, which is a potential target for DN therapy.

Author contributions

Y-JL: Investigation, Writing – original draft. H-JZ: Conceptualization, Resources, Validation, Writing – review &

editing. HW: Conceptualization, Investigation, Resources, Validation, Writing – review & editing. C-MC: Conceptualization, Formal Analysis, Resources, Validation, Writing – review & editing. G-JY: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Epigenetics and endoplasmic reticulum in podocytopathy during diabetic nephropathy progression

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Proteinuria or nephrotic syndrome are symptoms of podocytopathies, kidney diseases caused by direct or indirect podocyte damage. Human health worldwide is threatened by diabetic nephropathy (DN), the leading cause of end-stage renal disease (ESRD) in the world. DN development and progression are largely dependent on inflammation. The effects of podocyte damage on metabolic disease and inflammatory disorders have been documented. Epigenetic and endoplasmic reticulum (ER) stress are also evident in DN. Targeting inflammation pathway and ER stress in podocytes may be a prospective therapy to prevent the progression of DN. Here, we review the mechanism of epigenetics and ER stress on podocyte inflammation and apoptosis, and discuss the potential amelioration of podocytopathies by regulating epigenetics and ER stress as well as by targeting inflammatory signaling, which provides a theoretical basis for drug development to ameliorate DN.

KEYWORDS

epigenetics, endoplasmic reticulum, podocyte, diabetic nephropathy, inflammation signaling

Introduction

Diabetes mellitus (DM) is characterized by systemic hyperglycemia and can lead to a wide range of complications. It has seriously threatened the quality of life of millions worldwide. Although intensive measures have been taken to control blood glucose, the number of people with DM worldwide was half a billion in 2021 (1). Around 30–40% of type 1 or 2 DM patients are, unfortunately, prognosed with diabetic nephropathy (DN) and eventually develop end-stage renal disease (ESRD)

(2, 3), imposing an enormous economic burden on individuals, families, and societies. Therefore, novel treatments for DN are urgently needed.

The roles of various pathways in the pathogenesis of DN have been established. Activation of the renin-angiotensin-aldosterone system (RAAS) (4), reactive oxygen species (ROS) (5) and inflammation (6) are related to the onset and development of DN (7). Although treatment with angiotensin-converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB) slows the progression of diabetic nephropathy to ESRD, the risk of ESRD remains high and is associated with residual proteinuria (8), suggesting that better strategies are needed to prevent DN. There has been a growing consensus that inflammation pathways are important in DN progression. New therapeutic strategies could be developed by identifying novel inflammatory factors (9). In patients with diabetic kidney disease (DKD), glomerular macrophage accumulation correlates strongly with progression of kidney impairment (10). During infiltration, macrophages release cytokines such as TNF, IL-1, and IFN-γ, accelerating the progression of DKD (11). By altering the viability of podocytes, macrophages contribute directly to DKD in bone-marrow chimeric mice (12). Genetic deficiency or pharmacological blockade of C-C chemokine receptor type 2 impairs macrophage recruitment to DKD in mouse models, reducing albuminuria and urea nitrogen levels as well as improving histological parameters. Treatment with the CCR2 inhibitor CCX140-B for 52 weeks significantly reduced urinary albumin excretion in T2DM patients with proteinuria (13). Angiotensinogen and cytokines are implicated in DKD—NF-κB initiates inflammation and stimulates the transcription of genes encoding cytokines and adhesion molecules (14). T2DM patients with NF-κB activation in muscle are likely to develop peripheral-tissue insulin resistance (15). DKD patients with bardoxolone have shown renoprotective effects with inhibitors of NF-κB (16). Therefore, research on small molecular targets of inflammatory signaling pathways in DN is needed.

Epigenetic modifications play a role in DKD, by affecting gene expression in response to environmental factors (17, 18). Epigenetic modifications enable mitotically and/or meiotically heritable changes in gene function without altering the underlying DNA sequence (19). Most disease-associated loci and single-nucleotide polymorphisms (SNPs) are found in non-coding regions of the genome, including regulatory regions such as enhancers and promoters, as well as in non-coding RNAs (20), which can affect gene expression by altering transcription factor binding and chromatin accessibility.

Podocytopathies is a key factor in DN development. And podocytopathies can lead to destruction of the glomerular basement membrane (GBM), inducing proteinuria (21). Mice with podocyte-specific conditional disruption of slit molecule genes suffer severe proteinuria and sclerosis (22). Proteinuria is closely associated with foot process effacement in minimal change disease (23). Moreover, albuminuria is diagnostic of early DN. Although podocyte injury is associated with the progression of DN (24), the underlying mechanisms are unclear. Interestingly, ER stress accelerates DN progression by injuring podocytes, endothelial cells, and mesangial cells (25, 26). Hyperglycemia and proteinuria (27) reportedly induce ER stress in DN. Persistent ER stress could affect ER function and induce a maladaptive unfolded protein response (UPR), activating apoptotic signaling pathways and causing podocyte apoptosis (28).

Taken together, the interaction of epigenetic modifications and ER stress with inflammation and apoptosis in podocytopathies is unclear. Studies on the epigenetic mechanism of DN (17) have attenuated ER stress by targeting long non-coding RNA (lncRNA) and/or microRNA. And epigenetics may regulate diabetes-related diseases under inflammatory conditions. A review of recent research on epigenetic mechanisms and endoplasmic reticulum stress in inflammation or apoptosis in podocytopathy presented here. Looking forward to provide new drug treatment strategies for podocytopathy during DN progression.

Podocytopathies and DN

The glomerular filtration barrier is maintained by podocytes, highly specialized epithelial cells that contain critical molecules required for selective permeability (29). Loss of large numbers of podocytes leads to proteinuria, mesangial expansion, and glomerular sclerosis. Diabetic patients' podocytes are susceptible to injury due to apoptosis, which is the most common mode of podocyte loss due to glucose-induced oxidative stress and advanced glycation end products (28, 30). However, little is known of the underlying mechanisms (31).

Podocyte injury is characterized by hypertrophy, epithelial mesenchymal transition (32), detachment, and apoptosis (33). Glomerular hyperfiltration is accompanied by podocyte hypertrophy and abnormal podocyte function (34). Moreover, hyperglycemia could decrease the attachment of podocytes to the GBM—integrin α3β1 is an important receptor for podocyte–GBM attachment (35). The levels of podocyte cytoskeleton proteins, such as synaptopodin, podocin, and nephrin, were significantly reduced in diabetic kidneys (36). That is, a disordered podocyte cytoskeleton hampers adhesion, increasing GBM permeability and inducing proteinuria. Therefore, injury and depletion of podocytes is a crucial pathologic mechanism of albuminuria in DN. Proteinuria is also associated with a decrease in podocyte density and number in type 1 and type 2 diabetes. Indeed, podocytes in urine are an earlier marker of DN than proteinuria (37) (Figure 1).

Podocyte apoptosis contributes to filtration dysfunction in DN. As highly differentiated cells, podocytes have limited self-renewal *via* mitosis under adverse conditions, rendering them

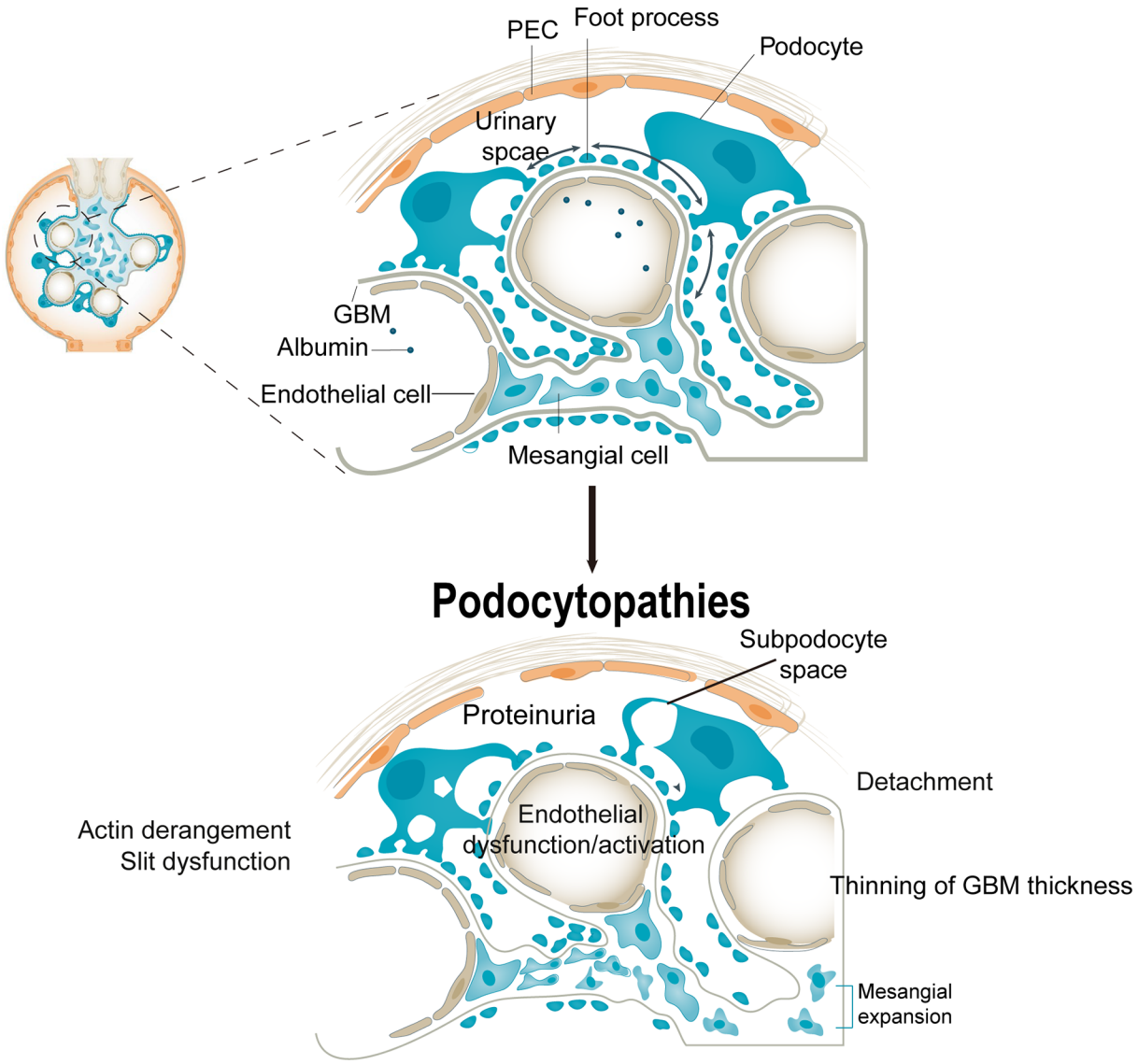

FIGURE 1
Consequences of podocytopathies. Podocyte injury causes cellular and structural responses in the glomerulus. An injured podocyte has cytoplasmic blebs, protein droplets, and an expanded subpodocyte space. Foot process effacement is associated with actin derangement, which induces dysfunction of slit molecules. The injured podocyte detaches from the glomerular basement membrane, which is associated with abnormalities of adhesion molecules, coagulation cascade, chemokine receptor expression, and lipid peroxidation. These changes may depend on podocyte detachment but also on aberrant factors from injured podocytes. PECs, parietal epithelial cells; VEGF, vascular endothelial growth factor.

vulnerable to damage by stress (28, 38). Therefore, podocytes are dependent on clearance mechanisms such as the adaptive UPR and autophagy to restore intracellular hemostasis (38). Podocyte autophagy deficiency triggers proteinuria and glomerulosclerosis, and maintaining a certain level of autophagy ensures normal physiological function (39). In aldosterone-induced ER stress and podocyte injury, inhibition and induction of ER stress suppress and enhance, respectively, autophagy (40). Hence, restoration of podocyte structure and function after injury is important.

Epigenetics in podocytopathies during DN progression

Histone modifications

Histone modifications (acetylation, methylation, phosphorylation, ubiquitination, and carbonylation) are important in epigenetics; histone acetylation and methylation predominate. Inhibitors of histone deacetylases (HDACs) (such as valproic acid) and SAHA (such as pan-HDAC inhibitors)

reduce proteinuria and glomerulosclerosis, thereby increasing survival (41). Epigenetics are implicated in the progression of various kidney diseases, including DN. Histone acetyltransferases' acetylation activities are balanced by HDACs, which modulate physiological and pathological gene transcription. A class III NAD^+-dependent HDAC, sirtuin6 (Sirt6), belongs to the sirtuin family, which consists of Sirt1 to Sirt7 that differ in their cellular and tissue distributions. Genes involved in glucose metabolism, DNA repair, telomerase activity, genomic stability, and cellular senescence are regulated by Sirt6-dependent deacetylation of H3K9 or H3K56. Several phenotypes of premature aging have been observed in mice lacking Sirt6. Podocytopathies were associated with a reduced level of Sirt6 in renal biopsies and a reduced glomerular filtration rate (42). In a DN mouse model, Sirt6 deletion exacerbated podocyte injury and proteinuria. Sirt6 suppresses *Notch1* and *Notch4* transcription, negatively regulating Notch signaling. Therefore, Sirt6 has potential as a therapeutic target for proteinuric kidney disease. In addition, many cellular biological processes regulated by Sirt1 are involved in kidney diseases, such as autophagy and apoptosis (43). Enhanced Sirt1 function in podocytes protects diabetic kidneys (44). Hence, histone modifications protect podocytes in hyperglycemia.

DNA methylation

Differences in DNA methylation are associated with predisposition to disease or treatment response. Inter-individual epigenetic differences among individuals can be used as predictive biomarkers of disease susceptibility (45). Thus, DNA methylation levels can be used to distinguish diabetic ESRD and renal complications without diabetes. The METTL3-mediated m6A modification contributes to podocyte injury in DN and targeting m6A by METTL3 has therapeutic potential (46) (Figure 2). Sirt1 mRNA was degraded in podocytosis by METTL14-mediated m6A modification. By upregulating Sirt1, podocytes activate autophagy and reduce apoptosis and inflammation, thereby alleviating proteinuria and delaying the progression of podocytopathies. Dysregulation of the RNA m6A modification may be mediated by METTL14 and may be a therapeutic target for podocytopathies (Figure 2).

When Sirt6 is reduced under pathological conditions, H3K9ac levels increase in the promoters of *Notch1* and *Notch4*, thereby increasing transcription. Sirt1 mRNA m6A modification and degradation are elevated when podocyte injury induces the expression of METTL14. As a result of Notch-signaling activation, podocyte injury is triggered by inflammation, apoptosis, actin-cytoskeleton degeneration, and autophagy inhibition.

Aberrant epigenetic alterations are implicated in the pathogenesis of DKD and modifying the podocyte injury response. DACH1 and Pax transactivation-domain interacting protein (PTIP) target genes are similar. PTIP is recruited by Pax proteins to promoter Pax response elements (PREs), increasing the levels of H3K4Me3 and activating transcription (47). PTIP is also recruited by sequence-specific binding of DACH1 to its target gene promoters, which suppresses transcription and reduces promoter H3K4Me3 levels. When podocyte DACH1 expression is reduced, PTIP is less likely to be recruited to target gene promoters by DACH1. Decreased DACH1 expression in podocytes under hyperglycemic conditions reduces the expression of multiple target genes and downstream signals, thereby damaging podocytes. Downregulation is caused by reduced recruitment of PTIP by the DACH1 target gene promoter, leading to excessive podocyte epigenome activity. Therapeutic interventions aimed at improving podocyte DACH1 activity will correct multiple dysfunctional signals simultaneously. Therefore, testing and development of drugs for DACH1 should be prioritized (Figure 3).

Non-coding RNA

Epigenetic determinants such as DNA methylation, histone modification, or RNA can be transferred to the next generation through meiotic products (gametes). DNA methylation and chromatin histone modifications are epigenetic mechanisms, but lncRNAs and microRNAs can also be modified post-transcriptionally (17). MicroRNAs are endogenous non-coding single-stranded RNAs of about 20-24 nucleotides, which can degrade or silence the translation of target gene mRNA by base-pairing with the 3'-UTR sequence (48). MicroRNA regulation of podocyte apoptosis has been a focus of research (48, 49). MicroRNAs regulate autophagy genes, affect the level of autophagy, and are implicated in DN (50, 51). Various miRNA regulatory mechanisms are involved in DN physiological and pathological processes, affecting podocyte apoptosis (49), and are targets for DN prevention and treatment (Figure 4).

Long noncoding RNAs (lncRNAs), as competing endogenous RNAs (ceRNAs), confirm the interaction and competitive regulatory mechanisms between lncRNA and microRNA (52, 53, Wang et al., 2022). Enzymes including DNMTs regulate a variety of miRNAs associated with Alzheimer Disease development and progression, including miR-34 a/b/c, miR-107, miR-124, miR-125b, and miR-137 (54). Upregulation of about 40 miRNAs in a lncRNA in the kidneys of diabetic mice promoted DN. lncRNAs have not been systematically studied in podocytopathy to date. miRNAs and epigenetic enzymes play important roles in a variety of diseases.

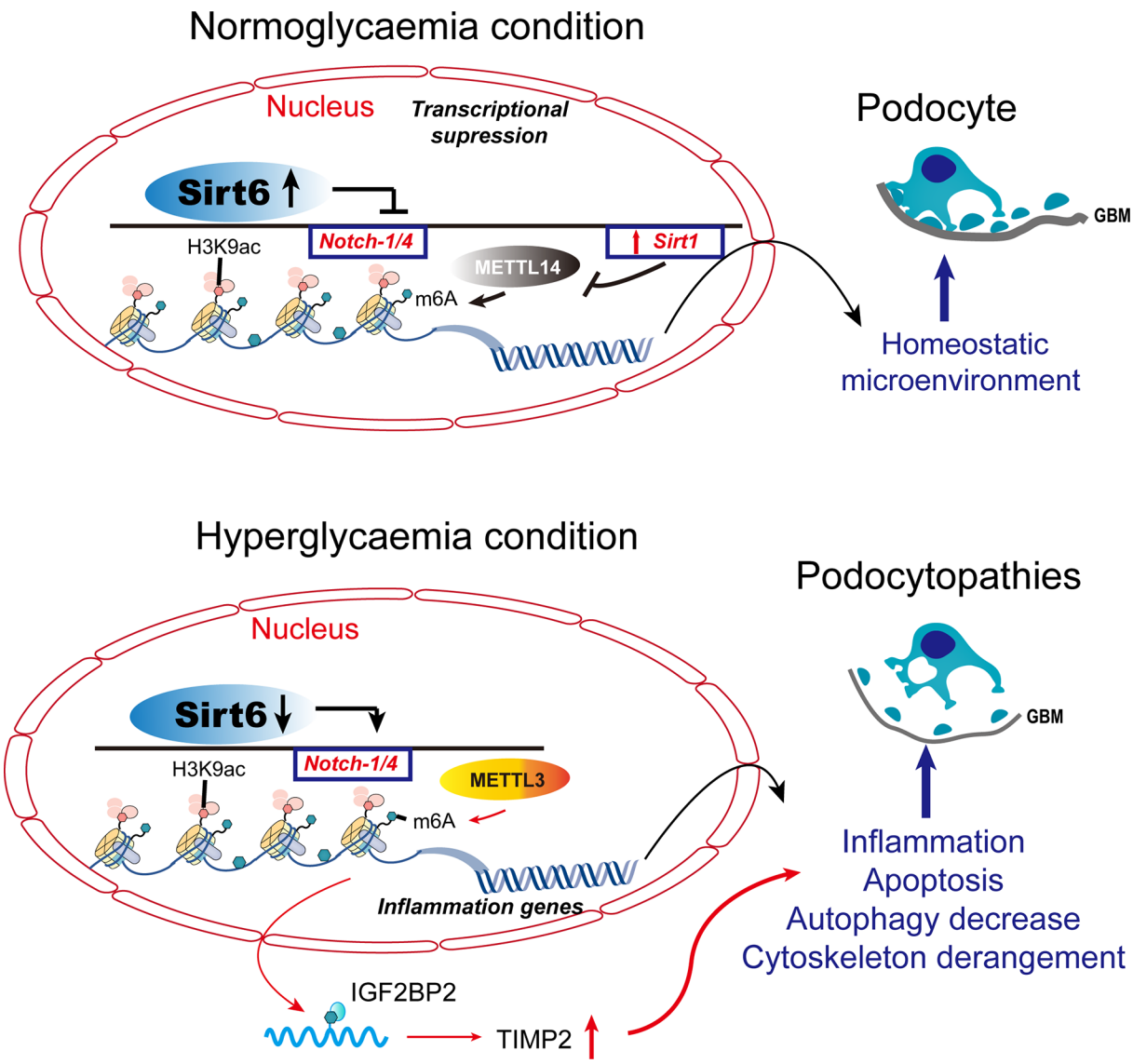

FIGURE 2
Histone modifications and DNA methylation in podocytopathies. Under normal conditions, Sirt6 inhibits the transcription of *Notch1* and *Notch4* by decreasing H3K9ac levels in the promoter regions of *Notch1* and *Notch4*. METTL14 deficiency inhibits Sirt1 mRNA m6A modification, thereby increasing Sirt1 levels in normal podocytes. METTL3 modulates the m6A modification of TIMP2 in an IGF2BP2-dependent manner and exerts pro-inflammatory and -apoptotic effects. TIMP2, tissue inhibitor of metalloproteinase 2; IGF2BP2, insulin-like growth factor 2 mRNA binding protein 2.

ER in podocytopathy during DN progression

Although podocytes play a crucial role in glomerular filtration by forming a slit diaphragm between interdigitating foot processes, the molecular details of protein folding and degradation in the ER are unclear. The ER plays a vital role in maintaining protein synthesis and hemostasis. Two studies have evaluated the pathophysiological roles of the ER in kidney dysfunction (55, 56). ER stress is characterized by the UPR. Sustained ER stress causes cell death, inflammation, and autophagy in the renal tubules. Therefore, inhibiting ER stress may have therapeutic potential for DN (57).

The upregulation of ER stress markers and downstream signaling molecules has been reported in cells, animals, and individuals with DN (55). CHOP, a key regulator of ER stress, was significantly increased in renal biopsies of type 2 DN patients (58, 59). High glucose induces ER stress, podocyte phenotypic switch, and podocyte loss in rat, effects inhibited by exogenous ER molecular chaperones (60, 61). ER stress also triggers podocyte apoptosis. ER markers such as GRP78 are upregulated in diabetic rats' podocytes (55, 62). Expression of

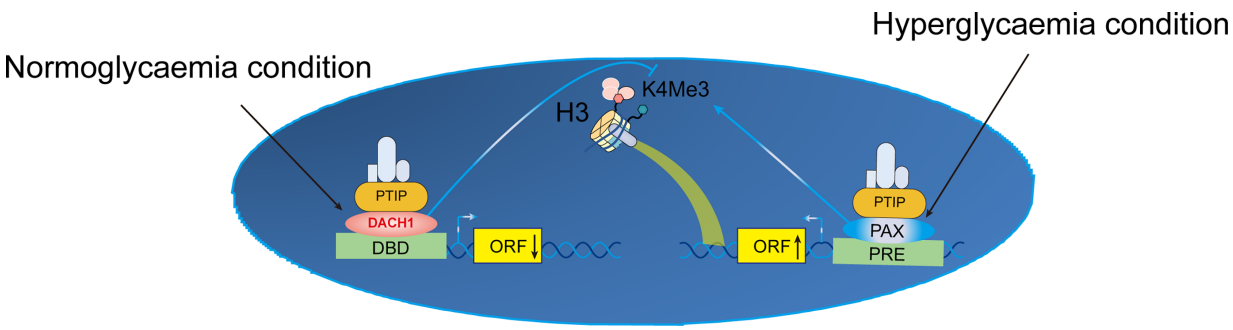

FIGURE 3
Proposed mechanism of transcriptional activation of K4Me in podocytes. Crosstalk between normal and hyperglycemic conditions in DN. ORF, open reading frame; DBD, DNA-binding domain; H3K4Me3, trimethylation of lysine 4 on histone H3 protein subunit; PRE, Pax response elements; DACH1, Dachshund homolog 1; PTIP, Pax transactivation-domain interacting protein.

GRP78 and other factors related to ER is increased significantly in HG-induced podocytes, leading to apoptosis (60). A newly hallmark, RTN1A, was found to be increased in the context of ER stress (58). Therefore, ER stress may be a key regulator of podocyte death. Here, based on the three arms of the UPR, we discuss the role of ER stress in inflammation and podocyte apoptosis.

Epigenetic modifications can affect endoplasmic reticulum stress response, resulting in disease risk. MicroRNA expression and DNA and histone methylation patterns are epigenetic phenomena associated with ER stress genes. Some results suggest that the methylation characteristics of leukocyte ER regulatory genes may be related to abdominal/central obesity markers (waist circumference), dyslipidemia and insulin resistance (63). However, the finding provides insight into the relationship between disorders and epigenetics as well as complications resulting from endoplasmic reticulum stress, further research is needed to confirm this.

Signaling pathways involved in inflammation and apoptosis

TLR4 knockout mice showed reduced albuminuria, renal dysfunction, inflammation, and fibrosis after STZ treatment. Diabetes-induced podocyte loss was reduced by deletion of Tlr4 despite tubular injury suppression (64, 65). High glucose upregulated *TLR4* expression in cultured podocytes by triggering ROS production and NF-κB activation (66). By activating the protein kinase C pathway and NADPH oxidase

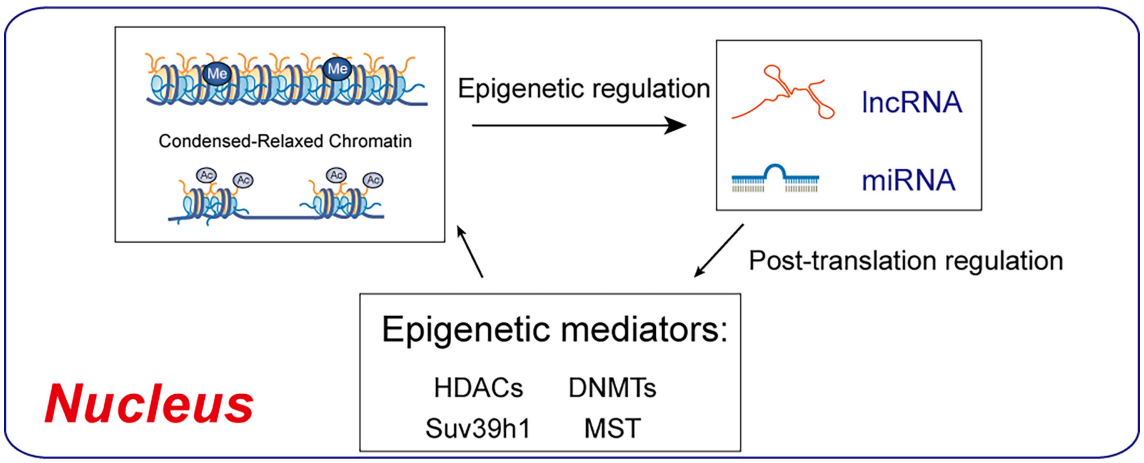

FIGURE 4
Regulatory interactions between epigenetic machinery and non-coding RNAs. DNMTs and HDACs are epigenetic mediators of DNA methylation and histone acetylation, which result in condensed-relaxed chromatin. lncRNAs and miRNAs modulate epigenetic mediators, thus regulating chromatin remodeling.

(NOX), high glucose increases TLR2 and TLR4 expression in monocytes (67). High glucose-induced upregulation of IL-6 and CCL2 was inhibited by silencing *TLR4*, while TLR2-deficient tubular cells showed no significant reduction in cytokine production. High glucose induced the expression of *TLR4* but not of *TLR2* in mesangial cells (68). GIT27, an anti-TLR agent, showed an anti-proteinuric effect in db/db mice treated with an immunomodulator that targets macrophages by blocking TLR2, TLR4, and TLR6 signals (69). Additionally, GIT27 inhibited cytokine production induced by glucose and free fatty acids in cultured podocytes (11). Therefore, TLR4 signaling is implicated in inflammation in hyperglycemic patients.

Akin to TLRs, NLRs play an important role in innate immunity by triggering pro-inflammatory cascades when pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) trigger innate immunity (70, 71). A variety of human diseases are caused by the NLRP3 inflammasome complex, including cancer, liver disease, and DKD. NLRP3 inflammasome activation is a two-step process. First, priming by PAMP- or DAMP-induced activation of TLR signaling, resulting in NF-κB-dependent expression of NLRP3, pro-IL-1β, and pro-IL-18. Second, various cellular mechanisms, such as potassium efflux, pore-forming toxins, calcium influx, mitochondrial dysfunction, and intracellular ROS production, trigger the formation of the NLRP3 complex and activation of CASP1 (70). Nod-like receptor protein 3 (NLRP3) inflammasome activation is responsible for the accumulation of intracellular or plasma S-adenosyl-homocysteine, promoting hyperglycemia-induced podocyte injury (72). Nephropathy in db/db mice was preceded by inflammasome activation in podocytes and endothelial cells (73). Diabetes-induced hyperglycemia increased thioredoxin-interacting protein (TXNIP) expression in the kidneys of diabetic mice, resulting in NOX and inflammasome activation (3, 74), podocyte loss and the onset of albuminuria (75). In mice, angiotensin II also activated NLRP3 inflammasomes, produced mitochondrial dysfunction, and led to nephron and podocin loss, as well as albuminuria (76). Therefore, NLRP3 inflammasome activation induces podocyte apoptosis and mitochondrial dysfunction, resulting in proteinuria. Additionally, *NLRP3*-knockout mice with STZ-induced DKD had improved renal function, glomerulosclerosis, tubulointerstitial inflammation, and renal fibrosis (77).

The ER chaperone immunoglobulin-binding protein (BiP) or glucose-regulated protein 78 (GRP78) mediates the UPR signaling network. The UPR consists of three major branches, which are initiated by the activation of three protein sensors—inositol-requiring enzyme 1α (IRE1α), activating transcription factor 6 (ATF6), and protein kinase RNA (PKR)-like ER kinase (78, 79) (Figures 5, 6). Under physiological conditions, the transducers are bound to BiP and are inactive. Accumulation of misfolded or unfolded proteins under stress detaches BiP from the transducers, simultaneously activating them (55). Subsequently, spliced Xbp1 (XBP1s), which encodes a potent transcriptional activator, is cleaved by IRE1-mediated sequence-specific cleavage (Figure 5A). Moreover, ER stress reduces insulin signaling during the development of diabetes *via* activation of JNK, plus it induces pancreatic apoptosis, which worsens diabetes complications (80). Insulin supplementation can inhibit the transcriptional regulation of XBP1s and thereby alleviate inflammation (Figure 6A).

After separating from BiP, UP-mediated phosphorylation of protein kinase RNA-like ER kinase (PERK) activates downstream activating transcript factor 5 (ATF5) (81–83) and its target genes such as *Txnip*, thus inducing inflammation of kidney cells, such as podocytes (84) (Figure 5E). PERK is also implicated in macrophage immunosuppression and its downstream targets include ATF4, ATF5, and TXNIP (85).

Eukaryotic translation initiation factor-2α (eIF2α) phosphorylation reduces the translation of most mRNAs by inhibiting the delivery of the initiator Met tRNAi to the initiation complex, leading to reduced protein translation and protein loading into the ER, thereby alleviating the early stage of ER stress (86–88). Acute diseases typically benefit from an adaptive UPR, but chronic diseases (such as hyperglycemia) may be adversely affected by long-term or continuous UPR activation (89). In fact, eIF2α promotes the translation of a number of mRNAs containing short upstream open reading frames such as ATF4, activating downstream factors (*e.g.*, *CHOP*, Bax/Bcl-2 transcriptional expression) and inducing apoptosis (86). Unfortunately, the mechanism responsible for the transition of the UPR from an adaptive to a cytotoxic response is unclear (Figure 6B).

In the Golgi apparatus, proteins are continuously cleaved to release cytosolic effectors or proteins from the membrane and allow them to enter the nucleus. Under ER stress, the transport of ATF6 from the ER to the Golgi apparatus is mediated by Golgi-resident site 1 protease (S1P) and S2P in an intramembrane region to release the cytoplasmic DNA binding protein portion, the ATF6 fragment. The ATF6 fragment translocates to the nucleus to induce gene expression (90), including UPR target genes (Figure 6C).

All branches of the UPR may be linked to podocyte apoptosis. The UPR is activated by increased expression of BiP, p-IRE1α, p-eIF2α, CHOP, ATF-6, and XBP1s in hyperglycemic podocytes *in vitro* and *in vivo* (91). ER stress activates the PERK pathway, upregulating the expression of downstream factors related to apoptosis such as Bax, leading to podocyte apoptosis. Increased phosphorylated PERK (p-PERK), phosphorylated eIF2α (p-eIF2α), ATF4 and CHOP increased the Bax/Bcl-2 ratio, promoting podocyte apoptosis (92, 93). By contrast, there are reversed results after using shRNA or knockdown PERK directly and thus podocyte apoptosis was controlled. Inhibition of PERK maintained the structure and function of the glomerular filtration barrier by increasing the production of the slit-diaphragm proteins podocin/nephrin (92). However, there is no information on

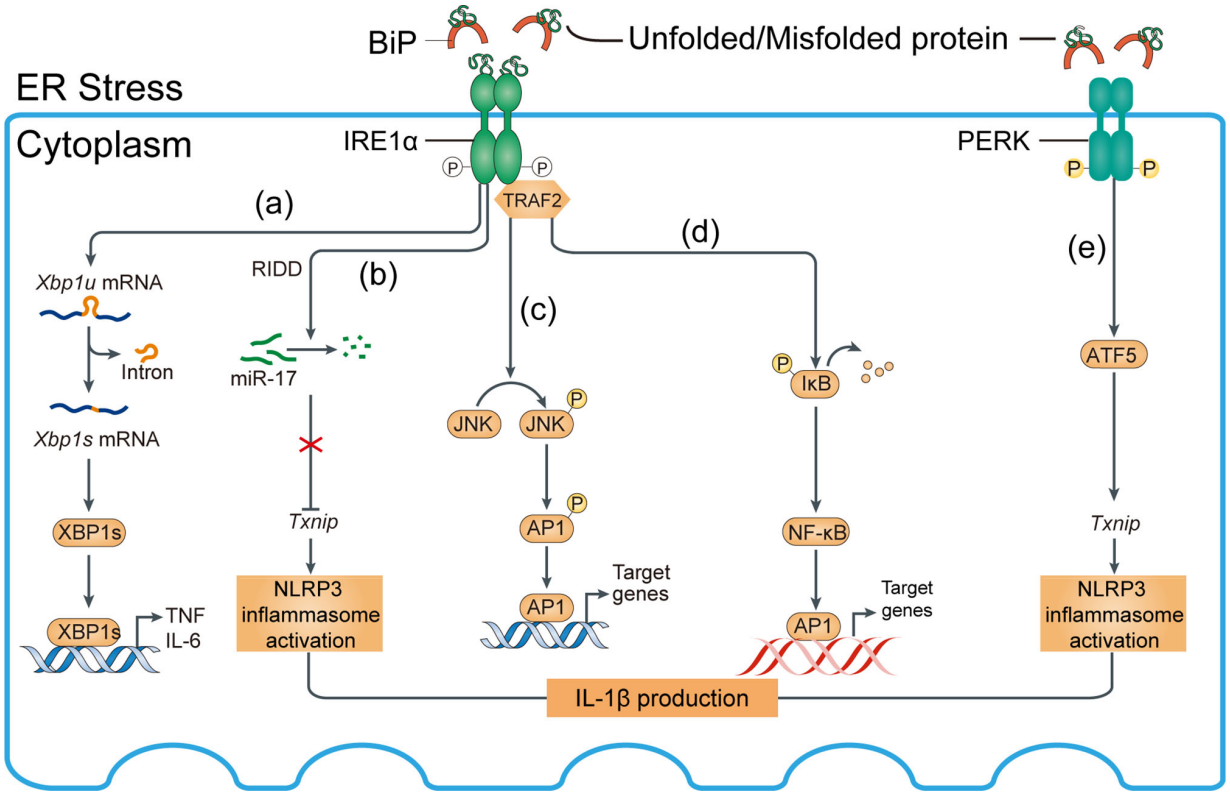

FIGURE 5
Mechanisms of ER stress-induced inflammation. Inositol-requiring enzyme 1α (IRE1α) and PKR-like ER kinase (PERK) activation induce inflammasome activation. **(A)** IRE1α activation and subsequent splicing of X-box binding protein 1 (Xbp1) produces the transcription factor XBP1s, which directly binds the promoters of tumor necrosis factor (TNF) and interleukin-6 (IL-6). **(B)** Regulated IRE1α-dependent decay (RIDD)-dependent degradation of miR-17, which in unstressed conditions represses thioredoxin-interacting protein (Txnip), increases the *Txnip* level, NLRP3 inflammasome activation, and IL-1β expression. **(C)** Activated IRE1α forms a complex with TNF receptor-associated factor 2 (TRAF2) to induce phosphorylation of Jun N-terminal kinase (JNK) and upregulation of proinflammatory genes *via* activated activator protein 1 (AP1). **(D)** IRE1α–TRAF2 complex recruits IκB kinase (IKK); subsequent phosphorylation and degradation of IκB releases nuclear factor-κB (NF-κB) for nuclear translocation. BiP, binding immunoglobulin protein. **(E)** *Txnip* can be induced by the PKR-like ER kinase (PERK)-activating transcription factor 5 (ATF5) pathway to induce inflammasome activation.

the other two UPR branches. Confusingly, suppression of the PERK pathway under ER stress exacerbated β-cell apoptosis in thapsigargin-induced primary human islets, mouse islets, and INS-1 beta cells (94). It is possible that activated PERK induces phosphorylation of eIF2α, suppressing protein translation and loading into the ER and thereby alleviating ER stress. Therefore, PERK is implicated in podocyte apoptosis in DN, but the mechanism warrants further research.

Targets for podocytopathy therapy

Epigenetic mechanisms

Histone modification and DNA methylation status are altered by hyperglycemia, causing chromatin structure to change from concentration (K9Me and K27Me) to relaxation (K4Me), activating the expression of pathogenic genes (Figure 4) (95). Various mechanisms can enable transcription factors and cofactors to access promoters and enhancers through histone acetylation, to enhance gene expression (96). However, insulin or anti-diabetic drugs continue to promote the expression of pathogenic genes after normoglycemia. The use of epigenetic drugs or induction of site-specific changes, such as locus modification or DNA methylation, may eliminate podocytopathies.

The structure-activity relationship (SAR) approach based on the resveratrol structure showed BF175 to be an effective SIRT1 agonist, activated by the downstream transcription factor PGC-1α. The protective effect of BF175 on diabetes podocytes was SIRT1 dependent (44). METTL14 expression was highest in mice with Adriamycin-induced DNA and an elevated m6A RNA level. Furthermore, METTL14 levels were increased in renal biopsies from diabetics, models of DN, and human podocytes cultured with advanced glycation end products. Mice with

podocyte-specific METTL14 deletions had improved glomerular function and alleviated podocyte injury, which was characterized by activation of autophagy and inhibition of apoptosis and inflammation (97).

There is an increase in METTL3 in podocytes from renal biopsy samples from patients with DN, which is associated with renal damage, in addition to METTL14 (46). *METTL3 Flox/Flox* (*METTL3*$^{fl/fl}$) mouse lines have been used as *METTL3*-deficient models (97). Further, STZ-induced diabetic mice with METTL3 podocyte-conditional knockouts showed significant improvement of podocyte injury and albuminuria. As a result of METTL3, TAB3 was modified by m6A, and its stability increased in a manner dependent on IGF2BP2. Genetic and pharmacological inhibition of METTL3 can attenuate renal injury and inflammation, suggesting that the METTL3/TAB3 axis is a potential therapeutic target in podocytopathies (98).

TXNIP-mediated oxidative stress and NLRP3 inflammasome activation has been decribed previously. A study by Yunjun Xiao et al. has shown that H3K27me3 is reduced trimethylated and its occupancy at promoters of early growth response 1 (EGR1) is enhanced by histone methyltransferase enhancer of zeste homolog 2 (EZH2). S-adenosylhomocysteine (SAH) is hydrolyzed by SAH hydrolase (SAHH) to homocysteine and adenosine. Adenosine dialdehyde (ADA), an inhibitor of SAHH, accumulates intracellular or plasma levels of the SAH levels and may exert as a regulator to hyperglycaemia-induced podocytopathy (72).

KLF4 epigenetics regulate podocyte phenotype and function, and the podocyte epigenome can be used to ameliorate proteinuria. A ChIP analysis of DNA methyltransferases (DNMTs) showed that DNMT1 binding to the nephrin promoter region was significantly reduced in KLF4-overexpressing podocytes, but its binding to the vimentin promoter was unaffected (99). To confirm that the transient upregulation of KLF4 has an extended effect on podocyte function, the *Tet-On* gene induction system induced by doxycycline was used to construct mice with KLF4-inducible and podocyte-specific overexpression. Doxycycline increased the expression of KLF4, and KLF4 was co-located with the podocyte marker nephrin, but not with the endothelial or mesangial marker desmin. Therefore, doxycycline may play an important role in the epigenetic regulation of KLF4 in podocytes.

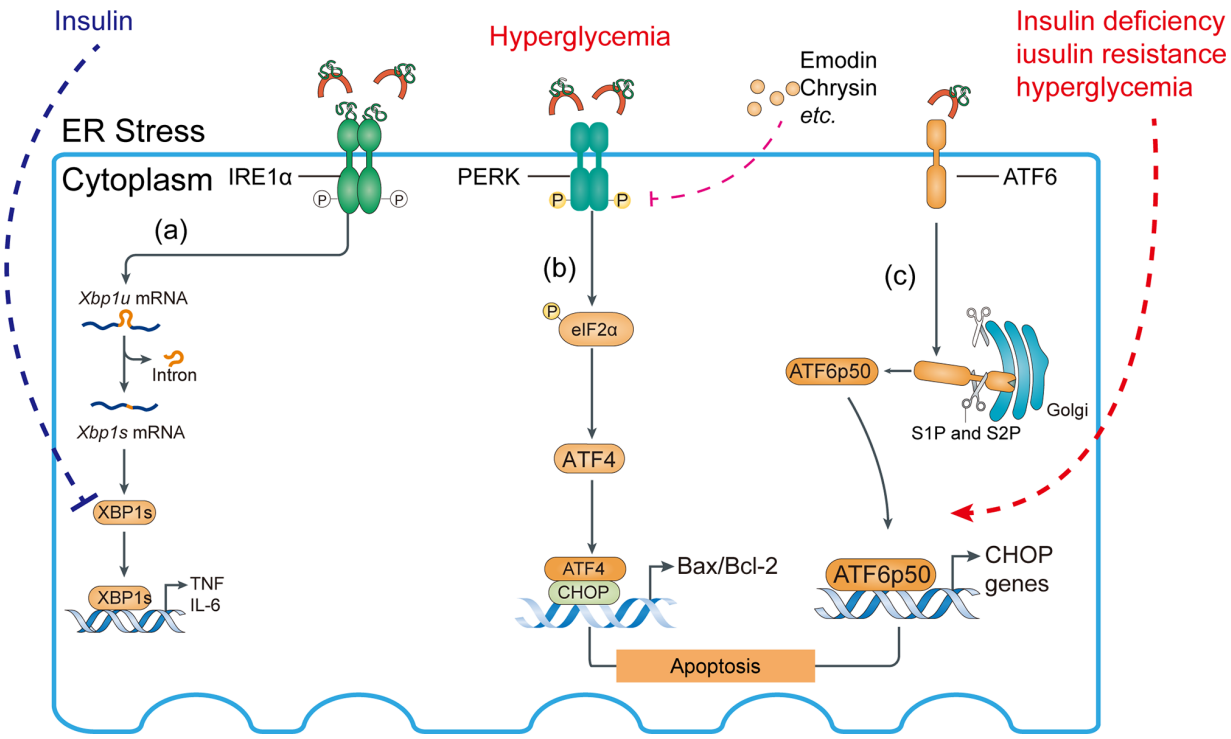

FIGURE 6
Activation of ATF6 pathway and abnormal IRE1 pathway may aggravate ER stress response. **(A)** Role of the IRE1 pathway in the ER stress response. Insulin signaling inhibits the interaction of XBP1s with the phosphatidylinositol 3-kinase (PI3K) regulatory subunits p-85α and p-85β, suppressing proinflammatory cytokine production. **(B)** Compounds such as emodin and chrysin inhibit PERK activation and decrease the Bax/Bcl-2 ratio in hyperglycemia, preventing podocyte apoptosis. **(C)** Insulin deficiency, insulin resistance, and hyperglycemia aggravate site 1 protease (S1P)- and S2P-mediated cleavage of ATF6α, allowing their fragments to translocate to the nucleus.

Hyperglycemia suppresses RCAN1 expression in cultured podocytes, which is alleviated by pretreatment with the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine. Mechanistically, increased expression of RCAN1 decreased hyperglycemia-induced nuclear factor of activated T cells (NFAT) transcriptional activity. Increased RCAN1 expression also stabilized actin cytoskeleton organization, consistent with inhibition of the calcineurin pathway (100). Hence, 5-Aza-20-deoxycytidine may suppress the expression of CXCL2, NFAT, and Wnt6 in hyperglycemic podocytes (Table 1).

miRNA-mediated epigenetic regulation of inflammatory gene expression is implicated in diabetes complications (101). MicroRNA-10 targets the NLRP3 inflammasome to suppress inflammation in diabetic kidneys. Patients with DKD and diabetic mice with miR-10a/b had low levels of miR-10a and -10b, which are expressed predominantly in podocytes. When miR-10a and b were delivered to the kidneys, NLRP3 inflammasomes were not activated and renal inflammation was prevented. By contrast, miR-10a/b knockdown increased activation of the NLRP3 inflammasome. Thus, upregulation of miR-10a/b could prevent podocytopathy in hyperglycemics (102) (Figure 7).

miRNA-27a is upregulated in podocytes of DN patients, possibly enhancing podocyte migration, diffusion, and apoptosis (103). Furthermore, miRNA-27a causes podocyte injury and apoptosis by targeting ER stress (52). The expression of miR-378 was downregulated in DN rats and high glucose-mediated podocytes, promoting podocyte apoptosis, which was reversed by inhibiting the expression of tumor-necrosis factor receptor associated factor 5 (TRAF5) by miR-378 (104). Moreover, miRNAs may be related to DN pathogenesis because they affect the expression of genes in the UPR signaling pathways. MiR-204 directly inhibits its target PERK under ER stress, exacerbating ER stress-induced apoptosis (105). MiR-204 targets the insulin transcription factor MAFA and thereby inhibits insulin translation and synthesis, indirectly activating ER stress (105–107). MiR-15b-5p was downregulated in patients with DN and could cause increased apoptosis in human kidney cells supported by elevated active caspase-3 and decreased viability and proliferation (108), linking miRNAs to the pathogenesis of DN.

As a competitive RNA (ceRNA), lncRNAs interact with and competitively regulate microRNAs (52, 53, 109). When lncRNA MALAT1 is decreased, microRNA let-7f is upregulated and KLF5 is inhibited, reducing podocyte damage in DN (110). miR-130a-3p competes with TLR4 as an endogenous sponge, as well as TLR4 as a miR-130a-3p target gene. As a result of miR-130a-3p/TLR4 crosstalk, MIAT knockdown protected podocytes from hyperglycemia-induced damage. Reduced MIAT expression restores slit-diaphragm integrity, attenuates foot process effacement, prevents dedifferentiation, and suppresses mitogenic catastrophe in podocytes during hyperglycemia. In response to ER stress, lnc-MGC and miR-379 cluster miRNAs are increased, and DN phenotypes (hypertrophy and fibrosis) are aggravated (111). Those findings show that miRNAs and lncRNAs are related to ER stress.

The lncRNA plasmacytoma variant translocation 1 (PVT1; 1.9 kb) is linked to kidney disease and encodes many alternative transcripts but not a protein. Silencing of PVT1 inhibits

TABLE 1 Reports of epigenetic drug treatment on podocytes during DN progression.

No.	Agents	Target genes/proteins	Signaling Route	Trends	Experimental model	Cytokines	Epigenetic machinery in podocytes
1	BF175	Sirt1	Sirt1 PGC-1α	↑ ↓	Diabetic OVE26 mice	(NA)	Improving the mitochondrial function and homeostasis
2	Adriamycin	METTL14	Sirt1	↓	C57BL/6J mice	Inhibited MCP-1, IL-6 and TNF-α	Promoting autophagy and inhibiting apoptosis and inflammation
3	Anti-METTL3 antibody	METTL3	p-p65 NF-κB	↓	Mouse podocytes (MPC5)	Inhibited TNF-α, interleukin-1β (IL-1b), and MCP-1	Relaxed chromatin and increased cell content
4	Cpd-564	METTL3	NF-κB	↓	*METTL3* cKO mice	(NA)	Inhibiting the inflammatory response and programmed cell death
5	Adenosine dialdehyde (ADA)	S-adenosylhomocysteine hydrolase (SAHH) and H3K27me3 at EGR1 promoter	TXNIP signaling	↑	C57BL/6J mice and human podocyte cell line	NLRP3 inflammasome activation	Inhibiting S-adenosylhomocysteine but aggravated oxidative stress
6	Doxycycline	DNMT1/H3K9ac	KLF4 expression	↑	human podocyte cell line	Increased nephrin	Increases nephrin promoter activity
7	5-Aza-2'-deoxycytidine	*RCAN1*	Calcineurin-NFAT	↓	*RCAN1*$^{-/-}$ mice and human podocyte cell line	Decreased CXCL2	Reduces podocyte apoptosis and stabilized actin cytoskeleton organization

↑ Indicate increased expression, ↓ Indicate decrease.
NFAT, nuclear factor of activated T cells; RCAN1, regulator of calcineurin 1.

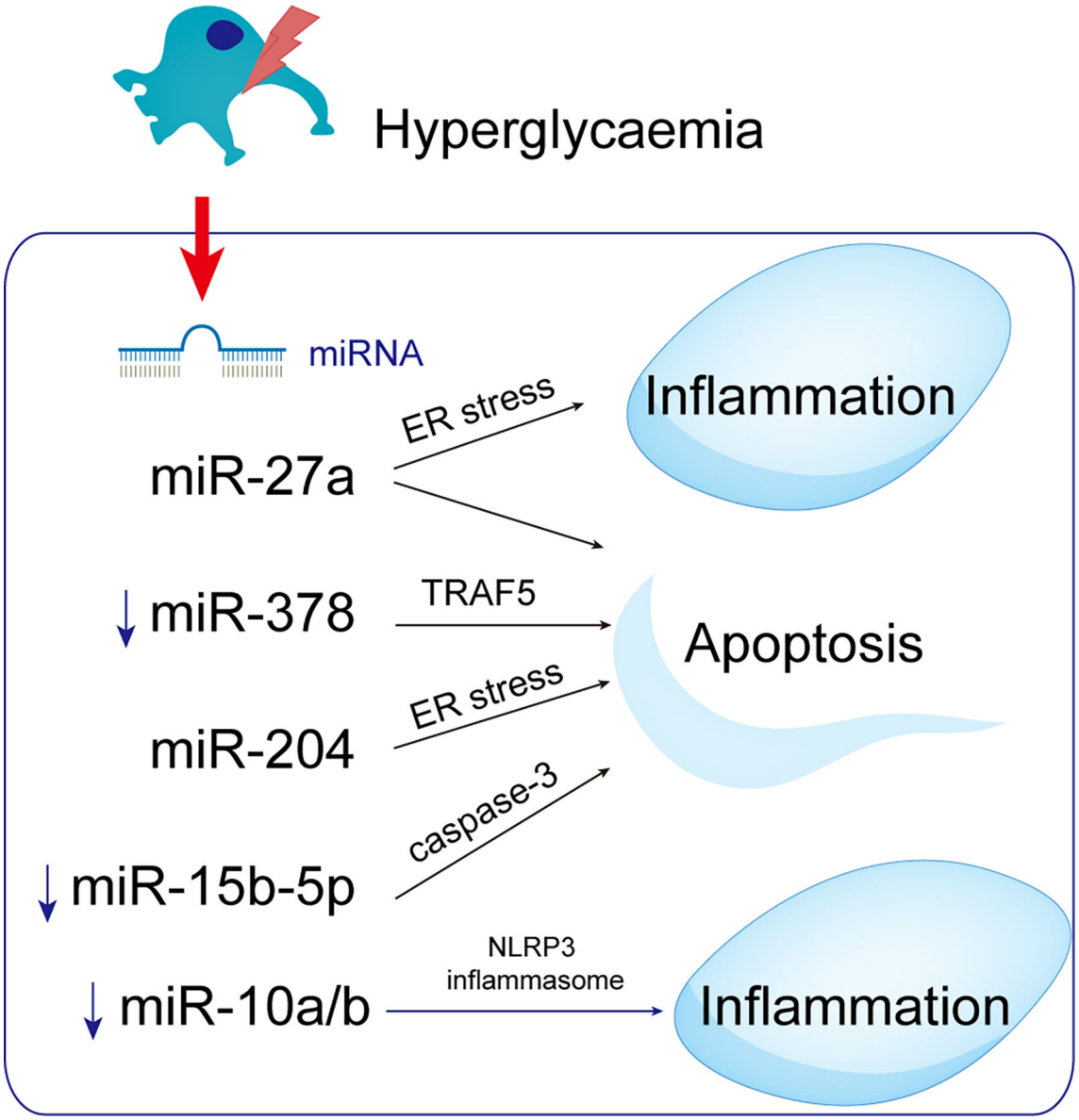

FIGURE 7
MicroRNAs with potential roles in podocytopathies. Hyperglycemia up- or down-regulates miRNAs in podocytes, resulting in podocytopathies. ↑ Indicate increased expression, ↓ Indicate decrease.

podocyte damage and apoptosis *via* the forkhead box A1 (FOXA1) in DN, which is of clinical importance (112). On human chromosome 20, small nucleolar RNA host gene 17 (SNHG17) is highly expressed in colorectal cancer tissues (113). SNHG17 controls mitophagy-induced apoptosis in diabetic podocytes. lncRNA SNHG17 knockdown promotes Parkin-dependent mitophagy and reduces apoptosis of podocytes by regulating the degradation of mammalian sterile 20-like kinase 1 (MST1) (114).

The AKT/mTOR pathway inhibits autophagy in a variety of cell types, including cancer cells, cardiomyocytes, and podocytes (115). The SPAG5/AKT/mTOR pathway inhibits podocyte autophagy and aggravates apoptosis. Therefore, SPAG5-AS1/SPAG5 has therapeutic potential for podocytopathies and DN (116) (Figure 8).

Therapies targeting in ER stress

There has been considerable interest in targeting ER stress as a therapy to improve DN. A variety of natural compounds

inhibit ER stress and so may have therapeutic value for podocytopathies.

Natural products that modulate ER stress are listed in Table 2. ER stress can be regulated by inhibiting UPR sensors and their downstream factors. Emodin (93), chrysin (92), berberine (119), huaiqihuang (118) and epigallocatechin 3-gallate (EGCG) (122) inhibit the PERK signaling pathway to alleviate ER stress and improve DN. MC-TG (117) reduces ER

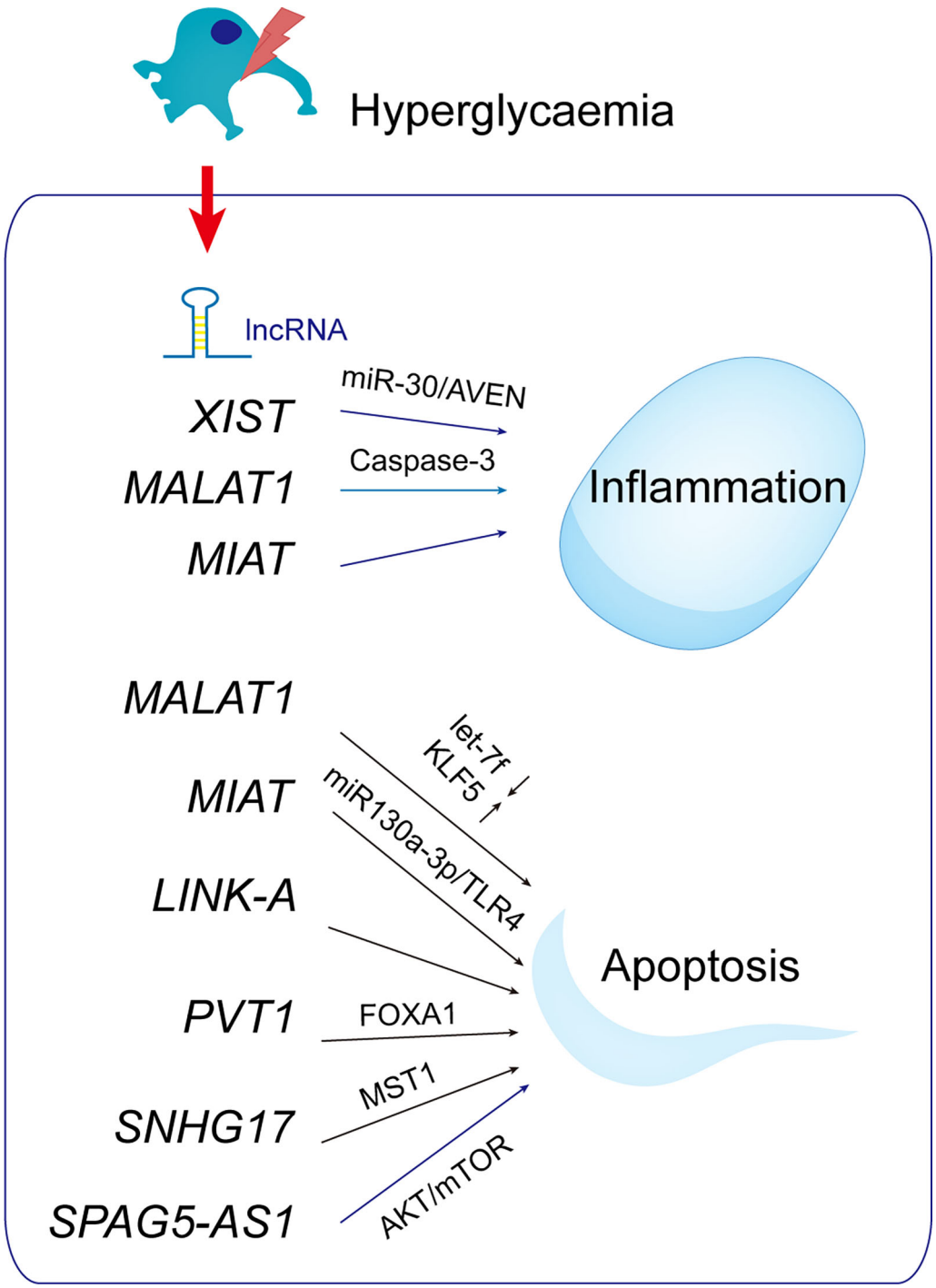

FIGURE 8
LncRNAs with potential roles in podocytopathies. Hyperglycemia up- or down-regulates lncRNAs in podocytes, resulting in podocytopathies.

TABLE 2 Therapies targeting ER stress.

Agents	Mechanisms
Emodin93	Inhibition of PERK
Chrysin92	
MC-TG117	Inhibition of IRE1α/NF-kB
Huaiqihuang118	Inhibition of GRP78, CHOP
Berberine119	
OA or NAC120	Suppression of ER stress
AS-IV121	Upregulation of SERCA
EGCG122	Inhibition of p-PERK, GRP78, CHOP
4-PBA and UDCA60, 123	Enhances protein folding efficiency

ER: endoplasmic reticulum; 4-PBA, 4-phenylbutyric acid; IRE1α, inositol-requiring enzyme 1α; PERK, PRKR-like ER kinase; UDCA, ursodeoxycholic acid; XBP1, X box-binding protein 1; NF-κB, nuclear factor-κB; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; EGCG, Epigallocatechin-3-gallate; OA, Oleanolic acid; NAC, N-acetylcysteine; MC-TG, Terpene glycoside component from Moutan Cortex; AS-IV, Astragaloside IV; Sarco/endoplasmic reticulum Ca^{2+}-ATPase2 (SERCA2) SERCA 2.

stress and inflammation, possibly by inhibiting IRE1/NF-κB. Also, OA and NAC (120) inhibit the three UPR sensors to suppress ER stress. ER stress is triggered by impaired SERCA2 activity or expression, hampering Ca^{2+} homeostasis (124). AS-IV may alleviate ER stress by increasing SERCA2 expression, thus restoring intracellular Ca^{2+} homeostasis (121). ER chaperones such as 4-phenylbutyrate (4-PBA) and ursodeoxycholic acid (UDCA) enhance the folding capacity of the ER (91, 123). Therefore, natural compounds have potential for inhibiting ER stress and thus attenuating DN.

