# EPIGENETIC ASPECTS OF AUTOIMMUNE DISEASES

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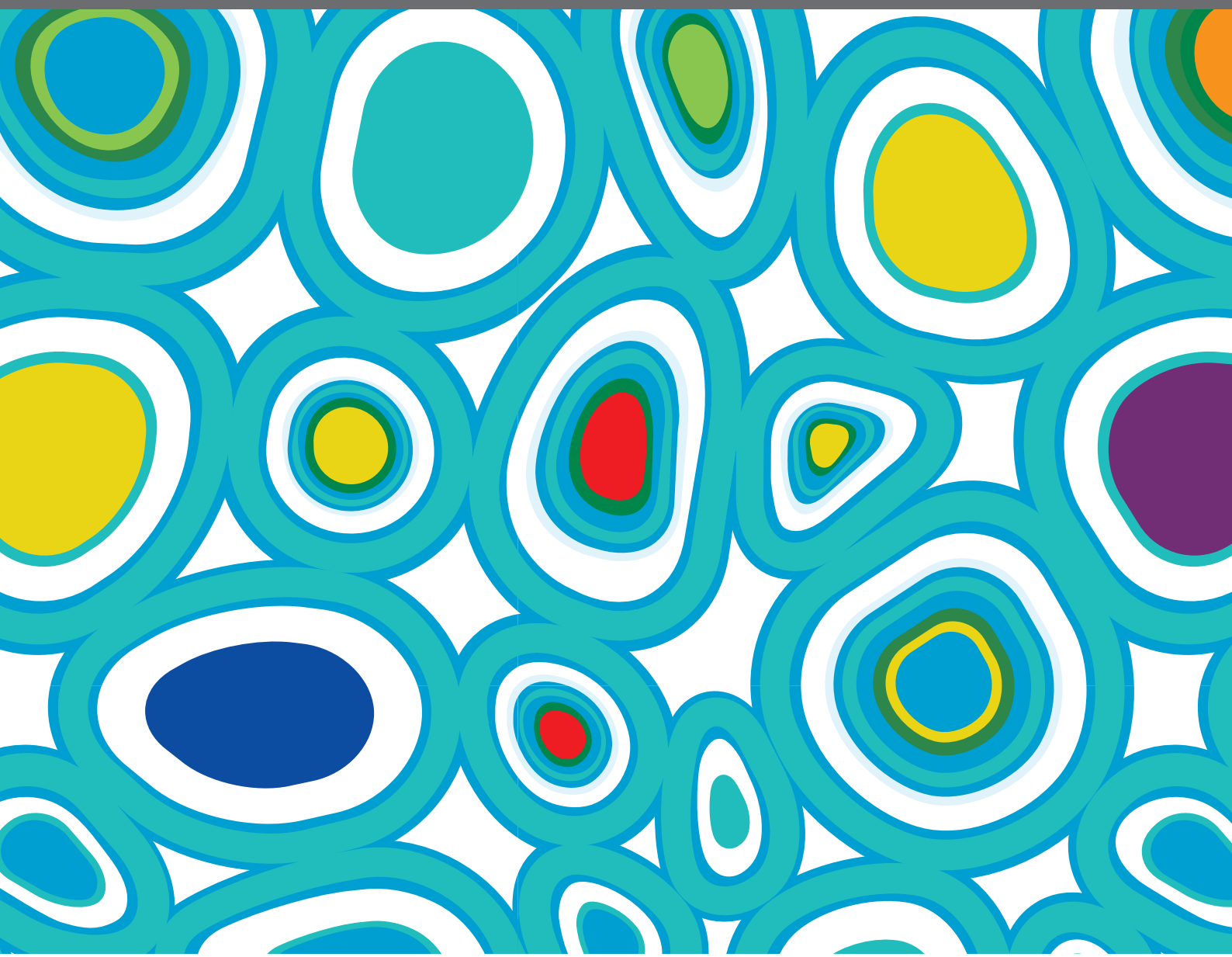

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# EPIGENETIC ASPECTS OF AUTOIMMUNE DISEASES

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# Editorial: Epigenetic aspects of autoimmune diseases

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**epigenetics, lupus (SLE), multiple sclerosis, rheumatoid arthritis, sjogren's syndrome**

Editorial on the Research Topic
Epigenetic aspects of autoimmune diseases

Genetics, environmental factors, and epigenetics all contribute to autoimmune disease onset and progression (Gulati and Brunner, 2018). Most of the earlier research on autoimmune diseases focused on genetics and environmental factors. Research into genetics, in attempting to identify specific risk alleles has had limited success, for example, identifying HLA-DRB1, a complex of genes coding for cell surface proteins, has emerged as a risk allele for autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Niu et al., 2015). However, genetic findings have not been sufficient to explain the majority of RA and SLE cases, autoimmune diseases in general, or the typical delay in initial onset, occurring later in life (early to mid-adulthood), in most autoimmune diseases. Even with identification of a risk allele, it is not clear if it is a cause of the disease or just a subsidiary factor. Likewise, environmental factors (e.g., viruses, toxins, bacteria, etc.) also present confusion as to their role since a variety of environmental factors and combinations can be involved in triggering onset of an autoimmune disease (Arleevskaya et al., 2016; Arleevskaya et al., 2020). For example, Epstein-Barr virus (EBV) is suspected of a role in autoimmune diseases, especially multiple sclerosis (MS) (Bjornevik et al., 2022). However, almost all adults have had exposure to EBV but only a small percentage develop MS and onset can be many years after initial infection with EBV. It may be that another environmental factor and/or a genetic risk allele needs to be involved, such as an especially heavy cellular viral load of EBV that may occur by viral binding and entry using HLA-DR cell surface proteins as opposed to other HLA types (Agostini et al., 2018).

Research into the involvement of epigenetics in autoimmune diseases has been steadily increasing in the past two decades. Epigenetics is the control of gene expression or suppression without changing the underlying DNA sequence of the gene (Renaudineau et al., 2011). Epigenetics can involve methylation of DNA, which typically suppresses the underlying gene, or demethylation of the DNA as a step towards expression (Fali et al., 2014). Coordinated with the DNA methylation state are

modifications to histone residues based on the "histone code" that further suppresses or opens the gene by altering how tightly the DNA is held by nucleosomes. In addition, non-coding RNA transcripts (i.e., they do not code for a protein) can add to epigenetic control, such as the X Inactivation Specific Transcript (XIST) RNA involved in silencing of the extra X chromosomes in female cells (Bost et al., 2022). Such RNAs can add structural support to heterochromatin and/or recruit the enzymes that modify the DNA and histones.

In this Research Topic, we have gathered articles discussing epigenetics in a variety of autoimmune diseases. Due to autoimmune tautology (i.e., common characteristics among different autoimmune diseases) as described previously by Anaya, we believe autoimmune researchers focused on one disease can learn from the insights and findings of researchers working on other autoimmune diseases (Anaya, 2012; Anaya, 2017; Arvaniti et al., 2019). Li and colleagues present an overview of DNA methylation by discussing its involvement in development of immune cells and in autoimmune diseases. They also provide discussion of aberrant DNA methylation modifications discovered in important disease-related cell types. With regards to specific diseases, Kabeerdoss and colleagues discuss DNA methylation in Takayasu Arteritis, an autoimmune vasculitis of the aorta. In addition, Charras and colleagues discuss a correlation between DNA methylation patterns in $CD8^+$ T-cells and clinically active psoriasis in Psoriatic Arthritis, and Vecellio and colleagues present insights into DNA methylation patterns in monozygotic twins discordant for in psoriatic diseases.

RNA methylation and numerous other RNA modifications are important epigenetic biomarkers of interest in relation to normal cellular functions and diseases. Such modifications are especially important in T cell maturation and can impact RNA localization, translation, and alternate splicing among other functions (Chao et al., 2021). Wang and colleagues present a discussion of the m6A RNA methylation with regards to autoimmune diseases and the immune system. Lv and colleagues discuss current understanding of RNA methylation in systemic lupus erythematosus, and Yao and colleagues present expression profiles of mRNAs and long non-coding RNAs in Graves' disease.

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

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REVIEW
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# RNA Methylation in Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is an autoimmune disease with complicated clinical manifestations. Although our understanding of the pathogenesis of SLE has greatly improved, the understanding of the pathogenic mechanisms of SLE is still limited by disease heterogeneity, and targeted therapy is still unavailable. Substantial evidence shows that RNA methylation plays a vital role in the mechanisms of the immune response, prompting speculation that it might also be related to the occurrence and development of SLE. RNA methylation has been a hot topic in the field of epigenetics in recent years. In addition to revealing the modification process, relevant studies have tried to explore the relationship between RNA methylation and the occurrence and development of various diseases. At present, some studies have provided evidence of a relationship between RNA methylation and SLE pathogenesis, but in-depth research and analysis are lacking. This review will start by describing the specific mechanism of RNA methylation and its relationship with the immune response to propose an association between RNA methylation and SLE pathogenesis based on existing studies and then discuss the future direction of this field.

**Keywords: RNA methylation, SLE, m6A, m5C, immune**

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a common chronic systemic autoimmune disease that mainly affects young or middle-aged women, with a male-to-female incidence ratio of approximately 1:9 (Stojan and Petri, 2018); men are affected by more severe renal injury (Ramírez Sepúlveda et al., 2019). The incidence rates for SLE range from 0.9 to 7.4 per 100,000 persons/year and differ by sex, region and population (Gergianaki et al., 2017; Stojan and Petri, 2018; Tanaka et al., 2020). For the Asia Pacific region, a meta-analysis showed that the incidence rates of SLE (per 100,000 per year) ranged from 0.9 to 8.4 (Tanaka et al., 2020).

Both environmental factors and genetic susceptibility play vital roles in SLE development. Collectively, evidence has shown that the sustained production of autoantibodies, complement activation, immune complex deposition, neutrophil extracellular traps, lymphocyte signaling and inflammation are all the key mechanisms underlying the loss of tolerance and tissue damage (Tsokos et al., 2016). Patients with SLE can present with diverse organ involvement, such as skin,

musculoskeletal, kidney, heart, and lung involvement (Smith and Gordon, 2010; Tsokos et al., 2016). Moreover, patients with SLE have a high overall risk of malignancy (Choi et al., 2017). At present, the early diagnosis of SLE is still based on clinical symptoms and signs, a laboratory examination and biopsy. A recent study compared three classification criteria for evaluating SLE: the European League Against Rheumatism (EULAR)/American College of Rheumatology (ACR)-2019 (Aringer et al., 2019), Systemic Lupus International Collaborating Clinics (SLICC)-2012 (Petri et al., 2012) and ACR-1997 criteria (Hochberg, 1997). An early SLE cohort study found that using these three evaluation criteria might miss or delay the classification and diagnosis of a significant subset of patients with moderate/severe SLE. The general therapeutic approach mainly relies on glucocorticoids and immunosuppressive drugs (ISDs) (Kuhn et al., 2015) with unavoidable side effects, and thus targeted therapy remains an urgent need. Although some progress has been achieved in the early diagnosis and treatment of SLE, the prognosis of patients with SLE is still not optimistic, and the medical cost is high. Therefore, further in-depth exploration of the pathogenesis of SLE is very important for its early diagnosis and the search for new therapeutic targets.

In recent years, an increasing number of scholars have focused their attention on the field of epigenetics, such as DNA methylation, which has led to a better understanding of the pathogenesis of SLE (Xiao and Zuo, 2016). However, with advances in RNA methylation research, scholars have found that RNA methylation is very important in the immune response and is closely related to SLE. RNA, an important intermediate product of gene expression, is also regulated by chemical modification after transcription and during translation through a process collectively referred to as epitranscriptomics (Saraceno et al., 2016). Similar to chemical modifications of DNA and histones, chemical modifications of RNA also regulate gene expression through mechanisms such as changing the structural properties of RNA or changing the affinity of mRNA for ribosomes. The discovery, continuous exploration, and in-depth study of chemical modifications in RNA have led epigenetic research to a new level and provided a new direction for further exploring the pathogenesis of diseases and developing new therapeutic strategies.

Therefore, this article will start by describing RNA methylation, summarize the application of RNA methylation in immunology, and speculate on the relationship between RNA methylation and the pathogenesis of SLE to provide more ideas for future research.

## THE SPECIFIC MECHANISM OF RNA METHYLATION

Chemical modifications of cellular RNAs are natural and abundant. The dynamic nature and increasing number of RNA modifications provide new possibilities for rapidly altering gene expression to adapt to specific environments. Due to the development of genomic approaches, various modifications on RNA have been identified and investigated. As of 2017, more than 150 distinct chemical modifications on RNA have been detected (Boccaletto et al., 2018).

$N^6$-methyladenosine (m6A) is the most widespread and investigated modification of mammalian mRNA and has a broad range of critical functions in development (Kasowitz et al., 2018), cancer (Ma S. et al., 2019), and viral infection (Williams et al., 2019). Pseudouridine (Ψ) was the first structurally modified nucleoside identified in the 1950s, and is known as the fifth nucleoside (Cohn and Volkin, 1951). Ψ generates an extra hydrogen bond donor at the newly formed N1 position, which increases the structural stability of the RNA and translation efficiency and accuracy (McKenney et al., 2017). The presence of N1mΨ (N1-methyl-pseudouridine (N1mΨ) in mRNA favors ribosome recycling on the same mRNA or *de novo* ribosome recruitment (Svitkin et al., 2017). 5-Methylcytidine (m5C) has long been studied in DNA. In RNA, m5C levels are 3–10-fold rarer than m6A levels (Legrand et al., 2017). Although the biological function of m5C in eukaryotic mRNA is just beginning to become clear, it is postulated to have a powerful function in regulating cellular processes (Trixl and Lusser, 2019).

Three types of molecules are involved in RNA methylation: writers, erasers and readers. Writers, namely, methyltransferases, transfer methyl groups to RNA in the form of protein complexes, individual proteins of which might have specific functions or integrate different signals. Erasers, namely, demethylases, erase the RNA methylation modification to convert m6A into RNA. Readers recognize the RNA methylation modification information to guide and participate in the translation and degradation of the modified downstream RNA sequence. In general, RNA methylation is first performed by writer complexes at different RNA sites and then can be demethylated by erasers, which makes RNA methylation a reversible process. The modified base sites are recognized by specific readers to mediate specific biological functions. Readers located in the nucleus might affect mRNA splicing or other nuclear processes, while those located in the cytoplasm might affect the stability, translation, or location of mRNAs. These three types of molecules are indispensable to achieving the regulatory functions of RNA methylation. We list the most well-studied writers, erasers and readers for m6A in **Table 1** and m5C in **Table 2**.

Studies have identified important roles for m6A and m5C in the development and regulation of many organs and systems, especially in the immune system. Therefore, we should focus on the specific mechanism of RNA methylation at m6A and m5C and the potential mechanism in the pathogenesis of SLE.

### m6A

The methylation of the sixth position of the RNA adenine ring and occurs in the sequence context Pu[G > A]m6AC[U > A > C](Pu = purine) (Schibler et al., 1977). It was first discovered in 1974 (Desrosiers et al., 1974) and plays a conservative role in the evolution of meiosis and cell differentiation in yeast, plants and mammals (Yue et al., 2015). Although m6A may exist in the primary transcript, in mammals and yeast, m6A is mainly located in genes, namely, the mRNA protein coding region (CDS) near the termination codon and 3′ untranslated region (3′ UTR) (Dominissini et al., 2012;

**TABLE 1 |** Writers, erasers, and readers for m6A.

| Effect | Protein name | Cellular location | Effect on RNA methylation and its mechanisms | Evidence related to SLE |
|---|---|---|---|---|
| Writers (methyltransferase) | METTL3/MTA70 | Nucleus and cytosol | The central methyltransferase that installs m6A residues on mRNAs and lncRNAs in eukaryotes Lin et al., 2019 | The levels of the METTL3 mRNA are significantly decreased in the peripheral blood of patients with SLE compared with healthy controls Luo et al., 2020a. |
| | METTL5 | | Mediates the m6A modification of human 18S rRNA with the activation of TRMT112 van Tran et al., 2019; Ignatova et al., 2020 | – |
| | METTL14 | Nucleus and cytosol | Forms a stable heterodimer with METTL3 and enhances the methylation activity of METTL3 | The expression of the METTL14 mRNA is decreasing in patients with SLE compared with healthy controls, which was associated with white blood cell count and monocyte count Luo et al., 2020b |
| | WTAP | Nucleus | Changes the alternatively spliced mRNA model Ping et al., 2014; shows no methyltransferase activity, but potentially enhances methyltransferase activity of the METTL3-METTL14 heterodimer Liu et al., 2014 | The levels of the WTAP mRNA are significantly decreased in the peripheral blood of patients with SLE compared with healthy controls Luo et al., 2020a. |
| | TRMT112 | | Forms a stable heterodimer with METTL5 and enhances the methylation activity of METTL5 van Tran et al., 2019; Ignatova et al., 2020 | – |
| | VIRMA/KIAA1429 | | Assembles core components through its N-terminus Yue et al., 2018 | – |
| | RBM15/15b | | Mediates the m6A modification of lncRNA-XIST Patil et al., 2016. | – |
| | ZCCHC4 | | Mediates the m6A modification of human 28S rRNA Ma H. et al., 2019; Ren et al., 2019; Pinto et al., 2020 | – |
| | ZC3H13 | Nucleus | Improves the catalytic function of WTAP and MTC, which are retained in nuclear speckles, by interacting *via* its low-complexity (LC) domains Knuckles et al., 2018; Wen et al., 2018 | – |
| | CBLL1 | Nucleus | Assists in the activation of WTAP by forming stable interactions Figueroa et al., 2009 | – |
| Erasers (demethylase) | FTO | Mainly in the nucleus | Removes m6A from mRNA and m1A from tRNA through its C-terminus Jia et al., 2011 | The levels of the FTO mRNA are significantly decreased in the peripheral blood of patients with SLE compared with healthy controls Luo et al., 2020a |
| | ALKBH5 | Mainly in the nucleus | Removes m6A from mRNA Zheng et al., 2013. | The levels of ALKBH5 mRNA in the peripheral blood of patients with SLE are related to anti-dsDNA antibodies, antinucleosome antibodies, rash, and ulceration. Based on this evidence, the ALKBH5 mRNA level might be involved in the pathogenesis of SLE Luo et al., 2020a,b. |
| Readers (methylation recognition protein) | YTHDC1 | Nucleus | Stimulates splicing and mRNA export (direct binding to m6A) Roundtree and He, 2016; Roundtree et al., 2017; reduces the rRNA synthesis Chen et al., 2021 | – |
| | YTHDC2 | Nucleus and cytosol | Stimulates mRNA decay and translation (direct binding to m6A) Hsu et al., 2017; Wojtas et al., 2017 | – |
| | YTHDF1 | Cytosol | Stimulates translation (direct binding to m6A) Wang et al., 2015 | – |
| | YTHDF2 | Cytosol | Stimulates RNA decay and translation (direct binding to m6A) Wang et al., 2014 | The levels of YTHDF2 mRNA are decreased in peripheral blood from patients with SLE Luo et al., 2020a,b, which might be risk factors for SLE Luo et al., 2020b. |
| | YTHDF3 | Cytosol | Stimulates RNA decay and translation (direct binding to m6A) Li A. et al., 2017; Shi et al., 2017. | – |
| | HNRNPA2B1 | Nucleus | Mediates m6A-dependent miRNA processing and may affect splicing (binding regulated by m6A-induced structural changes) Alarcón et al., 2015 | – |
| | HNRNPC | Nucleus | Affects mRNA splicing (binding regulated by m6A-induced structural changes) Liu et al., 2015 | – |
| | IGF2BP1-3 | Nucleus and cytosol | Increases mRNA stability (binding regulated by m6A-induced structural changes) Huang et al., 2018 | – |
| | FMRP | Nucleus and cytosol | Directly or indirectly maintains the stability of m6A-containing mRNAs by directly binding to YTHDF2 (binding to bona fide m6A-binding proteins) Edupuganti et al., 2017; Huang et al., 2018 | – |

*WTAP, Wilms' tumor 1-associated protein; TRMT112, tRNA methyltransferase 11-2 (TRMT112); VIRMA/KIAA1429, vir-like m6A methyltransferase-associated protein; RBM15/15b, RNA-binding motif protein 15/15b; ZCCHC4, zinc finger CCCH-type containing 4; ZC3H13, zinc finger CCCH-type containing 13; CBLL1, Cbl proto-oncogene like 1; FTO, obesity-associated protein; HNRNPA2B1, heterogeneous nuclear ribonucleoproteins A2B1; HNRNPC, heterogeneous nuclear ribonucleoproteins C; IGF2BP1-3, insulin-like growth factor 2 mRNA-binding protein1-3; FMRP, fragile X mental retardation protein.*

**TABLE 2 |** Writers, erasers, and readers for m5C.

| Effect | Protein name | Cellular location | Effect on RNA methylation and its mechanisms | Evidence related to SLE |
|---|---|---|---|---|
| Writers (methyltransferase) | DNMT2/ TRDMT1 | Nucleus and cytosol | Mainly mediates the m5C modification of DNA and tRNA Raddatz et al., 2013 | – |
| | NSUN2 | 1. G1 phase: nucleolus<br>2. S phase: between nucleolus and nucleoplasm<br>3. G2 phase: cytoplasm, M phase: centrioles | Modifies some non-coding small RNAs and tRNA Khoddami and Cairns, 2013 | The expression of NSUN2 was decreasing in $CD4^+$ T cells from patients with SLE compared with healthy controls Guo et al., 2020. |
| | NSUN5 | | Mediates the m5C modification of human 28S rRNA Janin et al., 2019 | – |
| | TRM4B | | Involved in tRNA methylation and recognition Cui X. et al., 2017; David et al., 2017 | – |
| Erasers (demethylase) | TET2 | Nucleus | Involved in 5-methylcytidine oxidation Xue C. et al., 2020 | Silencing of the TET2 gene obviously diminishes follicular helper T cell polarization *in vitro*, which plays a critical role in SLE Wu et al., 2016. |
| Readers (methylation recognition protein) | ALYREF | Nucleus | Involved in mRNA nuclear-cytoplasmic shuttling, viral RNA export and replication Xue C. et al., 2020 | |
| | Cytoplasmic YBX1 | Cytoplasm | Involved in mRNA stabilization, embryogenesis and tumorigenesis Xue C. et al., 2020 | |
| | TRM4B | | Involved in tRNA methylation and recognition Cui X. et al., 2017; David et al., 2017 | |

*DNMT2/TRDMT1, DNA methyltransferase E2/tRNA aspartic acid methyltransferase 1; NSUN2, NOL1/NOP2/SUN domain methyltransferase family2; NSUN5, NOL1/NOP2/SUN, domain methyltransferase family5; TRM4B, tRNA-specific methyltransferase 4B; TET2, ten-eleven translocation 2; ALYREF, Aly/REF output factor; YBX1, Y-box binding protein 1.*

Ke et al., 2015). Coding RNAs and non-coding RNAs, including tRNAs, rRNAs, small nuclear RNAs, microRNA (miRNA) precursors and long non-coding RNAs (lncRNAs), are modified with m6A in a variety of tissues.

## m6A Writers

The m6A methyltransferase complex transfers a methyl group from the donor substrate S-adenosyl methionine (SAM) to adenine nucleotides in the recipient RNA subunit (Bokar et al., 1997). The complex consists of METTL3 (also known as MTA70) (Lin et al., 2019), METTL5 (van Tran et al., 2019; Ignatova et al., 2020), METTL14 (Yue et al., 2015; Liu et al., 2016), Wilms' tumor 1-associated protein (WTAP) (Ping et al., 2014), tRNA methyltransferase 11-2 (TRMT112) (van Tran et al., 2019; Ignatova et al., 2020), vir-like m6A methyltransferase-associated protein (VIRMA, originally known as KIAA1429) (Yue et al., 2018), RNA-binding motif protein 15/15b (RBM15/15b) (Patil et al., 2016), zinc finger CCCH-type containing 4 (ZCCHC4) (Ma H. et al., 2019; Ren et al., 2019; Pinto et al., 2020), zinc finger CCCH-type containing 13 (ZC3H13) (Knuckles et al., 2018; Wen et al., 2018), and Cbl proto-oncogene like 1 (CBLL1) (Figueroa et al., 2009), among which the most common molecular components are METTL3 and METTL14.

METTL3 is considered the central methyltransferase because of its ability to bind SAM, and it is highly conserved in eukaryotes (Lin et al., 2019). The discovery of METTL3 initiated research on the relationship between m6A and cellular physiology. Differences in the METTL3 expression levels reflect changes in the total m6A level. METTL3 can be modified by SUMO1 at lysine residues $K^{177}$, $K^{211}$, $K^{212}$, and $K^{215}$, significantly repressing the methyltransferase activity of METTL3 (Du et al., 2018). SUMOylation is a reversible posttranslational modification process that attaches small ubiquitin-like modifiers to protein substrates (Hay, 2005). Moreover, sentrin/SUMO-SPECIFIC PROTEASE 7 is significantly upregulated in patients with SLE (Cui Y. et al., 2017), and levels of the METTL3 mRNA are significantly decreased in the peripheral blood of patients with SLE compared with healthy controls (Luo et al., 2020a). Thus, we speculate that SUMOylation might play roles in reducing the expression of METTL3 in patients with SLE.

METTL14 is highly homologous to METTL3. It forms a stable heterodimer with METTL3, enhancing the methylation activity of METTL3. Together, the heterodimer of METTL3-METTL14 forms the catalytic core of the m6A methyltransferase complex (Yue et al., 2015; Liu et al., 2016). Sanna Bystrom found that the expression profile of the METTL14 protein was altered in patients with multiple sclerosis (Byström et al., 2014). Qing Luo et al. observed decreased expression of the METTL14 mRNA in patients with SLE compared with healthy controls ($p < 0.001$), which was associated with white blood cell count and monocyte count (Luo et al., 2020b). Evidence also shows that the production of type 1 interferon (IFN I), the most important cytokine involved in SLE pathogenesis, its production triggered by dsDNA or human cytomegalovirus is controlled by cellular METTL14 and ALKBH5 demethylases (Rubio et al., 2018). METTL14 depletion increases both the production and stability of the nascent IFN β1 mRNA in response to dsDNA (Rubio et al., 2018). This phenomenon represents a potential mechanism by which METTL14 participates in the development of autoimmune diseases, especially SLE.

WTAP is also a core component of the m6A methyltransferase complex that interacts with METTL3 and METTL14 (Ping et al., 2014). The intracellular m6A abundance was markedly decreased when WTAP was knocked out compared with METTL3 or METTL14, which might result from changes in the alternative splicing of the mRNAs to which WTAP binds (Ping et al., 2014). Liu et al. found that the m6A level in polyadenylated RNA was decreased by ∼30, ∼40, and ∼50% in HeLa cells with knockdown of cellular METTL3, METTL14, and WTAP, respectively (Liu et al., 2014). Moreover, WTAP itself showed no methyltransferase activity but dramatically enhanced the methyltransferase activity when interacting with the METTL3-METTL14 heterodimer (Liu et al., 2014). WTAP is upregulated in many tumors, where it functions as an oncogene by interacting with different proteins involved in RNA processing (Sorci et al., 2018).

### m6A Erasers

The m6A demethylase is responsible for the selective removal of SAM from the adenine nucleotides of RNA to regulate gene expression and cell fate. Fat mass and obesity-associated protein (FTO) was the first RNA demethylase discovered, and its C-terminal structure demethylates mRNA, mainly in the nucleus (Jia et al., 2011; Bartosovic et al., 2017). Several studies have shown that FTO might not have a physiological function toward m6A because the FTO knockout transcriptome does not contain an increased number of m6A sites (Hess et al., 2013; Mauer et al., 2017; Garcia-Campos et al., 2019). Instead, FTO appears to function in specific tissues or under specific conditions. For example, FTO regulates the expression of oncogenes, namely, an ∼20% increase, by reducing the m6A modification in mRNA to enhance leukemic oncogene-mediated cell transformation and leukemogenesis (Li Z. et al., 2017). As shown in the study by Qing Luo, FTO expression positively correlates with SLE in patients. The mRNA levels of FTO in the peripheral blood of patients with SLE are significantly decreased compared with those of healthy controls (Luo et al., 2020a).

ALKBH5, the second RNA eraser identified, was shown to affect mouse spermatogenesis (Zheng et al., 2013). ALKBH5 is primarily colocalized with nuclear speckles and affects mRNA export and RNA metabolism in a demethylation-dependent manner (Zheng et al., 2013). Rubio et al. (2018) found that IFN I production triggered by dsDNA or human cytomegalovirus is affected by ALKBH5. ALKBH5 depletion reduces nascent IFN β1 mRNA production without detectably influencing IFN β1 mRNA decay (Rubio et al., 2018). Moreover, the levels of ALKBH5 mRNA in the peripheral blood of patients with SLE are related to anti-dsDNA antibodies, antinucleosome antibodies, rash and ulceration (Luo et al., 2020a). Based on this evidence, the ALKBH5 mRNA level might be involved in the pathogenesis of SLE (Luo et al., 2020a).

### m6A Readers

The downstream function of the presence of m6A on mRNA molecules is closely related to the recognition and binding of m6A-methylated recognition proteins, termed m6A readers. Various m6A readers have been identified, but their mechanism is similar: m6A readers recognize and bind the RNA decorated by m6A. The regulatory function of m6A is achieved by enhancing or weakening the recruitment of different RNA-binding proteins (RBPs) to target mRNAs or directly inducing secondary structural changes in target mRNAs to influence the interaction between RNAs and RBPs (Adhikari et al., 2016; Wu B. et al., 2017).

The most important m6A recognition protein in eukaryotes is the YTH domain-containing family proteins, which comprise the conserved C-terminal RNA recognition and binding domain YTH and the N-terminal variable region. This protein family is also considered the most primitive m6A reader, falling into three classes: YTHDC1, YTHDC2, and the YTHDF family (Zhang et al., 2010). Among them, YTHDF1, YTHDF2, and YTHDF3, which exist in humans, have been studied more extensively and have different cellular localizations but similar functions (Liao et al., 2018; Patil et al., 2018). Other recognized m6A recognition proteins include heterogeneous nuclear ribonucleoprotein A2B1 (HNRNPA2B1) (Alarcón et al., 2015), heterogeneous nuclear ribonucleoproteins C (HNRNPC) (Liu et al., 2015), fragile X mental retardation protein (FMRP) (Edupuganti et al., 2017) and insulin-like growth factor 2 mRNA-binding protein1-3 (IGF2BP1-3) (Huang et al., 2018).

### Effects of m6A Methylation

The m6A modification on RNA regulates the transcription of genes to achieve functional regulation at the cellular or tissue level, which is mainly achieved by the functions of various m6A methyltransferases, m6A demethylases and m6A recognition proteins. m6A is mainly modified on mRNA, and thus the main effects of the m6A modification include pre-mRNA shearing, the stability of mRNA, nuclear transfer of mRNA, and translation of mRNA, thus achieving the regulation of mRNA function (Meyer et al., 2015; Gan et al., 2019; Zhang et al., 2019a). For example, protein translation typically begins with the recruitment of the 43S ribosomal complex to the 5′ cap of mRNAs by a cap-binding complex. Evidence has shown that mRNAs containing m6A in their 5′ UTR are translated in a cap-independent manner (Meyer et al., 2015).

In addition, m6A also modifies non-coding RNAs, such as rRNA, miRNAs, and lncRNAs. m6A methylation of rRNA is dispensable in cell growth and ribosome biogenesis, but plays important roles in increasing translation efficiency and cell proliferation and differentiation (van Tran et al., 2019). Increasing translation efficiency is achieved by the regulation of the kinetics of translation rather than rRNA processing (van Tran et al., 2019; Pinto et al., 2020). Some researchers an increasing polysome/monosome ratio *via* m6A methylation of rRNA (Ma H. et al., 2019), but this phenomenon has not been widely recognized in other studies (van Tran et al., 2019; Pinto et al., 2020). The positive effects on cell proliferation and differentiation have been shown by knocking down the m6A writers of rRNA (Ma H. et al., 2019; Ignatova et al., 2020; Xing et al., 2020). High m6A methylation levels in rRNA have also been found in cancer (Ma H. et al., 2019). Meanwhile, METTL3 may play a positive role in prerRNA processing by influencing relevant protein expression or modifying snoRNA (Sergeeva et al., 2020). The regulation of m6A methylation is also be embodied in premiRNA

processing and lncRNA processing. Meanwhile, by sequence pairing with mRNAs containing miRNA target sites, miRNAs regulate the binding of METTL3 to target RNAs, leading to an increase in the m6A modification (Fazi and Fatica, 2019).

## m5C

m5C is the best-understood epigenetic modification of DNA (Jones, 2012) and is also present in RNAs that are more diverse and complex (Gilbert et al., 2016) The m5C methyltransferase catalyzes the methylation of the 5th position of the cytosine pyrimidine ring at a specific site (Schosserer et al., 2016). The function of m5C has been unclear for many years. However, with the continuous development of high-throughput sequencing technology, the specific locations and related functions of m5C have gradually become clear. Researchers have found that m5C is distributed across coding RNA sequences and all types of non-coding RNAs, such as tRNA and rRNA (Squires et al., 2012; Gilbert et al., 2016). Moreover, the m5C modification of RNA is an important regulator of many aspects of gene expression, including RNA processing and degradation, ribosomal assembly, translation, and RNA stability (Tuorto et al., 2012; Blanco et al., 2016; Nakano et al., 2016).

### m5C Writers

Among higher eukaryotes, the most frequently studied m5C methyltransferases are DNA methyltransferase E2/tRNA aspartic acid methyltransferase 1 (DNMT2/TRDMT1) (Tang et al., 2003) and the NOL1/NOP2/SUN domain methyltransferase family (NSUN) (Bohnsack et al., 2019). Mechanistically, both DNMT2 and NSUN form covalent intermediates through the interaction of a cysteine and cytosine of the target RNA to promote nucleophilic attack at C5 of the pyrimidine ring by SAM to form the m5C modification. However, the difference is that a single cysteine in DNMT2 molecules forms a covalent intermediate, with cytosine Myc-induced SUN domain-containing protein (Misu/NSUN2) molecules formed by two cysteine and cytosine in the covalent intermediate (King and Redman, 2002).

DNMT2 is a widely conserved member of the eukaryotic cytokine-5-DNA methyltransferase protein family, which is widely distributed in the nucleus and cytosol (Tang et al., 2003). Although DNMT2 mainly mediates m5C methylation in DNA, many studies have recently shown that DNMT2 could mediate the m5C modification of tRNA (Raddatz et al., 2013; Genenncher et al., 2018). According to recent studies, DNMT2 mainly mediates the methylation of tRNA at C38 in eukaryotic cells (Schaefer et al., 2010), and DNMT2-mediated RNA methylation has been detected in some eukaryotic organisms, such as zebrafish (Rai et al., 2007) and *Drosophila* (Schaefer et al., 2010). DNMT2-mediated RNA methylation exerts an important effect on organ differentiation and environmental tolerance (Schaefer et al., 2010). Evidence has shown that DNMT2 is required for an efficient innate immune response in *Drosophila*, which is possibly mediated by RNA methylation (Durdevic et al., 2013).

NSUN2 is a member of the protein family containing the NOL1/NOP2/SUN domains, which are mainly located in the nucleus. NSUN2 modifies some non-coding small RNAs and mRNAs in addition to tRNA (Khoddami and Cairns, 2013; Li Q. et al., 2017; Yang et al., 2017). A recent study showed substantially reduced NSUN2 expression levels in $CD4^+$ T cells from patients with SLE compared with healthy controls, which might be due to RNA methylation (Guo et al., 2020).

tRNA-specific methyltransferase 4B (TRM4B) is also an important m5C methyltransferase, and it has been proven to be relevant to tRNA methylation and root growth in *Arabidopsis* (Cui X. et al., 2017; David et al., 2017), but this result still needs to be confirmed in mammalian cells.

### m5C Erasers

The modification of m5C is reversible. Recent studies identified the m5C demethylase ten-eleven translocation 2 (TET2) (Wu and Zhang, 2011; Xue M. et al., 2020). Silencing of the TET2 gene obviously diminishes follicular helper T cell polarization *in vitro*, which plays a critical role in SLE. Because TET2 functions in both DNA demethylation and RNA demethylation, the specific mechanism of m5C in SLE deserves further study (Wu et al., 2016).

### m5C Readers

To date, three m5C-binding proteins have been identified as m5C readers: Aly/REF output factor (ALYREF, an mRNA transport adaptor) (Yang et al., 2017), cytoplasmic Y-box binding protein 1 (YBX1), and TRM4B (Cui X. et al., 2017; Chen X. et al., 2019). The ALYREF-dependent pathway potentially represents one of the main mechanisms for the selective export of m5C-modified mRNAs in mammals (Yang et al., 2017).

### Effects of m5C Methylation

Similar to m6A methylation, the effect of m5C is mainly achieved by influencing the process of protein translation. Studies have shown that m5C induces ribonuclease activity to promote tRNA degradation and affect protein translation; moreover, the modification of m5C on rRNA also affects protein translation (Burgess et al., 2015), and some experiments have suggested that m5C affects the stability of mRNA (Hussain et al., 2013). Although a variety of molecules are modified with m5C, in eukaryotes, the m5C modification is mainly detected on tRNA (Squires et al., 2012). With the exception of $tRNA^{LeuCAA}$, the m5C modification of tRNA mainly occurred outside anticodon rings (Chan et al., 2012). Therefore, the main effect of the CAA m5C modification is to regulate the translation efficiency by affecting CAA oscillation (Chan et al., 2012), while the m5C modification outside the anti-codon ring mainly affects the structure and stability of tRNA for the purpose of regulation (Vare et al., 2017; Janin et al., 2019). By regulating protein translation, m5C also plays a role in many normal physiological processes and abnormal diseases.

# POTENTIAL LINKS BETWEEN RNA METHYLATION AND SLE

## RNA Methylation in T Cells

T cells are regarded as a central component of the pathogenesis of SLE (Tsokos et al., 2016). T cell homeostasis is the key process

in maintaining the T cell pool size, and its imbalanced state is essential in the pathogenesis of SLE (Oster et al., 2019; Katsuyama et al., 2020). Evidence has shown that the m6A modification plays an important role in maintaining T cell homeostasis. Li HB and colleagues found that the m6A modification controls the differentiation of naïve T cells (Li H.-B. et al., 2017). Conditioned knockout of the *Mettl3* gene in mouse $CD4^+$ T cells reduces the m6A methylation level in naïve T cells, which leads to an increase in Th2 cells and a decrease in Th1 and Th17 cells, but has no effect on cell apoptosis or TCR-mediated proliferation (Li H.-B. et al., 2017). Researchers have inferred that naïve T cells lacking METTL3 or METTL14 does not undergo homeostatic amplification and remain naïve, mainly because SOCS family genes (*Socs1*, *Socs3*, and *Cis*) with less m6A modification exhibit slower mRNA decay and increased protein expression levels (Li H.-B. et al., 2017). Therefore, the increased SOCS family activity inhibits the IL-7/STAT5 signaling pathway and activates the TCR/ERK/AKT pathway, which inhibits T cell proliferation and differentiation (Palmer and Restifo, 2009; Li H.-B. et al., 2017). Afterward, RNA methylation was also shown to regulate T cell homeostasis through a repressive loss of function of regulatory T cells (Tregs) (Tong et al., 2018). The authors generated $Mettl3^{f/f}$; Foxp3Cre mice to determine the role of m6A methylation in T cell homeostasis *in vivo*. Inflammatory Th1 and Th7 responses were significantly increased in spleen and peripheral lymph nodes compared with wild-type mice at 60 days after birth (Tong et al., 2018). In $Mettl3^{-/-}$ Tregs, the reduction in m6A levels increases the mRNA stability of *SOCS* genes, including *Cish, Socs1, Socs2, Socs3,* and *Asb2*, and increased levels of SOCS proteins block signal transduction from the IL2-STAT5 pathway, which is essential for Treg function and stability (Shi et al., 2018; Tong et al., 2018). Thus, a loss of Treg function eventually leads to excessive inflammation, inhibiting the function of Tregs and playing an important role in regulating T cell homeostasis (Li and Rudensky, 2016; Tong et al., 2018). In addition to METTL3, METTL14 deficiency in T cells also induces unbalanced T cell homeostasis. A METTL14 deficiency in T cells induces spontaneous colitis in mice, manifesting as increased inflammatory cell infiltration and cytokine production from Th1 and Th17 cells (Lu T. X. et al., 2020). The Mettl14 deficiency also caused impaired induction of the differentiation of naïve T cells into induced Tregs (Lu T. X. et al., 2020). Therefore, the m6A modification exerts a positive regulatory effect on T cell differentiation and development and is one of the important regulatory mechanisms of adaptive immunity, as shown in **Figure 1**.

Evidence related to the role of m5C in T cells is limited. The m5C modification stabilizes mRNA expression, leading to an

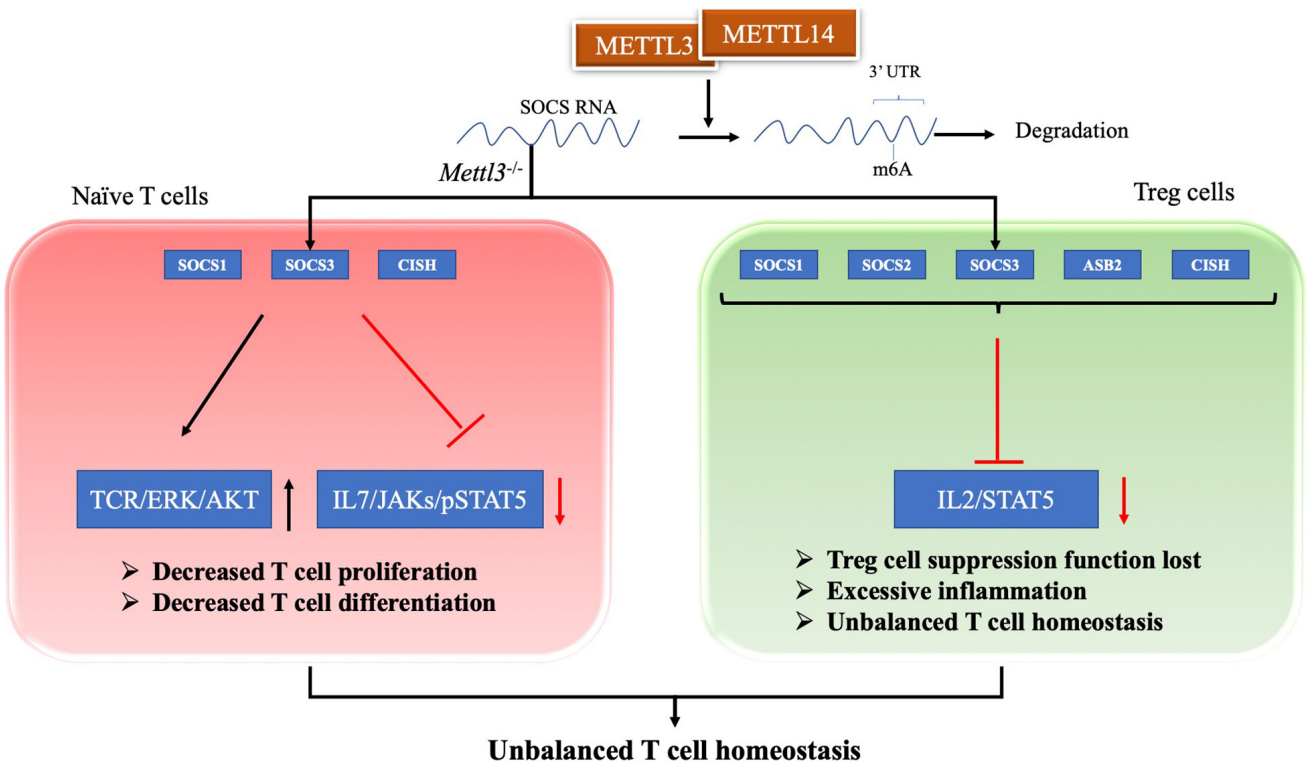


**FIGURE 1 |** RNA methylation in T cell homeostasis. For naive T cell, SOCS family genes with m6A modification exhibit slower mRNA decay and increased protein expression levels. The increased SOCS family activity inhibits the IL-7/STAT5 signaling pathway and activates the TCR/ERK/AKT pathway, which lead to decreased T cell proliferation and differentiation. For Treg cell, reduction in m6A enhances the mRNA stability of SOCS genes and the proteins levels. The increased SOCS family activity blocks signal transduction of the IL2-STAT5 pathway, which lead to Treg cell suppression function lost. These two evidences shows that m6A modification plays an important role in maintaining T cell homeostasis.

imbalance in Th17/Treg differentiation, which may reveal the potential mechanism of m5C in SLE pathology (Guo et al., 2020). The specific mechanism is worth further exploration.

## RNA Methylation in Dendritic Cells

Dendritic cells (DCs) play important roles in the innate and adaptive immune responses, as well as in the progression of SLE (McHugh, 2019; Xiao et al., 2020). A study revealed that DCs exposed to m6A- or m5C-modified nucleosides expressed significantly fewer cytokines and activation markers than those treated with unmodified RNA (Karikó et al., 2005). Mammalian total RNA, but not bacterial RNA, is abundant in modified nucleosides (Karikó et al., 2005). The innate immune system selectively responds to bacteria or necrotic tissue by detecting RNA lacking modification (Karikó et al., 2005). However, METTL3-mediated m6A methylation in DCs is required for the activation and function of DCs. METTL3 induces the phenotypic and functional maturation of DCs, and the expression of CD40, CD80, and IL-12 and the ability to stimulate T cells both *in vitro* and *in vivo* are promoted in *Mettl*3KO DCs (Wang H. et al., 2019). As shown in the study by Han D., knockdown of YTHDF1, an m6A-binding protein, in classic DCs enhanced the antitumor immune response *in vivo* by enhancing the cross-presentation of tumor antigens and the cross-priming of $CD8^+$ T cells (Han et al., 2019). Thus, RNA methylation also regulates the function of DCs in the immune response.

## RNA Methylation in Inflammation

RNA methylation is also an essential mechanism in the process of inflammation, and it is a key component of the pathogenesis of SLE (Frangou et al., 2019). The dynamic regulation of these inflammatory factors contributes to the susceptibility to SLE but is more strongly implicated in the loss of tolerance and end organ effects (Hu et al., 2015). Patients with SLE usually present increased serum levels of inflammatory factors, such as IL-6, TNF-α, and IL-1β, which are related to various immune processes in SLE pathogenesis (Wu Y. et al., 2017; Paradowska-Gorycka et al., 2019; Uzrail et al., 2019). For example, the elevated levels of IL-6 and TNF-α expression in PBMCs from patients with SLE are closely associated with the Th1/Th2/Th17 inflammatory response, which is positively correlated with SLE disease activity (De la Cruz-Mosso et al., 2018). Increased levels of IL-6, IL-10, and TNF-α are consistent with B cell proliferation and activation in patients with SLE (Fleischer et al., 2015). Thus, RNA methylation participates in SLE pathogenesis by regulating inflammatory factor expression, but further evidence is needed. To date, *in vitro* and *in vivo* studies have shown that RNA methylation regulates the occurrence of inflammation through several signaling pathways.

First, the level of RNA methylation regulates the expression of inflammatory factors through the MyD88 pathway. METTL3 knockdown decreases the lipopolysaccharide-induced expression of inflammatory cytokines, including IL-6, IL-8, GRO, Gro-α and RANTES (Feng et al., 2018; Zhang et al., 2019b), by facilitating the expression of MyD88S, a splice variant of myeloid differential protein-88 (MyD88) (Feng et al., 2018). Moreover, RNA methylation regulates inflammation by influencing the NF-κB and MAPK signaling pathways through effects on the phosphorylation of related molecules. METTL3 knockdown decreases the phosphorylation of IKKα/β, p65 and IκBα in the NF-κB signaling pathway and p38, ERK, and JNK in the MAPK signaling pathway in LPS-induced inflammation (Feng et al., 2018; Zhang et al., 2019b). Analogously, YTHDF2 knockdown increases the phosphorylation of p65, p38 and ERK1/2 in the NF-κB and MAPK signaling pathways (Yu et al., 2019). In addition, RNA methylation of the forkhead box O (FOXO) mRNA is also involved in the inflammation process. METTL14 and METTL3 separately increase m6A methylation of the FOXO1 and FOXO3 mRNAs and increase their stability *via* the interaction of YTHDF1 (Jian et al., 2020; Lin et al., 2020). YTHDF3 promotes FOXO3 translation by binding to the translation initiation region of the FOXO3 mRNA (Zhang et al., 2019c). As an important transcription factor, FOXO1 promotes the expression of VCAM-1 and ICAM-1 by directly binding to their promoter regions, which leads to endothelial inflammation and atherosclerosis development (Jian et al., 2020). The FOXO3 levels in B cells from patients with SLE are inversely correlated with disease activity and reduced in patients with elevated anti-dsDNA Ab levels (Ottens et al., 2018).

The regulatory effect on these inflammasome pathways in different autoimmune diseases has been reported. *In vitro* and *in vivo* experiments using samples from patients with rheumatoid arthritis (RA) revealed significantly elevated METTL3 levels in patients with RA that played an important role in LPS-induced inflammation in macrophages *via* the NF-κB signaling pathway (Wang J. et al., 2019). In the development of osteoarthritis (OA), METTL3 expression increases and subsequently regulates inflammation *via* the NF-κB signaling pathway and the degradation of extracellular matrix (ECM) (Liu et al., 2019). These findings not only provide new insights into the pathogenesis of SLE but also facilitate the identification of therapeutic targets and provide new directions for future research. For example, oleuropein (OL) regulates the activation of the JAK/STAT, MAPK, NF-κB and NLRP3 inflammasome pathways and exert its therapeutic effect on patients with SLE (Castejon et al., 2019).

## Role of RNA Methylation in IFN I Production

IFN I is a vital component of the antiviral innate immune response and is also the most important cytokine involved in SLE pathogenesis. We outline the studies that revealed the role of RNA methylation in IFN I production to determine the potential correlation between RNA methylation and SLE.

First, the m6A modification plays an important role in the regulation of IFN I production during virus recognition (**Figure 2**). The dead-box (DDX) helicase family plays an important role in identifying viral nucleic acids and regulating downstream pathways (Parvatiyar et al., 2012). DDX3 and DDX46 recruit and interact with ALKBH5 *via* the DEAD helicase domain (Shah et al., 2017; Zheng et al., 2017). The complex of DDX46 and ALKBH5 demethylates m6A-modified antiviral transcripts, which increases antiviral transcript (Mavs,

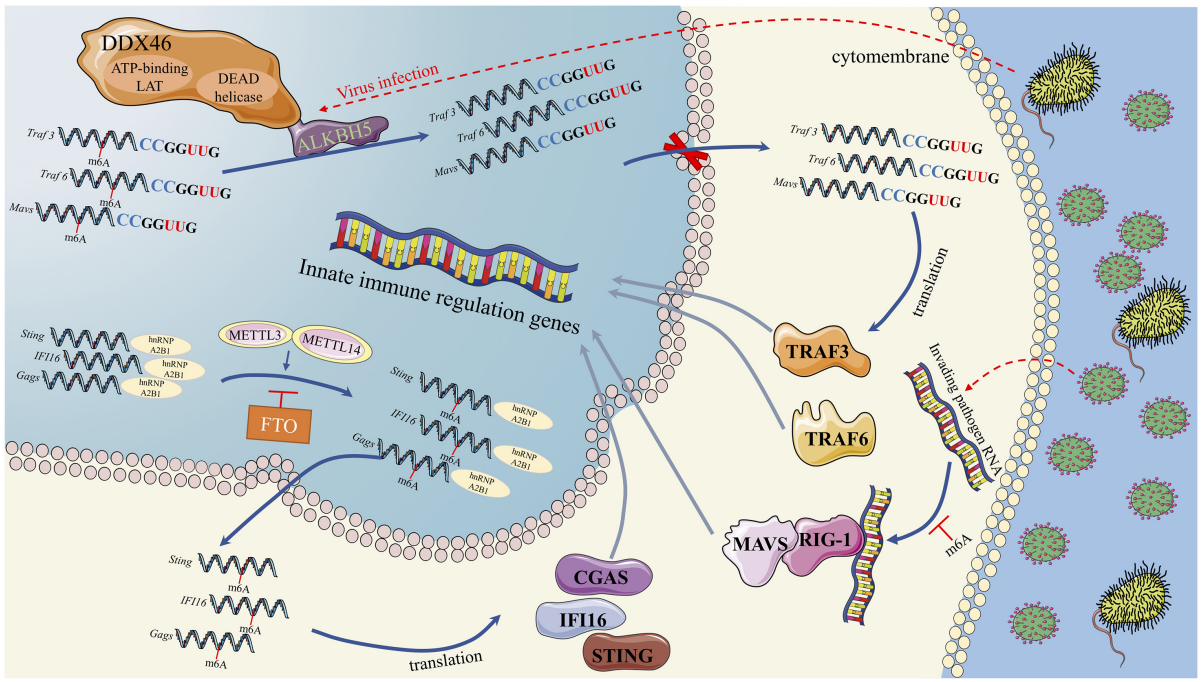


**FIGURE 2 |** RNA methylation in Interferon production in process of virus recognition. RNA methylation in Interferon production were mainly through three pathways. First, DDX46 and ALKBH5 complex could demethylate m6A-modified antiviral transcripts, which lead to antiviral transcript retention in the nucleus and decreases the IFN I protein level. Second, HNRNPA2B1 could activates the TBK1-IRF3 pathway by binding to CGAS, IFI16, and STING to promote IFN I production. METTL3 and FTO could regulate the function of HNRNPA2B1 by m6A methylation. Third, m6A modified transcripts could interrupts the RIG-I like innate immune activation, which could mediate the activation of transcription factors and interferon-stimulated genes.

Traf3, and Traf6) retention in the nucleus and decreases the expression of these proteins and IFN I (Zheng et al., 2017). In addition to DDX46, the function of HNRNPA2B1, a DNA virus sensor, in activating the TANK-binding kinase 1–interferon regulatory factor 3 (TBK1–IRF3) pathway and subsequent IFN-α/β production is also regulated by the m6A modification (Wang L. et al., 2019). In the cytoplasm, HNRNPA2B1 activates the TBK1-IRF3 pathway by binding to CGAS, IFI16, and STING to promote IFN I production (Wang L. et al., 2019). In this process, an RNA writer (METTL3) promotes, while an RNA eraser (FTO) inhibits, the function of HNRNPA2B1 (Wang L. et al., 2019). The m6A modification influences the function of retinoic acid-induced gene I (RIG-I). RIG-I plays a key role in recognizing viral infection, and its activated conformer engages the adaptor mitochondrial antiviral signaling protein (MAVS) to mediate the activation of transcription factors and interferon-stimulated gene (ISG) (Durbin et al., 2016). RNAs containing modified nucleotides interrupt signaling at early steps of the RIG-I-like innate immune activation pathway, and nucleotide modifications with similar chemical structures are organized into classes that suppress or evade innate immune signaling (Durbin et al., 2016). This result is consistent with the mechanism we discussed above in DCs, in which the innate immune system may selectively respond to invading pathogenic nucleic acids by detecting RNA lacking modification (Karikó et al., 2005). For example, human metapneumovirus (HMPV) RNA is modified by m6A, which promotes HMPV replication and gene expression. However, if the m6A modification of HMPV RNA is demethylated, the production of IFN I is increased by the high expression of RIG-I (Lu M. et al., 2020). Thus, if viruses or other invading pathogens acquire m6A in their RNA to mimic cellular RNA, they might avoid detection by the innate immune system (Lu M. et al., 2020).

In addition, the m6A modification of ISGs is logical. The m6A machinery, such as METTL3, YTHDF2, and YTHDF3, has been reported to regulate IFN β levels *via* the m6A modification of ISGs (Winkler et al., 2019a,b; Zhang et al., 2019c). YTHDF2 assists ISG20 in degrading HBV transcripts by selectively recognizing and binding HBV transcripts with the m6A modification (Imam et al., 2020), as the m6A modification participates in IFN α-induced viral RNA degradation. A study conducted by Rubio R. M. et al. revealed that the writer METTL14 and eraser ALKBH5 negatively regulate IFN β production through the m6A modification of the coding sequence and the 3′ untranslated region of the IFN β mRNA (Rubio et al., 2018).

Methylation of host non-coding RNAs also exerts an effect. The m6A modification of lncRNAs plays an important role in antiviral innate immunity based on IFN I signaling (Wang et al., 2020). Studies have revealed that m6A on circular RNA (circRNA) inhibits innate immunity (Chen Y. G. et al., 2019). CircRNAs lacking the m6A modification directly activate the RNA pattern recognition receptor RIG-I in the presence of lysine-63-linked polyubiquitin chains to cause filamentation of

the adaptor protein MAVS and activation of the downstream transcription factor IRF3, potentially inducing IFN I production (Chen Y. G. et al., 2019).

## Role of RNA Methylation in the Antiviral Immune Response

RNA methylation also plays an important role in the antiviral immune response, which is regulated by two systems: the innate immune response and adaptive immune response. In addition to IFN I production, the m6A modification has also been shown to regulate the innate immune response through other mechanisms. Toll-like receptors (TLRs) recognize viral RNA, which is an important information transmission process in the innate immune response (Karikó et al., 2005). Methylated RNA destroys the stability of double-stranded RNA bodies (Yang et al., 2020), which restrains the activation of TLRs (Kierzek and Kierzek, 2003).

RNA methylation is also important in specific antiviral immune responses. For example, in HIV-infected T cells, both host and viral RNA methylation levels are elevated (Lichinchi et al., 2016). Knockdown of METTL3 or METTL14 alone results in reduced viral replication, while ALKBH5 silencing results in significantly increased viral replication, indicating that m6A methylation exerts a positive regulatory effect on HIV replication (Lichinchi et al., 2016). YTHDF3 overexpressed in $CD4^+$ T cells recognizes the m6A modification on HIV and limits its infection (Jurczyszak et al., 2020). YTHDF1-3 also restrains HIV-1 reverse transcriptase (Tirumuru et al., 2016). In HIV-1-infected cells, 56 genes modified by m6A methylation were identified, 19 of which are related to HIV replication and mainly encode functional proteins. The m6A modification of these 56 genes is mainly involved in the T cell response to viral infection by changing their RNA metabolism (Lichinchi et al., 2016). At the same time, the m5C modification also plays a role in antiviral immunity. Experiments have proven that m5C regulates the splicing of HIV-1 mRNA and posttranscriptional function, which affects the expression of viral genes (Courtney et al., 2019). In a direct homologous RNAi screen of host factors regulating HIV-1 replication, the m5C methyltransferase NOP2/NSUN1 was found to limit HIV-1 replication in the nucleus. NOP2 is associated with the HIV-1 5′ LTR and interacts with the HIV-1 TAR RNA through its competition with HIV-1 Tat protein and contributes to the methylation of TAR m5C, which also proves that m5C methylation promotes HIV-1 transcription and virus entry into the incubation period (Kong et al., 2020). RNA chemical modification plays an important role not only in the specific immune response to HIV but also in the responses to other viruses, such as Kaposi's sarcoma-associated herpes virus (KSHV), and m6A has become one of the new targets for the treatment of KSHV (Ye, 2017; Tan and Gao, 2018).

## RNA Methylation in Autoimmune Responses

SLE is a typical systemic autoimmune disease characterized by various autoantibodies, a loss of tolerance and tissue damage. While less evidence is available for the regulation of m6A in the autoimmune response, the role of m6A in autoimmunity is non-negligible. In patients with SLE, the levels of ALKBH5 mRNA are associated with anti-dsDNA and antinucleosome antibodies, which are typical autoantibodies detected in patients with SLE (Luo et al., 2020a). IL-17 participates in various autoimmune diseases and in autoantibody production in patients with SLE (Ma et al., 2020). Wang et al. (2017) found that NSun2 methylates the IL-17A mRNA at cytosine C466 *in vitro* and *in vivo*, which promotes the translation of IL-17. Evidence has shown that m6A RNA methylation participates in coeliac disease, a complex autoimmune disorder (Olazagoitia-Garmendia et al., 2021). Higher m6A methylation in the 5′ UTR of the XPO1 RNA results in increased XPO1 protein concentrations that lead to nuclear factor kappa B activation and inflammation (Wang et al., 2017). Although we did not obtain direct evidence for the relationship between RNA methylation and autoantibodies in patients with SLE, these findings suggested that the topic deserves in-depth study.

# RNA METHYLATION AND ORGAN DAMAGE IN PATIENTS WITH SLE

Evidence shows that the loss of tolerance and tissue damage are distinct processes. Tissue effects might be major contributors to organ damage in patients with SLE independent of the effects of blood cells (Tsokos et al., 2016). These effects might also be mediated by RNA methylation.

## Nephritis

Renal tubulointerstitial fibrosis is one of the typical features of chronic kidney disease (CKD). In individuals with lupus nephritis, interstitial fibrosis is associated with CKD progression (Morales et al., 2021). A mouse fibrotic kidney disease model induced by UUO exhibited significant increases in the total m6A level, and ALKBH5, the eraser, suppressed fibrosis in this model (Ning et al., 2020). In an *in vitro* study, overexpression of METTL3 and METTL14 increased m6A levels and subsequently increased p53 mRNA and protein levels in cisplatin-treated HK2 cells (Zhou et al., 2019).

## Skin

Damage to stem cells in follicles might be one process leading to permanent loss of follicles in patients with SLE or cutaneous lupus erythematosus (CLE) (Al-Refu and Goodfield, 2009), which might also be regulated by RNA methylation. RNA methyltransferase (NSUN2) is required to balance stem cell self-renewal and differentiation in skin (Blanco et al., 2011). Compared with wild-type mice, an increase in the number of quiescent bulge stem cells was observed in $Misu^{-/-}$ mice, along with delayed exit from the bulge, increased self-renewal, and aberrant hair cycling (Blanco et al., 2011).

## Bone

Mesenchymal stem cells originally isolated from the bone marrow stoma are multipotent and possess strong immunomodulatory activities that interact with multiple

immune cells, including DCs, neutrophils, NK cells, T cells, and B cells. Bone marrow-derived mesenchymal stem cells (BM-MSCs) from patients with SLE exhibit defective immune regulation, which might contribute to the imbalance between Treg and Th17 cells in patients with SLE (Geng et al., 2020). On the other hand, the capacity of osteogenic differentiation of BM-MSCs from patients with SLE is reduced compared with cells from healthy controls, which contributes to osteoporosis in patients with SLE (Tang et al., 2013). This outcome might be due to increased IFN β production and activation of the NF-κB pathway in BM-MSCs from patients with SLE (Tang et al., 2013; Gao et al., 2020). According to recent studies, METTL3 silencing reduces mA methylation levels and inhibits the osteogenic differentiation of bone marrow-derived mesenchymal stem cells (Wu et al., 2018; Yan et al., 2020). Thus, the METTL3-mediated m6A modification might induce the dysfunction of BM-MSCs in patients with SLE.

## CONCLUSION

We discussed the role of RNA methylation in the pathogenesis of SLE, including innate immunity and adaptive immunity. Although the evidence has generally indicated a potential relationship between RNA methylation and SLE, few studies have interpreted a direct relationship between them. Therefore, in the next step, we must investigate the direct relationship by quantitatively measuring m6A levels and their association with these existing mechanisms to establish a stronger causal link. Moreover, we investigated the function of RNA methylation in a cohort with SLE to obtain more biomarkers for the diagnosis, treatment, and prognosis of SLE and related complications. Further in-depth research on RNA methylation may clarify the pathogenesis of SLE and provide additional insights into diagnostic and therapeutic strategies.

## AUTHOR CONTRIBUTIONS

XLV and XLI analyzed the literature and studies and wrote the manuscript. MZ, HW, and WZ assisted with constructing figures and polishing the language. QL and XC revised the manuscript. All authors listed have made substantial, direct and intellectual contributions to the work, and approved the article for publication.

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REVIEW
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# Critical Role of Gut Microbiota and Epigenetic Factors in the Pathogenesis of Behçet's Disease

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Behçet's disease (BD) is a chronic refractory multisystem autoinflammatory disease, characterized by typical clinical features of non-specific vasculitis, oral and genital ulcers, uveitis, as well as skin lesions. The exact etiopathogenesis of BD remains unknown, existing studies have indicated that genetics and environmental factors contribute to the increased development of BD. Recently, several studies have shown that external environmental factors can affect the process of epigenetic modification, and abnormalities of epigenetic factors have been confirmed to be involved in the occurrence of BD. At the same time, abnormalities of gut microbiota (GM) in the body, have also been confirmed to participate in the pathogenesis of BD by regulating the balance of Th17/Tregs. This article reviews the pathogenesis of BD and summarizes numerous clinical studies, focusing on the mechanism of GM and epigenetic factors impacting on BD, and providing new ideas for further elucidating the pathogenesis of BD.

**Keywords: Behçet's disease, gut microbiota, epigenetics, DNA methylation, histone modification, microRNA**

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## INTRODUCTION

Behçet's disease (BD) is a recurrent, chronic, multisystem autoinflammatory disease, characterized by recurrent stomatitis, uveitis, genital ulcer, oral ulcer, and skin damages (Alipour et al., 2017; Ortiz-Fernández and Sawalha, 2021). There are obvious regional and gender differences in the incidence of the disease. BD is most common along the ancient Silk Road stretching from China to the Middle East, such as the Mediterranean, Middle East, and far East (Cho et al., 2012; Chen et al., 2017). A meta-analysis showed that the highest prevalence rate (expressed as cases/100,000 inhabitants) is 119.8 for Turkey, 31.8 for the Middle East, 4.5 for Asia, and 3.3 for Europe (Maldini et al., 2018). In the same Asian region, the incidence rate in South Korea is 1.51/100000, which is common in women (Jun et al., 2020). While it is about 14/100000 in China, the prevalence rate is significantly higher in males than in females (Yang et al., 2021). These differences may be due to the inconsistency of other factors such as research methods. Although accumulating evidence has shown that many genetic factors, such as *HLA-B51*, *IL1A-IL1B*, *CEBPB-PTPN1*, *IRF8*, *ADO-EGR2*, *RIPK2*, and *LACC1*, are involved in the susceptibility of BD, the exact etiology of BD remains unclear (Takeuchi et al., 2017).

A number of studies have shown that the differentiation of helper T cells and the expression of corresponding inflammatory cytokines are abnormal in patients with BD. Recently, several

studies have shown that the number of regulatory T cells (Tregs) decreases in patients with BD, and the corresponding main anti-inflammatory cytokine interleukin 10 (IL-10) and TGF-β are also significantly decreased. This abnormality may lead to damage of the immunosuppressive state, which leads to the autoimmune environment in the pathogenesis of BD. In addition, the proportion of T helper 17 (Th17) cells and the level of IL-17 and IL-23 in patients with active BD are significantly increased (Hamzaoui et al., 2011; Zhu et al., 2017; Vural et al., 2021). Studies have found that serum IL-26 levels are significantly increased in patients with active BD (Lopalco et al., 2017). A study proposed that IL-26 can promote the production of Th17 (IL-17A, IL-23) and inhibit the production of Treg (IL-10, TGF-β) by stimulating CD4 + T cells and monocytes (Kaabachi et al., 2017). The expression of proinflammatory cytokines IL-6, IL-1 β, and tumor necrosis factor alpha (TNF-α) in dendritic cells (DCs) of active BD patients was significantly higher than that of healthy controls (Liang et al., 2021). Even in the peripheral circulation, the level of IL-9 mRNA in BD patients was higher than that of healthy controls, which was positively correlated with the level of IL-17 (Kaabachi et al., 2019). The proportion of Th17/Tregs in BD patients is much higher than that in healthy controls (Acosta-Rodriguez et al., 2007; Wilson et al., 2007). Therefore, the pathogenesis of BD may be due to immune tolerance deficiency caused by the decrease of Tregs, while the increase of Th17 cells promotes inflammation. Recent studies have confirmed that changes of intestinal microorganisms participate in the occurrence and development of BD by regulating Tregs, while external environmental factors also regulate the expression of Th17/Tregs ratio through epigenetic processes and are closely related to BD. Therefore, this article reviews the role of the association of intestinal microbiota and epigenetic factors with the etiology of BD.

## THE GUT MICROBIOTA AND BEHÇET'S DISEASE

Gut microbiota (GM), which is considered a metabolic organ, are involved in regulating host metabolism and is a vital factor in maintaining human health and balance in body. Short-chain fatty acids (SCFAs) are the downstream mediators of GM anti-inflammatory activity, which can regulate the mucosal immune system (Consolandi et al., 2015; Shimizu et al., 2019). The synergistic effects of these metabolites are the basis for maintaining immune homeostasis and host immune system function (Arpaia et al., 2013). The imbalance of GM may lead to pro-inflammatory responses, and changes in GM composition regulate the progression of many human inflammatory autoimmune diseases, such as systemic lupus erythematosus (He et al., 2020), psoriasis (Zhang et al., 2021), and rheumatoid arthritis (Brandl et al., 2021). Abnormal activities of Th1 cells, Th17 cells, and Tregs have been observed in patients with BD, and changes in the composition and metabolism of GM play a role in immune abnormalities in BD through the imbalance between Th17 cells and Tregs (Round and Mazmanian, 2009; Ye et al., 2018; Oezguen et al., 2019; van der Houwen et al., 2020; Yan et al., 2020). Therefore, the changes in GM are closely related to BD.

A research team from Italy analyzed the total bacterial DNA in the feces of 22 BD patients and 16 matched healthy controls. It was reported for the first time that a peculiar dysbiosis of the GM is present in BD patients, with a significant decrease in butyric acid production. *Roseburia* and *Subdoligranulum* in the GM were also significantly depleted. Butyric acid is a beneficial metabolite of SCFAs, which can protect the integrity of the intestinal epithelial barrier and affect immune regulation and mucosal immune response by inducing Tregs differentiation. Intestinal butyrate can also inhibit local pro-inflammatory cytokines. The reduced level of butyric acid leads to intestinal epithelial barrier dysfunction, promotes the expression of various inflammatory components, and reduces the level of Tregs which may promote an abnormal immune response (Consolandi et al., 2015). A study of the Japanese population found that the relative abundance of *Clostridia* in patients with BD was decreased. *Clostridia*, including SCFA-producing bacteria. The decrease in its abundance led to the reduced of SCFAs concentration, resulting in dysregulation of immune function in patients with BD. In addition, it was also found that the species of megalomonas and Vibrio butyricum producing SCFAs were also decreased, which may lead to the depletion of SCFAs in the intestine. The data show that the abnormality of GM in BD changes the synthesis of nucleic acids and fatty acids, and these changes in composition and function may be accompanied by adverse molecular exchanges between intestinal immunoreactive cells and intestinal microorganisms, which may be related to immune abnormalities in patients with BD (Shimizu et al., 2019). A study in a Dutch cohort found that in patients with BD, the abundance of *Barnesiellaceae* and *Lachnospira* was decreased. *Barnesiellaceae* may exert protective anti-inflammatory effects by reducing the level of TNF-α, one of the key and targeted cytokines of BD, and the decrease in butyric acid production may be regulated by reducing the abundance of *Lachnospira*, thereby affecting T-cell differentiation and causing inflammation in BD. GM participate in the occurrence and development of BD mainly by Tregs and affecting the balance of Th17/Tregs, but there are also some bacteria that play a role through other mechanisms (van der Houwen et al., 2020).

A report proposed that the relative abundance of *Eggerthella lenta*, *Acidaminococcus* species, *Lactobacillus mucosae*, *Bifidobacterium bifidum*, *Lactobacillus*, *Streptococcus*, and *saliva Lactobacillus* in the feces of patients with BD was significantly increased (Shimizu et al., 2019). In another study, it was found that at the genus level, *Eggerthella* was significantly increased in BD patients, whereas the relative abundance of *Megamonas* and *Prevotella* was significantly decreased. The role of Bacilli in inducing systemic inflammation was consistent and *Lactobacillus* plays a relatively large role in the BD microbiota (Shimizu et al., 2016). The researchers used IgA sequencing analysis to reveal that the species of *Bifidobacterium*, *Dorea*, and *Ruminococcus* coated with IgA increased, indicating that these microorganisms drive specific immunostimulatory responses, which may be pathogenic symbiotic bacteria in BD, reflecting the retention of anti-inflammatory species and neutralization of pathogenic

symbiotic bacteria in BD. IgA coating of *Bifidobacterium* and *Brominated rumencocci* induced in BD may effectively retain bacteria in intestinal mucosa and promote a sustainable dynamic balance by inhibiting pro-inflammatory signals in the host (van der Houwen et al., 2020). A research team from China analyzed the fecal and saliva samples of active BD patients and healthy controls, and found that several opportunistic pathogens were enriched in BD patients, while methanogens and butyrate-producing bacteria (BPB) were enriched in healthy controls. The overgrowth of conditional pathogenic bacteria may disrupt the balance of GM, leading to the decrease of BPB and methanogens. These abnormalities may lead to damage of the intestinal epithelial barrier, and promote the entry of effector molecules or MAMP/PAMP (PGN/LPS) into intestinal epithelium. At the same time, this process induces the overexpression of the corresponding pattern recognition receptor (TLR2/TLR4), which leads to a series of inflammatory reactions, including systemic vasculitis of BD (Ye et al., 2018). In another study, hypomethylated *TLR4* promoter and increased *TLR4* expression were found in BD patients, which suggests that there may be a common pathogenic pathway between GM and epigenetic factors (Kolahi et al., 2020). To determine whether GM play a role in the development of BD, researchers transplanted mixed fecal samples from patients with active BD into mice with experimental autoimmune uveitis (EAU) and observed increased intraocular inflammation; a large amount of inflammatory cell infiltration throughout the retina, choroid, and vitreous cavity; and increased production of inflammatory cytokines including IL-17 and interferon gamma (IFN-γ) (Ye et al., 2018). These findings further confirmed that imbalance of the GM may indeed be involved in the occurrence and development of BD. From the above, it can be seen that the decrease in the abundance of SCFAs play a particularly important role in the special flora imbalance of BD.

Recent studies have reported that fecal microbiome transplantation (FMT) can promote the increase of butyric acid-producing bacteria, so as to achieve the effect of treatment. The researchers transplanted human feces with normal glucose tolerance into type 2 diabetic mice. After FMT treatment, the level of SCFAs in diabetic mice increased, and the level of butyric acid increased significantly after 10 weeks of treatment. It is speculated that the mechanism may be that it can increase the diversity of GM in diabetic mice, affect different kinds of microorganisms in the intestinal tract of mice, and increase the number of bacteria that produce SCFAs, thus increasing the content of SCFAs in the feces of diabetic mice, and regulating dysfunctional glucose and lipid metabolism (Han et al., 2021). Therefore, the in-depth study of GM and FMT in patients with BD may provide new methods and ideas for the treatment of the disease. In addition, a study has found that patients with BD have special flora disorders, and the comparative analysis of the GM in BD, familial Mediterranean fever and Crohn's disease, which have the similar innate and autoinflammatory features in the pathogenesis, showed that *Succinivibrio* and *Mitsuokella* were "BD specific genera" (Tecer et al., 2020). Therefore, the study of GM may also provide a new direction for the differential diagnosis of BD from other inflammatory diseases.

Gut microbiota plays a key role in the immune response mainly by regulating the differentiation of T cells. Changes in the number and types of GM lead to immune abnormalities and diseases. The decrease in relative abundance of *Clostridium* can reduce the production of SCFAs, such as butyric acid, thus reducing the differentiation of Tregs, disrupting the balance of Th17/Tregs, and playing a role in the onset and development of BD. In addition, GM also participate in the BD process by driving specific immunostimulatory responses. The related research of GM is currently a hot spot, and further research in this area will promote our understanding of the pathogenesis of BD.

## EPIGENETICS AND BEHÇET'S DISEASE

Epigenetics refers to the heritable changes of gene expression without changing the DNA sequence, that is, phenotypic changes without changing genotypes (Alipour et al., 2017). It plays an important role in controlling gene expression and regulating cell development, differentiation, and activity (Allis and Jenuwein, 2016). It can not only maintain specific cell lines stably but also dynamically respond to developmental and environmental signals (Muhammad et al., 2019). Epigenetics are collection of mechanisms by which environmental stressors affect gene expression rather than potential genetic sequences (Barrere-Cain and Allard, 2020). Environmental factors and genetic polymorphisms of inflammatory cytokines induce susceptibility to the disease. Some studies have found that epigenetics is a bridge between environment and heredity. It is an important link among the genome, environment and disease, that is, genetic factors control individual susceptibility to disease, while epigenetics ultimately determine the occurrence and phenotype of disease through environmental factors (Hanson and Gluckman, 2008). Epigenetic processes including DNA methylation, histone modification, non-coding RNA (ncRNA), and especially microRNA (miRNA) are thought to be associated with the pathogenesis of BD (Leccese and Alpsoy, 2019). Some genetic or epigenetic factors combined with imbalances in immune regulation lead to the development of BD (Hou et al., 2020). Changes in the methylation level of IRS elements in diffuse repeat sequences, histone modification, and miRNA regulation-mediated gene silencing play a role in the control of immune and inflammatory responses and are related to the pathogenesis of autoimmune diseases, but the specific mechanisms are unknown (Alipour et al., 2017; Leccese and Alpsoy, 2019).

Epigenetics are reversible, and the study of cellular and molecular epigenetic changes associated with BD will provide novel targets for treatment and may address ethnic differences in genetic research verification. Therefore, this paper reviews the role of epigenetic abnormalities in the occurrence and development of BD, such as DNA methylation, histone modification, and ncRNA.

### DNA Methylation and Behçet's Disease

DNA methylation is the main epigenetic modification of a stable autoimmune disease. In mammals, using S-adenosylmethionine as a methyl donor, methyl is added to the fifth carbon of the

cytosine base under the catalysis of DNA methyltransferase, mainly in CpG dinucleotides (Ehrlich et al., 1982; Angeloni and Bogdanovic, 2019). It is generally believed that genetic and environmental factors can change the state of DNA methylation, and the loss of methylation is related to several cancers, autoimmune diseases and inflammation. Animal experiments have shown that DNA methylation inhibitors, such as 5-azacytidine, can control autoimmune diseases, so the study of methylation will provide a better understanding of the disease as well as ideas for treatment (Alipour et al., 2017; Muhammad et al., 2019). In recent years, many studies have confirmed that aberrant DNA methylation is involved in the pathogenesis of BD.

Hyperactivity of neutrophils is an important factor in the immune disorder of BD. Increased levels of cytokines produced by T cells, such as IL-2, IL-12, IL-18, and TNF-α, may lead to the activation of endothelial cells and neutrophils, so the inflammatory response of BD can be attributed to the excessive production of pro-inflammatory cytokines and a decrease in anti-inflammatory factors (Aziz et al., 2020). At present, research on cytokine gene expression is mainly focused on the DNA methylation status of the CpG site (Alipour et al., 2018). A study found that the expression of IL-6 in the peripheral blood mononuclear cells (PBMCs) was significantly higher in patients than in the healthy control group, while the relative promoter methylation level of IL-6 mRNA was significantly decreased. IL-6 is an important cytokine in the pathogenesis of BD and plays a key role in the differentiation of $CD4^+$ T cells into Th17 cells. It may affect the expression of effective genes by changing the DNA methylation pattern, thus stimulating the immune response, lowering the methylation level of the IL-6 promoter, and increasing the prevalence of BD (Alipour et al., 2020). Therefore, IL-6 can be used as a molecular marker for the diagnosis of BD, and this difference can be used for the early diagnosis and rapid treatment of disease. Meanwhile, hypermethylation of IL-10 and low levels of gene expression in the PBMCs of BD individuals and reduced serum levels have been confirmed, suggesting that the abnormality of DNA methylation may lead to inactivation of the IL-10 gene in patients with BD. IL-10 can inhibit the proliferation of $CD4^+$ T cell clones and reduce inflammation, thus controlling the immune response. At the same time, the study found that the level of methylation was significantly different among different age groups and disease severity. The level of IL-10 methylation is significantly increased in people over 45 years old and is more obvious in patients with severe versus non-severe BD. Demethylation therapy can regulate the expression of IL-10 and control the progression of BD disease (Alipour et al., 2018). And the methylation level of TNF-α in patients with BD was significantly lower than that in normal controls, and the methylation level was significantly different among the subgroups of age, ocular involvement and severe ocular involvement. The more serious the ocular involvement, the lower methylation level of TNF-α. IL-10 and TNF-α are mainly secreted by Tregs, so abnormalities in their methylation may also cause immune disorder by regulating the level of Th17/Tregs, leading to the occurrence of BD (Aziz et al., 2020).

A two-stage study on venous blood samples from 100 Chinese Han patients with active BD and 100 normal controls was found that five differentially methylated CpG loci showed significant hypomethylation of four different genes. Pyrophosphate sequencing verified that cg03546163, cg25114611, cg23261343, and cg142905764 CpG sites with abnormal methylation status can be used as diagnostic markers of BD, in which the hypomethylation of *FKBP5* promoter is the most significant, while the expression of *FKBP5* gene is high. And studies have shown that the hypomethylation of *FLJ43663* and *NFIL3* are associated with BD in Chinese Han population for the first time, but the exact effects of these genes need to be confirmed by further study (Yu et al., 2019). A study found that the methylation level of the *Bax* gene in the PBMCs of patients with BD was significantly higher than that in healthy subjects, while the average value of gene expression decreased. *Bax* is a pro-apoptotic gene that promotes physiological cell death, and the expression level is decreased in patients with severe disease. In addition, the regulation of decreased gene expression caused by methylation occurs under 45 years, but the specific mechanism is not clear (Asadi et al., 2020). In another study, researchers detected suppressor cytokine signaling 1 (*SOCS1*) methylation level in the PBMCs of 50 BD patients and 60 healthy subjects. *SOCS1* methylation level was higher in the patients than in the controls, while the change in *SOCS1* gene expression was less than that in the normal control group. *SOCS1* hypermethylation can activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway and directly influences the effects of IL-6 and other cytokines on cell function. *SOCS1* also inhibits the production of IFN-γ and IL-17A driven by STAT1 and STAT3 by maintaining the expression of forkhead box protein 3 (FoxP3), which plays an important role in the function and accuracy of Tregs. *SOCS1* plays a complex role in regulating IL-4, IL-12, and IL-15. Therefore, aberrant methylation of *SOCS1* may be involved in the occurrence of BD through these regulatory actions (Abdi et al., 2018).

Changes in innate immune function play an important role in initiating the BD inflammatory response. TLR is the main regulator of the innate immune system. The epigenetic mechanism, especially DNA methylation, controls TLR-related immunity (Muhammad et al., 2019). The expression of *TLR4* mRNA in the PBMCs of patients with active BD was significantly higher than that of the control group, and the methylation rate of the *TLR4* gene promoter in the active and inactive BD groups was significantly lower than that in the control group (Kolahi et al., 2020). Studies have shown that *TLR4* can promote the differentiation of initial $CD4^+$T cells into Th17 cells, so the hypomethylation of *TLR4* gene may participate in the pathogenesis of BD by increasing the expression of *TLR4* (Bartlett et al., 2018). In addition, a study found that the methylation levels of CG-7.8.9 unit of *GATA_3*, CG-2 site of *IL-4*, CG-2.3.4.5, and CG-10.11 sites of *TGF*-β in $CD4^+$T cells of active BD patients were significantly increased. As an important transcription factor that regulating the differentiation of Th2 cells and the expression of Th2 cytokines, the ablation of *GATA_3* leads to an increase in DNA methylation at the IL-4 gene site and reduced Th2 cytokine production. *TGF*-β is an important multipotent cytokine, which can induce inflammation by promoting the development of Th17 cells and inhibit the immune response by promoting the

development of Tregs. Aberrant methylation of *GATA_3*, *IL-4*, and *TGF*-β promoters may participate in BD by regulating T-cell differentiation, but whether it may become a potential biomarker for the disease remains to be further studied (Zhu et al., 2017). Recently, a genome-wide DNA methylation study demonstrated that the reversal of DNA methylation changes in some cytoskeleton-related genes is related to the remission of the BD. Studies have confirmed that there is a dynamic relationship between genetic susceptibility and environmental inducements. These environmental factors can transmit their destructive effects by affecting intracellular epigenetic events (Renauer et al., 2016).

The epigenetic remodeling of cytoskeleton genes underlying the pathogenesis and treatment response of BD provides new and specific molecules, which can be used as therapeutic targets and may develop into biomarkers of disease.

## Histone Modification and Behçet's Disease

Histone modification refers to the process of histone methylation, acetylation, phosphorylation, adenylation, ubiquitin, or small ubiquitin-like modifier (SUMO) modification under the action of related enzymes, and then regulates the dynamic chromatin structure and gene expression (Bannister and Kouzarides, 2011; Alipour et al., 2017). The most frequently studied histone modification is the modification of the N-terminal H3 (H3K) lysine residue of the histone, which acetylates to neutralize the positive charge, thus reducing the affinity between histone and DNA and promoting transcription in most cases. Histone methylation generally occurs at the lysine and arginine residues of histones H3 and H4, which can be monomethylated, dimethylated (lysine and arginine), or trimethylated (lysine). Misregulation of the methylation process may lead to some diseases, such as cancer and autoimmune diseases (Hanson and Gluckman, 2008; Alipour et al., 2017; Silva et al., 2020). Histone modification plays an important role in a variety of autoimmune diseases, including BD.

Sirtuin 1 (Sirt1) is a histone deacetylase dependent on $NAD^+$ coenzyme. It can inhibit T-cell proliferation and proinflammatory cytokine production by modifying histone deacetylation, regulating gene expression, and then regulating cell function and inflammation (Chadha et al., 2019). Researchers treated Sirt1 activator *in vitro* with mouse pLN cells and PBMCs from normal people in patients with BD ophthalmopathy. The proportion of Tregs in the retina decreased after Sirt1 activation. Inhibition or deletion of Sirt1 allowed accumulated acetylated FoxP3 to be protected from proteasome degradation, which enhanced the inhibition of Tregs *in vitro* and *in vivo* (Gardner et al., 2013). SIRT1-deficient mice showed maladjusted peripheral T-cell tolerance (Beier et al., 2011). Related studies have shown that cytokines, such as IL-6 and IL-17, play an important role in the pathogenesis of BD, and that IL-6 particularly plays a key role in promoting Th17 cells to produce IL-17, TNF-α, and IL-6. The ability of Sirt1 activation to inhibit IL-6 production in EAU indicates that Sirt1 activation has a potential limiting effect on Th17 cells and emphasizes that Sirt1 activation may directly or indirectly affect leukocyte recruitment and migration (Gardner et al., 2013). Resveratrol, a small molecule agonist of Sirt activity, can enhance chromatin-related Sirt1 protein in the CIAP-2 promoter region and correct local tissue inflammation and neurotoxicity by inhibiting the activation of microglia (Chen et al., 2005). By feeding resveratrol to endotoxin-induced uveitis mice with lipopolysaccharide-induced uveitis, a study team found that resveratrol could significantly enhance the expression of *Sirt1* gene in retinal pigment epithelial cells and choroid, and inhibit the occurrence of ocular inflammation. This effect was related to the loss of NF-κB regulated gene expression and the sensitivity of cells to TNF-α induced apoptosis (Kubota et al., 2009). The activation of SIRT1 promotes TNF-α induced apoptosis and inhibits NF-κB transcription by inhibiting the transactivation potential of RelA/p65 protein (Yeung et al., 2004; Guenane et al., 2006). Therefore, the regulation of histone acetylation, especially the activation of SIRT1, is a feasible target for the generation of new anti-inflammatory therapies, and the future targeted activation of SIRT1 is expected to become a potential treatment for non-infectious diseases such as BD-related uveitis.

In addition, ubiquitination reactions are involved in the regulation of receptor tyrosine kinase signal and may play important roles in the TNF-α, IL-1β, and TCR-mediated NF-κB activation pathway. Meanwhile, NF-κB regulates apoptosis-related factors and increases T-cell resistance to apoptosis (Hou et al., 2012). There is a strong Th1 cell immune response in active BD. IL-12 can prevent spontaneous and CD95-induced cell death, while the production of IL-12 is directly regulated by NF-κB (Todaro et al., 2005). Therefore, the ubiquitin-related pathway may play a protective role in the occurrence and development of BD, and ubiquitin deficiency may be involved in the pathogenesis of BD. The ubiquitin-related domain coding gene *UBAC2* is associated with susceptibility of the Chinese Han people to BD (Nakamura et al., 2019). SUMO4 participates in autoimmunity and inflammation by regulating NF-κB and activating heat shock transcription factors, resulting in the decreased transcription of proinflammatory cytokines (Hou et al., 2008), some studies have shown that SUMO4 +438 C and −847 G alleles seem to be associated with susceptibility to BD, and their gene polymorphisms may be involved in the development of skin lesions, vascular BD, as well as the severity of the disease (Kamoun et al., 2010). However, how the two participate in the occurrence and development of BD has not been clarified.

In summary, histone modification is involved in the occurrence and development of BD. Sirt1 inhibits the differentiation of Tregs and disrupts immune tolerance by inducing histone acetylation, which leads to the occurrence of BD. In addition, its methylation and ubiquitin abnormalities are also related to BD, although this needs to be confirmed. The specific mechanism of its participation in the occurrence of BD also needs to be further studied.

## Non-coding RNA and Behçet's Disease

Non-coding RNA refers to RNA, which does not encode proteins. The common characteristic is that it can be transcribed from the

genome but not translated into proteins, and can perform their biological functions at the RNA level. NcRNA is an important regulator of the inflammatory immune response, and its genetic variation may affect this biological function. NcRNA involved in epigenetic processes varies according to sequence length. It can be divided into short ncRNA (<30 nucleotides) and long ncRNA (lncRNA) (>200 nucleotides) (Hanson and Gluckman, 2008; Alipour et al., 2017; Hou et al., 2020). NcRNA may be used for disease diagnosis and/or treatment in the future (Akbaba et al., 2020).

## Long Non-coding RNA and Behçet's Disease

Long ncRNA is a ncRNA, with a length of more than 200 base pairs that regulates the transcriptional activity of specific genes and even chromosomal regions (Akbaba et al., 2020). It plays a key role in different biological processes, including chromatin remodeling, transcription and epigenetic regulation, as well as the development of various immune cells.

Many studies have shown that there are several specific expressions of lncRNA in cells related to immune response, which may be involved in the pathophysiology of the diseases, including cancer and nerve, autoimmunity and eye diseases, providing significant improvements in elucidating RNA-based mechanisms in gene expression control (Yamazoe et al., 2017; Yue et al., 2018). An allele association analysis showed that the rs9517723 locus located in lncRNALoc107984558 had the strongest association, and single nucleotide polymorphism (SNP) rs9517723 was recessively associated with the risk of eye and central nervous system damage. It was also confirmed that the homozygous risk allele (TT) of lncRNA LOC107984558/rs9517723 was significantly associated with the increased expression of *UBAC2*. Gene expression analysis showed that the expression of rs9517723 TT homozygous *UBAC2* was significantly increased. Increase in *UBAC2* expression of homozygous risk allele (TT) in rs9517723 can induce overactivation of ubiquitin-related pathways, leading to eye and central nervous system lesions in BD. In the future, rs9517723 may become a useful genetic marker for the diagnosis of BD, especially in patients with central nervous system diseases (Yamazoe et al., 2017). Another study found that *lncRNA-CD244*, *lncRNA-Cox2*, and *THRIL* are expressed in immune cells, which may regulate the immune response and the production of cytokines, such as IFN-γ, TNF-α, IL-4, and IL-12, which are related to the pathogenesis of many uveitis entities (Yue et al., 2018).

At present, there are few studies on lncRNA, and research on the participation of ncRNA in BD is mainly focused on miRNA.

## MicroRNA and Behçet's Disease

The three main types of short ncRNA are miRNA, siRNA, and Piwi-interaction RNA; miRNA are important mediators of mammalian epigenetic gene regulation and the key regulatory factors of immune response. Mature miRNA inhibits protein synthesis and negatively regulates gene expression by recognizing the 3′UTR region of the target mRNA, degrading the target gene, or inhibiting its translation (Yao et al., 2019). A great number of studies have shown that miRNA plays a critical role in the regulation of immune response and immune cell development. A miRNA can regulate hundreds of target genes by inhibiting translation, mediating mRNA fragments, or causing RNA instability. Multiple miRNAs can cooperatively bind and regulate a single target gene (Deng et al., 2015). MiRNA regulates T-cell differentiation and plasticity by targeting its corresponding mRNAs and plays an important role in many autoimmune diseases (Lu et al., 2020).

Uncontrolled miRNA targeting is involved in signaling pathways in the pathogenesis of BD, such as TNF-α, IFN-γ, and the VEGF–VEGFR signal cascade. Downregulated miRNA targets differentially expressed genes (DEGs), associated with the adaptive immune response, including genes that play a role in T-cell and B-cell immune response, and controls several genes and transcripts associated with Th17 cells, which are involved in IFN-I response, which may indicate that BD has lost control of the two synergistic mechanisms usually associated with autoimmune response (Puccetti et al., 2018). MiR-155 is related to inflammation, effectively upregulates many immune cell lines through TLR ligands, and promotes the expression of many immune cell lines through the expression of TLR ligands and precursors. Many studies have found that miR-155 is significantly downregulated in patients with BD, which is closely related to the onset and development of BD (Karasneh et al., 2005; Alipour et al., 2017; Ahmadi et al., 2019). A study analyzed the role of miRNAs in two common uveitis: BD and Vogt-Koyanagi-Harada (VKH) syndrome, and found that in PBMCs and DCs of patients with active uveitis BD, only miR-155 expression was significantly decreased, while there was no significant difference in miRNA expression in PBMCs and DCs in patients with VKH syndrome compared with control groups. In addition, it was found that the expression of TGF-β-activated kinase 1 binding protein 2 (TAB2) increased in DCs, and luciferase reporter gene detection showed that *TAB2* is the target gene of miR-155 (Zhou et al., 2012), and downregulation of miR-155 negatively regulate the inflammatory cytokines produced by DCs (Morton et al., 2016). DCs with reduced expression of miR-155 can promote the secretion of IL-6 and IL-1 but inhibits the production of IL-10. Because DCs mainly regulate the function of T cells, the downregulated expression of miR-155 can promote the secretion of IL-17, which is negatively correlated with the production of IL-17 in allogeneic $CD4^+$ T cells. Some studies have found that miR-155 regulates the Th17 immune response in patients with active BD (Na et al., 2016). The increase of Th1/Th17 ratio is usually related to BD, and Th17 cells response plays an important role in the pathogenesis of BD (Deng et al., 2015). In addition, the overexpression of miR-155 also significantly inhibits apoptosis, and the level of miR-155 is often lower in BD patients with severe clinical manifestations and higher BDCAF scores (Hatemi et al., 2019). And miR-155 targeting FoxP3 can regulate the differentiation and function of Th17 cells by inducing their differentiation into Tregs. However, miR-155 cannot effectively secrete Tregs-related cytokines, which may play a role by regulating the frequency, transcription factors, and cytokine levels of Tregs (Ahmadi et al., 2019).

In addition, studies have found that miRNA, which controls members of the TLRs and JAK/STAT pathways, is downregulated.

These two molecular signals are involved in autoimmune diseases, and are also active in BD (Puccetti et al., 2018). Some studies have found that the miR-146a variant rs2910164 is closely associated with BD in Chinese population (Deng et al., 2015). The protein levels of mature miR-146a transcripts and IL-17, TNF-α, and IL-1β in the PBMCs of BD patients with rs2910164 CC genotype are lower than those of GG genotype (Coit et al., 2018), which is more common in BD patients. The CC genotype of rs2910164 has a protective effect on BD. In BD patients, miR-146a is a negative regulator of innate immunity (Morton et al., 2016), and is also a key regulator of IFN-I pathway (Yokota et al., 1992). It is highly expressed in Tregs, which is considered necessary for the inhibitory function of Tregs, and affects the ability of these cells to inhibit Th1 cell's response through STAT1. STAT1 is an important transcriptional factor in the differentiation of Th1 cells. MiR-146a can inhibit the transition of Tregs to Th1-like cells (Ahmadi et al., 2019). A previous study confirmed that miR-146a plays a negative feedback regulation role in TLR signals by targeting TRAF6 and IL-1R related kinase (IRAK) (Taganov et al., 2006), which downregulation may play an important role in the occurrence of BD. In the miRNA study of BD, the regulation of Th17 cellular activity has become a popular topic. MiR-23b can reduce the production of IFN-γ and IL-17 by reducing the expression of Notch pathway genes (Coit et al., 2018). The expression of miR-23b in $CD4^+$ T cells of active BD patients was significantly lower than that of normal controls or inactive BD patients, and the active forms of Notch target genes Hes-1 and Notch1 were significantly increased in PBMCs and $CD4^+$ T cells. Reduced miR-23b expression may contribute to the activation of Notch pathway and the increase of Th1/Th17 cells in BD patients. Because STAT3 plays a key role in the differentiation of Th17 lymphocytes, the increased activation of Notch pathway associated with STAT3 phosphorylation may promote Th17 cell response in patients with active BD (Qi et al., 2014). Studies have also confirmed that the level of STAT3 phosphorylation is increased in patients with BD. Therefore, the regulation of Notch pathway may provide a novel treatment for BD. In addition, miR-23b is one of the miRNA involved in the differentiation of Tregs. The low expression of miR-23b is consistent with the finding of decreased Tregs levels in BD patients in early studies (Nanke et al., 2008), which was confirmed in another study (Morton et al., 2016).

SNPs may change the properties of miRNA by changing the expression or maturation of miRNA. Therefore, SNP in the process of mature miRNA may be related to autoimmune or autoinflammatory diseases. Researchers found that the frequencies of miR-196a2/rs11614913 TT genotype and T allele in BD patients with arthritis were significantly higher than those in non-arthritis BD patients. The expression of miR-196a is decreased in individuals with rs11614913 TT genotype, while the expression of Bach1 is increased. Luciferase report experiment confirmed that Bach1 was the target gene of miR-196a, and there

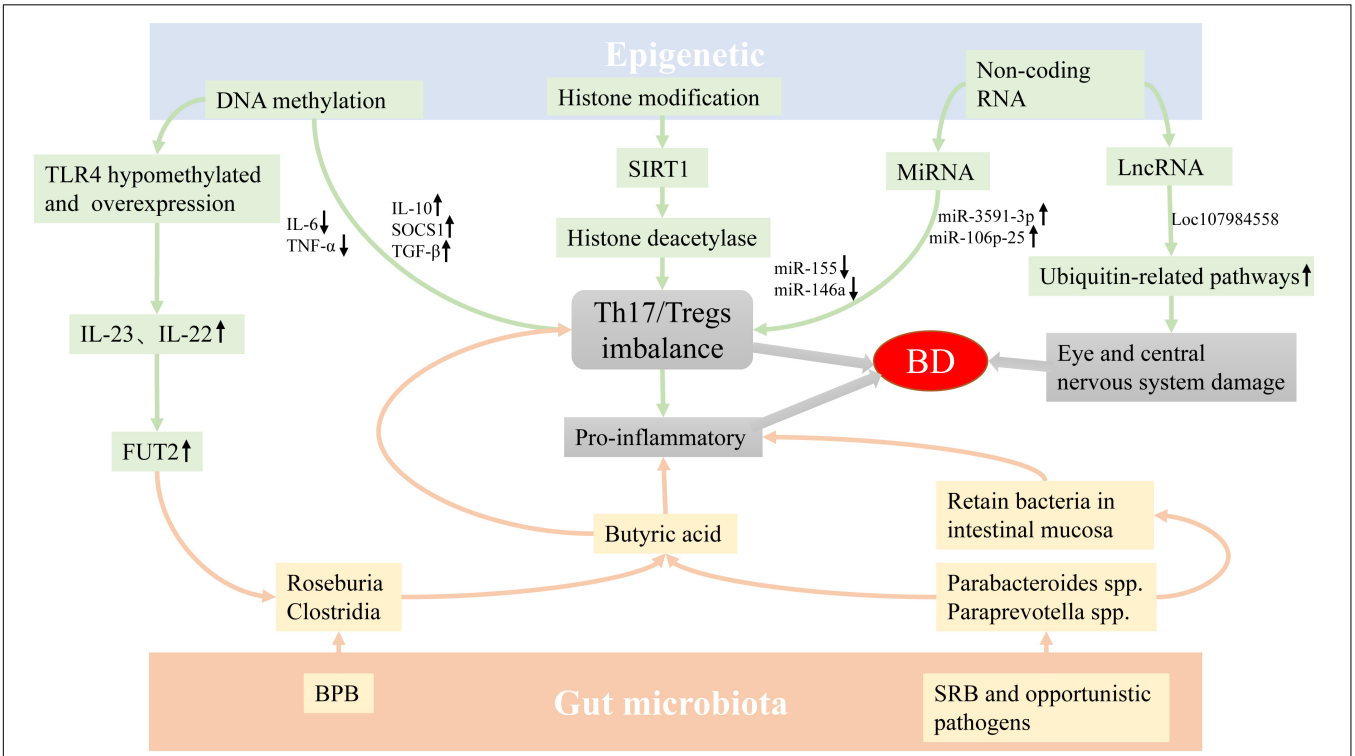


**FIGURE 1 |** The possible mechanism of epigenetics factors and GM involving the pathogenesis of BD. The imbalance of GM and epigenetics abnormality may be involved in the occurrence of BD by regulating the differentiation of T cells. In addition, the abnormality of conditional pathogenic bacteria may cause inflammation by retaining bacteria in intestinal mucosa, and the abnormality of LncRNA can also cause eye and central nervous system damage in patients with BD. Abbreviation: GM: Gut microbiota, BD: Behçet's disease, BPB: butyrate-producing bacteria, SRB: sulfate-reducing bacteria.

was a negative correlation between miR-196a and Bach1 (Qi et al., 2013). Bach1 is a mammalian transcriptional inhibitor of heme oxygenase-1 (HO-1) (Sun et al., 2002). The expression of HO-1 in the PBMCs of patients with BD is decreased. Bach1/HO-1 is a well-known oxidative stress signal pathway and participates in the pathogenesis of several inflammatory diseases. Rs11614913 may lead to the imbalance of Bach1/HO-1 pathway through the change of miR-196a expression. It affects the expression of pro-inflammatory cytokines and leads to the pathogenesis of BD. In addition, researchers used quantitative PCR to detect the expression levels of four selected miRNAs (miR-638, miR-4488, miR-3591-3p, and miR-1915), and to explore their relationship with TNF-α and IL-6 production. It was found that the expression of miR-638 and miR-4488 in the PBMCs of patients with stable BD was significantly lower than that of healthy controls. Stimulation of LPS can increase the level of miR-4488 in the PBMCs of patients with stable BD to the level of healthy controls. By contrast, the expression of miR-3591-3p in PBMC of active BD patients was significantly higher than that of BD patients in remission stage. Transfection of miR-3591-3p mimic could increase the IL-6 mRNA level of THP-1 cells stimulated by LPS, but the specific mechanism has not been elucidated (Woo et al., 2016). In other studies, it has also been found that some of the upregulation of miRNA is also related to BD.