Preventing ER stress can improve DN and attenuate oxidative stress. The effect of hyperglycemia-mediated oxidative stress on podocytes suggests that podocyte oxidation is activated by upregulating ER markers after exposure to high glucose for 24 hours, whereas inhibition of ER stress by ER inhibitors diminishes oxidative stress and exerts a renoprotective effect (60). Similarly, crosstalk between ER stress and oxidative stress mediated by aldosterone contributes to podocyte injury, which can be ameliorated by berberine (119). Therefore, novel therapeutic strategies aimed at attenuating mitochondrial dysfunction may ameliorate palmitic acid-induced podocyte injury.

Autophagy delivers proteins and damaged organelles to lysosomes for degradation and recycling to maintain intercellular hemostasis. Autophagic self-repair is important in neurons, podocytes, and other cells in the anaphase of division because their differentiation and proliferation are limited (125). Resveratrol attenuated hyperglycemia-induced apoptosis by activating autophagy in db/db mice and podocytes (51). miR-383-5p overexpression significantly inhibited autophagy and enhanced apoptosis, effects reversed by resveratrol. Therefore, modulation of autophagy may be a novel therapeutic approach for DN. Activated eIF2α upregulates the mRNA level of ATF4, which transcriptionally regulates autophagy factors to induce autophagy. Also, phosphorylation-dependent selective translation upregulates Atg12 expression and stimulates Atg5-Atg12-Atg16 complex formation, thereby initiating autophagy. This could promote cell survival *via* autophagic decomposition of cellular components to generate energy under adverse conditions (126). Therefore, modulation of the crosstalk between ER stress and autophagy may have therapeutic potential for DN.

Conclusion

A hyperglycemic environment can induce podocytopathy. As podocyte function declines, so does glomerular function. Podocyte inflammation and apoptosis are the main causes and characteristics of early proteinuria in DN. Although epigenetic mediators (*e.g.*, HDACs and DNMTs) are not genes, they regulate inflammation and apoptosis, and modulate the inflammatory response and apoptosis induced by ER stress in hyperglycemia. Therefore, protecting podocytes from deleterious effects can maintain the filter membrane, and thereby prevent DN. Natural drugs may also be useful for treating podocytopathy by transcriptionally regulating downstream factors of ER stress. We reviewed the epigenetic mechanisms and natural substances that regulate ER stress and inflammatory signaling pathways in podocytosis, with the aim of identifying new therapeutic targets in progressive DN.

Author contributions

XW and JR reviewed the literature and drafted the manuscript. JZ and YL participated in the conception and interpretation of the relevant literature for the manuscript. The final version of this review has been edited, revised critically, and approved by all authors.

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Conflict of interest

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

Publisher's note

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DNA methylation change of HIPK3 in Chinese rheumatoid arthritis and its effect on inflammation

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Introduction: Homeodomain-interacting protein kinase 3 (HIPK3) plays an important role in cell proliferation, apoptosis, and inflammation. Over-expression of HIPK3 in immune cells in rheumatoid arthritis (RA) has been reported. In this study, we investigated blood methylation levels and clinical characteristics of RA in a Chinese population.

Methods: A total of 235 patients with RA, 30 with osteoarthritis (OA), and 30 matched healthy controls were recruited. The methylation status of seven CpGs in the differentially methylated region of HIPK3 (cg05501357) was measured using targeted methylation-sequencing technology. The association between methylation haplotypes and the overall methylation status of HIPK3 with clinical characteristics was assessed using generalized linear regression.

Results: All seven CpGs showed hypomethylation status in RA blood compared with OA and normal individuals (overall p= 1.143×10^{-8} and FDR= 2.799×10^{-7}), which is consistent with the previously reported high expression of HIPK3 in RA immune cells. Among all seven CpGs, 33286785 showed the highest predictive power with an area under the curve (AUC) of 0.829; we received a higher AUC=0.864 when we combined HIPK3 with anti-citrullinated protein antibodies (ACPA -) and rheumatoid factor (RF +) in the prediction model, indicating that when a patient's ACPA is negative, HIPK3 can assist RF as a new clinical index for the diagnosis of RA. We also found that HIPK3 methylation levels were negatively correlated with C-reactive protein (CRP; r= -0.16,

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p= 0.01). Methylation haplotypes were analyzed, and the full methylation haplotype (FMH; r= 0.16, p= 0.01) and full non-methylation haplotype (FNH; r= 0.18, p= 0.0061) were negatively correlated with CRP.

Conclusion: Circulating blood methylation levels in the protein region of HIPK3 can be utilized as a supportive diagnostic biomarker and CRP level indicator for RA.

KEYWORDS

DNA methylation, rheumatoid arthritis, inflammation, HIPK3, methylation haplotypes

Introduction

Rheumatoid arthritis (RA) is characterized by an abnormal or excessive inflammatory response. Under the action of certain disease factors, patients trigger an inflammatory response in the body and form a storm of inflammatory factors (1). Persistent inflammation can cause joint pain, progressive destruction, and joint dysfunction (2). In addition, inflammation may cause various complications, such as cardiovascular diseases (3). Therefore, effective control of inflammation is key to the treatment of RA, and can even be used as an indicator to evaluate the effectiveness of treatment. Doctors can also decide whether to adjust the treatment plan according to the progress of inflammation. It is important to study the specific molecular biological mechanisms of RA and identify inflammation-related biomarker.

Epigenetic mechanisms (4), such as deoxyribonucleic acid (DNA) methylation, participate in many important life activities (5), including cell differentiation and proliferation, organism aging (6), and tumorigenesis (7), and play an important role in the regulation of gene expression (8). In recent years, the potential role of DNA methylation in RA development has attracted the attention of researchers (9, 10). DNA methylation affects different aspects of RA and modulates epigenetic silencing of genes and cellular behavior, especially in fibroblast-like synoviocytes (FLS), whose abnormal proliferation promotes persistent inflammation and joint damage (11, 12). Thus, aberrant DNA methylation occurs in the pathogenesis of RA and contributes to its development.

Homeodomain-interacting protein kinase3 (HIPK3) is a group of conserved serine/threonine kinases that can regulate different transcription factors that to influence developmental processes, such as cell proliferation, differentiation, apoptosis and inflammatory responses (13). The conservation of HIPK3 is relatively low and it shares many functional domains with HIPK2 (14). A study found that the expression levels of HIPK3 mRNA and protein were significantly down-regulated in human non-small cell lung cancer (NSCLC) tissues, and HIPK3 silencing promoted the invasion and metastasis of NSCLC (15). Thus, HIPK3 may be a valuable biomarker for predicting the prognosis of patients with non-small cell lung cancer. In addition, HIPK3 may also be a novel kinase regulator of autophagy in Huntington disease (HD) cells, contributing to the accumulation of proteins and disease progression. Targeting HIPK3 may provide drug discovery opportunities for the treatment of HD and similar diseases (16).

Currently, there are few studies on HIPK3, particularly regarding its relationship with RA. In this study, we explored the relationship between HIPK3, methylation haplotypes in whole blood, and clinical indicators in patients with RA, especially the correlation between the inflammatory indicator CRP and erythrocyte sedimentation rate (ESR). The results may provide potential value for clinical applications to predict the degree of inflammation in patients with RA.

Materials and methods

Participants and peripheral blood collection

We recruited participants (235 RA patients, 30 with OA patients, and 30 healthy controls) between October 20 and November 30, 2021, from the Guanghua Hospital Precision Medicine Research Cohort [PMRC] (17). The inclusion criterion for RA was the American College of Rheumatology (ACR) 2010 criteria (18). The clinical data of all participants were recorded, and whole blood samples were collected. This study was approved by the Ethics Committee of the Guanghua Hospital (Approval No. 2018-K-12), and all participants provided written informed consent.

DNA methylation detection for HIPK3

Genomic DNA was extracted from the peripheral blood of patients with RA, patients with OA, and healthy controls.

Sample integrity: For agarose electrophoresis detection, the main strip was required to be clear, with no obvious dispersion and tailing. DNA was extracted using the EZ DNA Methylation-Gold Kit (ZYMO, CA, USA), as recommended by the manufacturer. Subsequently, a single PCR step was used to amplify the target region of the transformed sample. The primer sequences for HIPK3 were as follow that primer F, ATTTTGTTTTGATTTTGTGGTAGTTGTT; and primer R, CAAAAATCATAACAACTCAAACACAAC. Then, dilute the mixed single PCR products 10-20 times and specific tag sequences were added. Using primers with an index sequence, a specific tag sequence compatible with the illumina platform was introduced at the end of the library *via* PCR amplification. Finally, FastQ data were obtained by high-throughput sequencing using the 2×150 bp double-terminal sequencing mode on an Illumina Hiseq (Illumina, CA, USA).

Statistical analyses

Spearman's rank correlation analysis was used to assess the association between DNA methylation level and clinical data of patients with RA. Using One-Way ANOVA test to calculate the P values in baseline analysis. Using Kruskal-Wallis rank sum test to analysis the methylation difference level between RA, OA and healthy groups. Clinical significance of HIPK3 methylation level analyzed by receiver operating characteristic curve (ROC curve). All statistical analyses were performed by the IBM SPSS version 20.0 software (IBM Inc, Armonk, New York, USA), GraphPad Prism software (version 9.0), and Sangerbox (19).

Results

Basic information of participants

The study included 235 patients with RA, 30 patients with OA, and 30 healthy individuals, who were divided into RA, OA, and healthy groups. In the RA group, 203 cases were RF (+), accounting for 86.38% of the total, and 213 cases were ACPA (+), accounting for 90.64%. In baseline analysis, using One-Way ANOVA test to calculate the P values showed that there were no significant differences in age (p= 0.460), height (p= 0.376), and weight (p= 0.525) among the three groups, but there were significant statistical differences in erythrocyte sedimentation rate (ESR; p= 0.000) and C-reactive protein (CRP; p= 0.000) (Supplement Table 1). The levels of CRP and ESR are important indicators for the diagnosis of OA and RA, and the degree of inflammatory activity. The increase of ESR and CRP levels in patients with active OA and RA can not only aggravate joint swelling, but also indirectly reflect the degree of bone, joint and organ damage, which is one of the important indicators for the diagnosis of OA and RA.

Differences in methylation level and correlation of HIPK3

Using the Kruskal-Wallis rank sum test to calculate the P value between RA, OA and healthy groups. The whole blood methylation study found that HIPK3 (cg05501357) was down-regulated in patients with RA ($P= 1.143\times10^{-8}$; Figure 1A). Further analysis showed that HIPK3 has seven CpGs, including 33286847, 33286832, 33286800, 33286785, 33286752, 33286747, and 33286724. Using the Kruskal-Wallis rank sum test to calculate the P values between RA, OA and healthy groups. The difference analysis showed that the methylation levels of these CpGs in patients with RA were down-regulated (Figure 1B). The internal distribution can be perfectly shown by scatter plot, fitting curve and matrix diagram. Spearman's rank correlation analysis was used to assess the association between two groups of data, and Based on the network data matrix, the correlation values between CpGs are analyzed and displayed visually by using the matrix correlation analysis and visualization function of Sangerbox (19) tool. An association analysis among all CpGs showed that 6847 and 6832, 6800 and 6752 have strong correlation ($|r|>0.8$), and 6847 and 6785, 6832 and 6785, 6800 and 6785, 6724 and 6785, 6752 and 6724 have weak correlation. In general, there is a relatively close relationship between these CpGs (Figure 1C). In the correlation analysis between the methylation level of HIPK3 and clinical indicators, it was found that HIPK3 was negatively correlated with CRP (r= -0.16, p= 0.01), and its seven CpGs also showed the same trend (Table 1). After that, the grouping analysis of RA patients showed that there was a stronger correlation between HIPK3 and CRP (r= -0.49, p= 0.00049) in patients with $2.6 \leq$ DAS28-CRP ≤ 3.2, indicating that HIPK3 has a higher clinical value in predicting CRP levels in these patients (Figure 1D). In addition, the receiver operating characteristic curve (ROC) analysis of HIPK3 and the seven CpGs showed AUC scores were of 0.821, 0.823, 0.817, 0.818, 0.829, 0.798, 0.788, and 0.821 (Supplement Figure 1A). RF and ACPA are unable to play a role in diagnosing RA in patients with RF (–)/ACPA (-) (AUC=0.023; Supplement Figure 2). In this context, the detection of HIPK3 methylation can play a role in diagnosing RA to a certain extent. After joint analysis with RF (-)/ACPA (-) (AUC= 0.742; Figure 1E), which has better clinical diagnostic significance. Subsequently, multi-factor joint analysis showed that HIPK3 combined with RF and ACPA, and when the patient's ACPA was negative, HIPK3 could assist RF as a new clinical index for the diagnosis of RA (AUC= 0.864; Figure 1E).

Differences in methylation level and correlation of haplotype sites

Similarly, the haplotype sites were also found to have significant differences in methylation in RA patients, OA

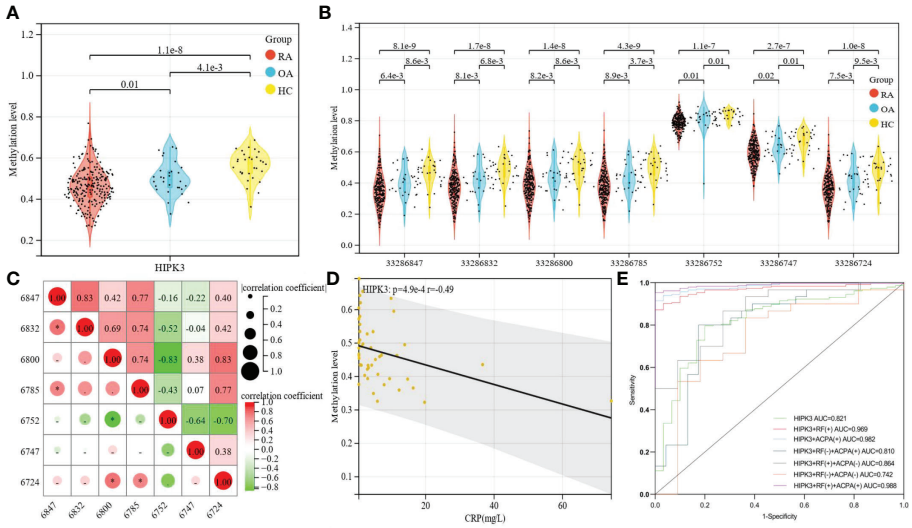

FIGURE 1
Differences in methylation level and correlation of HIPK3. **(A)**, Methylation level of HIPK3 between RA, OA patients and healthy controls; The result found that the methylation level of HIPK3 was down-regulated in patients with RA (P= 1.143×10^{-8}). **(B)**, Methylation level of seven CpGs in different patients; Further analysis showed that HIPK3 has seven CpGs, including 33286847, 33286832, 33286800, 33286785, 33286752, 33286747, and 33286724; The difference analysis showed that the methylation levels of these CpGs in patients with RA were down-regulated, and the statistical difference of 33286785 is the most significant. **(C)**, Correlation analysis in different CpGs; The association analysis among seven CpGs showed that 6847 and 6832, 6800 and 6752 have strong correlation (|r|>0.8), and 6847 and 6785, 6832 and 6785, 6800 and 6785, 6724 and 6785, 6752 and 6724 have weak correlation. In general, there is a relatively close relationship between these CpGs. **(D)**, Correlation between HIPK3 and CRP in patients with 2.6 ≤ DAS28-CRP ≤ 3.2; In patients with 2.6 ≤ DAS28-CRP ≤ 3.2, there was a stronger correlation between HIPK3 and CRP (r= -0.49, p= 0.00049), indicating that HIPK3 has a higher clinical value in predicting CRP levels in these patients. **(E)** Multi-factor joint ROC analysis of HIPK3 combined with RF and ACPA; In multi-factor joint analysis, the results showed that compare with RF/ACPA, HIPK3 has more valuable clinical diagnostic significance in patients with RF (-)/ACPA (-) (AUC=0.742 vs 0.023). In addition, in patients with RF (+)/ACPA (+), adding the detection of HIPK3 methylation level was helpful to improve the diagnosis of RA (AUC=0.988), and HIPK3 could assist RF/ACPA as a new clinical index for the diagnosis of RA. *: P<0.05.

TABLE 1 Correlation analysis between the methylation level of different CpGs and clinical data.

Group	HIPK3	33286847	33286832	33286800	33286785	33286752	33286747	33286724
Age	0.05(0.42)	0.05(0.45)	0.05(0.46)	0.05(0.48)	0.05(0.46)	0.11(0.08)	0.10(0.14)	0.04(0.54)
BMI	0.02(0.78)	0.03(0.61)	0.02(0.73)	0.02(0.80)	0.02(0.72)	0.02(0.71)	0.03(0.70)	0.0022(0.97)
Course of disease	-0.04(0.58)	-0.04(0.51)	-0.04(0.55)	-0.04(0.52)	-0.04(0.55)	-0.03(0.66)	-0.004(0.95)	-0.04(0.54)
Age of onset	0.07(0.31)	0.07(0.29)	0.07(0.30)	0.07(0.30)	0.07(0.29)	0.09(0.16)	0.07(0.27)	0.06(0.33)
TJC	-0.02(0.74)	-0.02(0.76)	-0.03(0.69)	-0.02(0.76)	-0.02(0.73)	-0.01(0.83)	0.0092(0.89)	-0.02(0.77)
SJC	-0.02(0.77)	-0.03(0.70)	-0.01(0.86)	-0.01(0.85)	-0.02(0.81)	-0.04(0.50)	-0.0003(1)	-0.03(0.69)
VAS	-0.08(0.22)	-0.08(0.20)	-0.07(0.27)	-0.07(0.25)	-0.08(0.23)	-0.11(0.08)	-0.06(0.35)	-0.08(0.24)
RF	-0.02(0.75)	-0.0079(0.90)	-0.0053(0.93)	-0.005(0.94)	-0.01(0.85)	-0.06(0.39)	-0.06(0.39)	-0.02(0.74)
ACPA	0.04(0.57)	0.03(0.60)	0.04(0.55)	0.04(0.57)	0.04(0.55)	0.09(0.16)	0.02(0.81)	0,0.02(0.8)
ESR	-0.10(0.13)	-0.11(0.09)	-0.10(0.14)	-0.08(0.24)	-0.09(0.19)	-0.15(0.02)	-0.09(0.17)	-0.08(0.23)
CRP	-0.16(0.01)	-0.18(0.0061)	-0.16(0.02)	-0.14(0.04)	-0.15(0.02)	-0.18(0.0071)	-0.16(0.01)	-0.16(0.02)
DAS28-ESR	-0.08(0.20)	-0.09(0.17)	-0.08(0.22)	-0.07(0.27)	-0.08(0.23)	-0.11(0.08)	-0.05(0.41)	0.25 (-0.08)
DAS28-CRP	-0.09(0.16)	-0.10(0.14)	-0.09(0.17)	-0.08(0.21)	-0.09(0.19)	-0.11(0.10)	-0.06(0.35)	-0.09(0.18)

Correlation result format: r (p).

patients and healthy controls (Figure 2A). Correlation analysis showed that the methylation level of CCCCCCT was negatively correlated with CRP (r= -0.17, p= 0.01) and ESR (r= -0.20, p= 0.0023), and CCCCCCC was negatively correlated with CRP (r= -0.16, p= 0.01) (Table 2). TTTTTTT indicated that there was no methylation in the HIPK3 gene, and in this state, the TTTTTTT level was positively correlated with ESR and CRP. Subsequently, when CCCCCCT, CCCCTCC, and CCCCCCC methylation occurred, the correlation between ESR and CRP also changed, and compared with TTTTTTT, the correlation was different (Figures 2B, C). Further association analysis showed that in patients with RF (-)/ACPA (-), CCCCCCC was negatively correlated with CRP level (r= -0.73, p= 0.01; Figure 2D). However, in patients with RF (+)/ACPA (+), CCCCCCT was negatively correlated with CRP (r= -0.17, p= 0.02) and ESR (r= -0.27, p= 0.00018) (Supplement Figures 1B, C). In patients with 2.6 ≤ DAS28-CRP ≤ 3.2, CCCCCCC (r= -0.44, p= 0.0019) (Figure 2E) and CCCCCCT (r= -0.42, p= 0.0033) (Supplement Figure 1D) were also negatively correlated with CRP.

Discussion

Rheumatoid arthritis (RA) is a systemic autoimmune disease caused by genetic and environmental factors. Owing to its heterogeneity and multiplicity, the etiology of RA remains largely unknown (20). Numerous studies have explored the attribution of the development of RA to genetic, infectious, and environmental factors (21–23). However, an increasing number of studies have shown that epigenetics plays an important role in the pathogenesis of RA, especially DNA methylation (24). Many studies have explored the pathogenesis of RA, but the interference of different epigenetic alterations in RA is not fully understood.

In addition to the comprehensive effects of environmental factors, genetic factors, and immune system disorders, inflammation plays an important role in the pathogenesis and pathological progression of RA (25). For example, interleukins (IL) can activate and regulate immune cells and mediate the activation of T and B cells, and their proliferation and differentiation play an important role in the inflammatory response. IL-1 and IL-6 were found to play a positive regulatory role and participate in the regulation mechanism of bone destruction in RA (26), while IL-23 is highly expressed in the serum of RA patients and is positively correlated with CRP and DAS-28 scores (27). Therefore, inflammatory reactions have received considerable attention in pathological studies of RA. CRP and ESR are associated with infection and tissue injury. They are non-specific markers of inflammation and directly participate in a series of acute and chronic inflammatory diseases, such as RA (28, 29). In this study, the methylation level of HIPK3 was negatively correlated with CRP (r= -0.16, p= 0.01). This confirmed the close relationship between the methylation levels of HIPK3 and CRP levels. In addition to CpGs, haplotype sites also serve as a medium for DNA methylation. Correlation analysis showed that the methylation level of CCCCCCT was negatively correlated with CRP (r= -0.17, p= 0.01) and ESR (r= -0.20, p= 0.0023), and CCCCCCC was negatively correlated with CRP (r= -0.16, p= 0.01).

Rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) are autoantibodies used in patients with RA. A study found that RF and ACPA have a synergistic effect in predicting developmental and bone erosion phenotypes (30), and their discovery has greatly promoted the early diagnosis and treatment of RA (31). Based on the above theoretical studies, we combined HIPK3 with RF (+), ACPA (+), RF (-)/ACPA (-) and RF (+)/ACPA (+) for ROC analysis. The results show that combined analysis can improve the clinical diagnostic value of HIPK3, especially in patients with RF (-)/ACPA (-) and RF (+)/ACPA (+).

The DAS-28 score is commonly used to evaluate disease activity in patients with RA; 2.6 ≤ DAS28-CRP ≤ 3.2 means that the patient was in a state of mild disease activity. Further research found that in patients with 2.6 ≤ DAS28-CRP ≤ 3.2, the methylation levels of HIPK3 (r= -0.49, p= 0.00049), CCCCCCC (r= -0.44, p= 0.0019), and CCCCCCT (r= -0.42, p= 0.0033) were negatively correlated with CRP, and the correlation is extremely high. Therefore, we can infer that in patients with RA, the methylation levels of HIPK3, CCCCCCC, and CCCCCCT are more sensitive to CRP and have greater clinical significance in predicting the trend of inflammatory reactions.

HIPK3 has been reported to be associated with tumorigenesis and apoptosis. Studies have found that circHIPK3 can act as an oncogene and autophagy regulator in lung cancer, and may be used as a prognostic marker and therapeutic target for lung cancer (32). In NSCLC tissues, low HIPK3 expression is associated with poor survival. Therefore, HIPK3 is considered a biomarker for predicting the survival of patients with NSCLC (15). However, the relationship between HIPK3 and RA has not been studied. In the current study, we found that the CpGs/haplotype site methylation level of HIPK3 was correlated with clinical indicators such as ESR, CRP, DAS28-CRP and RF/ACPA in RA patients. The correlation between HIPK3/haplotype sites and ESR and CRP, which are closely related to inflammation, was explained in detail in different characteristics of RA patients, and the clinical value of these sites in predicting the levels of ESR and CRP was also evaluated. Finally, the methylation level of HIPK3 in whole blood was closely related to the levels of ESR and CRP, and there was a significant negative correlation.

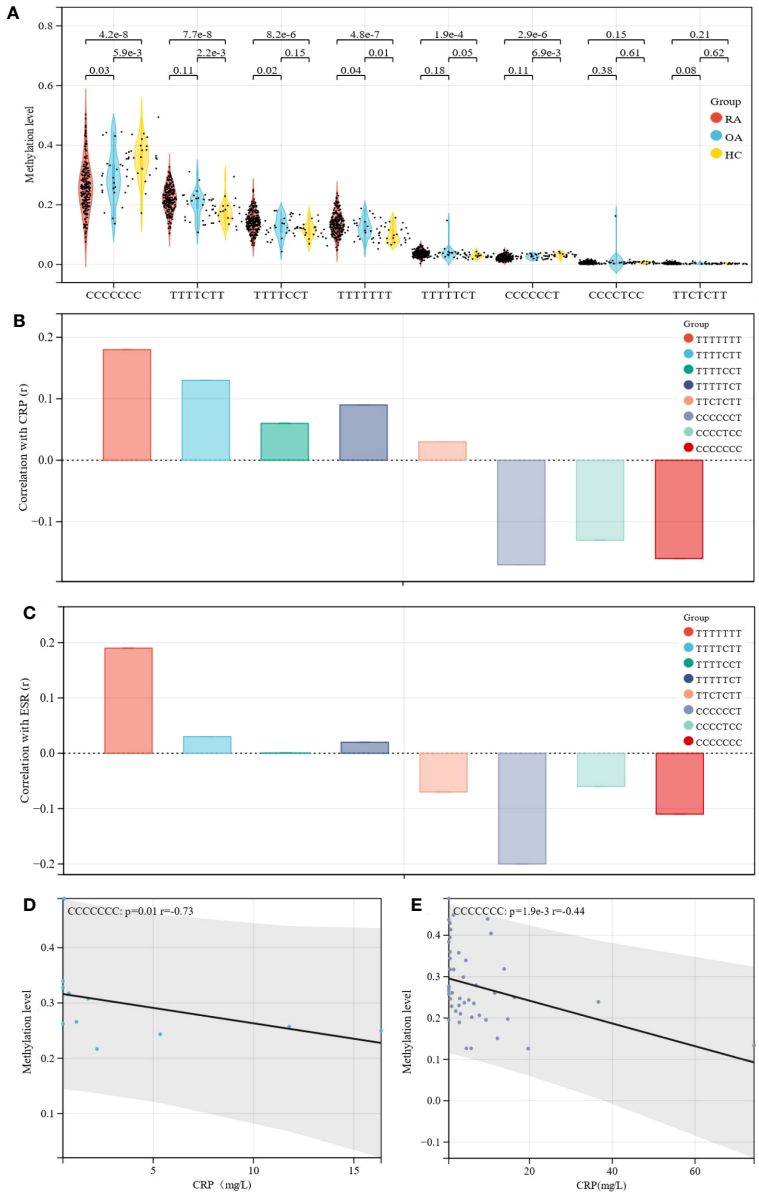

FIGURE 2
Differences in methylation level and correlation of haplotype sites. **(A)** Methylation level of different haplotype sites between RA, OA patients and healthy controls; Further analysis showed that HIPK3 has eight haplothpe sites, including TTTTTTT, TTTTCTT, TTTTCCT, TTTTTCT, TTCTCTT, CCCCCCT, CCCCTCC, CCCCCCC; The difference analysis showed that the methylation levels of these haplotype sites in patients with RA were different regulated, and among them, the methylation level of CCCCCCC in RA showed a downward trend, and the statistical difference was the most significant. **(B)** Correlation of haplotype sites with CRP were different compared with TTTTTTT; The correlation between different haplotype sites and CRP was analyzed, and the results were compared with TTTTTTT; Taking TTTTTTT as the standard, the trend of correlation between different haplotype sites and CRP was shown. **(C)** Correlation of haplotype sites with ESR was different compared with TTTTTTT; The correlation between different haplotype sites and ESR was analyzed, and the results were compared with TTTTTTT; Taking TTTTTTT as the standard, the trend of correlation between different haplotype sites and ESR was shown. **(D)** In patients with RF (-)/ACPA (-), CCCCCCC was negatively correlated with CRP; Further analysis of the correlation between haplotype sites and clinical indexes in different subgroups; The results showed that in patients with RF (-)/ACPA (-), correlation between CCCCCCC and CRP was the highest and the P value was statistically significant; CCCCCCC was negatively correlated with CRP level (r= -0.73, p= 0.01). **(E)** In patients with 2.6 ≤ DAS28-CRP ≤ 3.2, CCCCCCC were negatively correlated with CRP; Similarly, subgroup analysis also found that In patients with 2.6≤DAS28-CRP ≤ 3.2, there was also a statistically significant negative correlation trend between CCCCCCC and CRP (r= -0.44, p= 0.0019), and which was helpful to judge the change trend of clinical CRP level.

TABLE 2 Correlation analysis between the methylation level of different haplotype sites and clinical data.

Group	TTTTTTT	TTTTCTT	TTTTCCT	TTTTTCT	TTCTCTT	CCCCCCT	CCCCTCC	CCCCCCC
Age	-0.07(0.28)	-0.09(0.16)	0.003(0.96)	0.03(0.68)	0.0083(0.90)	0.07(0.30)	-0.05(0.47)	0.02(0.78)
BMI	-0.04(0.55)	-0.04(0.51)	0.0087(0.89)	0.02(0.82)	-0.01(0.84)	0.10(0.12)	0.08(0.26)	-0.005(0.94)
Course of disease	0.05(0.48)	-0.01(0.83)	0.00089(0.99)	0.08(0.21)	-0.13(0.05)	0.00057(0.99)	-0.04(0.60)	-0.04(0.50)
Age of onset	-0.88(0.22)	-0.06(0.35)	-0.06(0.36)	0.01(0.82)	0.11(0.10)	0.04(0.52)	0.04(0.56)	0.05(0.44)
TJC	0.0092(0.89)	-0.01(0.87)	0.06(0.34)	-0.02(0.81)	0.10(0.14)	-0.02(0.72)	0.03(0.63)	-0.03(0.66)
SJC	0.05(0.45)	-0.01(0.83)	0.02(0.77)	-0.0019(0.98)	-0.05(0.49)	0.06(0.36)	-0.01(0.88)	-0.04(0.57)
VAS	0.12(0.06)	0.01(0.84)	0.07(0.27)	0.04(0.56)	0.09(0.19)	-0.17(0.01)	-0.02(0.73)	-0.07(0.26)
RF	0.08(0.21)	0.04(0.59)	-0.04(0.59)	-0.03(0.61)	-0.02(0.77)	-0.02(0.81)	-0.05(0.45)	-0.0093(0.89)
ACPA	-0.08(0.23)	0.01(0.84)	-0.02(0.73)	-0.06(0.38)	-0.11(0.11)	0.07(0.26)	-0.02(0.79)	0.0012(0.99)
ESR	0.19(0.0038)	0.03(0.69)	0.00071(0.99)	0.02(0.76)	-0.07(0.28)	-0.20(0.0023)	-0.06(0.42)	-0.11(0.11)
CRP	0.18(0.0061)	0.13(0.04)	0.06(0.37)	0.09(0.16)	0.03(0.68)	-0.17(0.01)	-0.13(0.06)	-0.16(0.01)
DAS28-ESR	0.12(0.06)	0.01(0.82)	0.06(0.33)	0.02(0.80)	0.02(0.72)	-0.13(0.05)	0.0066(0.92)	-0.09(0.16)
DAS28-CRP	0.10(0.13)	0.04(0.55)	0.08(0.25)	0.04(0.51)	0.06(0.37)	-0.11(0.08)	-0.02(0.78)	-0.09(0.16)

Correlation result format: r (p).

In the ROC curve, through combinatorial analysis of HIPK3, RF (+) and ACPA (+), HIPK3 was found to have more clinical value in predicting the expression trend of inflammation. These results provide a basis and novel understanding for studying the relationship between inflammation and HIPK3 expression in RA.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: SRA, PRJNA918808.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Shanghai Guanghua Integrated Traditional Chinese and Western Medicine Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

The original manuscript was completed by PJ and KW. SG, CC, RZ, YJ and YQ were accountable for the statistical analysis of data. LXX, JZ, LSX, SS and YS collected samples and helped in the statistical analysis. RW, YQ and DH contributed to the conception, design, and final approval of the submitted version. All authors reviewed and accepted the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

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Mapping of DNA methylation-sensitive cellular processes in gingival and periodontal ligament fibroblasts in the context of periodontal tissue homeostasis

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Interactions between gingival fibroblasts (GFs) and oral pathogens contribute to the chronicity of inflammation in periodontitis. Epigenetic changes in DNA methylation are involved in periodontitis pathogenesis, and recent studies indicate that DNA methyltransferase (DNMT) inhibitors may protect against epithelial barrier disruption and bone resorption. To assess the impact of DNMT inhibition on GFs, cells were cultured with decitabine (5-aza-2'-deoxycytidine, DAC) for 12 days to induce DNA hypomethylation. We observed several potentially detrimental effects of DAC on GF biological functions. First, extended treatment with DAC reduced GF proliferation and induced necrotic cell death. Second, DAC amplified *Porphyromonas gingivalis*- and cytokine-induced expression and secretion of the chemokine CCL20 and several matrix metalloproteinases (MMPs), including MMP1, MMP9, and MMP13. Similar pro-inflammatory effects of DAC were observed in periodontal ligament fibroblasts. Third, DAC upregulated intercellular adhesion molecule-1 (ICAM-1), which was associated with increased *P. gingivalis* adherence to GFs and may contribute to bacterial dissemination. Finally, analysis of DAC-induced genes identified by RNA sequencing revealed increased expression of *CCL20*, *CCL5*, *CCL8*, *CCL13*, *TNF*, *IL1A*, *IL18*, *IL33*, and *CSF3*, and showed that the most affected processes were related to immune and inflammatory responses. In contrast, the genes downregulated by DAC were associated with extracellular matrix and collagen fibril organization. Our observations demonstrate that studies of DNMT inhibitors provide important insights into the role of DNA methylation in cells involved in periodontitis pathogenesis. However, the therapeutic potential of hypomethylating agents in

periodontal disease may be limited due to their cytotoxic effects on fibroblast populations and stimulation of pro-inflammatory pathways.

KEYWORDS

periodontitis, gingival fibroblast, periodontal ligament fibroblast, DNA methyltransferases, decitabine (DAC), *Porphyromonas gingivalis*

Introduction

Periodontitis is a chronic inflammatory diseases initiated by microbial imbalance (dysbiosis). In its severe form, periodontitis affects more than 10% of the population, causing an estimated loss of more than 150 billion EUR in Europe alone in 2018 (1). It is currently accepted that pathological changes in the gingival tissue are driven by the non-resolving host immune response to oral pathogens (2), among which *Porphyromonas gingivalis* plays a central role (3). Excessive production of inflammatory mediators by infiltrating immune cells and resident gingival cells drives connective tissue breakdown. As a consequence, chronic inflammation not only is the main cause of tissue damage and alveolar bone resorption, but also sustains dysbiotic microbiota by providing a source of nutrients for inflammophilic bacteria (4). Studies of individual cell populations in periodontitis have predominantly focused on gingival epithelial cells (GECs) as the natural first barrier protecting against oral pathogens (5). However, recent analysis of the human oral mucosal tissues at the single cell level identified immune functionality of stromal cells, in particular gingival fibroblasts (GFs) (6). Specifically, GFs play an essential role in recruiting neutrophils and lymphocytes into disease lesions (6).

Interactions with oral pathogens and the inflammatory tissue environment could lead to epigenetic changes in cells of the periodontium (7). Among the epigenetic regulatory mechanisms, methylation of cytosine residues at CpG dinucleotides is the most common and thoroughly characterized. Methylation marks on the DNA strand are established by DNA methyltransferases (DNMTs) in a site-specific manner and are typically associated with transcriptional repression due to chromatin condensation and/or disruption of interactions between transcription factors and DNA (8). While DNMT1 activity maintains DNA methylation profiles after replication, DNMT3a and DNMT3b are responsible for *de novo* DNA methylation (9). Facilitated by the rapid development of epigenomic technologies, dysregulated DNA methylation profiles, both globally and at individual gene promoters, have been identified in many pathologies, including inflammatory diseases and several types of cancer (10, 11). Importantly, aberrant DNA methylation can be targeted with small-molecule DNMT inhibitors. These compounds epigenetically reactivate silenced genes by inducing DNA hypomethylation and their potential to upregulate tumor suppressor genes has indicated their therapeutic potential in oncology. Indeed, 5-azacitidine (AZA) and 2'-deoxy-5-azacitidine (decitabine, DAC) are used for the treatment of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) (12, 13). However, the use of these compounds in clinical practice has been restricted to oncology due to their significant hematologic toxicity (14).

In recent years, several studies have identified epigenetic regulatory mechanisms as key factors in periodontitis pathogenesis and possible targets for therapeutic strategies aimed at the modulation of host responses (15). Alterations in the methylation status of genes associated with immune responses, such as *IL6*, *IL8*, *IFNG*, *TNF*, *PTGS2*, and *TLR2*, have been identified in gingival biopsies collected from patients with periodontal disease (16). However, many of the reported differences were not reproducible in independent studies or their biological relevance has not been verified in functional analyses (7). In contrast, *in vitro* studies have provided more consistent results. Exposure of cells of the periodontium to oral pathogens or inflammatory cytokines tends to induce promoter-specific hypermethylation. In GECs, chronic infection with *P. gingivalis* caused hypermethylation of the TLR2 promoter (17), while extended treatment of periodontal ligament fibroblasts (PDLFs) with *P. gingivalis* lipopolysaccharide (LPS) increased the methylation levels of genes encoding extracellular matrix (ECM) components (18). These observations provided evidence that oral pathogens directly influence host responses through the modulation of DNA methylation and suggested that DNMT inhibitors may be therapeutically beneficial in the context of periodontitis pathogenesis. Indeed, in a mouse model of periodontitis, DAC suppressed osteoclastogenesis and upregulated anti-inflammatory cytokines, which resulted in reduced bone resorption (19). GEC treatment with DNMT inhibitors reversed *P. gingivalis*-induced functional impairment of the gingival epithelial barrier, which was associated with increased promoter methylation and expression level of genes encoding proteins that form cell-cell junction complexes (20). GF treatment with AZA promoted bone morphogenetic protein-2-induced differentiation of GFs into osteoblasts that were capable of inducing bone formation *in vivo* (21). These findings indicate that DNA hypomethylating agents may reverse the pathological effects of dysbiosis and promote the resolution of inflammation in periodontitis. However, the biological consequences of extended treatment with DNMT inhibitors on GF responses in the context of chronic inflammation and constant exposure to oral pathogens in the inflamed gingival tissue remain unknown. In this study, we comprehensively analyzed the transcriptional and functional consequences of primary human GF and PDLF treatment with DNMT inhibitors, identifying several key cellular process that are affected by DNA hypomethylation.

Materials and methods

Subjects and cell isolation

Gingival tissue specimens were collected from 16 healthy individuals undergoing orthodontic treatment at the Department of Periodontology and Clinical Oral Pathology and Chair of Oral

Surgery, Faculty of Medicine, Jagiellonian University Medical College, Krakow, Poland. Teeth were collected from five patients referred for multiple adjacent tooth extractions due to gingivitis, periodontitis, or tooth decay at the Department of Oral and Maxillofacial Surgery at the OLVG hospital in Amsterdam, The Netherlands. GFs and PDLFs were isolated from gingival biopsies and teeth, respectively, according to the previously described protocols (22, 23), cultured in DMEM (Lonza) containing 10% fetal bovine serum (FBS) (Biowest), gentamicin (50 U/ml) and penicillin/streptomycin (50 U/ml) (both from Gibco), and used for experiments between passages 4 and 9.

Bacterial culture

Wild-type *P. gingivalis* (strain ATCC33277) were grown anaerobically on blood agar plates for 5-7 days before inoculation into brain-heart infusion (BHI) broth (Becton-Dickinson) with yeast extract containing 10 µg/mL hemin, 0.5 mg/mL L-cysteine, and 0.5 µg/mL vitamin K. Bacterial suspensions at optical density $(OD)_{600} = 1$ (corresponding to 10^9 colony-forming units (CFU)/ml) were prepared as described before (23).

Cell culture, treatment with DNMT inhibitors, stimulation, and infection

One day prior to treatment with inhibitors, cells were seeded in 10 cm dishes in medium supplemented with 10% FBS and antibiotics. Cells were cultured for 12 days in the presence of DMSO (control) or DNMT inhibitors: decitabine (DAC; 5 µM), 5-azacitidine (AZA; 5 µM) and 6-thioguanine (6-TG; 10 µM) (all from TargetMol). Every 4 days, the medium was replaced, and fresh portion of inhibitors was added. Alternatively, GFs were treated with DMSO or DAC for 12 days, with replacement of medium and compounds every day. In an independent set of experiments, GFs were cultured in the presence of DMSO or DAC for 3 days, either receiving a single dose of the compounds during treatment, or fresh portions of medium with DMSO or DAC every day. Cells were then split, counted, and seeded for infection or cytokine stimulation as described below.

Cell proliferation and viability assays

Cell proliferation was assessed using a Cell Proliferation ELISA BrdU kit (Roche). DAC- or DMSO-treated GFs were seeded in 96-well plates and after overnight culture a BrdU labeling reagent was added. Cells were then cultured for 24 h, fixed and incubated with anti-BrdU-POD antibody for 90 min. A Cytotoxicity Detection Kit (LDH) and Cell Death Detection ELISA (both from Roche) were used to assess cytotoxic and proapoptotic effects of DNMT inhibitors. After culture with DMSO or DAC, GFs were seeded in 96-well plates and cultured for 24 h. Staurosporine (STS, 1 µM) was used as a positive control for cell death induction. Next, supernatants were collected and the assays were performed according to the manufacturers' protocols. For the LDH assay, supernatants from lysis buffer-treated cells were used as a reference displaying 100% cytotoxicity. A FlexStation3 Multi-Mode microplate reader (Molecular Devices) was used for absorbance measurements.

ELISA

GFs or PDLFs seeded in 96-well plates were infected with *P. gingivalis* (MOI 10 or 50) for 1 h followed by 23 h of culture in fresh medium, or were stimulated with tumor necrosis factor (TNF) or IL-1β (both from BioLegend) at 10 ng/ml for 24 h. Concentrations of analytes were determined in cell-free supernatants using a CCL2 ELISA MAX Standard sets (BioLegend) or CCL20 and MMP1 ELISA Duo Sets (R&D Systems), according to the manufacturers' instructions.

RNA isolation and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using an ExtractMe Total RNA isolation kit (Blirt) and quantified using a BioPhotometer D30 (Eppendorf). A High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for RNA conversion to cDNA. qPCR reactions were performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) using PowerUp SYBR Green PCR mix (Applied Biosystems). The CFX Manager (Bio-Rad) was used for data analysis. mRNA expression relative to a housekeeping gene (*RPLP0*) was calculated using the ΔΔCT method. The sequences of the primers used in the study (purchased from Merck) are listed in Supplementary Data Sheet 1: Table S1.

Immunoblotting

Protein expression in cell lysates was determined by immunoblotting, as described previously (24) using antibodies recognizing ICAM-1 (#67836) or β-actin (#4967) (both from Cell Signaling Technology). A ChemiDoc MP Imaging System (Bio-Rad) was used for membrane visualization.

DNA isolation and analysis of global DNA methylation

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen) and quantified with a BioPhotometer D30. Global DNA methylation was assessed using a Global DNA Methylation LINE-1 Kit (Active Motif), according to the protocol provided by the manufacturer. Briefly, 1 µg of DNA from each sample was digested with *MseI* overnight at 37°C, and then the reaction was stopped by incubation of the samples at 60°C. Next, DNA was diluted to a final concentration 4 ng/ml and incubated with the LINE-1 Probe Solution. Samples and standards were then transferred to streptavidin-coated plates and incubated with a 5-methylcytosine antibody. Absorbance was measured at 450/655 nm and % 5-mC of CpG residues relative to the total cytosine content was determined.

Colony-forming assay

After 12-day culture with DMSO or DAC, GFs were seeded in duplicate wells in 12-well plates (10^5 cells per well) and infected with *P. gingivalis* (MOI 100) for 1 h. To discriminate between adhesion and internalization of bacteria, cells were washed three times with PBS and cultured for 1 h either in antibiotic-free fresh medium, or in medium with gentamicin (2.5 mg/ml) and metronidazole (2 mg/ml) for 1 h. Next, cells were lysed in sterile, distilled water for 40 min, 10-fold serial dilutions of cell lysates were prepared and 10 µl of each dilution was transferred to BHI blood agar plates in duplicate. *P. gingivalis* colonies were counted after plate incubation for 5 days at 37°C under anaerobic conditions.

Analysis of CCL20 degradation by *P. gingivalis*

P. gingivalis at 10^6, 2.5×10^6, 5×10^6 and 10^7 CFU/ml was incubated with 10 ng/ml CCL20 (BioLegend) in DMEM containing 2% FBS without antibiotics. Supernatants were collected after 2 h and 24 h and centrifuged to remove bacteria (10 000×g, 10 min, 4°C CCL20 concentrations were determined by ELISA.

P. gingivalis growth curve analysis

P. gingivalis cultures were inoculated into BHI broth containing 0.5 mg/ml L-cysteine, 10 µg/ml hemin and 0.5 µg/ml vitamin K at OD_{600} = 0.1. Bacterial suspensions were cultured anaerobically either with recombinant human CCL20 (BioLegend) at 0.2 or 1 µg/ml, or with bestatin (5 mg/ml) which was used as a control compound that inhibits the growth of *P. gingivalis*. Bacterial growth was determined by OD measurements at 0, 4, 8, and 24 h using a Cell Density Meter (Biochrom).

RNA sequencing

After 12-day culture with DMSO or DAC, GFs (n=5) were seeded in 6-well plates (5×10^5 cells per well) for 24 h and then were either left untreated or were infected with *P. gingivalis* (MOI 20) for 4 h. Quality of RNA extracted using an RNeasy Mini Kit (Qiagen) was assessed using an RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies). Library preparation, clustering, sequencing and data processing and analysis were performed at Intelliseq S.A. (Krakow, Poland) and are described in detail in Supplementary Data Sheet 1: Supplementary Materials and Methods.

Statistical analyses

Data are presented as the mean +SEM. Primary cells isolated from different donors were used in all experiments and the values of 'n' indicate the number of cell lines derived from independent donors used in each experiment. The ratio paired *t*-test was used for comparisons between groups unless otherwise indicated. *P* values <0.05 were considered statistically significant.

Results

DNMT inhibition reduces GF proliferation and viability

To verify how DNMT inhibition affects key biological functions of gingival stromal cells, we cultured primary human GFs with 5 µM DAC for 12 days to induce DNA hypomethylation. This experimental setup allows for multiple cell divisions that are required for efficient erasing of DNA methylation patterns that are not maintained during replication in the absence of DNMT1 activity. GF culture in the presence of DAC caused a 40% reduction of global DNA methylation (Figure 1A). During GF culture, we noted reduced density of cells that were cultured with DAC on day 7 and day 12 (Figure 1B), which was associated with significantly lower numbers of DAC-treated cells that were retrieved from culture dishes compared to DMSO-treated cells (Figure 1C). Reduction of GF cell numbers during culture with DAC was caused by reduced proliferation (Figure 1D) as well as cytotoxic effects of the DNMT inhibitor (Figure 1E) as determined by BrdU incorporation and the LDH release assay, respectively. The cytotoxic and anti-proliferative effects of DAC were dose-dependent and readily detectable upon GF treatment with 0.2 µM DAC (Supplementary Data Sheet 1: Figures S1A, B). Next, we analyzed the presence of nucleosomes in the cytosol and culture supernatants to identify the type of cell death induced by DAC in GFs. While DAC treatment did not cause the accumulation of nucleosomes in the cytosol, which is a hallmark of apoptosis, we detected significant levels of DNA-complexed histones in the supernatants, indicative of cell necrosis (Figure 1F). These results indicate that extended treatment with the DNMT inhibitor DAC has detrimental effects on GF proliferation and viability.

DNMT inhibitors promote the production of CCL20 and MMPs by GFs

Next, we determined the effects of DNA hypomethylation on GF inflammatory activation. After 12 d of culture in the presence of DMSO or DAC, equivalent numbers of GFs were plated and infected with *P. gingivalis* or stimulated with TNF. GF culture with DAC had no effect on both basal and inducible expression of *IL6*, *IL8*, *COX2*, and *CCL2* mRNA (Figure 2A). In contrast, transcript levels of the chemokine CCL20 induced by *P. gingivalis* infection or TNF stimulation were significantly higher in DAC-treated cells. Similarly, both basal and inducible mRNA expression of several MMPs, including *MMP1*, *MMP9* and *MMP13* were elevated in DAC-treated cells (Figures 2A, B). Strikingly, despite reduced cell viability, DAC-mediated induction of CCL20 and MMP1 was even more pronounced at the protein level. Compared to cells cultured with DMSO, GFs cultured in the presence of DAC released at least 10-fold higher amounts CCL20 upon infection with *P. gingivalis* or stimulation with TNF or IL-1β (Figure 2C). The augmentation of

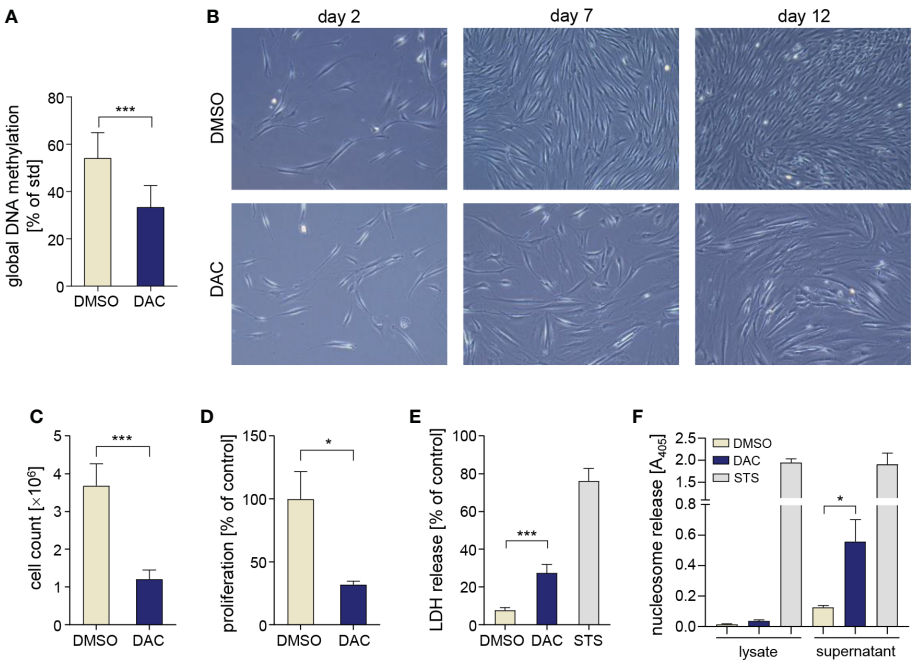

FIGURE 1
DAC induces global DNA hypomethylation and has anti-proliferative and cytotoxic effects on GFs. **(A)** Global DNA methylation in GFs cultured with DMSO or DAC for 12 days. **(B)** Representative micrographs of GFs cultured in the presence of DMSO or DAC for 12 days. Pictures were taken on days 2, 7, and 12 of the experiment using contrast-phase microscopy. **(C)** GF total cell counts (n=4) and **(D)** proliferation rate determined using the BrdU incorporation assay (n=8) after 12 days of culture with DMSO or DAC. Viability of GFs treated with DMSO or DAC for 12 days measured using **(E)** the LDH release assay (n=7) and **(F)** analysis of nucleosome release into cytosolic cell fractions (n=3) and culture supernatants (n=4). GFs treated with 1 μM staurosporine (STS) were used as a positive control. *P < 0.05, ***P < 0.001.

TNF-induced CCL20 production by GFs was also apparent at lower DAC concentrations (Supplementary Data Sheet 1: Figure S1C). Similarly, the levels of MMP1 produced by GFs cultured with DAC were significantly higher compared to DMSO-treated cells regardless of the absence or presence of cytokine stimulation or bacterial infection (Figure 2D). Consistent with mRNA expression data, secretion of CCL2 was largely unaffected by DAC (Figure 2E).

While the detrimental effects of MMPs in periodontitis have been well-documented (25), the role of CCL20 is controversial due to its dual biological activity as a chemokine and an antimicrobial peptide (26). To verify whether increased amounts of CCL20 produced by DAC-treated GFs could be sufficient for direct microbicidal effects, we cultured *P. gingivalis* anaerobically in the presence of recombinant CCL20 or bestatin as a control known to inhibit *P. gingivalis* growth (27). While bestatin had a strong bacteriostatic effect, CCL20 at either tested concentration had no effect on *P. gingivalis* proliferation (Figure 2F). The inability of CCL20 to affect *P. gingivalis* growth, in contrast to its antimicrobial activity on other Gram-negative bacteria (28), may be due to degradation by bacterial proteases, among which gingipains play the most prominent role (29). Indeed, incubation of recombinant CCL20 with *P. gingivalis* in conditions equivalent to those used in cell infection experiments resulted in rapid degradation of the protein (Figure 2G). These data indicate that CCL20 does not exert a direct antimicrobial effect on *P. gingivalis*. Therefore, increased production of CCL20 by DNMT-inhibitor treated GFs will likely have proinflammatory effects due to the recruitment of leukocytes. Collectively, these observations identify a cluster of inflammatory mediators that are dynamically regulated by changes in DNA methylation and suggest that DNMT inhibitors may amplify chronic inflammation in gingival tissue.

Degradation products or off-target effects are not responsible for DAC effects on GFs

To exclude the possibility that the effects of DAC may be caused by off-target activity of the inhibitor, we cultured GFs with another cytidine analogue, AZA, and the structurally unrelated compound 6-TG. *P. gingivalis*- and TNF-induced *CCL20* and *MMP1* expression levels were significantly increased in AZA-treated cells (Figure 3A) and both compounds enhanced basal MMP1 levels and IL-1β-induced production of CCL20 by GFs (Figure 3B).

DAC has a half-life of approximately 11 h at physiological temperature and pH, and decomposes into several degradation products, some of which display toxic properties independent of the parent compound (30). Therefore, we next tested whether the elimination of these DAC degradation products, by replacing culture media every day during treatment and/or by reducing the culture time to 3 days, would limit the detrimental effects of DAC on GF survival. Although a 20% reduction in GF proliferation was observed upon 3-day DAC treatment with daily change of culture media (Supplementary Data Sheet 1: Figure S2A), this effect was not statistically significant and was not accompanied by increased LDH release (Supplementary Data Sheet 1: Figure S2B). In contrast, GF treatment with DAC for 3 days without daily replacement of culture media caused a more pronounced reduction of cell proliferation and a clear increase in LDH release

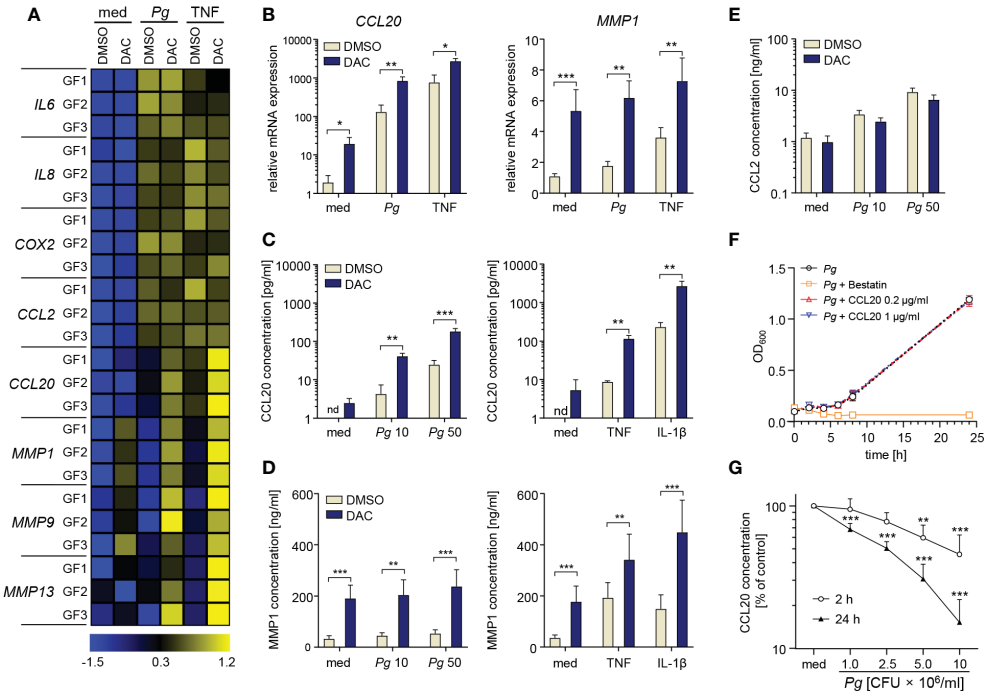

FIGURE 2
DAC promotes production of CCL20 and MMPs by GFs. **(A, B)** GFs treated with DMSO or DAC for 12 days were left unstimulated in medium (med), or were infected with *P. gingivalis* (*Pg*, MOI 20), or stimulated with TNF (10 ng/ml) for 4 h. **(A)** qPCR analysis of mRNA expression of inflammatory mediators in GFs from individual donors (GF1-GF3) shown on a heatmap as row Z-scores calculated from ΔCt values relative to a housekeeping gene (*RPLP0*). **(B)** Relative mRNA levels of *CCL20* and *MMP1* (n=5). **(C-E)** DMSO- or DAC-treated GFs were infected with *P. gingivalis* (MOI 10 or 50) for 1 h followed by washing and culture for another 23 h in fresh medium containing antibiotics or were stimulated with TNF or IL-1β (both 10 ng/ml) for 24 h. Production of **(C)** CCL20, **(D)** MMP1 and **(E)** CCL2 is shown as mean concentration +SEM (n=4-8). **(F)** Growth curve of *P. gingivalis* cultured anaerobically in the presence of recombinant human CCL20 (0.2 and 1 µg/ml) or bestatin (5 mg/ml) (positive control) (n=3). **(G)** Degradation rate of CCL20 by *P. gingivalis* at 10^6, $2.5x10^6$, $5x10^6$, and 10^7 CFU/ml after 2 h and 24 h of incubation (n=4). nd, not detectable; *P < 0.05, **P < 0.01, ***P < 0.001.

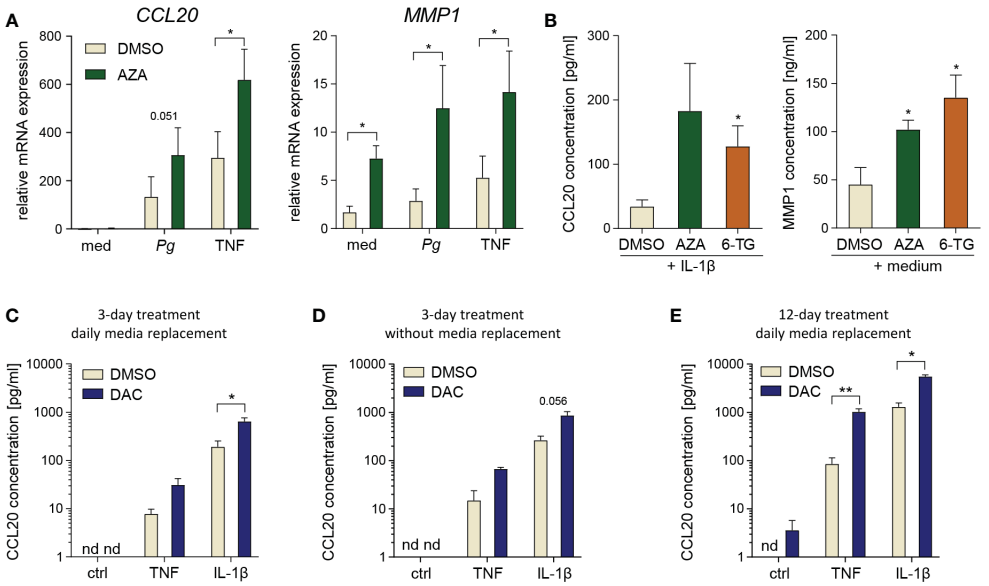

FIGURE 3
Degradation products or off-target effects are not responsible for DAC effects on GFs. **(A)** qPCR analysis of *CCL20* and *MMP1* mRNA expression in GFs treated with DMSO or 5-azacitidine (AZA) for 12 days followed by infection with *P. gingivalis* (*Pg*, MOI 20) or stimulation with TNF (10 ng/ml) for 4 h (n=5). **(B)** CCL20 and MMP1 production by GFs cultured with DMSO, AZA or 6-thioguanine (6-TG) prior to 24 h culture in medium alone or stimulation with IL-1β at 10 ng/ml h (n=5). *P < 0.05, RM One-way ANOVA followed by Bonferroni multiple comparison test. **(C-E)** CCL20 production by GFs (n=3-4) stimulated with TNF or IL-1β (both at 10 ng/ml) for 24 h after culture in the presence of DMSO or DAC: **(C)** for 3 days with replacement of medium and compounds every day, **(D)** for 3 days without replacement of medium and compounds, or **(E)** for 12 days with replacement of medium and compounds every day. nd, not detectable; *P < 0.05, **P < 0.01.