Compared with the control group, miR-25, miR-106b, miR-326, and miR-93 in peripheral blood of BD patients were significantly up-regulated. MiR-25, miR-106b, and miR-93 are located in the miR-106b-25 cluster and participate in the regulation of TGF-β pathway (Ahmadi et al., 2019). TGF-β plays an important role in the development of Tregs by inducing FoxP3. Therefore, the increased expression of miR-106b-25 associated with Tregs may disrupt the signal pathway of TGF-β and affect the differentiation of Tregs (De Santis et al., 2010). MiR-326 regulates the differentiation of Th17 cells by targeting Ets-1, a member of the ETS transcription factor family. The proportion of Th17 cells in BD patients is significantly increased, accompanied by increased gene expression levels of IL-17, IL-23, and retinoic acid-related orphan receptor. The balance of Th17/Tregs is broken, which may play a role in the occurrence and development of BD. The current evidence suggests that the impairment of inflammatory regulation in BD patients may be mediated by abnormal T-cell homeostasis. Genetic variations in the miRNA gene associated with BD have been shown to promote the phenotypic transformation of a disease, increase the production of pro-inflammatory cytokines, and reduce the expansion of anti-inflammatory Tregs. A study analyzed the association of miR-182/rs76481776 in 420 BD and 1200 controls, which confirmed that miR-182/rs76481776 is related to BD. MiR-182 can target the 3′UTR of *FoxO1*, resulting in the degradation of FoxO1, and FoxO1 controls the development and function of Tregs. Therefore, Tregs decrease and assist T cell clone proliferation. At the same time, miR-182 is IL-2-induced miRNA, which regulates the specialization and stability of Tregs, and is the key switch for Tregs differentiation. Treg is a subgroup of T cells, which regulates the immune system and maintains tolerance to autoantigens, thus controlling the occurrence of dominant autoimmune diseases (Yu et al., 2014).

## THE INTERACTION BETWEEN *TLR4* AND GUT MICROBIOTA MAY BE ASSOCIATED WITH THE DEVELOPMENT OF BEHÇET'S DISEASE

As mentioned earlier, *TLR4* showed hypomethylation and increased expression in Iranian BD patients (Horie et al., 2009; Kolahi et al., 2020). In addition, the researchers detected and analyzed the association between nine SNPs in *TLR4* and BD through direct sequencing, and found that the *TLR4* polymorphism may increase the risk of BD in the Japanese population (Meguro et al., 2008), which has also been confirmed in the Korean and Italian populations (Meguro et al., 2008; Boiardi et al., 2009). The expression of *TLR4* was also increased in intestinal lesions of BD patients, which may be related to intestinal abnormalities in BD patients (Nara et al., 2008). Interestingly, another study found that systemic exposure to TLR ligands caused rapid α (1,2)-fucosylation of small intestine epithelial cells (IECs) in mice, which needed the sensing of TLR agonists, the IL-23 production by DCs, activation of innate lymphoid cells and expression of fucosyltransferase 2 (FUT2) by IL-22-stimulated IECs (Pickard et al., 2014; Xavier et al., 2015). It can be seen from the above that the TLR4-IL-23-IL-22-FUT2 pathway may be a potential pathway for the interaction between genetics and GM to participate in the pathogenesis of BD, but the specific mechanism needs further studies.

## CONCLUSION

In summary, the abnormalities of GM and epigenetic factors may provide a susceptible background for the occurrence of BD through a variety of mechanisms (**Figure 1**). However, there are few related studies at present, and whether GM and genetic components affect the development of BD has not yet been clearly elucidated, and the specific mechanism of epigenetics on BD is also unclear. The future direction of study may be to combine environmental factors with genetic factors to explore whether they interact with each other and participate in the occurrence of diseases, so as to reveal the occurrence and development of BD as comprehensively as possible, thereby promoting the understanding of diseases, and providing new targets and approaches for disease prevention, diagnosis and treatment.

## AUTHOR CONTRIBUTIONS

HY and XM conceptualized and wrote this manuscript. XW, GZ, GT, WW, FZ, and DT had critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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# DNA Methylation Patterns in CD8$^+$ T Cells Discern Psoriasis From Psoriatic Arthritis and Correlate With Cutaneous Disease Activity

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**Background:** Psoriasis is a T cell-mediated chronic autoimmune/inflammatory disease. While some patients experience disease limited to the skin (skin psoriasis), others develop joint involvement (psoriatic arthritis; PsA). In the absence of disease- and/or outcome-specific biomarkers, and as arthritis can precede skin manifestations, diagnostic and therapeutic delays are common and contribute to disease burden and damage accrual.

**Objective:** Altered epigenetic marks, including DNA methylation, contribute to effector T cell phenotypes and altered cytokine expression in autoimmune/inflammatory diseases. This project aimed at the identification of disease-/outcome-specific DNA methylation signatures in CD8$^+$ T cells from patients with psoriasis and PsA as compared to healthy controls.

**Method:** Peripheral blood CD8$^+$ T cells from nine healthy controls, 10 psoriasis, and seven PsA patients were collected to analyze DNA methylation marks using Illumina Human Methylation EPIC BeadChips (>850,000 CpGs per sample). Bioinformatic analysis was performed using R (*minfi, limma, ChAMP*, and *DMRcate* packages).

**Results:** DNA methylation profiles in CD8$^+$ T cells differentiate healthy controls from psoriasis patients [397 Differentially Methylated Positions (DMPs); 9 Differentially Methylated Regions (DMRs) when ≥CpGs per DMR were considered; 2 DMRs for ≥10 CpGs]. Furthermore, patients with skin psoriasis can be discriminated from PsA patients [1,861 DMPs, 20 DMRs (≥5 CpGs per region), 4 DMRs (≥10 CpGs per region)]. Gene ontology (GO) analyses considering genes with ≥1 DMP in their promoter delivered methylation defects in skin psoriasis and PsA primarily affecting the BMP signaling pathway and endopeptidase regulator activity, respectively. GO analysis of genes associated with DMRs between skin psoriasis and PsA demonstrated an enrichment of GABAergic neuron and cortex neuron development pathways. Treatment with

cytokine blockers associated with DNA methylation changes [2,372 DMPs; 1,907 DMPs within promoters, 7 DMRs (≥5 CpG per regions)] affecting transforming growth factor beta receptor and transmembrane receptor protein serine/threonine kinase signaling pathways. Lastly, a methylation score including TNF and IL-17 pathway associated DMPs inverse correlates with skin disease activity scores (PASI).

**Conclusion:** Patients with skin psoriasis exhibit DNA methylation patterns in $CD8^+$ T cells that allow differentiation from PsA patients and healthy individuals, and reflect clinical activity of skin disease. Thus, DNA methylation profiling promises potential as diagnostic and prognostic tool to be used for molecular patient stratification toward individualized treatment.

**Keywords: psoriasis, psoriatic arthritis, $CD8^+$ T cell, chromatin, DNA methylation, patient stratification**

## INTRODUCTION

Psoriasis is a chronic systemic autoimmune/inflammatory disease that primarily affects the skin (Schön, 2019). An estimated 11–30% of psoriasis patients develop arthritis and are therefore diagnosed with psoriatic arthritis (PsA; Gladman et al., 2002). Notably, arthritis can precede skin disease, sometimes by several years, thereby complicating correct diagnosis and delaying the introduction of effective treatment (Diani et al., 2015). While psoriasis can generally occur at any age, and both sexes are equally affected, two peak ages have been suggested in adolescence/young adulthood (16–22 years) and later in life (57–60 years) (Perera et al., 2012). Thus, psoriasis can impact on almost any life stage and causes significant burden and cost for the individual and society.

The pathophysiology of psoriasis, both skin psoriasis and PsA, is incompletely understood. Multiple factors have been discussed, including genetic susceptibility, environmental triggers in combination with skin barrier disruption, and general immune dysfunction (Ayala-Fontánez et al., 2016). Skin psoriasis and PsA are characterized by a pathological activation of the TNF/IL-23/IL-17 cytokine axis, contributing to an immunological imbalance favoring effector T cell differentiation and activation, as well as their accumulation in affected tissues (Di Cesare et al., 2009; Hawkes et al., 2018; Carvalho and Hedrich, 2021). T cells play a pivotal role in disease pathogenesis at all stages, including breach of tolerance and the initiation of a pro-inflammatory phenotypes, the establishment of chronic inflammation, damage and amplification of self-reactivity, the maintenance of clinically established inflammatory tissue lesions, and "spreading" of inflammatory disease from initial sites (e.g., the skin) to secondary sites of inflammation (e.g., extra-cutaneous manifestations) (Casciano et al., 2018).

Epigenetic modifications orchestrate gene expression by regulating the accessibility of DNA to transcription factors and the transcriptional complex. Alterations to the epigenome have been linked with the molecular pathophysiology of autoimmune/inflammatory conditions, and link genetic predisposition with disease expression (Surace and Hedrich, 2019). Because of its stability in biological samples (Gosselt et al., 2021), DNA methylation is the most widely studied epigenetic mark. It contributes to effector phenotypes and inflammatory cytokine expression in T cells from patients with various autoimmune/inflammatory conditions (Brandt et al., 2018). In psoriasis, $CD4^+$, $CD8^+$ as well as $CD4^-CD8^-$ (double negative) T cells are involved in the molecular pathogenesis following their stimulation by (auto-)antigens (Brandt et al., 2018; Carvalho and Hedrich, 2021).

This study aimed to identify disease- and outcome-specific DNA methylation signatures in $CD8^+$ T cells from patients with skin psoriasis and PsA as compared to healthy controls.

## MATERIALS AND METHODS

### Patient Cohort

Samples were collected from 26 individuals: nine healthy controls, 10 plaque-type skin psoriasis (Kim et al., 2017), and seven PsA patients (Singh et al., 2019). Demographic and clinical characteristics of study cohorts are summarized in **Table 1**. Psoriasis Area and Severity Index (PASI) scores (Feldman, 2005) were collected at all study visits from patients with skin psoriasis and PsA.

### $CD8^+$ T Cell and Genomic DNA Isolation

Peripheral blood mononuclear cells (PBMCs) were *ex vivo* isolated from fresh blood samples obtained from patients and healthy controls using Biocoll (Merck) and Leucosep Tubes (Greiner, Bio-One), following standard protocols. Next, $CD8^+$ T cells were separated from PBMCs using flow cytometry and FACS sorting with the following antibodies: Pacific Blue anti-CD4 (OKT4; BioLegend); FITC anti-CD3 (OKT3; BioLegend); PE anti-CCR7 (G043H7; BioLegend); APC anti-CD45RA (HI100; BioLegend), APC-Cy7 anti-CD8 (SK1; BioLegend). Cells were stained with aforementioned fluorophore-conjugated antibodies in 2% BSA, 1 mM EDTA/PBS on ice for 30 min, phenotyped, counted and collected on a FACSAria II cell sorter (Becton Dickinson) by gating on viable $CD3^+$ and $CD4^-$, $CD8^{hi}$, $CD8^{lo}$, or $CD8^-$ T cell populations,

**TABLE 1 |** Participant demographics.

| Patient identification | Patient phenotype | Age | Gender | Ethnicity | PASI score | Systemic treatment |
|---|---|---|---|---|---|---|
| Control_1 | Healthy control | 27 | Male | Caucasian | – | – |
| Control_2 | Healthy control | 25 | Female | Caucasian | – | – |
| Control_3 | Healthy control | 35 | Male | Caucasian | – | – |
| Control_4 | Healthy control | 25 | Female | Caucasian | – | – |
| Control_5 | Healthy control | 51 | Female | Caucasian | – | – |
| Control_6 | Healthy control | 42 | Male | Caucasian | – | – |
| Control_7 | Healthy control | 26 | Female | Caucasian | – | – |
| Control_8 | Healthy control | 34 | Male | Caucasian | – | – |
| Control_9 | Healthy control | 25 | Female | Caucasian | – | – |
| Psoriasis_1 before treatment | Psoriasis | 33 | Female | Caucasian | 19.5 | – |
| Psoriasis_1 after treatment | Psoriasis | 33 | Female | Caucasian | 1.2 | Anti-TNFα |
| Psoriasis_2 before treatment | Psoriasis | 25 | Male | Caucasian | 16.3 | – |
| Psoriasis _2 after treatment | Psoriasis | 25 | Male | Caucasian | 3.1 | Anti-IL17 |
| Psoriasis_3 before treatment | Psoriasis | 30 | Male | Caucasian | 20.7 | – |
| Psoriasis_3 after treatment | Psoriasis | 30 | Male | Caucasian | 1.8 | Anti-IL17 |
| Psoriasis_4 before treatment | Psoriasis | 45 | Male | Caucasian | 21.8 | – |
| Psoriasis_4 after treatment | Psoriasis | 45 | Male | Caucasian | 4 | Anti-IL17 |
| Psoriasis_5 | Psoriasis | 51 | Male | Caucasian | 12.4 | – |
| Psoriasis_6 | Psoriasis | 49 | Male | Caucasian | 14.9 | – |
| Psoriasis_7 | Psoriasis | 37 | Female | Caucasian | 10.5 | – |
| Psoriasis_8 | Psoriasis | 27 | Female | Caucasian | 10.2 | – |
| Psoriasis_9 | Psoriasis | 51 | Male | Caucasian | 21.3 | – |
| Psoriasis_10 | Psoriasis | 20 | Male | Caucasian | 21.5 | – |
| PsA_1 before treatment | Psoriatic arthritis | 30 | Female | Caucasian | 25.1 | – |
| PsA_1 after treatment | Psoriatic arthritis | 30 | Female | Caucasian | 11.2 | Anti-TNFα |
| PsA_2 | Psoriatic arthritis | 40 | Male | Caucasian | 15 | – |
| PsA_3 | Psoriatic arthritis | 70 | Male | Caucasian | 7.6 | – |
| PsA_4 | Psoriatic arthritis | 49 | Male | Caucasian | 0.8 | – |
| PsA_5 | Psoriatic arthritis | 51 | Female | Caucasian | 0.8 | – |
| PsA_6 | Psoriatic arthritis | 81 | Female | Caucasian | 3.4 | – |
| PsA_7 | Psoriatic arthritis | 62 | Male | Caucasian | 6 | – |

*PsA, Psoriatic arthritis.*

as indicated (**Supplementary Figure 1A**). Datasets were analyzed using FlowJo software V10 (TreeStar).

Sorted $CD8^+$ T cells were collected and stored at −80°C. Isolation and separation of genomic DNA from sorted cells was performed using ZR-Duet DNA/RNA MiniPrep kits (Zymo Research) according to manufacturer's protocol (including DNAse step for RNA isolation). Genomic DNA quantity and quality was assessed using NanoDrop and Qubit (Thermo Fisher Scientific).

## DNA Methylation Profiling

The Illumina Infinium MethylationEPIC array BeadChip (850K) was carried out using Diagenode Epigenomic Services (Vienna, Austria, Cat No. G02090000). Genomic DNA samples from $CD8^+$ T were sent for bisulfite conversion [EZ-96 DNA Methylation Kit (Zymo Research)] and DNA methylation profiling using the Illumina Human Methylation EPIC platform to analyze the methylation status of more than 850,000 CpGs per samples. This microarray covers ∼96% of CpG Islands and 99% of annotated RefSeq genes.

## Quality Control, Data Normalization and Statistical Analysis of Differentially Methylated Positions and Differentially Methylated Regions

Methylation profiles of $CD8^+$ T cells were analyzed using R packages *Minfi* (Aryee et al., 2014) and *ChAMP* (Tian et al., 2017). Type 1 and type 2 probes were normalized using quantile normalization and BMIQ. The sex of donors was confirmed using the predictSex function of the *Minfi* package. The following probes were filtered out: (i) probes not passing the detection *p*-value cut-off of 0.01, (ii) probes with known SNPs, (iii) probes not in CpG context, and (iv) cross reactive probes [(McCartney et al., 2016; Pidsley et al., 2016) demonstrated that some probes map to multiple genomic sites and may therefore affect analysis]. Batch effects were corrected using the ComBat function of *ChAMP* package, which was derived from the *SVA* package (Johnson et al., 2007).

For downstream analysis and data visualization, M and Beta (β) values were generated: *M* values represent the base 2 log ratio of the intensities of the methylated and unmethylated probes;

β values are the ratio of methylated probe intensities to the overall intensity. For statistical purposes, *M* values were used as β value distribution displays heteroscedasticity in low and high methylation ranges (Du et al., 2010). However, β values, which range from 0 (0% methylation) to 1 (100% methylation) were used for data visualization.

Differentially methylated positions (DMPs) between groups were determined using empirical Bayes' moderated *t* test method, contained in the *limma* package (Ritchie et al., 2015). False discovery rates (FDR) <0.05 were used as significance threshold. Only probes with a difference in β values over 10% were kept for analysis ($|\Delta\beta| > 0.1$).

Differentially methylated regions (DMRs) were identified using the *DMRcate* package (Peters et al., 2015). The matrix of *M* value (logit transformation of beta) is annotated with the relevant annotation information about the probes such as their genomic position, gene annotation, etc. The limma pipeline was used for differential methylation analysis and to calculate moderated t-statistics; the dmrcate() function was used to combine CpGs to extract DMRs with a β value cut-off of 10% and FDR < 0.05 and a minimum number of CpG of 5, 10, and 20.

## Gene Ontology

In a first step, gene enrichment analysis was performed for genes presenting at least one promoter DMP (TSS1500, TSS200, 5′UTR), followed by analysis based on DMPs in promoters and gene bodies. Gene Ontology (GO) analysis for biological processes, cellular components and molecular functions and KEGG pathway analysis (Kyoto Encyclopedia of Genes and Genomes)[1] were performed using the R package *clusterProfiler* (Yu et al., 2012). Only significant GO terms and KEGG pathways are shown (Bonferroni corrected $p < 0.05$).

## Calculation of Methylation Scores (mDNA Scores)

To identify potential DMPs associating with disease activity before and after treatment, genes involved in TNF-α and IL-17 signaling pathways were identified through the WikiPathways database[2] (Martens et al., 2021). We focused on these genes, as patients involved in this target identification step of the study were treated with either TNF or IL-17A directed biopharmaceutical agents. Methylation scores were calculated as previously suggested by Björk et al. (2020). Briefly, means ($mean_{HC}$) and standard deviations ($SD_{HC}$) for each DMP involved in TNF-α and IL-17 signaling pathways in the healthy control group were used to achieve standardized values (SVs) for each individual according to the formula: $SV = (Value - Mean_{HC})/SD_{HC}$. Subsequently, SVs were summed up to total scores (Björk et al., 2020).

## Statistical Analysis

One-way 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 (normality and homoscedasticity were tested prior to these tests). Shapiro–Wilk normality tests were performed to assess Gaussian distribution before testing statistical associations between two variables using Pearson's correlation. Statistical tests were performed using GraphPad Prism (V.6.0, GraphPad).

[1] http://www.genome.ad.jp/kegg

[2] https://www.wikipathways.org/

# RESULTS

## Study Cohort

We investigated differential methylation patterns of $CD8^+$ T cells from patients with skin psoriasis ($N = 10$), PsA patients ($N = 7$), and healthy controls ($N = 9$). An imbalance existed in the distribution between women and men across sub-cohorts with more men in the skin psoriasis (70%) and PsA (57%) patient cohorts when compared to healthy controls (44%). Furthermore, the mean age of skin psoriasis (36.8 years) and PsA (54.7 years) patients was slightly higher when compared to healthy controls (32.2 years) (**Table 1**).

## Peripheral Blood Effector Memory $CD8^+$ T Cells Are Elevated in Psoriatic Arthritis Patients

$CD8^+$ T cells were FACS sorted and phenotyped (described in section "Materials and Methods") to distinguish the proportion of naïve, Effector Memory (EM), Central Memory (CM), and Effector memory cells re-expressing CD45RA (EMRA) sub-population by flow cytometry (**Supplementary Figure 1A**). While we observed comparable proportions of $CD8^+$ T cells across study cohorts (**Supplementary Figure 1B**), we detected significantly increased proportions of EM $CD8^+$ T cells in PsA patients when compared to healthy controls (**Supplementary Figure 1C**).

## Differentially Methylated CpG Positions Separate Psoriasis Patients From Controls, and Skin Psoriasis From Psoriatic Arthritis

Comparison between "all psoriasis" (combined skin psoriasis and PsA) patients versus healthy controls identified 397 differentially methylated positions (DMPs), including 195 hypermethylated and 202 hypomethylated CpGs (FDR < 0.05, $|\Delta\beta| > 0.1$) (**Table 2**). Differential global DNA methylation patterns (**Figure 1A**) and detailed examples of differentially methylated CpG sites (top four candidates, **Figure 1B**) are displayed in **Figure 1**.

Comparison of DNA methylation patterns in $CD8^+$ T cells from psoriasis vs. PsA patients allowed the identification of 1,861 DMPs, including 987 hypermethylated and 874 hypomethylated CpGs (**Table 2** and **Figure 2A**). Differential global DNA methylation patterns (**Figure 2A**) and detailed examples of differentially methylated CpG sites (top four candidates, **Figure 2B**) are displayed in **Figure 2**.

Overall numbers of DMPs identified across all aforementioned analyses are displayed in **Figure 3**. No DMPs (**Figure 3A**) were common to all four sub-analyses; 33 DMPs were shared

**TABLE 2 |** Differentially methylated positions (DMPs) in $CD8^+$ T cells from psoriasis patients and controls.

| | DMPs | | | Corresponding genes | | | |
|---|---|---|---|---|---|---|---|
| **Comparison** | **Hypermethylated** | **Hypomethylated** | **Total** | **Hypermethylated** | **Hypomethylated** | **Hyper and hypomethylated** | **Total** |
| Controls vs. Psoriasis | 110 | 224 | 334 | 73 | 140 | 1 | 212 |
| Controls vs. PsA | 957 | 1138 | 2095 | 602 | 644 | 24 | 1222 |
| Psoriasis vs. PsA | 987 | 874 | 1861 | 642 | 508 | 24 | 1126 |
| "All psoriasis" vs. Controls | 195 | 202 | 397 | 124 | 123 | 3 | 244 |
| Before vs. after treatment | 508 | 1864 | 2372 | 443 | 1460 | 59 | 1962 |

*DMPs were obtained between groups with criteria selection q value $< 0.05$ and $|\Delta\beta| \geq 0.1$ and genes concerned. PsA, Psoriatic arthritis; All psoriasis, Psoriasis and PsA patients combined.*

between "all psoriasis" patients versus controls and psoriasis versus PsA analyses (**Figure 3B**), accounting for 8% of DMPs identified in "all psoriasis" patient versus healthy controls, and almost 2% of DMPs identified in psoriasis versus PsA analyses. Results illustrate unique methylation profiles across patient and control cohorts.

## Genomic Distribution of Differentially Methylated Positions Across Study Cohorts

Considering DNA methylation patterns in $CD8^+$ T cells from "all psoriasis" patients versus healthy controls, DMPs were equally distributed among promoter regions (31.9%), gene bodies (33.9%), and intergenic regions (32.4%) (**Table 3**). Analyzing DNA methylation in the context of CpG density, the majority of DMPs were found in the "open sea" (53.5%), followed by CpG islands (20.4%).

Examination of DMP distribution between psoriasis patient sub-cohorts (skin psoriasis vs. PsA) revealed a similar distribution of DMPs between promoters (33.3%), gene bodies (34.3%), and intergenic regions (30.5%) (**Table 3**). As above, analysis of DMPs localization in relation to CpG density delivered a general predominance of DMPs in the "open sea" (53.1%) (**Table 3**). Notably, across all aforementioned comparisons, DMPs to almost equal extends exhibited hyper- or hypo-methylation between groups.

## Biological Pathway Analysis Separates Patients From Controls

Gene ontology (GO) analyses were performed to predict biological pathways affected by differential DNA methylation. First, analyses were limited to genes with at least one DMP in their promoter region. Analysis of 125 genes that included 154 DMPs (85 hypo and 69 hypermethylated) did not allow the identification of specific pathways between "all psoriasis" versus healthy controls. However, a total of 140 DMPs (98 hypo- and 42 hypermethylated CpGs) annotated to 111 unique genes were identified in skin psoriasis patients as compared to healthy controls. Gene ontology analysis of differentially methylated genes highlighted negative ($p = 1.82 \times 10^{-6}$) and positive ($p = 3.22 \times 10^{-5}$) regulation of the bone morphogenic protein (BMP) signaling pathway (**Figure 3C**). An even higher number of DMPs were identified between PsA patients and healthy controls. A total of 872 DMPs (482 hypo- and 449 hypermethylated CpGs) were identified and mapped to 667 unique genes linked to "endopeptidase regulator activity" ($p = 9.92 \times 10^{-5}$), "cysteine-type endopeptidase inhibitor activity" ($p = 1.03 \times 10^{-4}$), and "endopeptidase inhibitor activity" ($p = 2.09 \times 10^{-4}$) (**Figure 3D**).

Investigating variable DNA methylation in $CD8^+$ T cells from patients with skin psoriasis versus PsA, we discovered 813 DMPs (524 hypo- and 289 hypermethylated CpGs) that were uniquely annotated to 613 genes. Though a high number of DMPs were identified, no significantly enriched pathways have been found applying GO analysis.

Next, GO analyses were performed considering genes presenting DMPs in both promoters and gene bodies. Analysis of 516 DMPs uniquely associated to 279 genes allowed the identification of "synapse organization" ($p = 3.23 \times 10^{-5}$) and "cell junction assembly" ($p = 3.31 \times 10^{-5}$) as enriched biological processes between healthy controls and "all psoriasis" patients (**Supplementary Figure 2A**). However, GO analysis did not highlight pathways differentially affected between skin psoriasis patients and healthy controls, although 247 genes presented at least one DMP in their promoter and gene body. A meaningful number of DMPs (2,746) was observed between PsA patients and healthy controls, which mapped to 1,379 unique genes linked to a variety of biological processes, cellular components and molecular functions. Among the most significant enriched pathways there were: "synapse organization" ($p = 1.90 \times 10^{-8}$), "cell junction assembly" ($p = 2.34 \times 10^{-6}$), and "cell-cell adhesion via plasma-membrane adhesion molecules" ($p = 1.99 \times 10^{-5}$) (**Supplementary Figure 2C** and **Supplementary Table 1**).

Finally, investigating DMPs in $CD8^+$ T cells from patients with skin psoriasis versus PsA, a total of 2,457 DMPs were identified across 1,259 genes. DMPs corresponded also to a high number of biological processes associated, including "pattern specification process" ($p = 9.36 \times 10^{-8}$), "cartilage development" ($p = 9.34 \times 10^{-6}$), and "histone modification" ($p = 1.74 \times 10^{-5}$) (**Supplementary Figure 2B** and **Supplementary Table 2**).

## Identification of Differentially Methylated Regions

To identify larger differentially methylated genomic regions (DMRs), step-wise analysis including a minimum of 5, 10, or 20 CpGs per region was performed (**Supplementary Tables 3–5**).

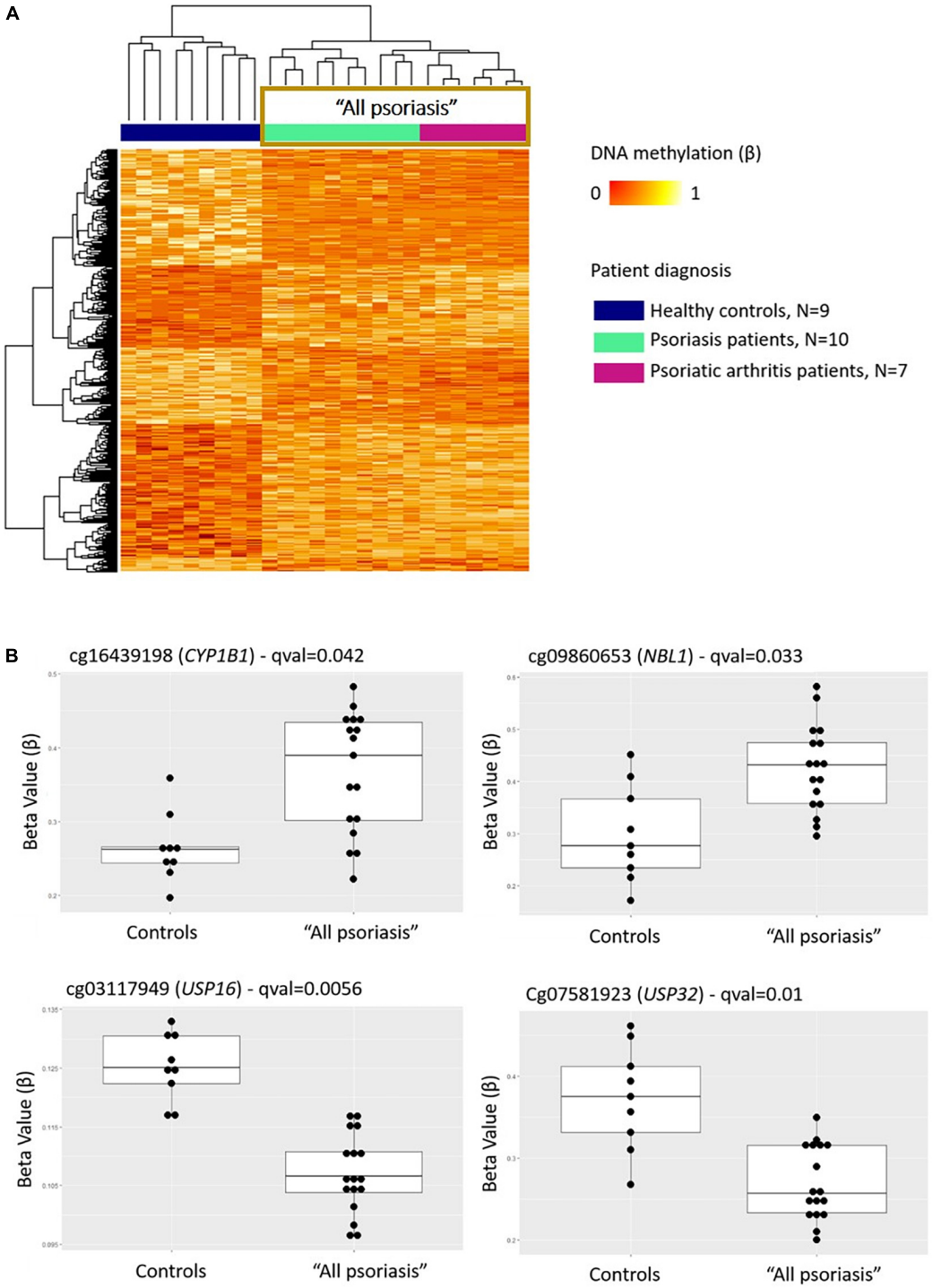


**FIGURE 1 |** DNA methylation profiles differentiate CD8$^+$ T cells from psoriasis patients and healthy individuals. **(A)** Heat map showing differentially methylated positions (DMP) between "all psoriasis" (combined psoriatic arthritis and psoriasis patients) patients and healthy controls (FDR $< 0.05$, $|\Delta\beta| > 0.1$). Normalized DNA methylation levels are displayed on the right with red indicating reduced methylation and yellow indicating increased methylation levels. **(B)** Differences in beta values of selected CpG sites identified as DMPs across "all psoriasis" patients versus healthy controls. "All psoriasis," Psoriasis and PsA patients combined.

Among DMRs identified between "all psoriasis" patients versus healthy controls, skin psoriasis patients versus healthy controls, PsA patients versus healthy controls, and skin psoriasis versus PsA patients with $\geq$5 CpGs, only 1 DMR within the *ZNF714* gene was common to all analyses (**Figure 4A**). No DMRs were common to all analyses when DMRs with a minimum of

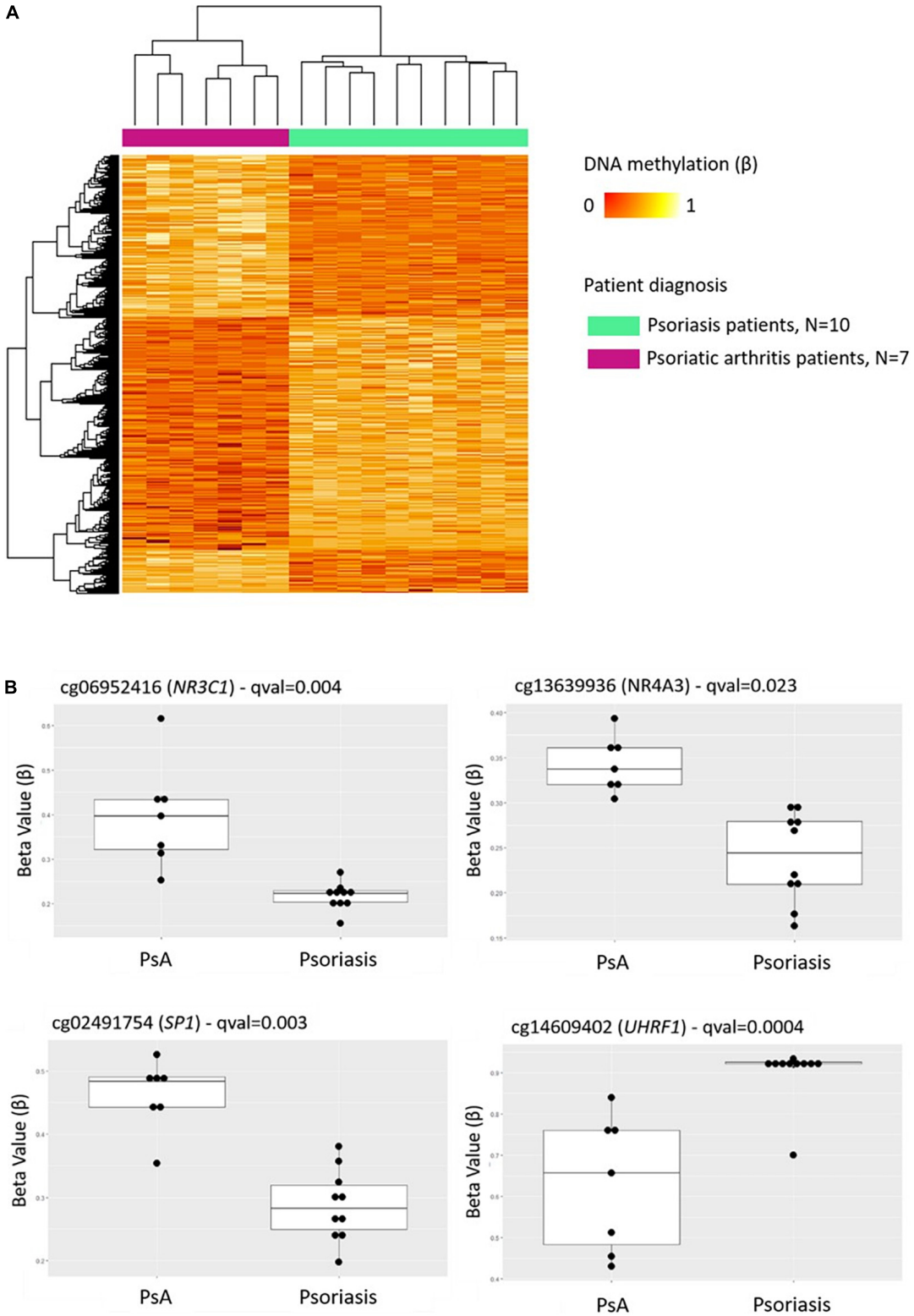


**FIGURE 2 |** DNA methylation profiles differentiate $CD8^+$ T cells from psoriatic arthritis and skin psoriasis patients. **(A)** Heat map showing differentially methylated positions (DMP) between psoriatic arthritis patients and psoriasis patients (FDR $< 0.05$, $|\Delta\beta| > 0.1$). Normalized DNA methylation levels are displayed on the right with red indicating reduced methylation and yellow indicating increased methylation levels. **(B)** Differences in beta values of selected CpG sites identified as DMPs across psoriasis and psoriatic arthritis patients. PsA, Psoriatic arthritis patient.

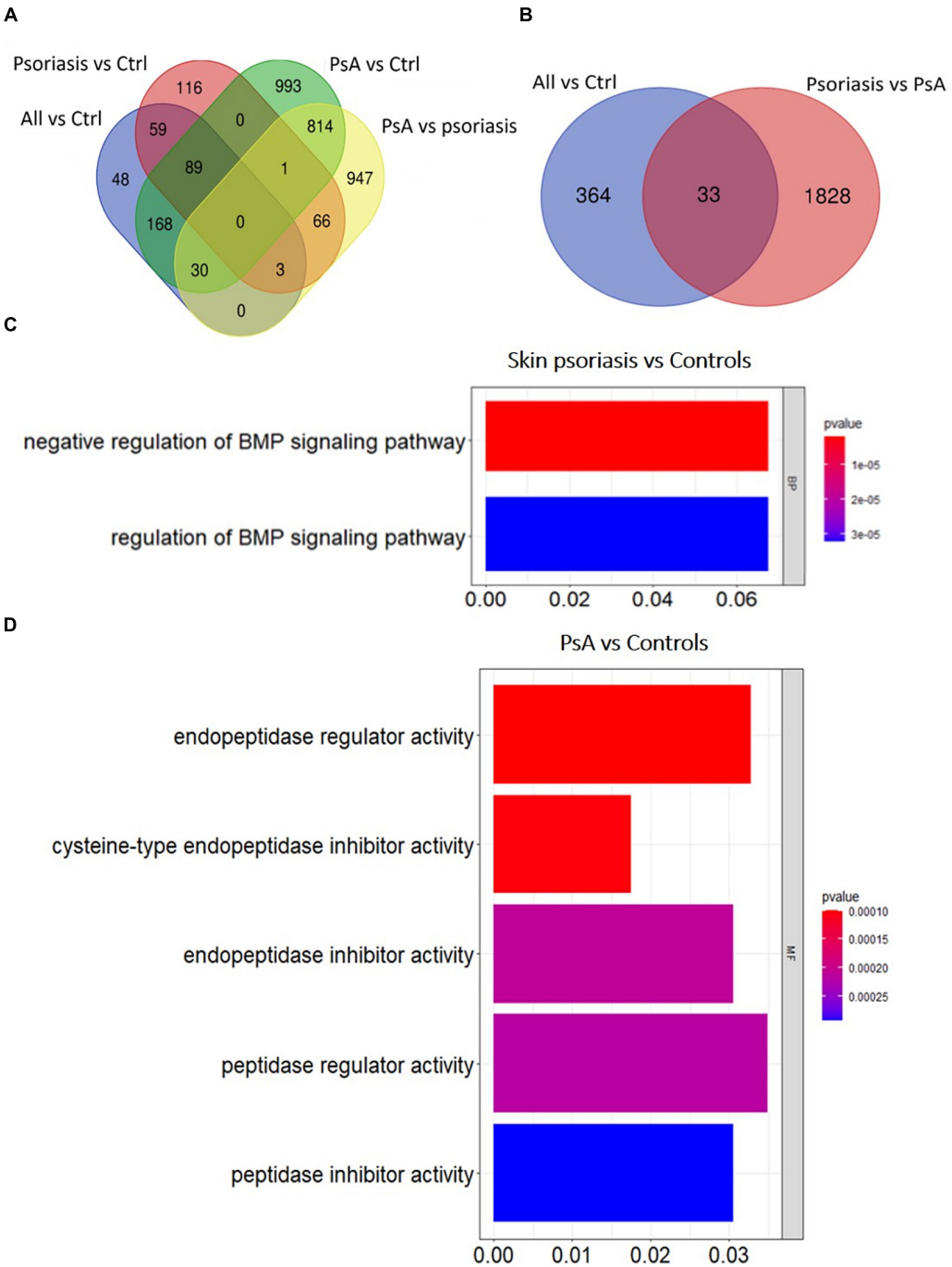


**FIGURE 3 |** Shared and unique methylation patterns in CD8$^+$ T cells from psoriasis patients and controls, and pathways affected. **(A)** Venn diagram showing overlapping and differentially methylated positions (DMP) found in psoriasis patients versus controls (Ctrl). Differences were tested between "All" (skin psoriasis and psoriatic arthritis combined) and Ctrl (healthy control), controls versus skin psoriasis patients, controls versus psoriatic arthritis (PsA) patients, and skin psoriasis versus PsA patients **(A)**. **(B)** Contrasts were assessed only between "All" and Ctrl and skin psoriasis versus PsA patients. **(C,D)** The bar plots show the results of the Gene Ontology (GO) analysis of genes which presented at least on DMP in their promoter. Only significantly enriched terms for Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) are represented ($P < 0.05$), in "skin psoriasis" versus Controls **(C)** and PsA versus Controls **(D)**.

10 CpG per region were considered (**Figure 4B**). One DMR with ≥20 CpGs was common to all analyses in the *LHX6* gene (**Supplementary Table 5**).

Subsequently, GO analysis was performed to predict biological pathways affected by DMRs. A total of 20 DMRs with ≥5 CpGs associated to 78 genes differentiated skin psoriasis

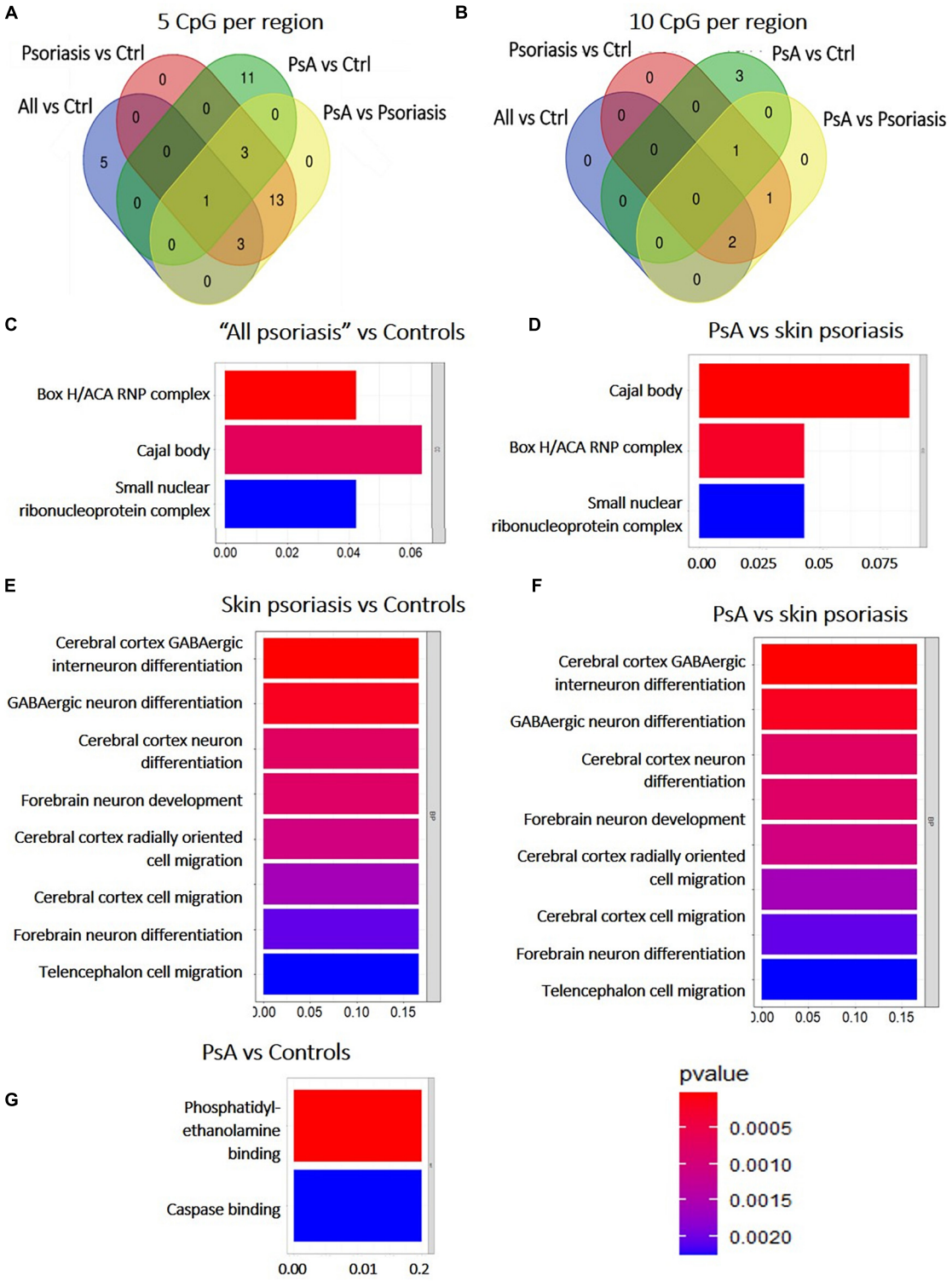


**FIGURE 4 |** Shared and unique Differentially Methylated Regions (DMRs) in CD8$^+$ T cells from psoriasis patients and controls, and pathways affected. **(A)** Venn diagram showing overlapping and differentially methylated regions (DMRs) with ≥5 CpGs **(A)** and ≥10 CpGs **(B)** per regions, in "all patients" (all, including psoriasis and psoriatic arthritis patients) versus controls (ctrl), controls versus psoriasis, controls versus psoriatic arthritis (PsA), and psoriasis versus psoriatic arthritis. **(C–G)** The bar plot shows the results of the Gene Ontology (GO) analysis of genes associated with DMRs with ≥5 CpGs per region between "all patients" versus controls **(C)** and PsA versus skin psoriasis **(D)**; and ≥10 CpGs for PsA versus skin psoriasis **(F)**, skin psoriasis versus controls **(E)**, and PsA versus controls **(G)**. Only significantly enriched terms for Biological Process (BP) and Cellular Component (CC) are represented ($P < 0.05$).

**TABLE 3 |** Functional genomic distribution of DMPs in CD8$^+$ T cells from psoriasis, psoriatic arthritis patients, and controls.

| | | "All psoriasis" vs. Controls | | | Skin psoriasis vs. PsA | | |
|---|---|---|---|---|---|---|---|
| | | All DMPs (%) | Hyper DMPs (%) | Hypo DMPs (%) | All DMPs (%) | Hyper DMPs (%) | Hypo DMPs (%) |
| In relation to gene region | Promoter | 31.9 | 29.5 | 34.2 | 34.3 | 39.6 | 28 |
| | 3′UTR | 1.6 | 2.3 | 0.9 | 1.8 | 1.8 | 1.9 |
| | Body | 33.9 | 34.1 | 33.8 | 33.3 | 30.8 | 36.3 |
| | ExonBnd | 0.2 | 0.5 | 0 | 0.1 | 0.1 | 0.2 |
| | Intergenic | 32.4 | 33.6 | 31.1 | 30.5 | 27.8 | 33.6 |
| In relation to CpG island | Island | 20.4 | 20 | 20.8 | 21.9 | 23.8 | 19.7 |
| | Shore | 16.9 | 13.3 | 20.3 | 17.4 | 18.8 | 15.7 |
| | Shelf | 9.1 | 9.2 | 8.9 | 7.7 | 6.8 | 8.7 |
| | Open Sea | 53.7 | 57.4 | 50 | 53.1 | 50.6 | 55.9 |

*Functional genomic distribution of differentially methylated positions (All DMPs), hypermethylated (hyper DMPs), and hypomethylated (hypo DMPs) DMPs is displayed between "All patients" (psoriasis and psoriatic arthritis combined) versus controls (left) and psoriasis versus psoriatic arthritis patients (PsA, right). Analyses were performed in relation to gene region [promoter, 3′UTR, gene body, exon boundaries (Bnd), and intergenic regions] and CpG island context (Island, shore, shelf, open sea). Values are percentages and color scale indicate a high percentage (red) or low percentage (blue) of the DMPs in each functionally annotated region.*

patients from healthy controls, and affected genes associated with the box H/ACA RNP complex ($p$ = 0.007), Cajal body ($p$ = 0.01), and the small nucleolar ribonucleoprotein complex ($p$ = 0.02) (GO analysis, **Figure 4C**). Similarly, 20 DMRs affecting 78 genes differentiated PsA and skin psoriasis patients, and affected Cajal body, box H/ACA RNP complex, and small nucleolar ribonucleoprotein complex ($p$ = 0.001, $p$ = 0.004, $p$ = 0.02, respectively) signaling pathways (GO analysis, **Figure 4D**).

Focusing on DMRs with ≥10 CpGs, a total of 4 DMRs affecting 11 genes differentiated skin psoriasis patients from healthy controls, and affected cerebral cortex GABAergic interneuron differentiation ($p$ = 0.04), GABAergic, cerebral cortex and forebrain neuron differentiation, forebrain neuron development and cerebral cortex radially oriented and telencephalon cell migration (all $p$ = 0.04) pathways (GO analysis, **Figure 4E**). Comparing PsA patients and healthy controls, 4 DMRs affecting 17 genes were identified and involved phosphatidylethanolamine and caspase binding (both $p$ = 0.03) (**Figure 4F**).

Lastly, 4 DMRS affecting 11 genes were identified when comparing PsA and skin psoriasis. Notably, GO analysis highlighted the same signaling pathways previously identified when comparing skin psoriasis to healthy control patients (**Figure 4G**).

**TABLE 4 |** Functional genomic distribution of DMPs in CD8$^+$ T cells in response to treatment.

| | | Before vs. after treatment | | |
|---|---|---|---|---|
| | | All DMPs (%) | Hyper DMPs (%) | Hypo DMPs (%) |
| In relation to gene region (%) | Promoter | 57 | 69.6 | 53.1 |
| | 3′UTR | 2 | 0.6 | 2.4 |
| | Body | 29.8 | 22 | 32.3 |
| | ExonBnd | 0.1 | 0 | 0.2 |
| | Intergenic | 11 | 7.7 | 12 |
| In relation to CpG island | Island | 68.1 | 52 | 72.5 |
| | Shore | 11.2 | 26.2 | 7.1 |
| | Shelf | 2.2 | 2.4 | 2.2 |
| | Open Sea | 18.5 | 19.5 | 18.2 |

*Functional genomic distribution of differentially methylated positions (All DMPs), hypermethylated (hyper DMPs), and hypomethylated (hypo DMPs) DMPs is displayed between psoriasis patients "before" and "after" treatment initiation with TNF inhibitors or inactivating IL-17A antibodies. Analyses were performed in relation to gene region (promoter, 3′UTR, gene body, exon boundaries, and intergenic regions) and CpG island context (Island, shore, shelf, open sea). Values are percentages and color scale indicate a high percentage (red) or low percentage (blue) of the DMPs in each functionally annotated region.*

## Therapeutic Cytokine Blockade Impacts DNA Methylation in Psoriasis Patients

To investigate the impact of treatment on DNA methylation patterns in CD8$^+$ T cells from psoriasis patients, DMP analysis was conducted before and after treatment initiation with cytokine blocking strategies (anti-TNF or anti-IL-17A treatment).

A comparable proportion of CD8$^+$ T cells was observed in patients before and after treatment. While no differences between the proportions of naïve, EM and CM CD8$^+$ T cells was observed, treatment with cytokine blockers associated with a larger proportion of effector memory re-expressing CD45RA CD8$^+$ T cells (EMRA) (**Supplementary Figure 3**).

A total of 2,372 DMPs (508 hyper- and 1,864 hypomethylated CpGs) were identified in relation to treatment initiation (FDR < 0.05, $|\Delta\beta|$ > 0.1) (**Table 2**). Differential global DNA methylation patterns (**Figure 5A**) and detailed examples of differentially methylated CpG sites (top four candidates, **Figure 5B**) are displayed in **Figure 2**. Generally, DMPs were enriched in promoters (57%) and CpG islands (29.8%, **Table 4**), with a mild predominance of hypermethylated positions in gene promoters (69.9%) and shore regions (26.2%), and more hypomethylation within CpG Island (72.5%).

As in GO analyses conducted above, initially only DMPs within promoter regions alone (TSS1500, TSS200, 5′UTR)

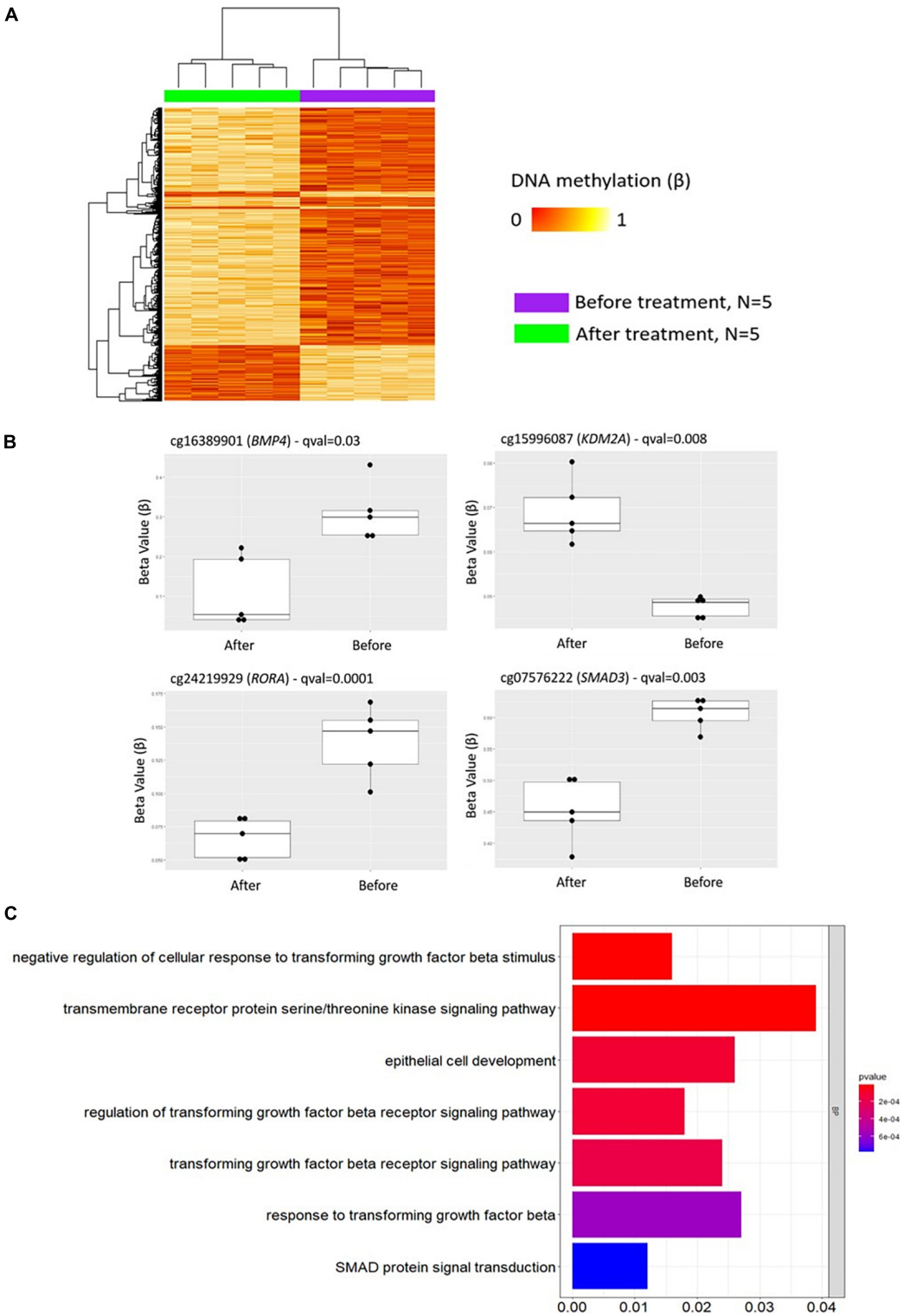


**FIGURE 5 |** DNA methylation profiles in CD8$^+$ T cells from patients with psoriasis change in response to treatment. **(A)** Heat map showing differentially methylated positions (DMP) in CD8$^+$ T cells from psoriasis ($N$ = 4 psoriasis and $N$ = 1 psoriatic arthritis) before and after treatment (FDR $< 0.05$, $|\Delta\beta| > 0.1$) with TNF inhibitors or inactivating IL-17A antibodies. Normalized DNA methylation levels are shown on the right red indicating reduced methylation and yellow indicating increased methylation levels. **(B)** Differences in beta values of selected CpG sites identified as DMP comparing status "before" versus "after" treatment initiation. **(C)** The bar plot shows the results of the Gene Ontology (GO) analysis of genes which presented at least on DMP in their promoter. Only significantly enriched terms for Biological process (BP) are represented ($P < 0.05$).

were considered. Comparing DNA methylation profiles of "all psoriasis" patients before and after treatment with anti-TNF or anti-IL17A directed agents, we identified 1,907 DMPs (1,359 hypo- and 548 hypermethylated) annotated to 1,380 genes. GO pathway analysis revealed an enrichment of genes involved in a total of 95 biological processes (**Supplementary Table 6**). In **Figure 5C**, signaling pathways associated with psoriasis (skin psoriasis or PsA) are displayed and include "negative regulation of cellular response to transforming growth factor beta stimulus" ($p = 1.54 \times 10^{-5}$), "transmembrane receptor protein serine/threonine kinase signaling pathway" ($p = 1.69 \times 10^{-5}$), "epithelial cell development" ($p = 1.14 \times 10^{-4}$), and "SMAD protein signal transduction" ($p = 7.72 \times 10^{-4}$). Additionally, GO analyses considering genes with at least one DMP in their promoter and gene body were performed, and delivered 3,883 DMPs uniquely associated to 2,055 genes which linked to multiple pathways, including "embryonic skeletal system development" ($p = 1.32 \times 10^{-8}$), "histone deacetylase complex" ($p = 2.75 \times 10^{-5}$) and "transcription regulator complex" ($p = 1.69 \times 10^{-5}$) (**Supplementary Figure 4** and **Supplementary Table 7**).

Considering wider DMRs, differentially methylated regions were identified in patients before and after treatment only with a minimum number of CpG of 5 (7 DMRs, **Table 5**).

## DNA Methylation Correlates With Skin Disease Activity

To develop a tool to measure skin psoriasis disease activity, DNA methylation changes in response to treatment were investigated and correlated with skin disease activity as assessed by PASI scores. As anti-TNF and anti-IL-17A treatments were applied and both cytokines play a key role in the pathophysiology of psoriasis (Blauvelt and Chiricozzi, 2018), the search was focused on these two biological pathways. Genes involved in TNF-α and IL-17 signaling were identified using the WikiPathways database (see text footnote 2) (Martens et al., 2021). Following this, 10 DMPs previously identified and associated with these genes were considered. Beta values were collected, and exclusively DMPs for which β values strongly correlated with skin disease activity/PASI scores (correlation coefficient above |0.8|) were used to calculate methylation scores (Björk et al., 2020). Notably, methylation scores increased in response to treatment becoming comparable to methylation scores in healthy individuals (**Figure 6A**). Indeed, methylation scores inversely correlated with PASI scores ($r = -0.696$ and $p = 0.025$, **Figure 6B**).

To assess whether methylation scores correlate with skin disease activity of psoriasis patients independent of treatment initiation, they were tested in the sub-cohort of patients with skin psoriasis. Indeed, a strong negative correlation was observed between PASI and methylation scores ($r = -0.893$, $p = 0.003$, **Figure 6C**).

# DISCUSSION

We, for the first time, show that patients with psoriasis exhibit DNA methylation patterns in $CD8^+$ T cells that allow differentiation from healthy controls. Furthermore, we demonstrate that DNA methylation marks in $CD8^+$ T cells from PsA patients and patients with skin psoriasis are distinct, suggesting DNA methylation screening as a promising candidate in the search for diagnostic and predictive biomarkers. This is of high potential value for translation into clinical care, as up to 25–50% of patients with PsA develop arthritis sometimes years before the onset of skin involvement (Busse and Liao, 2010). Furthermore, as joint disease in PsA is destructive and results in significant damage and disability, early recognition, delay of its onset or even prevention may be possible through identification of individuals at risk (Scher et al., 2019). However, at this stage, only samples from patients with current skin psoriasis or PsA were included in this study, and prospective monitoring of patients some of who will develop joint involvement is necessary to reliably answer the question of whether DNA methylation patterns change prior to disease progression from initial skin disease to PsA.

In addition to potential clinical applications as disease biomarkers, DNA methylation patterns also offer insights into the molecular pathophysiology of autoimmune/inflammatory conditions (Hedrich and Tsokos, 2011; Charras and Hedrich, 2019; Surace and Hedrich, 2019). Identification of molecular pathways affected may therefore offer insights into the pathogenesis of disease and direct future experimental work. Thus, functional gene ontology analyses were conducted examining genes containing at least one DMP in their promoter region. Interestingly, we only observed significant pathway associations between skin psoriasis or PsA patients and controls, but not between psoriasis sub-cohorts. This likely highlights skin psoriasis and PsA representing clinical phenotypes within the spectrum of "psoriatic disease" rather than distinct conditions (Sakkas and Bogdanos, 2017). Differences between all psoriasis patients and controls, but not between disease sub-types, may also reflect the importance of immunological interplay between immune cell populations beyond $CD8^+$ T cells (Teschendorff and Zheng, 2017).

Comparing skin psoriasis and healthy controls, GO analysis revealed DMP enrichment affecting distinct pathways, including negative and positive regulation of bone morphogenic protein (BMP) signaling. The BMP signaling pathway is an important regulator of epidermal homeostasis, hair follicle growth, melanogenesis and has previously been linked with the pathobiology of psoriasis (Botchkarev, 2003). Notably, Sconocchia et al. (2021) recently suggested functional links between BMP signaling and regulatory $CD4^+$ T cell (Treg) accumulation in psoriatic skin lesions. This is further supported by reports of dysregulated BMP-4 expression in psoriatic skin lesions that recover after 16 weeks of treatment with the TNF inhibitor adalimumab (Di Costanzo et al., 2019). Lastly, another BMP family member, BMP7 was suggested to locally promote Treg differentiation (Borek et al., 2020).

When comparing DMPs in $CD8^+$ T cells from PsA versus healthy controls, "endopeptidase regulator activity," "cysteine-type endopeptidase inhibitor activity," and "endopeptidase inhibitor activity" were among candidate pathways identified.

**TABLE 5 |** DMRs in $CD8^+$ T cells from patient before versus after treatment with a minimum number of 5 CpG per region.

| | Seq. names | Start | End | Width | Strand | No. CpGs | min_smoothed_fdr | Stouffer | HMFDR | Fisher | Max diff | Mean diff | Overlapping genes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | chr7 | 4829256 | 4831038 | 1783 | * | 7 | 0 | 2.29E-06 | 7.79E-11 | 1.60E-10 | 0.743623188 | 0.12051242 | AP5Z1 |
| 2 | chr1 | 1.6E + 08 | 1.6E + 08 | 1366 | * | 6 | 0 | 1.54E-07 | 1.60E-09 | 6.86E-10 | 0.616346078 | 0.113332116 | snoU13, Y_RNA, SCARNA16, SNORD112, SNORA63, U3, SNORA51, SNORA25, SNORD59, SCARNA20, SNORA67, U6, SNORA70, SNORA77, SNORA26, SNORA72, U8, SNORA31, SNORA40, CCDC19, ACA64, SNORD78, snoU109, SNORD60, SNORD116 |
| 3 | chr17 | 36622522 | 36623419 | 898 | * | 5 | 0 | 0.039037453 | 1.18E-10 | 1.33E-07 | 0.684386166 | 0.142011824 | SNORA69, ARHGAP23 |

*(Continued)*

**TABLE 5 |** (Continued)

| | Seq. names | Start | End | Width | Strand | No. CpGs | min_smoothed_fdr | Stouffer | HMFDR | Fisher | Max diff | Mean diff | Overlapping genes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 | chr1 | 33740732 | 33742369 | 1638 | * | 6 | 0 | 0.000709788 | 6.53E-10 | 1.38E-07 | 0.670186925 | 0.11635636 | snoU13, Y_RNA, SCARNA16, SCARNA21, U1, SCARNA17, SCARNA18, SCARNA24, ZNF362, SNORD112, SNORA62, SNORA63, SNORD46, SNORA2, SNORD81, U3, SNORA51, SNORA25, SCARNA20, SNORA67, U6, SNORA70, SNORA77, SNORA26, U8, SCARNA11, SNORA31, SNORA42, SNORA40, SNORD64, ACA64, snoU109, SNORD60 |
| 5 | chr11 | 1.34E + 08 | 1.34E + 08 | 1325 | * | 5 | 0 | 0.003851736 | 3.83E-09 | 1.58E-07 | 0.542962362 | 0.117641489 | B3GAT1 |
| 6 | chr10 | 1451166 | 1451609 | 444 | * | 6 | 0 | 0.080595592 | 6.16E-09 | 3.96E-06 | 0.60478433 | 0.105749797 | ADARB2 |
| 7 | chrX | 1.35E + 08 | 1.35E + 08 | 1187 | * | 7 | 1.43E-113 | 0.393049518 | 8.90E-06 | 0.002136723 | −0.806406846 | −0.133938921 | MMGT1 |

** is considered to belong to both strands.*

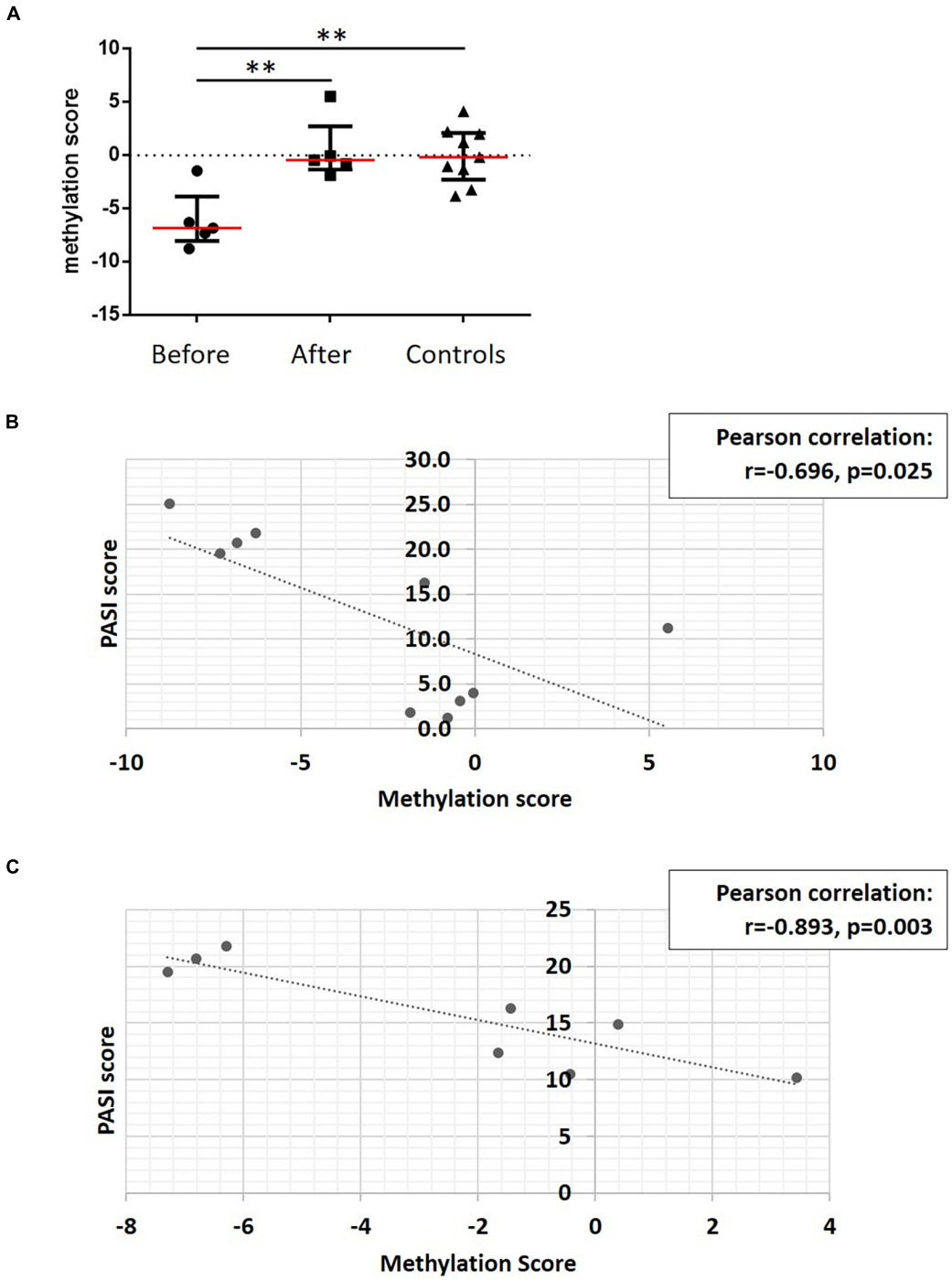


**FIGURE 6 |** A DNA methylation score associates with disease activity. **(A)** DNA methylation scores were calculated in patients before treatment, and after treatment and healthy control. $^{**}p \leq 0.01$, Dunn's multiple comparisons test. Median (red) and interquartile range are represented in this scatter plot. **(B)** Correlation analysis between methylation scores and PASI scores in patient cohort before and after treatment. After assessing Gaussian distribution, Pearson was used to measure the correlation. **(C)** Correlation analysis between methylation scores and PASI scores in the psoriasis patient cohort. After assessing Gaussian distribution, Pearson was used to measure the correlation. Dotted lines indicate a trendline.

Cysteine proteases are a group of endopeptidases that contain a Cys-His-Asn triad at the active site (Verma et al., 2016). Notably, cathepsin K plays a crucial role in bone remodeling and is predominantly expressed in osteoclasts (Rengel et al., 2007). Cathepsin K plays a key role in the development of psoriasis-like lesions in mouse models of psoriasis by

affecting Th17 polarization (Hirai et al., 2013). Notably, PsA patients exhibit increased cathepsin K serum levels when compared with psoriasis patients and controls, which negatively correlates with calcitonin serum levels (Cubillos et al., 2016). Calcitonin is a hormonal calcium sensor that reduces bone resorption and remodeling by osteoclasts (Xie et al., 2020). Furthermore, Cysteine endopeptidases are involved in extracellular-matrix remodeling and their dysregulation has been linked to various diseases, including fibrosis and osteoarthritis (Bonnans et al., 2014).

The inclusion of DMPs in both promoters and gene bodies allowed the identification of an even higher number of pathways affected by DNA methylation defects, some of which have been linked with disease pathophysiology and clinical outcomes. Comparing all sub-types of psoriasis with healthy controls and PsA patients with healthy controls, the involvement of "cell junction assembly" and "cell-cell adhesion via plasma-membrane adhesion molecules" were highlighted. Currently available literature suggests that alterations of tight junctions proteins play a role in the pathophysiology of psoriasis (Kirschner et al., 2009), and antigens related to endothelial cell activation are important for the maintenance of cell aggregates in inflamed skin and infiltration of leukocytes (Lee et al., 1994; Veale et al., 1995). Notably, "cartilage development" was among the most relevant pathways observed when comparing PsA and skin psoriasis patients. Cartilage damage is one of the main characterizing features of PsA through disease stages, and proteoglycan loss is strictly associated to periarticular inflammation and synovitis (Abrar et al., 2020).

Differentially methylated regions (DMRs) are composed of multiple consecutive CpG sites that can regulate cellular functions including cell differentiation, proliferation, and aging (Reik et al., 2001; Bjornsson et al., 2008; Bock et al., 2008). Inter-individual variability in these processes is associated with differential methylation patterns in DMRs of multiple genetic loci (Lee et al., 1994; Bonnans et al., 2014). Moreover, DMRs are tissue specific and associate with disease state and may reflect autoimmune/inflammatory disease stages (Eckhardt et al., 2006). As coordinated DNA methylation changes in wider genomic regions are more likely to have downstream biological effects and implications for diseases development and progression than methylation changes in a single CpG, we analyzed DMRs across study sub-cohorts (Hotta et al., 2018; Spindola et al., 2019). Indeed, findings from DMP analyses were largely confirmed investigating wider DMRs, and additionally allowed differentiation between skin psoriasis vs. PsA in subsequent GO analyses. DMRs analysis highlighted an enrichment of pathways linked to cellular components between all psoriasis patients and healthy controls, including the Small nuclear ribonucleoprotein (snRNP) complex, for which a class of autoantigens known as RNA-associated molecules and autoantibodies recognizing snRNPs has been described in a variety of autoimmune/inflammatory diseases, including systemic lupus erythematosus (SLE), systemic sclerosis and mixed connective tissue disease (Kattah et al., 2010). Moreover, Cajal bodies are involved in the biogenesis of snRNPs and autoantibodies against them have been observed in SLE (Vázquez-Talavera et al., 2004).

One of the targets identified to distinguishing PsA and psoriasis from healthy controls, GABAergic neuron and cortex neuron development pathways, may appear surprising at first. However, increased GABA ligand and the $GABA_A$ receptor expression has been previously reported in psoriatic skin lesions (Nigam et al., 2010). Indeed, the sensory nervous system may alter immune functions in the skin, thereby contributing to inflammatory disease (Ayasse et al., 2020). A cross-talk between the immune and the nervous system through neurotransmitters has recently been proposed in psoriasis (Chen et al., 2020). In this context, GABA may have immunoregulatory potential as it stimulates the expression of FoxP3, a transcription factor essential for regulatory T cell differentiation and the expression of immune regulatory cytokines IL-10 and TGF-β, the T cell regulatory co-receptor CTLA4, and the myeloid regulatory membrane glycoprotein SIRP-α (Bajić et al., 2020). Furthermore, neurotransmitters may affect the composition of the gut microbiome, a recently appreciated factor in psoriasis (Chen et al., 2020). Lastly, decreased GABA serum levels were noted in a majority of psoriasis patients with psycho-emotional disorders, a common comorbidity affecting psoriasis patients (Matiushenko et al., 2020). However, in the here presented study, no information on emotional health was collected.

Measuring disease activity is a challenge in systemic autoimmune/inflammatory disease, including psoriasis (Ballestar et al., 2020; Calle-Fabregat et al., 2020; Carvalho and Hedrich, 2021). Clinical scores, including PASI, are available but limited by their time-consuming and inter observer variability, especial in less experienced/specialized institutions (Fink et al., 2018). To develop tools to reliably and reproducibly monitor treatment response and gain additional insights into molecular mechanisms on inflammation in psoriasis, we investigated whether cytokine blockade (anti-TNF or IL-17A directed) and induction of clinical remission impact on DNA methylation patterns in $CD8^+$ T cells from psoriasis patients. Indeed, in response to treatment, we identified DMPs previously associated with epithelial cell development, TGF-β, and SMAD pathways. SMAD is a pivotal intracellular effector for TGF-β and BMP signaling (Dituri et al., 2019), both involved in the pathophysiology of psoriasis (Borek et al., 2020; Sconocchia et al., 2021).

Provided treatment-associated changes to methylation marks, we wondered whether the calculation of targeted methylation scores correlate with skin disease activity (PASI). We focused our efforts on genes involved in TNF and IL-17 signaling, as both pathways have previously been linked with the molecular pathophysiology of psoriasis and are targeted by (also here applied) available treatment options (Sakkas and Bogdanos, 2017). Indeed, methylation scores based on the formula suggested by Björk et al. (2020), discriminated between patients with disease activity versus the achievement of remission. Furthermore, methylation scores were comparable between healthy controls and psoriasis

patients after treatment initiation. To assess whether these methylation scores may be used to assess inflammatory activity of skin disease in an unbiased manner, we tested their association with PASI scores in the sub-cohort of skin psoriasis patients prior to the initiation of systemic treatment, and identified close inverse correlation between DNA methylation and PASI scores. While this requires to be confirmed in larger independent cohorts, including longitudinal follow-up of individuals, this promises potential for the use as prognostic biomarker.

Differential methylation status of $CD8^+$ T cells may be affected by differences in subset distribution across disease groups. Indeed, PsA patients included in this study exhibited higher proportions of EM $CD8^+$ T cells when compared to healthy controls. EM $CD8^+$ T cells are memory cells that have lost the constitutive expression of CCR7, heterogeneously express CD62L, and express chemokine receptors and adhesion molecules that are required for homing to inflamed tissues (Sallusto et al., 2004). Their increased proportion in PsA patients is in agreement with previous observations in blood and synovial fluid of PsA patients and their association with systemic inflammation (Diani et al., 2019). Furthermore, differential DNA methylation in response to treatment initiation also associated with changes in $CD8^+$ T cell subset distribution. We observed a higher proportion of CD45RA re-expressing EMRA $CD8^+$ T cells in response to treatment with cytokines blocking therapies. This relatively recently described memory T cells subset can express a variety of pro-inflammatory cytokines. Previous reports suggest that their increase may be the result of persistent exposure to pathogens, reactivation of latent viruses, and an increased levels of oxidative stress (Philippe et al., 2019). In the context of our study, the cause and effects of their increase in psoriasis patients after treatment initiation remains unclear and requires future studies.

While the work presented here delivers new insights into the molecular pathophysiology of skin psoriasis and PsA, and suggests the use of DNA methylation mapping as a diagnostic and/or prognostic biomarker, it has limitations. Sample size is limited because of the relative rarity of PsA, and findings require to be confirmed in larger independent cohorts. Furthermore, it remains unclear whether some of the differences in DNA methylation marks between disease sub-cohorts are (at least partially) caused by variable effector $CD8^+$ T cell distribution. This question will be addressed in future studies using single-cell DNA methylation screening. Lastly, the current work does not allow the assessment of DNA methylation impacting on gene transcription as RNA sequencing was not performed.

## CONCLUSION

Disease and subtype-specific DNA methylation patterns in $CD8^+$ T cells from psoriasis patients suggest that, similarly to what is already part of routine care in some cancers, DNA methylation profiling may allow for patient stratification toward individualized treatment, risk assessment and care. Targeted methylation scores closely correlating with skin disease activity (PASI) may represent a promising tool for monitoring disease activity and treatment response. While findings are encouraging, they require to be prospectively and independently confirmed in larger cohorts.

## DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the GEO repository, accession number: GSE184500.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the 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

AC and JG performed the DNA methylation, GO analysis, and wrote the first draft of the manuscript. SH and SR isolated immune cells and DNA. SH, SR, and EC analyzed flow cytometric datasets. SA consented patients and collected clinical data and biospecimen. CH oversaw all experimental and analytic steps and wrote the first draft of the manuscript. CH, SH, and SA planned the study. AC, JG, EC, CC, SR, 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.

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REVIEW
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# Insights Into the Role of DNA Methylation in Immune Cell Development and Autoimmune Disease

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To date, nearly 100 autoimmune diseases have been an area of focus, and these diseases bring health challenges to approximately 5% of the population worldwide. As a type of disease caused by tolerance breakdown, both environmental and genetic risk factors contribute to autoimmune disease development. However, in most cases, there are still gaps in our understanding of disease pathogenesis, diagnosis, and treatment. Therefore, more detailed knowledge of disease pathogenesis and potential therapies is indispensable. DNA methylation, which does not affect the DNA sequence, is one of the key epigenetic silencing mechanisms and has been indicated to play a key role in gene expression regulation and to participate in the development of certain autoimmune diseases. Potential epigenetic regulation via DNA methylation has garnered more attention as a disease biomarker in recent years. In this review, we clarify the basic function and distribution of DNA methylation, evaluate its effects on gene expression and discuss related key enzymes. In addition, we summarize recent aberrant DNA methylation modifications identified in the most important cell types related to several autoimmune diseases and then provide potential directions for better diagnosing and monitoring disease progression driven by epigenetic control, which may broaden our understanding and contribute to further epigenetic research in autoimmune diseases.

**Keywords: DNA methylation, T/B cell development and differentiation, cell memory, autoimmune diseases, DNA methyltransferases**

## INTRODUCTION

Autoimmune diseases, which represent a family of almost 100 conditions, have received mounting and widespread attention due to their complex etiologies and the life-long threat they pose. The initial study of autoimmune disease prediction can be traced back to the late 20th century and demonstrated that early risk factors for inducing autoimmune responses exist in the genes of the major histocompatibility complex (MHC) (Weetman and McGregor, 1984). The etiology of autoimmune diseases is multifactorial. In addition to variants in immune genes and environmental

factors, certain internal factors, including sex, age, and mental and emotional status, can also affect autoimmune responses, thus changing the possibility of developing clinical disease. The incidence rate of autoimmune diseases is high in industrialized countries, and females are predominantly affected, which is partially due to parent-of-origin differences in DNA methylation of the X chromosome (Golden et al., 2019). Although the clinical characteristics are diverse, all of these diseases have a basic etiology: a self-reactive adaptive immune response in which many lymphocytes participate (Rose, 2016) and a break of immune tolerance is the main character. Since most autoimmune diseases may have caused severe tissue damage before clinical diagnosis, it is necessary to make efforts to diagnose and treat them as soon as possible before irreversible damage occurs (Christen, 2019).

Epigenetics refers to heritable changes in gene expression separate from the DNA sequence that are mediated through a series of mechanisms regulated by environmental signals (Zheng et al., 2008). The major epigenetic regulation mechanisms include DNA methylation, histone modification and non-coding RNA regulation. To date, several lines of evidence confirm the important functions of epigenetic modifications in autoimmune diseases, especially DNA methylation, shedding light on disease pathogenesis, progression and activity to a certain extent (Wang et al., 2015).

In this review, DNA methylation, one of the major epigenetic adjustment mechanisms, will be reviewed, with particular attention on the function of DNA methylation in the types of cells involved in autoimmune diseases, the genomic methylation patterns involved in differentiation/development events and the dysregulated immune responses in specific autoimmune diseases. Moreover, the potential for epigenetic regulators as biomarkers and therapeutics for these diseases will be discussed.

## OVERVIEW OF DNA METHYLATION

One of the earliest discovered (∼1969) and intensely studied epigenetic regulation mechanism is DNA methylation, which functions in producing heritable phenotypic changes without affecting the DNA sequence (Bird, 2002; Xie et al., 2018). Thus, unlike genetic changes, epigenetic aberrations are reversible, which provides a direction for disease treatments by pharmaceutically inhibiting dysregulated epigenetic regulation (Zhang et al., 2020). The spectrum and distribution of methylation levels and patterns can vary between populations. Both nematodes and the insect *Drosophila melanogaster* have been reported to lack methylation due to their undetectable m5C expression level and absence of DNA methyltransferases (DNMTs) (Gowher et al., 2000). In mammals, the majority of DNA methylation mainly occurs on cytosine-guanine dinucleotide (CpG) sites, and the percentage of methylated CpG sites in the human genome is 70%∼80%. However, evidence has shown that a level of methylation on non-CpG sites exists in mouse and human embryonic stem cells (ESCs) (Xie et al., 2012). A proportion of unmethylated CpG dinucleotides are enriched mainly in gene promoter regions and are always located in clusters called CpG islands (CGIs) (Husquin et al., 2018; Li et al., 2021). In addition, there are regions called CGIs shore that are located no more than 2 kb from CGIs, which have strongly conserved tissue-specific methylation patterns. The methylation of both CGIs and CpG shores is strongly related to gene expression reduction (Irizarry et al., 2009). Moreover, the pattern and level of DNA methylation are influenced by the complex interplay of environmental and genetic factors. For example, some deleterious factors including toxic, radiation, drugs, and pollution. Moreover, lifestyles such as diet (folate uptake), smoking and stress are also typical environmental factors. Additionally, viral or bacterial infection, inflammatory cytokines induction (Rui et al., 2016; Sanderson et al., 2019; Zouali, 2021; **Figure 1**). As one of the most important and well-known epigenetic mechanisms, DNA methylation has been proposed to be involved in gene expression regulation and cell differentiation by cooperating with other regulators (Klutstein et al., 2016), as well as in chromatin structure. Subsequent chromatin remodeling can affect the production of many key proteins required for the normal function of the immune system (Lal et al., 2009).

The DNA methylation process involves a chemical modification in which specific bases in the DNA sequence are catalyzed by DNMTs and S-adenosyl methionine (SAM) is recruited as a methyl donor to obtain a methyl group for 5mC formation via covalent bonding (Moore et al., 2013). DNA methylation plays a maintainable role during normal development and functions in gene repression through silent chromatin reconstruction during each round of replication (Cedar and Bergman, 2012). With development, a substantial portion of DNA methylation in the blastocyst is gradually removed, and an epigenetic ground state is formed. Then, a wave of *de novo* methylation is established during X-chromosome inactivation, and almost all CpGs in the genome are modified at that time except protected CGIs (Cedar and Bergman, 2012). This brings about gene silencing on the inactivated chromosome, and housekeeping genes are expressed in all cells. After stage- and/or tissue-specific methylation changes, the epigenetic patterns of each individual cell type are ultimately molded (Meng et al., 2015). The DNA sequence information leads to this change, which serves as an important functor in the aspect of long-term expression stability.

The reversible function of epigenetic modification is due to the presence of enzymes that catalyze the apposition of posttranslational regulation, including histone methyltransferases and histone acetylases, which are recognized as epigenetic writers, and enzymes that act in the demethylation and deacetylation of histones, which are considered as epigenetic erasers (Renaude et al., 2021). A group of DNMTs (DNMT3a, DNMT3b, and DNMT1 are dominant) function in the establishment and maintenance of DNA methylation patterns in mammals. Both DNMT3a and DNMT3b enable the construction of a new methylation pattern for unmodified DNA, which is essential for their roles in transferring methyl groups during *de novo* methylation (Feng et al., 2005). Evidence in mouse embryonic stem (ES) cells has shown that the genomic enrichment pattern of DNMT3a is not consistent with that of DNMT3b, which reveals a phenomenon in which each

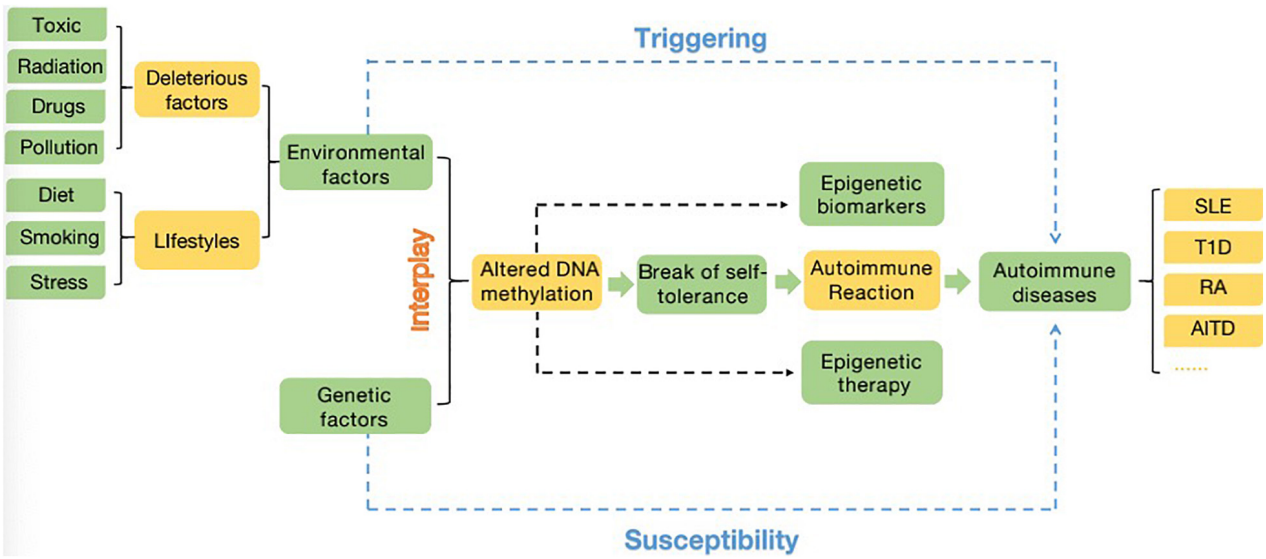


**FIGURE 1 |** A close interplay between the environment (deleterious factors and lifestyles) and genetic factors (related genes, SNPs, copy number variation. . .), the altered DNA methylome is responsible for the break of self-tolerance, leading to autoimmune reaction and then account for the emergence and/or progression of autoimmune diseases. The changed DNA methylated profiles can also provide new insights into diagnostic and therapeutic methods in autoimmune diseases. The major potential for epigenetics applying in clinical can be summarized as epigenetic biomarkers and epigenetic therapy. Additionally, environmental factors have ability to trigger autoimmune disease, while genetic factors provide susceptibility to autoimmune diseases (Zouali, 2021).

DNMT has specific targets reflecting their unique N-terminal domains during the development process. Genetic ablation of DNMT3a and DNMT3b leads to lethal phenotypes at different developmental stages. DNMT3a is required for establishing maternal imprints in differentially methylated regions (DMRs), and DNMT3b plays a leading role in inactivation of X chromosomes (Manzo et al., 2017; Yagi et al., 2020). DNMT1 is localized at replication foci and always acts in cell division. As a maintenance enzyme, DNMT1 crucially functions in preserving the stability of established DNA methylation patterns (Goll and Bestor, 2005; **Figure 2**). Furthermore, as an essential cofactor for *de novo* methyltransferase in ES cells, DNMT3L is highly expressed in ES and germ cells and plays a key role in the methyltransferase activity of DNMT3a and DNMT3b via a physical interaction (Ooi et al., 2010). Additionally, research has demonstrated that the methyltransferase activity of DNMT2 is weak *in vitro*, and deletion of DNMT2 has little effect on CpG methylation levels or developmental phenotypes (Goll and Bestor, 2005). Moreover, the harmony of the DNA methylation level requires balanced control between DNA methylation and demethylation. Replication-independent active DNA demethylation and replication-dependent passive DNA demethylation are two major pathways to reverse repressed gene expression. Ten-eleven translocation (TET) demethylases are key DNA demethylation enzymes (Lio and Rao, 2019). In addition, the existence of DNA methylation variability, which is due to polymorphisms or mutations in target genes, has the ability to influence the phenotype of an individual (Imgenberg-Kreuz et al., 2018). Aberrant methylation may serve as a risk factor for some autoimmune diseases and may be caused by the influence of aging or the environment (**Figure 3**).

## DNA METHYLATION IN T CELL DEVELOPMENT

T cells are regarded as key mediators in immunity and immunologic memory. Their differentiation fates can be partially regulated by epigenetic mechanisms, such as DNA methylation (Liu et al., 2019). Recently, genome-wide methylation analyses have demonstrated dynamic changes in the methylome during different development and differentiation processes and that some DNA regulators are involved in controlling various aspects, including cell fate decisions, function, and stability (Ji et al., 2010). Based on the fact that dysregulated T cells participate in different disease states, including autoimmune diseases, chronic inflammatory diseases and cancer, more detailed knowledge of how epigenomic programming functions in these pathologic states is critical (McLane et al., 2019; Correa et al., 2020).

### DNA Methylation in T Helper Cell Development and Function

While genetic and environmental factors are known risk factors for autoimmune diseases, incomplete disease concordance between identical twins supports the notion that other factors play a role in disease development and progression. Recently, convincing evidence has indicated that epigenetic modifications, particularly impaired T cell DNA methylation, contribute to this additional factor (Makar et al., 2003; Generali et al., 2017). Naïve $CD4^+$ T cells are characterized by high plasticity and have the ability to differentiate into discrete lineages with unique functions in the immune response. Then, differentiated T helper (Th) cells can maintain their original lineage selection

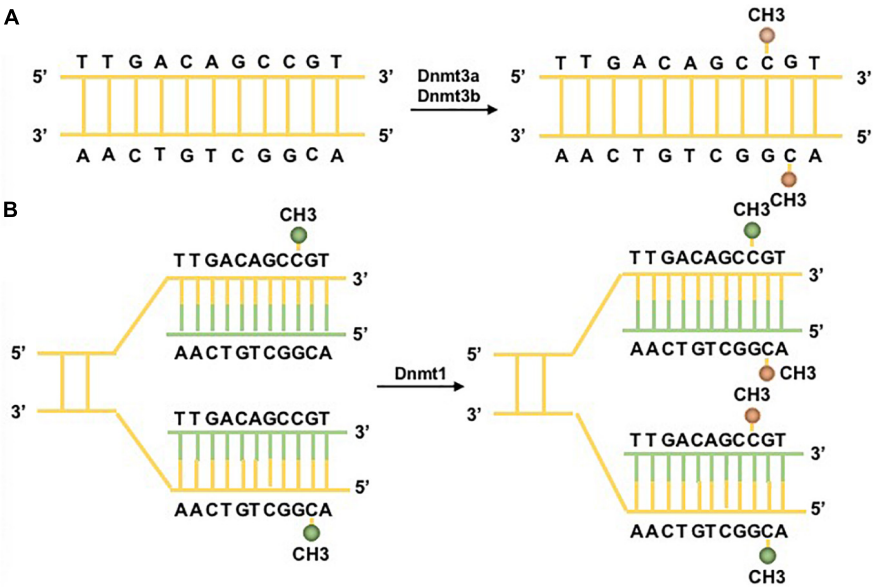


**FIGURE 2 |** DNA methylation pathways. Two major DNA methyltransferases (DNMTs) participate in the formation of 5-methylcytosine (5mC). **(A)** DNMT3a/3b/3L are the *de novo* DNMTs and transfer methyl groups (yellow) onto naked DNA. **(B)** DNMT1 is the maintenance DNMT and plays roles in maintaining the DNA methylation pattern during replication. Under the situation of DNA semiconservative replication, the parental DNA strand retains the original DNA methylation pattern (green). DNMT1 links to replication foci and precisely replicates the original DNA methylation pattern by adding methyl groups (yellow) onto the newly formed daughter strand (green) (Moore et al., 2013).

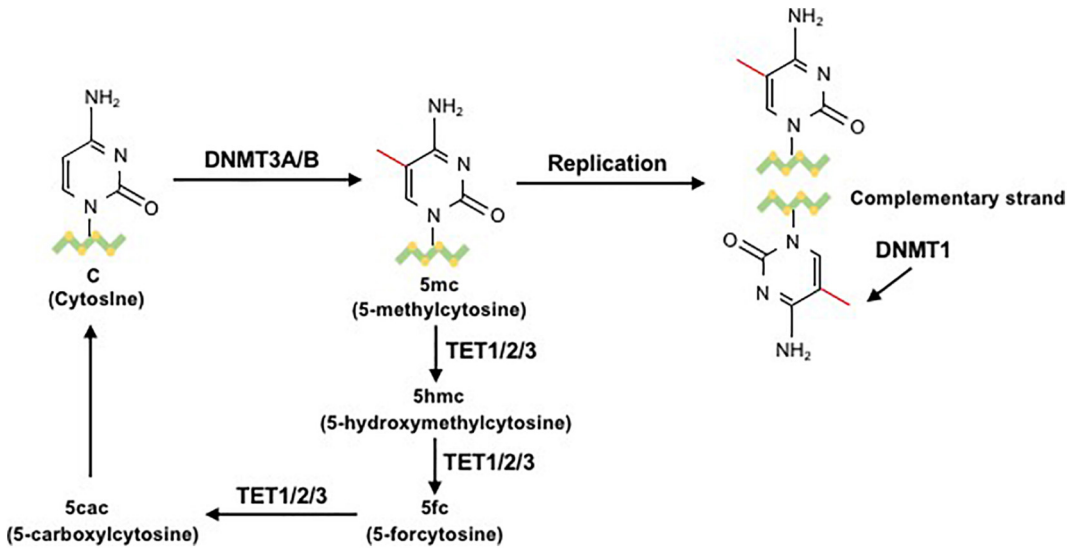


**FIGURE 3 |** The process of DNA methylation and demethylation. In the presence of the cofactor S-adenosyl methionine (SAM), the unmodified fifth carbon of cytosine resides in the DNA sequence to form a 5mC methyl group through the action of DNMT3a/b. 5-Methylcytosine (5mC) is mainly located on CpG dinucleotides in somatic cells. During the replication process, DNMT1 methylates the daughter chain to maintain 5mC. 5mC can be sequentially oxidized to 5hmC, 5fC, or 5caC by ten-eleven translocation (TET) cytosine dioxygenase enzymes. Then, 5fC and 5caC can be converted to unmodified cytosine (Correa et al., 2020).

under the condition of stable transcriptional memory to resist redifferentiation (Thomas et al., 2012a). During thymic development, the interleukin 4 (IL4) expression of naïve $CD4^+$ T cells has been reported to depend on epigenetic programming, which is consistent with the CD4/CD8 lineage. In the subsequent steps of peripheral maturation, mechanisms involving DNA methylation at the IL4-IL13 locus can partially suppress this IL4 expression potential (Makar et al., 2003). Transcriptional permission of the IL4–IL13 locus in naïve $CD4^+$ T cells still exists and is not affected by the accumulation of repressive DNA methylation marks (Baguet and Bix, 2004). Moreover, the process by which Th cells differentiate into mature Th1 and

Th2 fates is epigenetically regulated. DNMT1 plays an important role in repressing cytokine production, and depletion of DNMT1 mediated by CD4Cre brings about increased expression of the cytokines interferon-γ (IFN-γ), IL2, IL3, and IL4 in activated $CD4^+$/$CD8^+$ T cells and decreased proliferation of peripheral T cells (Lee et al., 2001). Th1 cells show an IFN-γ-demethylated promoter and act in fighting against bacteria by producing IFN-γ. As the key lineage marker for Th1 cells, the Ifng genomic locus is hypomethylated, and this pattern is maintained under Th1 polarized conditions *in vitro*, which is opposite to the conditions in Th2 cells, which show a hypermethylated Ifng locus and hypomethylated IL4 locus in $CD4^+$ T cells (Santangelo et al., 2002). Furthermore, the disassociation of DNMT1 and effector cytokine IL4 loci is the crucial step for IL4 expression during Th2 differentiation (Makar et al., 2003). The conditions of low IL4 expression in Th2 cells can be changed using 5-azacytidine (a hypomethylating agent), which has demonstrated that the magnitude of cytokine production in $CD4^+$ T cells can be regulated by the degree of DNA methylation (Guo et al., 2002). In contrast to DNMT1, *de novo* methylation mediated by DNMT3a is unable to affect the initial differentiation of Th1 and Th2 cells but is required to restrict Th lineage plasticity (Thomas et al., 2012a).

Epigenetics also participate in and provide molecular regulators for the progression of human naïve T cells (Tn) to differentiate into distinct types of memory cells and their long-term maintenance (Durek et al., 2016). Data from comprehensive epigenome and transcriptional analyses of the human $CD4^+$ T cell population have shown that there is progressive DNA methylation loss during the transition from the naïve to memory stages. This loss of methylation tends to occur in "partially methylated domains (PMDs)" (Hon et al., 2012) and serves as a common characteristic in B cell differentiation. Moreover, evidence has shown that there is an association between PMDs and heterochromatic histone signatures, as well as regions replicated in late S phase, and gradually lose methylation during excessive proliferation (Aran et al., 2011). In addition, a dynamic change in methylation states also participates in the differentiation of $CD4^+$ T cells to Th17 cells. Cooperation between DNA methylation and conserved intergenic elements contributes to control of transcription at the IL17 locus (Thomas et al., 2012b). DNMT3a is required for the stability of the Th17 program by suppressing the production of IFN-γ (Thomas et al., 2012a). Therefore, DNA methylation controls Foxp3 expression and plays an important role in T cell fate and function.

## DNA Methylation in Regulatory T Cells Development and Function

As a subset of $CD4^+$ T cells, regulatory T cells (Tregs) play roles in limitation of inflammatory reactions and immune responses. As the "master regulator" of Tregs, Foxp3 expression is crucial for the development and function of Tregs and is present in the thymus in natural Tregs (nTregs) (Josefowicz et al., 2009; Li et al., 2014). Conserved non-coding sequence (CNS) 2, one of the major CNSs controlling Foxp3 expression, is made up of numerous CpG elements and is especially controlled by DNA methylation (Correa et al., 2020). Evidence has demonstrated that there is a unique and evolutionarily conserved CpG-rich island in the Foxp3 non-intronic upstream enhancer that is excessively methylated in conventional $CD4^+$ T cells, activated $CD4^+$ T cells, and peripheral TGF-β-induced Tregs but demethylated in nTregs (Floess et al., 2007). In addition to the Foxp3 locus, the establishment of a Treg cell-specific CpG hypomethylation pattern also led to Treg cell development in a Foxp3-independent manner (Ohkura et al., 2012). Notably, DNMT1 may provide a possibility for DNA methylation to act in maintaining suppression of Foxp3 in thymic and peripheral Foxp3-negative $CD4^+$ T cells upon T cell receptor (TCR) stimulation. Although Tregs with DNMT1 deficiency are unable to change the methylation of CNS2 in Foxp3, global changes in DNA methylation are related to the deletion of several genes crucial to Treg function and an increase in inflammatory gene expression (Kim and Leonard, 2007; Polansky et al., 2008).

## DNA Methylation in T Cell Differentiation and Memory

DNA methylation is suitable for a particular cellular memory function in development due to its features of methylation state heritability and the secondary nature of the decision to include or exclude methylation (Radbruch et al., 2021). The epigenome and transcriptome of human $CD4^+$ T cells suggests that progressive changes in DNA methylation loss exist in the memory development of $CD4^+$ T cells, with a linear pattern in the order of Tn-T central memory (Tcm) – T effector memory (Tem) – T $CD45RA^+$ memory (Temra), while tissue-resident bone marrow- long-lived memory (Tmem) cells branch off with a unique epigenetic profile (Durek et al., 2016). Based on the differential methylation spectrum, differentiated $CD4^+$ memory cells can be distinguished, especially in the context of Th1 and T follicular helper (Tfh) committed cells (Hale et al., 2013). A study using TCR transgenic $CD4^+$ T cells pointed out that gene-specific DMRs are positioned at related gene-enhancer regions and that these regions are related to different expression levels of memory-associated genes (Hashimoto et al., 2013); moreover, there is a similar situation in $CD8^+$ T cells (Scharer et al., 2013).