(Supplementary Data Sheet 1: Figures S2C, D). Interestingly, 3-day treatment of GFs with DAC, with or without daily replacement of culture media, was sufficient to increase cytokine-induced CCL20 protein production (Figures 3C, D). However, these effects were less pronounced compared to those observed after 12-day cell exposure to DAC (Figures 2B, C). We also tested whether replacement of media with DAC every day during 12-day culture would limit the cytotoxicity of the compound. In this experimental protocol, the anti-proliferative and cytotoxic effects of DAC were comparable to those observed without daily change of DAC-containing media (Supplementary Data Sheet 1: Figures S2E, F), and so was the augmentation of cytokine-induced CCL20 production (Figure 3E). These results indicate that, whereas the early cytotoxic and anti-proliferative effects of DAC may be partly mediated by its degradation products, the long-term effects of DAC on GF viability occur despite regular removal of these products. Therefore, increased CCL20 production, as well as the cytotoxic effects observed after extended GF exposure to DAC, are likely a direct consequence of DNA hypomethylation induced by DAC and the contribution of compound decomposition products may be negligible.

DNMT inhibition promotes *P. gingivalis* adhesion to GFs

To gain more insight into the influence of DNA hypomethylation on *P. gingivalis* interactions with host cells, we analyzed *P. gingivalis* adhesion to and intracellular survival in GFs treated with DAC for 12 days. DAC treatment had no significant effect on the numbers of live *P. gingivalis* detected inside the cells after antibiotic treatment which removes adherent bacteria (Figure 4A). However, we noted increased CFU counts in DAC-treated GFs cultured without antibiotics after infection. This result reflects the combination of bacteria that adhered to and entered the cells and suggests that DAC treatment may promote bacterial adhesion to GFs (Figure 4A). To verify whether this effect may be caused by changes in expression of proteins that are involved in *P. gingivalis* adhesion to host cells (31, 32), we analyzed mRNA levels of integrin subunit β1 (*ITGB1*), transglutaminase-2 (*TG2*), and intercellular adhesion molecule-1 (*ICAM1*). Among these genes, only ICAM1 expression was significantly elevated in DAC-treated GFs (Figure 4B). Induction of ICAM1 expression by DAC was also evident at the protein level: GFs cultured with DAC expressed 6-fold higher levels of the ICAM-1 protein compared to cells treated with DMSO (Figures 4C, D).

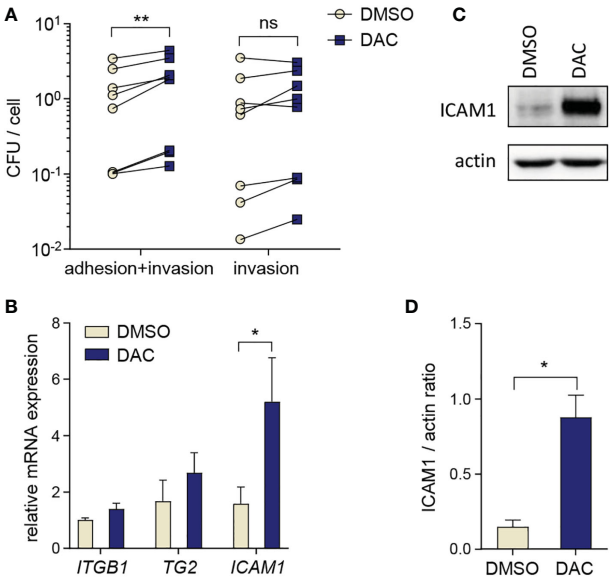

FIGURE 4
DAC promotes *P. gingivalis* adhesion to GFs. **(A)** Bacterial adhesion and intracellular survival determined by the colony forming assay in GFs (n=8) treated for 12 days with DMSO or DAC. Individual values of CFU counts per cell with lines connecting DMSO- and DAC-treated cells from individual donors are shown. **(B)** qPCR analysis of *ITGB1*, *TG2* and *ICAM1* in DMSO- and DAC-treated GFs (n=5). **(C, D)** Western blot analysis of ICAM1 and actin in GFs after 12 days of culture with DMSO or DAC. A representative of 4 independent experiments **(C)** and the ratio of ICAM1/actin signal intensity from densitometric analysis (n=4) **(D)** are shown. ns, not significant; *P < 0.05, **P < 0.01.

Global transcriptomic analysis of DAC-treated GFs identifies cellular processes affected by DNMT inhibition in GFs

To obtain a complete overview of cellular processes affected by DNMT inhibition, we performed a global transcriptomic analysis of GFs cultured with DMSO or DAC for 12 days prior to *P. gingivalis* infection. DAC induced major transcriptional changes: expression of approximately 500 genes was significantly modulated in DAC-treated cells compared to cells treated with DMSO both in the presence and absence of subsequent infection with *P. gingivalis* (Figures 5A, B and Supplementary Table 2). Nearly 80% of the affected genes were upregulated, consistent with the notion that DNA hypomethylation is typically associated with increased gene expression. Pathway analysis of significantly upregulated genes in DAC-treated GFs infected with *P. gingivalis* (including *CCL20*, *CCL5*, *CCL8*, *CCL13*, *TNF*, *IL1A*, *IL18*, *IL33*, and *CSF3*) showed that the most affected processes were related to immune and inflammatory responses. Interestingly, apart from lymphocyte migration and LPS signaling, several genes related to type I interferon signaling and responses to viral infection were upregulated after GF culture with DAC (*APOBEC3C*, *IFITM1*, *RSAD2*, *IFI27*, *OAS2*, *MX1*, *IFI6*, *ISG15*, *SAMHD1*) (Figure 5C). In contrast, the genes significantly downregulated by DAC were predominantly associated with the ECM and collagen fibril organization (*COL1A1*, *COL5A1*, and *SERPINH1*) (Figure 5D). These results not only substantiate but also extend our previous findings that the overall effect of DNMT inhibitors on GFs may be detrimental in the context of periodontitis pathogenesis due to the stimulation of inflammatory processes and dysregulation of ECM homeostasis. At the same time, the transcriptomic data reveal how broadly changes in DNA methylation affect key cellular processes in GFs.

DMNT inhibition has anti-proliferative and proinflammatory effects in PDLFs

Finally, we tested whether DNMT inhibition has similar effects on other stromal cells of the periodontium using PDLFs as a model. DAC significantly reduced PDLF proliferation (Figure 6A), but caused only a minor increase in LDH release (Figure 6B), suggesting that, in contrast to GFs, PDLFs are more resistant to the cytotoxic activity of DAC (or its metabolic products). After 12-day culture of PDLFs with DAC, both

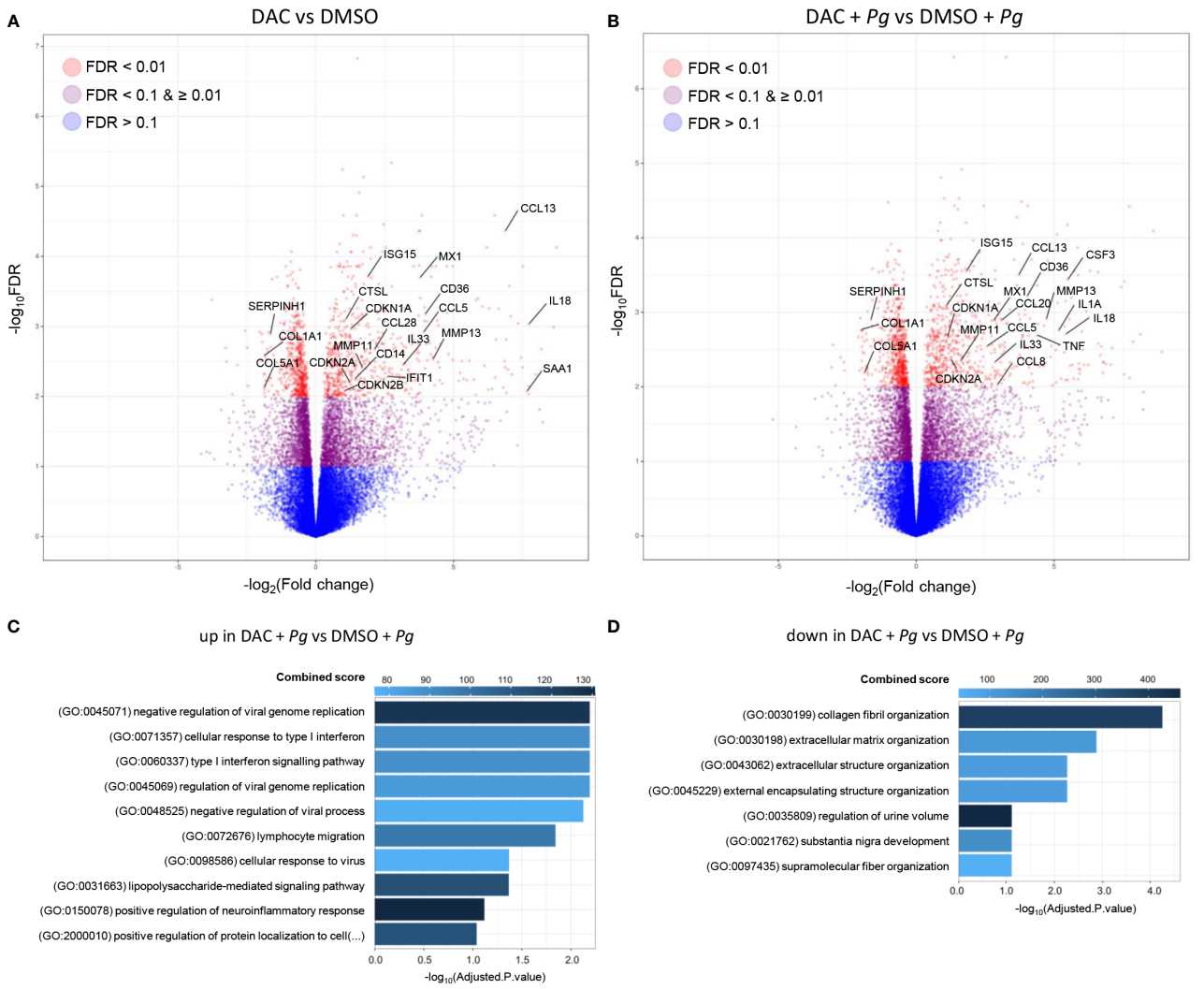

FIGURE 5
DAC induces global transcriptional changes in GFs. GFs (n=5) treated with DMSO or DAC for 12 days were left unstimulated in medium or were infected with *P. gingivalis* (*Pg*, MOI 20) for 4 h and mRNA expression was profiled by next generation sequencing. **(A, B)** Volcano plots of RNA-seq data showing the $\log_2$FC and $-\log_{10}$(FDR) of DMSO- and DAC-treated GFs in the absence **(A)** and presence **(B)** of *P. gingivalis* infection. **(C, D)** Pathway enrichment analysis of significantly upregulated **(C)** and downregulated **(D)** genes in DAC-treated GFs infected with *P. gingivalis*. Top hits based on Combined Score and adjusted *P*-value < 0.1 are presented.

basal and inducible mRNA expression levels of CCL20 and MMP1 were significantly increased (Figure 6C). In line with the mRNA data, trends towards elevated CCL20 and MMP1 production by DAC-treated PDLFs were observed after cytokine stimulation or *P. gingivalis* infection, though the differences reached statistical significance only in some of the tested conditions (Figure 6D). Collectively, these results demonstrate that the potentially detrimental effects of DNMT inhibition on cell proliferation and inflammatory responses are not restricted to GFs and at least partly occur in other periodontium-derived stromal cells, such as PDLFs.

Discussion

As one of the most prevalent chronic diseases, periodontitis represents a significant burden for healthcare systems (33). Uncontrolled inflammation of the periodontium not only causes tooth loss, but also leads to an increased risk of developing many systemic diseases, including rheumatoid arthritis, atherosclerosis, (RA), and malignancy (34, 35). Since the currently used treatment strategies focusing on reducing the bacterial challenge may be insufficient to restore microbial homeostasis and resolve chronic inflammation, there is a need for identifying new therapeutic options for the management of periodontitis (2). The idea of "host modulation therapy", where medications aimed at the damaging aspects of the immune response support conventional periodontal treatment, has received much attention in recent years (36). Based on their ability to suppress inflammation and modulate host responses to pathogenic bacteria (37, 38), many epigenetic drugs have been evaluated in preclinical models of periodontitis. We have previously demonstrated that histone deacetylase inhibitors (HDACi) and BET bromodomain inhibitors suppress the inflammatory activation of GFs without affecting cell viability and susceptibility to bacterial invasion (23, 39). These observations, in combination with previous evidence that BET inhibitors and HDACi reduce inflammation and ameliorate alveolar bone resorption in animal models of periodontitis

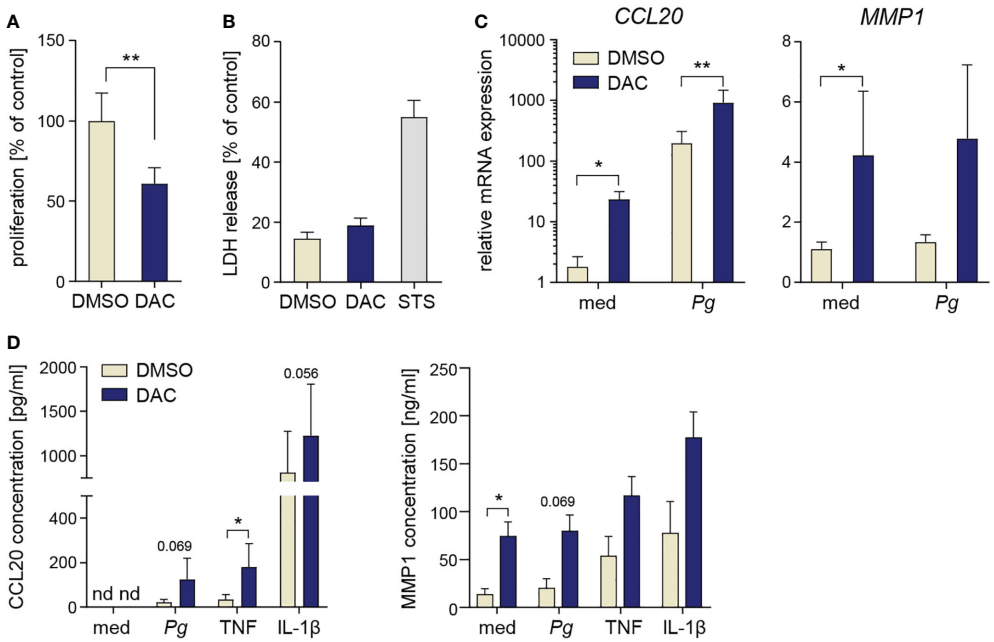

FIGURE 6
DAC has anti-proliferative and pro-inflammatory effects on PDLFs. **(A)** Proliferation rate (n=5) and **(B)** LDH release (n=4) in PDLFs treated with DMSO or DAC for 12 days. **(C)** qPCR analysis of *CCL20* and *MMP1* mRNA expression in PDLFs (n=5) treated with DMSO or DAC for 12 days prior to infection with *P. gingivalis* (*Pg*, MOI 20) for 4 h; 'med' refers to uninfected control. **(D)** CCL20 and MMP1 production by DMSO- or DAC-treated PDLFs that were infected with *P. gingivalis* (MOI 50) for 1 h followed by 23 h of culture in fresh medium, or were stimulated with TNF or IL-1β (both at 10 ng/ml) for 24 h (n=4). nd, not detectable; *P < 0.05, **P < 0.01.

(40, 41), identified histone acetylation as a potential target for epigenetic host modulation therapy (7). In the present study, we show that major transcriptional changes induced by DNMT inhibitors affect the viability and inflammatory activation of GFs and PDLFs in ways that can be detrimental in the context of the pathogenesis of periodontitis. In light of the well-documented hematologic toxicities of hypomethylating agents (14), recent evidence of DAC genotoxicity (42), and our observations reported here, these compounds are unlikely candidates for host modulation therapy in periodontitis.

Nonetheless, our observations reveal the scope of cellular process in GFs that are regulated by DNA methylation. Among the 400 genes significantly upregulated in DAC-treated GFs infected with *P. gingivalis*, we identified several pro-inflammatory cytokines and chemokines, including *CCL20*, *CCL5*, *CCL8*, *CCL13*, *TNF*, *IL1A*, *IL18*, *IL33*, and *CSF3*. Elevated levels of many of these mediators have been detected in gingival crevicular fluid or tissue from patients with periodontitis (43), and their activity contributes to the chronicity of inflammation and alveolar bone resorption. For example, the levels of IL-33 and granulocyte colony-stimulating factor (G-CSF), encoded by the *CSF3* gene, are increased in mice with experimentally induced periodontitis and in gingival epithelial cells from periodontitis patients (44–46). *In vivo* experiments confirmed the pathological roles of these cytokines: treatment of mice with IL-33 exacerbates bone loss (44), whereas neutralization of G-CSF with a blocking antibody alleviates bone resorption (46). While our *in vitro* observations are not consistent with the study of Tanaka et al., who demonstrated immunomodulatory effects of DAC in experimental periodontitis (19), it should be noted that the influence of DNMT inhibitors on inflammatory processes is highly disease- and cell type-specific. DAC has been shown to promote cytokine production by human dental pulp cells and bronchial epithelial cells (47, 48), and upregulate pro-inflammatory pathways in an epithelial breast cancer cell line (49). In contrast, DAC treatment had anti-inflammatory effects in mouse models of atherosclerosis (50), acute respiratory distress syndrome (51), and arthritis (52).

The combination of transcriptomic and functional studies allowed us to identify multiple aspects of GF biology other than inflammatory pathway activation that are regulated by DNA methylation. Although pathway analysis did not reveal gene clusters involved in the regulation of cell survival, DAC treatment significantly upregulated cyclin-dependent kinase inhibitors *CDKN1A* ($p21^{CIP1}$), *CDKN2A* ($p16^{INK4a}$), and *CDKN2B* ($p15^{INK4b}$), which are key regulators of cell proliferation. Upregulation of these cell cycle inhibitors is associated with GF senescence and impairment of ECM production (53, 54). Pathway analysis of DAC-induced genes also identified several processes related to interferon signaling and antiviral responses. While the functional consequences of this effect require further research, it should be noted that GF stimulation with bacterial cyclic dinucleotides results in the induction of a similar cluster of interferon signaling effectors, including *ISG15*, *MX1*, and *IFIT1* (55). Finally, several DAC-inducible genes in GFs overlap with the transcription profiles observed in gingival biopsies from patients with chronic periodontitis. Out of the 20 most upregulated genes in periodontitis tissues compared to controls (56), five were induced in DAC-treated GFs in our experiments (*CSF3*, *GLDC*, *SAA1*, *SAA2*, *GDF15*), highlighting the notion that DNMT inhibitors upregulate several genes that are associated with periodontitis pathology.

Although verification of the changes in DNA methylation profiles at individual gene promoters is beyond the scope of this study, many of the identified DAC-sensitive genes are reportedly regulated by changes in methylation of their promoter regions and sensitive to

hypomethylating agents. Differential *CCL20* promoter methylation was identified in different T cell subsets (57) and in hepatocellular carcinoma (HCC) samples (58), correlating with mRNA expression levels. In line with our observations in GFs and PDLFs, *CCL20* expression was strongly induced by DAC treatment in HCC cells (58). Similarly, DAC-induced upregulation of *ICAM1* has been reported in several cancer cell lines, including cutaneous melanoma (59), pediatric sarcoma (60), and glioma cells (61). *MMP* genes are also regulated by promoter methylation (62–64). Interestingly, MMP1 is regulated by DNMT inhibitors in fibrosarcoma cells through a transcription-dependent mechanism that involves recruitment of the transcription factor Sp1 (65). This suggests that other mechanisms of action of hypomethylating agents may also be responsible for the observed induction of MMPs.

In our experiments, the chemokine CCL20 was highly susceptible to regulation by DNMT inhibitors. Because CCL20 shares common structural characteristics with β-defensins and its antimicrobial activity against many Gram-negative and Gram-positive bacteria has been confirmed *in vitro* (26), it was postulated that increased production of CCL20 may enhance innate immune responses by directly eliminating oral pathogens (66). We tested this possibility, and our analysis indicates that even at concentrations exceeding those released by DAC-treated GFs CCL20 is unable to affect *P. gingivalis* growth. It is also unlikely that local accumulation of CCL20 at high concentration will exert antimicrobial activity because CCL20 is rapidly degraded by *P. gingivalis* proteolytic enzymes. Consistently, *P. gingivalis* can potently degrade other chemokines with antimicrobial properties (28), such as CXCL10 (67). Instead, the amounts of CCL20 released by DAC-treated GFs are well within the range required for its chemotactic activity (26). Elevated production of CCL20 by cells treated with DNMT inhibitors may therefore contribute to the accumulation of leukocytes expressing CCR6, such as dendritic cells, B cells, and specific T cell subsets, including Th17 cells. It is likely that increased infiltration of CCR6-expressing leukocytes caused by augmented CCL20 release would contribute to exacerbation of chronic inflammation. However, formal verification of this possibility would require analysis of gingival tissue composition after DAC treatment in an animal model of periodontitis (68).

While global epigenomic studies are needed to characterize promoter-specific changes in DNA methylation profiles induced by DAC in stromal cells of the periodontium, these results show that hypomethylating agents are a useful tool to identify the genes and processes regulated by DNA methylation in cells involved in the pathogenesis of periodontitis. Conversely, the detrimental effects of DNMT inhibitors on GF and PDLF proliferation and viability, as well as the activation of multiple pro-inflammatory pathways associated with the pathobiology of periodontal disease, may greatly limit the therapeutic potential of these compounds.

Data availability statement

The RNA-Seq dataset of DMSO- and DAC-treated GFs presented in this study has been deposited in the Gene Expression Omnibus (GEO) and is available under the accession number: GSE216757.

Ethics statement

The studies involving human participants were reviewed and approved by Bioethical Committee of the Jagiellonian University in Krakow, Poland (opinion number 1072.6120.104.2019) and the research ethics committee of the OLVG, Amsterdam, The Netherlands (protocol-ID: WO 17.194). The patients/participants provided their written informed consent to participate in this study.

Author contributions

KBL-C and MM performed experiments and contributed to study design, data acquisition, analysis and interpretation. EN, AS, AB, and SD contributed to data acquisition. WL, MK, MT, DD, and MG contributed to recruitment of study participants, collection of samples and clinical data. TK, TJdV, and JP contributed to study conception and design. AMG conceived and designed the study, acquired funding, contributed to data analysis and interpretation, and drafted the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Conflict of interest

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

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Supplementary material

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Understanding the function of the GABAergic system and its potential role in rheumatoid arthritis

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Rheumatoid arthritis (RA) is a highly disabling chronic autoimmune disease. Multiple factors contribute to the complex pathological process of RA, in which an abnormal autoimmune response, high survival of inflammatory cells, and excessive release of inflammatory factors lead to a severe chronic inflammatory response. Clinical management of RA remains limited; therefore, exploring and discovering new mechanisms of action could enhance clinical benefits for patients with RA. Important bidirectional communication occurs between the brain and immune system in inflammatory diseases such as RA, and circulating immune complexes can cause neuroinflammatory responses in the brain. The gamma-aminobutyric acid (GABA)ergic system is a part of the nervous system that primarily comprises GABA, GABA-related receptors, and GABA transporter (GAT) systems. GABA is an inhibitory neurotransmitter that binds to GABA receptors in the presence of GATs to exert a variety of pathophysiological regulatory effects, with its predominant role being neural signaling. Nonetheless, the GABAergic system may also have immunomodulatory effects. GABA/GABA-A receptors may inhibit the progression of inflammation in RA and GATs may promote inflammation. GABA-B receptors may also act as susceptibility genes for RA, regulating the inflammatory response of RA *via* immune cells. Furthermore, the GABAergic system may modulate the abnormal pain response in RA patients. We also summarized the latest clinical applications of the GABAergic system and provided an outlook on its clinical application in RA. However, direct studies on the GABAergic system and RA are still lacking; therefore, we hope to provide potential therapeutic options and a theoretical basis for RA treatment by summarizing any potential associations.

KEYWORDS

rheumatoid arthritis, gamma-aminobutyric acid(GABA)ergic, GABA relatedreceptors, GABA transporter (GAT) systems, inflammation

Introduction

Rheumatoid arthritis (RA) is an autoimmune-mediated chronic inflammatory joint disease characterized by pain and swelling in the joints of the hands and feet (primarily in the toe), proximal interphalangeal joints, and wrists. Unlike the "hard" swelling of osteoarthritis, this swelling is usually "soft", and is attributed to synovitis and fluid accumulation (1, 2). The global incidence of RA is approximately 0.24% (3), with a positive correlation with age. Furthermore, RA is a chronic, long-term joint disorder that may persist for decades and even for life (4, 5); however, the pathogenesis of RA remains unclear. Nonetheless, it is understood that in RA, immune tolerance to autologous proteins (such as collagen, wave proteins, and fibrinogen) is disrupted for various reasons, resulting in the formation of autoantibodies against autoantigens, such as anti-citrullinated peptide antibodies, anti-immunoglobulin G antibodies, and autoantigens that cross-react with bacterial or viral antigens (6, 7). It occurs when the immune system mistakenly attacks the tissues in the joints, leading to inflammation, swelling, and destruction of the joint structure (8).

Neovascularization is another hallmark of RA synovitis, in which multiple synovial infiltrates of the joint occur and endothelial cells are activated; furthermore, an expansion of synovial fibroblasts and macrophage-like cells leads to the proliferation of the supra-synovial layer and invasion of the periarticular bone at the cartilage junction, ultimately leading to bone erosion and cartilage degeneration (9). RA is incurable and patients must be treated with disease-modifying anti-rheumatic drugs (DMARDs) to relieve clinical symptoms, improve somatic function, and inhibit the progression of joint damage (10). Commonly used DMARDs include methotrexate, leflunomide, and sulfonamides. Early treatment with combined methotrexate and glucocorticoids followed by other DMARDs with the inhibitors of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), or Janus kinase can improve prognosis (11). Treatment aims to reduce disease activity by at least 50% within three months and achieve remission or reduced disease activity within six months, and can prevent RA-related disability with sequential drug therapy if necessary (12, 13). Therefore, understanding and identifying novel targets is important for the clinical management and treatment of RA. There is some evidence to suggest that the GABAergic system may be involved in the development and progression of RA (14). Some studies have found that gamma-aminobutyric acid (GABA) levels are lower in people with RA compared to healthy individuals (15). Additionally, GABA has anti-inflammatory and pain-relieving effects, and some researchers have suggested that abnormal GABA signaling may contribute to the immune system dysregulation that occurs in RA (16).

The GABAergic system is a network of neurons in the brain and central nervous system that produce and use the neurotransmitter GABA (17). GABA is an inhibitory neurotransmitter that helps to regulate the activity of neurons and maintain balance in the nervous system (18). It acts by binding to specific receptors on neurons, causing them to reduce their activity. Some studies have explored the potential role of GABA signaling in RA-related inflammation and pain. For example, some research has suggested that GABA signaling may be involved in the downregulation of T-cell autoimmunity and antigen-presenting cells (APC) activity, which play a role in the development of RA-related inflammation (19). Other studies have found that GABA agonists, which are medications that enhance GABA signaling, may be effective in reducing inflammation and pain in animal models (20). It is important to note that the relationship between the GABAergic system and RA is complex and not fully understood. More research is needed to fully understand the function of the GABAergic system in RA and its potential as a target for treatment. However, the findings of some studies suggest that medications that enhance GABA signaling may be a promising approach for managing RA-related inflammation and pain.

GABAergic system

The GABAergic system comprises the following components: GABA, glutamate decarboxylase (GAD), GABA-A receptors, GABA-B receptors, and GABA transporters (GATs). GAD is predominantly composed of two isoforms, GAD65 and GAD67. The genes of these isoforms are distributed differently and have multiple functions, and GABA acts by binding to their receptors (17, 21). The balance of the GABAergic system is essential for many physiological aspects, including blood pressure, baroreceptor function, human growth hormone release, weight regulation and feeding, respiratory function, brain function, kidney function, vision, and pancreatic function (22). The most prominent feature of RA is synovial inflammation, which is closely associated with abnormal expression of immune cells and inflammatory factors. Pro-inflammatory cytokines, including tumor necrosis factor (TNF) and IL-6, induce RANKL, prostaglandins, and matrix metalloproteinases, which mediate the pain and swelling of the disease; furthermore, RANKL, TNF, and IL-6 stimulate bone and cartilage (23). Recent studies have shown that immune system cells can produce GABA and express GABA-A ion channels, GATs, and GABA-B receptors (24). The synovium also contains a GABAergic system; specifically, synovial macrophage-like A cells possess a GABA-producing system alongside GAD and GABA-B receptor subunits. Furthermore, the GABAergic system appears to play a functional role in the synovium (14). GABAergic components have been reported to negatively regulate the immune response by affecting the production of pro-inflammatory cytokines and activation of signaling pathways (16, 25). Overall, GABAergic components may

Abbreviations: GABA, gamma-aminobutyric acid; RA, rheumatoid arthritis; GAT, GABA transporter; DMARDs, disease-modifying anti-rheumatic drugs; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; GAD, glutamate decarboxylase; BGT-1, betaine/gamma-aminobutyric acid transporter; CNS, central nervous system; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; MAPK, mitogen-activated protein kinase; TAK, transforming growth factor-β activating kinase; STAT4, signal transducer-activator of transcription 4; MMP3, matrix metallopeptidase 3; MHC, major histocompatibility complex; GABBR-1, gamma-aminobutyric acid type B receptor subunit 1; mTOR, mammalian target of rapamycin; Th17, T helper 17; IFN, interferon; ZNF354C, zinc finger protein 354C; KCNN4, potassium calcium-activated channel subfamily N member 4; CXCL5, C-X-C motif chemokine ligand 5; CCL20, C-C motif chemokine ligand 20; APC, antigen-presenting cells.

offer a new therapeutic approach for inflammatory and autoimmune diseases, including RA. However, the GABAergic system in the synovium has yet to be studied. In this article, we explore the role of GABAergic components in the regulation of inflammation and pain in RA by summarizing the major components of the GABAergic system and their physiological functions, alongside the potential links that exist with RA.

GABA receptors are widely expressed in the central nervous system (CNS) and occupy approximately 20% of the synapses in the cerebral cortex, hippocampus, thalamus, and cerebellum. These are closely linked to the control of cognitive functions such as memory, language, and attention (17); they are also located in the retina, thalamus, hippocampus, pituitary, and gastrointestinal tract, and are associated with visual processing, sleep-wake rhythm regulation, pain perception, memory, learning, hormone regulation, and neuroendocrine gastrointestinal secretion (26). GATs are present in the presynaptic membrane. GAT-1 and GAT-3 are abundantly expressed in the CNS, whereas GAT-2 and betaine/gamma-aminobutyric acid transporter (BGT-1) are expressed in tissues such as the liver, kidney, and intestine (26–28).

Physiological synthesis of GABA

GABA is the main inhibitory neurotransmitter in the CNS and plays a key role in controlling excitability, plasticity, and network synchronization in the CNS. GABA is present in the CNS alongside many other organs, such as the pancreas, pituitary gland, testes, gastrointestinal tract, ovaries, placenta, uterus, and adrenal medulla (29, 30). GABA synthesis requires GAD, which consists of two isomers (GAD65 and GAD67), encoded by a single gene on chromosomes 10 and 2, respectively, and is the rate-limiting enzyme that catalyzes the conversion of glutamate to the inhibitory neurotransmitter GABA (31). GABA is primarily synthesized *via* two pathways: direct synthesis from glutamic acid catalyzed by GAD65 or GAD67, and synthesis from glutamic acid produced by trichloroacetic acid catalyzed by GAD67. GABA acts through its receptors, primarily GABA-A and GABA-B (32). The GABA-A receptor is an ion-affinity receptor that binds to GABA and opens its complete chloride channel. In contrast, GABA-B receptors are G protein-coupled metabotropic receptors that have a negative effect on presynaptic voltage-activated calcium channels and a positive effect on postsynaptic inwardly rectifying potassium channels (33). GABA signaling in the synaptic cleft can be reuptaken by high-affinity GATs into the presynaptic membrane, thus avoiding GABA overexpression (34).

GABA receptors

GABA receptors are the major inhibitory neurotransmitters of the vertebrate CNS and are expressed by many neurons in the CNS, as well as other cell types in the periphery, with two main types: GABA-A and GABA-B receptors (35). GABA-A receptors (ionotropic receptors) are ligand-gated ion channels that bind to GABA and open the Cl^- channel. GABA-A receptors include many subunit types: α (one-six), β (one-three), γ (one-three), δ, ϵ, θ, π, and ρ (one-three). The most common GABA-A receptor subunit types in the brain are α1, β2, and γ2 (36). GABA-A receptors are recruited to increase acetylcholine release and facilitate transmission; however, GABA-B receptors are activated at high GABA concentrations, thereby decreasing acetylcholine release and transmission (36, 37).

GABA-B receptors (metabotropic receptors) are G-protein coupled receptors that have a negative effect on presynaptic voltage-activated Ca^{2+} channels but a positive effect on postsynaptic inwardly corrected K^+ channels (38). GABA-B receptors consist of B1 and B2 subunits, and it is generally believed that B1a/B2 heterodimers are localized to presynaptic neurons, while B1b/B2 heterodimers are localized to postsynaptic neurons. GABA-B receptors bind to G protein subsets, which consequently regulate specific ion channels, such as calcium channels, and trigger cyclic adenosine monophosphate cascade responses (39). GABA-B receptors modulate their inhibitory effects by activating the inwardly regulated K^+ channels, inactivating voltage-stalled Ca^{2+} channels, and inhibiting adenylyl cyclase. Postsynaptic receptors induce slow inhibitory postsynaptic currents that hyperpolarize the membrane and shunt excitatory currents by gating a particular type of K^+ channel (39, 40).

GATs

The GAT family, which includes GAT-1, GAT-2, GAT-3, and BGT-1, are important regulators of intracellular and extracellular GABA concentrations; specifically, these transporters mediate the secondary active transport of ion-coupled GABA across the plasma membrane (41). GATs inhibit GABA signaling by translocating GABA to cells and reducing extracellular GABA concentrations, and are potential drug targets in various diseases associated with dysregulated GABA delivery. GAT-1 and GAT-3 are predominantly expressed in the CNS where they regulate GABA activity (42), whereas GAT-2 is mainly located in peripheral organs, especially in the liver and kidney, and is thought to be a GABA and taurine transporter in the liver (43). GAT-1 is a highly conserved molecule encoded by solute carrier family 6 member 1, which transports GABA *via* Na^+ and Cl^-. As the major GAT in the brain, in addition to being involved in a wide range of brain functions, GAT-1 has been implicated in the pathophysiology of several neuropsychiatric disorders, including anxiety disorders, depression, epilepsy, Alzheimer's disease, and schizophrenia (44).

Potential link between GABAergic system and RA

The GABAergic system may act as a bridge between the nervous system and the immune system. As an inhibitory neurotransmitter in the CNS, the main role of GABA is to reduce the excitability of neurons throughout the nervous system (18). Activation of GABA and other ligands by the GABA receptor induces a conformational change in the receptor that increases axonal K^+ conductance, thereby accelerating action potential repolarization and leading to transient secondary inhibition of calcium by decreasing the degree of calcium channel activation (45, 46). GABAergic system plays an important

role in the function of immune cells mainly being influenced by GABA signaling. Recent studies have highlighted the immune function of GABA, suggesting cross-talk between the nervous and immune systems (47, 48). T cells express many neurotransmitter receptors, and their expression is regulated by T cell receptor activation, cytokines, and neurotransmitters themselves (49). The GABAergic system can negatively regulate immune responses, especially T cell–mediated immune responses, by affecting the production of pro-inflammatory cytokines and the activation of signaling pathways, such as mitogen-activated protein kinase and nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) pathways (50). These results may indicate that the GABAergic system offers a new therapeutic approach for inflammatory and autoimmune diseases and requires further evaluation, especially regarding RA treatment.

GABA/GABA-A receptor may inhibit inflammation in RA

Central and peripheral GABA-A receptors play a major role in inflammation; specifically, GABA inhibition reverses the pathological pain state in mice, and GABA-A reduces the production of pro-inflammatory mediators, thereby ameliorating the symptoms of inflammation (51). GABA/GABA-A receptors may induce RA inflammation by affecting T cell and macrophage populations. Mechanistically, GABA receptors firstly interact with KCNN4 to induce Ca^{2+} entry, resulting in activation of nuclear factor κB signal, and finally promotes macrophage invasion by inducing CXCL5 and CCL20 expression (52). This functional GABAergic system acts as a regulator of T cell activation (16). GABA is not only an inhibitory neurotransmitter but also an immunomodulator. GABA binding to GABA-A receptors directly affects the function of antigen-presenting cells and inhibits the production of inflammatory cytokines by T cells (53). Binding of GABA to GABA-A receptors also inhibits the proliferation of antigen-specific T cells and suppresses the production of IL-6, IL-12, inducible nitric oxide synthase, IL-1β, and TNF-α (52, 54). Furthermore, activation of GABA-A receptors inhibits the release of TNF-α and IL-6 from alveolar macrophages induced by lipopolysaccharide (55).

P38/MAPK may be a key player in the link between the GABA/GABA-A axis and inflammation in RA. P38 is a tyrosine phosphoprotein kinase isolated and purified from mammalian cells stimulated by endotoxin; this enzyme is the most important member of the mitogen-activated protein kinase (MAPK) family in terms of inflammatory response mediation (56). Key enzymes of the P38/MAPK pathway include mitogen-activated protein kinase kinase (MKK)-3, MKK6, and transforming growth factor-β activating kinase (TAK) (57). TAK activates MKK4, which in turn activates P38/MAPK that phosphorylates and activates many protein kinases and transcription factors (58). P38/MAPK activates signal transducer-activator of transcription 4 (STAT4) and NF-κB and promotes the release of inflammatory factors such as TNF-α, IL-1β, and IL-6 (59). Inflammatory stimuli, such as TNF-α, platelet-activating factor, and interleukins, induce P38 activation in endogenous immune cells such as monocytes, endothelial cells, and neutrophils (60, 61). When synovial disruption begins, somatic afferent pain signals received by the spinal cord could lead to stress-induced kinase release; these pain and cytokine signals activate P38/MAPK, which induces the upregulation and release of pro-inflammatory cytokines such as IL-1, IL-6, and matrix metallopeptidase 3 (MMP3) into the periphery (62, 63). A relationship exists between the CNS and peripheral immune response; specifically, afferent pain signals help the CNS propagate the inflammatory response, which may influence the development of peripheral arthritis (64). GABA is a major inhibitory neurotransmitter in the CNS that downregulates P38 activity to reduce the production of proinflammatory cytokines. For example, GABA prevents IL-6 release by inhibiting P38/MAPK in glioma cells, potentially affecting the inflammatory response in RA (65, 66).

The GABAergic system may be involved in regulating the pain response in RA

The GABAergic system has been suggested to be involved in the pain response in RA. Pain is one of the most important manifestations of RA, and is thought to be caused by joint inflammation (67). Inflammatory pain is predominantly due to local joint synovial inflammation caused by pro-inflammatory cytokines. This inflammation leads to activation of afferent nociceptive fibers and transmission of "pain" signals to the dorsal horn of the spinal cord, *via* the spinothalamic tract, to the thalamus (68, 69). However, clinical studies have shown that even when inflammation is controlled, patients with RA may continue to experience non-inflammatory pain (70). The main factors causing non-inflammatory pain are yet to be determined. Nonetheless, they are understood to be related to structural changes in the patient's joint environment, continued disease progression, ectopic secretions, and peripheral nerve damage and dysfunction due to increased excitability of damaged afferent injury receptors, which manifest as neuropathic pain (71, 72). GABA is an inhibitory neurotransmitter; therefore, selective loss of GABAergic interneurons after peripheral nerve injury is thought to be the underlying cause of inhibitory signal loss in RA. Furthermore, reduced inhibitory neurotransmission is a key feature of chronic pain states (73). There are two possible causes for this decrease in inhibitory neurotransmission: a decrease in GABA and its synthase caused by apoptosis of GABAergic neurons in the spinal cord (74, 75) and depletion of GABA in the synaptic gap (76). GABA-A and -B receptors and GAT proteins appear to be involved in the pathophysiology of the chronic pain associated with RA (73). Currently, several studies have investigated the positive modulators of these two receptors and used them for various types of pain; furthermore, a successful testing phase in animal models has recently been achieved (77). GABA-A and glycine receptors are key elements of the spine that control injury perception and pain. Impaired function of these receptors may contribute to the development of chronic pain; therefore, restoring their normal function through aggressive modulators has become an effective treatment for chronic pain syndromes (78). In the context of RA, the pain response in patients with RA may be modulated through the GABAergic system. Therefore, some small-molecule inhibitors or activators currently targeting the GABAergic system may be potentially beneficial in RA.

GABA-B receptors may be associated with inflammation in RA

Neutrophil is one of the key inflammatory effector cells of RA.GABA-B receptors are expressed in neutrophils and play an important role as chemotactic receptors in the inflammatory response (79). GABA-B receptors are also metabotropic receptors. Unlike ionotropic GABA-A receptors, which utilize rapid synaptic transmission, GABA-B receptors are heterodimers consisting of subunits encoded by *GABBR1* and *GABBR2* (80). The coding region of GABA type B receptor subunit *(GABBR)-1* is located on chromosome 6, 6p21.3, and the major histocompatibility complex (MHC) located in this region is associated with multiple sclerosis, Alzheimer's disease, schizophrenia, narcolepsy, epilepsy, and RA (81). The specific MHC, class II, DR beta 1 alleles are strongly associated with susceptibility to RA; this susceptibility is likely due to their role in presenting arthritogenic polypeptides. However, the linkage disequilibrium of the *GABBR1* gene polymorphism with these alleles is not as expected, as the distance between the two motifs is three Mb (82). Thus, the observed genetic association with this region suggests that *GABBR1* plays an independent role in genetic susceptibility to RA (83).

In yeast glycan-induced arthritis, GABA-B receptors are involved in neutrophil migration to the knee, similar to GABA; this migration may be associated with P38/MAPK activation in the spinal cord (84). Spinal cord inhibitory signaling may downregulate P38/MAPK and reduce pro-inflammatory cytokine production. Furthermore, any changes that affect this negative regulation, such as SNP alleles or haplotypes in *GABBR1*, may allow P38/MAPK to continue and worsen RA pathology in an uncontrolled manner (82). Although *GABBR1* polymorphisms have not been experimentally characterized in RA patients, computational analysis suggests that *GABBR1* encoding multiple isoforms, mutations, and several genes that potentially affect selectively spliced protein structures may be associated with RA progression (85).

GATs promote inflammation

Members of the GAT family, including GAT-1, GAT-2, GAT-3, and BGT-1, may contribute to inflammation. Multiple inflammatory factors, such as IL-6, IL-1β, and TNF-α are present in RA (86). The expression of GAT-1 and GAT-3 is closely associated with inflammatory factors, such as IL-6, IL-1β, and TNF-α, and may show a positive correlation with inflammation (87). IL-1β and TNF-α were found to upregulate GAT-1 and GAT-3 expression through the MAPK pathway, which subsequently increased IL-6 levels and further upregulated GAT-1 and GAT-3 expression; alternatively, inhibition of IL-1β and TNF-α receptors attenuated this GAT-1 and GAT-3 expression (88). GATs may be associated with subpopulations of lymphoid T cells and have been determined to regulate cytokine production and T cell proliferation. The gene transcripts of two cotransporters, GAT-1 and GAT-2, have been detected in immune cells and identified in human peripheral blood lymphocytes (89). GABA inhibits Th1 cell–mediated DTH responses *in vivo* and participates in T cell immunity *via* the GAT and GABA receptors. For example, GAT-1 is expressed only on antigen-activated T cells and downregulates the proliferation and expression of $CD4^+$ T cells (90). These findings suggest that GAT-1 is a key regulator of the T cell–mediated immune response. Additional evidence suggests that T cells expressing GAT-2 and GAT-2 deficiency promote T helper 17 cell (Th17) responses through the activation of GABA-mammalian target of rapamycin signaling; specifically, in a mouse model of infection, GAT-2 deficiency was observed to enhance the differentiation of naive T cells into Th1 cells (91).

GAT-2 is primarily pro-inflammatory in RA. Furthermore, Interferon (IFN)-γ is an important effector of RA (92) that induces GAT-2 expression in macrophages (93). A systematic evaluation and meta-analysis of common trace metals in RA found that serum copper levels were elevated in RA and higher in patients with active RA. These levels were positively correlated with erythrocyte sedimentation rate and morning stiffness, and were negatively correlated with hemoglobin levels and considered to be adjunctive markers for disease assessment (94, 95). Therefore, GAT-2 may promote inflammatory responses by being associated with abnormally high copper levels in RA. GAT-2 deficiency attenuates macrophage-mediated inflammatory responses *in vivo*, including lipopolysaccharide-induced sepsis, infection-induced pneumonia, and high-fat diet-induced obesity (25, 96). GAT-2 deficiency also decreases IL-1β production in pro-inflammatory macrophages. This mechanism may involve enhancement of the betaine/S-adenosylmethionine/hypoxanthine metabolic pathway by increasing DNA methylation in its promoter region to inhibit the expression of the transcription factor zinc finger protein 354C (*ZNF354C).* This zinc finger protein suppresses inflammasome formation and inhibits M1 macrophage polarization by targeting the expression of oxidative phosphorus-related genes (93). According to previous reports, activation of the GABAergic system in macrophages enhances autophagy activation, phagosome maturation, and antimicrobial response to *Mycobacterium* infection (97). Macrophage autophagy in the context of RA is an important mechanism that may co-mediate the abnormal immune response of macrophages with the GABAergic system (98).

Future perspectives and challenges: Clinical trials related to GABAergic system components

Relevant clinical trials of the major components of the GABAergic system mainly involve GABA, GABA receptor agonists and antagonists, and GABA agonists and antagonists (Table 1). Although there have been no direct clinical studies in the context of RA, ongoing clinical trials are currently providing information regarding the development of clinical small-molecule GABA drugs for RA. First, GABA and GABA-A receptor agonists can inhibit the immune response of immune cells to stimuli, which may be important for remission of inflammation (99). For example, honokiol (a GABA-A receptor modulator) relieves inflammatory arthritis and allergic asthma by affecting cytokine expression of cytokines (100). Additionally, a series of immunological abnormalities have been reported in T1DM patients, involving production of autoantibodies, glutamic acid decarboxylase (GAD-

TABLE 1 Clinical trials of GABAergic system components.

Name	Condition or disease	ClinicalTrials.gov number	Sponsor	Interventions	Primary outcomes	Phase
GABA	Pain Healthy subjects	NCT02928328 NCT04086108	Aalborg University Wageningen University	GABA/ lorazepam GABA/Tomato	Pain Intensity Rating Area under the plasma concentration versus time curve (AUC) of plasma-time curves of GABA	Not Applicable Not Applicable
	Diabetes Mellitus, Type 1 Diabetes Mellitus, Type 1 Healthy subjects Diabetes Mellitus, Type 1 Sleep Disturbance Type I Diabetes Epilepsy	NCT04375020 NCT03635437 NCT04303468 NCT03721991 NCT04857021 NCT02002130 NCT04144439	Ministry of Health and Population, Egypt Uppsala University Hospital Wageningen University Steno Diabetes Center Copenhagen Amorepacific Corporation University of Alabama at Birmingham Tanta University	GABA GABA/ Alprazolam GABA/Placebo GABA/Placebo GABA/Placebo GABA/Placebo/ GAD-alum GABA	GABA decrease anti gad antibodies GABA improve c peptide levels Adverse events possibly or probably related to GABA treatment Postprandial glycemic response during a 2-hour oral glucose tolerance test (OGTT) Insulin production, c peptide production during meal stimulation Change of total sleep time of polysomnography. Change of sleep latency of polysomnography. Change of NonREM stage 3 of polysomnography Compare the effect of oral GABA or oral GABA/GAD combination administration on pancreatic beta cell function by quantitative C-peptide secretion Number of seizures [Time Frame: 6 months]	Not Provided Phase 1 Not Applicable Not Applicable Not Applicable Phase 1 Phase 4
AZD7325	Healthy subjects	NCT02135198	University College, London	AZD7325/ Placebo AZD7325/ Placebo AZD7325/ Placebo AZD7325/ Placebo Baclofen/Placebo Baclofen Baclofen/Placebo Arbaclofen/ Placebo Clobazam/ Clonazepam/ Tolterodine	Change in conventional measure of percentage short interval intracortical inhibition (SICI) at an interstimulus interval (ISI) of 2.5 ms and conditioning stimulus intensity of 70 percent of resting motor threshold	Phase 1 Phase 1 Phase 2 Not Applicable Not Applicable Phase 2 Phase 1 Not Applicable Phase 3
	Healthy subjects Autism Spectrum Disorder Autism Spectrum Disorder	NCT02530580 NCT01966679 NCT03678129	University College, London University of California, Los Angeles King's College London Imperial College London National Institute on Drug Abuse Assistance Publi que-Hôpitaux de Paris King's College London University Hospital Inselspital, Berne		Change in peak grip force in an object manipulation task [Time Frame: from baseline at 1, 2, and 3 hours after the study medication] Electroencephalogram [Time Frame: week 6] Neurochemical response to GABAergic stimulation.	
Baclofen Arbaclofen Clobazam	Healthy subjects Cocaine-Related Disorders CHEYNE Stokes Respiration Autism Spectrum Disorder Pain	NCT01563224 NCT00218166 NCT01095679 NCT03594552 NCT01011036			EEG spectral power in theta band [Time Frame: Change from baseline to 4 hours after dosing] Progressive-ratio break point [Time Frame: Measured during each experimental session] Decrease in the coefficient of variation of the period of the ventilatory cycle Neurochemical response to GABAergic stimulation Area of hyperalgesia on the forearm	
Flumazenil Iomazenil	Fragile X Syndrome or Idiopathic Intellectual Developmental Disorder Primary/Idiopathic Hypersomnia Cognitive Dysfunction	NCT04308954 NCT01183312 NCT00611572	Stanford University Emory University Yale University	Flumazenil Flumazenil Iomazenil	Non-displaceable binding potential of F18 FMZ GABA (A) receptor density in fragile X syndrome (FXS) patients relative to control group comprising individuals with IDD Change in Psychomotor Vigilance Task (PVT) Median Reaction Time P300 as an ERP measure Mismatch Negativity	Phase 1 Phase 2 Phase 1

65), tyrosine phosphatase-associated islet antigen 2, zinc transporter protein 8, and insulin; furthermore, there is an altered ability of regulatory T cells (Treg cells) to inhibit the action of effector T cells, which play a key role in the immune destruction process (101). GABA also acts on GABA-A receptors in pancreatic alpha cells, thereby inhibiting glucagon secretion, suppressing inflammation, and increasing the number of regulatory T cells. In RA, Treg cells are important for the proliferation of inflammatory cells (Th17) and suppression of inflammatory factor secretion. Therefore, GABA has potential therapeutic implications for RA by increasing the number of regulatory T cells. Furthermore, the application of activators or inhibitors of the GABAergic system may have potential therapeutic effects on the pain response in RA. For example, studies based on animal models of inflammatory or neuropathic pain have found that selective positive modulators of α2 and α3 subtypes of GABA-A receptors may reverse the loss of postsynaptic GABA-A receptor-mediated spinal cord inhibition, leading to analgesia (102). GABA-B receptors are highly expressed in the structure of the pain pathway, suggesting their involvement in different levels of pain signaling; thus, they have long been considered valuable targets for the treatment of chronic pain (103). Activation of GABA-B receptors leads to hyperpolarization and reduced firing frequency, increased mediation of inhibitory neurotransmission, and production of analgesia in the brain and spinal cord (104). The GABA-B receptor agonist, baclofen, is widely used in clinical practice for the treatment of chronic pain. In the presence of reduced endogenous GABAergic tone, the GABA-B receptor agonist baclofen exerts an anti-injury sensory effect and releases GABA from the cortical precursors of GABAergic interneurons transplanted into the medial ganglion bulge, ultimately reversing neuropathic abnormal pain (105). GAT proteins are highly dynamic in different cell types and are responsible for the recycling and reuptake of GABA, thereby terminating inhibitory signaling (106). Studies have reported that inhibition of GAT reuptake enhances GABAergic neurotransmission and has an inhibitory effect on the release of aspartate and glutamate in the dorsal spinal cord, thereby inhibiting the release of pro-nociceptive neurotransmitters to achieve analgesia (107).

Discussion

This article focuses on the physiological role of the GABAergic system, its components, and the possible mechanisms of its influence in RA pathology (Figure 1). RA is an inflammatory disease that manifests in peripheral joints and connects the nervous system to the immune system *via* spinal P38/MAPK. GABA is not only a neurotransmitter, but also an immunomodulator that downregulates P38/MAPK activity to reduce the production of pro-

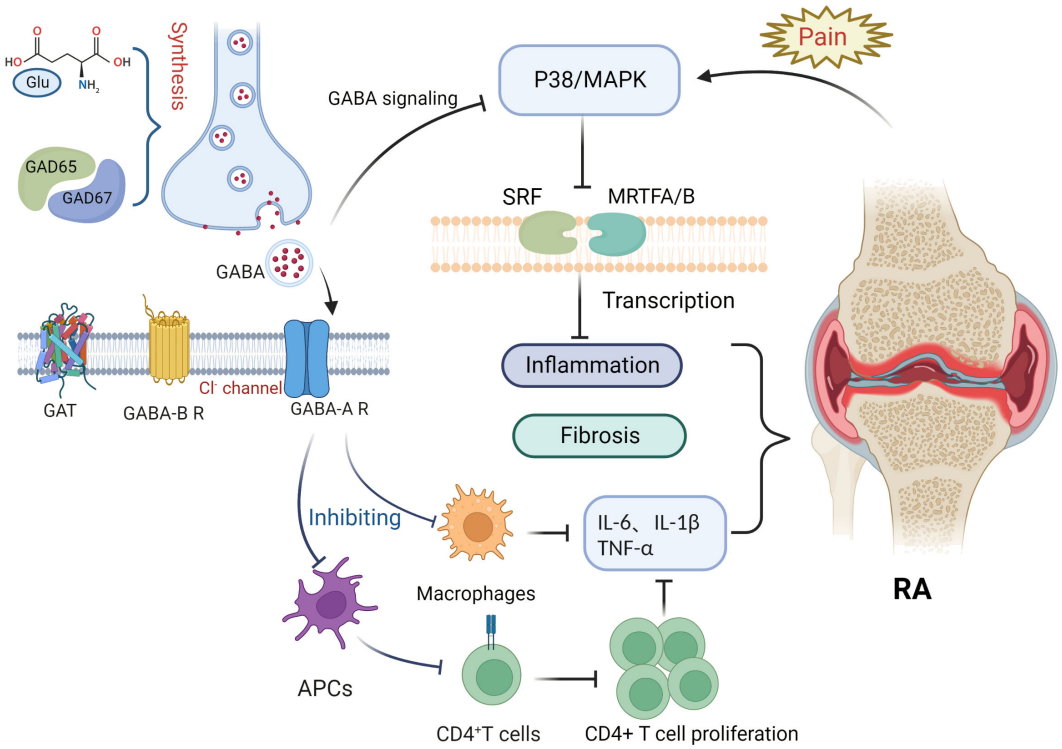

FIGURE 1
Potential role of the gamma-aminobutyric acid (GABA)ergic system in rheumatoid arthritis. Glutamate interacts with glutamate decarboxylase (GAD65 and GAD67) to produce gamma-aminobutyric acid (GABA). Binding of GABA to GABA-A receptors inhibits macrophage activation and decreases the release of inflammatory factors such as IL-6, IL-1β, and TNF-α. Antigen presentation by antigen-presenting cells, however, is impaired, inhibiting $CD4^+$ T cell proliferation and differentiation and reducing the expression of inflammatory factors such as IL-6, IL-1β, and TNF-α. Pain signaling activates the P38/MAPK pathway, whereas GABA binding to GABA-A receptors inhibits P38/MAPK. The P38/MAPK signaling pathway contributes to inflammation and is involved in the activation of myocardin-related transcription factor A (MRTFA), myocardin-related transcription factor B (MRTFB), and serum response factor (SRF) that played key roles in fibroblast activation.

inflammatory cytokines in RA joints, inhibit pro-inflammatory T cell value, and increase the number of regulatory T cells. Through MAPK phosphorylation, the GABA-A receptor inhibits the production of inflammatory cytokines *via* T cells and suppresses the proliferation of effector T cells, thereby inhibiting the inflammatory progression of RA. In contrast, the GABA-B receptor may be relevant to the pathogenesis of RA due to its unique coding region. GAT expression is positively correlated with inflammatory factors, with an increase in GATs promoting high expression of inflammatory factors and vice versa. GAT-2 deficiency enhances the differentiation of naive T cells into Th1 cells. The GABAergic system also plays an important role in analgesia, as GABA is an inhibitory neurotransmitter that negatively regulates pain signaling; similarly, its derivative, gabapentin, is also used in the treatment of pain. Both GABA receptors and its modulators are potential targets for pain treatment; consequently, studies surrounding the use of GAT inhibitors for pain treatment are ongoing. Therefore, we can see that the GABAergic system plays an important role in the pathogenesis and disease progression of RA, and in addition, GABA has excellent prospects for application in the control of RA-related pain. Nonetheless, there is still no relevant research about the direct link between GABA and the pathogenesis of RA. Further exploration regarding other aspects of the research, such as the nervous and immune systems, could improve our understanding of the overproduction of cytokines, underlying genetic information, and related signaling pathways in RA or neurodegeneration, which will ultimately provide a multidisciplinary understanding of RA and neurological diseases.

Author contributions

YS and JZ are responsible for the collection, collation, and writing of the original manuscript. YZ is responsible for the collection. SG, SS, and DH are responsible for the concept development, revision, and manuscript review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Role of the granzyme family in rheumatoid arthritis: Current Insights and future perspectives

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Rheumatoid arthritis (RA) is a complex autoimmune disease characterized by chronic inflammation that affects synovial tissues of multiple joints. Granzymes (Gzms) are serine proteases that are released into the immune synapse between cytotoxic lymphocytes and target cells. They enter target cells with the help of perforin to induce programmed cell death in inflammatory and tumor cells. Gzms may have a connection with RA. First, increased levels of Gzms have been found in the serum (GzmB), plasma (GzmA, GzmB), synovial fluid (GzmB, GzmM), and synovial tissue (GzmK) of patients with RA. Moreover, Gzms may contribute to inflammation by degrading the extracellular matrix and promoting cytokine release. They are thought to be involved in RA pathogenesis and have the potential to be used as biomarkers for RA diagnosis, although their exact role is yet to be fully elucidated. The purpose of this review was to summarize the current knowledge regarding the possible role of the granzyme family in RA, with the aim of providing a reference for future research on the mechanisms of RA and the development of new therapies.

KEYWORDS

rheumatoid arthritis, granzymes, apoptosis, inflammation, biomarker

Abbreviations: RA, Rheumatoid arthritis; CD, Cluster of Differentiation; Gzms, Granzymes; GzmA, Granzyme A; GzmB, Granzyme B; GzmH, Granzyme H; GzmK, Granzyme K; GzmM, Granzyme M; CLs, Cytotoxic lymphocytes; CTLs, Cytotoxic T lymphocytes; NK, Natural killer; NKT, Natural killer T; IL-1b, Interleukin-1b; TLR, Toll-like receptor; TNF, Tumor necrosis factor; RF, Rheumatoid factor; IL-6, Interleukin-6; IL-8, Interleukin-8; IL-1, Interleukin-1; IL-10, Interleukin-10; Il-15, Interleukin-15; IL-29, Interleukin-29; PAR, Proteinase-activated receptor; GSDMB, Gasdermin B; OA, Osteoarthritis; DAS28, Disease activity score; OCs, Proliferation Osteoclasts; ECM, Extracellular matrix; ACPAnegative, Anti-citrullinated protein antibody-negative; EVs, Extracellular vesicles; ROS, Reactive oxygen species.