As one of the most indispensable components of long-lived T cell immunity, there is still a long-standing debate centered on the formation of memory $CD8^+$ T cells, while the specific mechanism by which memory $CD8^+$ T cells retain naïve and effector characteristics remains unclear (Ahmed et al., 2009). A series of studies have demonstrated that genome-wide epigenetic reprogramming is involved in the differentiation of $CD8^+$ T cells. Upon infection with LCMV-Armstrong, T cells experience dynamic DNA remodeling during the transition from naïve to effector $CD8^+$ T cells. Moreover, related gene expression during this transition is negatively correlated with DNA methylation localized in proximal promoter regions. Both enhancer and gene promoter regions showing differential methylation are enriched for functional transcription factor motifs (Scharer et al., 2013). Recently, a study found that the coupled process of the inhibition of a naïve transcriptional programmer in memory precursor effector cells and *de novo* DNA methylation of the gene could be

eliminated in a cell division-independent process due to the cells reacquiring re-expression of naïve-associated genes (Youngblood et al., 2017). Given the known understanding of *de novo* methyltransferase activity, DNMT3a serves as a critical director in early $CD8^+$ T cell effector and memory fate commitments. Further, conditional deletion of DNMT3a has been found to promote the kinetics of memory cell development. One study showed that memory precursor cells could obtain *de novo* methylation programs mediated by DNMT3a at critical loci, and the obtained methylation programs could be erased, leading to re-expression of naïve genes during the development of memory $CD8^+$ T cells (Youngblood et al., 2017). However, inconsistent with this report, another study found that terminal effectors obtain *de novo* programs at critical loci, while these *de novo* programs are absent in memory precursor cells. Furthermore, DNMT3a-deficient T cells prefer to produce more memory precursors and fewer terminal effector cells in a T-cell internal manner instead of enhancing the plasticity of differentiated effector $CD8^+$ T cells. Additionally, DNMT3a depletion tends to differentiate early effector cells into memory precursor cells without *de novo* methylation programs (Ladle et al., 2016). All these results support the idea that DNA methylation functions in $CD4/CD8^+$ T cell differentiation and memory.

## DNA METHYLATION IN B CELL DEVELOPMENT

B cells serve as essential actors in the initialization and acceleration of autoimmune diseases (Ceccarelli et al., 2016). Once mature naïve B cells migrate to the peripheral lymphoid system and are exposed to self- and/or foreign antigens, the corresponding antigen-specific B cells are activated through signals from Th cell-produced cytokines and the help of Tfh cells. Then, activated B cells differentiate into plasma cells or memory B cells by undergoing a series of processes, which provide humoral immune functions (Alt et al., 2013). Several lines of evidence have demonstrated that epigenetic regulation is involved in the somatic hypermutation (SHM) and class switch DNA recombination modifications under the condition of B cell activation and differentiation. Thus, any abnormal regulation involved in these processes may provide the possibility of aberrant antibody production and lead to the pathogenesis of autoimmune diseases (Wu et al., 2018). Therefore, it is essential to summarize current research progress in epigenetic regulation that promotes B cell activation and differentiation to better comprehend B cell biology and its role in autoimmune development.

### DNA Methylation in Germinal Center B Cells

The formation of germinal centers (GCs) is attributed to activated B cell proliferation under the promotion of cytokines originating from Th cells and Tfh cells (Alt et al., 2013). Rapid proliferation tolerance and the mutagenic actions of activation-induced cytosine deaminase (AICDA) are the typical phenotypes of GC B cells (Klein and Dalla-Favera, 2008). Based on the knowledge that DNA methylation patterns act as important regulators in determining cellular phenotypes (Wu et al., 2018), one study aimed to explore DNA methylation and the function of DNMTs in GC formation. The results from DNA methylation profiles reflected a significant shift in the DNA methylation pattern in GC B cells compared with resting/naïve B cells. Overall, 223 differentially methylated genes were involved and were relatively hypomethylated in GC B cells compared with resting/naïve B cells. Except for some B cell lineage genes, such as *Pax5, Ebf1, Cd19,* and *Spib*, which show a continuous active epigenetic status during B cell activation, almost all genome-wide DNA is hypomethylated. Moreover, greater DNA methylation heterogeneity was present in GC B cells, and the binding sites of AICDA were overexpressed at hypomethylated loci. The genes showing differential methylation predominately represent components of NF-κB and MAP kinase signaling. Accumulated evidence has suggested that differentially methylated genes are related to specific biological functions, such as metabolic regulation, and synthase, synthetase, chaperone and transporter enrichment. Additionally, the results revealed that DNMT1 was the only DNMT that was significantly upregulated in GC B cells. An animal study found that DNMT1 hypermorphic mice exhibit GC formation deficiency; once mice were treated with the DNMT inhibitor decitabine, GCs were unable to form after stimulation (Shaknovich et al., 2011). Interestingly, evidence from GC B cells of DNMT1 hypomorphic animals has demonstrated the dual effects of DNMT1 in DNA methylation and break repair of double-stranded DNA (Shaknovich et al., 2011). Furthermore, epigenetic regulation, including DNA methylation and histone modification, plays an important regulator at the SHM stage involved in B cell activation, which targets V(D)J DNA via transcription (Cui et al., 2016). Notably, the fact that a demethylated allele is the only allele that can be hypermutated in comparable transcription of both alleles further suggests a critical role for DNA methylation in SHM (Odegard and Schatz, 2006).

### DNA Methylation in B Cell Memory

Memory formation serves as a critical hallmark of adaptive immunity. In addition to T cells, epigenetic regulation also contributes to the differentiation of memory B cells. A series of studies have suggested that another epigenetic modification, histone modification, plays an important role in this process, for example, by controlling the hallmark genes of memory B cells, such as CD27 in humans and CD38 in mice (Zan and Casali, 2015), and can also inhibit *Irf4* and *Prdm-1* transcription by catalyzing H3K27me3, thereby regulating the percentage of memory B cells, GC reactions and antibody responses (Good-Jacobson, 2014).

To begin to comprehend how DNA methylation acts in the formation of memory B cells, one study has shown that a large proportion of DNA methylation loss induced by activation is mapped to transcription factor binding sites. An extra level of demethylated loci mapped to *Alu* elements, with the help of the genome and coexisting DNMT3a suppression. Activation-dependent DNA methylation changes in the offspring of activated B cells contribute a comparable epigenetic characteristic to downstream memory B cells and plasma cells with diverse

transcriptional programs (Lai et al., 2013). These results revealed the methylation dynamics of the genome during cellular differentiation in an immune response.

## DNA METHYLATION IN AUTOIMMUNE DISEASES

The fundament of autoimmunity is self-tolerance. Although there is a growing body of research exploring the immune regulation related to autoimmunity, the specific mechanism that results in tolerance loss remains difficult to elucidate (Shoenfeld et al., 2008). Given that concordance rates in monozygotic (MZ) twins are no more than 50%, it is reasonable to speculate that there are other complementary mechanisms that participate in gene expression regulation, which eventually leads to dominant autoimmunity (Hewagama and Richardson, 2009; Meda et al., 2011). Additionally, whether in clinical settings or experimental models, an increasing number of studies have demonstrated that the epigenome is a critical actor in better understanding the initiation and perpetuation of autoimmunity (Meda et al., 2011).

Currently, an increasing number of studies have aimed to explore the effect of epigenetics in complicated disorders and to improve understanding of its distinct function within the field of medicine. Some hypotheses have noted that epigenetic modification, including DNA methylation, is considered a bridge connecting environmental stimulation and genetic factors in the pathogenesis of autoimmune diseases (Dupont et al., 2009).

Furthermore, the development of immune cells serves as a well-defined process in which progenitor cells produce progeny cells through a given differentiation pathway. The correctness of this process of differentiation and lineage commitment guarantees the establishment of immune tolerance. Thus, as one of the key regulators in immune cell differentiation and development, specific impairments in DNA methylation profiles could result in immune cell autoreactivity and predispose an individual to autoimmune dysregulation and risk for autoimmune diseases (Wang et al., 2015; Cumano et al., 2019). There is a relationship between DNA methylation defects and autoimmune disease pathogenesis. A genome-wide DNA methylation study quantified more than 4485,00 methylation sites across the genome (Coit et al., 2013). Thus, understanding the aberrant expression of DNA methylation mediators is critical for deciphering concurrent epigenetic alterations in various autoimmune diseases and for the development of new therapeutic strategies. In this section, we focus on the common autoimmune diseases systemic lupus erythematosus (SLE), type 1 diabetes (T1D), rheumatoid arthritis (RA), Graves' disease (GD), and Hashimoto's disease (HD), with the aim of clarifying the role of DNA methylation in disease pathogenesis and development (**Table 1**).

### DNA Methylation in Systemic Lupus Erythematosus

Systemic lupus erythematosus is a multiorgan autoimmune disease characterized by the presence of an autoantibody to nuclear and/or cytoplasmic antigens. Abnormal differentiation and activation of immune cells induced by factors associated with genetic susceptibility and epigenetic modification play an unequivocal role in SLE etiology (Miao et al., 2014). In recent years, it has been increasingly appreciated that abnormal DNA methylation is involved in the pathophysiology of SLE, and one view suggests that DNA hypomethylation and demethylated DNA fragments may influence the structure of T cell chromatin, leading to cellular hyperactivity and inducing the production of anti-DNA antibodies, thereby participating in the pathogenesis of SLE (Meda et al., 2011; Miao et al., 2014). A study in lupus T cells revealed that altered T cell DNA methylation in SLE is regulated by the extracellular signal-regulated kinase (ERK) signaling pathway. Furthermore, impairment of the ERK signaling pathway predominantly leads to SLE susceptibility in females, which is supported by a study in which only female mice with ERK impairment showed SLE-like symptoms versus male mice under the same conditions (Strickland et al., 2012). In murine models, this pathway is decreased, which leads to overexpression of methylation-sensitive autoimmune genes and downregulation of DNMT1 expression (Gorelik and Richardson, 2009). In addition, similar conclusions were also found in $CD4^+$ T cells, $CD19^+$ B cells, $CD14^+$ monocytes, and neutrophils from SLE patients. Several methylation-sensitive genes were found to be hypomethylated in $CD4^+$ T cells, such as lymphocyte function-associated antigen-1 *(LFA1)*, *CD70 (TNFSF7)*, *CD11a* (integrin alpha L, *ITGAL)*, *CD40 ligand (TNFSF5),* and *perforin (PRF1),* leading to overexpression, similar to that observed in $CD8^+$ T cells. All of these genes have a positive correlation with lupus disease activity. Furthermore, when compared with patients with inactive lupus and healthy individuals, the promoter regions of the genes mentioned above seem to be significantly hypomethylated in active lupus T cells (Richardson et al., 2012; Relle et al., 2015). Moreover, the promoter methylation of *IFI44L*, which is a blood biomarker for monitoring activity changes in SLE, has the ability to distinguish SLE patients from healthy controls with high sensitivity and specificity (Zhao et al., 2016). The use of an inhibitor of DNA methylation can result in hypomethylation of genes at the promoter region, and the corresponding genes are significantly upregulated (Sawalha et al., 2008). Another study using bisulfite sequencing showed a novel methylation-sensitive gene, serine/threonine-protein phosphatase 2A catalytic subunit α (*PP2Acα*), which is induced by oxidative stress, shows increased expression in SLE T cells and contributes to the pathogenesis of SLE. Mechanistically, CpG methylation occurs in the cAMP response element (CRE) motif, which ultimately results in hypomethylated expression of the activity of the *PP2Acα* promoter (Sunahori et al., 2011; Deng et al., 2019). Notably, an association between DNA methylation of type 1 IFN-related genes and autoantibody positivity has been identified in SLE. One study found that female SLE patients with and without a history of anti-dsDNA antibody positivity exhibit differentially methylated profiles (Yang et al., 2017). Furthermore, CD40L overexpression and corresponding demethylated genes on the inactive X chromosome are thought to be responsible for the female bias observed in SLE (Hewagama et al., 2013). On the other hand, the interplay between DNA methylation and microRNAs (miRNAs) in SLE has also been explored. Evidence

**TABLE 1 |** Available evidence on DNA methylation changes involved in SLE, T1D, and other common autoimmune diseases.

| | Specific target | Types of cells | Main findings | References |
|---|---|---|---|---|
| | ERK pathway signaling Methylation-sensitive autoimmune genes | Lupus T cells | ERK pathway signaling and chromatin structure impairments LFA1, *CD70 (TNFSF7)*, *CD40LG (TNSF5)*, *CD11a (ITGAL),* perforin (*PRF1*) ↑ DNMT1 ↓ | Gorelik and Richardson, 2009; Relle et al., 2015 |
| | *IFI44L* | Whole blood | *IFI44L* promotor methylation ↓ | Zhao et al., 2016 |
| *SLE* | *PP2Acα* | SLE T cells | DNMT1 ↓ *PP2Acα* promotor hypomethylation, *PP2Acα*↑ | Sunahori et al., 2011 |
| | Type 1 IFN-related genes | PBMCs | *IFIT1, IFI44L, MX1, RSAD2, OAS1, EIF2AK2,* and *NLRC5* are associated with autoantibody positivity | Yang et al., 2017 |
| | T1D-MVPs | Purified $CD14^+$ monocytes | 58 hypermethylated and 74 hypomethylated genes, typically *HLA-DQB1, GA62, TNF,* and *TRAF6* | Rakyan et al., 2011; Cerna, 2019 |
| | T1D-associated DNA methylation profiles | EBV immortalized B cells | 88 significant changes at CpG sites, typically in *HLA-E, HLA-DOB, HLA-DQ26, INS, IL2RB,* and *CD226* | Stefan et al., 2014 |
| | MHC region and T1D-associated CpG sites | Peripheral blood | Mostly methylation of *MAGI2, FANCC* and *PCDHB16* Modest methylation of *BACH2, INS-IGF2,* and *CLEC16A* | Elboudwarej et al., 2016 |
| *T1D* | T1D-related MVPs | $CD4^+$ T cells; $CD19^+$ B cells; $CD14^+CD16^-$ monocytes | T1D-related MVPs positioned at genes involved in immune cell metabolism and cell cycle, including mTOR signaling | Paul et al., 2016 |
| | *IL2RA* | Whole blood cells (WBCs) | *IL2RA* promoter is associated with methylation of CpG site; 349 differential CpG methylation sites in T1D patients with PDR and without PDR; 19 potential CpG sites associated with the risk of T1D-related DR | Bell et al., 2010; Belot et al., 2013; Agardh et al., 2015 |
| | Human *IGFBP1* gene | Whole peripheral blood cells | DNA methylation levels in the *IGFBP1* gene ↓; circulating IGFBP-1 levels in T1D patients ↑ | Gu et al., 2014 |
| | | T cells and monocytes | Global hypomethylation | Nakano et al., 2013 |
| | Genome-wide DNA methylation profiles | $CD19^+$ B cells, synovial fibroblasts and PBMCs | An altered pattern of DNA methylation and reduced 5mC expression *CD1C, TNFSF10, PARVG, NID1, DHRS12, ITPK1, ACSF3,* and *TNFRSF13C* are signatures in SLE patients | Ai et al., 2018; Rodriguez-Ubreva et al., 2019 |
| *RA* | *CD40L* | T cells | CD40L promoter demethylation in silenced X chromosomes caused CD40L overexpression, which plays a role in RA development | Lu et al., 2007 |
| | *IL2RA (CD25)* and *CTLA-4* | Treg cells | SNPs of *IL2RA (CD25)* and *CTLA-4* are associated with RA susceptibility, and their aberrant DNA methylation pattern affect Foxp3 reactivation and impair the normal function of Treg cells | Ohkura and Sakaguchi, 2020 |
| *GD and HD* | 27728 annotated CGIs and 22532 promoters | Peripheral blood cells | 132 hypermethylated and 133 hypomethylated regions in GD patients *ADRB2, B3GNT2, PADI4, TNFRAF25 (DR-3), ICAM1, MECP2,* and DNMT1 are regulated by DNA methylation and involved in GD development | Cai et al., 2015; Guo et al., 2018 |
| | *IL6* | Peripheral blood cells | *IL6* methylation level is related to GD intractability and HD susceptibility | Hirai et al., 2019 |
| | DNA methylation-related genes | | *DNMT, MTHFR,* and *MTRR* are related to AITD risk | Cai et al., 2016 |

has shown that the status of DNA methylation is regulated by some lupus-related miRNAs via targeting of DNA methylation enzymes or proteins associated with methylation pathways, such as genetic imprinting of Dlk1-Dio3 miRNAs (Lu et al., 2007; Dai et al., 2021).

## DNA Methylation in Type 1 Diabetes

Type 1 diabetes is a chronic, immune-mediated complex disorder caused by destruction of islet β cells that results in insulin deficiency, and both genetic and environmental factors are contributors to the pathogenesis of T1D (Xie et al., 2014, 2018; American Diabetes Association, 2020). A series of mechanisms linked to epigenetic regulation have been suggested to be involved in the development of T1D. One of the major mechanisms is regulation of lymphocyte maturation and cytokine gene expression, particularly for the differentiation of Th cell subtypes, which is regarded as the most complex immune process controlled by epigenetic regulation. Studies of genome-wide DNA methylation suggest that both dysregulated autoimmunity and primitive pancreatic damage are associated with abnormal DNA methylation (Xie et al., 2014; Elboudwarej et al., 2016; Paul et al., 2016). MZ twins are always employed to investigate the effect of epigenetic factors on disease development due to their almost identical genetic background and environmental exposures. A genome-wide DNA methylation profile for which purified $CD14^+$

monocytes were collected from 15 MZ twins with discordant T1D onset identified 132 T1D-related methylation variable positions (T1D-MVPs), consisting of 58 hypermethylated and 74 hypomethylated MVPs. The strongest T1D susceptibility genes *HLA-DQB1* and *GA62* (encodes GAD65), the T1D-related inflammatory cytokine *TNF* and the TLR receptor signaling pathway-related protein *TRAF6* are representative MVPs found in this analysis; additionally, some of these MVPs were found to be altered prior to overt T1D onset and maintained temporal stability over many years, which may provide a potential possibility of early clinical diagnosis of T1D (Rakyan et al., 2011; Cerna, 2019). Similar studies have explored the DNA methylation patterns between MZ twins with discordant T1D onset and MZ twins with concordant T1D in Epstein Barr virus (EBV)-immortalized B cells (Stefan et al., 2014), peripheral blood (Elboudwarej et al., 2016), $CD4^+$ T cells, $CD19^+$ B cells, $CD14^+CD16^-$ monocytes (Paul et al., 2016), whole blood cells (Belot et al., 2013), whole peripheral blood and $CD14^+$ monocytes (Cepek et al., 2016). The main findings and/or significantly changed CpG sites in all MZ twin pairs discordant for T1D are shown in **Table 1**. In addition, decreased immune tolerance is regulated by DNA methylation, which was found in $CD4^+$ T cells from latent autoimmune diabetes in adults (LADA) and peripheral blood mononuclear cells (PBMCs) from fulminant type 1 diabetes (FT1D) (Wang et al., 2013; Agardh et al., 2015). Collectively, these findings further help to characterize the T1D risk conferred by the information encoded by the DNA methylome, which supports the notion that alterations in DNA methylation are involved in the pathogenesis of T1D.

Furthermore, a growing number of studies have supported the association between DNA methylation and diabetes complications in T1D patients, such as diabetic nephropathy (DN) and proliferative diabetic retinopathy (PDR) (Gu et al., 2014; Agardh et al., 2015). A study on T1D and DN revealed that 19 potential CpG sites are associated with DN risk, including one CpG site localized in *UNC13B*, which itself is related to DN (Agardh et al., 2015). A similar study also identified some CpG sites that are involved in transcription regulation and are related to DR risk (Bell et al., 2010). Based on the fact that the serum concentration of insulin-like growth factor binding protein-1 (IGFBP-1) is correlated with T1D, one study first found downregulated methylation of the *IGFBP1* gene in T1D patients, and T1D patients with DN showed a higher concentration of IGFBP-1 than the other two groups (Gu et al., 2014). In addition, another study focused on T1D and PDR showed that CpG sites with hypomethylation accounted for approximately 80% of the differentially methylated CpG sites found in T1D patients with PDR, which predicts that DNA methylation may be a potential biomarker for T1D with PDR. A series of studies have shown that these T1D-related MVPs are often positioned at gene regulatory elements of genes engaged in the immune cell cycle, cell metabolism and immune and defense responses (Stefan et al., 2014; Paul et al., 2016). Generally, these results support the idea that epigenetic modification plays a functional role in the pathogenesis of T1D.

## DNA Methylation in Rheumatoid Arthritis, Graves' Disease, and Hashimoto's Disease

Similar to SLE, RA is also recognized as a common autoimmune disease influenced by epigenetic regulation. Aberrant epigenomes, including DNA methylation, influence a series of inflammatory and matrix-related pathways and contribute to the pathogenesis of RA. Multiple studies have demonstrated that RA patients show global T cell and monocyte hypomethylation; an altered pattern of DNA methylation in $CD19^+$ B cells, synovial fibroblasts and PBMCs; and reduced 5mC expression in synovial tissues compared with healthy controls (Nakano et al., 2013; Ai et al., 2018; Rodriguez-Ubreva et al., 2019; Fang et al., 2021). These hypomethylated genes are enriched in crucial ways associated with cell migration (Nakano et al., 2013). Furthermore, some of these genes, such as *CD1C, TNFSF10, PARVG, NID1, DHRS12, ITPK1, ACSF3,* and *TNFRSF13C*, also show a differentiated methylation signature in SLE patients (Ballestar et al., 2020; Fang et al., 2021). In addition, promoter region demethylation of *CD40L* in silenced X chromosomes leads to *CD40L* overexpression, which plays a role in RA development (Lu et al., 2007). Moreover, *IL2RA (CD25)* and *CTLA-4* are associated with RA susceptibility. As important Treg signature genes, single nucleotide polymorphisms (SNPs) in Treg-specific demethylated DNA regions of these two genes can affect Foxp3 reactivation and thereby impair the normal function of Treg cells (Ohkura and Sakaguchi, 2020).

DNA methylation modification is also a possible mechanism providing novel insight into autoimmune thyroid diseases (AITDs), which include Graves' disease (GD) and Hashimoto's disease (HD). Recently, attention has been given to the significance of DNA methylation in GD. A genome-wide methylation analysis covering 27728 annotated CGIs and 22532 promoters in peripheral blood cells uncovered an altered DNA methylation profile in GD patients, including 132 hypermethylated and 133 hypomethylated regions. Moreover, known candidate genes that were previously identified in GD or other autoimmune diseases were also found, such as *ADRB2, B3GNT2, PADI4, TNFRAF25 (DR-3)*, *ICAM1*, *MECP2,* and *DNMT1,* all of which are regulated by DNA methylation and involved in the development of GD (Cai et al., 2015; Guo et al., 2018). Moreover, another study showed that methylation levels of the *IL6* gene are linked to the intractability of GD and to susceptibility to HD (Hirai et al., 2019). In addition, a relationship between polymorphisms of genes involved in DNA methylation [such as *DNMT*, methylenetetrahydrofolate reductase *(MTHFR)* and methionine synthase reductase (*MTRR*)] and AITD risk has been demonstrated (Cai et al., 2016; Coppede, 2017).

# CONCLUSION AND PERSPECTIVES

Over the past years, a large number of studies have explored the epigenetic regulation patterns that occur during the development of autoimmunity. As one of the three major epigenetic regulation patterns, the potential epigenetic modifications caused by

DNA methylation have garnered more attention in recent years. Significantly, epigenetics can provide new insights into diagnostic and therapeutic methods for autoimmune diseases. The major potential for epigenetic application in the clinic can be summarized as epigenetic markers and epigenetic therapy. A series of epigenetic biomarkers, especially those associated with DNA methylation, are associated with clinical outcomes and provide an alternate stability profile rather than conventional testing based on DNA and RNA sequencing (Garcia-Gimenez et al., 2017; **Table 1**). Samples of blood, tissue, body fluid and secretions can be used to detect epigenetic biomarkers at the early stage of disease, which provides superiority compared with testing that is dependent on RNA and protein abnormalities (Zhang et al., 2020). On the other hand, a great deal of attention has been focused on epigenetic therapy, which is a novel option for disease treatment that employs epigenetic drugs or non-medical clinical management. For example, the first epigenetic drugs, azacytidine (5-AZA) and decitabine (5-AZA-CdR), were approved for clinical application in 2004 and have gradually been utilized for therapy targeting hematologic malignancies (Egger et al., 2004). Furthermore, a large number of epigenetic modifiers have been developed, and these modifiers can reprogram and reshape epigenetic patterns by reducing the level of DNA methylation and generating or removing epigenetic markers; thus, allowing full use of them would contribute to the treatment of diseases (Ballestar et al., 2020).

Although many lines of evidence have demonstrated that DNA methylation plays indispensable roles in autoimmune diseases by regulating immune cell differentiation and function, the specific mechanism by which it participates in the pathogenic states of autoimmune diseases still needs to be explored. In this review, we described DNA methylation to clarify its basic function and distribution, its ability to mediate gene expression, and the key working enzymes. Moreover, we introduced the role of DNA methylation in the development and differentiation of all types of T and B cells, discussed the controversial epigenomic differentiation models of T cells during memory development, and focused on discoveries of epigenetic control mechanisms in which the DNA methylation state is changed in both basic biological processes and the pathogenesis of a series of human autoimmune diseases. DNA methylation regulates the expression of genes that determine cell fates, predominantly via DNMTs. These genes encode key transcription factors, such as Ifng in Th1 and IL4 in Th2 cells and AICDA in GC B cells. By affecting related factor expression, dysregulated DNA methylation indirectly influences the regulatory networks in which these factors are involved, leading to amplification of effects and further deregulation of cell type-specific gene expression programmers. Although the important functions of DNA methylation in gene modification, cell differentiation and disease regulation have been confirmed, some questions still need to be clarified. For example, although it has been verified that DNA methylation plays an important role in globally controlling $CD4^+$ memory differentiation, the function of individual DNMT or TET family members in directing or maintaining $CD4^+$ T cell memory remains to be elucidated. In conclusion, DNA methylation is a promising field that links the roles of genetics, gene expression regulation, and environmental risk factors in autoimmune diseases. To beneficially give full play to the role of DNA methylation, comprehending the definite mechanisms and critical modifications of DNA methylation and discovering strategies to alter and achieve the desired magnitude and direction of immune responses, thereby providing a potential direction for better diagnosing, monitoring and treating the progression of diseases driven by epigenetics, is essential (**Figure 1**).

## AUTHOR CONTRIBUTIONS

JL performed the literature search, wrote the first draft of the manuscript, and revised the manuscript. YW, GH, XL, and ZZ critically revised the manuscript and provided substantial scientific contribution. LL and ZX proposed the project and revised the manuscript. All authors approved the final version of the manuscript.

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REVIEW
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# The Emerging Role of m6A Modification in Regulating the Immune System and Autoimmune Diseases

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Over the past several decades, RNA modifications have rapidly emerged as an indispensable topic in epitranscriptomics. N6-methyladenosine (m6A), namely, methylation at the sixth position of an adenine base in an RNA molecule, is the most prevalent RNA modification in both coding and noncoding RNAs. m6A has emerged as a crucial posttranscriptional regulator involved in both physiological and pathological processes. Based on accumulating evidence, m6A participates in the pathogenesis of immune-related diseases by regulating both innate and adaptive immune cells through various mechanisms. Autoimmune diseases are caused by a self-destructive immune response in the setting of genetic and environmental factors, and recent studies have discovered that m6A may play an essential role in the development of autoimmune diseases. In this review, we focus on the important role of m6A modification in biological functions and highlight its contributions to immune cells and the development of autoimmune diseases, thereby providing promising epitranscriptomic targets for preventing and treating autoimmune disorders.

**Keywords: RNA modifications, N6-methyladenosine, autoimmune disorders, innate immunity, adaptive immunity**

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## INTRODUCTION

Epigenetics, a link between genetic factors and environmental factors, refers to heritable modifications that regulate gene expression in the absence of nucleotide sequence alterations. Classical epigenetic mechanisms comprise DNA modifications, histone modifications and noncoding RNAs (ncRNAs). Over the past several decades, RNA modifications have emerged as new epitranscriptomic modifications, enriching the regulatory mechanisms of gene expression and providing novel insights into and strategies for exploring the underlying pathogenesis of diseases. N6-methyladenosine (m6A), the most abundant and widespread RNA modification, has been identified in coding RNAs (messenger RNAs, mRNAs) and ncRNAs, including transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs), small nuclear RNAs (snRNAs), long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs) (Niu et al., 2013; Chen et al., 2019; Lence et al., 2019; Li et al., 2020a). m6A is highly conserved and is installed predominantly in specific regions near stop codons, in internal long exons and in 3′ untranslated regions (3′UTRs) (Dominissini et al., 2012; Meyer et al., 2012; Wang et al., 2020a). More specifically, m6A is preferentially installed at the consensus motif RR-m6A-CH (R = G/A; H = A/C/U)(Batista, 2017).

**TABLE 1 |** Main functions of the three major groups of m6A-related proteins.

| Category | Proteins | Main functions | References |
|---|---|---|---|
| Writers | METTL3 | Catalyzes m6A formation | Tong et al. (2018a); Zhang et al. (2019) |
| | METTL14 | Promotes the catalytic activity of METTL3 | Wang et al. (2016); Batista (2017); Tong et al. (2018a); Zaccara et al. (2019); Zhang et al. (2019) |
| | WTAP | Ensures proper localization of the METTL3-METTL14 complex | Ping et al. (2014); Zhang et al. (2019) |
| | | Recruits target RNAs and other functional factors | Zhang et al. (2019) |
| | VIRMA | Mediates m6A deposition at specific regions of target mRNAs | Yue et al. (2018) |
| | RBM15/15B | Recruits the writer complex to specific regions | Patil et al. (2016) |
| | | Modulates m6A deposition on XIST | Patil et al. (2016) |
| | METTL16 | Installs m6A on snRNAs and mRNAs to regulate SAM homeostasis | Pendleton et al. (2017) |
| | CBLL1 | Interacts with WTAP | Růžička et al. (2017) |
| | ZC3H13 | Facilitates the nuclear localization of the writer complex | Knuckles et al. (2018); Wen et al. (2018) |
| | ZCCHC4 | Installs a single m6A modification on rRNA | Pinto et al. (2020) |
| | | Modulates mRNA translation | Ma et al. (2019) |
| Erasers | FTO | Removes m6A | Jia et al. (2011) |
| | | Removes m6Am | Mauer et al. (2019) |
| | | Demethylates other sites in certain snRNAs and specific tRNAs | Wang et al. (2014); Mauer et al. (2019) |
| | ALKBH5 | Removes m6A | Zheng et al. (2013) |
| | | Targets ncRNAs separately from mRNAs | Wang et al. (2014) |
| | | Participates in germ cell development | Zheng et al. (2013) |
| | | Modulates RNA metabolism and nuclear RNA export | Zheng et al. (2013); Zhang et al. (2017a) |
| | FMN | A newly identified artificial molecular demethylase | Xie et al. (2019) |
| Readers | YTHDF1 | Promotes the efficient translation of target mRNAs | Meyer et al. (2015); Wang et al. (2015) |
| | YTHDF2 | Accelerates RNA degradation and inhibits the translation of mRNAs | Meyer et al. (2015); Wang et al. (2015) |
| | YTHDF3 | Promotes RNA translation with YTHDF1 | Shi et al. (2017) |
| | | Inhibits the translation of mRNAs with YTHDF2 | Shi et al. (2017) |
| | YTHDC1 | Modulates alternative RNA splicing | Xiao et al. (2016) |
| | | Regulates nuclear export | Xiao et al. (2016) |
| | | Accelerates the decay of transcripts to maintain SAM levels | Shima et al. (2017) |
| | | Suppresses gene expression involving X chromosome inactivation | Patil et al. (2016) |
| | YTHDC2 | Promotes the efficient translation of its target transcripts | Kretschmer et al. (2018) |
| | | Mediates the subsequent degradation of its target transcripts | Kretschmer et al. (2018) |
| | IGF2BPs | Regulates the stability, localization and translation of target RNAs | Degrauwe et al. (2016); Huang et al. (2018) |
| | EIF3 | Initiates and promotes cap-independent translation | Meyer et al. (2015) |
| | HNRNPA2B1 | Modulates alternative splicing | Alarcón et al. (2015a) |
| | | Promotes mature miRNA biogenesis | Alarcón et al. (2015a); Coker et al. (2019) |
| | HNRNPC | Involved in premRNA processing, including splicing | Liu et al. (2015) |
| | HNRNPG | Involved in premRNA processing, including splicing | Liu et al. (2017) |

*ALKBH5, ALKB homolog 5; CBLL1, Casitas B-lineage lymphoma-transforming sequence-like protein 1; FMN, flavin mononucleotide; FTO, fat mass and obesity-associated protein; HNRNPA2B1/C/G, heterogeneous nuclear ribonucleoprotein A2B1/C/G; METTL3/14/16, methyltransferase-like 3/14/16; IGF2BPs, insulin-like growth factor 2 binding proteins; RBM15/15B, RNA binding motif protein 15/15B; VIRMA, Vir-like m6A methyltransferase associated; WTAP, Wilms tumor 1-associated protein; YTHDF1/2/3, YTH domain-containing family 1/2/3, YTHDC1/2, YTH domain-containing 1/2; ZC3H13, zinc finger CCCH domain-containing protein 13; ZCCHC4, zinc finger CCHC-type containing 4.*

The regulatory proteins involved in m6A modification fall into three categories: "writers," "erasers" and "readers" (**Table 1**; **Figure 1A**). m6A writers are methyltransferase complexes containing multiple subunits that install m6A cotranscriptionally at specific sites in target mRNAs. Methyltransferase-like 3 (METTL3), the only active catalytic component of the writer complex, exhibits catalytic activity independently and functions synergistically with METTL14 by forming a stable heterodimer. Many auxiliary subunits ensure efficient installation of m6A modification and determine the specificity of writers, including Wilms tumor 1-associated protein (WTAP), Vir-like m6A methyltransferase associated (VIRMA), and RNA binding motif protein 15/15B (RBM15/15B) (Shi et al., 2019). Erasers are RNA demethylases that remove the methyl group from m6A. The discovery of RNA demethylases suggested that m6A modification may be a reversible and dynamic process. Only two natural RNA demethylases have been identified to date, namely, fat mass and obesity-associated protein (FTO) and ALKB homolog 5 (ALKBH5), both of which belong to the ALKB family of

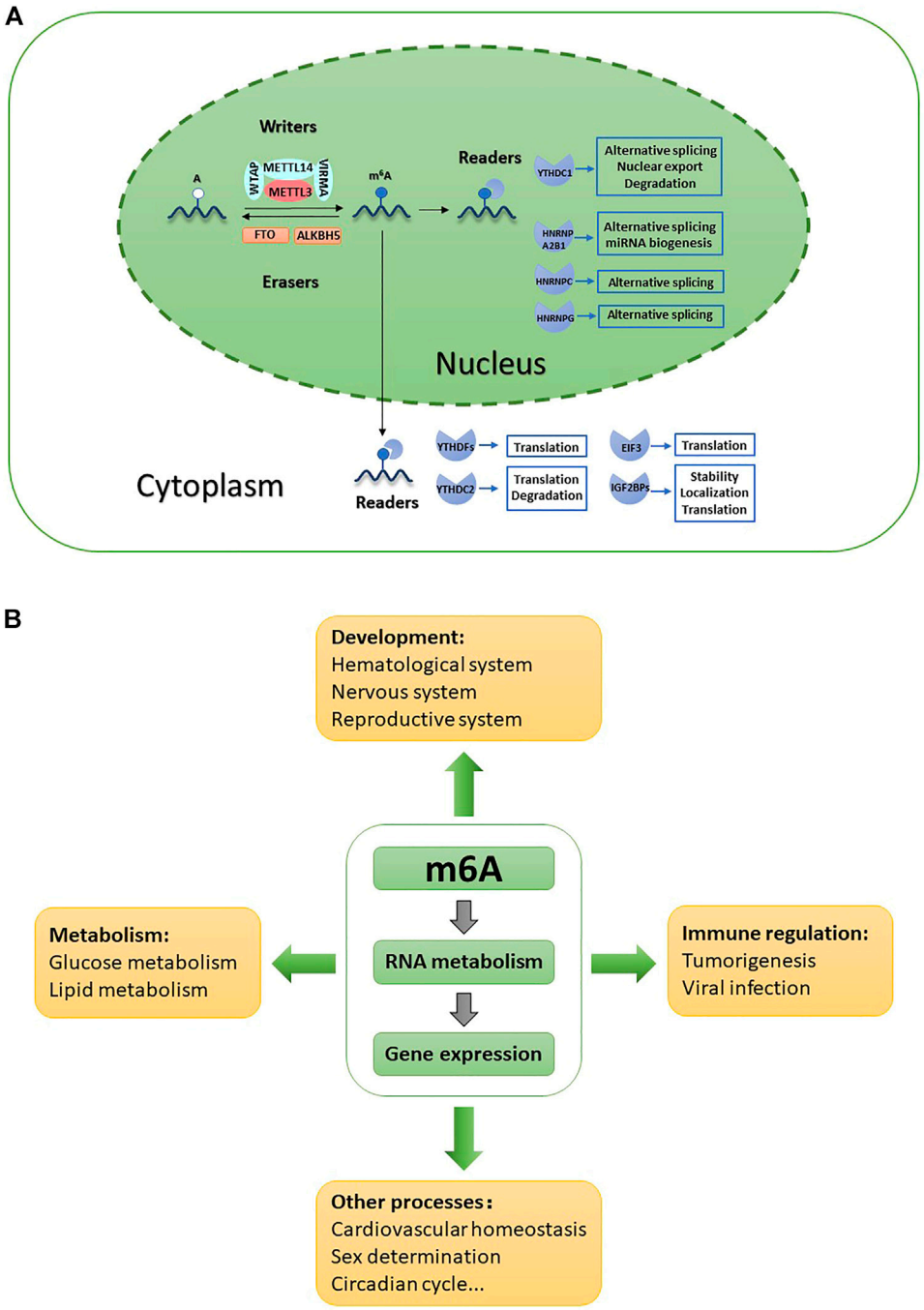


**FIGURE 1 |** Role of m6A in various biological functions. **(A)** m6A is installed by writers, removed by erasers and recognized by nuclear readers and cystoplasmic readers. m6A is involved in all aspects of RNAs metabolism and activity. **(B)** m6A is involved in both physiological and pathological processes through modulating gene expression.

proteins (Jia et al., 2011; Zheng et al., 2013). Recently, flavin mononucleotide (FMN) was identified as a novel artificial molecular demethylase (Xie et al., 2019). Readers are RNA binding proteins that mediate the fate of target transcripts and regulate downstream biological functions by preferentially recognizing and binding to modified sites (Batista, 2017; He et al., 2019; Zhang et al., 2019). Readers are classified as direct readers or indirect readers according to their interaction patterns with RNAs. Direct readers, such as YTH family members, selectively and directly bind to m6A sites, while indirect readers, such as heterogeneous nuclear ribonucleoprotein G (HNRNPG), indirectly bind to m6A

sites based on the "m6A switch" mechanism (Dai et al., 2018; Zaccara et al., 2019).

Autoimmune diseases result from a self-destructive immune response initiated by an impaired immune tolerance mechanism. This group of diseases imposes a substantial burden on health services, economic development and quality of life due to their slow progression, the difficulty in diagnosis because of their heterogeneous clinical manifestations and the numerous side effects occurring during immunosuppressive therapy (Anaya, 2012; Wardowska, 2021). However, the precise cellular and molecular mechanisms underlying autoimmune diseases have remained poorly understood until recently. Epitranscriptomic mechanisms have been widely recognized to play fundamental roles in the pathogenesis of immune-related diseases (Wu et al., 2017; Li et al., 2021a). Both innate and adaptive immunity clearly participate in the occurrence and progression of autoimmune diseases (Bluestone and Bour-Jordan, 2012; Wahren-Herlenius and Dörner, 2013). Numerous studies have recently characterized an essential role for m6A in many aspects of the immune system, including cell development, differentiation, activation, migration and function, indicating that m6A may contribute to the pathogenesis of autoimmune disorders.

In this review, we summarize the crucial role of the m6A modification in regulating cellular biological functions and highlight its contributions to the immune system and the development of autoimmune diseases, thereby providing novel insights into the pathogenesis of autoimmune disorders and potential targets for epitranscriptomic therapy.

## ROLES OF M6A IN VARIOUS BIOLOGICAL FUNCTIONS

m6A modification is precisely regulated by writers, erasers and readers and is involved in all aspects of RNA metabolism; moreover, its effects are not limited to mRNAs (**Figure 1A**). Through regulating gene expression, m6A is involved in diverse biological process including development, metabolism, immunity regulation, sex determination, circadian rhythms, and cardiovascular system homeostasis (Hu et al., 2020; Gu et al., 2021) (**Figure 1B**).

### Roles of m6A in RNA Metabolism and Activity

m6A and related proteins regulate almost all aspects of RNA metabolism and activity, thus modulating gene expression under physiological and pathological conditions. On the one hand, m6A is involved in the processing, alternative splicing, nuclear export, translation and degradation of mRNAs. FTO cooperates with METTL3 to regulate poly(A) sites and change the length of the 3′UTR (Bartosovic et al., 2017). In addition, FTO regulates alternative mRNA splicing not only by inhibiting the binding ability of serine and arginine-rich splicing factor (SRSF) 2 protein in an m6A-dependent manner but also by targeting m6Am during the biogenesis of snRNAs, which are integral spliceosome components and are involved in regulating premRNA splicing (Zhao et al., 2014; Mauer et al., 2019). YTH domain-containing 1 (YTHDC1) regulates alternative splicing by recruiting SRSF3 to promote exon inclusion and facilitates the binding of methylated mRNAs to nuclear export factor 1 (NXF1) to modulate their nuclear export (Xiao et al., 2016). YTH domain-containing family 1 (YTHDF1) promotes the efficient translation of target mRNAs in a cap-independent manner, particularly through its interaction with eukaryotic initiation factor 3 (eIF3), while YTHDF2 accelerates RNA degradation and inhibits protein translation by preferentially binding m6A in the 3' UTR and then recruiting the CCR4-NOT complex (Meyer et al., 2015; Wang et al., 2015; Du et al., 2016).

On the other hand, m6A is involved in regulating ncRNA metabolism and activity, including miRNA biogenesis, circRNA translation, and lncRNA stability and localization. With the assistance of METTL3 methylation activity, HNRNPA2B1 promotes mature miRNA biogenesis by recruiting DiGeorge syndrome chromosomal region (DGCR) 8 to primary miRNAs(Alarcón et al., 2015a; Alarcón et al., 2015b). METTL14 promotes the processing of pri-miR126 by directly recruiting DCGR8 (Ma et al., 2017). METTL3 has been reported to indirectly regulate miRNA expression and facilitate the translation initiation of circRNAs through an m6A-dependent mechanism (Karthiya and Khandelia, 2020). RBM15/15B alters the deposition of m6A on X-inactive specific transcript (XIST) by promoting the methylation of XIST, resulting in X-chromosome inactivation and gene silencing (Patil et al., 2016). METTL3 overexpression significantly increases the localization of the lncRNA RP11 in the nucleus, indicating that the localization of lncRNAs may also be regulated by m6A (Wu et al., 2019). ALKBH5 maintains the stability of the lncRNA GAS5-AS1, while YTHDF2/3 reduces its stability and accelerates lncRNA decay (Wang et al., 2019a; Ni et al., 2019). METTL16 introduces m6A into the U6 snRNA and regulates subsequent processing, thus regulating SAM homeostasis (Pendleton et al., 2017).

### Role of m6A in the Development of Multiple Organs

The dynamic m6A modification precisely regulates mRNA translation and degradation during early development. METTL3 mutations lead to early developmental stagnation, defects in the transition from mother to zygote and even embryonic lethality (Gu et al., 2021). Recent studies have focused on the role of m6A in the development of three main systems: the hematological system, nervous system and reproductive system. Further investigations are needed to determine whether m6A affects other systems. METTL3 deficiency affects hematopoietic development by significantly inhibiting the transition from endothelial cells to hematopoietic stem cells (HSCs) (Zhang et al., 2017b). In vascular endothelial cells, METTL3 knockout inhibits the function of hematopoietic stem/progenitor cells (HSPCs), while METTL3 knockout in HSPCs promotes differentiation (Vu et al., 2017; Lv et al., 2018).

The development of the nervous system depends on the specific expression of m6A modulators in different regions, cell subtypes and developmental stages of the brain. YTHDF2 deficiency in the embryonic neocortex impairs the self-renewal of neural stem/progenitor cells and special patterns of brain cell generation, leading to a failure of neural development (Yoon et al., 2017). METTL3 overexpression leads to structural disorders in both Purkinje and glial cells, and low METTL3 expression results in severe developmental defects in the cerebellum, indicating that a delicate m6A balance is essential for normal development (Ma et al., 2018; Wang et al., 2018). FTO knockout inhibits the proliferation and neuronal differentiation of adult neural stem cells and suppresses the expression of several crucial proteins involved in the brain-derived neurotrophic factor pathway, indicating its essential role in regulating adult neurogenesis (Li et al., 2017a).

Gametogenesis, a key step in reproductive system development, is also regulated by m6A modifications at the posttranscriptional level. YTHDC1/2 is required for spermatogenesis and oogenesis. YTHDC1 deficiency alters the length of the 3′ UTR by causing extensive alternative polyadenylation and impairs alternative splicing by inhibiting factors associated with premRNA 3′ end processing (Kasowitz et al., 2018). These events block oocytes at the primary follicle stage and eventually result in defective oogenesis. YTHDC2 knockout in germ cells leads to a failure to develop past the zygotene stage, thus resulting in male and female infertility (Hsu et al., 2017).

## Roles of m6A in Metabolism and Energy Homeostasis

m6A plays important roles in nutritional metabolism and energy balance, which are related to the pathogenesis of metabolism-related diseases, including type 2 diabetes and obesity (Gu et al., 2021). m6A activates glucose oxidation in rat adipocytes, suggesting that appropriate m6A levels may be essential for maintaining certain blood glucose concentrations (Souness et al., 1982). METTL3 knockout suppresses the expression of genes related to insulin secretion, thus inducing islet β-cell failure (Li et al., 2021b). METTL3 deficiency in mouse hepatocytes improves glucose tolerance and insulin sensitivity and decreases lipid accumulation (Li et al., 2020b). Acute deletion of METTL14 in β-cells reduces insulin secretion by increasing the activity of the IRE1a/sXBP-1 signaling pathway, finally leading to glucose intolerance (Men et al., 2019). Based on these results, METTL3/4 is essential for islet β-cell biology and maintaining the glucose balance. FTO is associated with carbohydrate and lipid metabolism and is involved in energy homeostasis. According to recent evidence, FTO participates in glucose metabolism through both m6A-dependent and nonm6A-dependent pathways (Wu et al., 2020). FTO also regulates adipogenesis by mediating the spicing of adipogenic regulatory factor RUNX1 translocation partner 1 (RUNX1T1), and AMPK positively regulates m6A levels in mRNAs to negatively regulate lipid accumulation in skeletal muscles (Zhao et al., 2014; Wu et al., 2017).

## Role of m6A in Immune Regulation

m6A has been reported to exert dual effects on regulating the immune response during both tumorigenesis and viral infection (Li et al., 2021a; Gu et al., 2021). m6A inhibits or promotes cancer progression by exerting dual-directional regulatory effects on apoptosis, autophagy, angiogenesis and the epithelial-mesenchymal transition (EMT). m6A modulators inhibit apoptosis by increasing oncogene expression levels and inhibiting tumor suppressor expression. In contrast, m6A modulators promote apoptosis by inhibiting the expression of oncogenes and promoting the expression of tumor suppressors (Li et al., 2021a). FTO silencing decreases the expression of light chain 3B (LC3B), a membrane marker of autophagy, but increases the expression levels of autophagy substrates by demethylating the UNC-51-like kinase 1 (ULK1) mRNA (Jin et al., 2018). Conversely, FTO promotes autophagy by directly demethylating autophagy-related (ATG) 5 and ATG7 (Wang et al., 2020b). In hepatocellular carcinoma, METTL3 knockdown induces the expression of some angiogenic biomarkers and increases the formation of tubes, indicating that METTL3 inhibits angiogenesis (Lin et al., 2020). IGF2BP3 recognizes m6A sites catalyzed by METTL3 and promotes angiogenesis by increasing the stability of HDGF transcripts (Wang et al., 2020c). Moreover, METTL3 overexpression decreases the expression of vimentin, β-catenin and N-cadherin and increases E-cadherin accumulation in renal cell carcinoma cells, thus promoting the EMT. Accordingly, METTL3 loss resulted in opposite alterations (Li et al., 2017b). Moreover, modulators alter the tumor microenvironment by regulating the expression of regulatory factors, such as lysosomal cathepsins and the TLR4 adaptor protein TIRAP, thus affecting immune escape and cancer immunotherapy (Li et al., 2021a). Based on the mechanism described above, m6A plays dual regulatory roles in tumorigenesis. However, the precise mechanism of the m6A modification in tumorigenesis remains to be fully elucidated.

Similarly, m6A promotes and inhibits immunity against viruses by modulating the lifecycles of viruses and immune responses of hosts (Gu et al., 2021). METTL3 increases replication efficiency by increasing the SUMOylation and ubiquitination of RNA polymerase 3D and is recruited to replication sites of viral RNAs(Hao et al., 2019). Moreover, writer and eraser knockdown promotes and inhibits the infection rate of the hepatitis C virus by increasing or decreasing the production of infectious viral particles, respectively (Gokhale et al., 2016). High METTL14 expression maintains the stability of latent Epstein-Barr virus transcripts (Lang et al., 2019). In hosts, METTL3 regulates innate immunity and adaptive immunity, including macrophages, dendritic cells (DCs), to exert a regulatory effect on viral infection (Gu et al., 2021).

# ROLE OF M6A IN IMMUNE CELLS

The precursor hematopoietic stem cells located in the bone marrow are the original source of all immune cells in the

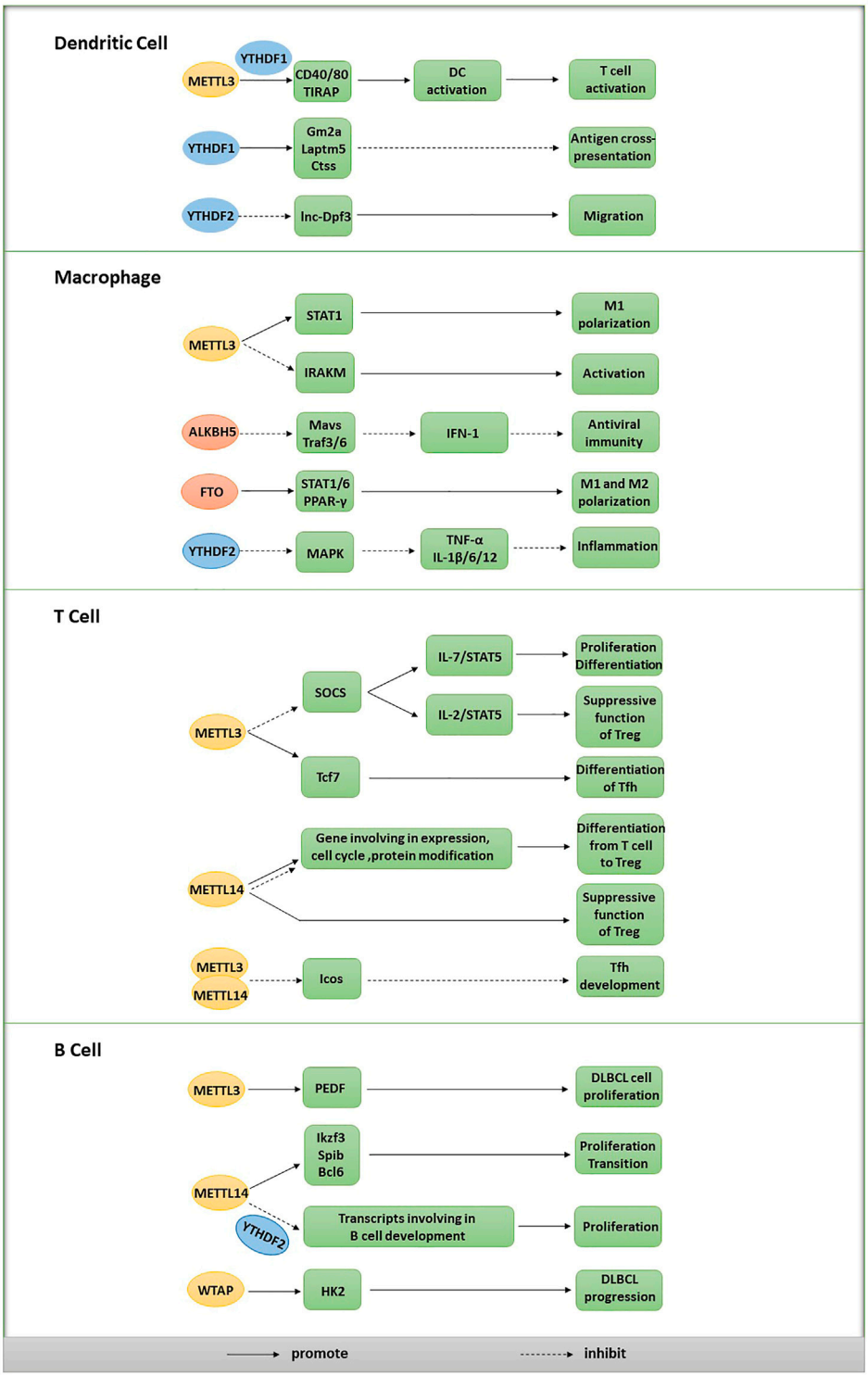


**FIGURE 2 |** Effects of m6A on different types of immune cells.

blood, lymph and immune organs. Immune cells are classified as innate immune cells and adaptive immune cells according to their functions and patterns in the immune response. Innate immune cells primarily include DCs, macrophages, granulocytes and mast cells that respond rapidly to antigenic stimuli. Adaptive immune cells primarily include T lymphocytes ($CD4^+$ T cells and $CD8^+$ T cells) and B lymphocytes that initially exhibit a delayed response but are involved in the formation of immunological

memory to respond strongly and rapidly to repeated stimulation with the same antigen. In addition, other types of immune cells, such as natural killer (NK) cells and NK-T cells, are components of both immune systems (McComb et al., 2019). Based on accumulating evidence, m6A is required for many processes in immune cells, including development, differentiation, activation, migration and polarization, thereby modulating the immune response (**Figure 2**).

## Role of m6A in DCs

Antigens are efficiently phagocytosed, processed and presented by DCs, leading to the activation of T cells and initiation of the immune response. METTL3, the catalytic subunit of the writer complex, is essential for the maturation and functional activation of DCs. METTL3 in DCs promotes T cell activation by catalyzing the formation of m6A in signaling molecule transcripts, including CD40, CD80 and TLR4 signaling adaptor (TIRAP). Then, these m6A-modified transcripts are recognized by YTHDF1 to increase their downstream translation, thus promoting DC activation and subsequent T cell responses (Wang et al., 2019b). On the other hand, YTHDF1 recognizes m6A-modified transcripts encoding lysosomal proteases and promotes their translation, thus limiting antigen cross-presentation by degrading protein antigens (Han et al., 2019). Therefore, YTHDF1 deficiency in DCs enhances antitumor immunity by promoting the cross-presentation of tumor antigens and cross-priming of $CD8^+$ T cells, indicating that YTHDF1 is likely to become a promising antitumor target (Karthiya and Khandelia, 2020). CCR7 induces lnc-Dpf3, a lncRNA, to bind HIF-1α and suppress HIF-1-dependent transcription of glycolytic genes, thus suppressing CCR7-dependent DC migration. YTHDF2 recognizes m6A-modified lnc-Dpf3 and accelerates its degradation, which may exacerbate the inflammatory response and disrupt immune homeostasis by promoting DC migration (Liu et al., 2019a).

## Role of m6A in Macrophages

Macrophages perform various functions, including removing damaged, dead or dying cells and other debris, presenting antigens to cells and producing cytokines and other regulatory factors, similar to secretory cells, to modulate the immune response. Type I interferon (IFN-1) production is inhibited by DDX46, a DDX helicase, through its interaction with Mavs, Traf3 and Traf6 transcripts, which encode signaling molecules essential for IFN-1 production under viral stimulation. In infected macrophages, DDX46 recruits ALKBH5 through its DEAD helicase domain to catalyze the demethylation of these methylated transcripts, which leads to the retention of the unmodified transcripts in the nucleus, prevents their efficient translation, inhibits IFN-1 production and finally suppresses antiviral immunity (Zheng et al., 2017).

Macrophages can be polarized into the M1 and M2 phenotypes; M1 macrophages produce interferon $\gamma$ (IFN-$\gamma$) to mediate proinflammatory activities, while M2 macrophages produce the cytokine interleukin-4 (IL-4) to mediate anti-inflammatory activities. The functional status of macrophages changes substantially with alterations between M1 and M2 polarization. The level of the METTL3 protein was reported to be specifically upregulated after M1 polarization of mouse macrophages. Furthermore, METTL3 directly methylates the mRNA encoding signal transducer and activator of transcription 1 (STAT1), an essential modulator of M1 polarization. Then, the stability of the methylated STAT1 mRNA is increased, and the STAT1 protein level is accordingly upregulated, thus driving M1 macrophage polarization. However, METTL3 deletion exerts opposing effects on macrophage polarization, decreasing M1 polarization but increasing M2 polarization and thus promoting an anti-inflammatory response (Liu et al., 2019b). In another study, FTO silencing induced the downregulation of STAT1 expression in M1-polarized macrophages and decreased the expression of STAT6 and PPAR-$\gamma$ in M2-polarized macrophages. Specifically, FTO knockdown suppressed NF-κB signaling by downregulating the phosphorylation of related proteins and decreasing the mRNA stability of STAT1 and PPAR-$\gamma$ through a mechanism dependent on the effect of YTHDF2, thereby preventing both M1 and M2 polarization of macrophages (Gu et al., 2020).

IRAKM is postulated to negatively regulate TLR4 signaling, which promotes macrophage activation. In another study, METTL3 deficiency led to impaired m6A formation in the IRAKM mRNA and slowed IRAKM degradation, ultimately inhibiting macrophage activation mediated by the suppression of TLR signaling (Tong et al., 2021). Upon lipopolysaccharide (LPS) stimulation, YTHDF2-deficient macrophages exhibited increased stability of the MAPK mRNA and its upregulated expression; the MAPK and NF-κB signaling pathways were subsequently activated to increase the expression levels of signaling molecules, including TNF-α, IL-1β, IL-6 and IL-12 (Liu et al., 2021).

## Role of m6A in T Cells

$CD4^+$ T cells and $CD8^+$ T cells are the primary classes of T cells. $CD8^+$ T cells (cytotoxic T lymphocytes, CTLs) secrete cytotoxic granules and perforin into the immune synapse to induce apoptosis in target cells, including infected cells and tumor cells. $CD4^+$ T cells (helper T cells, Th) differentiate into different phenotypes upon stimulation with different cytokines, and the differentiated cells contribute to cellular immunity or humoral immunity by secreting different cytokines. $CD4^+$ T cells are activated by IL-12 and IFN-$\gamma$ stimulation to differentiate into Th1 cells and secrete IFN-$\gamma$ and lymphotoxin-alpha (LT-α) to induce inflammation and support cellular immunity, while $CD4^+$ T cells are activated by IL-4 stimulation to differentiate into Th2 cells and secrete IL-4 to support humoral immunity and antibody production (McComb et al., 2019). In summary, Th1 cells induce a proinflammatory response, while Th2 cells induce an anti-inflammatory response. As another Th cell subtype, Th-17 cells produce IL-17 and promote inflammation and autoimmunity, while regulatory T cells (Tregs) promote immune tolerance, maintain immune homeostasis and suppress autoimmunity (Bettelli et al., 2007; Sakaguchi et al., 2008; Wan, 2010).

SOCS family proteins compete with IL-7 for binding to the IL-7 receptor, resulting in a failure to activate STAT5 and downstream signals that are important for the differentiation

and proliferation of naïve T cells. METTL3 deletion reduces the m6A level in the SOCS mRNA and decreases SOCS mRNA degradation, leading to increased SOCS mRNA and protein levels. Accordingly, upregulated SOCS family activity inhibits STAT5 activation mediated by IL-7, eventually preventing the normal proliferation and differentiation of T cells. Moreover, m6A may contribute to the induction of SOCS mRNA degradation upon IL-7 stimulation to promote proliferation and differentiation by reprogramming naïve T cells (Li et al., 2017c). Another study showed that the depletion of METTL3 in Tregs increased the mRNA levels of SOCS family genes, suppressed the IL-2/STAT5 signaling pathway and impaired the suppressive function of Tregs (Tong et al., 2018b). Based on these results, the m6A RNA modification regulates the differentiation of naïve T cells and sustains the suppressive functions of Tregs by specifically targeting the same family of genes in different T cell subtypes. Additionally, in a mouse model of colitis, METTL14 deficiency in T cells increases inflammatory cell infiltration, increases cytokine release from Th1 and Th17 cells and prevents the differentiation of naïve T cells into Tregs (Lu et al., 2020).

T follicular helper (Tfh) cells are critical for the formation of germinal centers (GCs) and effective humoral immunity. METTL3 has been suggested to play a key role in modulating the expression of important Tfh signature genes, including Tcf7 and Icos, which are related to the development and differentiation of Tfh cells. The m6A modification was reported to increase the stability of Tcf7 transcripts to promote Tfh cell differentiation programs in a METTL3-dependent manner (Yao et al., 2021), and the METTL3/METTL14 complex was shown to catalyze m6A installation on the Icos mRNA and subsequently cause GAPDH protein-induced suppression of Icos expression, thereby inhibiting Tfh cell development (Zhu et al., 2019).

### Role of m6A in B Cells

B cells are the main cells mediating humoral immunity. B cells depend on their B cell receptors (BCRs) to recognize specific antigens and differentiate into plasma cells, which produce and secrete specific antibodies that bind to the target antigen (McComb et al., 2019).

According to recent studies, the m6A modification and its regulators may be involved in the early development and proliferation of B cells. For example, METTL14 deficiency dramatically decreases the m6A level in mRNAs and causes the aberrant expression of genes essential for B cell development, eventually inhibiting IL-7-induced pro-B cell proliferation and the transition from large pre-B cells to small pre-B cells (Zheng et al., 2020). Moreover, IL-7-induced pro-B cell proliferation depends on transcriptional suppression mediated by YTHDF2, while the failure to transition from large pre-B cells to small pre-B cells is independent of both YTHDF1 and YTHDF2 (Zheng et al., 2020). METTL3 is expressed at high levels in diffuse large B cell lymphoma (DLBCL) cell lines and patient tissues and increases the m6A levels in the pigment epithelium-derived factor (PEDF) transcript to promote DLBCL cell proliferation (Cheng et al., 2020). Similarly, WTAP upregulation induced by piRNA-30473 promotes DLBCL progression by increasing the m6A level in HK2 transcripts and the subsequent expression of HK2(Han et al., 2021).

## ROLE OF M6A IN AUTOIMMUNE DISEASES

The role of immune cells in the pathogenesis of autoimmune diseases has been extensively studied, and these cells have proven to be involved in the development of autoimmune disorders. Interestingly, some immune cells, including macrophages, T cells and NK cells, exert dual effects: disease promotion and disease prevention (**Table 2**). This dual function may be attributed to differences in subsets of immune cells, the tissue microenvironment, and stages of autoimmune disease and to interactions between immune cells. Recent evidence shows that m6A may be involved in the development of autoimmune diseases. Moreover, some studies strongly indicate that m6A regulates the functions of immune cells, thereby affecting autoimmune diseases.

### Rheumatoid Arthritis

High-throughput m6A sequencing revealed a potential relationship between RNA methylation and rheumatoid arthritis (RA)-related genes, suggesting that m6A may contribute to the initiation and development of RA. Indeed, the global m6A content in peripheral blood is significantly increased in patients with RA compared to healthy people. Quantitative real-time polymerase chain reaction showed that the mRNA expression levels of ALKBH5, FTO and YTHDF2 were decreased in peripheral blood mononuclear cells (PBMCs) isolated from patients with RA. However, ALKBH5 expression is upregulated in patients with RA after treatment with the appropriate drug therapy. In addition, associations were identified between FTO mRNA expression and some indicative markers of RA activity, including the IgG level, C3 level, disease activity score 28 (DAS28) score and lymphocyte-to-monocyte ratio (LMR). Moreover, associations were observed between YTHDF2 mRNA expression and the red blood cell (RBC) count, lymphocyte percentage (L%), neutrophil percentage (N%), neutrophil-to-lymphocyte ratio (NLR), and LMR. In summary, m6A and regulators such as ALKBH5, FTO and YTHDF2 may be promising candidates for assessing the risk and progression of RA (Luo et al., 2020a).

METTL3 expression is significantly upregulated in patients with RA. In addition, positive correlations were found between METTL3 expression and biochemical indexes, including the C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR), which suggested alterations in RA disease activity. LPS stimulation of macrophages increases the expression and biological effects of METTL3. Moreover, METTL3 overexpression significantly inhibits the LPS-induced inflammatory response in macrophages through the NF-κB pathway (Wang et al., 2019). However, METTL3 may promote the activation of fibroblast-like synoviocytes (FLSs) and the inflammatory response through the NF-κB pathway, thus accelerating the initiation and progression of RA (Shi et al.,

**TABLE 2 |** Contribution of immune cells to the development of autoimmune diseases.

| Cell type | Contribution | Autoimmune disease | References |
|---|---|---|---|
| T cell (except Treg) | Promotion | Rheumatoid arthritis | Cope et al. (2007); Han et al. (2015) |
| | | Systemic lupus erythematosus | Han et al. (2015); Shao et al. (2020) |
| | | Sjogren's syndrome | Han et al. (2015) |
| | | Inflammatory bowel disease | Han et al. (2015) |
| | | Multiple sclerosis | Han et al. (2015) |
| | | Type 1 diabetes | Shao et al. (2020) |
| Treg | Protection | Rheumatoid arthritis | Cope et al. (2007) |
| | | Systemic lupus erythematosus | Ohl and Tenbrock, (2015) |
| | | Sjogren's syndrome | Alunno et al. (2015) |
| | | Multiple sclerosis | Danikowski et al. (2017) |
| | | Inflammatory bowel disease | Műzes et al. (2012) |
| | | Type 1 diabetes | ElEssawy and Li, (2015) |
| B cell | Promotion | Rheumatoid arthritis | Rubin et al. (2019) |
| | | Systemic lupus erythematosus | Rubin et al. (2019) |
| | | Sjogren's syndrome | Rubin et al. (2019) |
| | | Type 1 diabetes | Rubin et al. (2019) |
| | | Multiple sclerosis | Sabatino et al. (2019) |
| Dendritic cell | Promotion | Rheumatoid arthritis | Worbs et al. (2017) |
| | | Systemic lupus erythematosus | Worbs et al. (2017) |
| | | Psoriasis | Worbs et al. (2017) |
| | | Type 1 diabetes | Diana et al. (2013) |
| | | Multiple sclerosis | Mohammad et al. (2012) |
| Macrophage | Promotion | Rheumatoid arthritis | Funes et al. (2018); Shapouri-Moghaddam et al. (2018) |
| | | Systemic lupus erythematosus | Funes et al. (2018) |
| | | Autoimmune neuritis | Funes et al. (2018) |
| | | Inflammatory bowel disease | Funes et al. (2018) |
| | | Systemic sclerosis | Funes et al. (2018) |
| | | Autoimmune hepatitis | Shapouri-Moghaddam et al. (2018) |
| | | Crohn's disease | Shapouri-Moghaddam et al. (2018) |
| | | Multiple sclerosis | Shapouri-Moghaddam et al. (2018) |
| | Protection | Systemic lupus erythematosus | Funes et al. (2018) |
| | | Autoimmune neuritis | Funes et al. (2018) |
| | | Inflammatory bowel disease | Funes et al. (2018) |
| NK cell | Promotion | Multiple sclerosis | Fogel et al. (2013) |
| | | Rheumatoid arthritis | Palm et al. (2015) |
| | | Psoriasis | Ottaviani et al. (2006) |
| | | Primary biliary cirrhosis | Shimoda et al. (2011) |
| | | Type 1 diabetes | Dotta et al. (2008) |
| | Protection | Multiple sclerosis | Shi et al. (2011) |
| | | Type 1 diabetes | Dotta et al. (2008) |

2021). Therefore, the precise role of METTL3 in the pathogenesis of RA remains to be further investigated.

## Systemic Lupus Erythematosus

A comprehensive review first proposed that a link between the m6A modification and systemic lupus erythematosus (SLE) is reasonable based on the observation that m6A effectively regulates gene expression and the immune system (Li et al., 2018). Other scientists then observed downregulated mRNA expression of m6A regulators, including METTL3, METTL14, WTAP, FTO, ALKBH5 and YTHDF2, in patients with SLE (Luo et al., 2020b; Luo et al., 2020c). These decreases correlated with the index used to predict SLE disease activity. Specifically, levels of the METTL14 and YTHDF2 mRNAs in patients with SLE were associated with CRP and C3 levels, while the ALKBH5 mRNA levels of in patients with SLE were associated with C3, CRP and autoantibody levels and skin manifestations. In addition, positive correlations were observed among mRNA levels of three different regulators in PBMCs from patients with SLE (Luo et al., 2020b; Luo et al., 2020c). In addition, logistic regression and multivariate logistic regression analyses revealed that downregulated expression of the YTHDF2 or ALKBH5 mRNA may be associated with an increased risk of developing SLE (Luo et al., 2020b; Luo et al., 2020c). These findings indicate that the m6A regulators ALKBH5 and YTHDF2 are likely to be involved in the pathogenesis of SLE and are expected to be effective biomarkers to assess the SLE risk and disease activity (Luo et al., 2020c).

## Multiple Sclerosis

In a comprehensive analysis of DNA methylation and gene expression data, Mo et al. identified rs923829 in METTL21B and rs2288481 in the DKKL1 gene as strongly correlated with multiple sclerosis (MS). An analysis of the HaploReg database showed that these two m6A-related SNPs regulate the expression of the METTL21B and DKKL1 genes. Then, the researchers selected PBMCs from a small group of Chinese participants to

validate the association between rs923829 and METTL21B expression and between rs2288481 and DKKL1 expression. Importantly, rs923829 is strongly associated with METTL21B expression, while a significant statistical association is not observed between rs2288481 and DKKL1 expression. This group proved that these m6A-related SNPs may be related to the pathogenesis of MS(Mo et al., 2019).

Experimental autoimmune encephalomyelitis (EAE) is internationally recognized as an animal model for studying MS. More recently, specific ablation of ALKBH5 in T cells conferred protection against EAE. Mechanistically, the m6A eraser ALKBH5 decreased the m6A levels in the CXCL2 and IFN-$\gamma$ mRNAs and subsequently increased their transcript stability and protein expression, thereby enhancing $CD4^+$ T cell-mediated responses and inflammatory cell infiltration in the central nervous system to induce neuroinflammation (Zhou et al., 2021). This study was the first to prove a direct link between autoimmunity and m6A-mediated functions of immune cells.

### Psoriasis

Transcriptome-wide m6A profiling revealed that transcripts from psoriatic skin had the fewest m6A peaks and lowest m6A peak density compared with transcripts from uninvolved psoriatic skin and healthy skin. Bioinformatics pathway analyses indicated that transcripts that were hypermethylated in psoriatic skin were primarily correlated with inducing various responses, including immune responses, cytokine production and olfactory signal transduction, while transcripts that were hypomethylated in psoriatic skin were strongly related to the Wnt signaling pathway and development-related processes. Transcripts with lower expression levels were preferentially modified with m6A. Moreover, gene expression was upregulated in psoriatic skin, accompanied by increased m6A levels, indicating that alterations in m6A methylation affect the gene expression pattern (Wang and Jin, 2020).

### Other Autoimmune Diseases

T cell-specific METTL14 deficiency prevents the differentiation of naïve T cells into Tregs, leading to an imbalance in Th17 cells and Tregs, and thereby inducing spontaneous colitis. Considering that dysregulation of the balance between Th17 cells and Tregs is strongly associated with the initiation of inflammatory bowel disease (IBD), a reasonable assumption is that METTL14 may be involved in IBD development (Lu et al., 2020). Another study showed that $CD4^+$ T cells induce autoimmune colitis, an ability that might be controlled by ALKBH5. Therefore, ALKBH5-deficient naïve $CD4^+$ T cells failed to migrate into colon tissue, and their ability to promote colitis was reduced (Zhou et al., 2021).

Variants in IGF2BP2 were shown to decrease glucose-stimulated insulin secretion in the first phase of diabetes development. Additionally, IGF2BP2 was found to be downregulated and linked to diabetic nephropathy in male patients with type 1 diabetes (T1D), as well as impaired glucose tolerance in patients with type 2 diabetes (T2D) (Wang et al., 2021b). Correlations were observed between polymorphisms in the ALKBH5 gene, including rs9913266 and rs12936694, and the development of autoimmune thyroid disease (AITD). Therefore, ALKBH5 might be a candidate susceptibility gene for AITD (Song et al., 2021).

## CONCLUSIONS AND FUTURE PERSPECTIVES

Over several decades, studies of m6A modifications have resulted in substantial progress in epitranscriptomics. Convincing evidence suggests that reversible m6A modification may be involved in regulating many processes in immune cells, including development, differentiation, activation, and migration. Based on these results, m6A may participate in the pathogenesis of immune-related diseases, including cancers, viral infections, and inflammatory and autoimmune diseases. Currently, the relationships between m6A and cancer and viral infection have received extensive research attention. However, studies examining the essential role of the m6A modification in the pathogenesis of autoimmune diseases are lacking, although existing evidence strongly indicates that m6A may be involved in the development of autoimmune diseases. Direct studies assessing the mechanisms by which and to what extent m6A contributes to autoimmune diseases are urgently needed. In addition to the pathogenesis of autoimmune diseases, we should focus more on achieving the transition from mechanistic research to clinical applications, including diagnosis and treatment. Therefore, the following research gaps still remain to be filled to provide new opportunities for the treatment of immune-related diseases, including autoimmune diseases.

1. Innovative and more advanced technology. Various limitations in current technology exist, including low precision, poor calculation methods, high complexity, low repetition. Scientists must develop more convenient and accurate sequencing and imaging technology for the rapid and quantitative detection of the m6A modification, perform functional analysis and understand the dynamic mechanisms of modified RNAs.

2. Precise regulatory mechanisms among m6A modulators. Although numerous findings related to the function of m6A modulators have been reported, many knowledge gaps remain to be filled. The dynamic expression pattern of modulators makes functional identification more complicated. We should understand the mechanisms mediating the spatiotemporal specificity of m6A, how the function of regulatory proteins is regulated in different cell types, how to precisely regulate different target RNAs, how mediators regulate interactions with other regulatory proteins to perform their respective functions or exert their comprehensive effects, and how their unbalanced deposition leads to pathological processes.

3. Complicated network between m6A and other regulatory factors. m6A modification and other epigenetic regulatory mechanisms, including chromatin state interaction and histone modification, are emerging as a new area in the epitranscriptomic field. In addition, m6A exerts a decisive effect on the fate of noncoding RNAs, including microRNAs, lncRNAs and circRNAs. Their interaction will prompt more studies to obtain

an in-depth understanding of m6A. Moreover, the network between m6A and other RNA modifications, including 5-methylcytosine (m5C) and pseudouridine (Ψ), should be further explored.

4. Promizing but difficult clinical applications. m6A provides novel insights into the diagnosis, treatment and prognosis of diseases, especially autoimmune diseases. However, the use of modulators as therapeutic agents remains an important challenge. First, in the present review, m6A exerts dual effects on immune-related diseases, indicating a lack of consistent and consolidated evidence. Second, its safety has not been guaranteed. Finally, few small-molecule stimulants or inhibitors targeting m6A are available. Therefore, efforts are urgently needed to screen molecular drugs targeting m6A. An understanding of the mechanisms by which RNA modifications are introduced, removed or read will reveal the underlying pathogenesis and provide therapeutic targets for autoimmune diseases in the future.

## AUTHOR CONTRIBUTIONS

YW and LL performed the literature search, wrote the first draft of the article, and revised the text. JL, BZ, GH, XL, and ZZ critically revised the text and provided substantial scientific contribution. ZX proposed the project and revised the article. All authors approved the final version of the article.

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REVIEW
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# Epigenetic Modifications and Therapy in Uveitis

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Uveitis is a sight-threatening intraocular inflammation, and the exact pathogenesis of uveitis is not yet clear. Recent studies, including multiple genome-wide association studies (GWASs), have identified genetic variations associated with the onset and progression of different types of uveitis, such as Vogt–Koyanagi–Harada (VKH) disease and Behcet's disease (BD). However, epigenetic regulation has been shown to play key roles in the immunoregulation of uveitis, and epigenetic therapies are promising treatments for intraocular inflammation. In this review, we summarize recent advances in identifying epigenetic programs that cooperate with the physiology of intraocular immune responses and the pathology of intraocular inflammation. These attempts to understand the epigenetic mechanisms of uveitis may provide hope for the future development of epigenetic therapies for these devastating intraocular inflammatory conditions.

**Keywords: uveitis, epigenetic regulation, DNA methylation, microRNAs, epigenetic therapy**

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## INTRODUCTION

Epigenetics refers to stable and heritable alterations in gene expression without involving changes in the nucleotide sequence and was first proposed by Dr. Waddington in the 1940s (Bird, 2007; Waddington, 2012). Epigenetic phenomena are of remarkable importance for gene expression patterns in normal physiological functions such as cellular development and differentiation, as well as in response to environmental factors (Jeffries, 2020). The epigenome serves as a critical interface between the environment and genome. It comes into play during development and works in a highly organized way to modulate the landscape of gene expression in various cell types (Guerrero-Preston et al., 2011). The epigenetic regulation of gene expression leads to typical growth through the dynamic transcription of gametogenesis in the embryonic and neonatal stages and lasts for a lifetime (Meissner et al., 2008; Guerrero-Preston et al., 2011). In the whole life cycle, in response to various endogenous and exogenous factors, epigenetic regulation undergoes various changes that are transient or permanent (Kaminsky et al., 2009; Grolleau-Julius et al., 2010; Romani et al., 2015). Disruptions in epigenetically controlled gene expression patterns can lead to autoimmune diseases, cancer and a variety of other diseases (Zhang et al., 2020a; Safi-Stibler and Gabory, 2020; Tzika et al., 2020). Over the past decade, the effects of epigenetic modifications on innate and adaptive immunity have been studied intensively, especially in autoimmune diseases (Brooks et al., 2010; Long et al., 2016; Agudelo Garcia and Berger, 2020).

Uveitis is serious intraocular inflammation that occurs worldwide and can lead to visual impairment and even blindness. Approximately 25% of the irreversible blindness is caused by uveitis and its complications in the developing countries (Nussenblatt, 1990; Suttorp-Schulten and Rothova, 1996; Rao, 2013). Uveitis frequently occurs among people aged 20–50 years and causes

considerable economic burden (Rothova et al., 1992). The pathogenesis of uveitis has been considered to depend on complex interactions between multiple genetic substances and environmental risk factors. Genome-wide association studies (GWASs) have provided a powerful tool for the genome-wide analysis of genetic susceptibility to uveitis, revealing several genes associated with uveitis, including IL23R/C1ORF141, STAT4, and ADO/ZNF365/Egr2 (Hou et al., 2020). However, there are still some mechanisms that cannot be solved by genetics, and it is necessary to further study epigenetic modifications to explore the lack of heritability in uveitis risk (Hou et al., 2020).

In this Review, we introduce the main principles of epigenetic regulation, explain the possible association between epigenetic regulation and uveitis, and investigate how epigenetics could contribute to the understanding of the complex pathogenesis of uveitis. Finally, treatments for uveitis by manipulating epigenetic aberrations are discussed.

## EPIGENETICS AND UVEITIS?

Over the past 2 decades, twin studies have been performed to dissect the causal relationship between genetic and environmental factors in complex phenotypic traits and disease pathogenesis, including those in the eye (Montezuma et al., 2007; Sanfilippo et al., 2010; Tan et al., 2015). With the exception of a few cases, no consistency of uveitis has been reported between identical twins, implying that environmental factors associated epigenetic alterations may be important in the initiation of uveitis. Stress, lifestyle, nutrition/diet, and behaviors have been reported to change the susceptibility to disease induction (Sobrin and Seddon, 2014). At cellular level, there is considerable evidence that $CD4^+$ T cell-dependent immune responses are important in the pathogenesis of uveitis (Lee et al., 2014). Of interest, the activation and differentiation of $CD4^+$ T cells are typically epigenetic processes. In the process of differentiation from naive T cells into Th1, Th2, Th17, Treg and Tfh cells, the cell volume increases significantly, the morphology changes significantly, and the ability to secrete various cytokines and express immune functional proteins is obtained. It should be noted that during these processes there is no change in DNA sequence. On the other hand, epigenetic phenomena such as DNA methylation and histone modifications that occur during the regulation of chromatin recombination are key molecular bases for the major changes in gene expression and $CD4^+$ T cell function (Wei et al., 2009; O'Shea and Paul, 2010). Therefore, we must seriously consider the role of epigenetics in the pathogenesis of uveitis.

## ENVIRONMENTAL RISK FACTORS UNDERLYING EPIGENETIC MODIFICATIONS IN UVEITIS

BD and VKH syndrome have been proposed to be initiated by environmental factors in genetically predisposed individuals (Xu et al., 2021); however, the role of environment-cued epigenetic modifications in uveitis has not been studied carefully. The epigenetic machinery is widely influenced by the environment, as well as the environmental microbiome, which affects the host microbiome. In addition to seasonality, allergies and vitamin D being known environmental risk factors for uveitis in juvenile idiopathic arthritis (Clarke et al., 2021), emerging evidence has shown that host microbiomes, especially gut microbiomes, can influence the progression of uveitis (Horai and Caspi, 2019).

Both infectious uveitis caused by bacteria, viruses, fungi, or parasites and noninfectious uveitis are associated with a series of dysregulations in inflammatory genes and microRNAs (Wei et al., 2020). The expression of these genes sometimes requires chromatin remodeling, which can be modulated by both commensal and pathogenic bacteria. For example, mycobacteria have been demonstrated to be able to interfere with chromatin remodeling and inhibit IFN-$\gamma$-induced gene expression (Arbibe, 2008). IFN-$\gamma$ is one of the major cytokines released by $CD4^+$ Th1/Th17 cells to activate inflammatory cascades and cause local tissue damage in uveitis (Diedrichs-Mohring et al., 2018). During bacterial infection, the expression of several inflammatory genes can also be modulated at the epigenetic level (Zur Bruegge et al., 2017). The molecular mechanisms underlying microbiome-induced epigenetic alterations have not yet been understood; however, a growing number of studies suggest that microbial metabolites may have strong chromatin-modulating effects (Krautkramer et al., 2017). Butyrate, a major product of gut microbial fermentation, has been demonstrated to inhibit histone deacetylases (HDACs) (Chang et al., 2014). Other short-chain fatty acids (SCFAs), such as lactate, acetate, and propionate, could also exert effects on host chromatin, implying that the gut microbiota may be an important regulator of host epigenetic events (Chang et al., 2014).

In recent decades, there has been ample evidence documenting a role for the gut microbiota in uveitis (Molzer et al., 2020). A dogma in ophthalmic research is that the intraocular environment is always sterile under physiological conditions. Nevertheless, emerging evidence argues against intraocular sterility. As the first and foremost finding, our group identified the presence of an intraocular microbiota via quantitative PCR, transmission electron microscopy (negative staining), direct culture, and high-throughput sequencing technologies (Deng et al., 2021). A disruption of the intestinal barrier or blood-retina barrier could result in the leakage of bacteria or their metabolites into the circulatory system and the eye, leading to subsequent chromatin modifications and altered inflammatory gene expression (Smith et al., 2013).

## MECHANISMS OF EPIGENETIC REGULATION

Epigenetic mechanisms have been widely reviewed for their ability to regulate gene transcription and genomic stability. Epigenetics is a key factor in maintaining normal cell growth, development and differentiation (Esteller, 2007; Goldberg et al., 2007; Jones and Baylin, 2007; Peixoto et al., 2020). The term "epigenetics" can be summarized as follows: the meiosis/mitosis

of gene expression can be genetically changed, which is related to environmental factors, but the base sequence in DNA does not change (Bird, 2007). Since genome-wide analyses cannot provide enough answers to explain the complex biological processes in autoimmune diseases in some cases, epigenetic modifications retain additional regulatory factors in immune responses (Moosavi and Motevalizadeh Ardekani, 2016). Recognizing the complexity of the interaction between epigenetic regulation and changes in the immune system in autoimmune diseases is a prominent challenge for discovering new potential therapeutic strategies. The main mechanisms of epigenetic regulation are DNA methylation and histone modifications. In addition, RNAs, such as microRNAs (miRNAs), are now considered an additional layer of gene expression regulation (Vukic et al., 2019).

## DNA Methylation

DNA methylation is one of the earliest and most studied epigenetic regulatory mechanisms (Okano et al., 1999; Wildner and Diedrichs-Mohring, 2020). Studies have shown that DNA methylation largely occurs on CpG nucleotides, and approximately 70–80% of CpG islands are methylated in mammals (Jabbari and Bernardi, 2004; Law and Jacobsen, 2010). DNA methylation is an epigenetic mechanism mediated by DNA methyltransferase (DNMT), in which a methyl group is added to the fifth carbon atom of the cytosine ring, with S-adenosine methionine (SAM) as the methyl donor. There are five members of DNA methyltransferases (DNMTs), DNMT1, DNMT2, Dnmt3a, DNMT3b and DNMT3L, which play different roles either in maintaining DNA methylation (DNMT1, DNMT2) or acting as *de novo* DNA methylators (Dnmt3a, DNMT3b and DNMT3L) (Deplus et al., 2002; Dawson and Kouzarides, 2012; Jones, 2012). DNMTs can interact with each other and participate in the addition and removal of DNA by methyl groups. DNA methylation can be removed passively or actively (Reik and Walter, 2001; Seisenberger et al., 2013). DNA methylation affects a variety of biological processes, such as transcriptional inhibition, reversible promoter silencing and chromosomal instability (Bjornsson et al., 2004; Vaissiere et al., 2008). Studies have linked altered levels of DNA methylation to a variety of autoimmune diseases (Liu et al., 2011; Calabrese et al., 2012; Qiu et al., 2017a; Qiu et al., 2017b).

## Histone Modifications

Histones are basic proteins that bind to DNA in the nuclei of eukaryotes, and they are one of the highly conserved gene families in eukaryotes. There are five major histone families in most eukaryotes: H1/H5, H2A, H2B, H3, and H4. Two copies of histones H2A, H2B, H3, and H4 constitute an octamer core, which is the core part of the nucleosome, and the function of H1 is to combine with linear DNA to help form higher-order structures (Wong et al., 1998; Jenuwein and Allis, 2001). A growing body of evidence has confirmed that at least 12 specific modifications occur on the N-terminal amino acid residues of histones that affect the binding of nucleosomes to DNA and the three-dimensional structure of chromosomes and regulate gene expression. The histone modification model is as follows: acetylation (lysine), methylation (lysine and arginine), sumoylation (lysine), phosphorylation (serine and threonine), ADP ribosylation, ubiquitylation (lysine), citrullination, butyrylation, crotonylation, proline isomerization, formylation, serotonylation, propionylation, and dopaminylation (glutamine) (Tan et al., 2011; Nfonsam et al., 2020). These posttranslational modifications play an important role in regulating gene expression, DNA repair, chromatin dynamics and genome stability (Berger, 2007; Kouzarides, 2007). Among them, histone acetylation is an important type of histone modification, which means that the improvement in gene transcription activity and epigenetic markers is related to dynamic chromatin changes. Histone acetylation refers to HAT activating gene transcription through the acetylation of lysine residues in histones, while HDAC deacetylates histones and inhibits gene transcription. Histone acetylation is catalyzed by histone acetyltransferase (HAT) and HDACs (He et al., 2018). In addition, many amino acid residues in histones can be methylated, and different types of methylation, such as monomethylation (me 1), dimethylation (me 2) and trimethylation (me 3), show a variety of valence states. Changes in histone methylation patterns may promote or inhibit gene expression (Geng et al., 2021). Different histone modification types also influence each other to jointly regulate the expression of specific genes.

## MicroRNAs

MicroRNAs are a class of noncoding small RNAs with a length of 18–23 nucleotides located in the intragenic and intergenic regions of the genome. They are responsible for regulating the expression of approximately 60% of protein-coding genes in the human genome at the translation level. There is increasing experimental evidence that miRNA genes are distributed throughout the genome (Ambros, 2001; Ling et al., 2013; Vega-Tapia et al., 2021). It is well known that microRNAs can regulate various biological processes, such as proliferation, differentiation, apoptosis, the immune response and homeostasis (Chamorro Petronacci et al., 2019; Ashrafizadeh et al., 2020a; Ashrafizadeh et al., 2020b). These small dynamic RNA molecules regulate gene expression by binding to the 3′-untranslated region (3′-UTR) of the target mRNA, resulting in posttranscriptional inactivation of the target gene through mRNA degradation or the inhibition of translation (Catalanotto et al., 2016).

The role of histone modification in major rheumatic diseases such as rheumatoid arthritis has been considered (Li et al., 2009; Pedre et al., 2011; Singhal et al., 2015; Aslani et al., 2017), but there are few studies on histone modifications in uveitis. Therefore, we mainly discuss the role of DNA methylation and microRNAs in uveitis (**Table 1**).

# EPIGENETIC MODIFICATIONS IN DIFFERENT TYPES OF UVEITIS

## Epigenetic Modifications and Experimental Autoimmune Uveitis

Experimental autoimmune uveitis (EAU) is an animal model of human uveitis that has been extensively used in preclinical

**TABLE 1 |** Epigenetic alterations in Uveitis.

| Disease | Epigenetic change | Sample | Outcome | References |
|---|---|---|---|---|
| Experimental autoimmune uveitis | DNA methylation | Retinas and the RPE-Choroid Complexes | Tbx21 and Rorc showed dynamic methylation changes during EAU | Qiu et al. (2018) |
| | MicroRNA | PBMCs | 36 miRNAs were upregulated and 31 miRNAs were downregulated | Guo et al. (2015) |
| | | Retina | Upregulated: miRNA-223, 142-5p, 142-3p, 21, 146a, 146b, 1949, 1188-3p and 193<br>Downregulated: miRNA-181a, 183*, 124* and 331 | Watanabe et al. (2016) |
| | | Spleen, lymph nodes, and eye tissues | Downregulated: rno-miR-30b-5p | Sun et al. (2018) |
| | | Eye tissues | Upregulated: miRNA-142-5p and miRNA-21 Downregulated: miRNA-182 | Ishida et al. (2011) |
| | | $CD4^+$ T cells | Upregulated: miR-155 | Escobar et al. (2013) |
| | | Th17 cells | Upregulated: Mir-223-3p | Wei et al. (2019) |
| Behçet's disease | DNA methylation | Monocytes and $CD4^+$ T cells | 383 CpG sites in monocytes and 125 CpG sites in $CD4^+$ T cells differentially methylated | Hughes et al. (2014) |
| | | PBMCs and Neutrophil | Unmethylated (uCuC) Alu allele frequency increased in inactive BD patients | Yuksel et al. (2016) |
| | | Blood | 4,332 differentially methylated CpG sites were associated with BD and the most significant locus was located in the 5′UTR of FKBP5 | Yu et al. (2019) |
| | | $CD4^+$ T cells | Hypomethylation of GATA3, IL-4 and TGF-β promoter | Zhu et al. (2017a) |
| | | PBMCs | Hypomethylation of TLR4 promoter | Kolahi et al. (2020) |
| | | PBMCs | Hypermethylation of IL-10 promoter | Alipour et al. (2018) |
| | | PBMCs | Hypomethylation of IL-6 promoter | Alipour et al. (2020) |
| | | PBMCs | Hypomethylation of TNF-α promoter | Aziz et al. (2020) |
| | MicroRNA | PBMCs | Upregulated: miR-155 | Kolahi et al. (2018) |
| | | PBMCs | Upregulated: miR-326<br>Downregulated: miR-21, miR-146b | Jadideslam et al. (2019) |
| | | PBMCs | Upregulated: miR-3591-3p<br>Downregulated: miR-638, miR-4488 | Woo et al. (2016) |
| | | PBMCs and dendritic cells | Downregulated: miR-155 | Zhou et al. (2012) |
| | | $CD4^+$ T cells | Downregulated: miR-23b | Qi et al. (2014) |
| | | PBMCs | Upregulated: miR-25, miR-106b, miR-326, miR-93<br>Downregulated: miR-146a, miR-155 | Ahmadi et al. (2019) |
| Vogt–Koyanagi–Harada Disease | DNA methylation | $CD4^+$ T cells | Hypermethylation of GATA3, IL-4 and TGF-β promoter | Zhu et al. (2017b) |
| | | monocyte-derived dendritic cells | Hypermethylation of IRF8 | Qiu et al. (2017a) |
| | MicroRNA | Serum | 153 upregulated and 35 downregulated | Asakage et al. (2020) |
| | | $CD4^+$ T cells | Downregulated: miR-20a-5p | Chang et al. (2018) |
| Anterior uveitis | MicroRNA | PBMCs | Downregulated: miRNA146a, miRNA155 and miRNA125a5p | O'Rourke et al. (2019) |
| Experimental autoimmune anterior uveitis | MicroRNA | Iris, ciliary bodies, and popliteal lymph nodes | Upregulated: miR-182-5p, miR-183-5p and miR-9-3p<br>Downregulated: miR-146a-5p, miR-155-5p, miR-147b and miR-223-3p | Hsu et al. (2015) |

research (Caspi et al., 1988; Nussenblatt, 1991). Interphotoreceptor retinoid-binding protein (IRBP), a specific retinal antigen, can be used to induce EAU in mice (Caspi et al., 2008). In a highly susceptible B10R.III mouse strain, IRBP 161–180 peptide-induced EAU showed severe inflammation, including inflammation in the anterior and posterior segments of the eye, very similar to the clinical abnormalities observed in human panuveitis (Jiang et al., 1999).

T helper cell subsets and their featured transcription factors are related to various autoimmune diseases (Rahimi et al., 2019; Sakaguchi et al., 2020). To investigate whether the methylation of these main transcription factors is related to the development of EAU, Qiu Y et al. investigated whether the methylation of the T cell transcription factors TBX21 and RORC changed significantly during the development of EAU. They also provided evidence that DNMT1 may play an important role in regulating the DNA methylation of these two transcription factors (Qiu et al., 2018).

Mounting evidence has also demonstrated that certain miRNA imbalances are related to the progression of EAU. In EAU rats, it

was shown that 36 miRNAs were upregulated, and 31 miRNAs were downregulated, all of which are closely associated with immune signaling pathways (Guo et al., 2015). Watanabe et al. found that the expression of 9 miRNAs (miR-223, 142-5p, 142-3p, 21, 146a, 146B, 1949, 1188-3p and 193) was significantly increased and that the expression of 4 miRNAs (miR-181a, 183 *, 124 * and 331) was decreased in the retinas of EAU rats on the 14th day after immunization. Among them, the expression of miR-223 and miR-146a was consistent with the increase in IL-1β/McP-1 in the eye with EAU (Watanabe et al., 2016). Furthermore, Sun et al. demonstrated that rno-miR-30b-5P expression was decreased in the spleen, lymph nodes and eye tissues of EAU rats, and rno-miR-30b-5P played a role in the pathogenesis of uveitis by directly regulating the levels of IL-10- and TLR4-positive cells to affect the development of uveitis (Sun et al., 2018). Th17 cells play a key role in the pathogenesis of autoimmune uveitis (Peng et al., 2007). Several studies have revealed that miRNAs influence the pathogenesis of EAU by regulating the Th17 cell response. Significantly increased levels of miR-142-5p and miR-21 were detected in mouse eye tissues 7 days after EAU induction, while significantly decreased levels of miR-182 were detected. The dynamic changes in these miRNAs were similar to those of IL-17. These results suggest that miRNAs regulate EAU development by influencing IL-17 expression (Ishida et al., 2011). In addition, Escobar et al. demonstrated that STAT3 directly binds to the miR-155 locus and forms a STAT3 and miR-155 axis that amplifies pathogenic Th17 cells and exacerbates EAU inflammation (Escobar et al., 2013). In another study, miR-223-3p was confirmed to be significantly upregulated in interphotoreceptor retinoid-binding protein-specific Th17 cells. Mechanistic studies showed that miR-223-3p directly inhibited the expression of FOXO3, which negatively regulated the pathogenic Th17 response partially by inhibiting il-23R expression (Wei et al., 2019). Together, these results suggest that miRNAs play an important role in the pathogenesis of EAU.

## Epigenetic Modifications and Behcet's Disease

BD is a chronic multisystem disease characterized by recurrent inflammation, and its underlying histopathology is occlusive vasculitis (Mendes et al., 2009). It is characterized by ocular lesions, oral ulcer, genital ulcer, and multiple skin lesions (Mishima et al., 1979; Yang et al., 2008; Zeidan et al., 2016). The ocular involvement is found in 83–95% in males and 67–73% in females, and BD is the cause of blindness in about 12% of acquired blindness in adults. (Mishima et al., 1979). The eye involvement mainly manifests with chronic, recurrent bilateral non-granulomatous uveitis with necrotizing retinal vasculitis (Davatchi et al., 2017), which may lead to severe vision loss (Park et al., 2014; Ksiaa et al., 2019). According to the most widely accepted criteria for BD published by the international research group (ISG) in 1990, uveitis is an important diagnostic criterion for (Criteria for diagnosis of Behcet's disease, 1990). Furthermore, BD and VKH disease are the most common non-infectious uveitis entities seen in Asia (Pineton de Chambrun et al., 2012; Hou et al., 2020). Although there is increasing evidence about the pathogenesis of BD, its actual etiology is still unclear. Previous studies have shown that genetic and epigenetic factors play an important role in BD. The incidence and clinical manifestations of BD are related to epigenetic factors such as age, sex and smoking, as well as exogenous factors such as diet, infection and stress (Saadoun and Wechsler, 2012; Demirelli et al., 2015; Alipour et al., 2018; Farhadi et al., 2019).

The first epigenome-wide study in BD was carried out by Hughes T et al. In their study, they analyzed the genome-wide DNA methylation patterns of monocytes and $CD4^+$ T cells and found significantly different methylation sites between untreated BD patients and controls: 383 CpG sites in 228 genes in monocytes (129 hypermethylated and 254 hypomethylated) and 125 CpG sites in 62 genes in $CD4^+$ T cells (67 hypomethylated and 58 hypermethylated). Furthermore, they performed bioinformatics analysis to reveal the abnormal DNA methylation pattern between genes that regulate cytoskeleton dynamics and indicated that the multiclass structure of the cytoskeleton and the abnormal DNA methylation of regulatory proteins may contribute to the pathogenesis of BD (Hughes et al., 2014). In another study, Yüksel et al. evaluated the epigenetic changes of interspersed repetitive sequences (IRSs) in BD using combined bisulfite restriction analysis-interspersed repetitive sequences (COBRA-IRS). They found that the unmethylated (uCuC) Alu allele frequency increased in the peripheral blood mononuclear cells (PBMCs) and neutrophils of inactive BD patients, while the hypomethylation frequency did not differ significantly between active BD patients and controls. Therefore, the pathogenesis of BD may involve changes in IRS element methylation levels (Yuksel et al., 2016). Yu H et al. found 4,332 differentially methylated CpG sites associated with BD in a recent genome-wide DNA methylation profile study. Further validation experiments showed that the most significant locus was located in the 5′-UTR of FKBP5 (cg03546163, $p$ = 3.81E-13) (Yu et al., 2019). In addition, Zhu Y et al. revealed that the hypermethylation of GATA3 and TGF-β may lead to gene transcriptional silencing, which may play a role in the pathogenesis of BD (Zhu et al., 2017a). Kolahi S et al. suggested that hypomethylation of the TLR4 gene may be involved in the pathogenesis of BD by increasing TLR4 expression (Kolahi et al., 2020). Alipour S et al. found that the expression level of the IL-10 gene in BD patients was significantly decreased, while the promoter methylation rate in patients with low IL-10 mRNA expression was significantly higher than that in controls (Alipour et al., 2018). In contrast, the expression of the IL-6 and TNF-α genes was significantly increased in BD patients, and the methylation levels of the IL-6 promoter and TNF-α were significantly decreased (Alipour et al., 2020; Aziz et al., 2020). Combing these data suggests that DNA methylation is involved in the development of BD. Of note, all of the BD patients in the studies carried out by Yuksel et al. (2016), Yu et al. (2019), Zhu et al. (2017a) had uveitis. In the other studies, such as studies performed by Hughes et al. (2014), Kolahi et al. (2020), Alipour et al. (2018), Alipour et al. (2020), Aziz et al. (2020), only part of the BD patients had ocular symptoms which presumably involved uveitis.

MicroRNA expression disorders have been widely studied in BD patients, and some miRNAs are considered biomarkers for disease diagnosis. For example, Kolahi et al. found in the PBMCs of BD patients with uveitis in Iran that the expression of miR-155 was significantly increased compared with that in healthy volunteers, and there was no significant difference in the expression of miR-146a (Kolahi et al., 2018). In addition, Jadideslam et al. confirmed that the expression of miR-21 and miR-146b decreased significantly in BD patients from Iran, while the expression of miR-326 increased significantly. They proposed that the expression rate of miR-326 could be used as a biomarker to predict uveitis and severe ocular involvement in BD patients (Jadideslam et al., 2019). Moreover, Woo et al. observed that the expression of miR-638, miR-4488, and miR-3591-3p in the PBMCs of BD patients, part of them suffered from ocular symptoms, was altered, which is associated with the production of IL-6, a pleiotropic cytokine implicated in the pathogenesis of many immune-mediated disorders including several types of non-infectious uveitis (Woo et al., 2016). Uveitis associated with BD and VKH syndrome is likely also involved (Perez et al., 2004; Mesquida et al., 2014; Lin, 2015).