Introduction

Rheumatoid arthritis (RA) is a common, long-term autoimmune disease that causes chronic inflammation of synovial tissues in multiple joints. This inflammation can damage cartilage and bone and cause disability (1, 2). RA is more common in women than that in men and occurs at any age (1). Approximately 1% of the population is affected by RA, which significantly affects individuals and society (3, 4). Therefore, it is important to develop novel strategies for timely diagnosis and treatment to reduce inflammation and prevent further damage. Genome-wide association studies have linked the immunopathogenesis of RA to *HLA-DRB1*, a class II major histocompatibility gene (5). Other genes and loci also play a role in the development of RA, including co-stimulatory receptors molecules, cytokine receptor signaling pathways, and activation of the innate immune response (6). Multiple factors, including genetic and epigenetic modifications, immunity, inflammation, microorganisms, metabolism, and other mechanisms, constitute the pathological mechanism responsible for RA in which various immune cells and molecules interact with each other to mediate the autoimmune reaction, eventually causing bone and joint destruction or even disability (7–13).

Despite significant progress in understanding the inflammatory processes involved in RA, the exact mechanism underlying its development and progression is still not fully understood. However, recent studies have suggested that members of the granzyme family play an important role in the immunopathology of RA. Zhang F et al. (14) applied single-cell RNA sequencing, mass cytometry, bulk RNA-sequencing, and flow cytometry to identify the cell populations contributing to joint inflammation in RA. They applied intracellular staining to tissues from RA samples and RNA-seq to sorted CD8 T cells. Intracellular staining of GzmK and GzmB proteins in disaggregated tissue samples from patients with RA revealed that the majority of CD8 T cells in synovial tissue express GzmK. Furthermore, most HLA-DR CD8 T cells express both GzmB and GzmK by intracellular protein staining. Therefore, they defined distinct subsets of CD8 T cells characterized by a GzmK, GzmB phenotype. Defining key cellular subsets and their activation states in the inflamed tissue is a critical step to define new therapeutic targets for RA. Gzms are proteases produced and released by certain immune cells, including cytotoxic T cells (CTLs) and natural killer (NK) cells (15, 16). There are five human Gzms, namely granzyme A (GzmA), granzyme B (GzmB), granzyme H (GzmH), granzyme K (GzmK), and granzyme M (GzmM). Gzms are released into the immune synapse between CLs and target cells, enter target cells with the help of the pore-forming protein perforin, and activate various pro-apoptotic pathways by breaking down intracellular substrates (15, 17). Perforin and granulysin are two pore-forming proteins of cytotoxic granules of human killer cells, and they have significant roles in mediating Gzm responses to infection (18). There's work showing the role of granulysin as a biomarker and pathogenic factor in RA (19). In addition to playing a role in the process of apoptosis or programmed cell death, Gzms are also involved in the immune response to infection and tissue damage (20, 21). Some studies have shown that Gzms are elevated in the synovial fluid and synovial tissue of patients with RA and may contribute to inflammation and joint damage (22, 23). The known extracellular activities of Gzms suggest a proinflammatory effect in RA. This review aims to summarize the current knowledge on the possible roles of the granzyme family in RA, with the goal of providing a reference for further research into the disease mechanism of RA and the development of targeted therapies.

GzmA-mediated proinflammatory cytokine-induced bone destruction in RA

Considering all types of killer cells, GzmA is the most abundant Gzm as it is widely expressed in both CD8 CTLs and NK cells (24). GzmA is a serine protease secreted by various CLs, such as NK cells (25), natural killer T (NKT) cells (26), CTLs (27), and CD4 CTLs (28, 29). It plays a key role in the cell death pathway by targeting the endoplasmic reticulum-associated oxidative stress response complex called the SET complex. The SET complex contains at least two GzmA substrates, the nucleosome assembly protein SET (also known as 12PP2A), and the DNA binding protein HMG2. GzmA-mediated cleavage of SET cause inhibition of GzmA-activated DNase NM23-H1 and leads to single-stranded DNA damage (25). GzmA has been shown to have a variety of proinflammatory mechanisms. Hildebrand D et al. (30) suggested that GzmA enters target cells independently and functions as a mediator for inflammation *via* interleukin (IL)-1β cleavage. Wensink AC et al. (31) discovered that treatment of monocytes with GzmA in combination with toll-like receptor-2 (TLR2)- and TLR4-agonists markedly increases the release of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), IL-6, and IL-8. GzmA also promotes inflammation *via* extracellular activities, such as extracellular cleavage of urokinase (32), proteinase-activated receptor-1 (PAR-1), and PAR-2 (33–36). It is an important proinflammatory mediator in RA (37, 38), psoriasis (39), and osteoarthritis (40). Additionally, NK cells and CTLs transport GzmA into the cytoplasm of target cells through the perforin–granzyme system, and GzmA can cleave the gasdermin B (GSDMB) protein into GSDMB-N and GSDMB-C (at sites K229/K224), releasing its N-terminal pore-forming active fragment, thereby inducing pyroptosis (41).

Abnormal GzmA expression has been linked to inflammatory reactions (23, 42, 43). GzmA and GzmB levels in the plasma and synovial fluid are significantly increased during active periods of RA compared to those during osteoarthritis (OA) (23). GzmA stimulates peripheral blood mononuclear cells to produce TNF-α, IL-6, and IL-8 (36) and stimulates fibroblasts to produce IL-6 and IL-8 (44). These cytokines are largely expressed in the synovium and are mainly produced by macrophages and fibroblast-like synoviocytes (45–47). Therefore, high levels of GzmA in RA joints can promote synovial inflammation owing to its influence on cytokine production. The abnormal expression of GzmA may be related to its abnormal expression in various immune cells, including T cells, NK cells, and NKT cells. Perforin is a 70 kDa glycoprotein that is responsible for the formation of pores on the membrane of target cells (48) and participates in cytotoxic reactions in target cells (49). CD4+ perforin+ and GzmA+ cells have been observed in RA synovial samples (50, 51). Nanki T et al. (52) used flow cytometry to analyze

the expression of GzmA and perforin in peripheral blood CD4+ and CD8+T cells of patients with RA and healthy people. GzmA and perforin were mainly expressed by CX3CR1 CD4+ and CD8+T cells in patients with RA and healthy people, with increased expression in patients with RA. In addition, Aggarwal et al. (53) found elevated levels of GzmA in NK and NKT cells and GzmB in NK cells of venous blood samples in patients with RA. Elevated GzmA and GzmB levels are associated with disease severity, tissue damage, and joint damage in RA. Correlation studies showed that the disease activity score (DAS28) is positively associated with enhanced levels of GzmA-expressing NK and NKT cells, perforin-GzmA dual-positive NK, NKT cells, and GzmB-expressing NK cells. Loetscher P et al. (54) analyzed chemokine-mediated enzyme release from cytotoxic lymphocytes using cloned and freshly isolated human blood NK cells and CD8+ T cells. They found that GzmA from CD8+T and NK cells can be activated by chemokines, suggesting chemokines may be involved in regulating cytotoxicity in lymphocyte. Many chemokines capable of inducing Gzms release have been shown to be upregulated in the synovial tissue of patients with RA (55, 56). Therefore, GzmA overexpression in CD8+T and NK cells may be related to chemokines upregulated in RA. Moreover, GzmA and GzmB degrade ECM proteins *in vitro* (20). Santiago L et al. (37) evaluated inflammatory arthritis induced by type II collagen in wild-type, GzmA-deficient, and perforin-deficient mice, and found that GzmA is more closely associated with cartilage and bone injury in mouse paws and knees than with inflammatory signs and synovial cells. Proliferating osteoclasts (OCs), which are primary bone-resorbing cells, are hematopoietic in origin and have a monocyte/macrophage lineage. The formation and activation of OCs are tightly regulated by systemic and pericellular factors (57). GzmA activates monocytes and other OC precursors to secrete TNF, thus increasing proinflammatory cytokine-induced bone destruction observed in RA. However, the mechanism involved seems to be complex and may be either direct by promoting OC differentiation or indirect *via* other inflammatory responses (37).

GzmB-mediated inflammation and ECM degradation in RA

GzmB is a granzyme family member with the strongest apoptotic activity because of its caspase-like ability to cleave substrates at aspartic acid residues, thereby activating procaspases directly and cleaving downstream caspase substrates (58). GzmB is a 32 kDa serine protease that is secreted by NK cells and CTLs (59, 60). When released into the gap between those cells and target cells, GzmB can enter the cytoplasm of target cells in the presence of perforin. Subsequently, apoptosis is induced by cleaving various intracellular substrates (61) associated with DNA maintenance, such as inhibitors of caspase-activated DNase, poly (ADP-ribose) polymerase, DNA-dependent protein kinase, and lamin B (62–65). GzmB can be produced by various immune and non-immune cells, including T and B cell subsets, monocytes/macrophages, mast cells, basophils (66–71), vascular smooth muscle cells, lung cells, keratinocytes, chondrocytes, and various types of cancer cells (70, 72–79). GzmB can also have extracellular functions, including the degradation of ECM components, cytokines, cell receptors, and clotting proteins (21, 22, 80). The potential pathophysiological consequences of their cleavage constitute the basis for envisaging a crucial proinflammatory role for GzmB in the pathogenesis of inflammatory diseases (81). In the extracellular pathway, direct processing of caspase-3 and caspase-7 by GzmB promotes caspase-mediated degradation of hundreds of protein substrates, resulting in rapid apoptosis (82).

Abnormal expression of GzmB has been observed in the synovial tissues of patients with RA (83). Studies have shown that most CD8T cells in the synovial tissues of patients with RA express GzmK and GzmB proteins (14). Although Gzms are expressed by CTL, only a small percentage of granzyme-positive cells in the synovial membrane are CD8+ and CD4+ T cells, with the majority being NK cells (84). Elevated levels of GzmB have been found in blood and synovial fluid of patients with RA, which may be a result of GzmB release from inflamed joints (23, 84). Tripathy A et al. (85) indicated that RA patients express functional P2X4 and P2X7 receptors on peripheral CD8+T cells which when ligate with ATP produce high amounts of GzmB. When the ATP molecules induce purinergic signaling and activate T cells *via* P2X receptors (86), the excess extracellular ATP acts as a self-adjuvant to generate abnormal immune responses (87) and triggers inflammation (88, 89). In the case of RA, the release of ATP and its downstream binding to the purinergic receptors is a key regulator of the inflammatory activity (90, 91). The CD8+T cells from RA patients released significant amounts of GzmB in comparison to the CD8+T cells from HCs when stimulated with extracellular ATP. Moreover, the CD8+T cells from RA patients were increasingly activated over time and hence released greater concentrations of GzmB. GzmB is a specific activation marker protein for CD8+T cells. It thus implies that the excess extracellular ATP in the plasma of RA patients can activate immune cells rapidly and hence can be afflictive for the patients.

Goldbach-Mansky R et al. (92) explored the diagnostic and prognostic value of serum GzmB in patients with a diverse spectrum of early inflammatory arthritis and found that GzmB concentrations were significantly higher in rheumatoid factor positive (RF +) RA than those in RF-RA. Patients with joint erosions had significantly higher levels of GzmB than those without, indicating the independent value of GzmB in the prediction of erosive disease. GZMB+CD4 and CD8 CTL cells have also been found to be upregulated in the peripheral blood of active patients with RA (93), potentially reflecting an autoimmune response. Elevated levels of GzmB in blood may result from extracellular GzmB not taken up by the receptor during the induction of apoptotic cell death (92).

GzmB is a multifunctional proinflammatory molecule (94). It can process and activate proinflammatory, pro-fibrotic, and senescence mediators belonging to the IL-1 cytokine family (95, 96). GzmB can process IL-1α into potent proinflammatory fragments, enhancing inflammation. It stimulates interstitial collagenase production by fibroblasts and ECM remodeling, thereby regulating both, normal and aberrant tissue repair (96, 97). Among proinflammatory cytokines, IL-1α/β and TNF-α can trigger the intracellular molecular signaling pathway responsible for RA pathogenesis, which activates mesenchymal cells and synoviocytes and recruits innate and adaptive immune system cells. Synoviocytes, in turn, activate various mediators, including TNF-α, IL-1, IL-6, and IL-8, resulting in synovium inflammation, increased angiogenesis, and decreased lymphangiogensis (98). Therefore, GzmB may be involved in the inflammatory response of RA by regulating IL-1α expression. The

role of GzmB in bone destruction in RA has also been suggested by other studies. Single nucleotide polymorphisms in the GzmB gene have been found to influence the joint destruction rate of RA (99). H. K. Ronday et al. (100)found that GzmB can degrade proteoglycan components in cartilage and contribute to the destruction of articular cartilage in RA. Additionally, GzmB is a potential biomarker for RA diagnosis, with higher levels of GzmB in serum being correlated with increased disease activity as measured by the DAS28-CRP score (101). In summary, GzmB may contribute to inflammation and joint destruction associated with RA through its proinflammatory and tissue-degrading effects.

While several studies have reported the role of GzmB as a proinflammatory molecule in the progression of RA proinflammatory, Xu et al. (102) found the frequency of GzmB production by regulatory B cells (Bregs) in patients with RA to be significantly reduced compared to that in healthy controls. The expression of IL-21 receptor in B cells in patients with RA was also significantly reduced, which may contribute to the reduction in GzmB-producing Bregs in these patients. Further analysis showed that the number of GzmB-producing Bregs was negatively correlated with erythrocyte sedimentation rate tender joint count, and disease activity score DAS28. The number of GzmB-producing Bregs increased significantly after RA treatment. A reduction in Bregs, especially those that produce IL-10 has been shown to be negatively correlated with disease activity in RA (103). Those cells may help maintain immune balance by inhibiting proinflammatory cytokine production and T cell differentiation (104). Whether GzmB has cell-specific functional differences remains to be determined.

GzmH and GzmB are structurally similar with 71% amino acid identity and belong to a gene cluster located on chromosome 14, which also includes cathepsin G and mast cell chymase. Although they have high sequence homology, these enzymes have distinct enzymatic activities (105). GzmH has not been detected in NKT cells, monocytes, or neutrophils (106). High levels of human GzmH mRNA have been found in the peripheral blood lymphocytes, lungs, spleen, and thymus (107, 108). Hou et al. (109) discovered that GzmH can induce rapid apoptosis in target cells, resulting in mitochondrial damage, nuclear condensation, and DNA breakage. GzmH-induced apoptosis depends on caspase activation and cytochrome c release. To date, no research has been conducted on GzmH in the context of RA. GzmH is predominantly expressed at high levels in NK cells (110), and GzmH mRNA has also been detected in activated human T cells (107, 111). IL-15 has significantly higher levels in the serum and synovial fluid of patients with RA than those with OA and healthy control groups (112), and plays key roles in promoting activation of NK and CD8 T cells (113). Zhang B et al. (114) stimulated NK-92 cells with IL-15, and it was found that IL-15 significantly up-regulated GzmA and GzmB gene expression, but GzmH transcripts were down-regulated. Therefore, higher levels of IL-15 in patients with RA might regulate Gzms expression. However, the specific role of GzmH in RA requires further investigation, and IL-15 may be a potential target to focus on.

GzmK- and GzmM-mediated cytokine-based inflammation in RA

GzmK is a trypsin-like molecule in the granzyme family that is expressed by CTLs, NKT, γδ T cells, and CD56bright+ NK cells (110, 115–117). Besides being a member of the granzyme family, little is known about the function of GzmK (118). *In vitro* studies have demonstrated that GzmK can induce non-apoptotic cell death through the production of reactive oxygen species (ROS) and mitochondrial dysfunction when combined with perforin (119). Further studies have shown that GzmK activates caspase-independent apoptosis by cleaving the SET complex, leading to SET destruction. This results in unleashing GzmA-activated DNase NM23H1, which translocates to the nucleus and nicks DNA (120). GzmK may also cleave the tumor suppressor p53, thus sensitizing tumor cells for apoptosis induction (121) and process a vasolin-containing protein, thus contributing to endoplasmic reticulum stress and caspase-independent cytotoxicity (122). GzmK inhibits influenza virus replication in mice (123) and has an immunoregulatory function in multiple sclerosis (124).Cooper DM et al. (125) demonstrated GzmK-induced activation of both ERK1/2 and p38 MAP kinase signaling pathways and significantly increased fibroblast proliferation in patients with sepsis and acute lung inflammation. Wensink AC et al. (126) demonstrated that extracellular GzmK potentiates the lipopolysaccharide-induced release of inflammatory cytokines from monocytes and that this effect is independent of the catalytic activity of GzmK.

GzmK levels in synovial tissue samples from patients with RA are higher than the levels in those with OA (127). GzmK may have proinflammatory effects and can activate PAR-1, a family of G protein-coupled receptors that mediate the physiological response to serine proteases (125, 128). PAR-1 is activated by thrombin and trypsin and can induce the production of inflammatory cytokines, such as TNF-α, IL-1, IL-6, and monocyte chemotactic protein 1 (129, 130). CD8T cells primarily release GzmK, whereas CD4 T cells primarily release GzmB (131). In the context of RA synovium inflammation, GzmK can act as a key inflammatory agent, inducing synovial fibroblasts to activate proinflammatory pathways, including IL-6, CCL2, and ROS production. This effect does not require perforin or any other agent to induce internalization of GzmK, indicating that GzmK has a proteolytic target on the surface of these cells (129). The protease activity of GzmK can also promote degradation of the ECM, leading to inflammatory cell infiltration and tissue destruction. Blocking GzmK or cytokines that activate CD8T cells, such as IL-12 or IL-15, may be an effective treatment for RA. Anti-citrullinated protein antibody-negative (ACPA-negative) RA comprises up to one-third of patients with RA, whereas lack of biomarkers in ACPA-negative RA poses a big challenge to early diagnosis (132). Lu J et al. (133) reintegrated across the GSE89408 dataset to evaluate the performance of GzmK in the diagnosis of ACPA-negative RA. The expression levels of GzmK in the ACPA-negative RA group were significantly higher than that in the normal and OA groups, and the area under the curve of GzmK expression level was 0.916, suggesting its potential as a biomarker.

GzmM is a trypsin-folding serine protease found specifically in the granules of NK cells (134). High levels of GzmM protein and mRNA have been detected in NK, NKT, γδT, and CD8+T cells (118). Studies have shown that human GzmM promotes cell death in a manner similar to GzmB, including caspase-3 activation, DNA fragmentation, ROS production, and the mitochondrial release of cytochrome c (135, 136). Cytoskeletal components, such as α-tubulin and ezrin, nucleolar phosphoprotein nucleophosmin, and apoptosis-

associated p21-activated protein kinase 2, have been identified as direct GzmM subunits and are cleaved during GzmM-induced cell death and cytotoxic lymphocyte-induced cell death (137, 138). Synovial fluid-derived mononuclear cells show GzmM expression, with the highest expression in CTLs and NK cells. Elevated levels of GzmM in synovial fluid from patients with RA compared to OA controls have been shown to stimulate human fibroblasts to release IL-29, a proinflammatory cytokine, and type III interferon (IFN-λ1), suggesting that GzmM may play a local role in the pathophysiology of RA (139). Further studies are needed to fully understand the specific role of GzmM in RA.

Perspectives and challenges

Gzm-inhibiting serpins are believed to act as a fail-safe mechanism for CLs to avoid self-injury during granule exocytosis (140). In recent years, the prevailing theory has been that although circulating Gzms might not be able to enter cells without a high local perforin concentration to induce cell death, they could proteolyze cell surface receptors or extracellular proteins to cause destruction. Particularly when Gzms present at high concentrations at inflamed sites in the absence of natural inhibitors (24). To date, SERPINB12, SERPINB9, SERPINB4, SERPINB1, and inter-alpha inhibitor proteins, have been identified as intracellular inhibitors of GzmA, GzmB, GzmM, GzmH, and GzmK (141–146). Although physiological inhibitors of Gzms are known, no clinical trials have been reported for their use as treatments. Researchers believe that the development of GzmA inhibitors for the treatment of RA may have beneficial effects compared to other commonly used anti-inflammatory drugs, such as corticosteroids or TNF blockers (37). Some studies have suggested that cyclosporine and zidovudine may be potential target drugs for RA treatment in combination with GzmA (43). Zidovudine was developed as an anti-cancer agent in the 1960s and was later approved by the US FDA as an anti-HIV therapeutic drug in the late 1980s after fast track clinical trials (147). Nowadays, this drug is commonly used in the prevention of perinatal HIV-1 transmission (vertical transmission) that consists of the use of this drug by the mother before and during delivery, and treatment of the newborn (148). New potential inhibitors of GzmB, such as tannic acid, mupirocin, cefpiramide, xenazoic acid, vidarabine and phytonadiol sodium diphosphate, have been identified (149). Mi-Sun Kim.et al (150) developed a novel class of weak small-molecule inhibitors against human GzmB by docking studies employing binding site hot spots and three constraints (hydrogen bonding with Arg226, and hydrophobic interactions for S2 and S4 subsites) based on computational solvent mapping using FTMAP. The most distinctive compounds identified were thiazolidinediones 8 (IC50 = 25 μM) and 9 (IC50 = 28 μM), triazole 6 (IC50 = 44 μM), and diazolidinedione 7 (IC50 = 44 μM). Ikram S et al. (151) identified 12 potential inhibitors of GzmH from two separate databases of small molecules. Currently, there are no Gzms inhibitors that are specifically approved for the treatment of RA. Understanding of the precise mechanisms by which granzymes contribute to the development and progression of RA is limited. Lack of clear evidence demonstrating that targeting granzymes is a viable therapeutic strategy for RA. RA is a complex and heterogeneous disease, and it is not clear whether the same granzyme-mediated mechanisms are involved in all patients with RA. There is currently no single, reliable biomarker to indicate patterns of Gzms in patients with RA. Given the intracellular, extracellular, and proinflammatory effects of Gzms on RA, Gzms and their physiological inhibitors may be potential therapeutic targets for RA treatment. It is worth noting that extracellular vesicles (EVs), as one of the important communication carriers between cells and host, may also be a potential contact media between the Gzms family and RA (152). EVs containing granzyme from NK cells and CTLs require Ca2+-dependent signals to release (153). The EVs from activated NK cells include a variety of Gzms, such as GzmA and GzmB, which have cytotoxic effects on tumor cells (154), inhibit cell proliferation, and promote cell death. They are considered a safe and effective immunosuppressive agent, which may have potential therapeutic significance for RA FLS (154, 155).

Discussion

In this review, we described the physiological function, cellular expression, and potential role of five members of the Gzms family in RA (Table 1). Gzms are involved in the induction of apoptotic cell death. In RA, Gzms demonstrate non-cytotoxic activities that include diverse biological effects, such as stimulation of proinflammatory cytokines and remodeling of extracellular matrices. Considering the extracellular and intracellular functions of Gzms, they have the potential to contribute to the pathogenesis of inflammatory diseases (Figure 1). First, GzmA level is significantly elevated in plasma and synovial fluid and can degrade ECM proteins, potentially contributing to bone destruction in RA. Higher levels of GzmB in serum are correlated with increased disease activity. GzmB can degrade proteoglycan components in cartilage and contribute to the destruction of articular cartilage in RA. A subgroup of B cells, Bregs that express GzmB, may inhibit proinflammatory cytokine production and abnormal autoimmune T cell differentiation in patients with RA. GzmM promotes inflammation mainly by stimulating the release of the proinflammatory cytokine IL-29 and is elevated in RA. GzmK is mainly associated with endothelial cells and fibroblasts, suggesting its role in abnormal angiogenesis and synovial hyperplasia in RA. However, the specific role of GzmH in RA requires further investigation. Our search for the latest clinical trials showed that few clinical inhibitors of Gzms have been identified. While the development of clinical drugs targeting the Gzms family is limited, evidence suggests that targeting these proteins may have potential value for the clinical treatment and management of RA. To further enhance our understanding of Gzms in RA, comprehensive use of molecular biology, cellular immunology, and other technologies is necessary. Notably, Gzms primarily play a biological role in cell perforation and target cells. The multiple potential roles of Gzms in RA may include an abnormal manifestation of uncontrolled or excessive cell death. Additionally, the known extracellular activities of Gzms suggest a proinflammatory effect in RA. Therefore, further research on the association between multiple cell death pathways and RA, and experiments defining Gzm-activated proinflammatory pathways may be a promising direction to determine the significance of Gzms as a proinflammatory mediator in future studies.

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TABLE 1 The physiological function, cellular expression, and potential role of the Gzms family in RA.

Granzyme	Cellular expression in RA	Granzyme relevance to RA
GzmA	CD4+T cell CD8+T cell NK cell NKT cell	1. Elevation of GzmA in NK and NKT cells associated with disease severity, tissue damage, and joint damage in RA. 2. GzmA activates monocytes and other OC precursors to secrete TNF, thus increasing proinflammatory cytokine-induced bone destruction observed in RA.
GzmB	CD4+T cell CD8+T cell NK cell	1. GzmB concentrations in RF+ RA are significantly higher than those in RF- RA, and patients with joint erosions have significantly higher levels of GzmB than those without, indicating the independent value of GzmB in the prediction of erosive disease. 2. GzmB can degrade proteoglycan components in cartilage and contribute to the destruction of articular cartilage in RA. 3. Higher levels of GzmB in serum are correlated with increased disease activity as measured by the DAS28-CRP score. 4. The expression of IL-21 receptor on B cells of patients with RA is significantly decreased, which may be a possible mechanism of reducing GzmB-producing Breg in patients with RA. Regulatory B cells (Bregs), particularly IL-10-producing Bregs, have been shown to be reduced in number and negatively correlated with disease activity in RA and may contribute to the maintenance of immune functions by inhibiting proinflammatory cytokine production and T cell differentiation.
GzmM	CD8+T cell NK cell	GzmM plays a role in the pathophysiology of RA by stimulating the release of proinflammatory cytokine IL-29, a type III interferon cytokine also known as IFN-λ1.
GzmK	CD8+T cell	In the case of synovial inflammation in RA, GzmK itself acts as a key inflammatory agent. GzmK induces synovial fibroblasts to activate proinflammatory pathways, including IL-6, CCL2, and ROS production. The protease activity of GzmK can also promote degradation of the ECM, leading to inflammatory cell infiltration and tissue destruction.
GzmH	The specific role of GzmH in RA requires further investigation.	

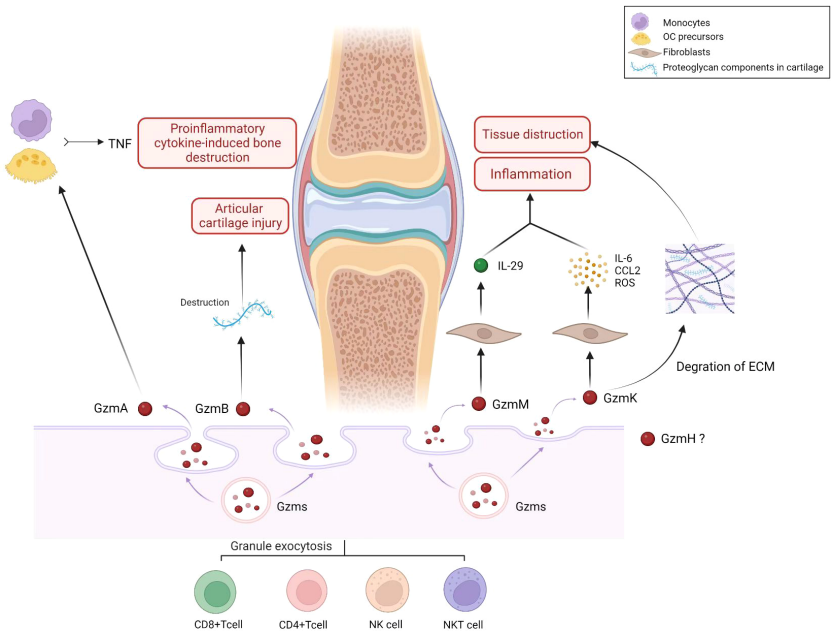

FIGURE 1
Role of Granzyme family (GzmA, GzmB, GzmH, GzmM, GzmK) in pathology and progression of RA. Gzms are produced and released by immune cells, such as cytotoxic T cells and natural killer cells. They play a role in the ability of the immune system to recognize and eliminate infected or damaged cells. The granzyme family includes several granzyme types, including granzyme A (GzmA), granzyme B (GzmB), granzyme H (GzmH), granzyme M (GzmM), and granzyme K (GzmK). In the context of RA, Gzms may contribute to the pathology and progression of the disease in several ways. RA is an autoimmune disorder characterized by chronic inflammation of the joints that leads to joint damage and deformity. Gzms may contribute to the inflammation and joint damage observed in RA by inducing programmed cell death (apoptosis) in cells within the joint tissue. This can lead to the destruction of joint cartilage and bone, resulting in joint deformity and loss of function. As the figure shows, GzmA activates monocytes and other OC precursors to secrete TNF, thus increasing proinflammatory cytokine-induced bone destruction observed in RA. GzmB can degrade proteoglycan components in cartilage and contribute to the destruction of articular cartilage in RA. GzmM plays a role in the pathophysiology of RA by stimulating the release of proinflammatory cytokine IL-29. GzmK induces synovial fibroblasts to activate proinflammatory pathways, including IL-6, CCL2, and ROS production. The protease activity of GzmK can also promote degradation of the ECM, leading to inflammatory cell infiltration and tissue destruction. (Created with BioRender.com).

Author contributions

YZ and JZ is responsible for the collection, collation, and writing of the original manuscript. YS is responsible for the collection. SG, SS, and DH are responsible for the concept development, revision, and manuscript review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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MicroRNA-183/96/182 cluster in immunity and autoimmunity

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MicroRNAs (miRNAs) are crucial post-transcriptional regulators of gene expression in ubiquitous biological processes, including immune-related pathways. This review focuses on the miR-183/96/182 cluster (miR-183C), which contains three miRNAs, miR-183, -96, and -182, having almost identical seed sequences with minor differences. The similarity among seed sequences allows these three miRNAs to act cooperatively. In addition, their minor differences permit them to target distinct genes and regulate unique pathways. The expression of miR-183C was initially identified in sensory organs. Subsequently, abnormal expression of miR-183C miRNAs in various cancers and autoimmune diseases has been reported, implying their potential role in human diseases. The regulatory effects of miR-183C miRNAs on the differentiation and function of both innate and adaptive immune cells have now been documented. In this review, we have discussed the complex role of miR-183C in the immune cells in both normal and autoimmune backgrounds. We highlighted the dysregulation of miR-183C miRNAs in several autoimmune diseases, including systemic lupus erythematosus (SLE), multiple sclerosis (MS), and ocular autoimmune disorders, and discussed the potential for utilizing miR-183C as biomarkers and therapeutic targets of specific autoimmune diseases.

KEYWORDS

microRNA, miR-183/96/182 cluster, immunity, autoimmune, autoinflammatory diseases, epigenetic regulation

1 Introduction

MicroRNAs (miRNAs) are small non-coding RNAs (mostly 20~24 nucleotides) that regulate gene expression at the post-transcriptional level (1, 2). Since the discovery of the first microRNA (miRNA), *lin-4*, in *C. elegans* by Lee et al. and Wightman et al., a large number of miRNAs have been identified in plants, animals, bacteria, and viruses, implying that miRNAs are conserved across species (3–8). Approximately 48,860 mature microRNAs in 271 organisms have been recorded on the miRbase database released in 2019, and it is predicted that this number will increase in the future (8). miRNAs have been shown to epigenetically regulate the expression of target mRNAs by binding to complementary sequences. Given that each miRNA targets many mRNAs, almost all cellular pathways are predicted to be subjected to miRNA-mediated regulation (1, 9–11). Various miRNAs, individually or cooperatively, regulate the normal development and

function of biological systems in animals (7, 12–15). Abnormal expression of miRNAs in multiple human diseases, including cancers and autoimmune diseases, have been reported, implying a conceivable role of miRNAs in human disease pathogenesis. The specific disease-related miRNAs could serve as diagnostic biomarkers and potential therapeutic targets of the disease (16–19).

The microRNA-183/96/182 cluster (miR-183C) is a highly conserved microRNA cluster containing three paralogous microRNAs (miR-183, miR-96, and miR-182), located on human chr7q32.2 and mouse chr6qA3 (20). The first identified miRNA of human miR-183C is miR-96 in human cancer HELA cells (21). Subsequently, miR-182 and miR-183 in humans were identified by different groups as a cluster (22, 23). miR-96 was later classified as a member of miR-183C due to their sequence similarity, co-expression, and close chromosomal location (20). The miR-183C miRNAs are markedly expressed in the sensory organs (such as eye and ear) and play an essential role in the normal development and function of sensory organs (20, 24). Interestingly, although miR-183C miRNAs are expressed at a low level in non-sensory organs, the upregulation of miR-183C miRNAs has been noticed in various cancers (such as prostate cancer, breast cancer, lung cancer, melanoma), and in autoimmune and inflammatory diseases (such as systemic lupus erythematosus or SLE and multiple sclerosis or MS) (25–31). The crucial role of miR-183C miRNAs in the immune system and autoimmune diseases has been reported in recent studies (32–35). In this review, we summarized previous research on miR-183C involvement in immunity and autoimmunity and discussed the current understanding of the regulatory effect of miR-183C on immune cell functions and autoimmune disease pathogenesis.

2 Expression and function of miR-183C in sensory organ

The expression of the polycistronic miR-183C microRNAs starts from the transcription of a long single hairpin-shaped pri-miR/183/96/182 transcript that contains three miRNAs with similar seed sequences (20, 31, 35). The miR-183C is highly conserved in various species, and the miR-183C is expressed at high levels in sensory organs, such as the retina, olfactory epithelia, and inner ear (20, 36). The crucial role of miR-183C miRNAs in sensory organs development and function has been reported by different groups (37–42).

The mutations of the miR-96 seed region are responsible for progressive hearing loss in both humans and mice (37, 38). The *Mir96*^{Dmdo} mice, which carry an *N*-ethyl-*N*-nitrosurea (ENU)-induced A to T mutation in the seed sequence of miR-96, had degeneration of hair cells in homozygotes by seven days and the loss of any detectable cochlear nerve activity by four weeks of age (37). By mapping the genes of an autosomal dominant deafness locus, Mencia et al. determined that the mutations at the human miR-96 seed region caused autosomal dominant progressive hearing loss in humans (38). Furthermore, a group of deafness-related genes was identified in the miR-96-mediated gene regulatory network, suggesting a vital role of miR-96 in inner ear development and progressive deafness (39). Further studies have shown that the mice with the inactivation of miR-183/96/182 cluster (miR-183C$^{GT/GT}$) or deletion of both miR-96 and miR-183 (*miR-183/96*^{dko}) had congenital deafness with severe defects in hair cell development and function (40, 41). The miR-182 single knockout (*miR-182*^{ko}) mice only exhibited progressive hearing loss (40). The expression of the three miR-183C miRNAs was progressively increased during mouse retinal development, and their expression was highly induced by the light (20, 43). Inactivation of the miR-183/96/182 cluster resulted in retinal dysfunction and degeneration with progressive electroretinogram defects and increased sensitivity to light damage in miR-183C$^{GT/GT}$ mice (42). Although deleting miR-182 in B6 mice did not induce any apparent histological changes in the retina, it led to the decline of retinal function (44, 45). Together, the above studies strongly demonstrated the essential role of miR-183C miRNAs, either individually or collectively, in the development and function of the retina and inner ear.

3 Expression and function of miR-183C in immune cells

Unlike in the sensory organs, miR-183C miRNAs expression was not detectable in unstimulated cells of the immune system (basal state) in normal mice (20). However, upon stimulation with different antigens and cytokines, the expression of miR-183C was highly upregulated in both adaptive and innate immune cells (33, 46). Figure 1 summarizes the current understanding of the role of miR-183C on the development and functions of different subpopulations of the cells of the immune system.

3.1 T cells

T lymphocytes (T cells) coordinate the activities in other white blood cells, either *via* secreting cytokines or by direct cell-to-cell contact. T cells are indispensable for the effective countering of intracellular infections, especially by secreting proinflammatory cytokines [interferon-gamma (IFNγ) or interleukin-17 (IL-17)] and inducing cytotoxicity of infected cells. Dysregulated T cell numbers and/or functions are evident in many autoimmune diseases (47, 48). These include enhanced pathogenicity of T helper 17 (Th17) cells or impaired function of regulatory T (Treg) cells (which normally hold-in-check autoreactive cells), or increased secretion of proinflammatory cytokines (such as IL-17 and IFNγ). Of note, the expansion and function of Th1/Th17/Treg cells were found to be regulated by miR-183C miRNAs *via* targeting different genes/signaling pathways in different experimental settings (Figure 2), suggesting a crucial role of miR-183C miRNAs in T-cell immunity and autoimmunity.

miR-182 expression was highly upregulated in the activated Th cell subsets (Th1, Th2, and Th17) to promote Th cell expansion *via* targeting Foxo1 (32). Th1 and Th17 are proinflammatory immune cells that are thought to promote autoimmune diseases (52, 53). Inhibition of miR-182 with antagomir-182 significantly reduced Th

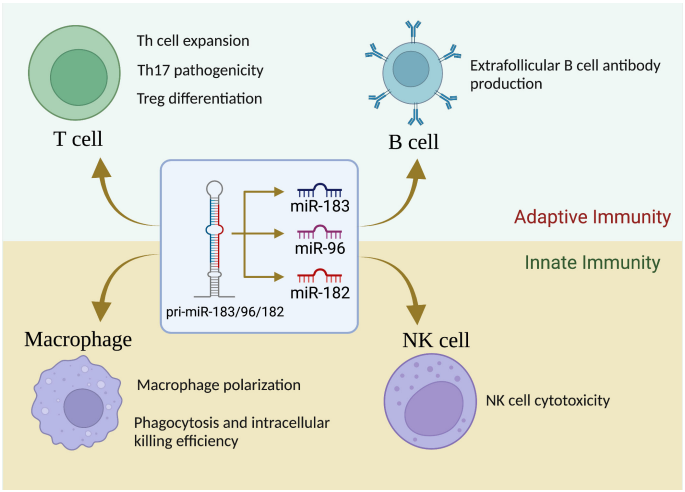

FIGURE 1
miR-183C is a crucial regulator of immune cell development, differentiation and function. While miR-183C miRNAs are dispensable for T-cell development, they play a vital role in Th1/Th17/Treg differentiation, proliferation, and function. miR-183C miRNAs have minimal effect on B-cell development, GC response, and antibody production, although miR-182 appears to have a promoting effect on extrafollicular B-cell response and antibody production. In addition, miR-183C miRNAs play an important role in regulating macrophage polarization, phagocytosis, and innate NK cell cytotoxicity.

cell expansion *in vitro* and *in vivo* due to the lower proliferation and higher cell death rates (32). In a murine model of ovalbumin (OVA)-induced arthritis, miR-182 has been shown to promote inflammation. The RAG1$^{-/-}$BALB/c mice receiving antagomir-182 transfected Th cells had reduced OVA-induced arthritis with amelioration of knee swelling and significantly reduced inflammation and tissue destruction (32). MiR-182 was also found to be upregulated in peripheral CD4$^+$ T cells from multiple sclerosis patients, which was associated with augmented production of proinflammatory cytokine IFNγ and IL-17 (27). In experimental autoimmune encephalomyelitis (EAE) mice, overexpression of miR-182 promoted the expansion of IFNγ- and IL-17- expressing T cells *in vivo* and exacerbated clinical score. Further *in vitro* experiments suggested that miR-182 promoted the differentiation of inflammatory T cells, especially Th1 cells *via* targeting hypoxia-inducible factor-1α (HIF-1α), a regulator of inflammation (27).

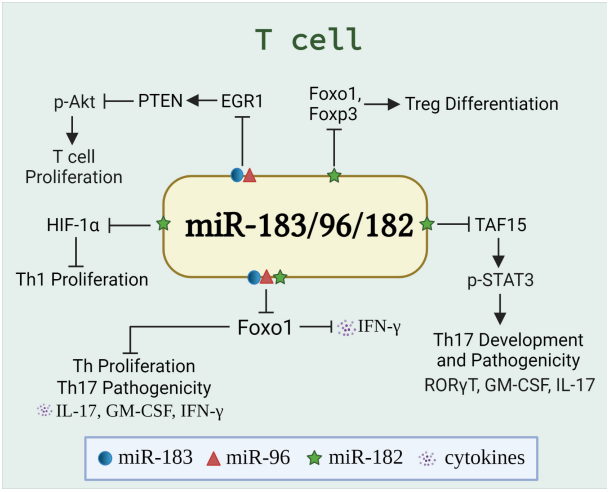

FIGURE 2
miR-183C regulates the differentiation, proliferation, and function of T cells. The schematic graph illustrates the miR-183/96/182-regulated genes and pathways, either individually or collectively, in T cells. The miR-183C promotes T cell proliferation and IFNγ production *via* targeting Foxo1 (32, 34), HIF-1α (27), or EGR1/PTEN/Akt pathway (49). In addition, miR-182 suppresses Treg differentiation by targeting Foxo1 and Foxp3 (50). While miR-183C has been shown to promote the pathogenicity of Th17 cells by inhibiting Foxo1 (33), miR-182 has been shown to inhibit the Th17 development and pathogenic response *via* targeting TAF15/STAT3 signaling pathway (51).

miR-183 and miR-96, akin to miR-182 also promote T cell proliferation. Both miR-96 and miR-183 were shown to regulate CD4$^+$CD25$^-$ T cell proliferation *via* the EGR1/PTEN/Akt pathway (49). Overexpression of miR-183 and miR-96 in CD4$^+$ T cells reduced Early growth response 1 (EGR1) expression directly, which led to reduced expression of Phosphatase and tensin homolog (PTEN) expression and consequently increased Protein kinase B (PKB or Akt) phosphorylation and T cell proliferation. Adoptively transferring of miR-183 and miR-96 overexpressing hemagglutinin (HA)-specific CD4$^+$CD25$^-$ T cells into autoimmune diabetes-prone INS-HA/Rag2KO mice resulted in the expansion of antigen-specific CD4$^+$ T cells and accelerated the development of autoimmune diabetes (49). Further, simultaneous inhibition of miR-183 and miR-96 in CD4$^+$CD25$^-$ T cells with specific antagomirs promoted EGR1 and PTEN expression and suppressed T cell proliferation. Adoptively transferring antagomir-183/96 transfected HA-specific CD4$^+$CD25$^-$ T cells into INS-HA/Rag2KO mice delayed the onset of autoimmune diabetes (49).

miR-183C miRNAs were highly induced in differentiated Th17 cells, particularly in pathogenic Th17 cells through the activation of the IL-6/STAT3 pathway (33). Notably, these investigators demonstrated that miR-183C miRNAs are essential for the pathogenic function of Th17 cells by positively regulating

cytokines involved in the pathogenicity of Th17 cells without affecting the differentiation of this subset. Overexpression of miR-183C miRNAs, especially miR-96, in primary $CD4^+$ T cells, promoted the production of inflammatory cytokines IL-17A, IL-17F, GM-CSF, IL-22, and IFNγ in differentiated pathogenic Th17 cells. In *miR-183C*-deficient pathogenic Th17 cells, the expression of these inflammatory cytokines was significantly depressed compared to *miR-183C*-sufficient controls (33). The clinical score of myelin oligodendrocyte glycoperotein$_{35-55}$ (MOG_{35-55})-induced EAE in the $Rag1^{-/-}$ mice that received $CD4^+$ T cells from miR-$183C^{-/-}$ knockout mice was significantly reduced compared to the $Rag1^{-/-}$ mice receiving $CD4^+$ T cells from control miR-$183C^{+/+}$ mice. Mechanistically, the authors demonstrated that the miR-183C miRNAs positively regulated the pathogenic function of Th17 cells partially by inhibiting the expression of Foxo1, a negative regulator of Th17 cell pathogenicity (33).

The above study demonstrated that all three miR-183C miRNAs had a promoting effect on the pathogenic function of Th17 cells derived from normal B6 mice without affecting the Th17 cell differentiation, with miR-96 being the most potent (33). However, in a mouse model of autoimmune disease experimental autoimmune uveitis (54), miR-182 had an inhibitory effect specifically on the Th17 cell development and pathogenic function (51). Zhang et al. reported that miR-182 was downregulated in the EAU mouse-derived Th17 cells. Inhibition of miR-182 significantly increased the expression of Th17 signature genes such as *IL-17*, *IL-22*, *GM-CSF*, and *RORγT* in EAU mice-derived Th17 cells. Mechanistically, miR-182 inhibited Th17 cell development by directly targeting and inhibiting the transcriptional initiator TATA-binding protein-associated factor 15 (TAF15). *In vitro* studies indicated that the overexpression of TAF15 was able to rescue the miR-182 overexpression-induced reduction of signal transducer and activator of transcription 3 (STAT3) phosphorylation, an important upstream regulator of Th17 (51, 55).

In several human autoimmune diseases, the proportion of proinflammatory Th17 cells was increased, while the immunotolerant Treg cell differentiation was suppressed. In contrast to its promoting role on Th17 cell differentiation, miR-182 plays a suppressing role in $CD4^+CD25^+Foxp3^+$ Treg differentiation. Both *in vitro* and *in vivo* studies demonstrated that the knockdown of miR-182 with a lentiviral vector expressing miR-182 inhibitor (anti-LV-miRNA182) promoted the differentiation of Tregs *via* upregulating the expression of *Foxo1* and *Foxp3*. The miR-182 knockdown mice were more resistant to MOG_{35-55}-induced EAE disease than control mice due to increased differentiation of $CD4^+CD25^+Foxp3^+$ Tregs in peripheral lymphoid organs of the miR-182 knockdown mice (50). However, an *in vitro* study demonstrated that overexpression of miR-182 in Jurkat T cells, a $CD4^+$ cell line derived from human acute T cell leukemia, downregulated Foxo1 expression and promoted the polarization of the transduced T cells to $Foxp3^+$ T cells, of which some were $Foxp3^+IL\text{-}17^+$ cells (56). The above studies indicated that the miR-182 could have either a positive or negative role in regulating Tregs/Th17 differentiation, depending on the pathological conditions, experimental settings, and types of cells.

In addition, the expression of miR-183C miRNAs was dynamically regulated in discrete phases of invariant natural killer T (iNKT) cell maturation. The expression of miR-183C miRNAs is crucial for the development, maturation, and function of iNKT cells (57). The miR-$183C^{-/-}$ knockout mice showed a reduced proportion and absolute numbers of iNKT cells in the thymus and spleen, depressed terminal maturation of thymic iNKT cells. Meanwhile, the percentages of IL-17-producing NKT cells (NKT17) out of both thymic and splenic iNKT cells were dampened in miR-$183C^{-/-}$ mice compared with wild-type mice (57). Mechanistically, Foxo1, Foxo3, EGR1, and EGR2 were the potential targets of miR-183C in iNKT cells. miR-183C miRNAs are thought to promote NKT17 effector function through inhibiting Foxo1 (57).

While miR-182 is highly upregulated in activated T lymphocytes, surprisingly, the systemic knockout of miR-182 in B6 mice had no obvious effect on the development and function of the T cell subsets such as $CD4^+$, $CD8^+$, $CD4^-CD8^-$ double negative (DN) T cells, and Tregs (58). Furthermore, the T cell-dependent immune response to *Listeria monocytogenes* in miR-$182^{-/-}$ mice was similar to that in the controls (58). The dispensable role of miR-182 in lymphocyte development and function of B6 mice is likely due to the compensation by miR-96 and miR-183 since the three miRNAs have similar seed sequences and overlaps of the target genes.

In summary, although the molecular mechanism needs to be further clarified, the current studies demonstrated an important role of miR-183C miRNAs in the regulation of Th cell expansion, Th17/Treg differentiation and function, and T cell-mediated inflammation in specific pathological conditions.

3.2 B cells

The critical role of miRNAs on B cell development and activation has been extensively discussed (59–61). Similar to T cells, the miR-183C is expressed at a very low level in naïve B cells and is markedly upregulated in activated B cells (58, 62). However, compared with T cells, there are fewer reports of the role of miR-183C miRNAs in B cells. The dispensable role of miR-182 and miR-183C miRNAs on B cell development and differentiation has been consistently documented in different studies (58, 62, 63). In miR-$182^{-/-}$ and miR-$183C^{-/-}$ mice, the proportions of B cells at different developmental stages in the bone marrow and differentiation stages in the spleens mainly remained unchanged when compared to wild-type control mice (58, 62, 63).

Nevertheless, inconsistent results were obtained with regard to the role of miR-182 on B cell response and function. Pucella et al. reported that miR-$182^{-/-}$ mice have a similar B cell response to the immunization with T cell-dependent (TD) antigen ($NP_{(30)}$-CGG) as the control B6 mice with similar fractions of germinal center (GC) B cells and normal production of both low-affinity and high-affinity NP-specific IgG1 at 21 days post-immunization (58). On the other hand, Li et al. reported that the production of NP-specific IgM and IgG1 was inhibited at early stages (7- and 14-days post TD antigen NP_{38}-CGG immunization) in miR-$182^{-/-}$ mice, although the formation of germinal center B cells (GC B) and the differentiation of follicular helper T cells (Tfh) were not compromised in miR-$182^{-/-}$ B6 mice (62). In miR-$182^{-/-}$ mice, the fraction of NP-specific IgG1-secreting cells in the spleen was

reduced compared to controls at five days post-immunization with NP_{38}-CGG but remained at a similar level at ten days (62). Immunization of mice with NP_{25}-Ficoll, a T cell-independent type II (TI II) antigen, confirmed that miR-182 deficiency affected extrafollicular plasma B cell generation. The miR-$182^{-/-}$ mice showed a more general reduction in various subtypes of anti-NP_{25}-Ficoll antibodies throughout all time points (4-, 7-, 14-, and 21-days post-immunization) and a reduction in IgG1 secreting cells in the spleen at day five (62). Therefore, these authors concluded that miR-182 plays an important role in driving extrafollicular B-cell response to T cell-dependent (NP_{38}-CGG) and independent (NP_{25}-Ficoll) antigens without affecting GC responses.

Considering the potential compensation effect of miR-96 and miR-183 on the deletion of miR-182 individually, Pucella et al. utilized a whole miR-183C cluster knockout (miR-$183C^{GT/GT}$) mouse model to investigate the effect of miR-183C on the humoral response to antigens (63). In this study, these workers further showed that miR-183C miRNAs were dispensable for B cell development and function. The miR-$183C^{GT/GT}$ mice had normal GC response to the immunization of TD antigens, either sheep red blood cell (SRBC) or NP-CGG, although there was a mild light zone GC skew in miR-$183C^{GT/GT}$ mice. In miR-$183C^{GT/GT}$ mice, $NP_{(31)}$-CGG immunization induced comparable levels of serum total IgM and IgG1 and serum NP-specific IgM as wild-type littermates at different time points, while a slight but significant reduction of NP-specific serum IgG1 was observed in 28 days after immunization. Further, the authors demonstrated that miR-183C miRNAs were not required for the extrafollicular humoral responses to both types I and II TI antigens at either early or late stages of immunization. There was no significant difference in the proportion of plasmablasts ($CD138^{+}B220^{lo}$) and intracellular IgM^{+} cells observed in TI-1 antigen LPS immunized miR-$183C^{GT/GT}$ and miR-$182^{-/-}$ mice compared to their respective control mice. Similarly, there were equivalent frequencies of IgM, IgG1, and IgG3 secreting plasmablasts and NP-specific IgM and IgG secreting cells in TI antigen NP-Ficoll immunized miR-$183C^{GT/GT}$ and miR-$182^{-/-}$ compared to control mice (63). Thus, Pucella et al. concluded that miR-183C miRNAs had a minimal role in primary B-cell antibody response to either TD or TI antigens, which are predominately involved in GC and extrafollicular B-cell response, respectively.

Together, current studies indicated that miR-183C miRNAs are largely dispensable for B cell development and GC B-cell response (62, 63). The reasons for the discrepancies between the two studies about the role of miR-182 on extrafollicular B-cell responses remain unclear, although it might be explained by the difference in NP-specific IgG1 antibody-secreting plasmablasts in control mice (63).

3.3 Macrophages

Macrophages are potent phagocytotic cells essential for the removal of aging and dying apoptotic cells. It is well-accepted that impaired phagocytosis of macrophages in autoimmune disease patients results in inadequate clearance of dead cell debris. Consequently, the accumulation of apoptotic debris provides a source of autoantigens that potentially contributes to the initiation of autoimmune diseases (64, 65). Macrophages also contribute to the development of autoimmunity through producing inflammatory cytokines/chemokines or shifting the macrophage polarization (66, 67). Recent *in vivo* and *in vitro* studies showed that miR-183C miRNAs regulate macrophage functions *via* targeting different genes and pathways (Figure 3). miR-183C regulated the number of $CD11b^{+}F4/80^{+}$ corneal resident macrophages and inhibited IL-17 and IL-10 production in corneal resident macrophages through targeting *Runx1* and *Maf* (68). The inactivation of miR-183C in miR-$183C^{GT/GT}$ mice led to increased corneal resident macrophages number and enhanced IL-17 and IL-10 production in corneal resident macrophages following *Pseudomonas aeruginosa* (PA) infection (68). Also, miR-183C provided a negative feedback regulation on TLR4 signaling pathway (68). Another study reported that PA or LPS treatment significantly induced miR-183 and miR-182, but not miR-96, in macrophage cell line RAW264.7 cells (69). Inhibition of miR-183C miRNAs in RAW264.7 cells significantly inhibited the inflammatory response to either LPS or PA treatment with reduced production of CCL2, CXCL2, and/or TNF-α when compared to control cells. The peritoneal macrophages from miR-$183C^{GT/GT}$ mice had higher basal levels of proinflammatory cytokines IL-1β, CCL2, and CXCL2 than controls, but had reduced responsiveness to *in vitro* treatment with LPS or PA (69). The

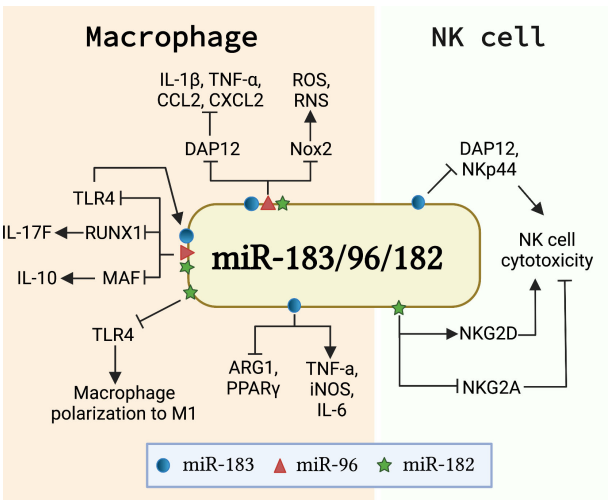

FIGURE 3
miR-183C is involved in the development and function of innate immune cells. The schematic graph illustrates the major genes/signaling pathways that mediate the function of miR-183C in macrophages and NK cells. In macrophages, miR-183C regulates the expression of IL-10 and IL-17F by targeting MAF and RUNX1, respectively, and provides a negative feedback regulation of TLR4 signaling (68). miR-183C also regulates the inflammatory responses and phagocytosis function in macrophages by inhibiting DAP12 and Nox2, respectively (69). In addition, miR-182 and miR-183 promote M1 macrophage polarization *via* different mechanisms. miR-182 targets TLR4 to regulate the macrophage polarization (70). miR-183 promotes M1 signature genes (TNFα, iNOS, IL-6) expression and inhibits M2 macrophage signature genes (ARG1 and PPARγ) expression (71). In NK cells, miR-182 and miR-183 have opposite effects on the cytotoxicity of NK cells. While miR-182 promotes cytotoxicity by suppressing the inhibitory receptor NKG2A and increasing activating receptor NKG2D expression (72), miR-183 inhibits the cytotoxicity of NK cells by targeting DAP12 and NKp44 (46, 73).

authors suggested that miR-183C potentially inhibited DNAX-activating protein of 12 kDa (DAP12), a negative regulator of TLRs responses, to regulate inflammatory responses in macrophages (69). In addition to regulating cytokine/chemokine production in macrophages, miR-183C also regulates the phagocytosis function of macrophages. A bioinformatics analysis approach revealed that the predicted target sites of miR-182 were over-presented in a set of macrophage intracellular pathogen response (macIPR) genes (74). Further, Gregory et al. demonstrated that overexpression of miR-182 in primary human alveolar macrophage-like monocyte-derived macrophages (MDM) increased proinflammatory cytokines genes expression and enhanced autophagy function of macrophages during infection with *F. tularensis* live vaccine strain (LVS) (74). Overall, this study showed an enhancing effect of miR-182 on the autophagy function of macrophages against infection. However, the suppressing effect of miR-183C miRNAs on phagocytosis has also been reported (75). Inactivating miR-183C significantly reduced the severity of PA-induced keratitis in miR-183C$^{GT/GT}$ with increased efficiency for bacterial cleaning. Overexpression of miR-183C miRNAs significantly inhibited phagocytosis function and intracellular killing efficiency in mouse macrophage cell line RAW264.7, while inhibition of miR-183C miRNAs increased phagocytosis function and intracellular killing efficiency. The increased phagocytosis capability and intracellular killing efficiency were also observed in the polymorphonuclear neutrophils (PMNs) from miR-183C$^{GT/GT}$ mice compared to the cells from the control mice (75). In a later study by the same group, the authors demonstrated that miR-183C limited the phagocytosis intracellular killing capacity of macrophages *via* targeting NADPH oxidase 2 (Nox2) to suppress the production of reactive nitrogen (RNS) and oxygen species (ROS), two key players in macrophage antimicrobial activity (69). Moreover, miR-183C miRNAs have been shown to regulate the polarization of macrophages. Inhibition of miR-183 in bone marrow-derived macrophages (BMDMs) dramatically decreased M1 macrophage-associated genes (*TNF-α, iNOS, IL-6*) expression in response to oxidized low-density lipoprotein (Ox-LDL) stimulation, while concomitantly increasing M2 macrophage-associated genes (*Arg1, TGF-β, PPARγ*) expression (71). Furthermore, miR-182 has been shown to be a major player in mesenchymal stem cells-derived exosomes (MSC-Exo)-mediated attenuation of myocardial ischemia/reperfusion (I/R) injury by modulating macrophage polarization from inflammatory M1 macrophage toward M2 (70). RAW264.7 macrophages receiving miR-182 inhibitor transfected MSC-Exo had reduced expression of M1 markers and increased expression of M2 markers compared to the cells receiving negative control inhibitor transfected MSC-Exo. Mechanistically, miR-182 regulates macrophage polarization by targeting TLR4 to influence the crosstalk between TLR4/NF-κB pathway and PI3K/Akt pathway (70).

3.4 NK cells

Nature killer (NK) cells are innate lymphocytes that play an important role in killing tumor cells and virus-infected cells (76, 77). Transforming growth factor β1 (TGF-β) has been shown to suppress natural killer (NK) cell function by inducing miR-183 to inhibit the expression of DAP12, a critical regulator of NK cell effector response (46, 78). Overexpression of miR-183 significantly suppressed DAP12 and surface receptor NKp44 expression in primary NK cells and NK cell line NK92 cells, which led to reduced cytotoxicity of NK92 cells (46). A separate study confirmed that TGF-β induced miR-183 expression in NK cells suppressed DAP12 (73). In this study, the researchers demonstrated that platelet-derived ectosomes (PLT-Ecto) transfused NK cells had a suppressed level of intracellular IFNγ and DAP12 *via* TGF-β mediated induction of miR-183 (73). Overall, the above two studies showed that miR-183 suppresses NK cell function by inhibiting the expression of DAP12. In addition, increased expression of miR-182 was found in NK cells of hepatocellular carcinoma patients (72). Overexpression of miR-182 in NK cells increased the expression level of activating receptor NKG2D and suppressed the inhibitory receptor NKG2A, leading to an enhanced NK cells cytotoxicity against tumorigenic Huh-7 cells (72). Together, these works suggested an opposite role of miR-182 and miR-183 in regulating NK cell function, potentially through the regulation of different genes and pathways (Figure 3).

4 miR-183C miRNAs in autoimmune disease and potential therapeutic application

The dysregulated expression of miR-183C miRNAs among different cell types has been found in various autoimmune diseases such as SLE, MS, RA, EAU, graves' orbitopathy, and sympathetic ophthalmia with uveitis and implicated in disease pathogenesis (Table 1). Given their altered expression in autoimmune diseases and crucial immune regulatory roles, miR-183C miRNAs have been considered as potential therapeutic targets for immune-related diseases in several studies. In the following section, we focus on the dysregulation and the potential for therapeutic intervention of miR-183C miRNAs in SLE, MS, and autoimmune-mediated eye diseases.