The inflammatory response, especially the Th17 cell-mediated inflammatory response, is the basis of BD (Chi et al., 2008; Hamzaoui, 2011; Alpsoy, 2016; Leccese and Alpsoy, 2019). Studies have shown that miRNAs are key modulators of the immune response in BD, and the regulation of Th17 cell activity has been the subject of miRNA studies in BD. miR-155 was found to inhibit dendritic cell-driven Th17 responses by targeting TAB2 in BD (Zhou et al., 2012). In addition, the balance of Th1/Th17/Treg cells and their transcription factor-related microRNAs has attracted attention in BD patients. For example, miR-23b levels in the $CD4^+$ T cells of patients with active BD decreased significantly, accompanied by increased Notch pathway activation and an active Th1/Th17 response (Qi et al., 2014). In another report, Ahmadi et al. observed that the proportion of Treg cells decreased significantly and the proportion of Th17 cells increased significantly in BD patients, along with significant upregulation of miR-25, miR-106b, miR-326 and miR-93 and downregulation of miR-146a and miR-155 (Ahmadi et al., 2019). In addition, all of the BD patients suffered from uveitis in the studies carried out by Zhou et al. (2012), Qi et al. (2014) and part of patients had eye involvement in the study performed by Ahmadi et al. (2019).

Given that the coding sequences of microRNAs are affected by genetic variation, similar to any other gene (Cammaerts et al., 2015), the genetic variation in BD has also been studied. Several such variants have been identified in patients with BD, such as rs2910164 (miR-146a), rs11614913 (miR-196a2), rs3746444 (miR-499), and rs76481776 (miR-182) (Qi et al., 2013; Yu et al., 2014; Zhou et al., 2014; Oner et al., 2015; Ibrahim et al., 2019; Kamal et al., 2021). Zhou et al. were the first to identify a strong association between rs2910164 of miR-146a and BD in the Chinese population, and the expression of miR-146a, interleukin (IL-17), tumor necrosis factor (TNF)α and IL-1β was decreased in individuals carrying the CC genotype (Zhou et al., 2014). Oner et al. proved that the homozygous CC genotype and C allele of the rs2910164 polymorphism are protective factors against BD in Turkey. In addition, they found that the miR-499 rs3746444 homozygous (TT) genotype significantly increased the risk of BD in individuals in Turkey (Oner et al., 2015). Additionally, Kamal et al. found that the miR-146a rs2910164 variant plays an important role in the development and clinical regulation of BD in Egyptian patients (Kamal et al., 2021). Moreover, the mutation of miR-196A2/RS11614913 is associated with the risk of BD by reducing the expression of miR-196a and increasing the secretion of the target protein Bach1 as well as the proinflammatory IL-1β and McP-1 cytokines (Qi et al., 2013). In addition, Yu et al. revealed that the frequency of the miR-182/rs76481776 CC genotype and C allele was significantly reduced in BD patients (Yu et al., 2014). MiRNAs and their single nucleotide polymorphisms affect BD-related cells and molecules, deepen our understanding of the pathogenesis of BD, and provide new ideas for the diagnosis and treatment of BD. Furthermore, all of the BD patients had uveitis in the studies carried out by Zhou et al. (2014), Qi et al. (2013), Yu et al. (2014) and part of BD patients had eye involvement in the studies performed by (Oner et al. (2015), Kamal et al. (2021).

## Epigenetic Modifications and Vogt–Koyanagi–Harada Disease

VKH syndrome is an autoimmune disease with multiple system involvement. The clinical changes are characterized by bilateral granulomatous panuveitis accompanied by other system damage, such as vitiligo, hearing loss and nervous system damage (Yang et al., 2007; Yang et al., 2019). However, in the early stage of the disease, patients usually show isolated ocular involvement, the choroid is the main site of ocular inflammation, and the iris and ciliary body may also be involved (Baltmr et al., 2016). At this stage, patients mainly complain of visual impairment, and most patients with bilateral posterior uveitis often manifest with severe retinal detachment, congestion and edema of the optic nerve papilla, posterior choroidal thickening, and an increased retinal choroid layer around the optic nerve papilla (Du et al., 2016). The exact molecular mechanism of VKH disease is still not known and needs further study.

Zhu Y et al. found that the promoter methylation levels of GATA3, IL-4 and TGF-β were remarkably increased in patients with active VKH (Zhu et al., 2017b). In another study, they found that decreased IRF8 mRNA expression in monocyte-derived dendritic cells (DCs) of patients with active VKH was associated with higher methylation levels (Qiu et al., 2017a).

In a recent study, Asakage et al. conducted advanced high-throughput, untargeted and unbiased comprehensive miRNA analysis using serum samples from patients with noninfectious uveitis. The results showed 153 upregulated miRNAs and 35 downregulated miRNAs in VKH patients, and let-7g-3p was determined to be the best predictor miRNA of VKH (Asakage et al., 2020). Other microRNAs have also been reported to be associated with VKH disease. miR-20a-5p was found to be expressed at low levels in $CD4^+$ T cells in patients with active VKH (Chang et al., 2018). Copy number variations in miR-146a, miR-23a, and miR-301a have been revealed to confer risk for VKH syndrome (Hou et al., 2016). Furthermore, miR-182 has

also been confirmed to be involved in the genetic susceptibility of VKH (Yu et al., 2014). VKH is a complex disease whose pathogenesis is not fully understood. Although scant, evidence supports a role for miRNAs in the development of VKH disease.

## Epigenetic Modifications and Anterior Uveitis

Anterior uveitis (AU) is the most common form of uveitis according to the majority of surveys worldwide and presents in up to 90% of cases of uveitis (Chang and Wakefield, 2002; Wakefield and Chang, 2005). AU is the most common presentation among patients with ankylosing spondylitis (AS), occurring in 90% of all uveitis cases in spondylitis (SpA) (Rosenbaum and Chandran, 2012). It is frequently characterized by sudden onset and is often unilateral or unilateral alternating, anterior and recurrent (Zeboulon et al., 2008; Canouï-Poitrine et al., 2012). Anterior uveitis associated with SpA is typically a nongranulomatous type of uveitis characterized by the presence of fine keratic precipitates visible on slit lamp examination of the anterior segment. Intraocular pressure is usually low due to severe inflammation of the ciliary body. In severe forms of acute AU (AAU), hypopyon and fibrin can be visualized as a white and dense clot in the anterior chamber (Agrawal et al., 2010). In addition, approximately 50% of all patients with acute AU (AAU) are human leukocyte antigen B27 (HLA B27) positive (Wakefield et al., 2020). Some authors speculate on the prognosis of HLA-B27-related AAU, reporting a higher frequency of recurrence and a worse outcome than HLA-B27-negative AAU (Rothova et al., 1987; Power et al., 1998). However, in a study based on meta-analysis conducted by D ′Ambrosia and colleagues, they concluded that there is no significant difference between HLA B27 positive and HLA B27 negative AAU with regard to the final visual acuity and structural complications, such as posterior synechiae, cataract, and macular oedema (D'Ambrosio et al., 2017).

Although the exact pathogenesis of AU remains unclear, large-scale genome wide association studies have confirmed that AU is a polygenic disease, with overlaps with the seronegative arthropathies and inflammatory bowel diseases, associations that have been repeatedly confirmed in clinical studies (Wakefield et al., 2020). Some evidence has proven that epigenetic modifications are involved in the regulation of AU development, such as microRNAs. O'Rourke et al. demonstrated increased expression of miR-146a, miR-155 and miR-125a5p in the PBMC of AU patients compared with healthy controls. The expression of miR-155 was increased following TLR1/2 and TLR4 stimulation and the expression of miR-146a was increased in response to IL1β. In a proinflammatory environment, miR-155 overexpression in THP1 cells yielded increased cytokine output whereas miR-146a overexpression showed decreased cytokine output. CD80, PRKCE and VASN were confirmed as novel targets for miR-146a and SMAD2, TYRP1 and FBXO22 for miR-155 (O'Rourke et al., 2019). In another study, Verhagen et al. designed a strategy to robustly identify changes in the miRNA profiles of two independent cohorts totaling 54 untreated patients (with one of three archetypical types of noninfectious uveitis: HLA-B27–associated acute AU, idiopathic intermediate uveitis, or Birdshot uveitis and eye-restricted disease) and 26 age-matched controls. Using stringent selection criteria, they identified and independently validated a miRNA cluster that is associated with noninfectious uveitis. Pathway enrichment analysis for genes targeted by this cluster revealed significant enrichment for the PI3K/Akt, MAPK, FOXO, and VEGF signaling pathways, and photoreceptor development. In addition, unsupervised multidomain analyses linked the presence of the uveitis-associated miRNA cluster to a different composition of leukocyte subsets, demonstrating systemic changes in epigenetic regulation underlying noninfectious uveitis (Verhagen et al., 2018). Furthermore, low gene copy numbers of miR-143, miR-146a, miR-9-3, miR-205 and high gene copy numbers of miR-301a and miR-23a were associated with susceptibility to AAU + AS + patients. A low copy number of miR-146a and a high copy number of miR-23a and miR-205 were associated with AAU + AS- patients (Yang et al., 2017). Based on these observations, it was speculated that these differentially expressed miRNAs might contribute to the pathogenesis of AU, but the potential roles of miRNAs in the AU are still in the early discovery stage and need to be fully explored in the future.

Experimental autoimmune anterior uveitis (EAAU) is an animal model of idiopathic human anterior uveitis in which inflammation is limited to the anterior segment and does not affect retinal tissue and photoreceptor cells (Broekhuyse et al., 1991). The pathogenesis of EAAU is largely unknown, but epigenetic mechanisms provide evidence for a better understanding of the disease. Hsu et al. Observed the dynamic changes of miRNA during EAAU, the down regulation of mir-146a-5p, mir-155-5p, mir-223-3p and mir-147b, and the up regulation of mir-182-5p, mir-183-5p and mir-9-3p. Meanwhile, cytokine analysis showed that IFN- γ, IL-17, IL-12A, IL- 1 β and IL-6 were overexpressed and IL-10 was down regulated. Summarizing these results, they speculated that these differentially expressed miRNAs may promote the dynamic changes of Th1/Th17 related cytokines, so as to promote the pathogenesis of EAAU (Hsu et al., 2015). However, the research of miRNA in EAAU is still in infancy, and future exploration needs to be fully developed.

# PROSPECTS FOR EPIGENETIC THERAPY IN UVEITIS

In recent decades, research on the role of epigenetic regulation in health and disease has increased explosively. Specific epigenetic clinical markers have attracted the attention of scientists, and epigenetic therapy has been proposed. Many epigenetic drugs, such as DNA methylation inhibitors and HDAC inhibitors, have been approved by the U.S. Food and Drug Administration and have been used in clinical treatment or are currently being tested in clinical trials, especially those on cancer treatment and the prevention and treatment of autoimmune diseases (Walton, 2016; Prachayasittikul et al., 2017; Jones et al., 2019; Morel et al., 2020).

**TABLE 2 |** Epigenetic therapies in experiments and animal model of Uveitis.

| Drug | Mechanism of action | Consequence | References |
|---|---|---|---|
| Zebularine | DNMT inhibitor | Downregulation of IL-17 and IFN-$\gamma$ expression repression the infiltration rate of inflammatory cells in intraocular of EAU | Zou et al. (2019) |
| Vorinostat | HDAC inhibitor | Repression the Th1 and Th17 cells as increase the Th0 and Treg cells. inhibition production of transcription factors, including STAT1, STAT3 and p65 in EAU. | Fang et al. (2016) |
| Curcumin | HDAC inhibitor | Suppression of inflammatory cytokines in M1 macrophages of BD patients | Palizgir et al. (2018) |
| miRNA mimic | miRNA replenishment | Targeting miR-155 leads to reduce TNF-α, IL-6, and IL-1β by affecting the Akt/mTOR signaling pathway and autophagy on DC form BD patients | Liang et al. (2021) |
| miRNA mimic | miRNA replenishment | Targeting miR-146a leads to reduce intraocular inflammation and leukocyte infiltration through the inhibition of NF-κB in EAAU. | Hsu et al. (2017) |
| anti-miRNA adenovirus | miRNA depletion | Targeting miR-21-5p leads to affect the balance of Th17 and Treg cells and reduce retinal cell apoptosis in EAU. | Shi et al. (2019) |
| miRNA mimic | miRNA replenishment | Targeting miR-182-5p leads to inhibit the pathogenic Th17 response by negatively regulation of TAF15 in EAU. | Zhang et al. (2020b) |
| miRNA mimic | miRNA replenishment | Targeting rno-miR-30b-5p leads to suppress IL-10 and TLR4 positive cell proportion and ameliorate the development of EAU. | Sun et al. (2018) |

*DNMT DNA, methyltransferase; HDAC, histone deacetylase.*

With regard to autoimmune uveitis, epigenetic therapy has been studied in animal models and *in vitro* trials and is expected to be used in clinical trials in the future (**Table 2**). The animal model of uveitis, EAU, is helpful for studying the pathogenesis of this disease as well as to design and examine treatment strategies.

In our previous study, we evaluated the efficacy of zebularine, a DNA methylation inhibitor with low cellular toxicity and a long half-life (Billam et al., 2010), in the course of EAU. Our results showed that zebularine inhibited the expression of the inflammatory cytokines IFN-$\gamma$ and IL-17 in human and mouse $CD4^+$ T cells *in vitro*. Importantly, zebularine also significantly reduced intraocular inflammation and retinal tissue damage in a mouse EAU model *in vivo*, indicating that zebularine is a new candidate therapeutic agent for uveitis (Zou et al., 2019).

In addition, studies have evaluated the potential therapeutic effects of HDAC inhibitors in EAU. In one study, Fang et al. observed that vorinostat alleviated the clinical and histopathological manifestations of EAU by inhibiting the production of Th1 and Th17 cells and increasing the production of Th0 and Treg cells. In addition, vorinostat treatment significantly reduced IFN-$\gamma$ and IL-17A expression levels but increased IL-10 levels. Mechanistic studies showed that vorinostat therapy greatly inhibited transcription factors, including STAT1, STAT3 and p65. These data suggest that vorinostat may be a potential anti-inflammatory drug for the treatment of uveitis (Fang et al., 2016). In another study, curcumin, a natural phenolic compound with an inhibitory effect on acetyltransferase activity (Radomska-Lesniewska et al., 2019), was used to study macrophages in patients with BD. The results showed that curcumin could inhibit the expression and production of inflammatory cytokines in the M1 macrophages of BD patients, suggesting that curcumin could better regulate inflammatory signals in macrophages from BD patients compared with macrophages from HCs (Palizgir et al., 2018).

In view of the important roles of microRNAs in disease, miRNA-based therapies have been widely used, and miRNA mimics/inhibitors, lentiviral overexpression plasmids and other approaches have also been attempted for the treatment of uveitis (Li and Rana, 2014; Lu et al., 2019; Takahashi et al., 2019). In a recent study, Liang et al. found that the transfection of miR-155 mimics into dendritic cells (DCs) from patients with BD reduced the expression of TNF-α, IL-6, and IL-1β in DCs (Liang et al., 2021). In addition, Hsu et al. found that the intravitreal injection of an appropriate concentration of nucleic acid miR-146a simulant effectively inhibited the intraocular inflammation of experimental autoimmune anterior uveitis (Hsu et al., 2017). Shi et al. showed that the subretinal injection of anti-miR-21-5p adenovirus attenuated EAU by inhibiting the inflammatory response and reducing retinal cell apoptosis. Their results demonstrate that miR-21-5p can be used as a therapeutic target for uveitis and other autoimmune diseases (Shi et al., 2019). Furthermore, Zhang et al. demonstrated that miR-182-5p mimicry inhibited the pathogenic Th17 response in EAU mice by directly inhibiting the transcription promoter TATA-binding protein-related factor 15 (Zhang et al., 2020b). In addition, Sun et al. revealed that rno-miR-30b-5p mimics play a role in the pathogenesis of uveitis by reducing the number of IL-10- and TLR4-positive cells and influencing the development of uveitis (Sun et al., 2018). However, research on miRNAs for the treatment of autoimmune uveitis is in its early stages and requires broader investigations.

## CONCLUSION

Uveitis is a complex multiple system disease, which is characterized by different clinical manifestations involving both extraocular and ocular sites. Infectious causes of uveitis accounts for a minority of cases, while idiopathic/non-infectious/autoimmune uveitis, which accounts for the majority, is sometimes associated with systemic diseases (Rosenbaum

et al., 2016). Although the exact pathogenesis of uveitis is unclear, accumulating evidence shows that the combination of certain genetic or epigenetic factors causes an imbalance in the regulation of the immune response, leading to the development of uveitis. Epigenetics is a rapidly expanding scientific field, and the research on epigenetic regulation of chronic diseases is rising. This work focused on the epigenetic mechanisms that regulate several autoimmune uveitis, aiming to provide new therapeutic ideas for this field. However, the epigenetic mechanisms of uveitis are still in their early stages. Identifying the spectrum of epigenetic changes in all cells contributing to the pathogenesis of uveitis, especially T cells and neutrophils, and translating the correlation of epigenetic changes into cellular and molecular pathways of uveitis is the main direction forward for the field. Epigenetics and its regulators bring new hope for the treatment of autoimmune uveitis, however, the research in this area is still in the stage of animal model, and its clinical application is still limited; thus, more profound research is warranted.

## AUTHOR CONTRIBUTIONS

YZ read the literature related to the topic and participated in drafting the manuscript. JL and WX participated in searching and archiving the literature related to the topic and prepared figures. XK and HD revised the manuscript. YL and LW participated in the design, revision, and final approval of the manuscript. All authors read and approved the final manuscript.

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# DNA Methylation Signature in Monozygotic Twins Discordant for Psoriatic Disease

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**Background:** Psoriatic disease is a multifactorial inflammatory condition spanning from skin and nail psoriasis (Pso) to spine and joint involvement characterizing psoriatic arthritis (PsA). Monozygotic twins provide a model to investigate genetic, early life environmental exposure and stochastic influences to complex diseases, mainly mediated by epigenetics.

**Methods:** We performed a genome-wide DNA methylation study on whole blood of monozygotic twins from 7 pairs discordant for Pso/PsA using the Infinium Methylation EPIC array (Illumina). MeDiP—qPCR was used to confirm specific signals. Data were replicated in an independent cohort of seven patients with Pso/PsA and 3 healthy controls. Transcriptomic profiling was performed by RNAsequence on the same 7 monozygotic twin pairs.

**Results:** We identified 2,564 differentially methylated positions between psoriatic disease and controls, corresponding to 1,703 genes, 59% within gene bodies. There were 19 regions with at least two DMPs within 1 kb of distance and significant within-pair $\Delta\beta$-values ($p < 0.005$), among them SNX25, BRG1 and SMAD3 genes, all involved in TGF-β signaling pathway, were identified. Co-expression analyses on transcriptome data identified IL-6/JAK/STAT3 and TNF-α pathways as important signaling axes involved in the disease, and they also suggested an altered glucose metabolism in patients' immune cells, characteristic of pro-inflammatory T lymphocytes.

**Conclusion:** The study suggests the presence of an epigenetic signature in affected individuals, pointing to genes involved in immunological and inflammatory responses. This result is also supported by transcriptome data, that altogether suggest a higher activation state of the immune system, that could promote the disease status.

**Keywords: DNA methylation, twins, psoriartic arthritis, psoriatic disease, transcriptomic (RNA-seq), epigenetics (DNA methylation)**

## INTRODUCTION

Psoriatic arthritis (PsA) and psoriasis (Pso) represent different manifestations of the same disease spectrum, i.e., psoriatic disease, a systemic chronic inflammatory condition, where genetic factors contribute to disease susceptibility and where biomarkers are virtually absent (Ritchlin et al., 2017). Monozygotic twins (MZ) exhibit a variable degree of concordance for several complex disorders, including autoimmune diseases (Ceribelli and Selmi, 2020), and twin studies contributed to identify susceptibility genes. MZ twins show concordance rates as high as 64% for psoriasis, while in the case of PsA these are almost equal in MZ and dizygotic (DZ) twins although these rates are difficult to compare, based on the bias related to the coexistence of Pso and PsA (Pedersen et al., 2008). Nonetheless, these observations suggest that epigenetic factors might play a major role (Ceribelli and Selmi, 2020), as supported by the experimental evidence in healthy MZ twins (Brodin et al., 2015; Generali et al., 2017) and by the phenotype changes according to age in MZ twins (Fraga et al., 2005). Further, DNA methylation patterns are associated with MZ twins' discordance in the imprinted regions of monogenic syndromes (Ollikainen and Craig, 2011) and in inflammatory diseases with largely incomplete MZ twin concordance, such as systemic lupus erythematosus, type-1 diabetes, rheumatoid arthritis, and systemic sclerosis (Absher et al., 2013; Coit et al., 2013; Liu et al., 2013; Ramos et al., 2019). Limited data from epigenome-wide association studies (EWAS) are available for Pso and PsA and a deregulated epigenetic machinery has been demonstrated in psoriatic skin and whole blood (Zhou et al., 2016).

We hypothesize that epigenetic changes, in specific DNA methylation, may contribute to the onset of Pso/PsA in discordant MZ twins. (Pai et al., 2015; Schübeler, 2015). We performed a peripheral blood EWAS on a cohort of 14 (seven pairs) MZ twins clinically discordant for Pso/PsA and identified DNA methylation changes with specific differentially methylated positions (DMPs) associated with psoriatic disorder. The characterization of a possible circulating psoriatic disease-associated methylome would identify specific candidate *loci* to be further investigated as molecular biomarkers.

## MATERIALS AND METHODS

### Study Participants

We enrolled pairs of MZ twins previously identified through the collaboration with the Italian Twin Registry—Istituto Superiore di Sanità and residing in the area surrounding the Humanitas Research Hospital in Rozzano (Milan, Italy). Participants were evaluated jointly by a rheumatologist and a dermatologist to define the diagnosis of Pso according to the physical examination and/or PsA according to the CASPAR classification criteria (Taylor et al., 2006). The study was approved by the local IRB and, following the signing of an informed consent, all participants provided peripheral whole blood samples at the time of the joint rheumatological and dermatological evaluations. Whole blood and peripheral blood mononuclear cells (PBMCs) were obtained from both twins at the same time using heparinised vacutainers and processed in parallel. In total, 7 MZ twin pairs discordant for Pso or PsA (the presence of either being defined psoriatic disease) were included. Epigenetic data were validated in an independent cohort of seven patients with Pso/PsA and 3 healthy controls. Disease subtype, current and/or past drug history of the affected twins, comorbidities, family history, smoking status and smoking behaviour, diet, alcohol consumption and allergies of both twins were recorded.

### DNA Extraction and Methylation Analysis

Genomic DNA was extracted from whole blood using an automated DNA extractor (Chemagic Star workstation; Hamilton, ON, Canada), following manufacturer's instructions. For each sample, 500 ng of genomic DNA was bisulfite converted using the EZ DNA Methylation Kit (ZymoResearch, Irvine, CA, United States). Bisulfite-treated samples were then processed using the EPIC array (Illumina, San Diego, CA, United States), according to manufacturer's instructions. The iScan system (Illumina) was used to scan the arrays and obtain the raw intensity data files. DNA methylation data were processed using the R package minfi (Aryee et al., 2014), to obtain DMPs. First, a quality control was performed to evaluate sample-specific methylation parameters, exploiting the presence of control probes on the EPIC array. Then, the mean detection *p*-value was evaluated across all samples, and those probes with a *p*-value > 0.01 in one or more samples were discarded from the dataset. We also removed those probes where SNPs may affect the CpG, and underperforming probes, as suggested by Zhou and others (Zhou et al., 2017). Finally, we also filtered out probes localized on the X and Y chromosome, to avoid sex-specific effects. After this QC step, the final dataset included 762,451 probes, which accounts for 88% of the EPIC probes. Finally, data were normalized using the minfi quantile normalization algorithm. DMPs were calculated using the R-package limma (Ritchie et al., 2015), the function lmFit, and eBayes, based on a paired statistics, and correcting for batch effects with the sva package (Leek et al., 2020).

### Regulatory Annotations

The eFORGE v2.0 (https://eforge.altiusinstitute.org/) (Breeze et al., 2016) tool was used to identify if the associated CpGs (top 1,000) were enriched in cell-specific regulatory elements, such as DNase I hypersensitive sites (DHSs) (active regulatory regions) and loci with overlapping histone modifications (H3K4me1, H3K4me3, H3K9me3, H3K27me3, and H3K36me3) across available cell lines and tissues from the Roadmap Epigenomics Project, BLUEPRINT Epigenome, and ENCODE (Encyclopedia of DNA Elements) consortia data. Chromatin Hidden Markov Models (ChromHMMs) bioinformatic approach was adopted to annotate Pso/PsA-associated DMPs through the combination of epigenetic maps across multiple cell types (Chen et al., 2013).

### Gene Ontology Analysis

Enrichment analysis for the host genes of the differentially methylated positions was performed using the "topGO" Bioconductor tool, the "biological processes," the "molecular functions" database, and the *elim* algorithm (Alexa et al., 2006). Gene ontology enrichment was performed on those genes containing at least two DMPs.

## MeDIP—qPCR

MeDIP was performed to detect immunoprecipitated methylated DNA with an anti-5′-methyl-cytosine antibody. The MagMeDIP qPCR kit (Diagenode, Belgium) was used following manufacturer's instructions. Briefly, genomic DNA was sheared using a BioRuptor sonicator (Diagenode, Belgium) to produce 400 bp fragments, which were checked by gel electrophoresis. The obtained fragments were immunocaptured with a monoclonal antibody specific for 5-methyl cytosine (supplied by the kit). Methylated DNA was then washed and purified from beads with a DNA Isolation Buffer (DIB) and proteinase K provided by the kit. Following an incubation at 55°C and at 100°C (for 15 min each), the supernatant containing the DNA was used for qPCR analysis to evaluate enrichment.

## RNA Sequencing

Whole blood was collected in a PAXgene Blood RNA Tube (PreAnalytiX, Switzerland) and RNA extraction performed using the Maxwell simplyRNA Blood Kit (Promega, United States), following the manufacturer's instructions. RNA quality was assessed by LabChip GX Touch (PerkinElmer, Waltham, MA). Libraries were prepared starting from 500 ng of total RNA, using the TruSeq Stranded mRNA Library Prep Kit (Illumina) and following the manufacturer's instructions. Samples underwent a paired-end 75 bp sequencing using a NextSeq 500 platform (Illumina).

## Data Analysis

Sequencing reads were mapped to the human genome (hg19) using STAR (version 2.5.2). Transcript quantification from mapped reads was performed using HTSeq-count (version 0.6.1p1) and the human transcripts annotations from Ensembl database (GRCh37 version). Differential expression analysis was carried out using R and the DESeq2 package, considering an FDR<0.1 as threshold.

The enrichment analysis was performed using the Enrichr online tool (Chen et al., 2013), the MSigDB Hallmark pathway, and the PheWeb databases. The co-expression of genes was evaluated starting from transcriptome data and the Coseq R package (Rau and Maugis-Rabusseau, 2018). We applied a centered log ratio (CLR) transformation to count data before fitting a Gaussian Mixture Model, in order to identify gene clusters. For the enrichment analysis we selected only those genes that were attributed to a cluster with a conditional probability>0.9. For each cluster, the average expression profile of all the genes belonging to the cluster was compared between affected and non-affected MZ twins by the Wilcoxon rank sum test.

The estimation of the cellular composition of the samples was performed using the immunedeconv R package, and the quantiseq method (Sturm et al., 2020).

## Quantitative Real-Time Polymerase Chain Reaction

Quantitative RT-PCR was performed following the protocol previously described (Vecellio et al., 2016). RT-PCR was performed in triplicate and the 2−ΔCt method was used to calculate the expression of SMAD3, BRG1 and SNX25 relative to $\beta$-Actin used as housekeeping gene (ID Assay qHsaCED0036269, Bio-Rad Laboratories, Kidlington, United Kingdom).

Forward and reverse (for and rev) primers are listed below:

SMAD3 for 5′- CATCGAGCCCCAGAGCAATA_-3'; SMAD3 rev: 5′- GTGGTTCATCTGGTGGTCACT_3'; BRG1 for: 5′- AGTGCTGCTGTTCTGCCAAAT - 3'; BRG1 Rev: 5′- GGCTCGTTGAAGGTTTTCAG -3'.

Primers for SNX25 (for 5′- CCGTTGTTCTCGTGCGTT AA_3′) and (rev: 5′- CCCACCTCGTTTACCACTCG-_3′) were derived from Kato L, et al. Nonimmunoglobulin target loci of activation-induced cytidine deaminase (AID) share unique features with immunoglobulin genes, *Proceedings of the National Academy of Sciences*, 2012, 201120791; DOI: 10.1073/pnas.1120791109.

## Statistical Analysis

We performed one sample *t*-test to determine statistically significance in RT-PCR and MeDIP followed by qPCR experiments. Significance values were set to $p < 0.05$. In **Figure 6** and related text, data are represented as mean ± SEM.

## Patient and Public Involvement

It was not appropriate or possible to involve patients or the public in the design, or conduct, or reporting, or dissemination plans of our research.

# RESULTS

## Pso/PsA-Associated Whole Blood Methylome Study

The genome-wide DNA methylation profile was investigated using the Illumina Inflnium MethylationEPIC BeadChips (EPIC arrays). The general demographic and clinical characteristics of the MZ twins for which methylation data were available are illustrated in **Table 1**. After performing the quality check and the exclusion of low-quality and unreliable probes, sites that overlap with SNPs, probes with a very low signal and those located on the X and Y chromosomes, methylation data for 762,451 sites were obtained. The experimental workflow and the analyses performed are shown in **Figure 1**.

## Identification of DMPs Between Psoriatic Disease Affected and Non-affected Twins

The principal component analysis (PCA) highlighted the high similarity between MZ twins (PC1 vs PC2, **Figure 2A**). However, when higher components were considered, a clear cluster separation between affected and non-affected twins emerged, suggesting a different methylation pattern related to the disease status (PC7 vs PC8, **Figure 2B**). The statistical analysis did not demonstrate genome-wide significant probes, but when a more stringent filter was applied to the analysis ($p < 0.005$), 2,564 DMPs were observed (**Figure 2C**), mapping to 1,703 genes (**Figure 2D**), in the majority of cases at the level of gene

**TABLE 1 |** Main demographic and clinical features of the 7 pairs of MZ twins discordant for Pso or PsA.

| | Pso/PsA affected MZ co-twins (*n* = 7) | Non-affected MZ co-twins (*n* = 7) |
|---|---|---|
| Median age, years (range) | 44 (25–68) | 44 (25–68) |
| Female/male | 1/6 | 1/6 |
| Pso only | 4 | — |
| PsA only | 2 | — |
| Pso + PsA | 1 | — |
| Family history for psoriatic disease | 4 | 4 |
| Current smoker | 2 | 2 |
| Former smoker | 1 | 2 |
| Years of co-living (average) | 25 | 25 |

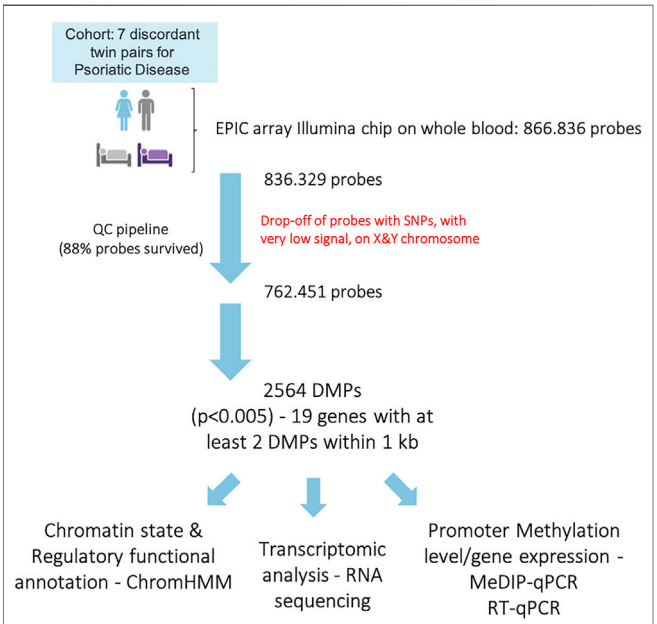


**FIGURE 1 |** Schematic workflow of the study and analysis conducted.

bodies (56%), less frequently in intergenic (27%) and promoter regions (16%), in line with the probe distribution in the array, where 51% of probes map to gene bodies, 29% to intergenic and 20% to promoter regions.

When evaluating whether the Pso/PsA-associated DMPs reside in genomic regulatory regions involved in transcriptional regulation (such as gene promoters, enhancers, transcriptional start sites, specific histone modifications) in different cell types and tissues we observed that the Pso/PsA-associated CpGs were enriched in H3K36Me3 specific histone modifications, especially in blood cells, such as monocytes, lymphocytes, NK cells and hematopoietic stem cells (FDR<0.01, **Supplementary Figure S1A**). To predict disease-relevant cell types, we investigated the ChromHMM state, which was suggestive of either strong or weak gene transcription overlapping associated DMPs in blood cells (**Figure 3**) and epithelial fibroblasts (**Supplementary Figure S1B**) among others (FDR<0.05, $p < 10^{-10}$).

We found 19 regions with at least two DMPs within 1 kb, significant within-pair $\Delta\beta$-values and a suggestive $p < 5 \times 10^{-3}$, as shown in **Figure 2C**. The 19 regions associated with the identified DMPs are listed in the **Supplementary Table S1** and include SF3B1, MAEA, STK32A, CUX1, NUBPL, CDC42BPB, SMAD3, SPNS3, SMARCA4, ARHGEF3, SNX25, EYA4/TARID, CAMK2B, NGDN, SECISBP2L, IGFALS, and MRPS23 genes and one non-coding RNA, SNHG23 as well as a region localized on chromosome 2, where no nearby genes are annotated. Seven of the 19 regions are characterized by a concordant $\beta$-value variation in all the probes that are distributed in the region (either increased or decreased methylation levels in patients compared to controls) as shown by the scatterplots in **Figures 4A–G**.

## Psoriatic Disease MZ Twins Have a Distinct Transcriptomic Profile

The RNA sequencing analysis, performed on the same cohort of MZ twins didn't highlight differentially expressed genes surviving the multiple testing correction. Nonetheless, we performed an enrichment analysis selecting those genes characterized by a significant unadjusted $p$-value ($N = 662$). Very interestingly, when considering deregulated genes, we observed an enrichment in the pathways related to "oxidative phosphorylation," "inflammatory response," and "MYC targets" (**Supplementary Figure S2**, **Supplementary Table S2**).

As a next step, we searched for gene clusters that were co-expressed with a different profile between discordant MZ twins. The analysis identified 20 clusters (**Figure 5A**), the majority showing a statistically significant difference in the average expression profiles between the two groups. We ranked the clusters based on the average expression ratio between affected patients and controls, and we selected the most dysregulated three (clusters 14, 19, 13) to perform an enrichment analysis considering the genes included in each cluster. Interestingly, cluster 19 genes, characterized by a higher average expression profile in affected MZ twins (average expression profile ratio: 1.14, Wilcoxon $p < 2.2e-16$), were associated to "Psoriasis vulgaris" and "Psoriasis" in the PheWeb database (**Figure 5B**). Moreover, the same set of genes showed a trend for enrichment in the "IL-6/JAK/STAT3" pathway (**Figure 5C**), a signaling axis that has been implicated in psoriasis (Andrés et al., 2013). Cluster

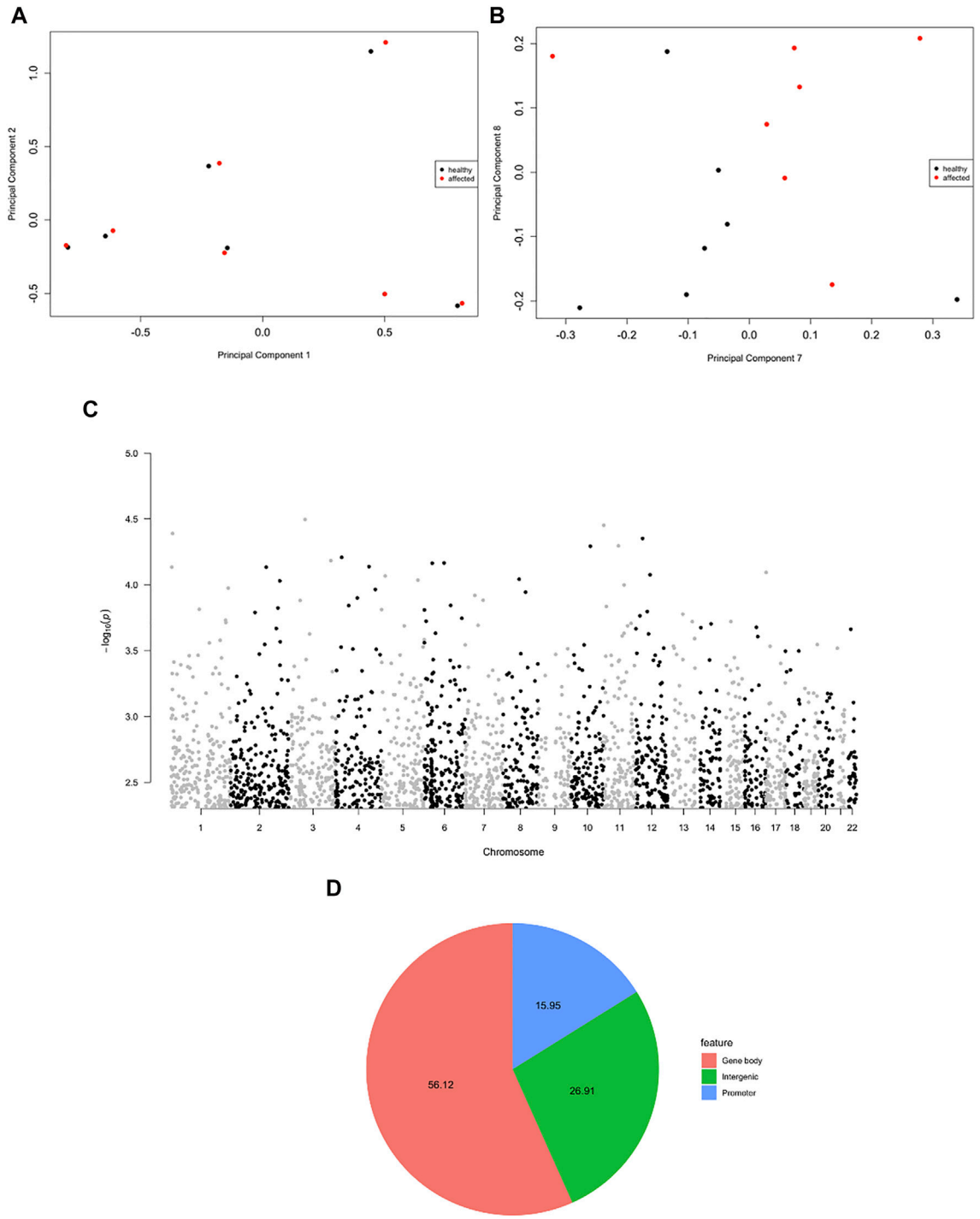


**FIGURE 2 |** Evidence for psoriatic disease-associated differential DNA methylation in whole blood MZ twins. **(A,B)** A multi-dimensional scale (MDS) plot of all samples is shown, considering the first two components **(A)** and the dimensions seven and eight **(B)**. **(C)** Manhattan plot showing the distribution of the significant DMPs ($p < 0.005$) in the genome. **(D)** Pie chart showing the genomic localization of the DMPs.

13, also showing a higher expression in patients compared to controls (average expression profile ratio: 1.1, Wilcoxon $p <$ 2.2e-16), confirmed the enrichment in genes involved in the "IL-6/JAK/STAT3" pathway, as well as in "inflammatory response" and "TNF-α signaling pathway" (**Figure 5D**). Cluster 14, characterized by a higher expression in controls (average expression profile ratio: 0.86, Wilcoxon $p <$ 2.2e-16), was instead mainly enriched in "oxidative phosphorylation"

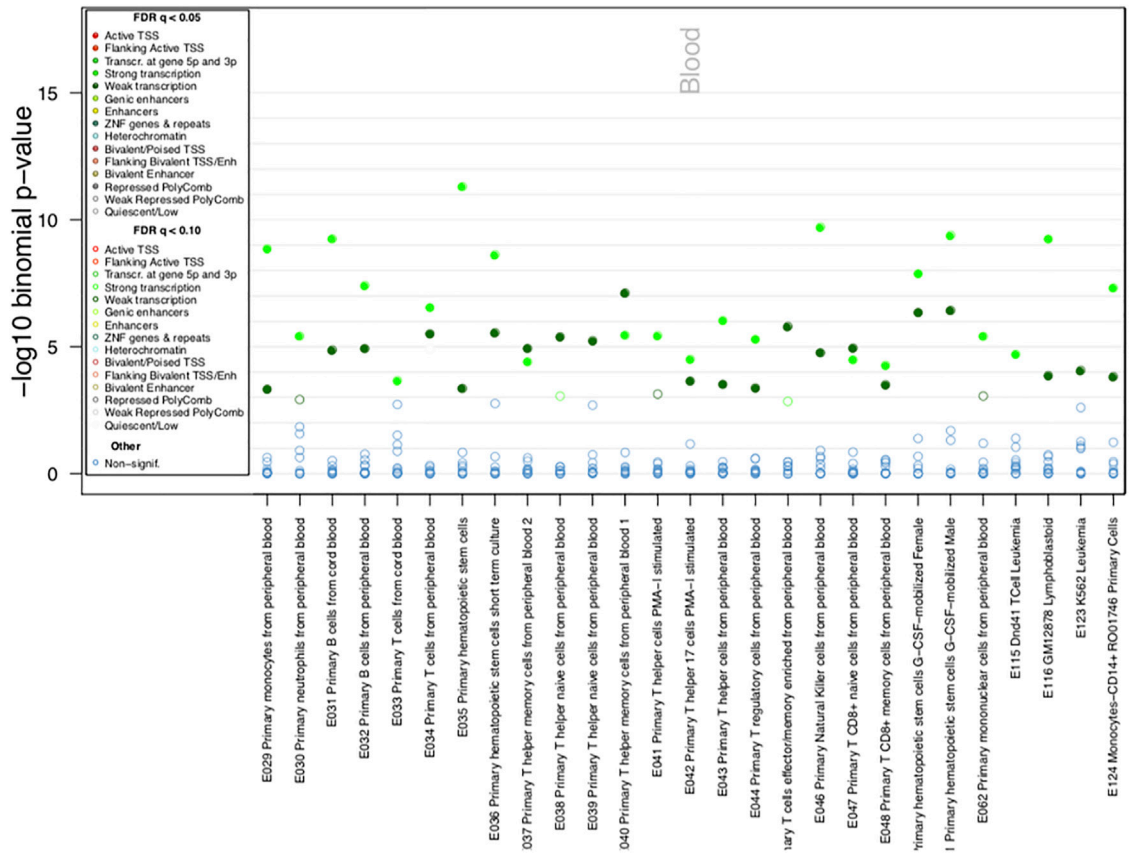


**FIGURE 3 |** ChromHMM Functional annotation of identified DMPs. Chromatin state enrichment in blood cells as calculated by eFORGE software.

pathway (**Figure 5E**). Finally, we evaluated through a deconvolution method, the cellular composition of the RNAseq samples, but no significant differences were observed between affected and non-affected twins (data not shown).

## Evidence for SNX25 Deregulation in Affected MZ Twins

Among the differentially methylated regions, only SNX25 resulted also nominally deregulated in MZ twins' transcriptome data. This gene is involved in the TGF-β pathway and potentially relevant in Pso and PsA pathophysiology. The observed downregulation (logFC = -0.48, $p = 0.02$) is concordant with the methylation profile, with affected MZ twins showing higher methylation levels (**Figure 4B**). To confirm this result, a quantitative PCR specific for SNX25 promoter was carried out after immunoprecipitation of the methylated DNA in PBMCs of MZ twins. The analysis showed the same trend, although not significant, observed in the methylation array (**Figure 6A**). No significant differences were observed in the PBMCs' SNX25 expression of affected and healthy twins (**Figure 6B**). However, when SNX25 expression was tested in PBMCs of an independent cohort of 7 patients with Pso/PsA and 3 healthy controls the same trend observed in RNAseq data was present (**Figure 6C**).

## SMARCA4/BRG1 and SMAD3 Show Methylation Differences with Discordant β-Value Variation

A Gene Ontology (GO) analysis of DMPs associated to the 19 regions showed an enrichment in transcription factor binding, transcription corepressor and transcription coactivator activity, SMAD binding and histone -lysine-N-methyltransferase activity ($p < 0.005$) (**Figure 7A**).

Among the 19 genes identified with the Infinium Methylation EPIC array, we found significant interactions for SMARCA4/BRG1 (transcription activator BRG1, brahma-related gene-1) and SMAD3 (mothers against decapentaplegic homolog 3), by performing protein-protein interaction analysis (String v11.2 database; www.string-db.org) (**Figure 7B**). The BRG1/SMAD3 interaction was indeed already demonstrated in the literature: immunoprecipitation from cells overexpressing FLAG epitope-tagged BRG1 and HA-tagged SMAD proteins showed that BRG1 is a direct interactor of SMAD3 (Xi et al., 2008). Based on this observation, we further analyzed the methylation status of these two genes. Methylation scatterplots showed a significantly different methylation level of the probes ($p < 0.005$) localized in the SMAD3 and SMARCA4 genes, in affected and healthy

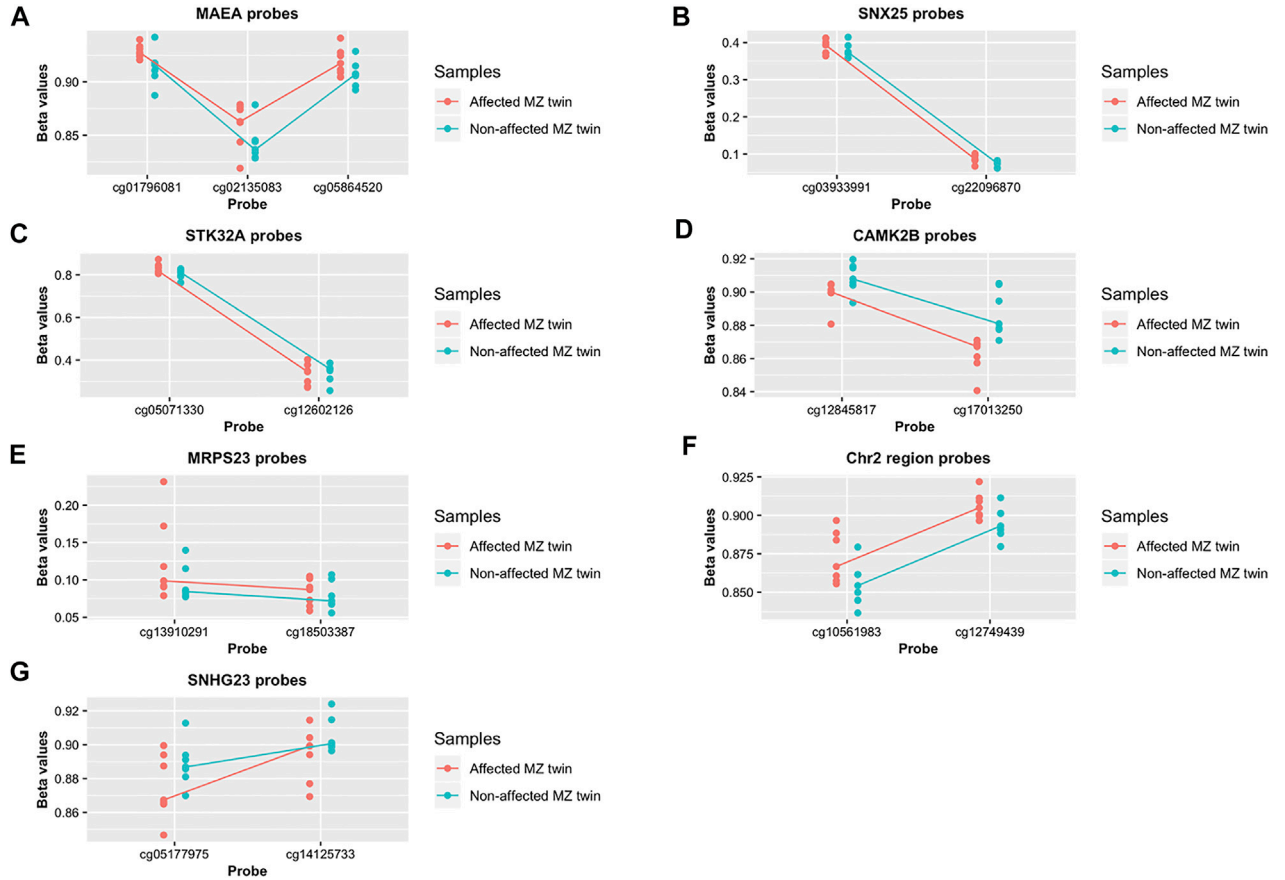


**FIGURE 4 |** Differential methylation pattern identified in whole blood psoriatic disease MZ twins **(A–G)**. Methylation level of the DMPs localized in the 7 regions characterized by concordant $\beta$-value variation. Data are presented as a scatterplot, including the individual data points (for the 7 monozygotic twin pairs) that represent the beta values of the significant DMPs. The lines connect the median methylation value of each CpG site, in affected and healthy twins separately (represented in pink and blue, respectively).

twins separately, represented in pink and blue, respectively (**Supplementary Figures S3A,B**) although with discordant $\beta$-value variation in the CG probes. We interrogated the promoter of SMARCA4/BRG1 and SMAD3 to evaluate any change in the level of methylation in whole PBMCs from affected and healthy twins. Immunoprecipitation of 5′-methylcytosine antibody followed by quantitative PCR was performed with specific primers for SMARCA4/BRG1 and SMAD3, but we could not identify significant differences between affected and healthy twins ($n$ = 4 twin couples, **Supplementary Figure S3C**). Further, no changes were found in SMARCA4/BRG1 and SMAD3 mRNA expression in PBMCs (**Supplementary Figure S3D**). We also evaluated the mRNA expression of SMAD3 and SMARCA4/BRG1 in the selected control cohort of 7 patients with Pso/PsA and 3 healthy controls, confirming no significant changes (**Supplementary Figures S3E,F**).

## DISCUSSION

Epigenetic changes have widely been proposed to be crucial in the pathogenesis of complex diseases (Petronis, 2010) and, more specifically, DNA methylation is linked to different immune-mediated disorders, from rheumatoid arthritis to multiple sclerosis (Liu et al., 2013; Dick et al., 2014). While epigenetic changes are ideal mechanisms to determine disease discordance in MZ twins, translating EWAS data into a quantifiable epigenetic difference and mechanistic understanding for complex diseases like Pso and PsA (herein cumulatively identified as psoriatic disease) is challenging, particularly in the absence of disease biomarkers. We performed the first genome-wide integrative approach to identify differential DNA methylation in whole blood from a selected cohort of MZ twins discordant for psoriatic disease, similar to what has been performed in other complex diseases (Javierre et al., 2010; Rakyan et al., 2011).