4.1 miR-183C in SLE

The overexpression of miR-183C miRNAs has been identified in splenic lymphocytes of different lupus-prone mice models, including MRL/lpr, C57BL/6-lpr, NZB/W$_{F1}$, and C3.MRL-Fas^{lpr}, and in peripheral blood mononuclear cells (PBMCs) of MRL/lpr mice (Table 1) (28, 79, 80). The upregulation of miR-182 has also been reported in PBMCs of human lupus patients (82). Inhibition of miR-182 alone or three miR-183C miRNAs simultaneously *in vitro* in splenocytes of MRL/lpr mice significantly inhibited either LPS or Concanavalin A (Con A) induced production of proinflammatory cytokines IFNγ and IL-6 (34). These *in vitro* data suggested a proinflammatory role of miR-182 and miR-183C in lupus. To further understand the effect of miR-182 and miR-183C miRNAs *in vivo* on lupus, we developed B6/lpr models with conditional deletion of miR-182 alone (miR-182$^{-/-}$B6/lpr) or whole miR-183C cluster (miR-183C$^{-/-}$B6/lpr) in CD2^{+} lymphocytes. Deletion of miR-

TABLE 1 Dysregulation of miR-183C in autoimmune diseases.

microRNA	Expression	Cell/ tissues	Species/ Strains	Disease	Potential pathogenic contribution	References
miR-183, miR-96, miR-182	Up	Splenocytes, PBMCs, $CD4^+$T	MRL/lpr, B6/lpr, NZB/W_{F1}, C3.MRL-Fas^{lpr}	SLE	Promoted inflammatory cytokine IFNγ and disease *via* targeting Foxo1	(28, 34, 79–81)
miR-182, miR-183	Up	PBMC	Human	SLE	Not assessed (N/A)	(82, 83)
miR-182	Up	kidney tissue	MRL/lpr	SLE/LN	Promoted renal fibrosis and LN development *via* targeting Foxo1	(81)
miR-183	Down	kidney tissue	MRL/lpr, human	SLE/LN	Promoted renal damage by blunting the inhibitory effect of miR-183 on mTOR or TGF-β/Smad/TLR3 signaling pathway.	(84, 85)
miR-183	Down	Plasma	Human	SLE/LN	N/A	(86)
miR-182	Up	$CD4^+$ T	Human, C57BL/6 mouse	RRMS, EAE	Enhanced Th1 expansion, IFNγ and IL-17 production *via* targeting HIF-1α; Suppressed Treg differentiation *via* targeting Foxo1 and Foxp3	(27, 50)
miR-96	Up	PBMC	Human	Remission MS	N/A	(29)
miR-96	Down	Plasma	Human	RA	N/A	(87)
miR-183-3p	Down	Psoriatic lesioned skin	Human	Psoriasis	Promoted proliferation and migration of keratinocytes by increasing miR-183 target gene, GAB1.	(88)
miR-96, miR-183	Up	$CD4^+$ T	Human	Graves' orbitopathy	Promoted T cell proliferation and activation *via* regulating EGR1/PTEN/AKT pathway	(49)
miR-182, miR-183	Up	Ocular tissue	Rat	EAAU	N/A	(89)
miR-182, miR-183	Down	Ocular tissue	Human	sympathetic ophthalmia	N/A	(90)
miR-182	Down	$CD4^+$ T	C57BL/6 mouse, Human	EAU, sympathetic ophthalmia	Enhanced Th17 development and pathogenicity by blunting the suppressive effect of miR-182 on TAF15/STAT3 pathway	(51)
miR-182, miR-96, miR-183	Down	Ocular tissue	B10.RIII mouse	EAU	N/A	(91)

EAAU, experimental autoimmune anterior uveitis; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis/uveoretinitis; LN, lupus nephritis; RA, rheumatoid arthritis; RRMS, relapse remitting multiple sclerosis; SLE, systemic lupus erythematosus. N/A, Not Assessed.

182 alone or miR-183C in B6/lpr mice did not exhibit an obvious effect on body and spleen weight, proteinuria level, and renal histopathological score but significantly inhibited IgG deposition in kidneys. However, in miR-$183C^{-/-}$B6/lpr mice, the production level of anti-dsDNA autoantibodies was significantly reduced in a time-dependent manner, suggesting the ameliorating effect of miR-183C deletion on lupus *in vivo* (34). Consistently, there was a significant reduction of IFNγ production in *in vitro* stimulated splenocytes from either miR-$182^{-/-}$B6/lpr or miR-$183C^{-/-}$B6/lpr mice compared to their respective controls. Further, we demonstrated that miR-183C regulated IFNγ production in splenocytes by targeting Foxo1.

The ameliorative effect of miR-182 inhibition on lupus has also been reported by Wang et al. (81). In this study, increased miR-182 expression in high Chronicity Index (CI) LN patients and in lupus-prone MRL/lpr mice was associated with the development of LN and reduced Foxo1 expression. *In vivo* inhibition of miR-182 in MRL/lpr mice with antagomir-182 delayed LN progression with attenuation of tissue damage and improved renal functions (81). Transforming growth factor-β (TGF-β) signaling plays a major role in driving tissue fibrosis, a pathological process during LN development (92, 93). The authors further demonstrated that TGF-β1 treatment promoted miR-182 expression in human renal tubular epithelial HK-2 cells and mouse glomerular mesangial SV40 MES 13 cells, suggesting a potential involvement of miR-182 in renal fibrosis during LN development.

Nevertheless, reduced miR-183 expression was identified in renal tissues of LN patients and lupus mice, which suggested a protective effect of miR-183 on LN (84, 85). One group reported that intraperitoneal injection of miR-183 mimic into MRL/lpr mice led to a significant reduction in serum anti-dsDNA levels, blood urea nitrogen (BUN) and urinary albumin levels (84). The MRL/lpr mice injected with miR-183 mimic also had reduced immunocomplex (IgG and C3) deposition in the kidneys and a prolonged survival rate compared to the mice receiving the control miRNA mimic (84). The

authors further demonstrated that miR-183 exhibited a protective effect on lupus *via* targeting mammalian target of rapamycin (mTOR) since there was reduced mTOR expression and activation in miR-183 mimic-treated cells. The ameliorating effect of mTOR inhibition on lupus has been well documented (94, 95). Another study reported that overexpression of miR-183 in MRL/lpr mice *via* caudal vein injection of miR-183 agomiR reduced renal injury with decreased renal histopathological scores, reduced expression of proinflammatory cytokines (IL-6 and TNF-α) and renal fibrosis-related factors (α-SMA and Vimentin) in the kidney (85). The inhibition of miR-183 *via* injecting the mice with antagomir-183 had opposite effects. Moreover, the inhibitory effect of miR-183 on proinflammatory cytokines and renal fibrosis-related factors was verified in human renal glomerular endothelial cells. Mechanistically, Qi et al. demonstrated that miR-183 attenuated LN by targeting transforming growth factor beta receptor 1 (Tgfbr1), an enhancer of the TGF-β/Smad/TLR3 pathway (85).

In summary, miR-182 and miR-183 demonstrated tissue-specific dysregulation in lupus and exhibited opposite roles on LN. It is noteworthy that a single base difference between seed sequences of miR-183 and miR-182 gives them distinct mRNA target genes. Thus, further investigations about the specific and overlapping target genes of individual miR-183C miRNAs under an SLE background would assist us in illuminating the similar and divergent roles of miR-183C miRNAs in autoimmunity and autoinflammation.

4.2 miR-183C in MS and EAE

The abnormal expression of miR-183C miRNAs has been identified in the immune cells of MS patients and the murine model of MS, EAE mice (Table 1) (27, 29). Several groups have evaluated the pathogenic role of miR-183C miRNAs in EAE. It has been reported that miR-182 was upregulated in a Chinese cohort of patients with relapsing and remitting multiple sclerosis (RRMS), which is associated with the increase of IFNγ-expressing Th1 cells (27). The *in vivo* study indicated that transgenic overexpression of miR-182 in mice promoted MOG_{35-55}-induced EAE with increased frequency of Th17 and Th1 cells, early onset of the disease, and severe symptoms. In contrast, the EAE mice with global knockdown of miR-182 showed mitigated disease symptoms with a significantly lower frequency of Th17 and Th1 cells compared to controls (27). The promoting effect of miR-96 and miR-183C on the onset of EAE in mice has been reported (33). miR-183C miRNAs, particularly miR-96, drove the Th17 pathogenicity in autoimmune disease. Compared to controls, the mice receiving miR-96-transduced MOG-specific TCR transgenic (2D2) Th17 cells developed much more severe EAE, accompanied by a higher frequency of IL-17 and GM-CSF expressing cells in the central nervous system. In addition, the authors showed that the *RAG1*$^{-/-}$ mice that received $CD4^+$ T cells from miR-183C$^{-/-}$ mice developed less severe EAE with reduced frequency of IL-17 and GM-CSF expressing T cells when compared to the *RAG1*$^{-/-}$ mice received $CD4^+$ T cells from miR183C$^{+/+}$ mice (33). These studies strongly suggested that miR-183C miRNAs have a promoting effect on the pathogenesis of MS and EAE by driving the pathogenicity of Th17 cells, a major pathogenic contributor in MS and EAE autoimmune disease.

4.3 miR-183C in autoimmune-mediated eye diseases

miR-183C miRNAs are critical for retinal development and function. Dysregulated miR-183C expression has also been implicated in the pathogenesis of autoimmune-mediated eye diseases such as autoimmune uveitis and Grave's orbitopathy (GO) (Table 1) (49, 51, 96). miR-183C miRNAs were downregulated in the ocular tissues of EAU mice, correlating with the upregulation of IL-17 in the eye (91). The downregulation of miR-182 was also detected in $CD4^+$ T cells of EAU mice and in the ocular tissues and $CD4^+$ T cells of human patients with sympathetic ophthalmia (SO) (51, 90). Zhang et al. further demonstrated that miR-182 negatively regulated Th17 development and pathogenicity in EAU *via* targeting TAF15 (51). The mice receiving miR-182 mimic transfected Th17 cells had ameliorated clinical EAU scores compared to the mice receiving control mimic transfected Th17 cells. This study suggested a therapy role of miR-182 mimic in the EAU (51).

In contrast to the downregulation of miR-182 in EAU mice and human patients with SO, miR-182 and miR-183 were upregulated in ocular tissues of experimental autoimmune anterior uveitis (EAAU) rat (89), and miR-96 and miR-183 were elevated in $CD4^+$ T cells of human patients with GO (49). Elevated miR-96 and miR-183 expression contributed to enhanced T cell proliferation and activation by targeting EGR1/PTEN/Akt pathway (49). The genetic variant of miR-182 (rs76481776 CC→TT/CT) was associated with the susceptibility of human Vogt-Koyanagi-Harada (VKH) syndrome and Behçet's disease, which are characterized by bilateral granulomatous and non-granulomatous uveitis, respectively (97). Further, Yu et al. demonstrated that miR-182 expression was significantly increased in the activated $CD4^+$ T cells from healthy controls with rs76481776 TT/CT genotype compared to rs76481776 CC genotypes (97).

5 Conclusions and perspectives

This review summarizes the complex role of miR-183C miRNAs, either individually or the whole cluster, in different types of immune cells and autoimmune diseases. Most, but not all, studies found that miR-183C miRNAs are proinflammatory and upregulated in autoimmune disorders, especially in SLE and MS/EAE (Table 1). These findings suggest that miR-183C miRNAs can potentially be diagnostic biomarkers and/or therapeutic targets for specific autoimmune diseases.

The therapeutic effects of inhibiting miR-183C miRNAs *in vivo* in animal models of autoimmune diseases (such as SLE and MS) have been confirmed by different research groups. In these studies, inhibition or deletion of miR-183C whole cluster or individual miR-183C miRNA *in vivo* led to the attenuation of disease symptoms in lupus mice and in EAE mice (27, 33, 34, 81). These data are highly promising for developing miR-183C miRNAs-based therapy for specific autoimmune disorders treatment. Given the importance of miRNAs in biological functions and the pathogenesis of different diseases, a number of miRNA-based therapeutic approaches are

being developed and entered into clinical trials. The major challenges to miRNA-based therapy include developing efficient and targeted delivery systems, dosage concerns, degradation caused by nucleases, and off-target effects (98, 99). ABX464, which upregulates miR-124, was approved for clinical trials to treat ulcerative colitis and rheumatoid arthritis (100–102). RG-012, an effective inhibitor of miR-21, is undergoing Phase 2 HERA trials for Alport syndrome; and MRG-201, the agonist to miR-29b, is in Phase 1 clinical trial for scleroderma (103, 104). These clinical trials may eventually pave the way for using other disease-specific miRNAs in autoimmune and inflammatory diseases.

Since miR-183C miRNAs are involved in embryonic development and regulation of various biological functions of other organs, it is imperative that miR-183C-based treatment requires achieving stable and cell-specific delivery to minimize potential unforeseen side effects. In general, the miR-183C miRNAs studies have been shown to regulate a broad range of immunological pathways, including the development, differentiation, and cytokine production of various immune cells. Each miRNA in miR-183C has both overlapping redundant and unique targets due to minimal differences in seed sequences. This allows miR-183C miRNAs to target gene expression cooperatively or individually. miR-183C's regulatory effects on the immune system or inflammatory diseases should not be generalized and need to be evaluated in the specific disease context as they were differentially dysregulated in different types of autoimmune diseases (Table 1). Thorough investigations of miR-183C miRNAs, both individually and collectively, in cell-specific and disease-specific contexts are essential prerequisites for developing innovative diagnostic and therapeutic alternatives.

Author contributions

ZW drafted and wrote the manuscript. RD assisted with the writing and critically reviewed and edited the manuscript. SA critically reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Insights into the role of nucleotide methylation in metabolic-associated fatty liver disease

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Metabolic-associated fatty liver disease (MAFLD) is a chronic liver disease characterized by fatty infiltration of the liver. In recent years, the MAFLD incidence rate has risen and emerged as a serious public health concern. MAFLD typically progresses from the initial hepatocyte steatosis to steatohepatitis and then gradually advances to liver fibrosis, which may ultimately lead to cirrhosis and carcinogenesis. However, the potential evolutionary mechanisms still need to be clarified. Recent studies have shown that nucleotide methylation, which was directly associated with MAFLD's inflammatory grading, lipid synthesis, and oxidative stress, plays a crucial role in the occurrence and progression of MAFLD. In this review, we highlight the regulatory function and associated mechanisms of nucleotide methylation modification in the progress of MAFLD, with a particular emphasis on its regulatory role in the inflammation of MAFLD, including the regulation of inflammation-related immune and metabolic microenvironment. Additionally, we summarize the potential value of nucleotide methylation in the diagnosis and treatment of MAFLD, intending to provide references for the future investigation of MAFLD.

KEYWORDS

MAFLD, DNA methylation, M 6 A modification, immune microenvironment, metabolomics, epitranscriptomics

1 Introduction

MAFLD, formerly termed non-alcoholic fatty liver disease (NAFLD), is defined based on the coexistence of hepatic steatosis with other metabolic diseases, including obesity, type 2 diabetes and metabolic dysfunction. With the in-depth research of disease heterogeneity and pathogenesis, NAFLD was renamed MAFLD, which more accurately reflects the current understanding of the disease (1). MAFLD is the most common chronic liver disease worldwide, affecting 25% of the population (2) and up to 55.5% (3) and 90% (4) of patients

with type 2 diabetes and morbid obesity, respectively. However, its specific pathogenesis still needs to be studied in depth. The "multi-hit" pathogenesis of MAFLD is now widely accepted, with insulin resistance, oxidative stress, epigenetics, lipotoxicity, inflammatory factors, altered intestinal flora, and other factors affecting hepatocyte fat content and the hepatic inflammatory milieu, thus contributing to the development of steatosis and liver inflammation (5, 6). With disease progression, MAFLD eventually advances to nonalcoholic steatohepatitis (NASH), fatty liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (7). There is no effective therapeutic drug for the treatment of MAFLD, although lifestyle changes, including weight loss and a balanced diet, may alleviate symptoms (8). Consequently, MAFLD has seriously impacted human health and caused a huge medical burden (9).

Epigenetics is defined as the study of the processes that modify gene expression without altering the DNA sequence directly. These processes mainly comprise nucleotide methylation, histone modification, chromosomal remodeling, and so on (10). Mounting evidence suggests that epigenetic modifications offer fresh insight into the pathophysiology of MAFLD (11–13). It has been shown that nucleotide methylation (14), histone modifications, and microRNAs (15) are involved in the pathogenesis of lipid metabolic disorder, inflammation, oxidative stress, and mitochondrial damage. Among them, DNA and RNA methylation are two of the most critical epigenetic alterations contributing to the development and progression of MAFLD (14, 16).

The immune system and inflammation also play a part in the onset of MAFLD. An inflammatory microenvironment constituted of liver resident immune cells like Kupffer cells (KCs), natural killer cells (NKCs), dendritic cells (DCs), and regulatory T lymphocytes (Tregs), characterizes NASH (17). Moreover, immune cells, working in tandem with other immune cells, can increase local inflammatory responses in the liver and exacerbate liver injury by secreting a wide variety of inflammatory factors and chemokines (18, 19). Notably, several investigations have shown that nucleotide methylation is critical in controlling the immunological response (20), suggesting that it may serve a role in the development and progression of MAFLD by triggering an inflammatory response. In addition to inflammation, the accumulation of triglycerides in liver tissue is a result of a disturbance in lipid synthesis or lipid catabolism, both of which contribute to hepatic lipid metabolic disorder (21). A growing number of studies illustrated that nucleotide methylation can impact the evolution of MAFLD through the regulation of both metabolic and immune aspects. Therefore, in this review, we aim to highlight the potential utility of regulating nucleotide methylation in diagnosing and treating MAFLD from the perspectives of the immune system and metabolism.

2 Background on the epigenetic function of nucleotide methylation

The accumulation of epigenetic changes, including nucleotide methylation, histone modification, non-coding RNAs, etc., is one of the fundamental processes driving cancer initiation and progression. It mainly affects the function and characteristics of genes by regulating gene transcription or translation. Nucleotide methylation, which includes DNA methylation and RNA methylation, is one of the most extensively researched epigenetic modifications.

DNA methylation, the conversion of cytosine into 5-methylcytosine (5-mC) by the addition of methyl groups provided by S-adenosyl methionine (SAM), is catalyzed by DNA methyltransferase (DNMTs) enzyme family and occurs most frequently on CpG dinucleotides. In mammals, coordinated and cooperative action between DNMTs and demethylases is essential for the regulation of DNA methylation. DNMT1 is primarily responsible for maintaining DNA methylation, while DNMT3A and DNMT3B usually carry out *de novo* methylation of unmethylated DNA or hemimethylated DNA (22). The deletion of DNMT1 and DNMT3B in mice leads to embryonic lethality, and the deletion of DNMT3A causes death within a few weeks after birth, demonstrating their vital involvement in survival and development (23). Additionally, DNMTs are intimately associated with the progression of tumors and other diseases (24). Demethylation enzymes consisting of ten-eleven translocation (TET) dioxygenases and thymine DNA glycosylase (TDG) are involved in active or passive DNA demethylation (22), and an abnormal expression of these enzymes has been linked to tumorigenesis, embryonic development and cell differentiation (25–27). In most cases, TET methylcytosine dioxygenases are responsible for the oxidation of 5-mC to 5-carboxycytosine (5caC), which is then identified and excised by TDG. Finally, the base excision repair (BER) pathway is triggered, which results in the reduction of 5-caC to unmodified cytosine. It was previously believed that DNA methylation was stable in the genome (28). However, with the development of sequencing technologies, it has been revealed that DNA methylation and demethylation are in dynamic change and balance (29, 30), which is crucial for their regulatory roles (31).

DNA methylation acts as a regulator in immune cell proliferation, differentiation, and response. Pluripotent hematopoietic stem cells can differentiate into lymphoid and myeloid stem cells. During the differentiation process, several genes of key regulatory transcription factors of myeloid stem cells, including GATA binding protein 2 (GATA2), T-cell acute leukemia 1 (TAL1), and LIM domain only 2 (LMO2), showed an increased level of DNA methylation, indicating that the differentiation of adult stem cells is regulated by DNA methylation (32). DNA methylation is intrinsically linked to cellular immunity because it is extensively reprogrammed during differentiation in functional $CD8^+$ T cells. Moreover, gene expression and promoter DNA methylation are inversely correlated (33). It has been found that DNA methylation can stimulate the signaling pathways involved in the inflammatory immunological response. The expression of the zinc finger transcriptional repressor Snail can be upregulated by the Hepatitis B virus (HBV) due to an increase in mitochondrial reactive oxygen

species. Together with DNMT1 and histone deacetylase 1 (HDAC1), snail binds to the promoter of suppressor of cytokine signaling 3 (SOCS3) and facilitates the epigenetic silencing of SOCS3 and prolonged activation of the IL-6/STAT3 pathway (34).

Aberrant DNA methylation is also associated with the development of cancer. Two types of DNA methylation abnormalities may exist in tumor cells: genomic hypomethylation (which enhances genomic instability and activates oncogene transcription to promote tumorigenesis), and CpG island DNA hypermethylation (which causes gene silencing and decreased expression) (35). Thus, both low and high DNA methylation levels can affect gene expression in ways that promote tumor growth. A group of genes expressed in the germline, called "cancer-testis genes" or "cancer germline genes" (CG), are usually expressed only in testicular germ cells and are silenced by DNA methylation in most somatic cells. However, aberrant activation of CG genes is a significant consequence of DNA hypomethylation in tumors. Several CG genes can trigger oncogenic pathways involved in cell proliferation and metastasis, thereby promoting tumorigenesis (36). In addition, hypermethylation of CpG islands in many genes was observed in HCC tumor samples; this process is known to repress the expression of the targeted genes. In particular, tumor growth and progression were linked to decreased expression of eyes absent 4 (EYA4) together with its hypermethylation (37).

In recent years, RNA modifications were identified to play a crucial function in regulating gene expression, with N6-methyladenosine (m^6A) alteration accounting for approximately 80% of RNA methylation modifications. Even though the biological role of m^6A alterations has been well reported, the effects and potential mechanisms of other RNA modifications such as N6, 2'-O-dimethyladenosine (m^6Am), N1-methyladenosine (m^1A), 5-methylcytosine (m^5C), and 5-hydroxymethylcytidine (hm^5C), remain poorly understood (16). Methylation at the N6 position of adenosine is a dynamic and reversible change that affects a wide range of biological functions (38). The regulatory proteins of m^6A can be classified as "Writers", "Readers" and "Erasers", depending on their roles in the regulation processes. "Writers" are m^6A methyltransferases mainly composed of methyltransferase-like protein 3 (METTL3) (39), methyltransferase-like protein 14 (METTL14) (40), and Wilms tumor 1 associated protein (WTAP) (41). "Readers" are m^6A binding proteins including YT521-B homology-domain-family protein (YTHDFs) (42), insulin-like growth factor 2 mRNA binding protein (IGF2BPs), and YT521-B homology-domain-containing protein (YTHDCs) (43). "Erasers" are m^6A demethylases, including fat mass and obesity-associated protein (FTO) (44) and alkB homolog 5 (ALKBH5) (45). They together constitute an efficient and regulatory network of m^6A RNA modifications.

An increasing number of studies have discovered that RNA-modified m^6A is critical in maintaining immunological homeostasis. Researchers revealed that knocking down the m^6A methyltransferase METTL3 or METTL14 reduced the proliferative activity of $CD4^+$ T cells. These findings suggest that m^6A modification is vital for maintaining T cell homeostasis (46). METTL3-mediated m^6A alternation has also been found to promote DC maturation by inducing cytokine production through the NF-κB signaling pathway. Furthermore, METTL3 could strengthen T cell activation by regulating the translation of CD40, CD80, and Tirap mRNA in DC cells (47).

There is growing evidence that m^6A modification also affects carcinogenesis and tumor growth. m^6A methylation can influence cancer cell metastasis by increasing epithelial-mesenchymal transition (EMT); conversely, knockdown of METTL3 significantly reduced the expression of Snail, a key transcription factor modulating EMT in cancer metastasis (48). Furthermore, through YTHDF2-dependent post-transcriptional silencing of suppressor of cytokine signaling (SOCS2), METTL3 was discovered to serve a pro-oncogenic role in human HCC by increasing proliferation and metastasis (49). Although some progresses have been made, it is still necessary to investigate the role of m^6A modification on specific mRNA in normal physiological activities and abnormal pathological changes. Currently, the commonly used methods to detect RNA m^6A methylation are m^6A-seq, methylated RNA immunoprecipitation sequencing (MeRIP-seq), and photo-crosslinking-assisted m^6A sequencing (PA-m^6A-seq) (50, 51). Figure 1 shows the process of DNA methylation and RNA methylation.

3 Mechanisms of nucleotide methylation in the regulation of fatty liver and steatohepatitis, liver fibrosis and cirrhosis, and HCC

Nucleotide methylation modifications can affect cell growth and homeostasis by promoting or repressing the expression of specific genes. Abnormal gene methylation may alter cellular physiology and functions, resulting in disease development. Nucleotide methylation has been linked to the development and progression of various chronic liver diseases, including fatty liver, steatohepatitis, liver fibrosis, cirrhosis, and HCC. In this section, we discuss how nucleotide methylation contributes to the etiology and progression of numerous liver illnesses by repressing or activating the expression of associated genes.

3.1 Mechanisms of nucleotide methylation in the regulation of fatty liver and steatohepatitis

DNA methylation and demethylation are involved in MAFLD and NASH. As liver inflammation and disease progressed, it was discovered that the total DNA methylation levels were considerably lower in the livers of MAFLD patients (52). The hypomethylated levels of protein kinase C epsilon (PRKCE) and sec14-likeprotein3 (SEC14L3) were related to body mass index (BMI), waist circumference, total triglycerides, high-density lipoprotein cholesterol, alanine aminotransferase and aspartate aminotransferase (53). In addition, a high-sugar diet increases nuclear 25-hydroxycholesterol (25HC) to activate DNMT1, which regulates cell lipid metabolism genes through DNA methylation,

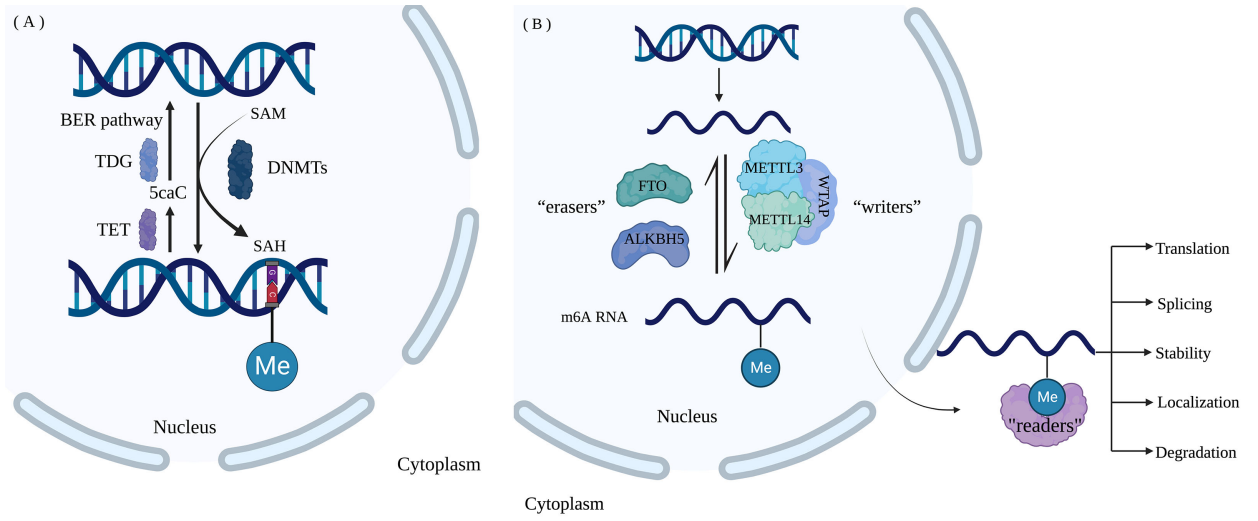

FIGURE 1
Nucleotide modification process **(A)** DNA methylation and demethylation. DNMTs catalyze the addition of methyl to C in the CG of DNA forms 5-mC, and methyl-group is provided by SAM. 5-mC undergoes oxidation by TET dioxygenase, recognition and excision by TDG and reduction to unmodified cytosine by the BER pathway. **(B)** M^{6}A RNA methylation. The dynamic m^{6}A modification of RNA is catalyzed by "Writers" consisting of methyltransferases METTL3, METTL14, and WTAP and is removed by "Erasers" including FTO and ALKBH5. "Readers" are m^{6}A- binding proteins, including YTHDFs, IGF2BPs, and YTHDCs, regulating RNA translation, localization, splicing, degradation, and stability. (Created with BioRender.com).

contributing to MAFLD and metabolic syndrome (54). Patients with NASH have greater methylation levels of the mitochondrial NADH dehydrogenase-6 (MT-ND6) gene than those with simple steatosis, which can lower mRNA expression levels. Of note, elevated methylation and transcriptional downregulation of MT-ND6 correlate with the severity of MAFLD (55).

M^{6}A methylation and its regulators also play a regulatory role in the development of MAFLD and NASH. The m^{6}A methylase METTL3 in liver tissue of T2DM patients was up-regulated, and METTL3 improved the expression level of fatty acid synthase (FASN) mRNA through m^{6}A modification, which in turn promoted fatty acid metabolism while inhibiting insulin sensitivity of the liver. It is indicated that m^{6}A and its regulators could participate in the occurrence of metabolic diseases by regulating fat metabolism (56). Mice overexpressing IGF2BP2 are more likely to develop steatohepatitis and progress to HCC. However, IGF2BP2-deficient mice show remarkable resistance to the diet-induced fatty liver *via* enhancing the expression of mRNAs encoding mitochondrial proteins, such as uncoupling protein-1 (UCP1) (57). Glucocorticoid receptor (GR)-dependent FTO transactivation and m^{6}A demethylation on FTO mRNA promote lipid accumulation in hepatocytes; FTO knockdown dramatically attenuates dexamethasone-induced fatty liver in mice, further supporting the role of m^{6}A on lipid synthesis. These results implicate FTO and m^{6}A demethylation as key players in the pathogenesis of MAFLD (58). FTO can also regulate lipid synthesis and increase lipid accumulation in hepatocytes by promoting the maturation of sterol regulatory element binding protein 1c (SREBP1c) and enhancing the transcription of cell death-inducing DFFA-like effector c (CIDEC) (59). It has also been shown that FTO can stimulate inflammation in the liver by blocking m^{6}A mRNA methylation of IL-17RA (60).

3.2 Mechanism of nucleotide methylation in the regulation of liver fibrosis and cirrhosis

Nucleotide methylation has been shown to exert a role in the initiation and progression of liver fibrosis and cirrhosis. Hepatic fibrosis is partly driven by the transdifferentiation of quiescent hepatic stellate cells (HSC) into active myofibroblasts; this process has been shown to significantly reduce the overall DNA methylation level, with over 20% of about 400 methylation regions being hypermethylated or hypomethylated (61). The DNA methylation reader methyl CpG binding protein 2 (MeCP2) can be recruited to the promoter region to inhibit transcription of peroxisome proliferator-activated receptor γ (PPARγ), thereby promoting the occurrence of liver fibrosis. PPARγ plays a negative role in regulating the transdifferentiation of myofibroblast into hepatic myofibroblast (62). The hypermethylation level of CpG dinucleotides within the promoter of the human PPARγ gene was found to be beneficial in distinguishing mild or severe fibrosis patients in a cohort analysis. This finding suggests that specific CpG islands' methylation status may be an important predictor of liver fibrosis progression (63). A recent study reported that, compared to healthy controls, patients with cirrhosis and HCC had considerably more hypomethylated sites (64) and hypermethylated sites (65) among 27578 CpG motifs studied (66). These findings suggest that DNA methylation may contribute to liver fibrosis and cirrhosis, but the underlying mechanisms need further investigation.

RNA methylation also has its unique regulatory role for liver fibrosis. METTL3 was discovered to be highly elevated in HCC. By m^{6}A methylation sequencing, one study identified SOCS2 as a target of METTL3-mediated m^{6}A modification (49). In general,

SOCS2 was hypermethylated in HCC and was associated with JAK-STAT pathway activation (67). Aside from its role in HCC, SOCS2 regulates liver fibrosis as well. Specifically, the LncRNA NEAT1/microRNA-129-5p/SOCS2 axis controls liver fibrosis in alcoholic steatohepatitis (68). Another study showed that acid-sensitive ion channel 1a (ASIC1a) promoted liver fibrosis by regulating miR-350 through METTL3-dependent m^6A modification. Liver fibrosis patients and animal models had considerably higher levels of ASIC1a expression, and METTL3 was linked to ASIC1a's promotion of liver fibrosis. METTL3-dependent m^6A modification could regulate the synthesis of miR-350 by combining with DiGeorge syndrome critical region gene 8 (DGCR8). At last, mature miR-350 promoted liver fibrosis through PI3K/AKT and ERK pathways (69). Additionally, studies have demonstrated that RNA methylation modifications contribute to fibrosis in cirrhosis (70).

3.3 Mechanism of nucleotide methylation regulating HCC

HCC typically develops in patients with preexisting liver diseases, including hepatitis virus infection, alcoholic liver disease, and MAFLD, among which the prevalence of MAFLD is growing rapidly and is expected to be the leading cause of HCC. Studies have shown that nucleotide methylation can trigger changes in the HCC genome, which in turn cause dysregulation of related oncogene expression, and boost the initiation, progression, and metastasis of HCC.

The hypomethylation or hypermethylation of DNA methylation both can affect hepatocarcinogenesis and metastasis differently. Knockdown of osteopontin (OPN) can decrease the methylation of specific tumor suppressor genes (TSG), resulting in reduced DNA methylation and down-regulation of DNMT1 to inhibit tumor development and metastasis (71). Metabolic reprogramming is a typical feature of cancer cells. Glycolysis enzyme phosphoglycerate kinase 1 (PGK1) can catalyze 1,3-diphosphoglycerate to generate 3-phosphoglycerate and ATP simultaneously. Reduced PGK1 promoter methylation leading to higher expression is substantially associated with shorter overall survival and worse prognosis in HCC, as well as with a wide range of other malignancies (72). Data on 369 HCC patients' genomics, methylomes, transcriptomes, proteomes, and clinical histories have been obtained and evaluated. It indicates that DNA methylation is connected with HCC prognosis (73). Angiogenesis plays an important role in the development, progression, and metastasis of HCC. MiR-378a-3p methylation induced by DNMT1 was shown to modulate the NF-κB signaling pathway, which promoted angiogenesis in HCC and was associated with a poor prognosis for HCC patients (74). In addition, methylation of mitochondrial fission regulator 2 (MTFR2) in HCC tissues causes aberrant expression and may contribute to HCC progression by influencing cell cycle progression, p53 signaling pathway, and DNA replication (75).

M^6A methylation is a well-known mRNA epigenetic modification involved in hepatocarcinogenesis. A high level of WTAP expression in HCC is linked to a worse prognosis. WTAP-directed m^6A modification has been reported to increase HCC advancement *via* the HuR-ETS1-p21/p27 axis (76). Downregulation of RAD52 motif 1 (RDM1), a key regulator of DNA double-strand break repair and recombination, suppresses tumor growth in HCC, whereas deletion of RDM1 promotes HCC cell proliferation. As the data reveal, RDM1 can work with the tumor suppressor gene p53 to increase p53's protein stability, suggesting that RDM1 has tumor-suppressive effects. However, overexpression of METTL3 can dramatically induce m^6A modification of RDM1 mRNA and decrease its expression, which is involved in tumorigenesis (77). The ability of M^6A mediated by YTHDF1 to accelerate the translation of snail mRNA suggests that m^6A modification has a significant effect on HCC progression, and overexpression of YTHDF1 is associated with poor prognosis in HCC patients (48). Reportedly, YTHDF2 can directly bind to the m^6A modification site of epidermal growth factor receptor (EGFR) to promote the degradation of EGFR mRNA in HCC cells. It is indicated that YTHDF2 may function as a tumor inhibitor by reducing the stability of EGFR mRNA in HCC cells, therefore suppressing cell proliferation and expansion (78). Nucleotide methylation's potential impact on MAFLD progression is depicted in Figure 2.

4 Role of nucleotide methylation in the remodeling of the inflammatory immune microenvironment during the progression of MAFLD

Liver is capable of producing immune-inflammatory responses, and the pathogenesis and disease progression of MAFLD are closely related to the activation of immune-inflammatory responses (79). Increasing evidence confirms that inflammation and immune responses, including innate immunity and adaptive immunity, are crucial to the development of MAFLD. In particular, inflammation becomes an indispensable component of disease progression. Since nucleotide methylation is involved in stimulating and maintaining immune activation of inflammation, we subsequently discuss the role of nucleotide methylation in remodeling the IME during MAFLD progression.

4.1 Remodeling of immune cells in the inflammatory microenvironment of MAFLD by nucleotide methylation

The liver is home to a wide variety of immune cells, including macrophages, KCs, NKCs, and natural killer T cells (NKTs). T cells, B cells, and innate immune cells are all examples of acquired immune cells that could have a role in the onset and progression of MAFLD. The regulation of immune cells by nucleotide methylation may have a role in MAFLD.

DNA methylation is a key control factor of the phenotype of macrophages. DNA methylation of the promoter regions of 26

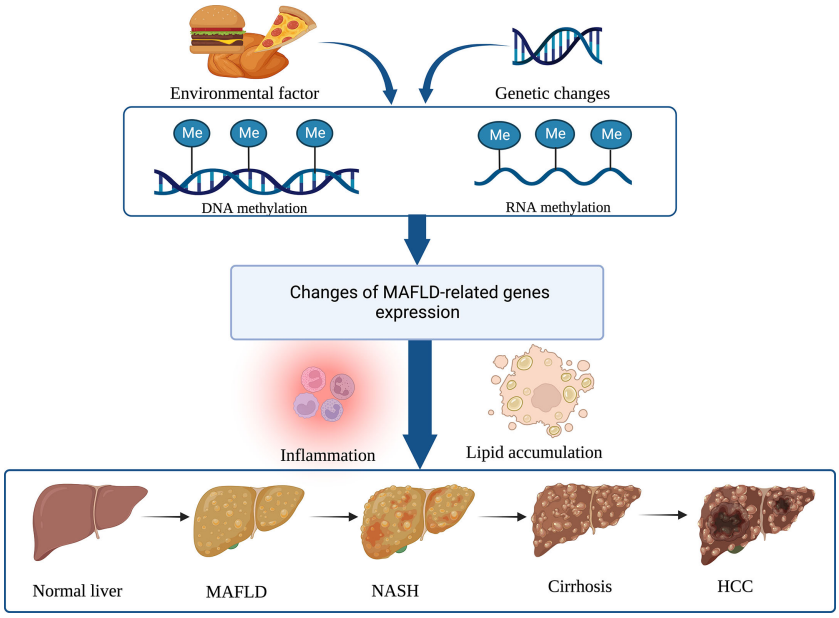

FIGURE 2
Nucleotide methylation regulates MAFLD and its progress. DNA and RNA methylations are the two main epigenetic modifications that alter gene expression involved in immune inflammation and lipid accumulation, and hence contribute to the development of MAFLD and its subsequent progression to NASH, cirrhosis, and HCC. (Created with BioRender.com).

genes in CpG islands was identified and validated in carbon tetrachloride (CCl4)-induced liver fibrosis mice. Methylation of proline-serine-threonine phosphatase interaction protein 2 (PSTPIP2) was found to lead to mixed induction of hepatic classical macrophages (M1) and replacement macrophages (M2). The results showed that overexpression of PSTPIP2 suppressed M1 expression by inhibiting signal transducer and activator of transcription 1 (STAT1) activity and enhanced M2 expression by promoting STAT6 activity. Conversely, the knockdown of PSTPIP2 promoted M1 polarization and inhibited M2 polarization (80). MAFLD, obesity, and metabolic syndrome are closely related to the inflammatory response. Research reports that adipose-associated macrophages (ATM) in obese patients can shift from M2-type anti-inflammatory macrophages to M1-type pro-inflammatory macrophages (81), contributing to obesity-induced inflammation and IR. Additionally, macrophage infiltration is associated with the proliferation of preadipocytes; this process might cause adipogenesis and thus aggravate obesity (82). Pharmacological DNMT1 deficiency promotes selective macrophage activation by blocking PPAR promoter DNA methylation, and DNMT1-deficient mice show improved M2 differentiation, reduced macrophage inflammation, and ameliorated obesity-induced inflammation and IR (83). These results suggest that obesity-related DNA methylation is a critical factor in macrophage-induced inflammation.

CpG island hypomethylation has been reported as the predominant form of genomic DNA methylation associated with human NK cell activation, suggesting a crucial role for methylation in NK cell formation and function (84). In a global DNA methylation test of obese and T2DM patients, DNA methylation was discovered to be elevated in the participants' B and NK cells. It may have contributed to the development of IR (85). In addition, the proliferation and function of macrophages and T cells are regulated in part by the ubiquitin-associated and SH3 domain-containing protein A (UBASH3A) and the tripartite motif protein 3 (TRIM3), both of which are hypermethylated in obese individuals (86).

METTL3 is specifically upregulated after M1 polarization in mouse macrophages, and further m^6A methylation experiments demonstrate that METTL3 promotes M1-type macrophage polarization through methylating STAT1 mRNA (87). M^6A is also involved in the regulation of T cell homeostasis and differentiation. Results show that METTL3 regulates $CD4^+$ T cell proliferation and differentiation *via* targeting the IL-7/STAT5/SOCS signaling axis to maintain T cell immune homeostasis (46). The specific knockout of METTL3 in Tregs can lead to severe autoimmune diseases or even death in mice. Exploring the mechanism reveals that m^6A modification can promote Treg-mediated suppression of immune cells *via* promoting degradation of SOCS mRNA in Treg cells. Decreased SOCS expression may subsequently activate the IL-2-STAT5 signaling pathway (88).

METTL14 deletion hinders B-cell development and significantly reduces the number of B cells. Results show that METTL14 deletion lowers IL-7-induced pre-B cell proliferation and induces aberrant gene expression linked to B cell development, suggesting that m^6A methylation regulates early B cell development (89). In addition, YTHDF1-deficient DC cells can enhance tumor antigen presentation and the anti-tumor capacity of

$CD8^+$ T cells (90). The above studies suggest that m^6A can regulate immune cells and play a regulatory role in inflammatory diseases, but most of these findings have not been considered in a liver-specific setting. Therefore, m^6A modification of immune cells in regulating liver homeostasis and liver disease should be further explored.

KCs are macrophages in the liver with the function of phagocytosis, antigen processing, presentation, and producing a variety of inflammatory factors, including tumor necrosis factor-α (TNF-α) and IL-6 (91). KCs are divided into two broad phenotypes, the classically activated M1 macrophage, and the alternative M2 phenotype. The M1 phenotype is considered pro-inflammatory and capable of producing cytokines and chemokines, and M2 is considered anti-inflammatory. KCs in steatotic livers contain lipid droplets, recruit more lymphocytes, and release large amounts of pro-inflammatory factors that promote immune responses to facilitate MAFLD (92). The accumulation of KCs in the portal vein is an early event of MAFLD, preceding the aggregation of other immune cells (93). However, the role of the M2 phenotype of KCs in MAFLD and NASH is unclear (94).

DCs are also present in the liver as antigen-presenting cells. Studies have shown that DCs can limit sterile inflammation by removing apoptotic cells and necrotic debris (91). Still, a current study found that increased cDC1 of the DC subtype may promote $CD8^+$ T cells, which can promote liver inflammation and injury in NASH, suggesting that cDC1 is a participant in the pathogenesis of NASH (95). Because the exact mechanism is not clear, continued research on the role of DCs in MAFLD is needed.

Invariant natural killer T (iNKT) cells are elevated in both NASH mouse models and NASH patients, where they secrete a variety of pro-inflammatory cytokines and boost OPN to exacerbate steatosis, NASH, and liver fibrosis, all of which contribute to the progression of MAFLD (96).

Neutrophil infiltration and neutrophil extracellular traps (NETs) are associated with chronic inflammation. in the early stages of fatty liver, NETs have been detected, indicating that neutrophil activation is a key factor in liver injury (97).

$CD4^+$ T_H cells can differentiate into T_H1, T_H2, and T_H17 cells with characteristic expression of interferon-γ (IFN-γ), IL-13, and IL-17, respectively (98), to participate in the process of NASH, liver fibrosis, and liver injury. T_H17 cells are a newly discovered subgroup of $CD4^+$ T cell characterized by the secretion of IL-17A, which plays a pathogenic role in promoting inflammatory diseases (65). It has been shown that T_H17 cells are increased in NASH mouse models, especially a pro-inflammatory $CXCR3^+$ T_H17 cell subpopulation, driving NASH pathogenesis (99). In MAFLD, $CD4^+$ memory T cell subsets are crucial in the transition from steatosis to fibrosis. After a high-fat diet (HFD), immunodeficiency mice implanted with human immune cells develop steatosis, liver inflammation, and fibrosis, along with increased liver and peripheral blood $CD4^+$ memory T cells. Depleting human $CD4^+$ cells in this model reduces liver inflammation and fibrosis, but not steatosis by secreting pro-inflammatory molecules such as IFN- γ and IL-17A (100).

$CD8^+$ T cells mainly produce cytotoxic molecules such as IFN-γ, TNF-α, and perforin, and the number of hepatic $CD8^+$ T cells is increased during NASH in mice and humans. Diet-induced NASH mouse models activate $CD8^+$ T cells and NK T cells, which promote NASH and HCC through their interactions with hepatocytes (101).

B cells with roles in antigen presentation, immunoglobulin production, and cytokine secretion are involved in immune-mediated inflammatory responses. It has been demonstrated that patients with MAFLD have an accumulation of B cells in the liver and that B2 lymphocytes can develop into mature plasma cells. In addition, B lymphocytes can contribute to the occurrence of MAFLD through the secretion of cytokines, IgG, and in response to oxidative stress (102). In the mouse model of NASH, B cells exhibit pro-inflammatory effects through B cell receptor-mediated adaptive immune mechanisms and MYD88-dependent innate immune mechanisms. Moreover, intestinal microbial-induced inflammatory mediators in NASH liver contribute to B cell activation and disease progression (103).

It is thus clear that the remodeling of immune cells significantly influences MAFLD in the IME of the liver, and nucleotide methylation also has some regulatory effects on immune cells in MAFLD. However, due to the lack of studies on the special IME of the liver, the specific effects need further investigation. Figure 3 shows the role of nucleotide methylation in remodeling immune cells during MAFLD.

4.2 Effect of nucleotide methylation on inflammatory factors in the inflammatory microenvironment of MAFLD

Nucleotide methylation affects inflammatory cytokines by regulating gene expression. DNA methylation regulates inflammatory cytokines, including IL-6, IL-11, and TNF-α, thus impacting the development of liver injury, diabetes, and obesity. It has been reported that the T-2 toxin, a highly toxic trichothecenes mycotoxins produced by Fusarium, can induce liver injury under inflammation. T-2 toxin can significantly increase the DNA methylase at the site of liver injury, inducing the expression of cytokines IL-11, IL-6, IL-1α, and TNF-α, thus aggravating the liver injury (104). A study has shown that the level of TNF-α gene methylation is related to the pathogenesis of T1DM (105). Another study discovered that obese males who lost weight had lower total TNF-α promoter methylation levels, suggesting that TNF-α promoter methylation can be utilized as a predictive biomarker of weight reduction (106). In obese patients, aberrant methylation of the IL-6 gene promoter is greatly enhanced, suggesting that it may be implicated in obesity's pathophysiology (107).

RNA methylation has been confirmed to affect the IME by regulating a range of inflammatory factors and related signaling pathways. YTHDF2 knockout significantly increases the expression of IL-6, TNF-α, IL-1β, and IL-12 in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, and activates the NF-κB signaling pathway, hence promoting inflammatory response (108). Furthermore, METTL3 deletion in mouse T cells affects T cells' homeostasis and differentiation *via* targeting the IL-7/STAT5/SOCS pathway (46). SOCS gene family expression is also found to be increased in METTL3-/- Treg cells and to reduce the suppressive function of Treg cells *via* IL-2/STAT5 (88).

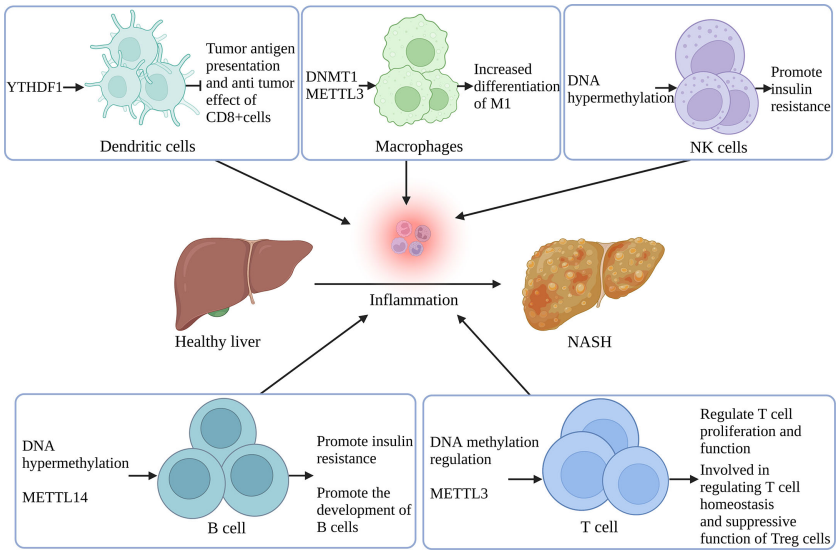

FIGURE 3
Remodeling of immune cells in the inflammatory microenvironment of MAFLD by nucleotide methylation YTHDF1 regulates tumor antigen presentation and anti-tumor effect in DCs. DNMT1 and METTL3 support M1 differentiation in macrophages. DNA hypermethylation in NK and B cells is associated with increased IR. METTL14 controls B cell development and maturation. DNA methylation also regulates T cell proliferation and function, and METTL3 participates in T cell homeostasis regulation and Treg's immune suppression. (Created with BioRender.com).

4.3 Effect of nucleotide methylation on chemokines in the inflammatory microenvironment of MAFLD

Chemokines are the primary immune response-related drivers of hepatic immune cell infiltration during the immune response. Upstream regulators of chemokines include inflammatory cytokines including TNF, IL-1, IFN-γ, IL-17, and IL-6, which can promote chemokines' transcription by stimulating nuclear factors and transcription factors (109). Additionally, as KCs in the liver are the primary producer of cytokines and chemokines in MAFLD, they can regulate hepatic inflammation *via* downstream proinflammatory signaling (110).

Nucleotide methylation may be involved in liver disease by influencing the expression of chemokines. In HCC samples showing vascular invasion, the methylation of C-X-C motif chemokine ligand 12 (CXCL12) gene is detecetd to be removed with a ratio of 22.1%, which could lead to increased expression. The CXCL12-CXCR4 axis expression is also significantly higher in HCC tissues, indicating that methylation deficiency of CXCL12 may be critical in vascular invasion of HCC (111). While preserving the activation and proliferation of $CD4^+$ and $CD8^+$ T cells and preventing the growth of colorectal tumors, METTL3 knockdown in colorectal cancer cells reduces myeloid-derived suppressor cell (MDSC) infiltration. Notably, METTL3 promotes the expression of BHLHE41 in an m^6A-dependent manner and subsequently induces CXCL1 transcription to promote colorectal cancer (112). Tristetraprolin (TTP) is an immunosuppressive protein. TTP is upregulated in acute liver failure (ALF), and the mRNA stability of C-C motif chemokine ligand 2 (CCL2) and C-C motif chemokine ligand 5 (CCL5) is weakened by m^6A modification, thus alleviating acute liver injury (113).

One study has found that CXC chemokine receptor 3 (CXCR3) is significantly elevated in the liver tissue of MAFLD patients and NASH mouse models. Moreover, induction of CXCR3 is related to the production of liver pro-inflammatory cytokines, T lymphocyte infiltration, endoplasmic reticulum stress (ER stress), and fatty acid synthesis (114). It has been discovered that the chemokine CCL2 is upregulated in the liver of MAFLD mice and that CCL2 enhances MDSC migration *in vitro*. MDSCs have been shown to inhibit T-cell immunity in tumor and inflammatory diseases. The findings imply that the CCL2-CCR2 pathway may contribute to the accumulation of MDSC in MAFLD, accelerating the disease's pathogenesis and progression (115). CXCL10, a chemokine induced by IFN-γ, is associated with obesity and T2DM as well as being involved in the pathogenesis of NASH. Mice fed with methionine and choline deficiency (MCD) can develop NASH and increase the expression of CXCL10 in the liver. Consistent with the observation that inhibiting CXCL10 suppresses the development of steatohepatitis in mice, the results reveal that CXCL10 is implicated in steatosis *via* up-regulation of SREBP1c and liver X-activated receptor (LXR) (116). This finding suggests a potential function for CXCL10 in the development of NASH. A recent sutdy reported that hepatic $CXCR6^+$ CD8 T cells are abundant in NASH mice and patients, and IL-5-induced downregulation of FOXO1 and upregulation of CXCR6 trigger hepatic $CXCR6^+$ CD8 T cells to engage in self-attack, thereby harming the liver (117).

In overview, multiple immune cell populations and their secreted cytokines and chemokines are involved in the pathogenesis of MAFLD. The action of immune cells may cause

the IME of MAFLD. At the same time, nucleotide methylation can also modulate immune cells, inflammatory factors, and chemokines; presumably, the two parts should be linked together. However, most studies on nucleotide methylation modifications of immune cells have not been performed in the liver-specific IME. Therefore, the specific effects and mechanisms of nucleotide methylation modifications of immune cells on the occurrence and development of MAFLD need to be studied in depth.

5 Effect of nucleotide methylation regulated metabolism on the IME in MAFLD

The liver is a crucial organ for glucose, lipid, and amino acid metabolism, and can maintain the body's metabolic homeostasis. Metabolic disorders, brought on by a diet heavy in fat and sugar, can cause a variety of health problems, including lipotoxicity and cell damage. Constant cell death and damage set off a chain reaction of oxidative stress and KC activation that compromises the immune system and speeds up the progression of liver disease. It has been proved that nucleotide methylation is involved in various life processes, including glycolipid and amino acid metabolism. Therefore, this part will describe the effects of nucleotide methylation on the IME of MAFLD from the perspective of how it affects metabolisms.

5.1 Nucleotide methylation and regulation of lipid metabolism

The hallmark of MAFLD is the abnormal accumulation of triglycerides in liver tissue, which results from an imbalance between lipid acquisition, primarily derived from fatty acid uptake and lipid synthesis from scratch, and lipid clearance (16). Nucleotide methylation can change the balance of lipid metabolism by participating in fat formation, storage, and clearance. In addition, metabolism can affect the liver's IME and promote the progression of MAFLD and HCC metastasis. Therefore, nucleotide methylation has the potential to impact the IME and the development of MAFLD *via* metabolic regulation.

Dysregulation of methylation of DNA is one of the major epigenetic changes that lead to MAFLD. DNA methylation regulates lipid metabolism by changing promoter methylation and participating in lipid synthesis, storage, and clearance. The methylated DNA donor is SAM, which can be obtained from food. One study has found that supplementing with methylated donors can alleviate the accumulation of triglycerides induced by an HFD in the liver. These results suggest that methyl-donor supplements may improve HFD-induced MAFLD by promoting hypermethylation of FASN genes (118). PPARs are nuclear receptors that can activate transcription factors, mainly PPARα, PPARβ, and PPARγ, and play physiological regulatory roles in the homeostasis of lipid and glucose metabolism, cell differentiation and development, and inflammation (119). The differentiation of preadipocytes can be promoted by the demethylation of the PPARγ promoter (120). Another study has found that an HFD during pregnancy increases PARAα methylation levels and reduces the expression of related proteins, resulting in obesity in offspring (121).

By decreasing methylation of the promoter for carnitine palmitoyl transferase 1 (CPT1A) and inducing hepatic lipid accumulation, an HFD leads to an increase in CPT1A, facilitating the transport of fatty acids into the mitochondria for β-oxidation (122). Increased methylation of ELOVL fatty acid elongase 2 (ELOVL2) in elderly fibroblasts reduces ELOVL2 expression, leading to ER stress and mitochondrial dysfunction and potentially accelerating aging *via* disruption of lipid metabolism (123). According to a gene cohort analysis, methylation changes of MAFLD-related genes are associated with lipid homeostasis, including apolipoprotein (APO) family for lipid transport, STAR-related lipid transfer domain 4 (STARD4) for cholesterol transport, STAR-related lipid transfer domain 10 (STARD10) for HDL metabolism, Niemann-Pick C1-Like 1 (NPC1L1) for cholesterol metabolism, and Solute Carrier family 47 member 1 (SLC47A1) for glucose and bile salt transport (124). Interestingly, the overall hypomethylation of circulating cell-free DNA in the plasma of individuals with MAFLD has been discovered. Decreased methylation of genes involved in lipid metabolism, such as Acyl CoA synthase long-chain family member 4 (ACSL4) and carnitine palmitoyltransferase1C (CPT1C), has been linked to the occurrence of MAFLD (125).

M^6A can participate in the regulation of lipid metabolism through m^6A regulatory proteins. Studies have investigated that genes with high m^6A methylation levels in the HFD-induced fatty liver are mainly concentrated in pathways related to lipid metabolism (126). The m^6A reading protein YTHDF2 recognizes and degrades the methylated mRNA of cyclin A2 (CCNA2) and cyclin-dependent kinase 2 (CDK2), and reduces the expression of CCNA2 and CDK2, thereby prolonging the cell cycle of adipocytes and inhibiting adipocyte differentiation (127). It has been reported that METTL3 inhibits bone marrow-derived stromal cell (BMSC) differentiation into adipocytes by targeting the JAK1/STAT5/C/EBPβ pathway in an m^6A-YTHDF2-dependent manner (128). One study showed that the expression of lipid and cholesterol metabolism genes, including enoyl-CoA hydratase and 3-hydroxy acyl CoA dehydrogenase (EHHADH), FASN, and Sirtuin1 (SIRT1), is significantly decreased in METTL3 knockout mice, especially FASN (56). FTO promotes fatty acid synthesis and inhibits TG hydrolysis through RNA demethylation, thus promoting liver fat accumulation Analysis has shown that the decrease of m^6A caused by the overexpression of FTO is associated with increased expression of genes related to lipid metabolism, including FASN, Stearoyl-CoA Desaturase1 (SCD1) and Monoacylglycerol acyltransferase 1 (MGAT1), and the decreased expression of genes related to lipid transport, including microsomal triglyceride transfer protein (MTTP), APOB and Hepatic Lipase (LIPC) (129). HFD-fed hepatocellular specific IGF2BP2 knockout mice have been

found to exhibit reduced liver fatty acid oxidation, increased triglyceride accumulation, and moderate fatty liver disease, possibly due to significantly reduced expression levels of the CPT1A and PPARα (130). M^6A methylation regulates the circadian rhythm of lipid metabolism. Loss of Bmal1 in the liver, an important component of the mammalian clock gene regulatory network, increases m^6A methylation of PPARα mRNA and affects lipid metabolism, while METTL3 knockout can increase PPARα mRNA expression and reduce lipid accumulation *in vitro* (131).

Metabolic regulation and immune response are closely linked. Adipose tissue dysfunction involves the dysregulation of adipokine and cytokine release, thus promoting diabetes, inflammation, and atherosclerosis. Immune signals can also induce metabolic disorders resulting in hepatic steatosis (132). In the tumor microenvironment (TME), Treg function depends on the energy supplement pathway involved in lipid metabolism, and glucose uptake may promote the fatty acid synthesis of Tregs. Thus, there are close connections between the expansion of Tregs and lipid and glucose metabolism in the TME (133). Figure 4 shows the effect of nucleotide methylation on lipid metabolism in MAFLD.

5.2 Nucleotide methylation and regulation of glucose metabolism

Glycolysis is the process of converting glucose to pyruvate to produce ATP and NADP after a series of metabolic reactions. Glycolysis has three rate-limiting enzymes: hexokinase (HK), phosphofructokinase (PFKM), and pyruvate kinase (PK), and insulin and glucagon are the main hormones that regulate blood glucose (134). Metabolites produced by glycolysis can enter the pentose phosphate, tricarboxylic acid cycle, lipid synthesis, and amino acid pathways. When glycolysis is dysregulated, the relevant metabolic pathways are also problematic (135).

As it is reported, IR is the main driving factor of MAFLD progress. Indeed, the essence of IR is that the peripheral tissues are less sensitive to insulin. In the IR state, the inhibition of insulin on hormone-sensitive lipase is weakened, thus promoting the continuous hydrolysis of fat, leading to the increase of free fatty acid (FFA). When the rise of FFA exceeds the compensatory capacity of the liver, it results in lipid metabolism disorder and hepatocyte steatosis. Elevated FFA levels in MAFLD patients negatively affect glucose metabolism, promote gluconeogenesis, and aggravate IR (136). Nucleotide methylation influences glucose metabolism by participating in glycogen synthesis and degradation, regulating islet development and differentiation, insulin secretion, and glucose metabolism-related pathways in MAFLD (137). Since glucose metabolism impacts the liver IME, nucleotide methylation has the potential to alter the liver IME by regulating glucose metabolism.

Recently, a comprehensive DNA methylation analysis was conducted on islets from T2D and non-diabetes. 276 CpG islands associated with 254 gene promoters were found to show significant differences in DNA methylation in T2D islets (138). It has been determined that hypomethylation of genes involved in hepatic glycolysis and IR leads to increased levels of expression products of genes participating in hepatic glycolysis and gluconeogenesis, which contributes to the development of obesity, IR, and T2DM (139).

DNA methylation may serve a role in the pathogenesis of MAFLD by modulating glucose metabolism, as it can influence enzymes involved in glycolysis. HFD in obese rats was discovered to

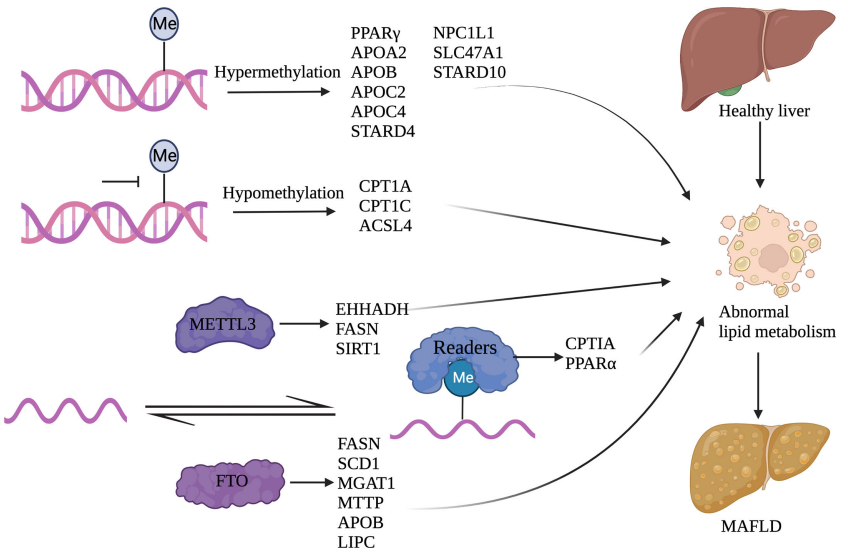

FIGURE 4
Nucleotide methylation and its regulation of lipid metabolism Abnormal lipid metabolism occurs in MAFLD. DNA methylation and RNA methylation can regulate gene expression involved in lipid metabolism. Among them, DNA hypermethylation genes include PPARγ, APOA2, APOB, APOC2, APOC4, STARD4, NPC1L1, SLC47A1, and STARD10; DNA hypomethylation genes include CPT1A, CPT1C, and ACSL4; METTL3 related genes include EHHADH, FASN, and SIRT1; FTO related genes include FASN, SCD1, MGAT1, MTTP, APOB, and LIPC; IGF2BP2 related genes include CPTIA and PPARα. (Created with BioRender.com).

cause aberrant DNA hypermethylation in the promoter regions of the liver glucokinase (GCK) and L pyruvate kinase (LPK) genes, leading to decreased production of GCK and LPK and an effect on blood glucose content (140). The Warburg effect, characterized by increased aerobic enzymes and lactate generation, is commonly seen in tumor cells. By modulating critical glycolysis enzymes, DNA methylation can directly regulate glycolysis and influence the Warburg effect (141). PK can be subdivided into PKM1 and PKM2 due to selective splicing. It has been found that PKM2 stimulates the Warburg effect by inducing DNA methylation, specifically related to the binding of DNA methylation-mediated brother of the regulator of imprinted sites (BORIS) at the PK exon. When DNA methylation is inhibited, glycolysis and the Warburg effect are both downregulated (142). One research reported that Hexokinase HK2 was upregulated in malignant glioma due to DNA methylation, and this upregulation was followed by increased aerobic glycolysis and tumor proliferation (143).

It is generally accepted that M^6A is vital for maintaining glucose homeostasis, and dynamic changes in m^6A modification alter the expression of signaling molecules and metabolism-related genes, leading to the development of metabolism-related diseases such as obesity, diabetes mellitus, and MAFLD. FTO, an m^6A demethylase, is widely expressed in a variety of tissues and is involved in the development of metabolic diseases. It has been shown that FTO-dependent m^6A modification regulates glucose metabolism by controlling the FTO-forkhead box O1 (FOXO1) axis in hepatic gluconeogenesis (144). METTL3 overexpression induced by an HFD exacerbates hepatic metabolic disorders and IR in hepatocytes. In contrast, METTL3 knockdown significantly improves HFD-induced metabolic disorders by slowing weight gain, reducing lipid accumulation, and improving insulin sensitivity (145). The expression of MELLT3 and the level of m^6A methylated RNA are increased in liver tissues of T2DM patients and mice with HFD. METTL3 knockout could reduce the expression and m^6A methylation levels of FASN, thus inhibiting the metabolism of fatty acids (56). Similarly, the m^6A RNA reading protein IGF2BP2 promotes islet β-cell proliferation and insulin secretion by enhancing pancreatic and duodenal homeobox 1 (PDX1) expression (146).

Glucose metabolism and the hepatic IME are also significantly associated. Hepatic steatosis and persistent inflammation in patients with MAFLD affect the normal glucose metabolism process and promote IR and T2DM. Correspondingly, the presence of IR will eventually act on the inflammatory pathway, resulting in MAFLD and forming a vicious cycle. In the liver TME, PKM2 expression and aerobic glycolysis of pro-inflammatory immune cells are increased, promoting the infiltration of various immunosuppressive cells. Meanwhile, PKM2 knockdown inhibits the proliferation, migration, and invasion of HCC cells. These findings support the hypothesis that PKM2 facilitates HCC progression by creating an immunosuppressive microenvironment (147). Additionally, enhanced glycolysis often results from the increased expression of glycolytic-related enzymes in mononuclear macrophages that have infiltrated HCC tumors and peritumoral tissues. Glycolysis induces the expression of programmed cell death 1 ligand 1 (PD-L1) in monocytes *via* the PFKFB3-PD-L1 axis, thereby inhibiting the cytotoxic activity of T lymphocytes against tumor cells and promoting the progression of HCC (148).

5.3 Nucleotide methylation and regulation of amino acid metabolism

In addition to producing proteins, peptides, and other molecules needed for life, amino acid metabolism also allows for their decomposition *via* deamination and transamination, as well as their conversion into sugars, lipids, and even some non-essential amino acids. Many studies have found a link between amino acid metabolism and MAFLD (149). Nucleotide methylation exerts a specific influence on amino acid metabolism, and thus participates in the occurrence and progression of MAFLD by influencing amino acid metabolism.

Amino acid metabolism plays a particular role in the occurrence and development of MAFLD. Researchers have found that genes involved in glycine biosynthesis, especially alanine glyoxalate aminotransferase 1 (AGXT1), are suppressed in the livers of both humans and animals with MAFLD, suggesting that impaired glycine metabolism may be an etiological factor in this disease. A glycine-containing tripeptide can significantly improve HFD-induced NASH mice, reducing circulating glucose and lipids, and improving steatohepatitis (150). The enhanced IR and protein catabolism seen in obese and MAFLD patients is thought to account for their high plasma amino acid concentrations, notably in branched-chain amino acids (151). It has been demonstrated that supplementation with branched-chain amino acids may have a positive impact on the improvement of glucose metabolism, reduction of fat accumulation in skeletal muscle, and prognosis in patients with cirrhosis (152). Furthermore, the serum glutamate to glutamine ratio is correlated with myofibroblast density and severity of liver fibrosis in NASH progression, suggesting that glutamate plays a critical role in amino acid metabolism in the liver (153). Another finding is that phenylalanine can promote IR by inactivating insulin receptor beta (IRβ). This has been shown in animals fed a diet rich in phenylalanine, who thereafter develop IR and type 2 diabetes symptoms (154).

6 Clinical translational study of nucleotide methylation and MAFLD

Currently, there are no effective drugs for the clinical treatment of MAFLD. Simple lifestyle intervention cannot alleviate the disease for patients progressing to NASH, cirrhosis, and HCC. Nucleotide methylation-induced epigenetic changes have a specific regulatory role in MAFLD and its development into HCC. Its potential reversibility provides new ideas for developing new biomarkers and drugs for treating MAFLD and HCC.

DNA methylation biomarkers of HCC help to improve the diagnostic accuracy of HCC and effectively predict prognosis. The

overall 5mC DNA methylation and the oxidized derivative of 5mC, 5-formylcytosine (5fC), are significantly reduced in the early stages of HCC. The genomic DNA content of 5mC and reduced 5fC levels are related to poor prognosis in HCC patients and may also be potential biomarkers predicting prognosis, whereas dynamic variations in 5mC can be utilized to differentiate the staging of HCC (155). CXCL2, an immune-related chemokine whose regulatory mechanism is related to DNA methylation, was significantly down-regulated in tumor tissues, and tumors with higher CXCL2 expression contained more multiple tumors than those with lower expression (156). P16 is a tumor suppressor gene associated with the cell cycle, and hypermethylation of the promoter region can lead to cancer development, so abnormal methylation of the p16 promoter may be a promising marker for detecting HCC (157). Keratin 19 (K19) promoter methylation is significantly associated with K19 deficiency, and K19-expressing HCC patients have a worse prognosis than K19-deficient patients. It is speculated that K19 expression is a potential predictor of poor prognosis in HCC patients (158).

The inositol 1, 4, 5-trisphosphate receptor (ITPR3) is absent or under-expressed in hepatocytes from the normal liver. However, multiple regions of the ITPR3 gene are hypomethylated in HCC samples from three separate patient cohorts, and higher ITPR3 expression is correlated with shorter survival times in HCC patients (159). Acyl-CoA dehydrogenase (ACADS) has been found to inhibit HCC carcinogenesis and metastasis. The methylation of ACADS, which is influenced by DNMTs, was shown to be upregulated in HCC, while DNMTs knockdown increased ACADS expression, implying that it could be helpful as a diagnostic or prognostic marker (160). HCC suppressor 1 (HCCS1) promoter methylation is frequently detected in the serum of HCC patients, and it is more likely to be seen in patients with advanced tumor lymph node metastasis (161). CCAAT/enhancer-binding protein-beta (C/EBPβ) enhancer hypomethylation, which activates C/EBPβ expression, is associated with poor prognosis. It is also discovered that the deletion of C/EBPβ enhancer reduces the tumorigenicity of HCC (162). Glycerol-3-phosphate dehydrogenase 1 like (GPD1L) expression is substantially increased in HCC patients with vascular invasion compared to those without; meanwhile, high GPD1L expression is linked to poor overall survival and HCC recurrence (111). All of the above mentioned have the potential to become biomarkers for HCC in connection with diagnosis, treatment or prognosis, as shown in Table 1.

Promoter hypermethylation is a biomarker of HCC, so it is also a potential therapeutic target. HFD mice can be detected DNA damage, DNA genome-wide hypomethylation, and promoter hypermethylation. Vitamin E treatment in HFD mice has been shown to reduce DNA damage and significantly reduce methylation of the same CPG in HFD mice given vitamin E compared to HFD controls (64). Recent research suggests that targeting epigenetic regulators can control the progression of HCC. 5-azacytidine (5-AZA) is a first-generation DNMT inhibitor (DNMTi) that binds to newly synthesized DNA, binds irreversibly to DNMT1, and induces DNMT1 degradation and DNA demethylation. The demethylated compound 5-AZA at non-cytotoxic doses promotes the anticancer response by inhibiting the tumorigenicity of HCC cells (163). Guadecitabine (SGI-110), a second-generation DNMTi, impedes tumor growth and inhibits angiogenesis in a xenograft HCC HepG2 model. Still, it fails to prevent liver fibrosis and inflammation in a mouse model of steatohepatitis (164). The results suggest that SGI-110 reverses most of the aberrantly transcribed genes in HCC tumors and stimulates the innate immune response, and it is hypothesized that SGI-110, in combination with other cancer

TABLE 1 Biomarkers of nucleotide methylation in HCC.

Nucleotide methylation types	DNA methylation biomarker	Function	Potential biomarker role in HCC	References
DNA methylation	CXCL2	Immunochemokine	Diagnosis and treatment of HCC	(156)
	p16	Cancer suppressor gene involved in cell cycle regulation	HCC detection marker	(157)
	ACAD	Involved in tumor proliferation and metastasis	Diagnosis and prognosis of HCC	(160)
	HCCS1	HCCS 1	Diagnosis and prognosis of HCC	(161)
	K19	Vascular invasion, poorly differentiated tumors, tumor recurrence after resection	Poor prognosis in patients with HCC	(158)
	ITPR3	Cell proliferation	Pathogenesis of HCC	(159)
	C/EBPβ	Early tumorigenesis	Poor prognosis in patients with HCC	(162)
	GPD1L	Vascular invasion	Poor outcomes in patients with HCC	(111)
RNA methylation	METTL3	Promote HCC progression through YTHDF2-dependent SOCS2 posttranscriptional silencing	Poor prognosis of patients with HCC	(49)

therapeutics, may be more effective in increasing sensitivity to immune checkpoint therapy and resensitizing drug-resistant tumor cells (165).

Decitabine (DAC), an inhibitor of DNA methylation transferase, is able to induce CXCL10 expression in tumor cells and suppress anti-tumor T-cell responses and hepatoma proliferation when administered to HCC mice (166). Doxorubicin (DOX) is a DNMT1 inhibitor that impacts telomerase activity and leads to the induction of apoptosis, thereby killing cancer cells (167). Zebularine (ZEB), a DNA methyltransferase inhibitor, can bind preferentially to DNA, reduce DNA methyltransferase levels, and alter p16 gene expression and demethylation, so displaying a more effective tumor cell growth suppression (168). The growth of tumors that overexpress the human organic cation transporter-1 (hOCT1, encoded by the SLC22A1 gene) is inhibited by sorafenib, although hOCT1 expression is low in HCC, and hOCT1 expression is negatively correlated with SLC22A1 promoter methylation. Therefore, increasing hOCT1 expression is a possible approach to improve the sensitivity of HCC to the pharmacological effects of sorafenib (169).

Since m^6A RNA methylation has a significant role in the progression of different liver diseases, targeting m^6A RNA methylation to treat liver diseases may provide potential therapeutic approaches. Several inhibitors targeting FTO, such as entacapone and meclofenamic acid (MA), have been identified. Entacapone has been shown to affect liver gluconeogenesis and adipose tissue by acting on the FTO-FOXO1 regulatory axis (144), and administration of entacapone reduces body weight and decreases fasting glucose concentrations in diet-induced obese mice. MA competes with FTO binding for m^6A-containing nucleic acids (170) and prevents the increase of total triglycerides in oleic acid/dexamethasone (OA/DEX) (58). The yellow polyphenolic compound curcumin can affect the expression of METTL3, METTL14, ALKBH5, FTO, and YTHDF2 mRNA and increase m^6A modification in the piglet liver. It could attenuate LPS-induced liver injury and hepatic lipid metabolism disorders by changing m^6A RNA methylation (171). In human HCC, METTL3 overexpression is associated with poor prognosis in HCC patients (49), so the expression of m^6A regulators may be a potential biomarker for prognosis prediction in HCC patients. METTL3 is associated with the occurrence and maintenance of acute myeloid leukemia (AML), so one study has explored whether STM2457, as a small molecule specific inhibitor of METTL3 enzyme, targeting METTL3 enzyme activity has anti-leukemia therapeutic potential. The results showed that STM2457 could reduce m^6A levels and lead to abnormal mRNA translation, prevent the progression of AML, and reduce key leukemia stem cells *in vivo*, demonstrating that the targeting of RNA modifying enzymes is a promising anticancer therapy pathway (172). Immune checkpoint therapy is currently a new hot topic in cancer therapy. By targeting programmed death 1 (PD1) in cytotoxic T cells or PD-L1 in cancer cells, immune checkpoint therapy activates the adaptive immune system to clear cancer cells. It is found that knockdown of YHTDF1 significantly improves the anti-tumor response to anti-PD-L1 therapy (90), so targeting m^6A regulatory factors may be a potential therapeutic approach to improve the immune potential therapeutic approach for checkpoint therapy. As a key m^6A reading protein, IGF2BP1 has been implicated in tumorigenesis. It has been found that IGF2BP1 knockdown can induce apoptosis of cancer cells, significantly activate immune cell infiltration, and also reduce the expression of PD-L1 in HCC. A drug called cucurbitacin B (CuB) was found to directly target IGF2BP1 and block its recognition of m^6A, resulting in the induction of apoptosis of cancer cells *in vivo*, the activation of immune cells into the TME and the reduction of PD-L1 expression in HCC, demonstrating substantial anti-HCC effects and improving the TME (173). Including the above studies, there are also related DNA methylation drugs in Table 2.

M^6A regulators are associated with tumor resistance, and patients exhibit higher tolerance to tyrosine kinase inhibitor (TKI) during TKI therapy because of reduced m^6A due to FTO overexpression in leukemia cells (174). Cells lacking METTL3 show higher sensitivity to anticancer agents such as gemcitabine, 5-fluorouracil, cisplatin, and radiotherapy (175). However, to develop assays that can identify cancer-specific m^6A modifications for early diagnosis and create new specific inhibitors that target m^6A or m^6A regulators, more research is required to investigate the role of m^6A modifications and m^6A regulators in the development of HCC and their potential as therapeutic targets.

In clinical treatment, epigenetic drugs can also be used in combination with traditional anti-liver cancer drugs, improving the efficacy and reducing the side effects of conventional anticancer drugs. Low-dose SGI-110 pretreatment with oxaliplatin can produce enhanced cytotoxicity. Compared with oxaliplatin alone, the combination of SGI-110 and oxaliplatin can significantly delay tumor growth in mice (176). Due to its severe toxicity and adverse reactions, cisplatin has a poor effect on HCC, but curcumin can improve the sensitivity of HCC to chemotherapy drugs. Studies have shown that cisplatin combined with curcumin in mouse HCC H22 and human HCC HepG2 xenograft models shows better antitumor effects and reduces side effects compared with monotherapy (177).

With the deepening of genomics and metabolomics research on the etiology of MAFLD and HCC, targeted drugs targeting the epigenetics related to MAFLD are also under continuous research. For patients who cannot alleviate the disease through lifestyle regulation, targeted drugs will be an effective way to improve or even reverse liver histology. Multigroup treatment will effectively prolong the survival time of patients with advanced MAFLD, improve the quality of life, and avoid related complications.

7 Discussion and conclusion

The accumulation of lipids in the liver can cause steatosis and even lipotoxicity. With the ER stress and mitochondrial dysfunction caused by steatosis, liver cells are damaged, and a large number of inflammatory factors are secreted, leading to damage or death of liver cells, which may further develop into NASH, liver fibrosis, and HCC (178). As an immune organ, the liver has many kinds of immune cells. At the initial stage of liver cell damage, residential DCs, KCs, and other immune cells release many proinflammatory cytokines and chemokines in response to stress. Chemokines will promote further infiltration of inflammatory cells, intensifying the inflammatory response, thus leading to cell apoptosis and inducing NASH (179). Damage-associated molecular patterns (DAMPs) are

TABLE 2 Drugs related nucleotide methylation for the treatment of HCC.

Nucleotide methylation types	Drugs	Substance of drug	Mechanisms of action	References
DNA methylation	5-AZA	Nucleotide analogues	Reduce DNA methylation by inhibiting DNA methylation transferase and re-expression of epigenetic silenced genes to promote anti-cancer effect	(163)
	SGI-110, a novel second-generation DNA methylation inhibitor	A second-generation DNA methylation inhibitor formulated as a dinucleotide of DAC and deoxyguanosine	Demethylate the promoter of tumor suppressor gene and inhibit tumor growth and angiogenesis	(164)
	5-AZA-DC: DAC	The DNA methyltransferase inhibitor	Directly incorporate DNA, cause DNA hypomethylation and activate silent TSG	(166)
	DOX	An inhibitor of DNMT1 and a hydroxyl derivative of daunorubicin	Affect telomerase activity, induce apoptosis, and kill cancer cells	(158)
	Vitamin E	Lipid-soluble vitamin	Reduce DNA damage, regulate expression of DNMT1 and reduce oxidative stress	(64)
	ZEB	A nucleoside analog of cytidine and a new DNMTi	Reduce DNA methylation by inhibiting DNA methylation transferase and prevent the occurrence of remethylating	(168)
RNA methylation	Entacapone	A chemical inhibitor of FTO	Bind directly to FTO and inhibits FTO activity *in vitro*, reducing body weight and fasting glucose concentration	(144)
	MA	A non-steroidal, anti-inflammatory drug	Compete with FTO binding for the m^6A-containing nucleic acid	(170)
	Curcumin	A natural compound with good anti-inflammatory and anticancer properties	Alleviate LPS-induced lipid metabolism disorders in liver through m^6A RNA methylation modification	(171)
	CuB	A class of tetracyclic triterpenoids isolated from Cucurbitaceae	Directly target IGF2BP1 and block its recognition of m^6A, thereby inducing apoptosis of cancer cells *in vivo*, activating immune cells into the TME and reducing PD-L1 expression in HCC	(173)

released by damaged or dead hepatocytes, continuing to induce strong inflammatory responses; these DAMPs include mitochondrial damage-related molecular patterns (mito-DAMP), in which mitochondrial DNA directly activates KCs, setting off an inflammatory cascade and driving the occurrence and development of liver fibrosis (180). An imbalance of the intrinsic immune regulatory system also has a facilitative role in the development of MAFLD, contributing to hepatic steatosis, IR, and fibrosis (181).

Epigenetic changes are closely linked to the pathogenesis of MAFLD. Epigenetic modifications are involved in lipid metabolism, IR and ER stress, mitochondrial damage, oxidative stress, and inflammation, which work in concert to induce MAFLD. Nucleotide methylation can regulate MAFLD from both aspects of immunity and metabolism. Nucleotide methylation can participate in pathways related to lipid metabolism, glucose metabolism, and amino acid metabolism; meanwhile, the metabolic environment can influence immune cells, cytokine release, and regulate signaling pathways such as the IL-2-STAT5 signaling pathway. Therefore, nucleotide methylation can regulate the IME of the liver by indirectly altering metabolism, thus participating in the development and progression of MAFLD. As MAFLD progresses, nucleotide methylation remodels the IME by regulating immune cells like macrophages, NK cells, B cells, and T cells, inflammatory factors like IL-11, IL-6, IL-α and TNF-α, and chemokines like CXCL2, CXCL3, CXCL10, as well as associated signaling pathways that are crucial to MAFLD pathogenesis.

Nucleotide methylation is an important determinant of MAFLD and is strongly associated with the progression of MAFLD, including NASH, liver fibrosis, cirrhosis, and HCC. Nucleotide methylation markers have the potential as therapeutic targets and staging biomarkers. They are expected to identify patients with early and progressive MAFLD by developing noninvasive operational screening techniques, such as blood-derived biomarkers (182). Because the nucleotide methylation or demethylation state is reversible, a shift from an HFD to a regular diet and weight loss after bariatric surgery can also partially normalize the DNA methylation profile in MAFLD. However, for patients whose disease cannot be alleviated through lifestyle modification, targeted drugs targeting nucleotide methylation may be a useful way to improve or even reverse liver histological status in addition to bariatric surgery. Improving the screening, diagnosis, and treatment of patients with NASH-related HCC can, to some extent, reduce the suffering of patients with MAFLD and delay the development of the disease, thus bringing benefits to patients with MAFLD (12, 183). Drugs targeting nucleotide methylation have opened up new ideas for MAFLD treatment. However, their role in liver disease needs to continue to be explored and these drugs' specificity and side effects

should be clarified. Therefore, more studies on these drugs are needed *in vitro* and *in vivo*. And combining drugs targeting nucleotide methylation with existing medical treatments, such as immune checkpoint blockade, chemotherapy, or radiotherapy, may lead to more effective treatments. In conclusion, a better understanding of the mechanisms of nucleotide methylation in the pathogenesis of MAFLD will lead to better diagnostic, prognostic, and therapeutic interventions, and promise great advances in personalized medicine.

Author contributions

Conceptualization, SJ and QL. writing original draft preparation, NZ. writing review and editing, SJ, QL, XT, HW, DZ, and TY. visualization: NZ and CL. supervision: SJ and QL. funding acquisition: SJ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Epigenetic control of type III interferon expression by 8-oxoguanine and its reader 8-oxoguanine DNA glycosylase1

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Interferons (IFNs) are secreted cytokines with the ability to activate expression of IFN stimulated genes that increase resistance of cells to virus infections. Activated transcription factors in conjunction with chromatin remodelers induce epigenetic changes that reprogram IFN responses. Unexpectedly, 8-oxoguanine DNA glycosylase1 (Ogg1) knockout mice show enhanced stimuli-driven IFN expression that confers increased resistance to viral and bacterial infections and allergen challenges. Here, we tested the hypothesis that the DNA repair protein OGG1 recognizes 8-oxoguanine (8-oxoGua) in promoters modulating IFN expression. We found that functional inhibition, genetic ablation, and inactivation by post-translational modification of OGG1 significantly augment IFN-λ expression in epithelial cells infected by human respiratory syncytial virus (RSV). Mechanistically, OGG1 bound to 8-oxoGua in proximity to interferon response elements, which inhibits the IRF3/IRF7 and NF-κB/RelA DNA occupancy, while promoting the suppressor NF-κB1/p50-p50 homodimer binding to the IFN-λ2/3 promoter. In a mouse model of bronchiolitis induced by RSV infection, functional ablation of OGG1 by a small molecule inhibitor (TH5487) enhances IFN-λ production, decreases immunopathology, neutrophilia, and confers antiviral protection. These findings suggest that the ROS-generated epigenetic mark 8-oxoGua via its reader OGG1 serves as a homeostatic thresholding factor in IFN-λ expression. Pharmaceutical targeting of OGG1 activity may have clinical utility in modulating antiviral response.

KEYWORDS

ROS, NF-κB, IRF, IFN-λ, small airway epithelium, innate immune response

1 Introduction

Many of the cellular effects of reactive oxygen species (ROS) are due to oxidation of the base composition of DNA. Among the DNA bases, the most susceptible base to oxidation is guanine (Gua). The generated 8-oxo-7,8-dihydroguanine (8-oxoGua) is a marker of oxidative stress intensity (1). 8-oxoGua is repaired through the evolutionally conserved base excision repair (BER) pathway (2), which is primarily initiated by 8-oxoguanine DNA glycosylase/lyase 1 (OGG1) (3). Recent genome-wide strategies have mapped 8-oxoGua accumulation preferentially within G:C-rich gene regulatory regions, like promoters and super-enhancers (4–6). These studies identified 8-oxoGua as an epigenetic-like mark rather than a mutagenic lesion in shaping gene regulation. Studies have reported that post-translationally modified OGG1 with or without enzymatic activity recognizes 8-oxoGua within and/or in close proximity to *cis* elements of transcription factor (TF) binding sites, like the NF-κB site, facilitating NF-κB binding to inflammatory gene promoters (7–9). Administration of the small molecule TH5487, which inhibits OGG1 recognition of 8-oxoGua, decreases pathophysiological consequences of infections and ameliorates the associated tissue injury (10–12).

Ogg1 knockout in mice or OGG1 depletion by siRNA in human cells lowered ROS and IL-4 levels but increased interferon (IFN) production following challenge with the potent allergen house dust mite extracts (13). Functional inactivation of OGG1 by the specific inhibitor SU0268 induces the release of type I IFNs (triggered by oxidatively damaged DNA via cGAS-STING-IRF3-IFN axis), which decreased *Pseudomonas aeruginosa* loads and halted progression of lung inflammation (14). Inhibition of OGG1 substrate binding by the active site inhibitor TH5487 led to increased expression of type I and type III IFNs, in RSV-infected lungs (15) and in primary alveolar macrophages infected by African swine fever virus (16).

Type III interferons (IFN-λ), consist of IFN-λ1 (IL-29), IFN-λ2, IFN-λ3 (IL-28A, IL-28B), and IFN-λ4 are primarily expressed in mucosal epithelial cells in response to microbial infections (e.g., viruses, bacteria). The detection of pathogen-associated molecular patterns (PAMPs) such as double stranded RNA, which is generated during the life cycle of virus replication, is sensed by the RIG-I like receptors (RLRs). This family includes retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). Activation of these receptors leads to the recruitment of the mitochondrial antiviral signaling protein, MAVS to mitochondrial associated membranes and peroxisomes. These signaling pathways lead to activation of the transcription factors NF-κB and interferon regulatory factors (IRFs) and expression of both type I and III IFN genes (17). ROS are also involved the expression of IFNs through protein kinase A-driven phosphorylation of NF-κB/RelA and via activation of the RIG-I-IFN pathway (18, Kim, 19–21). The released IFN-λ binds to the IFN-λ receptor, which consists of the IL28 receptor α chain and IL-10 receptor β chain to induce expression of IFN-stimulated genes (ISG). ISGs function to stop the spread of viruses in the host and are crucial in establishing the so called "antiviral state" in neighboring non-infected cells (22).

Here, we tested the hypothesis that the oxidatively-generated epigenetic mark 8-oxoGua and its reader OGG1 controls expression of epithelial type III IFNs in RSV-infected human small airway epithelial cells (hSAECs) and in the lungs of infected mice. We show that inhibition of OGG1 binding and genetic ablation of OGG1 leads to significant increases in mRNA and protein levels of IFN-λ. OGG1 controls IFN-λ expression through increasing DNA occupancy of the suppressor NF-κB1/p50-p50 homodimers, and thereby interfering with binding of IRFs and NF-κB/RelA on the IFN-λ gene promoter. Thus, activation of IFN-λ expression via inhibition or genetic ablation of OGG1 ultimately results in a boost in host antiviral and anti-inflammatory responses. This study advances our understanding of how OGG1 regulates host innate immune responses during pulmonary viral infections and demonstrates that modulation of OGG1 activity may be a druggable target with clinical utility.

2 Methods and materials

2.1 Cell culture and treatment

HEp-2 cell line (ATCC, CCL-23) was grown in Eagle's minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS). A549 cell line (ATCC, CCL-185) was grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) supplemented with 10% (v/v) FBS. FBS (HyClone, SH30084) was obtained from GE Healthcare Life Sciences. All media were supplemented with penicillin (100 units/mL; Gibco, Life Technologies, Inc.) and streptomycin (100 μg/mL; Gibco, Life Technologies, Inc.). Human small airway epithelial cell line (hSAEC) was cultured in small airway epithelial growth media (Promo Cell, C-21070), supplemented with supplement Mix (Promo Cell, C-39175) containing 0.004 μg/mL Bovine Pituitary Extract, 10 ng/mL Epidermal Growth Factor (recombinant human), 5 μg/mL Insulin (recombinant human), 0.5 μg/mL Hydrocortisone, 0.5 μg/mL Epinephrine, 6.7 ng/mL Triiodo-L-thyronine, 10 μg/mL Transferrin (recombinant human), 0.1 ng/mL Retinoic Acid, 2.5 mg/mL, Bovine Serum Albumin-Fatty Acid Free (BSA-FAF).

We confirmed that TH5487 (10 μM) inhibits RSV-induced cytokine production without inducing cell death at this concentration as measured by lactate dehydrogenase assay (10, 15). TH2840 (inactive analog of TH5487), or O8 (Sigma, SML1697) were added into culture media at 10 μM concentration 1 h prior and after RSV infection every 8 h. (αR)-α-[[(1,2-Dihydro-2-oxo-6-quinolinyl) sulfonyl] amino]-N-(2-furanylmethyl)-2-methoxy-N-(2-thienylmethyl)-benzeneacetamide (OSMI 1, Sigma-Aldrich, SML1621) was used at a concentration of 1 mM. OGG1 knockout was performed using CRISPR/Cas9 technology as described previously (10). Briefly, targeting sequences of OGG1 5′-GATGCGGGCGATGTTGTTGTTGG-3′ and 5′- AACAAC ATCGCCCGCATCACTGG-3′ were introduced into pSpCas9(BB)-2A-Puro expression vector. Following lipofectamine 2000 transfection (ThermoFisher Sci/Invitrogen, 11668027) into hSAECs, 3 μg/mL of puromycin (ThermoFisher, A1113802) was added. Cells were sub-cultured into 24-well plates, and clones were established. OGG1

knockout cultures were maintained in hSAECs growth medium, containing Growth Medium Supplement Mix in the presence of 2 μg/mL puromycin.

2.2 Animals

Eight-week-old female and male BALB/c mice (The Jackson Laboratory, Bar Harbor, ME, USA) housed in pathogen-free conditions in the animal research facility of the UTMB (Galveston, Texas) were used for these studies. Randomly selected groups of mice (50% ♂ and 50% ♀) were challenged intranasally (i.n.) with RSV (10^6 PFU) in 60 μL of pH-balanced saline solution (pH: 7.4) under mild anesthesia (23). RSV viral titers in the lungs were determined by plaque assay. Vehicle or TH5487 (30 mg/kg, in a 100 μL volume of solvent containing 5% DMSO, 10% Tween 80 in saline) was administered via the intraperitoneal (i.p.) route. In some experiments, mice were treated with recombinant IFN-λ2 protein (0.1 mg/kg, PeproTech, 250-33) or anti-IL-28A/IFN-lambda 2 (2.5 mg/kg, R&D Systems, MAB4635) intranasally.

Euthsaia of mice was performed by carbon dioxide (CO_2) inhalation. Bronchoalveolar lavage fluid (BALF) samples were obtained by infusing 0.7 mL of PBS into the lungs via the trachea, followed by aspiration into a syringe. BALF samples were centrifuged at 500×*g* for 5 min at 4°C, and the resulting supernatants were snap-frozen and stored at −80°C for further analysis. All experiments were performed according to the NIH Guide for Care and Use of Experimental Animals and approved by the University of Texas Medical Branch (UTMB) Animal Care and Use Committee (approval no. 0807044D).

2.3 siRNA depletion of gene expression

Triplicate cultures of hSAECs or A549 cells were transfected with siRNA targeting hOGG1 (Dharmacon, L-005147-00-0020), hMTH1 (Dharmacon, L-005218-00-0020), hNEIL1 (Dharmacon, L-008327-00-0020). The human NF-κB1/p50 siRNA (16708) and human NF-κB/RelA siRNA (AM16708) were purchased from ThermoFisher Scientific. siRNA transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (13778150, ThermoFisher Scientific) according to the manufacturer's instructions. In controls, non-targeting siRNAs were used. Depleted cells in fresh culture medium were infected with RSV (MOI = 3) for 24h.

2.4 Respiratory syncytial virus infection

The human RSV A2 strain (ATCC VR-1544) was propagated using HEp-2 cells (ATCC CCL-23) and then locally purified on discontinuous sucrose gradients as described previously (24, 25). Aliquots of sucrose purified (cytokine and lipopolysaccharide free) RSV virion suspensions were stored at −80°C. For experiments, cell monolayers (80 to 90% confluence) were infected with RSV at the pre-calculated multiplicity of infection (MOI = 3). After 1 h adsorption, the cell monolayer was washed, and culture medium containing 2% FBS was added.

2.5 Assessment of intracellular ROS levels

Amplex red assays were carried out as described previously (26, 27) with minor modification. Briefly, RSV-infected hSAECs were incubated for various lengths of time and equal numbers of cells were washed with PBS (pH 7.4), harvested and sonicated (3 × 30 sec) in a reaction buffer containing the Amplex UltraRed. Mixtures were incubated for 5 min; cell debris was removed (1 min 13,500 g) and changes in resorufin fluorescence were determined at 560 and 620 nm (excitation and emission) by using a Synergy H1 Hybrid Multi-Mode Reader (BioTek). To establish the standard curve, increasing concentrations of H_2O_2 (0 to 1000 nM) were used. Resorufin formation in cell extracts was inhibited by addition of catalase (5 U/mL, Sigma–Aldrich).

Intracellular ROS levels were also determined by using the fluorogenic probe 5- (and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA; C6827 Invitrogen, Eugene, OR) (15). In brief, hSAEC cells were mock or polyinosinic-polycytidylic acid [poly(I:C)] (Sigma-Aldrich, P1530) exposed ± phenyl-alpha-tert-butyl nitrone (100 μM, Sigma-Aldrich, B7263) and loaded with 10 μM CM-H_2DCF-DA at 37°C for 10 minutes. Cells were then washed with PBS and lysed (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40), then clarified by centrifugation. Changes in DCF fluorescence in supernatant fluids were determined by using a Synergy H1 Hybrid Multi-Mode Reader (BioTek) with excitation/emission at 485 nm/535 nm. Results are expressed as changes in fluorescence units (FU).

2.6 Enzyme-linked immunosorbent assay

Commercially available ELISA kits were used to quantify IFN-β (R&D Systems, DY8234-05), IFN-λ2/3 (R&D Systems, DY1789B-05), IFN-γ (BioLegend, 430804), Myeloperoxidase (R&D Systems, DY3667) and Neutrophil Elastase/ELA2 (R&D Systems, DY4517-05) in BALF following the manufacturer's protocol.

2.7 RNA extraction and qRT-PCR

Total RNAs were extracted using a RNeasy Mini kit (Qiagen, 74106) according to the manufacturer's instructions. Crude RNAs were DNaseI-treated and loaded onto a RNeasy column and subjected to washes with RW1 and RPE buffers. The RNA concentration was determined spectrophotometrically on an Epoch Take-3^{TM} system (Biotek, Winooski, VT) using Gen5 v2.01 software. The quality of the total RNA was confirmed via the 260/280 nm ratio, which varied from 1.9 to 2.0. 500 ng total RNA was used to generate cDNA using iScript reverse transcription supermix (Bio Rad, 1708840). qPCR was performed using specific primers, and cellular GAPDH as an internal control (sequences of primer are listed in Supplementary Table 1). Changes in mRNA levels were calculated using the 2-ΔΔCt method.

2.8 mRNA stability analyses

To determine decay of endogenous IFN-λ2/3 mRNAs, OGG1 proficient, OGG1 KO cells or hSEACs with functionally (TH5487) inactivated OGG1 were poly(I:C) treated for 30 min and monolayers were washed with PBS. Four hours later 20 μg/mL 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; D1916; Millipore-Sigma, Saint Louis, Missouri, USA) was added. Total RNAs were isolated at DRB addition (0 h), 2, 4, 8 and 18 h using a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Total crude RNAs were DNaseI-treated and loaded onto a RNeasy column and subjected to washes. RNAs eluted with the RNase-free water included in the kit. The RNA concentration was determined spectrophotometrically on an Epoch Take-3™ system (Biotek, Winooski, VT) using Gen5 v2.01 software. The quality of the total RNA was confirmed via the 260/280 nm ratio, which varied from 1.9 – 2.0. 0.5 μg RNA was used to generate cDNA with oligo-dT (Takara, RR037A). The quantities IFNL2/3 mRNAs at each time points were determined by qPCR by normalizing to 18S rRNA. The relative amount of *IFN-λ2/3* mRNA at time 0 h of DRB addition was set at 100% in each cell type (OGG1 proficient, OGG1 KO cells or hSEACs with functionally (TH5487) inactivated OGG1).

2.9 Western blot analysis

Cells were lysed in RIPA buffer (Pierce, 89900 ThermoFisher Scientific) containing Protease and Phosphatase Inhibitor Cocktail (78442, Thermo Fisher Scientific), and centrifuged at 15,000 g for 15 min at 4°C. Equal amounts of Proteins were separated by SDS-PAGE electrophoresis, transferred into nitrocellulose membranes, blocked with 5% non-fat dry milk in TBST (20 mM Tris-base, 500 mM NaCl, and 0.05% Tween-20, pH 7.5), incubated overnight at 4° C with primary antibody and subsequently with horseradish peroxidase-conjugated secondary antibody (1:4000 dilution; SouthernBiotech, Birmingham, AL, USA). Blots were visualized using the enhanced chemiluminescence (ECL) detection system (Bio-Rad, 1705061). To identify proteins in DNA-protein complexes, EMSA gels were transferred to PVDF membrane and performed immunoblotting. The following antibodies were used: OGG1 (Abcam, 124741), phospho-IRF3 (D601M) (Cell Signaling, 29047), phospho-IRF7 (Cell Signaling, 5184), NF-κB/p65 (D14E12) XP (Cell Signaling, 8242), NF-κB1 p105/p50 antibody (AF2697 Novus Biochem), β-Tubulin (Santa Cruz, SC-9104) or β-actin (1:500 dilution; sc-1615, Santa Cruz Biotechnology) was used as internal control. Chemiluminescent signals were visualized and quantified by using the Amersham Imager 680 (Global Life Sci. Sol. Marlborough, MA).

2.10 Identification of O-GlcNAcylated OGG1

The method for determination of OGG1-specific O-GlcNAcylation was described previously (28) with modifications. Briefly, 500 μg of total protein samples from FLAG-OGG1 expressing hSAECs were subjected to immunoprecipitation (IP) using FLAG antibody or β-actin (1:500 dilution; sc-1615, Santa Cruz Biotechnology) was used as internal control at 4°C overnight. 30 μL of Protein A/G Magnetic beads (Millipore, MAGNA0017) blocked with normal rabbit IgG (Santa Cruz Biotechnology, SC-2027) were added on next day and incubated for 3 h. Immunoprecipitants were washed extensively and analyzed by Western blotting using anti-O-GlcNAc (RL2) (Thermo Fisher Scientific, MAI-072) and anti-FLAG antibodies. Images were acquired with the Amersham Blot and Gel Imager 680 (Global Life Sci. Sol. Marlborough, MA).

2.11 Assessment of protein cysteine oxidation

To determine oxidation of OGG1 at cysteine residues cysteine sulfenic acid probe: DCP-Bio1 reagent (NS1226, Millipore Sigma) was used as we described previously (29). Briefly, hSAECs expressing FLAG-tagged OGG1 were RSV-infected (MOI = 3) for increasing lengths of time and lysed in 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, and 20 μg/ml aprotinin/leupeptin/PMSF buffer (pH: 5.5) and 100 μM DCP-Bio1 supplemented with in 200 units/ml Catalase, 100 μM Diethylene triamine penta-acetic acid, and 5 mM iodoacetamide (30, 31). To capture biotin-linked proteins (OGG1)-Cys-OH, lysates were incubated overnight with 20 μL of Streptavidin beads (Thermo Scientific, 11205D). The IPs were washed with lysis buffer and resolved by SDS–PAGE and then subjected to immunoblotting. OGG1 reactions with DCP-Bio1 were detected by enhanced chemiluminescence.

2.12 Chromatin immunoprecipitation assay

ChIP experiments were performed using the ChIP-IT Express Kit (53008, Active Motif, CA, USA). Briefly, 1×10^7 cells were cross-linked in 1% formaldehyde for 10 min followed by mixing with 1×Glycine for 5 min. After washing with chilled PBS, the cells were re-suspended in 300 μL of 1 x lysis buffer containing iron chelator desferioxamine (DFO, Millipore Sigma, 252750). DNA was sheared by sonication and chromatin was incubated with specific antibodies and isotype control IgG overnight at 4°C and collected using Protein A/G Magnetic beads (Millipore, MAGNA0017) for 3 h. ChIP quality antibodies were phospho-IRF3 (D601M, Cell Signaling, 29047), phospho-IRF7 (Cell Signaling, 5184), NF-κB/p65 (D14E12) XP (Cell Signaling 8242S), NF-κB1 p105/p50 antibody (Cell Signaling 3035). Magnetic beads were washed and the immunoprecipitated DNA was eluted in elution buffer (1% SDS and 100 mM $NaHCO_3$) at 65°C for 2 h. The precipitated DNA was phenol/chloroform-extracted, precipitated with 100% ethanol, and dried. qRT-PCR reactions were performed in triplicate using SYBR Green PCR Master Mix (Bio-Rad, 1725120) in a CFX 96 real-time PCR detection system (Bio Rad). Primer sequences are listed in Supplementary Table 2. ChIP-qPCR calculations were performed as

described previously (32). In brief, protein specific Ab ChIP-ed DNA signal strength value was divided by the intensity value of the IgG-ChIP-ed signal, representing the fold enrichment of the protein on the specific region of genomic DNA.

2.13 Electrophoretic mobility shift assay

Whole cell lysates (WCL) or nuclear extracts (NE) were prepared using buffer (Cell Signaling, 9803) and protein concentrations were quantified by a Pierce BCA Protein Assay Kit (Thermo Scientific, 23225). For each reaction, 450 fmol 5'-Cy5-labeled probe was incubated with WCL (or NE) in a total volume of 15 µL containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, 2 mM DTT, 5% glycerol, 0.5% NP-40, 10 µg/mL of BSA, 62.5 µg/mL of poly(I:C) for 20 min at room temperature. For recombinant His-IRF3, 1 pmol 5'-Cy5-labeled probe was incubated with increasing concentrations (0, 5, 10 pmol) of His-IRF3 in a total volume of 10 µL containing 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 1% glycerol, 5 mM $MgCl_2$, 0.05% NP-40, 50 ng/µL poly(I:C) for 30 min at room temperature. For competition assays, increasing concentrations (0, 2, 4, 8 pmol) of OGG1 (ProSpec, ENZ-253) were incubated with probe for 10 min, then WCL or His-IRF3 was added for 20 min at room temperature. Protein-DNA complexes were resolved on a 6% DNA retardation gel (Invitrogen, EC6365BOX) in 0.5 × TBE buffer (100 V for 2h) and visualized using the Amersham Imager 680 (Global Life Sci. Sol. Marlborough, MA). Oligonucleotide sequences from the *IFN-λ2/3* promoter containing consensus binding sites of IRF3 are shown in Table 1. Band intensities were quantified using the Image J v1.51 (U. S. NIH, Bethesda, Maryland, USA). In selected experiments DNA-protein complexes were transferred to PVDF membranes and performed immunoblotting using specific antibodies to RelA/p65, NF-κB1/p50, IRF and OGG1 (for antibody specifications, see 2.9. Western blot analysis). Membrane processing was performed as above and Chemiluminescent signals were visualized and quantified using the Amersham Imager 680 (Global Life Sci. Sol. Marlborough, MA).

2.14 Gelatin zymography

BALF from five mice were pooled, treated with protease inhibitors, and homogenized. Protein concentrations were quantified by Pierce BCA Protein Assay Kit. 2 µg of proteins were mixed with non-reducing sample buffer (final concentrations: 0.8% SDS, 5% glycerol, 0.002% bromophenol blue, 25 mM Tris-HCl, pH 6.8) applied directly to a 7.5% acrylamide gel containing gelatin. The gel was run at 150 V until good band separation was achieved. Then the gel was incubated twice with washing buffer (2.5% Triton X-100, 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) for 30 min at room temperature to remove SDS from the gel, and rinsed for 5–10 min in incubation buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 1 µM $ZnCl_2$) at 37°C. Incubation buffer was replaced, and the gel was incubated for another 24 h at 37°C. Subsequently, the gel was stained with staining solution (0.5% Coomassie Brilliant Blue R250 in 40% methanol and 10% acetic acid) for 30 min to 1 h and was then incubated with de-staining solution (40% methanol and 10% acetic acid) until bands were clearly resolved. The activities of MMPs were detected as clear bands against a blue background.

2.15 Statistical analysis

Bars represent means ± SD. Results were analyzed for significant differences using unpaired, two-tailed Student's t-tests. Differences were considered significant at $p<0.05$.

3 Results

3.1 Loss of OGG1 function increases *IFN-λ2/3* gene expression

To examine the role of OGG1, hSAECs were RSV-infected (MOI = 3) and IFNs expression was determined as a function of time. Results show a ~6-fold increase in *IFN-λ* mRNA levels

TABLE 1 Sequences of duplex oligonucleotides from IFN-λ promoter used for electromobility gel shift assay.

Pr0: 5'-CTGTGT**TTTCACTTTTC**CTACATCAGCT***GGGACTGCCC***TTCTGTCAGGGATAA-3' Cy5-3'-GACACAAAAGTGAAAAGGATGTAGTCGACCCTGAC**GGG**AAGACAGTCCCTATT-5'
Pr1: 5'-CTGTGT**TTTCACTTTTC**CTACATCAGCT/**G*/*GGACTGCCC***TTCTGTCAGGGATAA-3' Cy5-3'-GACACAAAAGTGAAAAGGAGTGTAGTCGACCCTGAC**GGG**AAGACAGTCCCTATT-5'
Pr1a: 5'-CTGTGT**TTTCACTTTTC**CTACATCAGCT***GGGACTGCCC***TTCTGTCAGGGATAA-3' Cy5-3'-GTCACAAAA/G*/TGAAAAGGATGAGTCGACCCTGAC**GGG**AAGTCAGTCCCTATT-5'
Pr2: 5'-CTGTGT**TTTCACTTTTC**CTACATCAGCT***GGGACTGCCC***TTCTGTCAG/**G***/GATAA-3' Cy5-3'-GACACAAAAGTGAAAAGGATGTGTCGACCCTGAC**GGG**AAGACAGTCCCTATT-5'
Pr3: 5'-CTGTGT**TTTCACTTTTC**CTACATCAGCT***TGGACTGCCC***TTCTGTCA**TGG**ATAA-3' Cy5-3'-GACACAAAAGTGAAAAGGATGTAGTCGACCCTGAC/**G*/GG**AAGACAGTCCCTATT-5'
Pr4: 5'-CTGTGT**TTTCACTTTTC**CTACATCAGCT***TGGACTGCCC***TTCTGTCA**T**GGATAA-3' Cy5-3'-GACACAAAAGTGAAAAGGATGTAGTCGACCCTGAC**TGG**AAGACAGTCACTATT-3'

The consensus binding site of IRF3 is underlined. NF-κB/p50-p65 binding site is italic and underlined. The 8-oxoGua modification is represented as G* in red. In selected Gua runs (bold), G was mutagenized using thymine (T).

between 2 and 3 hours post infection (hpi), which then reached over 100-fold by 24 hpi (Figure 1A). There was a transient increase in *IFN-α* mRNA levels (between 2 and 6h), while expression of *IFN-β* significantly increased from 12 hpi, reaching a ~30-fold elevation by 24 hpi. Low level expression was observed for *IFN-γ* (Figure 1A). Although extent of expression is different, similar results were obtained after RSV infection of hSAEC and A549 cells using higher MOI (MOI = 5, Supplementary Figures 1A, B).

Upon loss of OGG1 by CRISPR/Cas9-mediated knockout and siRNA, the mRNA level of *IFN-λ2/3* significantly increased (nearly 500-fold) compared to mock-infected cells to 3-times the level seen in OGG1 proficient hSAECs (Figure 1B). Compared to OGG1 proficient, in OGG1 knockdown cells RSV infection increased *IFN-α*, *IFN-β* and *IFN-γ* mRNA levels, but the extent is negligible compared to *IFN-λ* (Figures 1B, C). Minor enhancement was observed when knocking down Nei-like DNA glycosylase1 (NEIL1) or 8-oxo-2'-deoxyguanosine triphosphatase a MutT homolog 1 (MTH1) compared to non-targeting siRNA transfected controls (Figure 1C and Supplementary Figure 2A). Inhibition of OGG1 by the small molecule TH5487 (inhibits OGG1 binding to its DNA substrate) increased *IFN-λ2/3* expression after RSV infection (Figure 1D and Supplementary Figure 2B). Another nontoxic, selective OGG1 inhibitor O8 (33), which inhibits OGG1 lyase activity but not OGG1 substrate binding, had no effect on IFNs gene expression (Figure 1D). Similar data were obtained using hSAECs (Supplementary Figure 2A), and A549 cells (Supplementary Figure 2B) after infecting cells with RSV (MOI = 3). In controls, the inactive analogs of TH5487, TH2840 or O8, did not alter gene expression levels (Figure 1D). These data are in line with substrate binding and excision activity of OGG1 ± inhibitors (Supplementary Figures 3A, B).

To test if the modulatory effect of OGG1 on IFNs expression is limited only to virus infected cells, hSAECs were treated with poly(I:C), a synthetic dsRNA and a potent IFN inducer (34). Compared with OGG1 proficient hSAECs, poly(I:C) increased *IFN-λ2/3* mRNA levels from ~500 to ~3400 fold in OGG1 KO hSAECs (Figure 1E). Inhibition of OGG1 by TH5487, increased *IFN-λ2/3* mRNA levels from 300 ± 97 to 1700 ± 77-fold (Figure 1F). In OGG1 KO and TH5487-treated cells, poly(I:C) also induced expression of *IFN-β* to a lesser level compared to *IFN-λ2/3* (Figures 1E, F). These data can be explained by poly(I:C)-induced activation of shared signaling pathways and similarity of the promoter sequences between *IFN-λ2/3* and *IFN-β* (35). Introduction of poly(I:C) into cells increased cellular ROS, which were significantly lowered by the antioxidant phenyl-alpha-tert-butyl nitrone (PBN) (Supplementary Figure 4A), also shown previously (36). Poly(I:C) transiently increased 8-oxoGua levels and OGG1 binding as shown by ChIP assays (Supplementary Figure 4B). PBN significantly increased IFN-λ and IFN-β expression in poly(I:C)-treated cells by preventing generation of 8-oxoGua in the promoter region (Supplementary Figure 4C). Moreover, increased IFN-λ mRNA levels were due to the promoter-driven transcriptional regulation, as there was no difference in mRNA half-life among OGG1 proficient and OGG1 KO cells or TH5487-treated hSAECs (Supplementary Figure 2C). Collectively, these data suggest that OGG1 by binding to 8-oxoGua in promoter modulates *IFN-λ2/3* expression not only in RSV-infected, but also in poly(I:C) exposed hSAECs.

3.2 8-oxoGua enrichment on promoter, but not OGG1 correlates with *IFN-λ2/3* expression

To gain insight into the mechanism by which OGG1 regulates *IFN-λ2/3* repression, we first assessed whether 8-oxoGua, a specific substrate recognized by OGG1, is generated in sequences proximal to the transcription start site (TSS). To address this question, we used chromatin immunoprecipitation (ChIP) assays. As shown in Figure 2A, antibody (Ab) to 8-oxoGua extensively ChIP-ed to the TSS adjacent sequences of the *IFN-λ2/3* gene. 8-oxoGua was also increased in the *IFN-β* promoters (Figure 2A), at all-time points after RSV infection. As expected, OGG1 enrichment correlated well with the presence of 8-oxoGua in the promoter until 12 hpi, and then decreased on both *IFN-λ2/3* and *IFN-β* promoters at 24 hpi (Figure 2B). These data are consistent with increased ROS levels in RSV-infected cells (Figure 2C). Binding was specific as OGG1 enrichment can be inhibited by TH5487 (Figure 2D). Our data is consistent with previous work where OGG1 is enriched in the proximal promoter regions of *TNF*, *CXCL1* and *IL10* (15).

IRF3 enrichment on the *IFN-λ2/3* promoter was observed after 2 hpi (>3-fold), which continuously increased to 9- and 11-fold by 24 h (Figure 2E). Kinetic changes in IRF7 enrichment were similar to IRF3, but its extent is slightly lower (Figure 2E, right panel). Both IRF3 and IRF7 abundance on the *IFN-λ2/3* promoter was significantly increased by inhibition of OGG1 (Figure 2F). TH5487 has no effect either in IRF3 and IRF7 phosphorylation nor nuclear translocation (Figure 2G). To confirm these observations 8-oxoGua was placed in oligos at the 5' end of Gua runs in proximity of interferon response elements (IREs) (11 bases downstream of IREs, Table 1). The 3' end of oligomers were labelled with Cyanine-5 (Cy5) and EMSAs were performed. Recombinant OGG1 binds only to 8-oxoGua containing DNA in a concentration-dependent manner (Figure 2H). Using nuclear extracts (NE) from RSV-infected cells, EMSA analyses confirmed that 8-oxoGua in proximity to IRE (Pr1) or within IREs (Pr1a) did not alter IRF3 binding, and it was similar to that of probe Pr0 (without 8-oxoGua substitution) (Figure 2I). These results show that 8-oxoGua does not directly block IRF3 from binding to the *IFN-λ2/3* promoter, rather it is the occupation of OGG1 on the 8-oxoGua containing DNA motifs that blocks IRF3 binding to the *IFN-λ2/3* promoter.

3.3 Increased *IFN-λ2/3* expression after inhibition of OGG1 by host-directed O-linked N-glycation

To obtain insight into the mechanism by which OGG1 inhibition facilitates *IFN-λ2/3* expression, we transgenically expressed FLAG-tagged OGG1 in OGG1-knockout hSAECs, then infected these cells with RSV. The OGG1 level was not affected by RSV infection, but from 2 h onward, OGG1 reacted with DCP-Biol1, indicating oxidation of its cysteine residues ($OGG1^{S\text{-}OH}$) (Figure 3A). $OGG1^{S\text{-}OH}$ binds 8-oxoGua containing oligo with no base excision (Figure 3C), as shown previously (7, 9).

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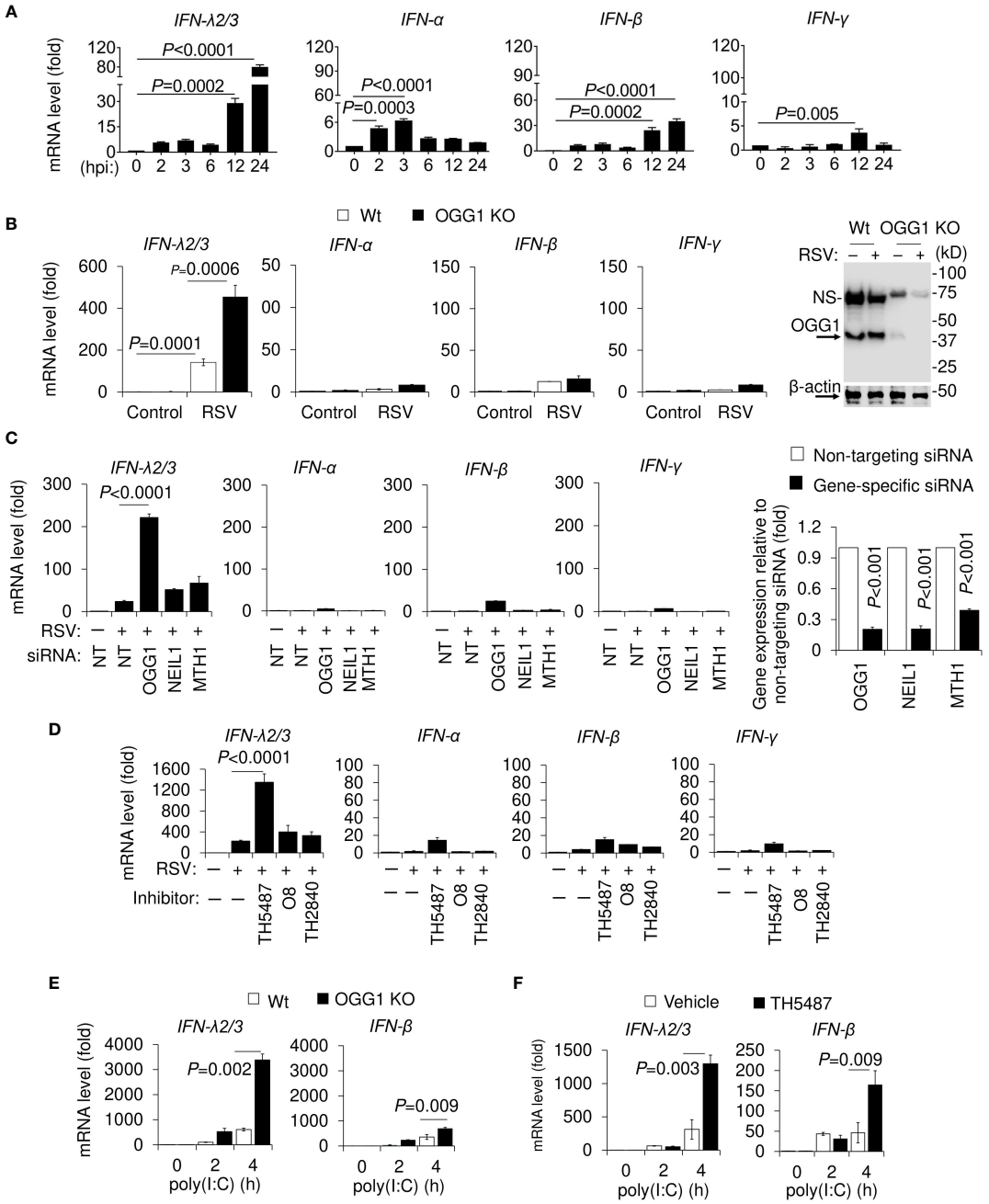

FIGURE 1
Time course analysis of RSV-induced expression of IFNs in cultured cells and effect of OGG1 knockdown. **(A)** Kinetic changes in mRNA levels of *IFN-λ2/3*, *IFN-α*, *IFN-β* and *IFN-γ* in hSAECs after RSV infection. **(B)** Expression levels of mRNA for *IFN-λ2/3*, *IFN-α*, *IFN-β*, and *IFN-γ* in Wt and OGG1 KO hSAECs, following RSV infection for 24h using an MOI of 3. Most right panel, OGG1 level in KO hSAECs. **(C)** OGG1, but not NEIL1 or MTH1 depletion by targeting siRNA affect *IFN-λ2/3* levels. Far right panel in C shows the mRNA levels of OGG1, NEIL1 and MTH1 after siRNA targeted depletion. **(D)** mRNA levels of IFNs after RSV infection in OGG1 inhibitor-treated hSAECs. **(E, F)** Effect of genetic ablation **(E)** and pharmacological OGG1 inhibition **(F)** on mRNA levels of *IFN-λ2/3* and *IFN-β* after poly(I:C) (100 μg/mL) treatment of cells. Bars represent means ± SD. Statistical analysis, Student's t-tests (unpaired). NEIL1, Nei Like DNA Glycosylase 1; MTH1, MutY homolog1 or 7,8-dihydro-8-oxoguanine triphosphatase.

In a recent study, we documented activation of the hexosamine biosynthetic pathway and increased activity of O-GlcNAc transferase upon RSV infection (37, 38). Therefore, an antibody (Ab-clone RL2) was utilized against O-linked N-acetylglucosamine that bound strongly to OGG1 from 12 h onwards (Figure 3B) and is strong indication for OGG1 inactivation by O-GlcNAcylation (28). Importantly, the inactivation timeline of OGG1 paralleled with the increase in *IFN-λ2/3* mRNA levels, which was shown in Figure 1A. The cell-permeable compound, OSMI-1, a specific inhibitor of O-GlcNAc transferase(s), prevents OGG1 O-GlcNAcylation (Figure 3D) and decreases *IFN-λ2/3* mRNA levels in RSV-infected cells (Figure 3E).

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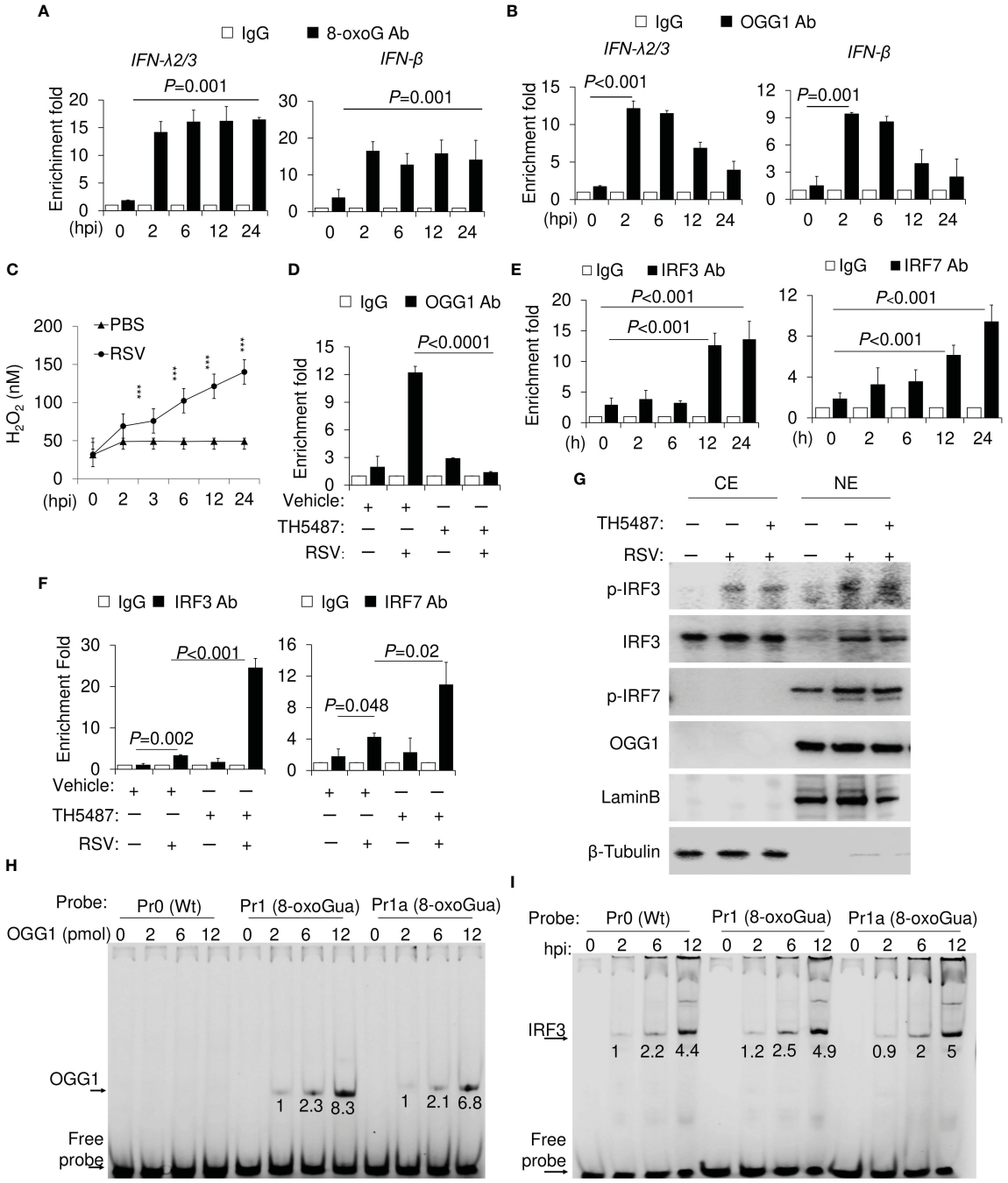

FIGURE 2
OGG1 and 8-oxoGua enrichment and binding of IRFs to promoters in RSV-infected cells. **(A)** Time course analysis of RSV-induced increase in 8-oxoGua levels in the proximal promoter of *IFN-λ2/3* and *IFN-β*. **(B)** Time course analysis of OGG1 enrichment in *IFN-λ2/3* and *IFN-β* promoter after RSV infection (MOI = 3). **(C)** RSV (MOI = 3) induced ROS levels as assessed by AplexRed assay. ***$p < 0.001$ **(D)** Effect of the OGG1 inhibitor TH5487 on the OGG1 enrichment on the *IFN-λ* promoter 6 h after RSV infection (MOI = 3). **(E)** Kinetics changes in IRF3 and IRF7 enrichment on IFN-λ2/3 promoter. **(F)** Enrichment of IRF3 and IRF7 on IFN-λ promoter 24 h after RSV infection ± TH5487. **(G)** Protein levels with indicated antibodies shown by western blotting (24 h after RSV infection). **(H)** DNA occupancy of OGG1 on wildtype and 8-oxoGua containing DNA. **(I)** Effect of 8-oxoGua on DNA occupancy of the IRF3 on the IFN-λ promoter. **(A-F)** cross-linked protein-DNA complexes were isolated, and ChIP assays were performed by using Ab to OGG1, 8-oxoGua, IRF3 and IRF7. Fold enrichments were normalized to IgG (Materials and Methods). Bars represent means ± SD. Statistical analysis in **(A-F)** One-way ANOVA (Signal-factor) with Tukey's multiple comparisons. CE, cytoplasmic extracts. NE, nuclear extracts.

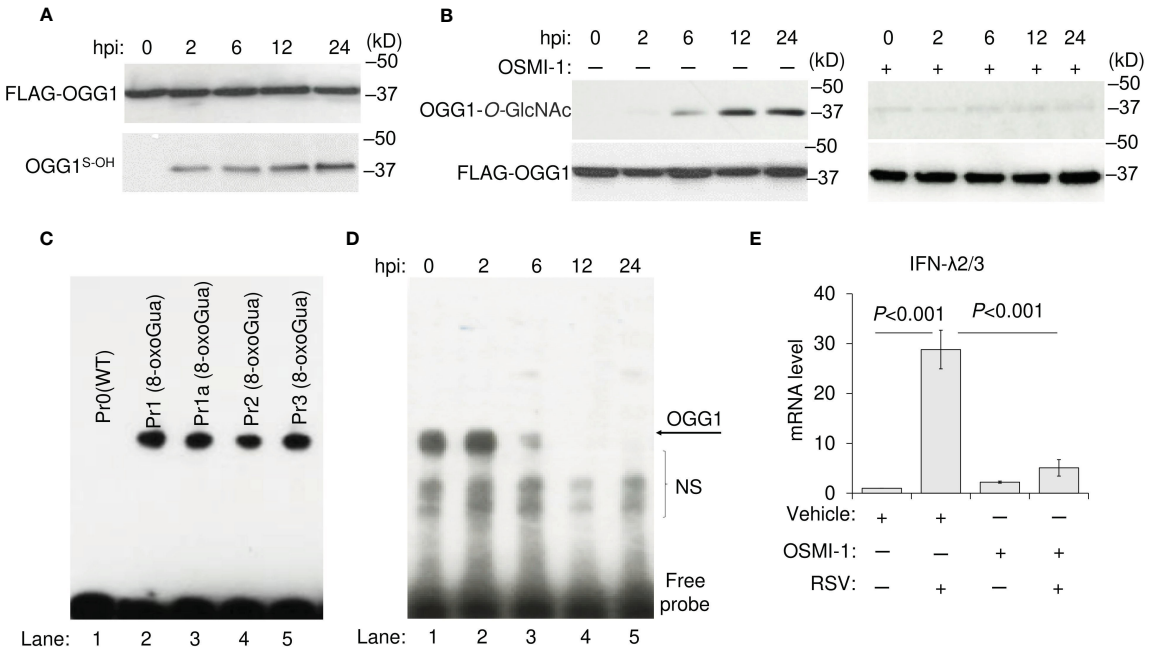

FIGURE 3
Effect of post-translational modifications of OGG1 on DNA binding and IFN-λ expression in RSV-infected hSAECs. **(A)** Oxidation of OGG1 at cysteine residues in RSV-infected cells as shown by DCP-Biol-1 assays. **(B)** Time course analysis of O-linked N-glycation of OGG1 and its inhibition by the OSMI-1 after RSV infection. **(C)** OGG1 binding in NE from RSV-infected hSAECs to 8-oxoGua-containing (lane 2-5), but not to Wt probes (lane 1). **(D)** OGG1 binding to Pr2 (8-oxoGua) in NE of RSV-infected hSAECs as a function of time. **(E)** IFN-λ2/3 mRNA expression levels in RSV (MOI = 3) infected ± OSMI-1 treated hSAECs as determined by qRT-PCR. In **(A, B)**, FLAG-tagged OGG1 expressing hSAECs cells were RSV infected (MOI = 3) and harvested as indicated, to determine levels of $OGG1^{S\text{-}OH}$ and O-linked N-Glycated OGG1 as in Materials and Methods. $OGG1^{S\text{-}OH}$: oxidized OGG1 at cysteine residues; NE, nuclear extract; OSMI-1, O-GlcNAc transferase inhibitor. Bars represent means ± SD. Statistical analysis, Student's t-tests (unpaired).

3.4 OGG1 does not directly control IRF binding to IRE

ChIP analysis strongly suggests an inverse correlation between OGG1 enrichment and DNA occupancy of IRF3 and 7 on the *IFN-λ2/3* promoter. Next, we examined whether OGG1 directly interferes with IRFs binding to IREs. EMSAs were performed using a probe containing IREs without 8-oxoGua (Pr0), and two probes containing 8-oxoGua, the first (Pr1) of which 8-oxoGua is 11 bases upstream from the IRFs, and the second (Pr2) 8-oxoGua is 29 bases upstream from the IREs. The oligomers used in these experiments were labelled at the 3'-end with Cyanine-5 (Cy5) (Table 1).

IRF3 bound efficiently along with NF-κB/p50-p65 to Pr0, and rOGG1 addition had no effect, when using NEs isolated from RSV-infected OGG1 KO hSAECs (Figures 4A, D, lane 1). With IRF3 bound to Pr1 containing 8-oxoGua, however, addition of OGG1 resulted in additional distinct complexes containing IRF-OGG1 (Figure 4B). Addition of OGG1 into NE, Pr2 resulted in three distinct bands (Figure 4C, D, lane 6). OGG1 decreases the IRF-DNA complex in an OGG1 concentration-dependent manner (Figure 4B, C). The higher shift indicates the molecular size equals to IRF3-OGG1-DNA complex. A 3rd complex was observed using Pr2, namely NF-κB1/p50-p50-DNA complex, accompanied by a drastic diminution in the DNA shifted by NF-κB/p50-p65 (Figure 4D, lane 6). Proteins in EMSA bands were identified as OGG1, IRF3, p50 and RelA/p65 by immunoblotting (Figure 4E). These data are in line with the presence of binding sites for IRF, NF-κB and OGG1 in the DNA probe.

In addition to immunoblot analysis, recombinant IRF3, OGG1 and NF-κB1/p50-p50 were used to identify binding characteristics. Addition of IRF3 without OGG1 to Pr0, Pr1 and Pr2 resulted in defined shifts (Figure 4F). OGG1 binds only to 8-oxoGua containing probes (Figure 4F, lane 8). When OGG1 was added along with IRF3, they formed a higher molecular size complex (Figure 4F, lane 6-7 and lane 10 to 11), implying that OGG1 and IRF3 bound to the same DNA. In support of these observations, the OGG1 inhibitor prevented OGG1 binding to DNA and only an IRF3-DNA complex could be seen with highest concentration of TH5487 (Figure 4G, densitometry values are under the corresponding bands). Together, these results suggest that OGG1 bound to 8-oxoGua indirectly inhibits expression from *IFN-λ2/3* and additionally, raising the existence of a suppressive protein, namely the NF-κB1/p50-p50 homodimer.

3.5 OGG1 8-oxoGua complex facilitates binding of NF-κB1 suppressor to Guanine islets in the *IFN-λ2/3* promoter

NF-κB1/p50-p50 extensively binds to 8-oxoGua containing oligos, in parallel with a decreased DNA occupancy of IRF3 and NF-κB/p50-p65 in the presence of OGG1 (Figures 4B-D). These results imply that OGG1 engaged with 8-oxoGua can allosterically

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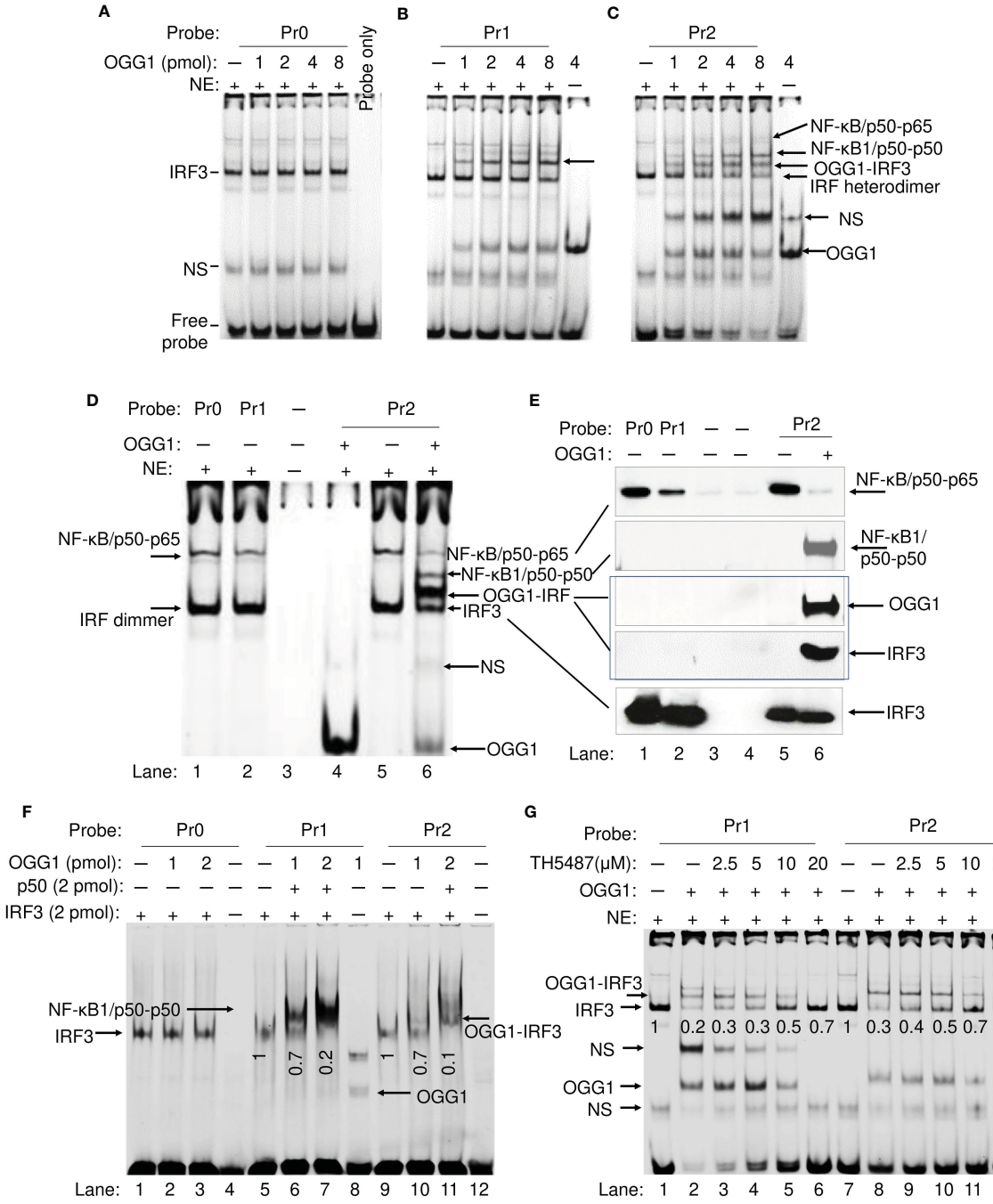

FIGURE 4
OGG1 indirectly inhibits IRF3 binding to the IRE of the IFN-λ2/3 promoter. **(A)** EMSA shows IRF3 DNA binding from nuclear extract (NE) to wildtype probe (Pr0) ± OGG1. **(B)** EMSA shows IRF3 binding to 8-oxoGua containing Pr1 probe ± OGG1. **(C)** EMSA shows DNA occupancy of IRF3, NFκB1/p50-p50 and NFκB/p50-p65 on 8-oxoGua containing Pr2 probe ± OGG1. **(D)** DNA-protein complexes in NE associated with Pr0, Pr1, and Pr2 ± rOGG1. **(E)** Immunoblot identification of proteins associated with the DNA probe (Pr0, Pr1 and Pr2). **(F)** Reconstruction experiments using OGG1, p50 (2 pmol), and IRF3 (2 pmol), as well as probes without and with 8-oxoGua located 11 and 29 base down-stream from IREs. **(G)** EMSA shows OGG1-IRF-DNA complex is inhibited by TH5487 in a concentration-dependent manner. Sequence of Pr0, Pr1 and Pr2 derived from the *IFN-λ* promoter (Table 1).

change the adjacent DNA helix, which was documented previously (39, 40). To test this possibility, ChIP assays using Ab to p50 were performed using RSV-infected OGG1 expressing and OGG1 KO cells. Results indicate significant levels of NF-κB1/p50-p50 enrichment on the *IFN-λ2/3* promoter from 2 hpi, which further increased by 6 hpi and then decreased significantly from 6, 12 hpi to near background level by 24 hpi in OGG1 proficient cells (Figure 5A). This decrease in NF-κB1/p50-p50 promoter enrichment parallels with the inability of increased O-GlcNAcylated OGG1 to bind the intrahelical DNA substrate (Figure 3D). In support of this observation, both in RSV-infected OGG1 KO hSAECs, as well as OGG1 proficient cells treated with TH5487, NF-κB1/p50-p50 enrichment was poor (Figures 5A, B). To test whether NF-κB1/p50-p50 is indeed suppressive on *IFN-λ2/*

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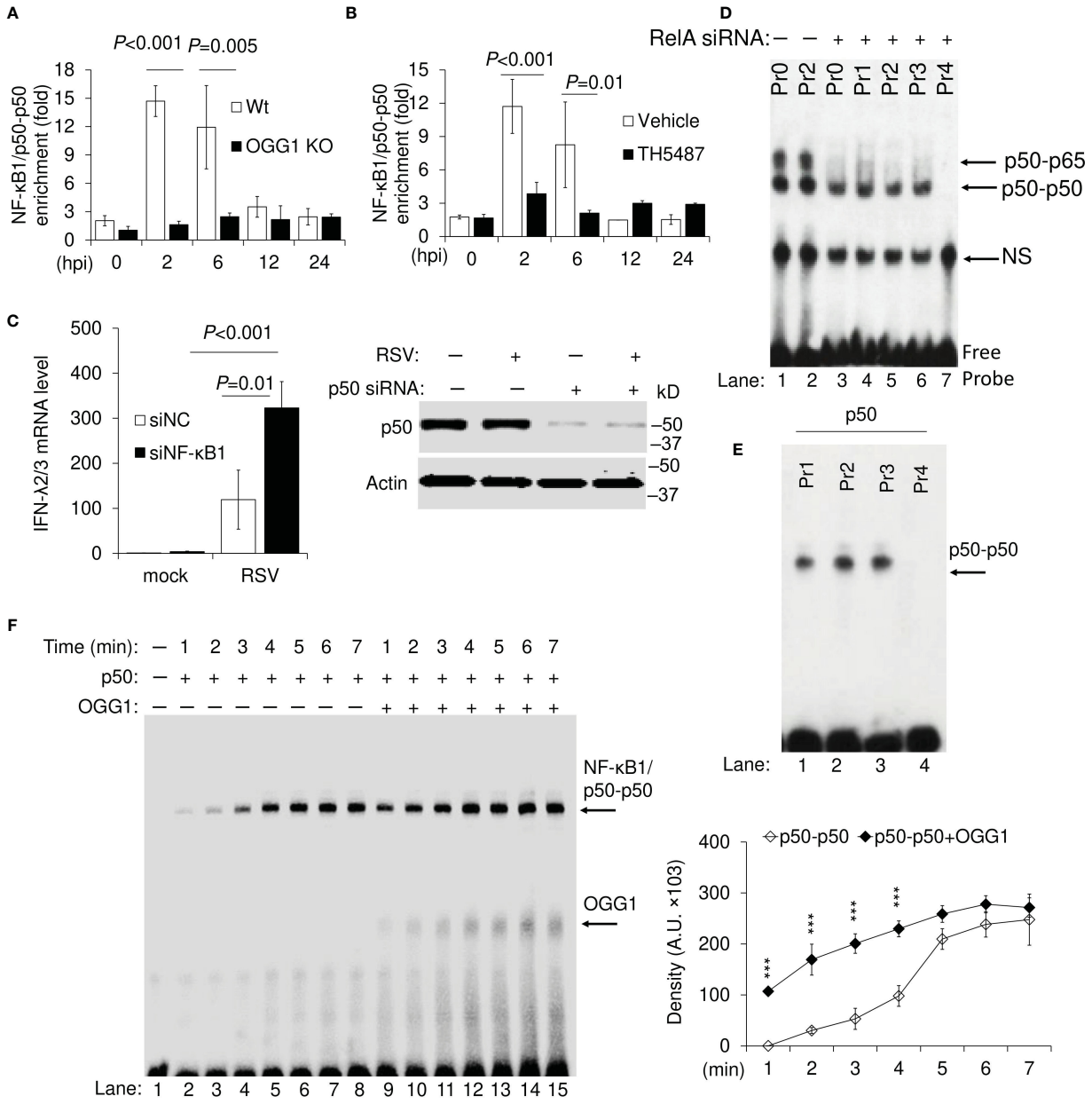

FIGURE 5
Binding of NF-κB1/p50-p50 suppressor to Gua islets in wildtype and OGG1 knockout RSV-infected cells. **(A)** NF-κB1/p50-p50 enrichment on the IFN-λ2/3 promoter in OGG1 proficient and OGG1 KO cells. **(B)** Effect of OGG1 inhibitor on NF-κB1/p50-p50 enrichment on the *IFN-λ2/3* promoter. **(C)** mRNA levels of IFN-λ2/3 in NF-κB1/p50-p50 proficient and depleted mock and RSV-infected hSAECs. Right panel: Western blot analysis of NF-κB1/p50-p50 in proficient and siRNA-depleted, mock- and RSV-infected hSAECs. **(D)** RelA was depleted by targeted siRNA in hSAECs, and infected with RSV. NE was isolated and incubated with probes to perform EMSA. **(E)** NF-κB1/p50-p50 binding to intact 8-oxoGua containing probes (Pr1,2 and 3), or the mutagenized probe (Pr4). **(F)** Time course analysis of NF-κB1/p50-p50 binding to Pr2 ± OGG1. OGG1 and NF-κB1/p50-p50 proteins were incubated with Pr2 probe for indicated time and subjected to EMSA. Right panel: graphical depiction of band densities. Bars represent means ± SD. Statistical analysis, Student's t-tests (unpaired). ***$p<0.001$.

3 expression, NF-κB1/p50-p50 was depleted by siRNA in hSAECs (Figure 5C, right panel), and cells were infected by RSV for 24 h. ChIP analysis failed to show significant NF-κB1/p50-p50 enrichment on the *IFN-λ2/3* promoter (not shown). In p50 siRNA-treated cells infected with RSV, there was a >300-fold increase in IFN-λ2/3 mRNA expression. This compares to ~100-fold increase in IFN-λ2/3 mRNA expression in non-targeting siRNA transfected cells (Figure 5C, left panel). These results suggest an inhibitory role of NF-κB1/p50-p50 on IFN-λ2/3 expression.

Next, we made attempts to identify the sequence requirement for NF-κB1/p50-p50 binding. Results show that OGG1 promotes NF-κB1/p50-p50 DNA occupancy. In both the *IL28A* and *IL28B* promoters, IRF binding sites are in proximity to NF-κB binding elements (5'-**GGG**ACTGCC-3'). There are Gua runs (5'-GGG-3') upstream and downstream to the NF-κB binding sites, and these

binding sites are present on both the sense and antisense DNA strands (Supplementary Figure 5). 8-oxoGua was placed individually in the 5' end of Gua runs (Table 1), including NF-κB binding elements as may occur in chromatin due to charge migration (41) and the potential NF-κB1/p50-p50 binding sites including Gua runs were also mutagenized (G→T, Pr3, Pr4). First, NE from nontargeting siRNA transfected RSV-infected from OGG1 expressing hSAECs showed binding of activated NF-κB/p50-p65 heterodimer to ±8-oxoGua probes consistent with NF-κB binding elements (Pr0 and Pr2) (Figure 5D, lanes 1-2). NEs from RelA-depleted cells (targeting siRNA to RelA) resulted in binding only of NF-κB1/p50-p50 to probes ±8-oxoGua (Figure 5D, lanes 3-4). In the probe where all Gua runs were mutated, no p50-p50 was captured from NEs (Figure 5D and 5E, last lanes, Pr4). The EMSA also showed that 8-oxoGua in proximity to the NF-κB site or in Gua runs (Pr1, Pr2, Pr3) had no effect on NF-κB1/p50-p50 binding, but substitution of Gua for T in G-runs (5'-GTG-3') eliminated the shift of Pr4 (Figure 5E, Lane 4). OGG1 binds to 8-oxoGua containing probes, and there is no difference in binding caused by 8-oxoGua being in the sense or in the antisense strand (Figure 3C). Results summarized in Figure 5F (Pr2, lanes 2-8 and lanes 9-15) clearly suggest that OGG1 increases NF-κB1/p50-p50 binding to 5'-GGG-3' in a time dependent manner. For example, in the presence of OGG1 at 1 min, levels of DNA-associated NF-κB1/p50-p50 were similar to that of 4 min without OGG1 (compare lane 5 to lane 9, in Figure 5F). Binding of NF-κB1/p50-p50 to Gua runs is in line with the strict conservation of the first three guanines in the NF-κB site as determined by structural studies (42).

3.6 Pharmacological inhibition of OGG1 increases IFN-λ expression during RSV infection

IFN-λ2/3 has the unique capacity to restrict viral invasion, increase virus clearance and decrease inflammatory responses to preserve the integrity of the mucosal epithelium (43). Therefore, we tested whether OGG1 inhibition altered expression of mucosal IFN-λ2/3 and lung pathology in mice infected with RSV (10^6 PFU per lung). Vehicle or TH5487 (30 mg/kg) was administered intraperitoneally (i.p.) prior and after infection, as outlined in Figure 6A. TH5487 prevented OGG1 binding to substrate-containing DNA in NE both *in vitro* (Supplementary Figure 6A) and *in vivo* as measured by ChIP assays (Supplementary Figure 6B). 8-oxoGua levels in the same promoter as OGG1 enriched were increased continuously (Supplementary Figure 6C). Compared with mock controls, the lungs of RSV-infected mice demonstrated increased mRNA expression of *IFN-λ2/3*. This effect was further enhanced in RSV-infected mice that were treated with TH5487 to inhibit OGG1 (Figure 6B). These data support the findings generated with cell culture and *in vitro* assays that showed that OGG1 presence in the promoter inhibits IRF binding via the suppressor NF-κB1/p50-p50 (Supplementary Figure 6D, lane 2 and 3). At 24 hpi, OGG1 inactivation by O-GlcNAcylation (Supplementary Figure 6E) corelated with increasing binding of IRF3 (Supplementary Figure 6D, lane 5 and 6). We note, *Ifn-α* and *Ifn-β* are not significantly increased until 24 hpi in line with accumulation of IFNα, and β expressing immune cells (Natural killer (NK) cells, B- and T-cells and macrophages). *Ifn-γ* expression was not significant during the early phase of RSV infection (Figure 6B) as it produced by NK T cells, CD4 Th1 and CD8 cytotoxic T lymphocyte, and effector T cells (44, 45), of which the expression is low even at 24 hpi.

IFN-λ2/3 expression correlates with a significant induction at the mRNA level of selected interferon stimulated genes (ISG), like Oas1a (2'-5'-Oligoadenylate Synthetase 1), Isg15 (Interferon-stimulated gene 15), and Rsad2 (Radical S-Adenosyl Methionine Domain 2) in the presence of TH5487 at 24 hpi (Figure 6C). We examined expression of IFN at protein levels in BALF. Mock- and RSV-infected mice were treated with vehicle or TH5487 (30 mg/kg), and BALF was collected at indicated time post-infection. We showed that treatment with 30 mg/kg TH5487 significantly increased levels of IFN-λ2/3 (Figure 6D). Hence, in mice treated with TH5487, IFN-λ2/3 expression in the airways is substantially altered, including a sustained peak response during RSV infection. In addition, we also observed less weight loss in TH5487-treated mice (Figure 6E). Furthermore, TH5487-treated mice had lower viral load than vehicle treated mice at day 4 post-infection (Figure 6F). These results demonstrate that TH5487 may have a protective role by enhancing host antiviral response upon RSV infection. There were no significant changes in expression of OGG1 mRNA (Figure 6G) or protein (Figure 6H) 6 days post-RSV infection, indicating that OGG1 function can be targeted at the level of substrate recognition. The decreases in RSV-mediated immune pathology are in line with those observed after IFN-λ addition to animals prior to infection with human metapneumovirus (HMPV) or African swine fever virus (16, 46).

The potent immunomodulatory activities of IFN-λ prompted us to further investigate its potential therapeutic activity *in vivo*. First, we blocked early IFN-λ release with anti-IL-28A/IFN-λ (2.5 mg/kg), an IFN-λ neutralizing antibody, during RSV-infection (Figure 7A). Then, we administered TH5487 (30 mg/kg) or recombinant IFN-λ2 (rIFN-λ) (0.1 mg/kg) to mock- and RSV-infected mice. Prophylactic treatment with TH5487 and rIFN-λ directly to the upper respiratory tract resulted in increased expression of *Isg15*, *Oas1*a and *Rsad2*. This effect was partially abrogated in mice treated with the neutralizing antibody on day 1 post-infection (Figure 7B). Neutrophils respond to cytokines to upregulate antimicrobial functions and exhibit pro-inflammatory activation, which is essential for confronting infection but also induces immunopathology (47). Mouse and human neutrophils both express the type III IFN receptor thus suggesting a possible conserved role for IFN-λ in regulating neutrophil activation. We then tested neutrophil infiltration into BALF of mock- and RSV-infected mice. We found a significant decrease in RSV-induced neutrophils in the presence of TH5487 and rIFN-λ. The neutralizing IFN-λ antibody elicits the highest neutrophil accumulation (Figure 7C).

Upon activation, neutrophil elastase (ELA2) and myeloperoxidase (MPO) enhances chromatin de-condensation and promotes formation of neutrophil extracellular traps (NETs) (48). Therefore, we measured levels of ELA2 and MPO in the BALF from mock- and RSV-infected mice. Treatment with TH5487 and rIFN-λ had similar effects in

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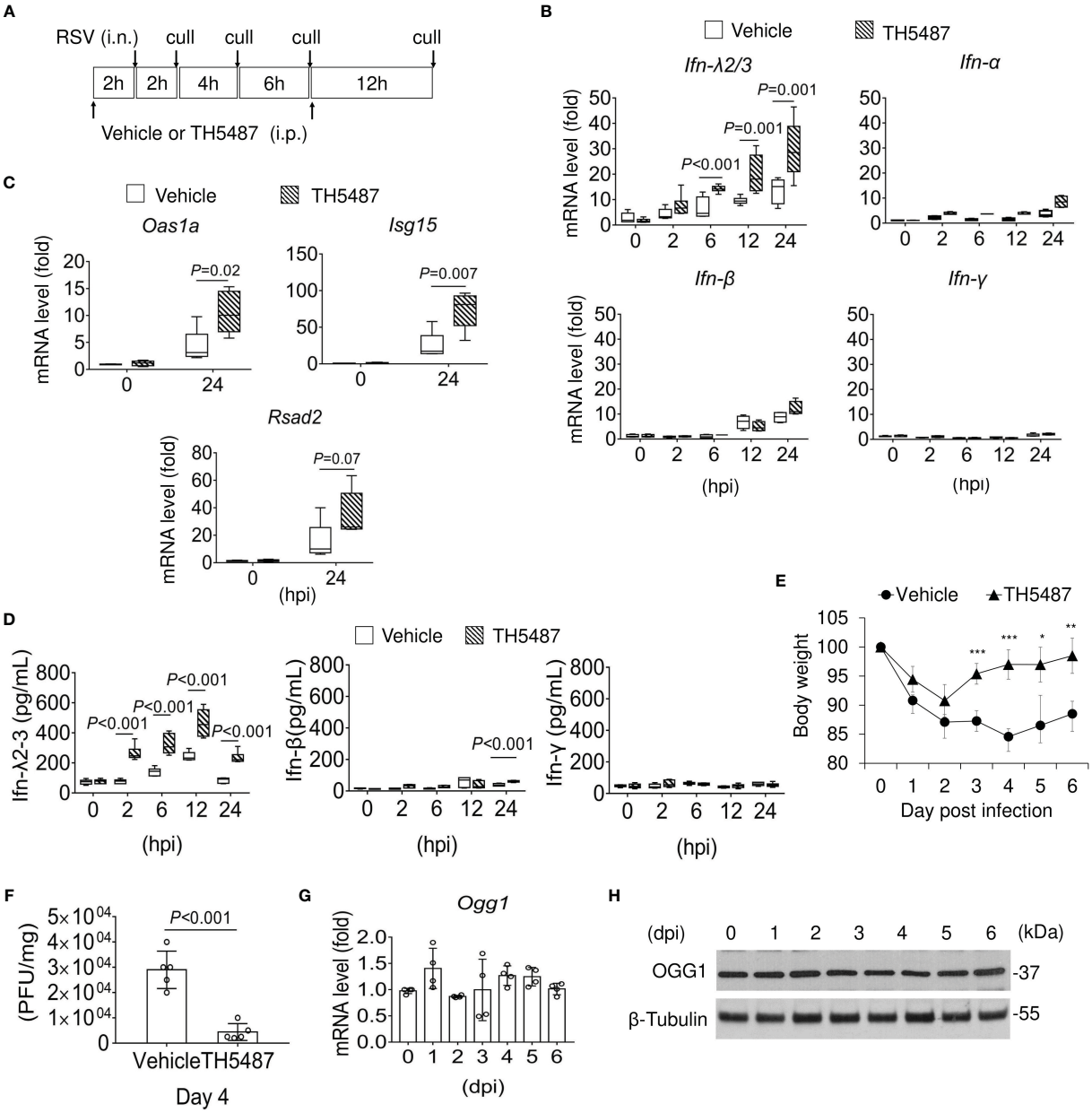

FIGURE 6
Expression of IFNs in the lungs of mock and RSV-infected mice with or without OGG1 inhibition. **(A)** Infection and treatment schedule of experiment. **(B)** mRNA levels of *Ifn*-λ2/3, *Ifn*-α, *Ifn*-β and *Ifn*-γ in RSV-infected lungs with or without TH5487 treatment. **(C)** mRNA levels of selected IFN-stimulated genes, *Oas1a*, *Isg15* and *Rsad2* in RSV infected lungs ± TH5487. **(D)** IFN-λ, IFN-β and IFN-γ protein levels in BALF as determined by ELISA (n=6). **(E)** Percentage weight loss of mice with and without TH5487 treatment after RSV infection. **(F)** Viral load in lung tissue of vehicle and TH5487 treated RSV infected mice was determined by plaque forming assays on day 4. **(G)** mRNA and **(H)** protein level of OGG1 in the lungs after RSV infection. In **(B-F)** groups of mice were mock or RSV infected (PFU = 10^6 per lung) and treated i.p with TH5487 (30 mg/kg) or equivalent volume of solvent daily. **(B, C)** mRNA levels were determined by qRT-PCR. Data is shown from three to four independent experiments. PFU, plaque forming unit; BALF, bronchoalveolar lavage fluid; *Oas1a*, 2'-5'-Oligoadenylate Synthase 1; *Isg15*, Interferon-Induced 17-kD/15-kD Protein; *Rsad2*, Radical S-Adenosyl Methionine Domain-Containing Protein 2. Data is expressed as means ± SD. Statistical analysis in B to F, Student's t-tests (unpaired). *p<0.05, **p<0.01, ***p<0.001.

lowering ELA2 and MPO levels in response to RSV infection (Figure 7D, left and right panels). In addition to elevated levels of ELA2, the activities of matrix metalloproteinase (MMP) have also been demonstrated in facilitating migration of neutrophils across basement membranes (49). Thus, we performed gelatin zymography to determine MMP activity (50). The degree of digestion of gelatin in BALF samples from RSV-infected mice showed that the proenzyme is converted to an active form, which was hardly seen in mice treated with TH5487 and rIFN-λ, suggesting that these compounds inhibit MMP activity (Figure 7E). Additionally, RSV-infected mice treated with anti-IL-28A/IFN-λ (2.5 mg/kg) had a more robust increase in neutrophil infiltration in both BALF (Figure 7F) and airway tissues (Figure 7G) as compared to TH5487- and rIFN-λ-treated mice. An exacerbated antiviral response is also associated with an increase in pro-

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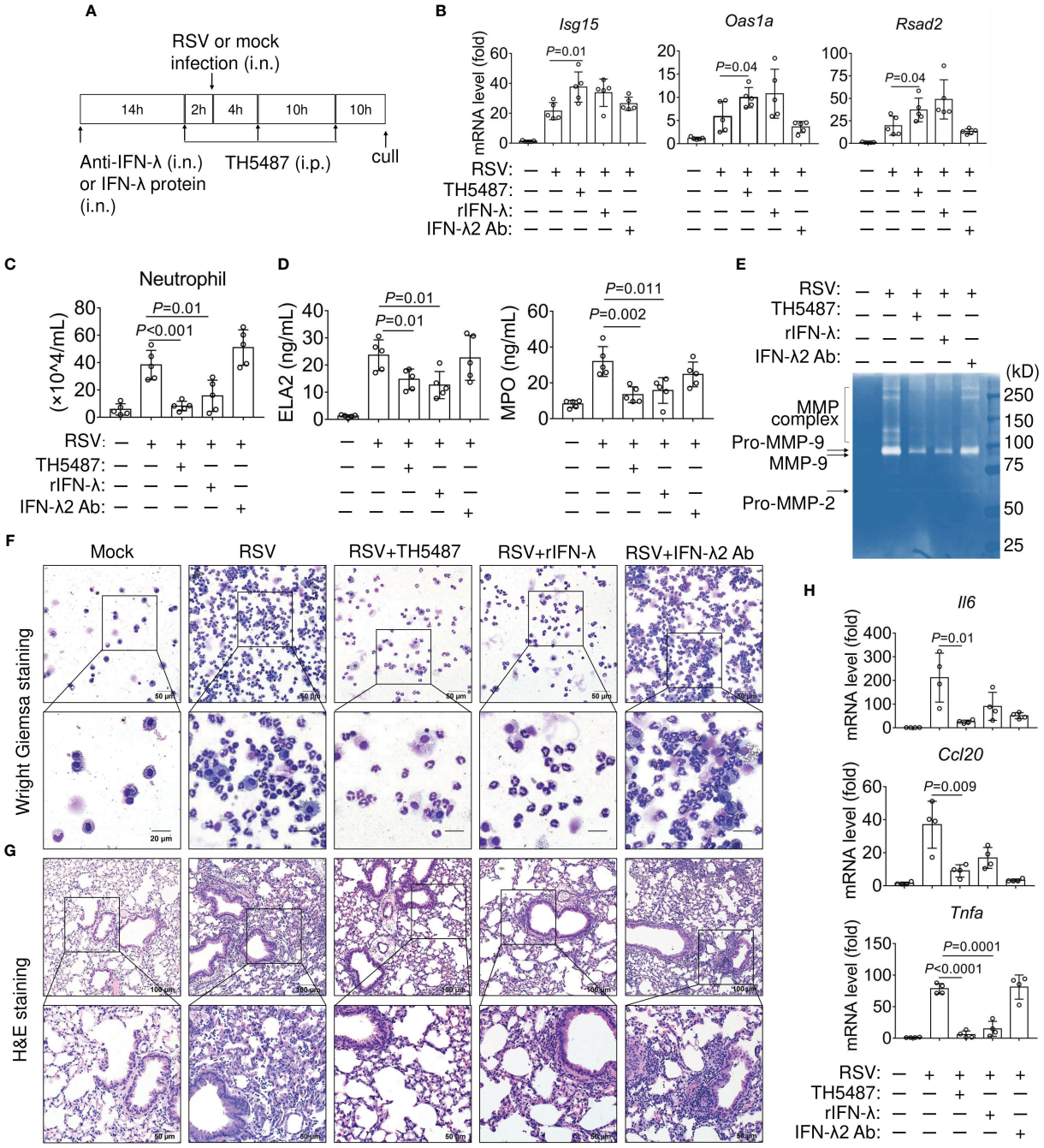

FIGURE 7
Effect of IFN-λ and TH5487 administration on expression of IFN-stimulated genes and airway inflammation in RSV-infected mice. **(A)** Schematic depiction of experimental design. **(B)** mRNA levels of IFN stimulated genes, *Oas1a*, *Isg15*, and *Rsad2* in the mouse lung as determined by qRT-PCR (n=5). **(C)** Numbers of neutrophils in the BALF. **(D)** Neutrophil Elastase/ELA2 and myeloperoxidase (MPO) in BALF were quantified by ELISA. **(E)** Matrix Metallopeptidase 9 (MMP-9) activity in the BALF from RSV-infected mice ± TH5487, IFN-λ protein or IFN-λ neutralizing antibody was analyzed by gelatin zymography. Pro-MMP-9, MMP-9 are indicated by arrows. **(F)** Representative images of Wright Giemsa-stained cells in BALF from mock- and RSV-challenged mice ± TH5487, IFN-λ protein or IFN-λ neutralizing antibody. **(G)** Representative images of hematoxylin and eosin-stained lung sections after RSV infection ± TH5487, IFN-λ protein or IFN-λ neutralizing antibody. **(H)** mRNA levels of inflammatory cytokines, *Il6*, *Tnfα* and *Ccl20* in the mouse lungs as determined by qRT-PCR. In (B-H) mice were RSV challenged (10^6 PFU) via the intranasal route, TH5487 (30 mg/kg daily). IFN-λ (0.1 mg per kg), antibody to IFN-λ (2.5 mg per kg) administered i.n. Symbols represent individual mice (n=5). Statistical analysis, One-way ANOVA with Tukey's multiple comparisons. In (C, D) Statistical analysis, Student's t-tests (unpaired). *Oas1a*, *Isg15*, and *Rsad2*, as in legend to Figure 6; *Il6*, interleukin 6 or interferon beta-2; *Tnfα*, tumor necrosis factor-alpha; *Ccl20*, C-C motif chemokine ligand 20.

inflammatory cytokines. Accordingly, RSV-infected mice demonstrated increased mRNA expression of *Il6*, *Tnfα* and *Ccl20* (Figure 7H). In contrast, the expression of these pro-inflammatory cytokines is substantially lower in TH5487- and rIFN-λ-treated mice. IFN-λ2/3 neutralization resulted in the upregulation of *Tnfα* mRNA (Figure 7H). Thus, pharmacological inhibition of OGG1 results in a beneficial outcome that is associated with increased IFN-λ2/3 expression. Taken together, these data indicate that OGG1 plays a crucial role in mediating the anti-viral immune response through regulation of IFN-λ2/3.

4 Discussion

Herein, we describe an unexpected regulatory circuit controlling IFN-λ expression in RSV-infected hSAECs and mice. Specifically, OGG1 interaction with its substrate inversely correlates with binding of the primary transcription factor(s) IRFs and NF-κB/RelA to IREs for expression of IFN-λs. Mechanistically, allosteric changes in DNA promote binding of the repressor NF-κB1/p50-p50 that decreases DNA occupancy of IRFs and NF-κB/RelA in the chromatin which negatively controls IFN-λ expression. This suppression is reversed by host-driven post-translational modification (O-GlcNAcylation) of OGG1, OGG1 knockdown, and OGG1 inhibition (Figure 8). OGG1 knockdown or pharmacological inactivation also increased poly(I:C) induced expression of IFNs, extending the range for OGG1 in controlling the inducible expression of these key immune regulators. We speculate that targeting OGG1 substrates-binding through TH5487 can be utilized to optimize expression of IFNs, thus controlling the extent of innate and adaptive immune responses.

Through specific post-translational modifications, OGG1 binds to covalently modified DNA base 8-oxoGua without excision to facilitate transcription factors binding to DNA motif, mediating timely cellular physiological responses (51). Conventionally, 8-oxoGua is a marker of oxidative stress as Gua has the lowest oxidation potential among nucleobases (52, 53). However, in gene regulatory sequences it can function as an epigenetic-like mark (51, 52, 54, 55) with OGG1 considered a reader. 8-oxoGua can be generated by ROS derived from receptor-driven targeted demethylation of histone H3 lysine 9 at both enhancer and promoter locations by the activation of resident lysine specific demethylase1 (56, 57) and ROS generated by other oxidoreductases (e.g., NADPH oxidases in RSV-infected cells) (58).

In contrast to IFN-λ expression, pro-inflammatory mediators (e.g., TNF, ILs, CCLs, CXCLs) correlated well with the transiently generated epigenetic mark, 8-oxoGua and with enrichment of the reader OGG1 in TSS adjacent promoter regions (9, 15). The TSS-proximal promoter region of cytokines/chemokines and IFN-λ2/3 are guanine-rich, containing Gua islands (e.g., 5'-GGG-3') and this π-stacking effect becomes more pronounced as the length of the G run increases (52, 59, 60). Therefore, it was not surprising to observe an 8- to 14-fold increase in 8-oxoGua enrichment in the promoters after RSV infection. Its enrichment took place in TSS proximal regions of *IFN-λ* (also *IFN-β*), which both contain a cluster of binding sites for IRFs, and NF-κB/p50-p65. Interestingly, in OGG1-enriched *IFN-λ2/3* promoter, IRFs and NF-κB/RelA enrichment was low or undetectable. However, treatment of RSV-infected hSAECs with the OGG1 inhibitor TH5487 or OGG1

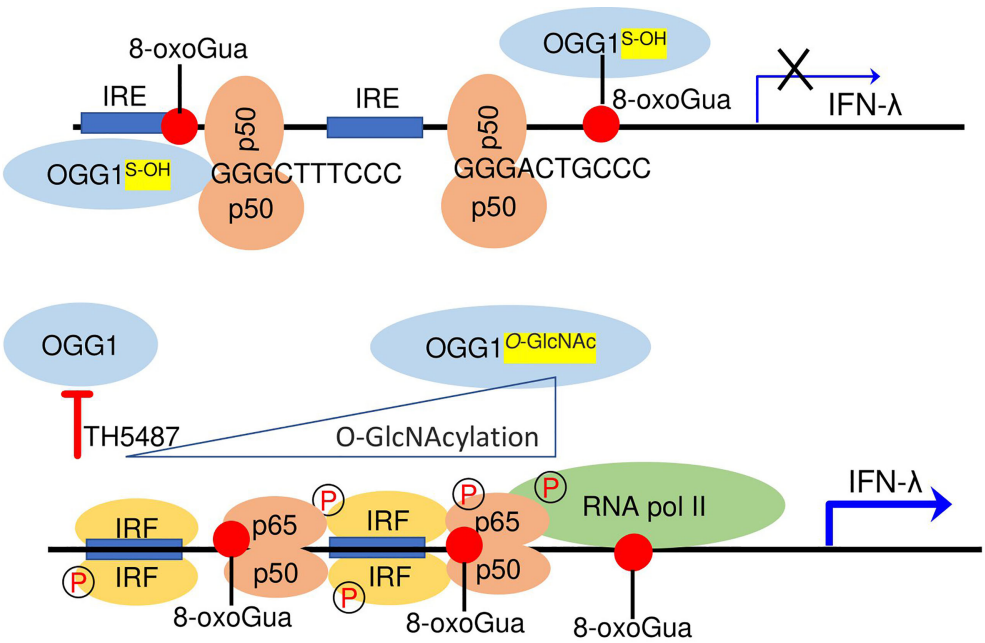

FIGURE 8
Epigenetic regulation of INF-λ expression in RSV-infected cells. Upper panel, OGG1 interaction with the epigenetic mark 8-oxoGua in promoter facilitates binding of the suppressor NF-κB1/p50-p50 to 5'-GGG-3' at early time of RSV infection. Lower panel, functional inhibition by TH5487 or O-GlcNAcylation prevent OGG1 to read 8-oxoGua allowing NF-κB/50-p65 and IRF dimer binding for transcriptional activation of IFN-λ in the late-phase of RSV infection.

knockdown decreased levels of proinflammatory mediators, while significantly increasing IRFs and NF-κB/p50-p65 promoter enrichment and IFN-λ2/3 expression. These findings imply that OGG1 has a differential effect on gene expression as shown previously (9). In support, ChIP-coupled sequencing studies have shown that stimuli-dependent enrichment of OGG1 occurred over thousands of enhancers and promoters in chromatinized DNA, however, a large portion of OGG1 enrichment was associated with gene activation and another portion with gene silencing (9).

Although an RSV dose added to cells (MOI = 3) activates both IRFs and NF-κB/p50-p65 at early time points, IFN-λ2/3 expression only increased abundantly at 24 hpi. Further investigation showed that there were no changes in OGG1 expression levels, while a post-translational modification by O-GlcNAcylation occurred only at later time points in infected cells. Note that at this time point (24 hpi) expression of selected inflammatory mediators (e.g., TNF, CXCL1) decreased (15), which can now be explained by OGG1 O-GlcNAcylation. This reversible post-translational modification primarily affected serine and threonine residues of OGG1, leading to decreased substrate recognition and damaged base excision (28). OGG1 also showed oxidation at its cysteine residues to sulfenic acid at all time points of infection, like those shown previously (15). Placing these modifications into the context of IFN-λ2/3 expression, $OGG1^{-S\text{-}OH}$ can bind to DNA via 8-oxoGua, while O-GlcNAcylated OGG1 poorly recognized its DNA substrate (28). Since the O-GlcNAcylation inhibitor (OSMI-1) suppresses *IFN-λ2/3* expression, it is likely that this decrease is due to the enhanced binding of OGG1 to 8-oxoGua in the *IFN-λ2/3* promoter. OGG1 O-GlcNAcylation correlated well with increased binding of NF-κB/p50-p65 and IRFs and enhanced IFN-λ2/3 expression. Therefore, in this context, OGG1 inactivation by O-GlcNAc transferase can be considered part of an antiviral response. OGG1 O-GlcNAcylation is in line with RSV-induced increase in uridine 5′-diphosphate-N-acetyl-D-glucosamine (UDP-GlcNAc) levels, a rate-limiting precursor of the O-GlcNAc transferase in the hexosamine biosynthetic pathway shown by our labs and others (37, 38). In addition, NF-κB O-GlcNAcylation robustly increases NF-κB/p65-dependent gene expression (61).

$OGG1^{S\text{-}OH}$ can bind 8-oxoGua and interact with the complimentary cytosine, which bends DNA and changes the adjacent DNA architecture. These changes in the DNA landscape are hypothesized to decrease the energy required for transcription factor binding including NF-κB/p50-p65, AP1, SMADs, which results in increased expression from proinflammatory and wound healing genes (9, 36, 39, 51, 53). We note that the DNA mismatch repair pathway permitted survival of influenza virus infected cells that also showed an increase in IFN expression and enabled transcription of host genes in OGG1 proficient cells (62, 63).

From our study it is obvious that OGG1 binding to its epigenetic mark in the *IFN-λ2/3* proximal promoter increases DNA occupancy of the constitutive repressors (e.g., NF-κB1/p50-p50) to Gua island (5'-GGG-3') within or adjacent to IREs in the *IFN-λ2/3* promoter. The NF-κB1/p50-p50 lacks a transactivation domain such as RelA, c-Rel or RelB and therefore, it has a repressive role on gene expression by interfering with transcription factor binding, thereby decreasing *IFN-λ* expression. Indeed, using wild-type and mutagenized oligo probes, we found that OGG1 increases DNA occupancy of NF-κB1/p50-p50 on Gua runs (5'-GGG-3'). This data can be explained by crystallographic studies showing that p50-p50 makes base-specific contacts with the first three Gua of the NF-κB consensus site (5'-GGGRNYYYCC-3') (8, 64). In further support, X-ray structures of the NF-κB1/p50-p50 DNA complex show the ability of p50-p50 to efficiently bind to Gua runs (5'-GGG-3') (42, 65). Specifically, the hydrogen bonds made by His 64 with the N7 group of G5, and by Arg 56 and Arg 54 with both the N7 and O6 groups of G4 and G3 (42, 64). Therefore, NF-κB1/p50-p50 can act as a transcriptional repressor on pro-inflammatory genes (66, 67). Previous studies using molecular, biochemical and structural analyses also showed that the NF-κB1/p50-p50 homodimer significantly decreased expression of a number of IFN-inducible genes via binding to a subclass of guanine-rich IRE sequences (68). Moreover, NF-κB1 knockout mice lacking the p50-p50 have highly increased inflammatory responses to various agents in their lungs, liver, and kidney implying a predominant role for NF-κB1/p50-p50 as a negative regulator of NFκB-driven pro-inflammatory gene expression (69, 70).

The receptor for IFN-λ is primarily expressed on epithelial cells (71) and neutrophils (72), thus suggesting that the protective effects of IFN-λ are prominent specifically in stimulating innate immune cells. Pharmacological inhibition of OGG1 by TH5487 decreased markers of lung injury in RSV-infected mice by IFN-λ-mediated inhibition of RSV replication, neutrophilia, or both. While we have no evidence for direct implication of OGG1 in RSV replication, there was decreased neutrophil numbers, decreased ELA2, MPO and MMP-9 expression. We also observed decreased weight loss and RSV viral titers in RSV-infected lungs. These results are in line with roles of IFN-λ in activation of antiviral defences of cells expressing corresponding receptors (73–75). In support, IFN-λ receptor KO mice showed higher RSV, human metapneumo- and influenza virus yields, and increased inflammatory responses compared to wild-type mice (76, 77). Neutrophils utilize three major protective mechanisms: phagocytosis, degranulation (emitting an array of anti-microbial substances, expression IFN stimulatory genes), and neutrophil extracellular traps (78). However, these defensive roles of neutrophils appear to be secondary due to their low numbers in lungs of OGG1 inhibitor-treated mice. Because the goal of this study was to elucidate the epigenetic role of 8-oxoGua in enhanced IFN-λ expression, its role (s) in anti-RSV infection still needs to be determined in future studies. The strategy of inhibiting OGG1 function by TH5487 will provide appropriate curtailment of the early response to prevent immune pathology (79).

In IFN expression, DNA cytosine methylation at CpG repeats are essential regulatory entities at sites where 8-oxoGua is generated. Oxidation of Gua to 8-oxoGua opposite to cytosine or in proximity (2-3 bases) inhibits the function of the DNA methyltransferases (DNMTs) (80, 81). Moreover, methyl binding proteins [MBP(s)] recognize Gua's O6 and N7; however, oxidation of Gua converts the N7 from a hydrogen acceptor to a hydrogen donor and replaces the proton-8 with an oxygen atom, resulting in interference with methyl-CpG dinucleotide recognition by MBPs (82–84). To this end, the family of Ten-eleven translocation (TET)

proteins are responsible for the enzymatic oxidation of 5mC into 5-hydroxymethylcytosine (5hmC), and 5-carboxylcytosine, which are substrates for thymidine DNA glycosylase and repaired via the BER pathway. The interference of 8-oxoGua function of MBPs imply that a group of genes may utilize OGG1 the reader of 8-oxoGua to override the repression by 5mC to execute a prompt cellular response to stimuli. While oxidative stress persists, OGG1 may further recruit TET1 for enzymatic catalysis of 5meC into 5hmC and thus the OGG1-initated pathway could be utilized to transfer the DNA oxidation signal to downstream of DNA demethylation enzymes (84). Gua oxidation is non-enzymatic thus, OGG1 complexed with its substrate might be the most economical strategy for cells to override repression by 5mC. The biological function of DNA methylation associated with 8-oxoGua generation in inflammatory processes and specifically with regulation of IFN-λ needs further characterization.

In this study, we found that RSV-infection generated oxidatively modified Gua lesions 8-oxoGua in promoters, and its reader, OGG1, impact antiviral gene expression. We propose that OGG1 by rotationally diffusing along the DNA helix, a mechanism facilitated by thermal activation locates its mark 8-oxoGua and may also influence chromatin remodelers. Also, it is yet to be discovered whether formation of the epigenetic mark 8-oxoGua and docking of the reader OGG1 precedes changes in chromatin architecture by histone modifications (such as acetylation, methylation, phosphorylation, and others) at the N- and C-terminal tails of histones (85). Given that the cellular redox state via posttranslational modifications affects OGG1 function, whether as a reader or eraser, both roles fit very well into the present hypothesis. We conclude that the epigenetic regulation is a highly dynamic process and, therefore, facilitates rapid phenotypic changes for the host. To this end, our work introduces a new concept –pharmacological regulation of OGG1 function as a reader and eraser to control the outcome of viral infections, which may also represent a general antimicrobial strategy in the future.

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 animal study was reviewed and approved by University of Texas Medical Branch (UTMB) Animal Care and Use Committee (approval no. 0807044D).

Author contributions

YX, LP, XZ, KW, SV, and IB designed, performed, analysed cell culture and animal experiments. LP and IB wrote the manuscript. KW helped XZ to perform binding assays and immunoprecipitation. KW, LP, and XZ generated and characterized expression vectors. KW and YX generated OGG1 knock out hSAECs using CRISPR/Cas9 genome editing and characterized isolated clones. SV, LT, ZR, and ARB helped with writing, experimental designed, performed, some of the experiments. ARB and XB advised ChIP experiments. All authors discussed results and approved the content of the manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1161160/full#supplementary-material

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DNA methylation patterns in CD4$^+$ T-cells separate psoriasis patients from healthy controls, and skin psoriasis from psoriatic arthritis

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Background: Psoriasis is an autoimmune/inflammatory disorder primarily affecting the skin. Chronic joint inflammation triggers the diagnosis of psoriatic arthritis (PsA) in approximately one-third of psoriasis patients. Although joint disease typically follows the onset of skin psoriasis, in around 15% of cases it is the initial presentation, which can result in diagnostic delays. The pathophysiological mechanisms underlying psoriasis and PsA are not yet fully understood, but there is evidence pointing towards epigenetic dysregulation involving CD4$^+$ and CD8$^+$ T-cells.

Objectives: The aim of this study was to investigate disease-associated DNA methylation patterns in CD4$^+$ T-cells from psoriasis and PsA patients that may represent potential diagnostic and/or prognostic biomarkers.

Methods: PBMCs were collected from 12 patients with chronic plaque psoriasis and 8 PsA patients, and 8 healthy controls. CD4$^+$ T-cells were separated through FACS sorting, and DNA methylation profiling was performed (Illumina EPIC850K arrays). Bioinformatic analyses, including gene ontology (GO) and KEGG pathway analysis, were performed using R. To identify genes under the control of interferon (IFN), the *Interferome* database was consulted, and DNA Methylation Scores were calculated.

Results: Numbers and proportions of CD4$^+$ T-cell subsets (naïve, central memory, effector memory, CD45RA re-expressing effector memory cells) did not vary between controls, skin psoriasis and PsA patients. 883 differentially methylated positions (DMPs) affecting 548 genes were identified between controls and "all" psoriasis patients. Principal component and partial least-squares discriminant analysis separated controls from skin psoriasis and PsA patients. GO analysis considering promoter DMPs delivered hypermethylation of

genes involved in "regulation of wound healing, spreading of epidermal cells", "negative regulation of cell-substrate junction organization" and "negative regulation of focal adhesion assembly". Comparing controls and "all" psoriasis, a majority of DMPs mapped to IFN-related genes (69.2%). Notably, DNA methylation profiles also distinguished skin psoriasis from PsA patients (2,949 DMPs/1,084 genes) through genes affecting "cAMP-dependent protein kinase inhibitor activity" and "cAMP-dependent protein kinase regulator activity". Treatment with cytokine inhibitors (IL-17/TNF) corrected DNA methylation patterns of IL-17/TNF-associated genes, and methylation scores correlated with skin disease activity scores (PASI).

Conclusion: DNA methylation profiles in $CD4^+$ T-cells discriminate between skin psoriasis and PsA. DNA methylation signatures may be applied for quantification of disease activity and patient stratification towards individualized treatment.

KEYWORDS

psoriasis, psoriatic arthritis, $CD4^+$ T-cell, epigenetics, methylation, interferon, biomarker

1 Introduction

Psoriasis is a systemic inflammatory disease that primarily affects the skin (1). Approximately one-third of psoriasis patients also develop joint involvement and are therefore diagnosed with psoriatic arthritis (PsA) (2). In adult-onset disease, PsA typically manifests within 10 years after the onset of psoriasis, but approximately 15% of patients experience arthritis either concurrently or prior to the onset of skin disease (3). As a result, the diagnosis of PsA may be missed, delaying the introduction of effective treatment and resulting in disease progression and damage accrual (4). To date, disease-, activity- and/or outcome-specific biomarkers are missing, impeding disease monitoring and individualized care. Though the pathophysiology of psoriasis/PsA is complex and remains incompletely understood (5), the role of effector $CD4^+$ T-cells has been established (6), and pathological activation of the Tumor Necrosis Factor (TNF)/Interleukin (IL-)23/IL-17 cytokine axis contributes to the differentiation and activation of effector T-cells and their accumulation in affected tissues (7–9).

Epigenetic modifications alter gene expression without affecting the underlying genomic sequence. Alterations to the epigenetic landscape have been observed in several autoimmune diseases, including psoriasis (10). The addition of a methyl group to the 5' position of cytosine in cytosine phosphate guanosine (CpG) dinucleotides through DNA methyltransferases (DNMTs) is a potent epigenetic mechanism inhibiting the recruitment of transcription factors and RNA polymerases (11). Due to its stability in biological samples, DNA methylation is the most commonly investigated epigenetic mark (12). DNA methylation has been linked with the establishment of pathological effector T-cell phenotypes and the expression of inflammatory cytokines (13) in systemic autoimmune/inflammatory diseases, including psoriasis (14).

The aim of this study was to identify disease-associated DNA methylation signatures in $CD4^+$ T-cells from patients with psoriasis and PsA that may be used as diagnostic and/or prognostic biomarkers to inform treatment and care.

2 Participants, materials and methods

2.1 Patient cohorts and healthy controls

Whole blood samples from patients with psoriasis limited to the skin, psoriatic arthritis (PsA) and healthy controls were collected. All PsA patients satisfied both Moll and Wright classification criteria and Classification criteria for Psoriatic Arthritis (CASPAR) (15, 16). All patients enrolled in this study developed skin psoriasis before the onset of PsA. At the time of study inclusion, patients were not receiving any relevant systemic immunomodulating therapy. The washout periods were at least 4 weeks for conventional systemic therapies or more than 2 half-lives for biologic drugs. For 5 psoriasis and 3 PsA patients, samples were collected before and after the administration of biologic disease-modifying antirheumatic drugs (bDMARDs) (IL-17 inhibitors, TNF-inhibitors, IL-23 inhibitors; see Supplementary Table 1). Study participants were enrolled at the Department of Dermatology, University Hospital Carl Gustav Carus, TU Dresden, Germany. Demographic data, treatment information and Psoriasis Area and Severity Index (PASI) scores (17) were collected at all study visits (Table 1).

The study was approved by the ethics committee of the Faculty of Medicine Carl Gustav Carus, TU Dresden, Dresden, Germany. Written informed consent was obtained from all participants.

2.2 Sample processing and genomic DNA isolation

Sample processing and analyses were performed as described previously (14). Briefly, peripheral blood mononuclear cells

TABLE 1 Demographic and clinical characteristics.

	Healthy controls, n = 8	Psoriasis, n = 12	PsA, n = 8	P-value
Men, n (%)	4 (50)	7 (58)	4 (50)	NS
Age, median (IQR), years	27.0 (25.2-32.5)	41.0 (27.7-51)	56.5 (42.2-72.2)	0.01*
PASI score, median (IQR)	NA	15.6 (10.6-20.8)	6.8 (1.45-13.8)	0.04
Global PASI score, median (IQR)	NA	11.6 (6.4-18.7)		NA
Patients receiving bDMARDs, n (%)	NA	8 (67)	3 (37)	NA
Of these:				
IL-17 inhibitors	NA	8	1	NA
TNF inhibitors	NA	0	1	NA
IL-23 inhibitors	NA	0	1	NA

Men, n (%): n refers to the absolute number of male patients, and the number in brackets refers to the proportion of male patients out of the total number of patients.
Patients receiving bDMARDs, n (%): n refers to the number of patients receiving biologic DMARDs (NA in case of healthy controls) and the number in brackets refers to the proportion of patients receiving bDMARDs out of the total number of patients.
PsA, psoriatic arthritis; n, number; NA, not applicable; NS, not significant; IQR, interquartile range; bDMARDs, biologic Disease-Modifying Antirheumatic Drugs; IL, interleukin; TNF, Tumor Necrosis Factor; PASI, Psoriasis Area and Severity Index. *post-hoc comparison: healthy controls vs. PsA (p=0.007).

(PBMCs) were isolated from whole blood samples. $CD4^+$ T-cells were separated through Fluorescence-activated cell sorting (FACS) using the following antibodies (BioLegend): Pacific Blue anti-CD4 (OKT4); Fluorescein Isothiocyanate (FITC) anti-CD3 (OKT3); Phycoerythrin (PE) anti-CCR7 (G043H7); Allophycocyanin (APC) anti-CD45RA (HI100), Allophycocyanin-Cyanine 7 (APC-Cy7) anti-CD8 (SK1). Flow cytometry results were analyzed using FlowJo™ v10 Software. Genomic DNA was extracted (All prep Kit, Qiagen), and Genome-wide DNA methylation profiling was realized by Infinium MethylationEPIC array BeadChip 850K (18). Methylation at individual CpG positions was expressed as Beta (β) and M values, which were used for data visualization and statistical analysis purposes, respectively (19).

2.3 Bioinformatic analyses

In this study, two analyses were conducted. First, skin psoriasis and PsA patients not receiving systemic immunomodulating treatments were compared with controls, then samples from psoriasis patients "before treatment" (escalation) and "after treatment" (for >3 months) were compared.

2.3.1 DNA methylation profiling, psoriasis versus controls

Methylation profiles of $CD4^+$ T-cells were analyzed using R packages *minfi* (20) and *ChAMP* (21). Quantile and Beta MIxture Quantile (BMIQ) normalization strategies (22) were applied to raw data. Poor-quality probes (p-value <0.01), probes associated with Single Nucleotide Polymorphism (SNPs), not in CpG context (CH), and cross-reactive were identified and excluded (23). The biological sex of patients and healthy controls was confirmed through the predictSex function of the *minfi* package. Batch effects and covariates were identified and corrected using the *ComBat* function from the *ChAMP* package (24). Corrections for age, sex and slide effects (p <0.05) were performed. Differentially Methylated Positions (DMPs) between healthy controls and patient groups were identified using the empirical Bayes' moderated t-test method (*limma* package) (25). Probe expression was defined as statistically different between the patient/control groups applying a false discovery rate (FDR) <0.05, and a $|\Delta\beta| > 0.1$. The *DMRcate* package (26) was used to identify Differentially Methylated Regions (DMRs). DMRs with FDR <0.05, $|\Delta\beta| > 0.1$, and a minimum number of CpGs of 5 were considered for further analyses.

2.3.2 "Before" versus "after treatment" analysis

A sub-analysis, including patients for whom samples collected before and after the administration of biologic DMARDs were available, was performed. Since the cohort was smaller and contained samples of the same patients before and after treatment, quantile normalization was performed. The probe filtering process was comparable to the main analysis (exclusion of probes in SNP and CH context, cross-reactive probes). Correction for sex was then applied. DMPs between "before" versus "after treatment", and "after treatment" versus healthy controls were identified (FDR <0.05, $|\Delta\beta| > 0.1$).

2.3.3 Gene Ontology analyses

DMPs in the context of promoters - Transcription Start Site (TSS)1500, TSS200, 5' Untranslated Region (5'UTR) - were considered and divided into hypomethylated and hypermethylated positions. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) analysis for biological process and molecular function were performed using the *EnrichR* (https://maayanlab.cloud/Enrichr/) (27). KEGG pathways and GO terms with a Bonferroni corrected adjusted p-value <0.05 are displayed. The top ten pathways and terms sorted by adjusted p-values ranking are displayed; the complete lists containing all enriched KEGG pathways and GO terms associated with DMPs in all comparisons are reported in the Supplementary Materials.

2.3.4 DNA methylation scores

To identify differentially methylated genes (considering DMPs) regulated by type I and type II interferons (IFN) and genes involved in TNF and IL-17 signaling pathways, the *Interferome* database

version 2.01 (28) and WikiPathways database (29) were consulted. Where necessary, the R package *org.Hs.eg.db* was used to convert gene symbol into their ENTREZ ID. DNA-based methylation scores were established as described by Björk et al. (30). Briefly, the mean ($Mean_{HC}$) and standard deviation (SD_{HC}) of each DMP associated with type I and/or type II IFN-regulated genes or genes involved in TNF/IL-17 pathways were calculated using the healthy control group. These values were used to obtain standardized values (SVs) for each study participant by using the formula: $SV = (Value\text{-}Mean_{HC})/SD_{HC}$. The SVs for each DMP were then summed up to get total scores.

2.3.5 Statistical analysis

Statistical analyses and figures were generated using R version 3.1.1 and GraphPad Prism software version 6.0. Shapiro–Wilk normality tests were used to assess if variables had Gaussian distribution. Student's t-test and Mann-Whitney U test were used in pairwise comparisons of parametric and nonparametric continuous data, respectively, and Fischer's exact or χ^2 test for categorical data. One-way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test and Kruskal–Wallis followed by Dunn's *post hoc* tests were used when comparing more than two groups in normally distributed and non-normally distributed data, respectively. After assessing the Gaussian distribution, statistical associations between variables were assessed through Pearson's correlation. P-values <0.05 were considered statistically significant.

3 Results

3.1 Patient characteristics

A total of 36 peripheral blood samples were collected from 29 participants. One study participant (Healthy Control_9) was excluded, as the sample did not pass quality control during the data pre-processing phase. After this, 35 samples from 28 individuals remained, including 12 patients with skin psoriasis, 8 PsA patients and 8 healthy controls (HC). Sexes were evenly distributed among groups. The median age at inclusion was 27.0 years (IQR 25.2-32.5) in the healthy control group, which compared to 41.0 years (IQR 27.7-51) in psoriasis patients, and 56.5 years (IQR 42.2-72.2) in PsA patients. The median age between healthy controls and PsA patients was statistically different (p=0.007), which was considered during bioinformatic analyses (below). All patients were of White European ethnicity. Median PASI scores were higher in psoriasis (15.6, IQR 10.6-20.8) when compared to PsA patients (6.8, IQR 1.45-13.8; p=0.04) (Table 1).

Among patients who received biologic DMARDs, 8 psoriasis patients and 1 PsA patient received IL-17 blockers, one PsA patient was treated with an IL-23 inhibitor, and another received a TNF-inhibitor (Table 1). Samples were collected from 8 patients before and/or after treatment: 3 patients provided pre- and post-treatment samples, 2 patients donated samples at different post-treatment time points, and in 3 patients only post-treatment samples were available (Supplementary Table 1).

3.2 $CD4^+$ T-cell distribution

Immune phenotyping of $CD4^+$ T-cells to identify the proportion of naïve ($CD3^+CD4^+CCR7^+CD45RA^+$; 48.0% in HCs, 58.4% in psoriasis, 51.6% in PsA), Central Memory (CM; $CD3^+CD4^+CCR7^+CD45RA^-$; 20.5% in HCs, 18.2% in psoriasis, 23.5% in PsA), Effector Memory (EM; $CD3^+CD4^+CCR7^-CD45RA^-$; 25.4% in HCs, 18.4% in psoriasis, 25.5% in PsA), and Effector Memory cells re-expressing CD45RA (EMRA; $CD3^+CD4^+CCR7^-CD45RA^+$; 1.1% in HCs, 1.4% in PsA and 1.9% in psoriasis) subsets did not deliver differences between controls and patients with skin psoriasis or PsA (Supplementary Figures 1, 2; Supplementary Table 2).

3.3 DNA methylation patterns distinguish psoriasis patients from healthy controls

After quality control and probe filtering, 791,852 of 1,051,943 probes (59%) were included in the statistical analysis. Comparison of DNA methylation patterns in $CD4^+$ T-cells from healthy controls with cells from "all" psoriasis patients (skin psoriasis and PsA) identified 883 DMPs (433 hypo-, 450 hypermethylated) affecting 548 genes (Table 2). Based on DNA methylation patterns, samples from "all" psoriasis patients can be discriminated from healthy individuals, and skin psoriasis samples cluster separately from PsA patients (Figure 1A).

To identify methylation signatures that separate groups, unsupervised principal component analysis (PCA) and supervised Partial Least-Squares Discriminant Analysis (PLS-DA) were performed (based on DMPs). Based on PCAs, "all" psoriasis

TABLE 2 Differentially methylated positions (DMPs) in $CD4^+$ T-cells.

Comparison	Hypomethylated	Hypermethylated	Total (number of genes)
Controls *vs.* skin psoriasis	272	353	625 (374)
Controls *vs.* PsA	6,362	2,107	8,469 (4,525)
Skin psoriasis *vs.* PsA	2,230	719	2,949 (1,829)
Controls *vs.* "all" patients	433	450	883 (548)
Before *vs.* after treatment	878	1,118	1,996 (1,438)

DMPs, Differentially Methylated Positions; PsA, Psoriatic Arthritis.

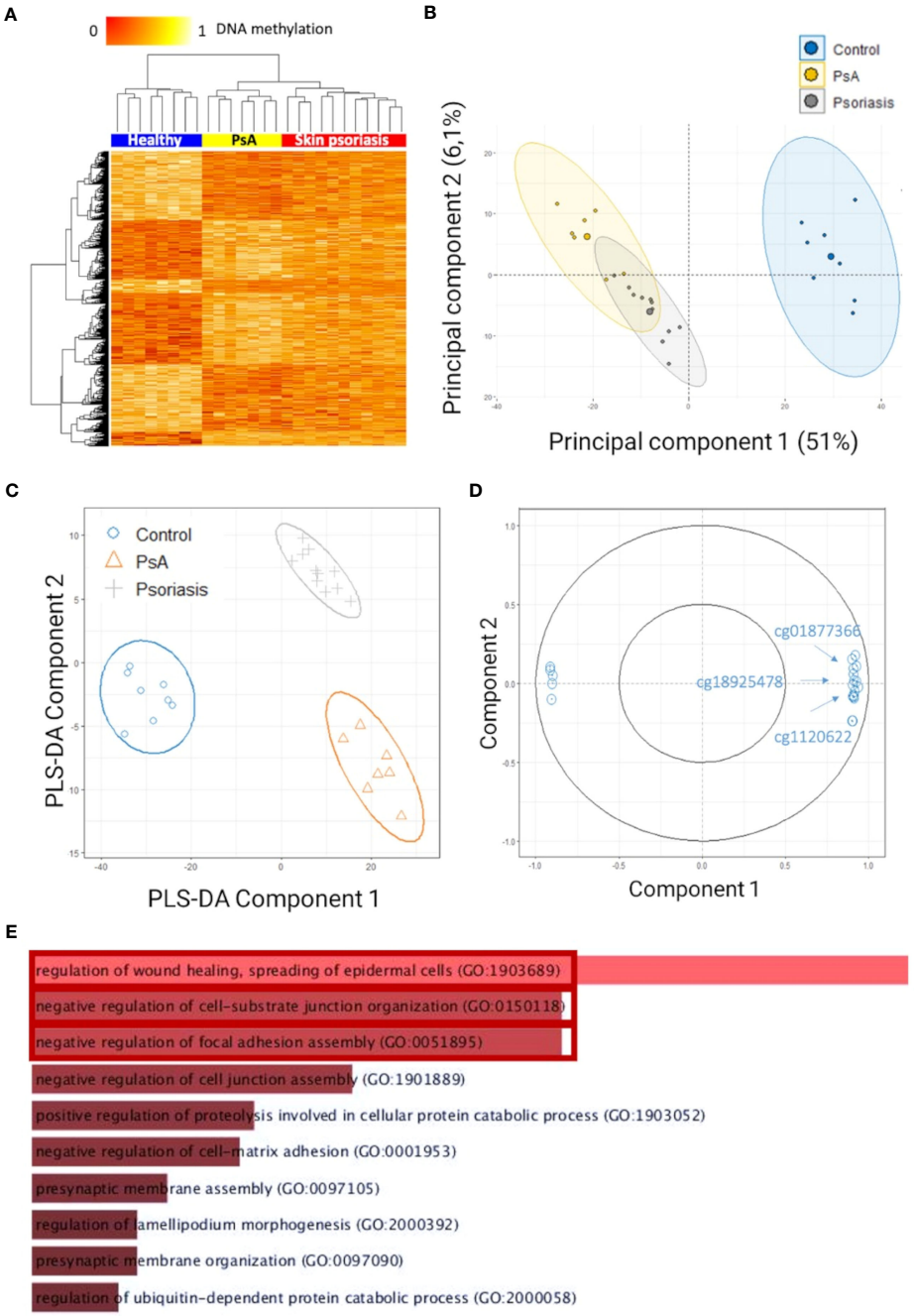

FIGURE 1
Differentially methylated CpGs in CD4$^+$ T cells distinguish psoriasis patients from healthy controls. **(A)** Heat map displaying differentially methylated positions (DMPs) between "all" psoriasis patients (skin psoriasis and PsA) and healthy controls (FDR < 0.05, |Δβ| > 0.1). Normalized DNA methylation levels are depicted on the top left, with red representing lower methylation and yellow indicating higher methylation levels. **(B)** Unsupervised Principal Component Analysis (PCA) of 883 DMPs identified between "all" psoriasis patients and healthy controls (FDR < 0.05, |Δβ| > 0.1). Principal Component (PC)1 and PC2 are displayed, explaining 51% and 6.1% of variance. **(C)** PLS-DA of 883 DMPs between "all" psoriasis patients and healthy controls (FDR < 0.05, |Δβ| > 0.1). **(D)** Correlation circle plot considering the "top" 20 CpGs with a correlation coefficient above|0.9| that primarily contribute to the definition of PLS-DA component 1 discriminating "all" psoriasis patients from healthy controls. **(E)** Bar diagram depicting results of Gene Ontology (GO) analysis considering hypermethylated genes containing at least one DMP in their promoter. Top 10 GO terms are represented, statistically significant pathways are framed in red.

patients can be separated from controls with an overlap between skin psoriasis and PsA (Figure 1B). Using supervised PLS-DA, samples from patients with skin psoriasis clustered independently from samples from PsA patients (Figure 1C). DMPs (N=20) (correlation cut-off 0.9) most predictive of psoriasis (PLS-DA component 1) included cg01877366 in Trafficking Protein Particle Complex Subunit 9 (*TRAPPC9*), cg1120622 in Reversionless 3-like (*REV3L*) and cg18925478 in Phosphatase and

Actin Regulator 2 (*PHACTR2*) (Figure 1D; Supplementary Table 3), all of which have previously been reported in the context of psoriasis, PsA (31, 32) or other autoimmune diseases (18).

3.4 Differential DNA methylation affects gene ontology and KEGG pathways

To identify biological processes associated with altered DNA methylation, KEGG pathway and GO analyses were performed. Only DMPs located in promotor regions (TSS1500, TSS200 and 5'UTR) were included in these analyses and have been divided into two categories: hypermethylated and hypomethylated. GO analysis delivered an enrichment of hypermethylated DMPs/genes, in biological processes involved in the regulation of cellular adhesion and signaling, including "regulation of wound healing, spreading of epidermal cells" (p=0.01), "negative regulation of cell-substrate junction organization" (p=0.03) and "negative regulation of focal adhesion assembly" (p=0.03) (Figure 1E; Supplementary Table 4).

Analysis of all genes with hypomethylated promoter regions failed to deliver statistically significant enrichment in GO and KEGG pathway analyses when comparing "all" psoriasis patients to healthy controls.

3.5 Differential DNA methylation discern skin psoriasis from PsA

Comparing DNA methylation profiles of patients with skin psoriasis with PsA patients, 2,949 DMPs uniquely associated with 1,084 genes were identified, including 2,230 hypomethylated and 719 hypermethylated CpGs (Table 2). Distinct methylation patterns were observed between psoriasis and PsA patients, and a higher number of DMPs were identified in comparison to the previous analysis that also included healthy controls (Figure 2A).

GO analysis for molecular function (but not KEGG pathway analysis) considering hypermethylated (but not hypomethylated) CpGs revealed enrichment of genes involved in "cyclic adenosine

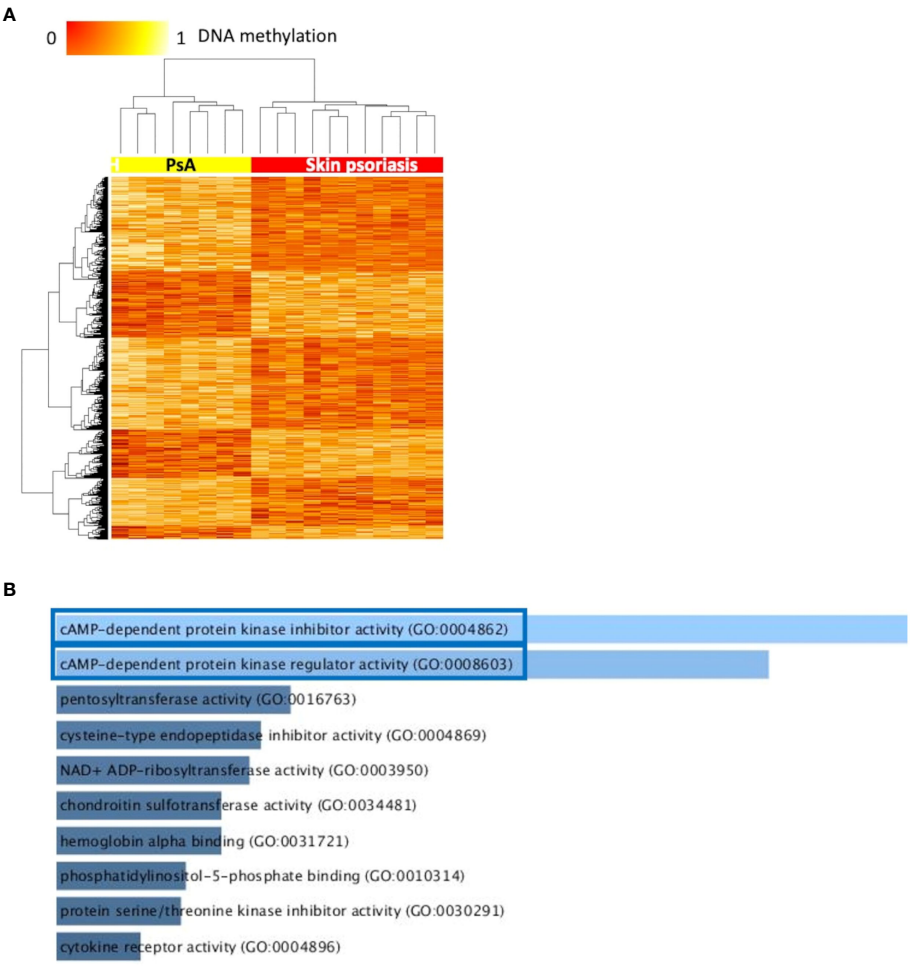

FIGURE 2
Differentially methylated CpGs differentiate skin psoriasis from PsA. **(A)** Heat map displaying differentially methylated positions (DMPs) between skin psoriasis and PsA (FDR < 0.05, $|\Delta\beta| > 0.1$). Normalized DNA methylation levels are displayed on the top left with red indicating reduced methylation and yellow indicating increased methylation levels. **(B)** Bar diagrams depict the results of Gene Ontology (GO) analysis of hypomethylated genes which presented at least one DMP in their promoter. Top 10 GO terms are represented, and the statistically significant pathways are framed in blue.

monophosphate (cAMP)-dependent protein kinase inhibitor activity" (GO:0004862, p=0.02) and "cAMP-dependent protein kinase regulator activity" (GO:0008603, p=0.03) (Figures 2B; Supplementary Table 5).

3.6 DNA methylation of IFN-associated genes differs between psoriasis patients and healthy controls

The IFN pathway plays a key role in the pathogenesis of psoriasis (33). Thus, we explored differential DNA methylation affecting IFN in $CD4^+$ T-cells (Supplementary Table 6). Genes associated with type I and/or type II IFNs and associated DMPs were identified using the *Interferome* database (28). Notably, 69.2% (379 of 548) of differentially methylated genes between "all" psoriasis patients and control are involved in IFN signaling with equal distribution between type I IFN (19.7%), type II IFN (21.9%), or both (21.9%) (Figure 3A; Supplementary Tables 6, 7). DNA methylation scores were calculated following the suggestion of Björk et al. (30). Scores were higher in "all" psoriasis patients when compared to healthy controls when considering genes related to type I IFNs (Figure 3B) and both type I and II IFNs (Figure 3D). No significant differences were seen when considering type II IFNs alone (Supplementary Figures 3A, B). Comparing skin psoriasis and PsA subgroups, significant differences were observed comparing psoriasis and healthy controls considering type I, type II and type I/II-IFN methylation scores (Supplementary Figures 4A–C). Considering IFN-associated methylation patterns across the three study subpopulations, displayed clustering of skin psoriasis patients separate from patients with PsA and/or healthy controls considering type I (Figure 3C) and type I/II IFN-related genes (Figure 3E).

3.7 Identification of differentially methylated regions

Larger differentially methylated genomic regions (DMRs) containing a minimum of 5 CpGs were investigated between groups. We identified 8 DMRs comparing "all" psoriasis patients to healthy controls, 27 DMRs when comparing skin psoriasis and healthy controls, and 5 DMRs in each of the comparisons of PsA versus healthy controls and skin psoriasis versus PsA (Supplementary Tables 8, 9). Two DMRs were shared between all comparisons (Figure 4A; Supplementary Tables 9): a first DMR in the 3' region of Growth Differentiation Factor 7 (*GDF7*) gene on chromosome 2, containing 9 CpGs located in the 3'-part of the gene, and 8 of which cover a CpG island (CGI) (Figure 4B, Supplementary Figure 5; Supplementary Table 9); a second DMR upstream of the Phosphatidylinositol Glycan Anchor Biosynthesis Class Z (*PIGZ*) gene, covering the 3' region of ENSG00000287265 described as PIWI-interacting RNA (piRNA; piR-53563), containing 6 CpGs (Figure 4C; Supplementary Figure 6, Supplementary Table 9). This first DMR extends into the 3'UTR of the *GDF7* gene, which can have an impact on gene expression of the transcript (34). The second DMR is distant from the transcriptional initiation site of the *PIGZ* gene but partially overlaps with a Histone H3 lysine 27 acetylation (H3K27ac) mark (associated with active enhancer function) in the human immortalized myelogenous leukemia cell line K562. This DMR also covers the 3'UTR of a piRNA which preserves the genomic integrity by suppressing mobile genetic element; its role in the context of psoriasis and PsA remains unclear.

Two DMRs separating "all" psoriasis patients from controls and skin psoriasis from PsA *vs.* control analyses appeared to be of special interest, region 1 encompassing cg04267224 to cg01516881 and region 2 cg07332563 to cg01171360. In fact, both regions overlap and cover the 5'UTR as well as a CGI and are enriched for the H3K27ac mark for the Dual Specificity Phosphatase 22 (*DUSP22*) gene, which may affect its transcription (Supplementary Figure 7; Supplementary Table 9). DMRs containing a minimum of 10 and 15 CpG per region are displayed in Supplementary Tables 10, 11, respectively.

3.8 DNA methylation signatures "normalize" in response to treatment

To explore effects of cytokine-blocking agents on DNA methylation patterns in $CD4^+$ T-cells from psoriasis and PsA patients, DMP analysis was performed in samples collected before and after treatment with either IL-17 or TNF inhibitors (Table 1). Comparing samples collected before versus after treatment initiation, a total of 1,996 DMPs affecting 1,438 genes were identified, including 878 hypomethylated and 1,118 hypermethylated CpGs (Table 2). DNA methylation patterns before treatment (naïve) were distinct from post-treatment patterns (Figure 5A), which were comparable to healthy controls. KEGG pathway and GO analyses were conducted considering only DMPs within promoter regions (TSS1500, TSS200, 5'UTR). Considering hypomethylated positions, KEGG pathway and GO analyses for biological processes and molecular function delivered enrichment of the "glutathione metabolism" pathway (Figure 5B; Supplementary Tables 12) (p=0.05). Analysis of all the genes with hypermethylated DMPs in their promoters failed to identify differences before versus after treatment initiation.

3.9 DNA methylation scores correlate with skin disease activity

Next, we explored the relationship between skin psoriasis disease activity, based on PASI scores, and changes in DNA methylation including matched samples from skin psoriasis and PsA patients before and after treatment. Because all patients were treated with IL-17 or TNF inhibitors, we focused on DMPs in genes related to the IL-17/TNF pathway using the *WikiPathways* database (29). Of 1,996 DMPs identified in the comparison before versus after treatment, 8 CpGs annotated to 7 unique genes were related to this pathway. Beta values were calculated and, for each position,

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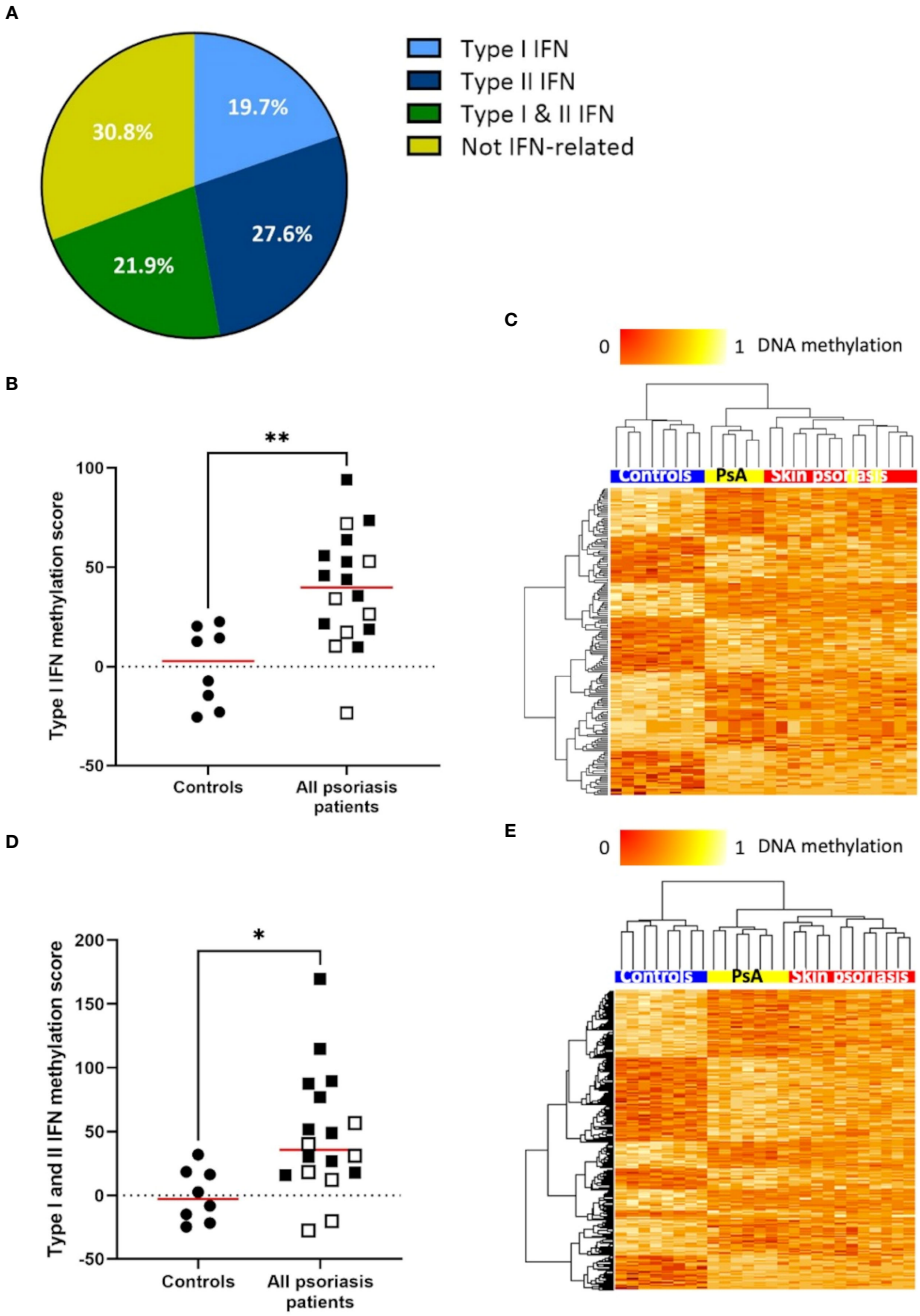

FIGURE 3
DNA methylation of IFN-associated genes differs between psoriasis patients and healthy controls. **(A)** Pie chart representing the proportion of genes (with a minimum of 1 DMP between "all" psoriasis patients and healthy controls (FDR < 0.05, $|\Delta\beta| > 0.1$) under the control of type I interferons (IFNs), type II IFNs, their combination, and not-IFN-related pathways. **(B)** Type I IFN methylation scores calculated in "all" psoriasis patients and healthy control according to the method reported by Björk et al. (30). The median is reported in red, skin psoriasis patients are reported by a black square and PsA patients by a white square, results from Mann-Whitney tests are displayed (**p < 0.01). **(C)** Heat map displaying differentially methylated positions (DMPs) of genes under the control of type I IFNs between "all" psoriasis patients and healthy controls (FDR < 0.05, $|\Delta\beta| > 0.1$). Normalized DNA methylation levels are displayed on the top left with red indicating reduced methylation and yellow indicating increased methylation levels. **(D)** Type I and II IFN methylation scores calculated in "all" psoriasis patients and healthy controls. The median is reported in red, skin psoriasis patients are reported by a black square and PsA patients by a white square, results from Mann-Whitney tests are displayed (*p < 0.05). **(E)** Heat map displaying DMPs of genes under the control of type I and II IFN between "all" psoriasis patients and healthy controls (FDR < 0.05, $|\Delta\beta| > 0.1$). Normalized DNA methylation levels are displayed on the top left with red indicating reduced methylation and yellow indicating increased methylation levels.

their correlation with PASI scores was estimated. Only positions with a Pearson correlation score above $|0.7|$ were kept to calculate methylation scores (30). After treatment initiation, methylation scores of genes related to the IL-17/TNF pathway increased significantly, reaching levels comparable to those observed in healthy individuals (Figure 5C) and an inverse correlation between methylation scores and PASI scores was observed ($R=-0.72$, $p=0.008$, Figure 5D).

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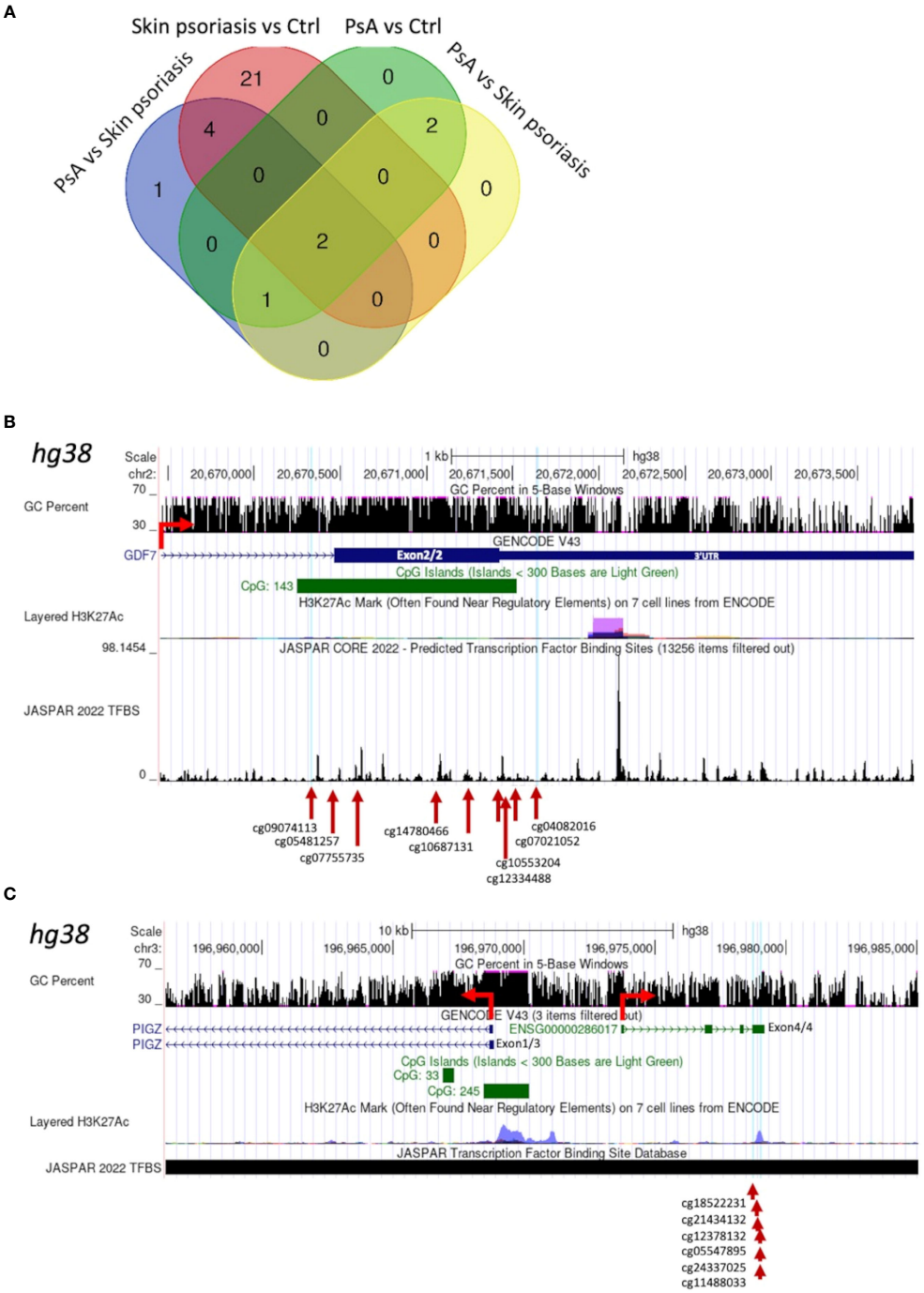

FIGURE 4
Shared Differentially Methylated Regions (DMRs) in CD4$^+$ T-cells from psoriasis patients and healthy control. **(A)** Venn diagram displaying overlapping and differentially methylated regions (DMRs) with ≥5 CpGs per region, in "all" psoriasis patients versus controls (ctrl), controls versus skin psoriasis, controls versus psoriatic arthritis (PsA), and skin psoriasis versus PsA. Map of a DMR within the 3'UTR of *GDF7* **(B)** and a DMR upstream of the Transcription Start Site (TSS) of *PIGZ* **(C)** using the UCSC genome browser database (https://genome.ucsc.edu). The GC content of this locus, the presence of CpG islands (green square), its H3K27ac active marker coverage and its TFBS (Transcription Factor Binding Site) coverage (from the JASPAR database) are shown. The red arrow indicates the sense of transcription. At the bottom of the image, the different CGs within DMRs are indicated.

4 Discussion

The prediction of joint disease in psoriasis patients who will develop PsA, as well as early and correct diagnosis of PsA patients in the absence of skin disease are as challenging as crucial. The importance of early diagnosis lies in the destructive nature of joint involvement in PsA and the concept of a therapeutic "window of opportunity" in inflammatory musculoskeletal diseases, in which disease progression and damage may be prevented by the termination of inflammation (35, 36). Failure to initiate appropriate treatment in a timely fashion may result in

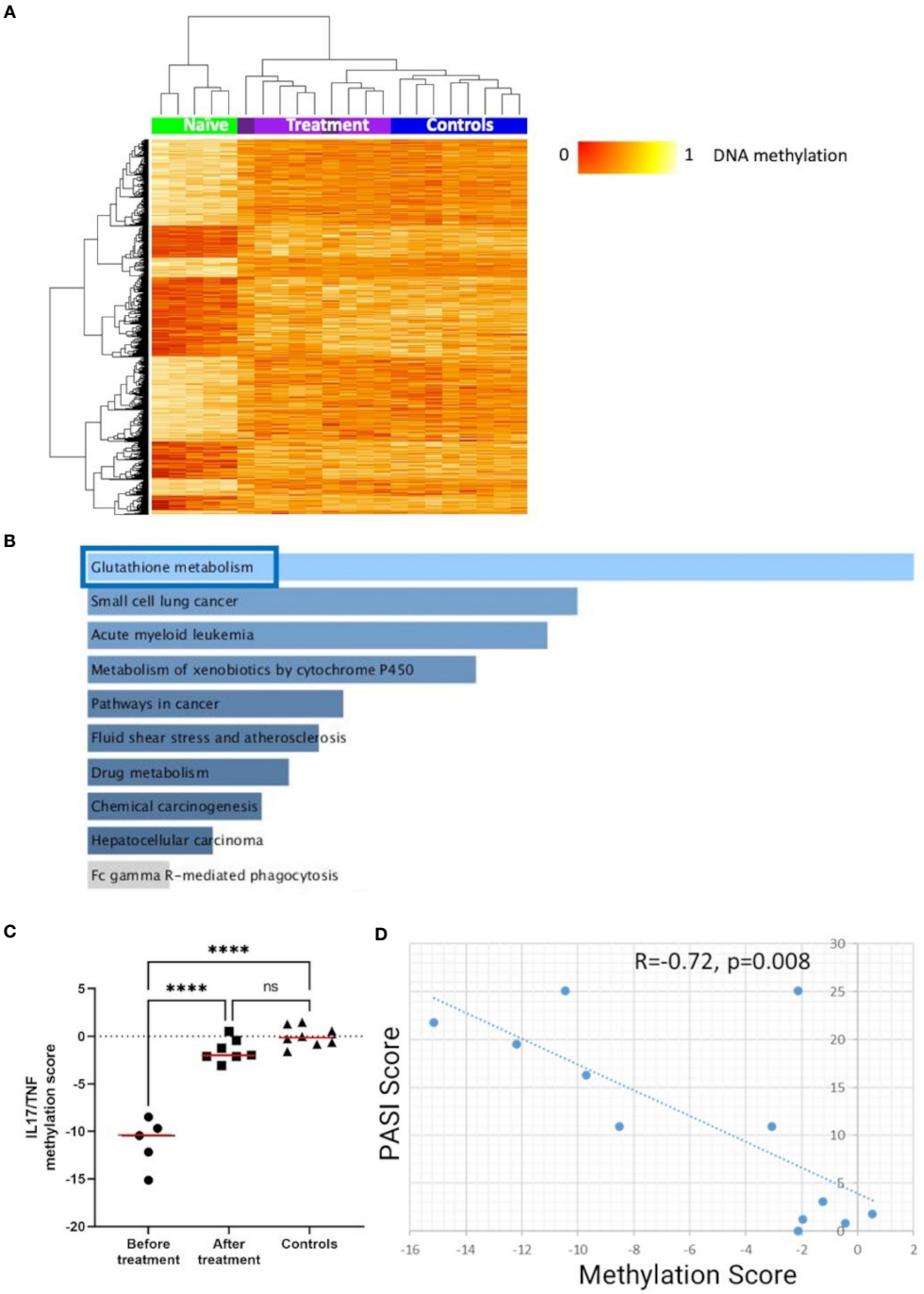

FIGURE 5
DNA methylation signatures "normalize" in response to treatment. **(A)** Heat map displaying differentially methylated positions (DMPs) in CD4$^+$ T-cells from "all" psoriasis patients (N = 4 psoriasis and N = 1 psoriatic arthritis) before and after treatment (N = 5 psoriasis and N = 4 psoriatic arthritis) (FDR < 0.05, $|\Delta\beta| > 0.1$) with TNF or IL-17A inhibitors. Light purple and dark purple indicate the first and (where available) the second time-point after-treatment. Normalized DNA methylation levels are shown on the right with red indicating reduced methylation and yellow indicating increased methylation levels. **(B)** Bar diagrams depict results of the KEGG pathway enrichment analysis of hypermethylated genes which presented at least one DMP in their promoter. Top 10 pathways are represented, and the statistically significant ones are framed in blue. **(C)** IL17A/TNF methylation scores were calculated in patients before and after treatment and. in healthy control. ****$p \leq 0.0001$, Anova and Tukey's multiple comparisons. Medians are reported in red. **(D)** Correlation analysis between DNA methylation and PASI scores in the patient cohort before and after treatment. After assessing the Gaussian distribution, Pearson was used to measure the correlation.

irreversible joint damage and deformities, leading to chronic pain, disability and decreased quality of life (4, 37).

While some clinical predictors of joint involvement have been proposed, including 3 affected sites or more, high severity of skin disease, onychopathy, scalp lesions and intergluteal/perianal psoriasis (38, 39), no reliable clinical or laboratory biomarkers for the development of PsA are available. This study revealed distinct DNA methylation profiles in CD4$^+$ T-cells that distinguish psoriasis

patients from healthy individuals and revealed additional differences between patients with skin psoriasis and PsA. These observations underscore the importance of altered epigenetic mechanisms in the complex pathophysiology of psoriasis and PsA (40–42) and promise potential as diagnostic and/or prognostic biomarkers and future treatment targets.

A growing body of literature suggests that epigenetic modifications are involved in the molecular pathogenesis of psoriasis and PsA, including dysregulation of adaptive immune responses (43, 44), T-cell differentiation and activation (45), and keratinocyte dysfunction (46). The development of targeted therapeutic interventions to correct epigenetic signatures, e.g., DNA methylation patterns, may therefore permanently resolve immune dysregulation and improve outcomes in psoriasis, PsA and other T-cell mediated autoimmune/inflammatory diseases (47).

Given the recognized role of effector T-cells in the pathophysiology of psoriasis and PsA (6, 48–50), the here presented study focused on examining DNA methylation patterns in $CD4^+$ T-cells. One crucial aspect of $CD4^+$ T-cell involvement in psoriasis and PsA is their differentiation into distinct effector subsets, particularly T-helper 17 (Th17) and T-helper 22 (Th22) cells (51, 52). The IL-23/Th17 axis has emerged as a key pathway in chronic inflammatory conditions, including psoriasis and PsA (53). IL-23 promotes the survival and maintenance of Th17 cells, which produce IL-17, IL-22 and other inflammatory effector cytokines involved in psoriasis (54, 55). High expression of IL-23 has been observed in psoriatic skin lesions, and experimental models have demonstrated that IL-23 injection can induce epidermal hyperplasia through keratinocyte proliferation in mice (56). To further support the importance of this pathway, blockade of IL-17 (57–59) or IL-12/IL-23 (60–62) are effective treatment options in psoriasis and PsA. The recently identified Th22 effector T cell subset, characterized by IL-22 and IL-13 production, has also been implicated in psoriasis (63). Similarly to IL-17, IL-22 can stimulate keratinocyte proliferation, while impeding keratinocyte differentiation and inducing neutrophil infiltration in mice (64, 65).

Using Gene Ontology enrichment analysis, this study observed differential DNA methylation between controls and "all" psoriasis patients in promoter regions (one or more DMPs) affecting "regulation of wound healing, spreading of epidermal cells", "negative regulation of cell-substrate junction organization" and "negative regulation of focal adhesion assembly". Previous studies reported an involvement of accelerated wound healing in psoriasis (66) underscoring the significance of this process in its pathogenesis. Differential methylation of genes associated with cell-substrate junction organization and focal adhesion assembly has previously been observed in association with Munro micro-abscess formation, a histological hallmark in early psoriatic lesions and during disease flares (67). Lastly, in $CD8^+$ T-cells, altered DNA methylation affecting genes associated with "cell junction assembly" was identified in psoriasis patients (skin psoriasis and PsA) when compared to healthy controls (14). These observations suggest that altered DNA methylation may affect biophysical properties of the skin and facilitate the recruitment of neutrophils to the epidermis.

To further explore differences between "all" psoriasis patients and controls, PLS-DA were performed and identified 20 DMPs (in component 1) that strongly contributed to the discrimination between groups. Among these, the CpG site cg01877366 located in *TRAPPC9*, encoding for trafficking protein particle complex subunit 9, was of special interest. *TRAPPC9* is involved in the activation of the transcription factor nuclear factor (NF)-κB through phosphorylation of the IκB kinase complex. A previous study investigating DNA methylation profiles in PsA patients identified *TRAPPC9* as a candidate gene for the prediction of failure to respond to TNF-inhibitors (68). Moreover, we identified cg1120622, located in the *REV3L* gene, encoding a DNA polymerase ζ catalytic subunit. In mice, deletion of *REV3L* resulted in impaired wound healing and excessive proliferation of the epidermis (69). Single nucleotide polymorphisms (SNPs) in the intergenic region between *REV3L* and the neighboring gene *KIAA1919* are associated with the development of rheumatoid arthritis (RA) in black South Africans (70). Furthermore, *REV3L* has recently been identified as a candidate for gene therapy for psoriasis and PsA using a genetics-dependent drug target prioritization approach (31). Another CpG site contributing to the separation of patients and controls was cg18925478 within the *PHACTR2* gene encoding for the phosphatase and actin regulator 2 that has previously been linked with the development of inflammatory bowel disease (71). Furthermore, the PHACTR2 protein was suggested as a potential marker of disease exacerbation in systemic lupus erythematosus (SLE) (72).

When comparing controls to "all" psoriasis patients, almost two-thirds of the genes associated with DMPs were involved in or affected by type I IFN, type II IFN, or both, and DNA methylation scores considering IFN-associated genes allow prediction of disease. These findings support previous reports implicating type I and type II IFN dysregulation in psoriasis (73, 74) and PsA (75) that may be the target of therapeutic interventions, e.g. with Janus kinase (JAK) inhibitors that are now approved for the treatment for moderate to severe psoriasis and PsA patients who have had an inadequate response or intolerance to methotrexate or other DMARDs (76–78) (79). Because of difficulties predicting disease progression from psoriasis to PsA and diagnosing PsA in the absence of skin involvement, DNA methylation patterns were investigated as possible predictors of disease phenotypes and outcomes. Approximately 3,000 DMPs were identified between skin psoriasis and PsA patients. However, because all patients enrolled in this study had developed skin disease prior to arthritis, it was not possible to determine whether the observed differences in DNA methylation profiles were present at disease onset and therefore predict the development of PsA or whether they developed with disease progression. Prospective studies are required to determine whether DNA methylation profiling can identify psoriasis patients at risk of developing PsA. Indeed, skin psoriasis patients treated with biologic DMARDs may be at a decreased risk of developing PsA (80) when compared to patients treated with other, less "aggressive" treatments, which underscores the significance of early diagnosis and therapeutic intervention.

Investigating possible functional implications of DMPs between patients with skin psoriasis and PsA, GO terms related to cAMP-dependent protein kinase (PKA) activity were enriched. Cyclic AMP is a key second messenger of extracellular ligands that is

involved in a wide range of cellular responses (81). The homeostasis of cAMP is primarily regulated by adenylate cyclases and phosphodiesterases (PDE), responsible for the conversion of adenosine triphosphate (ATP) into cAMP and the degradation of cAMP, respectively. High intracellular levels of cAMP trigger activation of PKA by binding to its regulatory region and mediating dissociation of the PKA catalytic subunit, which phosphorylates various target proteins. Furthermore, PKA phosphorylates and regulates the activity and expression of the cAMP-response element modulator (CREM) protein family, a group of transcription factors playing an important role in various cellular functions (82). Increased expression of CREMα in $CD4^+$ T-cells has been linked with effector cytokine expression in psoriasis, PsA (83) and other autoimmune diseases, including SLE (84) and juvenile idiopathic arthritis (85). The involvement of the cAMP-PKA pathway in the pathogenesis of psoriasis and PsA is further supported by the efficacy of PDE-4 inhibitors, with the European Medicines Agency (EMA) and Food and Drug Administration (FDA) having approved apremilast for the treatment of these conditions (86–88). Inhibition of PDE4 leads to high levels of cAMP, which in turn, activates PKA resulting in phosphorylation of transcription factors, such as cAMP Response Element-binding protein (CREB) and activating transcription factor-1 (ATF-1). As a result, phosphorylated CREB and ATF-1 promote the expression of anti-inflammatory cytokines, while inhibiting NF-κB activity. These findings highlight the importance of the cAMP-PKA pathway in psoriasis and PsA.

Contiguous differentially methylated CpG sites can compose differentially methylated regions (DMRs), which may exert regulatory effects on various biological processes, including cell function, proliferation or ageing (89–91). DMRs are tissue-specific and may be associated with disease stages and outcomes in several autoimmune/inflammatory conditions (92), such as RA (93), SLE (94) and Sjögren's syndrome (95). Since coordinated changes in DNA methylation in broader genomic regions may have greater downstream biological consequences and influence disease development and progression, we performed DMR analysis in the sub-cohorts of the study (96, 97). Among all DMRs identified here, two were shared between all comparisons (controls versus skin psoriasis versus PsA): *GDF7* on chromosome 2 and *PIGZ/piRNA* on chromosome 3. The *GDF7* gene encodes for a secreted ligand of the Transforming Growth Factor Beta (TGF-β) superfamily and enhances regulatory T-cell (Treg) function through the upregulation of Forkhead Box P3 (FOXP3) and Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA4) (98). Reduced expression of *GDF7* was observed in $CD4^+$ T-cells from SLE patients, suggesting its link to impaired function of Treg cells (99). The *PIGZ* gene encodes for a mannosyl-transferase involved in glycosylphosphatidylinositol (GPI)-anchor biosynthesis. GPI-anchored membrane proteins have been reported to be down-regulated in psoriatic skin lesions (100) and metabolites in GPI-anchor biosynthesis are altered in psoriasis patients (101).

Another DMP separating "all" psoriasis patients from controls was *DUSP22*, encoding for a protein phosphatase responsible for activation of the c-Jun NH2-terminal kinase (JNK) signaling pathway that is dysregulated in psoriasis (102), SLE (103) and ankylosing spondylitis (104). DUSP22 contributes to the inactivation of Lymphocyte Cell-Specific Protein-Tyrosine Kinase (Lck) during the deactivation phase of T-cell receptor signaling, thereby suppressing T-cell-mediated immune responses and inflammation (105). In RA, promoter hypomethylation of *DUSP22* associates with erosive disease (106).

Objective assessment and quantification of treatment responses can be challenging but is essential for disease monitoring in clinical practice and clinical trial settings (107, 108). Thus, we explored the impact of cytokine-blocking agents (IL-17 or TNF inhibitors) on DNA methylation patterns in "all" psoriasis patients. A total of 1,996 DMPs were identified between treatment naïve patients and those who received biological DMARDs, which compared to methylation in healthy controls. The observed shift in methylation patterns in response to treatment indicates a significant impact of these agents on the DNA methylation landscape. Remarkably, pathway analysis of DMPs associated with treatment delivered a significant enrichment of genes associated with glutathione (GSH) metabolism. GSH, the most abundant endogenous antioxidant, plays a role in mitigating oxidative stress and regulating immune function. It is crucial for effector T-cell functions through the regulation of metabolic activity and has been linked with the pathogenesis of several autoimmune diseases (109), including psoriasis (110). In a recent randomized clinical trial investigating the IL-17A inhibitor secukinumab, lesional skin transcriptomic profiles showed Glutathione-S-transferase α3 among the top 10 upregulated genes after 12 weeks of treatment (111). This suggests that GSH metabolism plays a key role in T-cell mediated autoimmune/inflammatory diseases that may be targeted directly by future therapeutic interventions.

Because all patients enrolled in this study with samples available before and after treatment initiation received IL-17 or TNF-inhibitors, we focused DNA methylation analyses on this pathway by calculating DNA methylation scores focused on DMPs in genes involved in the IL-17/TNF pathway. This approach delivered an inverse correlation between DNA methylation and skin disease activity, as measured by PASI scores. Comparable results were observed in a previous study investigating DNA methylation in $CD8^+$ T-cells in psoriasis and PsA (14). This suggests that cytokine-blocking agents can modulate DNA methylation patterns and restores "normal" profiles. DNA methylation profiles may therefore represent a potential biomarker for treatment response, but validation in larger prospectively assembled cohort is required.

Notably, numbers and proportions of $CD4^+$ T-cells and their subsets did not differ across psoriasis, PsA and healthy controls. Thus, the observed differences among cohorts reflect variations in DNA methylation profiles of $CD4^+$ T-cells more than "simple" shifts between their subsets.

While this study offers valuable insights into molecular mechanisms involved in skin psoriasis and PsA, it has limitations. The sample size was limited due to the relative rarity of PsA, especially of treatment naïve patients recruited here. Thus, we were also unable to enroll participants from minority ethnic groups that may exhibit differential DNA methylation patterns. Despite the potential bias introduced by age differences between psoriasis and PsA patients (112), normalization techniques and correction using

the *ComBat* function from the ChAMP package were applied to minimize the impact of age differences on DNA methylation. Because of lacking information regarding active joint counts and joint disease activity in the PsA patients, we were not able to correlate these with DNA methylation patterns. Furthermore, this study was not able to assess the impact of DNA methylation on gene transcription, as RNA sequencing was not performed. Validation of DNA methylation patterns as a tool for the prediction of disease progression from skin psoriasis to PsA requires larger unrelated cohorts. Furthermore, to gain deeper insights into the transition from skin psoriasis to PsA, it will be important to include a subgroup of patients with PsA without pre-existing skin involvement.

5 Conclusions

DNA methylation profiles in $CD4^+$ T-cells discriminate psoriasis patients from healthy individuals and skin psoriasis from PsA patients. DNA methylation may represent a stable and reliable biomarker candidate for predicting disease progression from skin psoriasis to PsA, monitoring disease activity and evaluating treatment response. Future application clinical practice requires prospective validation in independent cohorts.

Data availability statement

The datasets presented in this study can be found in online repository. The names of the repository/repositories and accession number(s) can be found below: GSE236694 and GSE236695 (Gene Expression Omnibus).

Ethics statement

The studies involving human participants were reviewed and approved by Faculty of Medicine Carl Gustav Carus, TU Dresden, Dresden, Germany. The patients/participants provided their written informed consent to participate in this study.

Author contributions

VN and AC analyzed flow cytometric datasets, performed the bioinformatic analysis and wrote the first draft of the manuscript. SH, FS, and SR isolated immune cells and DNA. SA consented patients and collected clinical data and biospecimen. CH oversaw all experimental and analytic steps and revised the first draft of the manuscript. CH, SH, LM, and SA conceived the study. VN, AC, LM, SH, SA, and CH were involved in individual or all steps of data analysis and overall data interpretation. All authors read, commented and agreed to the final version of the manuscript and the authors' list. All authors contributed to the article.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1245876/full#supplementary-material

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