Our EWAS approach identified distinct DNA methylation patterns at specific DMPs differentiating affected and healthy twins, mapping to the gene body of 1,703 genes while we did not observe genome-wide significant differences [$p < 3.6 \times 10^{-8}$ (Saffari et al., 2018)]. The ENCODE and Blueprint regulatory annotations showed an enrichment for H3K36me3 histone mark overlapping the significant DMPs. DNA promoters methylation is largely known for its repressive effect on transcription initiation of protein-coding genes, non-coding RNAs, or transposon

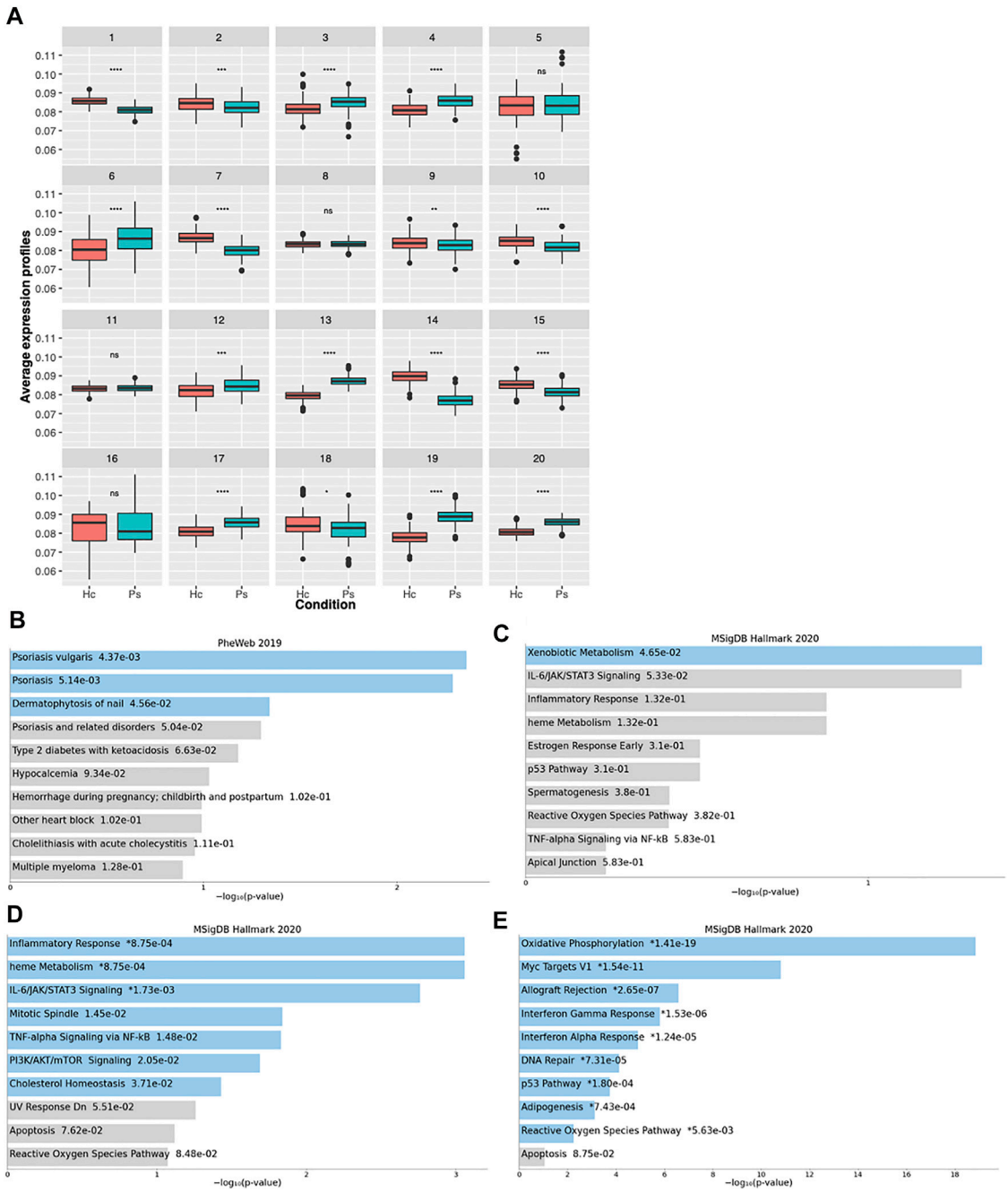


**FIGURE 5 |** Transcriptional landscape of psoriatic disease-affected MZ twins. **(A)** Boxplots indicating the clusters identified by Coseq2 software, characterized by co-expressed genes. For each cluster, the average expression profile of all the genes belonging to the cluster is shown in affected and non-affected MZ twins. The *p*-values, as calculated by the Wilcoxon rank sum test are displayed at the top of each boxplot. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ns not significant. Ps: Pso/PsA MZ twin, Hc: unaffected twin. **(B–E)** The bar charts show the top 10 enriched terms in the chosen library (MSig DB Hallmark or PheWas), along with their corresponding *p*-values, for clusters 19 **(B,C)**, 13 **(D)**, 14 **(E)**. Colored bars correspond to terms with significant *p*-values (<0.05). An asterisk (*) next to a *p*-value indicates the term also has a significant adjusted *p*-value (<0.05).

repeats (Teissandier and Bourc'his, 2017), while the function of intragenic methylation remains unclear (Baubec et al., 2015) and it may be involved in the modulation of alternative splicing, in exon definition and recognition (Maunakea et al., 2013). The commonly accepted dogma where methylation negatively correlates with gene expression as and vice versa, not always hold as, in different cancers, within promoter regions it has been observed a positive correlation between methylation and gene expression (Chatterjee and Vinson, 2012; Kim et al., 2016; Spainhour et al., 2019). H3K36me3 is required for preventing

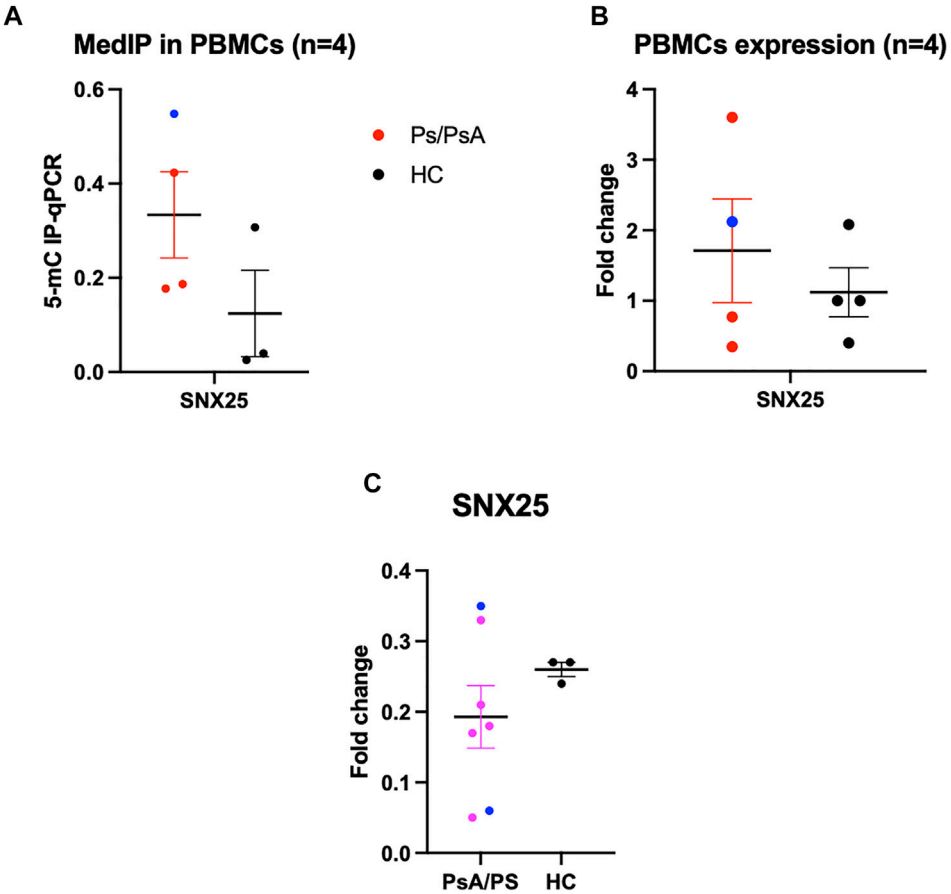


**FIGURE 6 |** Functional validation of SNX25 gene. **(A)** MeDIp of SNX25 promoter in PsA Twins. **(B)** mRNA expression evaluated with qPCR in PsA MZ Twins. **(C)** SNX25 expression evaluated in a selected cohort of Pso/PsA patients ($n$ = 7) vs healthy volunteers ($n$ = 3). Blue dots show PsA patients, red dots Pso patients.

spurious transcription initiation stemming from transposon fragments or cryptic promoters, preserving transcription process to canonical promoters (Verma et al., 2018). It has been recently demonstrated that H3K36me3 may act as a docking site for DNMT3B (DNA Methyltransferase 3 Beta), and together with reduced H3K4me3, altogether they repress the default activity of intragenic CpG islands and act as transcriptional promoters (Baubec et al., 2015). Further, the enrichment of methylated cytosines in regulatory regions that we identified in blood cell subsets suggests a dysregulation in psoriatic disease, which might be cell-type specific.

The enrichment analysis performed on transcriptome data suggested a significant role of "oxidative phosphorylation," "inflammatory response" and "myc targets" pathways in disease susceptibility. Indeed, these pathways have been already associated to autoimmune disorders, and also to Pso/PsA. Regarding the "oxidative phosphorylation" pathway, it was observed that there is a direct link between dysregulated glucose metabolism in lymphocytes and autoimmunity (Bantug et al., 2018). In fact, enhanced glycolytic activity is a common feature of pro-inflammatory effector CD4 lymphocytes such as T helper (Th) 1 and Th17, cells characterized by a Warburg-like glycolytic metabolism, since they require aerobic glycolysis for their differentiation and effector functions. On the other hand, Regulatory T (Treg) cells, which suppress inflammatory responses and promote tolerance, after the initial activation, are characterized by an enhanced oxidative metabolism (Kornberg, 2020). The co-expression analysis again evidenced the dysregulation of this pathway, since we found an enrichment of the "oxidative respiration" pathway in cluster 14, characterized by a higher gene expression in non-affected MZ twins. It is worth mentioning that one of the top dysregulated genes in the transcriptome data is PDK4, coding for Pyruvate Dehydrogenase Kinase 4, a protein implicated in glucose metabolism. The gene was upregulated in affected MZ twins (logFC = 0.64, unadjusted $p = 1.1*10^{-4}$). A recent study evaluated the effect of PDK4-deficiency on the development of experimental autoimmune encephalomyelitis, the mouse model of multiple sclerosis, another autoimmune disorder, induced by pathogenic Th17 cells. Very interestingly, knock-out mice developed a less severe disease and showed a decrease in Th17 cells and an increased infiltration of Foxp3+ Tregs in the central nervous system (Allon Wagner et al., 2020). All together these results suggest that in affected MZ twins there might be a higher

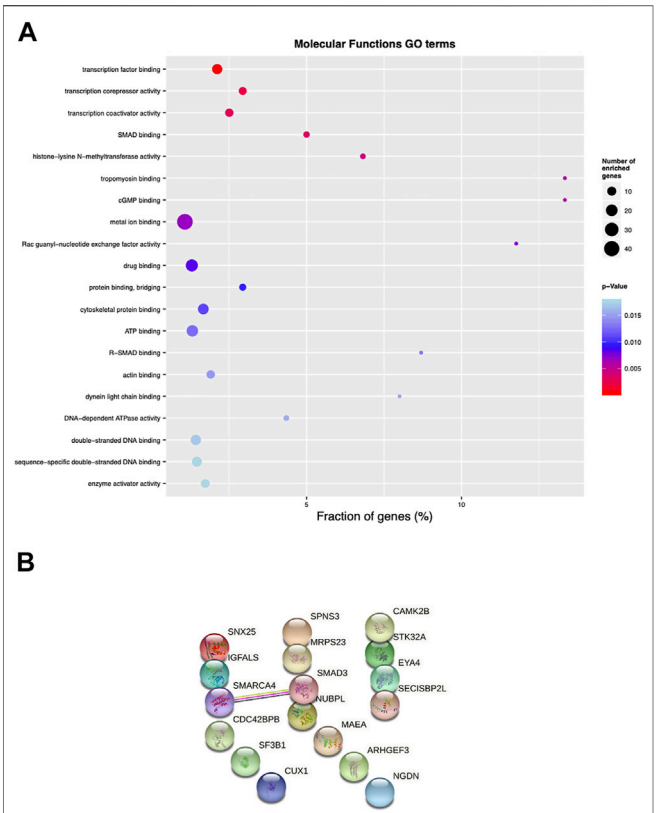


**FIGURE 7 |** Pathway enrichment and protein-protein interaction analysis of psoriatic disease MZ twins. **(A)** Top 25 differentially enriched GO terms identified by the "topGO" Bioconductor tool, using the "molecular functions" database, ordered by elimFisher *p* value. The width of the dots indicates the percentage of the enriched genes out of the total number of genes belonging to each term. **(B)** protein-protein interaction analysis evaluated with stringdb (https://string-db.org).

activation state of pro-inflammatory, and hence disease-supporting, Th lymphocytes.

Also MYC is one of the key players that coordinate metabolic reprogramming and activity in immune cells (Gnanaprakasam and Wang, 2017), which play a pivotal role in the development of inflammation and autoimmunity.

The finding that seven genomic regions are characterized by a concordant $\beta$-value variation in all the probes distributed, either with increased or decreased methylation level between affected and healthy twins, is of primary importance in order to define a specific methylation signature associated with psoriatic disease. Among these seven genes, SNX25, a negative regulator of TGFβ pathway and intracellular trafficking, was previously found associated among the differentially methylated sites between psoriatic and normal skin (Verma et al., 2018). Interestingly, transcriptome data pointed to a deregulation of this gene in affected MZ twins. Further analysis performed to replicate the results showed a trend, although not significant, both in methylation status and in expression level of the gene. Additional analyses are needed on a larger cohort of patients to confirm these results.

A small number of the 19 genes with at least two DMPs within 1 kb of distance and significant within-pair Δβ-values was found significantly associated with psoriatic disease. Among those, SMAD3, which is involved in the downstream signaling pathway of TGF-β, is associated with BRG1 to mediate TGF-$\beta$-induced transcriptional regulation at multiple genes, by means of chromatin remodeling and gene expression regulation. Although the methylation level of the CpG probes located in SMAD3 and SMARCA4/BRG1 was significant, this did not follow a consistent trend. Further, despite no significant changes in RNA expression were observed, a possible contribution of the differential methylation pattern in the definition of the alternative splicing profile of the gene cannot be ruled out.

A few studies evaluated the role of DNA methylation in the disease susceptibility specifically in psoriasis (Gervin et al., 2012; Li et al., 2020) at genome-wide level in immune cells. The most comprehensive one (Gervin et al., 2012), focused on lymphocytes, didn't disclose any differentially methylated regions between co-twins; however, when gene expression was also considered, different genes were identified. Among the 50 top hits, 7 of them (14%) were found differentially methylated in our dataset. Moreover, GO analysis revealed enrichment in processes associated with "immune response" and "psoriasis." This is in accordance with our results, since in the co-expression analysis we highlighted an enrichment in genes associated with "inflammatory response," "IL6/STAT/JAK signaling," as well as involved in psoriasis. The role of inflammatory response was also evidenced by another genome-wide transcriptome analysis (Rawat et al., 2020) where a consistent, though small, pattern of changes was observed for a set of genes associated with "neutrophil activation" and "inflammation." Even though all the data point to the involvement of the same pathways in disease susceptibility, the lack of a high degree of overlap in the identified genes can be due to the use of different starting samples.

We are aware of the strengths and limitations of our study. Among the former, the chosen model of MZ twins is a unique setting to investigate complex diseases, as hereditary factors are not the major determinants of immunological functions, as proven in healthy MZ twins (Brodin et al., 2015). Further, using twins can improve the statistical power of a genetic study as it reduces the amount of genetic and/or environmental variability. Conversely, the results obtained from twin studies cannot be universally correlated to the general population, due to lack of randomization (Sahu and Prasuna, 2016). We are aware that our twin's cohort is heterogenous (see **Table 1** for patients' characteristics), as it includes patients affected by psoriasis, psoriatic arthritis and both manifestations simultaneously. Further, the multitier approach with a small independent cohort of unrelated patients and controls also represents an advantage for our study. Among the study limitations, DNA methylation was measured in peripheral blood cells while epigenetic changes associated with psoriatic disease may be present in specific tissues such as the skin, but the collection of these samples through biopsies is invasive, and the collection of synovial fluid may not be feasible in most of PsA cases when synovitis is not the predominant domain. For this reason, peripheral blood is the best accessible alternative that reflects

different pathophysiological pathways and there is high potential clinical utility for any identified blood-derived epigenetic disease marker. Nevertheless, we are aware that the results we obtained in whole blood, could represent a limitation, as the observed changes cannot be associated with a specific cell type (i.e., T-cells, monocytes).

In conclusion, our genome-wide study on the unique model of MZ twins demonstrates high similarity in whole blood-based methylomes of psoriatic disease-discordant samples. However, we identified DMPs specific for psoriatic disease and few candidate loci that warrant to be further evaluated. The evaluation of the causal relation between genetic variants, biomarker levels and DNA methylation is becoming of undisputed importance. Their interplay is relevant to define plausible biological pathways in psoriatic disease that are regulated by epigenetic mechanisms.

## 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: GSE186713 for methylation data, GSE186724 for RNA-seq data.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Study-specific ethical approval was provided by the local ethics committee. All study participants provided written informed consent. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

All authors contributed to manuscript preparation. AC, EP, NI, FM, MV, MD, and CS actively participated to rheumatological evaluations and experiments, and supervised the whole project from the initial planning to the present manuscript; EP, GC, MR, RA, and SD performed and supervised the epigenetics and transcriptomics experimental parts; MS and SB enrolled twin couples involved in the study; FS and AC evaluated the enrolled patients from the dermatological point of view; MV, EP, AC, SD, and CS drafted the manuscript, and all the authors revised the final version prior submission.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.778677/full#supplementary-material

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# Identification of lncRNA and mRNA Expression Profile in Relapsed Graves' Disease

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**Background:** Graves' disease (GD) is a common autoimmune disease, and its pathogenesis is unclear. Studies have found that the occurrence of GD is related to the immune disorder caused by the interaction of genetic susceptibility and environmental factors. The $CD4^+$ T cell subset is closely related to the immune disorder of GD. LncRNAs are RNA molecules with a length of more than 200 nt and are involved in a variety of autoimmune diseases. However, the roles of lncRNAs in recurrent GD are still elusive. The purpose of this study is to identify lncRNA and mRNA expression profile in relapsed Graves' disease.

**Method:** $CD4^+$ T cells from 12 recurrent GD and 8 healthy controls were collected for high-throughput sequencing. The gene-weighted co-expression network analysis (WGCNA) was used to construct the co-expression module relevant to recurrent GD, and the key genes in the module were verified by RT-PCR.

**Results:** There are 602 upregulated lncRNAs and 734 downregulated lncRNAs in $CD4^+$ T cells in recurrent GD patients compared with the healthy controls. The module most relevant to GD recurrence was constructed using WGCNA, and the key genes in the module were verified by RT-PCR. We found that the expression of RPL8, OAS2, NFAT5, DROSHA, NONHSAT093153.2, NONHSAT118924.2, and NONHSAT209004.1 was significantly decreased in GD group ($p < 0.001$, $p < 0.001$, $p < 0.01$, $p < 0.05$, $p < 0.001$, $p < 0.05$, and $p < 0.01$, respectively).

**Conclusion:** LncRNAs are closely related to the recurrence of GD. For the first time, we constructed the expression profile of lncRNAs and mRNAs in $CD4^+$ T cells in recurrent GD patients.

**Keywords: lncRNAs, relapsed GD, WGCNA, NONHSAT093153.2, NONHSAT209004.1**

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## INTRODUCTION

Graves' disease, also known as toxic diffuse goiter, is characterized by the production of antibodies against thyroid stimulating hormone receptors (TRAb), leading to the hypertrophy and hyperfunction of the thyroid follicular cells (Morshed et al., 2012). GD is the most common cause of hyperthyroidism, and its incidence is about 0.5% (Brent, 2008). Hyperthyroidism caused by GD also increases the risk of atrial fibrillation, congestive heart failure, and miscarriage in pregnant women (Iddah and Macharia, 2013). However,

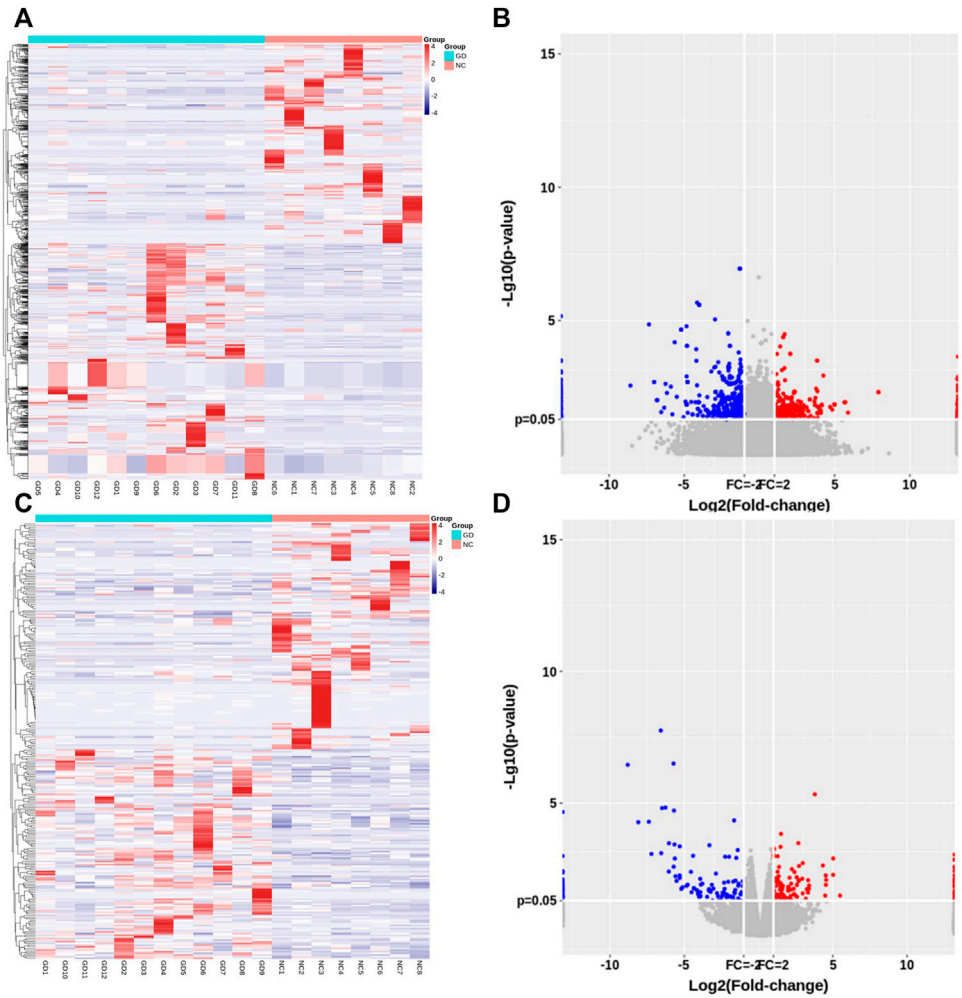


**FIGURE 1 |** Expression profile of lncRNAs and mRNAs in CD4+ T cells of relapsed GD patients. **(A)** Hierarchical clustering of differentially expressed lncRNAs between GD group ($n$ = 12) and normal controls (NC) ($n$ = 8). **(B)** Volcano plots of lncRNA expression levels between two groups. **(C)** Hierarchical clustering of differentially expressed mRNAs between GD group ($n$ = 12) and normal controls (NC) ($n$ = 8). **(D)** Volcano plots of mRNA expression levels between two groups. Each column represents a sample, and each row indicates one gene. Red indicates those genes with relatively high expression level, and blue indicates those genes with relatively low expression level.

the pathogenesis of GD is unclear, and its therapeutic effect is not satisfactory. At present, there are three main treatment methods for GD, including anti-thyroid drugs, radioactive iodine (RAI), and surgical resection. Each of the three methods has its own advantages and disadvantages. Compared with RAI and surgery, the disadvantage of anti-thyroid drugs mainly includes lower remission rate of hyperthyroidism and higher recurrence rate of patients with high TRAb titer. In addition, anti-thyroid drugs can also cause side effects such as skin rash, joint pain, agranulocytosis, and liver toxicity (Kotwal et al., 2018). Although radioactive iodine therapy has a higher cure rate, it has more chances of causing permanent hypothyroidism. Moreover, RAI is contraindicated in pregnant and lactating women and patients with active thyroid eye disease (Kotwal et al., 2018). Although surgical removal of the thyroid can quickly improve the symptoms of hyperthyroidism, patients need to take thyroid hormones throughout their lives, and the operation itself can cause a variety of complications such as hypoparathyroidism, recurrent laryngeal nerve injury, and neck hematoma. Therefore, it is urgent to further study the pathogenesis of GD and, on this basis, develop etiological treatment methods to reduce the serious harm of GD to public health.

Long non-coding RNAs (lncRNAs) are new members with more than 200 nucleotides in length of the non-coding RNAs (Caley et al., 2010). Although lncRNAs do not encode any protein products, they can regulate the gene expression at the transcriptional, post-transcriptional, and epigenetic level (Caley et al., 2010; Guttman et al., 2011). LncRNAs are also involved in functionally distinct biological and physiological processes such as chromatin remodeling, RNA junction, and protein transport (Mercer et al., 2009). Several studies have shown that lncRNAs are associated with

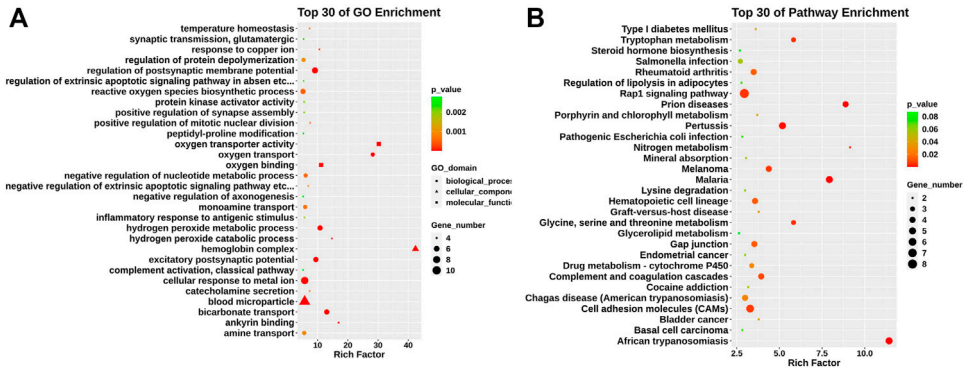


**FIGURE 2 |** GO and KEGG pathway analysis in relapsed GD CD4$^+$ T cells. **(A)** GO analysis of differentially expressed genes between GD group and contols. According to biological process (circle), cellular component (triangle), and molecular function (square). **(B)** KEGG pathway analysis for differentially expressed mRNAs.

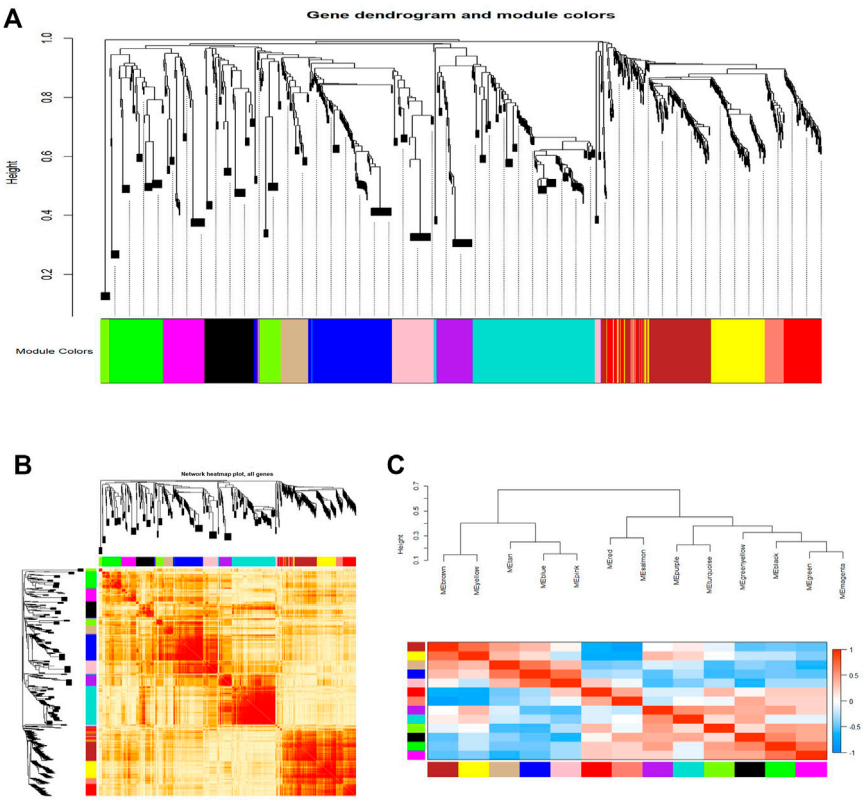


**FIGURE 3 |** WGCNA revealed gene co-expression modules in the CD4+ T cells of relapsed GD patients. **(A)** Clustering dendrograms of mRNAs and lncRNAs. Each color represents a co-expression module. **(B)** Network heatmap plot in the co-expression modules. **(C)** Eigengene dendrogram and eigengene adjacency heatmap.

autoimmune diseases, such as Crohn's disease (Qiao et al., 2013), systemic lupus erythematosus (SLE) (Zhang Y. et al., 2021), and rheumatoid arthritis (Zhang J. et al., 2021), but the association of lncRNAs with relapsed GD remains unclear. Here, this study explored the potential roles of lncRNAs in relapsed GD.

# MATERIALS AND METHODS

## Subjects

Forty-six relapsed GD patients and 33 age- and sex-matched normal healthy controls (NC) were enrolled from Zhoupu Hospital. Among them, 12 GD patients and 8 healthy controls were collected for

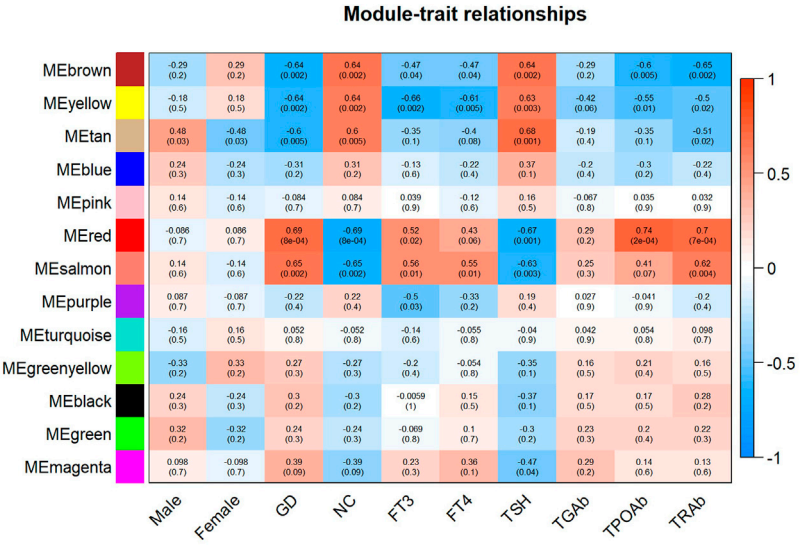


**FIGURE 4 |** Heatmap of the correlation between each coexpression module and relapsed GD and different phenotypes. The phenotypes mainly include sex, FT3, FT4, TSH, TGAb, TPOAb, and TRAb. (The correlation coefficient and corresponding *p* value were shown in each cell.)

lncRNA and mRNA sequencing, and the rest of subjects were recruited for the subsequent validation. Relapsed GD was diagnosed based on recurrence of clinical symptoms, elevated free triiodothyronine(FT3) or free thyroxine(FT4), suppressed thyroid-stimulating hormone(TSH), and positive anti-thyrotropin receptor antibody (TRAb) after a 12- to 18-month course of ATD treatment. We also detected the antibody against thyroglobulin (TGAb) or thyroid peroxidase (TPOAb) level of relapsed GD patients. Individuals without any acute or chronic autoimmune or allergic or infectious diseases or any acute or chronic visceral diseases were recruited as healthy controls or normal controls(NC). The study was approved by the Ethics Committee of Zhoupu Hospital. All subjects signed an informed consent form.

## $CD4^+$ T Cell Isolation

Firstly, peripheral blood mononuclear cells (PBMCs) of all subjects were isolated from freshly collected venous blood by lymphocyte separation medium (Sigma-Aldrich) according to the manufacturer's instruction. Then, the human CD4 Micro Beads (Miltenyi Biotec, Germany) was used to purificate $CD4^+$ T cells from fresh PBMCs. The $CD4^+$ T cells with a purity greater than 95% were used for further research. We calculated the purity of $CD4^+$ T cells by flow cytometer (BD Biosciences, USA).

## Differentially Expressed Gene Screening

$CD4^+$ T cells were isolated from PBMCs of 12 relapsed GD patients and 8 healthy controls. Then, we added 1 ml TRIzol to the $CD4^+$ T cells. Total RNA was extracted from the $CD4^+$ T cells using the TRIzol reagent (Takara) according to the manufacturer's protocol. The samples were preserved at −80°C for further analysis. These samples were then subjected to mRNA and lncRNA-seq on the Illumina HiSeq platform following the standard procedures. The raw data were cleaned to obtain the reads with high quality. The clean reads with high quality were then aligned to the reference genome. Subsequently, the differentially expressed lncRNAs and mRNAs between GD patients and NC were screened in the expressing data using the "edeg" R package. The significantly changed genes with $p < 0.05$ and log2 fold change (FC)≥1 were considered as differentially expressed genes. Gene ontology (GO) enrichment and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis was conducted by R package.

## Construction of Co-Expression Module

We constructed the co-expression module of the differentially expressed lncRNAs and mRNAs ($p < 0.05$ and average expression level >1) between relapsed GD group and healthy controls by the R package "WGCNA". We chose 5 as the soft-thresholding power, and 30 was chosen as the minimum number of modules.

## Hub Gene Identification

In the module-trait correlation analysis, the genes with gene significance greater than 0.4 and module group members (MM) greater than 0.9 were considered as hub genes, which are significantly associated with clinical phenotypes.

## Statistical Analysis

Software R3.5.3. was used to perform WGCNA analysis. The comparison between relapsed GD group and healthy controls was analyzed using non-parametric test. A *p* value less than 0.05 was considered statistically significant.

# RESULTS

## The Expression Profile of lncRNAs and mRNAs in Relapsed GD $CD4^+$ T Cells

To explore the crucial role of lncRNAs and mRNAs associated with the recurrence and development of GD, we performed RNA-Seq to detect the expression profile of lncRNAs and mRNAs in GD and NC

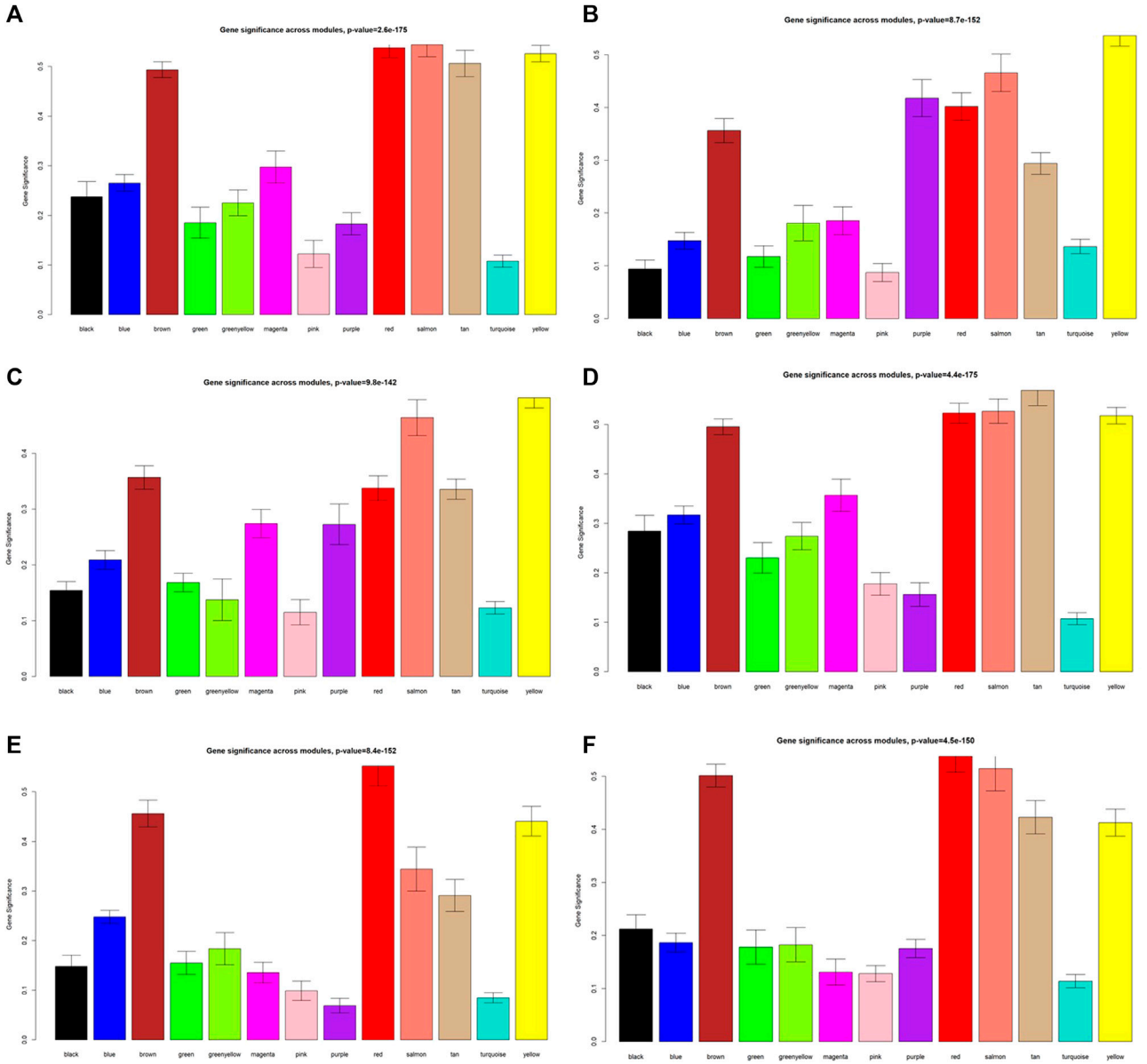


**FIGURE 5 |** Module significance values of co-expression modules related to different phenotypes. **(A)** GD; **(B)** FT3; **(C)** FT4; **(D)**, TSH; **(E)** TPOAb; **(F)** TRAb. Each color indicated one coexpression module.

group. Totally, we found that 1336 lncRNAs and 266 mRNAs were significantly differentially expressed between relapsed GD patients and healthy controls. Of the identified lncRNAs, 602 lncRNAs were significantly upregulated and 734 lncRNAs were significantly downregulated in the $CD4^+$ T cells of GD patients.

Of those detected mRNAs, 128 mRNAs were upregulated and 138 mRNAs were downregulated in $CD4^+$ T cells of GD patients. Hierarchical cluster analyses displayed lncRNA and mRNA expression profile in two groups (**Figures 1A,C**). Volcano plot analyses were also performed to visualize the differentially expressed lncRNAs and mRNAs (**Figures 1B,D**).

## GO Analysis and Pathway Analysis

We conducted Gene ontology (GO) and KEGG pathway enrichment analyses to further explore the function of those differentially expressed genes. The GO analysis found that differentially expressed genes identified were mainly enriched in hemoglobin complex, oxygen transporter activity, excitatory postsynaptic potential, and positive regulation of mitotic nuclear division (**Figure 2A**). KEGG pathway analysis revealed that those genes were mainly enriched in glycine, serine, and threonine metabolism, complement and coagulation cascades, and cell adhesion molecules (CAMs) (**Figure 2B**).

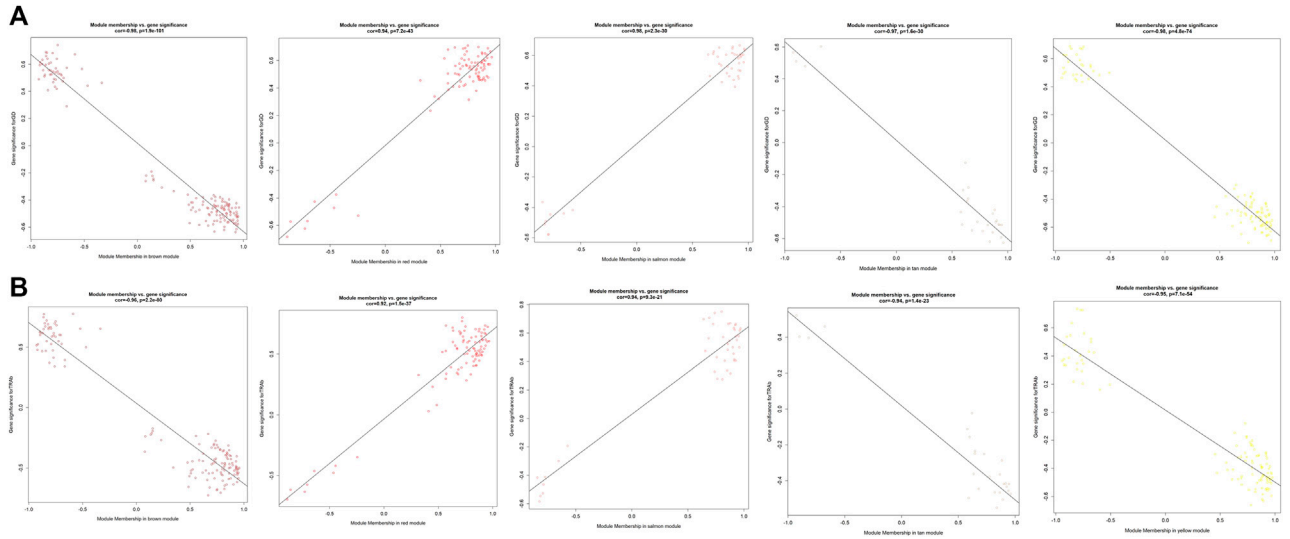

**FIGURE 6 |** The scatterplots of different modules highly related to GD and TRAb. **(A)** GD; **(B)** TRAb. The correlation coefficient and *p* value of module membership vs. gene significance were shown.

## WGCNA Analysis

As shown in **Figure 3**, we constructed a total of 13 co-expression modules by WGCNA analysis (**Figure 3A**). Moreover, these constructed modules were independent of each other (**Figure 3B**). **Figure 3C** shows an eigengene dendrogram and adjacency heatmap.

As displayed in **Figure 4**, module-trait correlations showed that five modules were related to GD, including red, salmon, brown, yellow, and tan module. Interestingly, all these modules are also related to TSH and TRAb. While brown, yellow, red, salmon, and purple modules were associated with FT3, three modules including brown, yellow, and salmon were related to FT4. The brown, yellow, and red modules were associated with TPOAb. **Figure 5** shows module significance values of co-expression modules associated with each phenotype, including GD (**Figure 5A**), FT3 (**Figure 5B**), FT4 (**Figure 5C**), TSH (**Figure 5D**), TPOAb (**Figure 5E**) and TRAb (**Figure 5F**). **Figure 6** shows the scatterplots of gene significance for GD (**Figure 6A**) and TRAb (**Figure 6B**) vs. MM in different modules.

## Functional Annotation of Key Co-Expression Modules

**Figure 7** shows the results of GO analysis about genes in different module. Genes in brown module were mainly enriched in negative regulation of gene expression and epigenetic and chromatin silencing (**Figure 7A**); genes in yellow module were enriched in viral transcription, viral gene expression, and nuclear-transcribed mRNA catabolic process (**Figure 7B**); genes in red module were mainly enriched in inositol phosphate-mediated signaling, histone H3-K4 methylation, calcineurin-NFAT signaling cascade, and calcineurin-mediated signaling (**Figure 7C**); the salmon module was mainly enriched in type I interferon signaling pathway, response to virus, etc (**Figure 7D**).

KEGG analysis (**Figure 8**) found that the genes in brown module were involved in alcoholism, systemic lupus erythematosus, and neutrophil extracellular trap formation (**Figure 8A**); genes in yellow module were involved in ribosome, coronavirus disease—COVID-19, RNA transport, etc. (**Figure 8B**). The genes in red module were involved in VEGF signaling pathway, viral carcinogenesis, and Yersinia infection (**Figure 8C**), and the genes in salmon module were involved in hepatitis C, influenza A, measles, human papillomavirus infection, and biosynthesis of cofactors (**Figure 8D**).

## Validation of Hub Genes

The gene with gene significance value greater than 0.4 and module membership greater than 0.9 is considered as the hub gene. We verified the expression of eight mRNAs and six lncRNAs of interest by PCR, including RPL8 in brown module, PARP9, RSAD2, OAS2, USP18, and IFIH1 in salmon module, NFAT5 and DROSHA in red module, and NONHSAT093153.2, NONHSAT209004.1, NONHSAT101116.2, NONHSAT161865.1, NONHSAT118924.2, and NONHSAT077537.2 in tan module. As shown in **Figure 9**, our results showed that the expression of RPL8, OAS2, NFAT5, DROSHA, NONHSAT093153.2, NONHSAT118924.2, and NONHSAT209004.1 was significantly decreased in GD group ($p < 0.001$, $p < 0.001$, $p < 0.01$, $p < 0.05$, $p < 0.001$, $p < 0.05$, and $p < 0.01$, respectively). However, there was no significant difference in the expression level of PARP9, RSAD2, OAS2, USP18 A, NONHSAT101116.2, NONHSAT077537.2 (data not shown), and NONHSAT161865.1 (data not shown) between the two groups (all $p > 0.05$.)

## DISCUSSION

Considering the high recurrence rate of GD after treatment, research on clarifying the pathogenesis of GD is an important but challenging

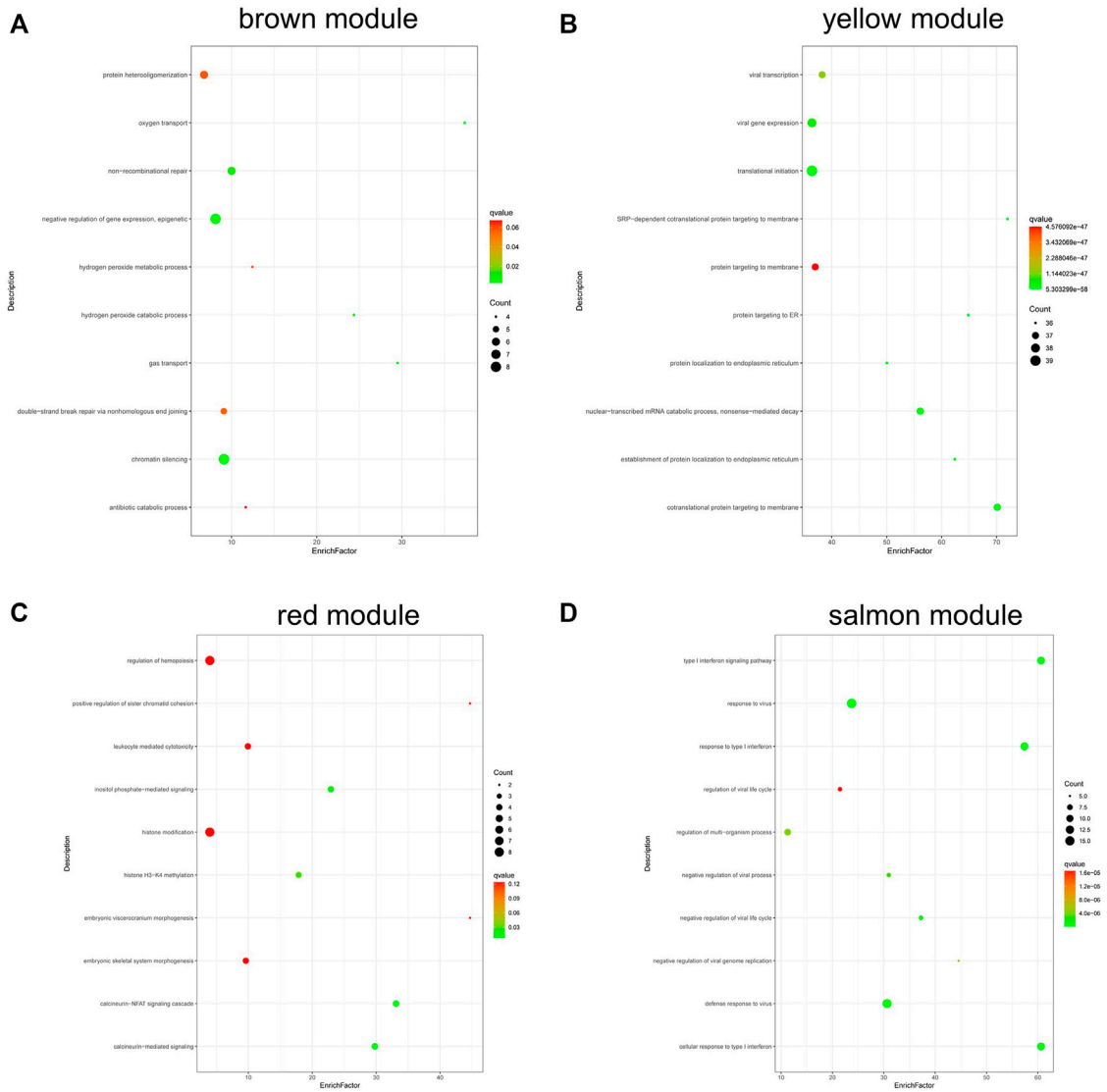


**FIGURE 7 |** GO analysis of genes in coexpression modules associated with relapsed GD. **(A)** Brown module; **(B)** Yellow module; **(C)** Red module; **(D)** Salmon module.

task. The lymphocyte infiltration results in the destruction of thyroid tissues and amplifies the extent of autoimmune response. Among the lymphocytes, $CD4^+$ T cells play an important role in the pathogenesis of GD, which mainly includes Th1, Th2, Th17, Th22, Tfh cells, and Treg. Emerging studies have shown that the imbalance between Th1 and Th2 cells leads to GD (Zemmour et al., 2017). Recent studies have revealed that the abnormal expression of Th17 (Su et al., 2020; Zake et al., 2021), Th22 (Peng et al., 2013; Vitales-Noyola et al., 2017), Tfh cells (Liu et al., 2018), and Treg (Chen et al., 2021a) is associated with GD pathogenesis. The above findings illustrated that the dysfunction of $CD4^+$ T cells plays a vital role in the development of GD. Nevertheless, the underlying mechanisms of $CD4^+$ T cell dysfunction need to be further clarified. In the present study, we generated a signature profile of numerous lncRNAs and mRNAs in $CD4^+$ T cells of relapsed GD patients compared with healthy controls by high-throughput sequencing technologies.

We obtained a total of 13 co-expression modules by WGCNA analysis. Among them, five modules including brown, yellow, tan, red, and salmon module were the main modules involved in GD, containing 144, 105, 49, 90, and 43 genes, respectively.

Currently, widely used GO analysis is very powerful in classifying various biological entities into functional related groups (Rue-Albrecht et al., 2016). In the present study, we also used GO analysis to study the biological functions of genes in the five modules.

Our results showed that the genes in brown module were mainly enriched in negative regulation of gene expression and epigenetic and chromatin silencing; the genes in red module were mainly enriched in inositol phosphate-mediated signaling, histone H3-K4

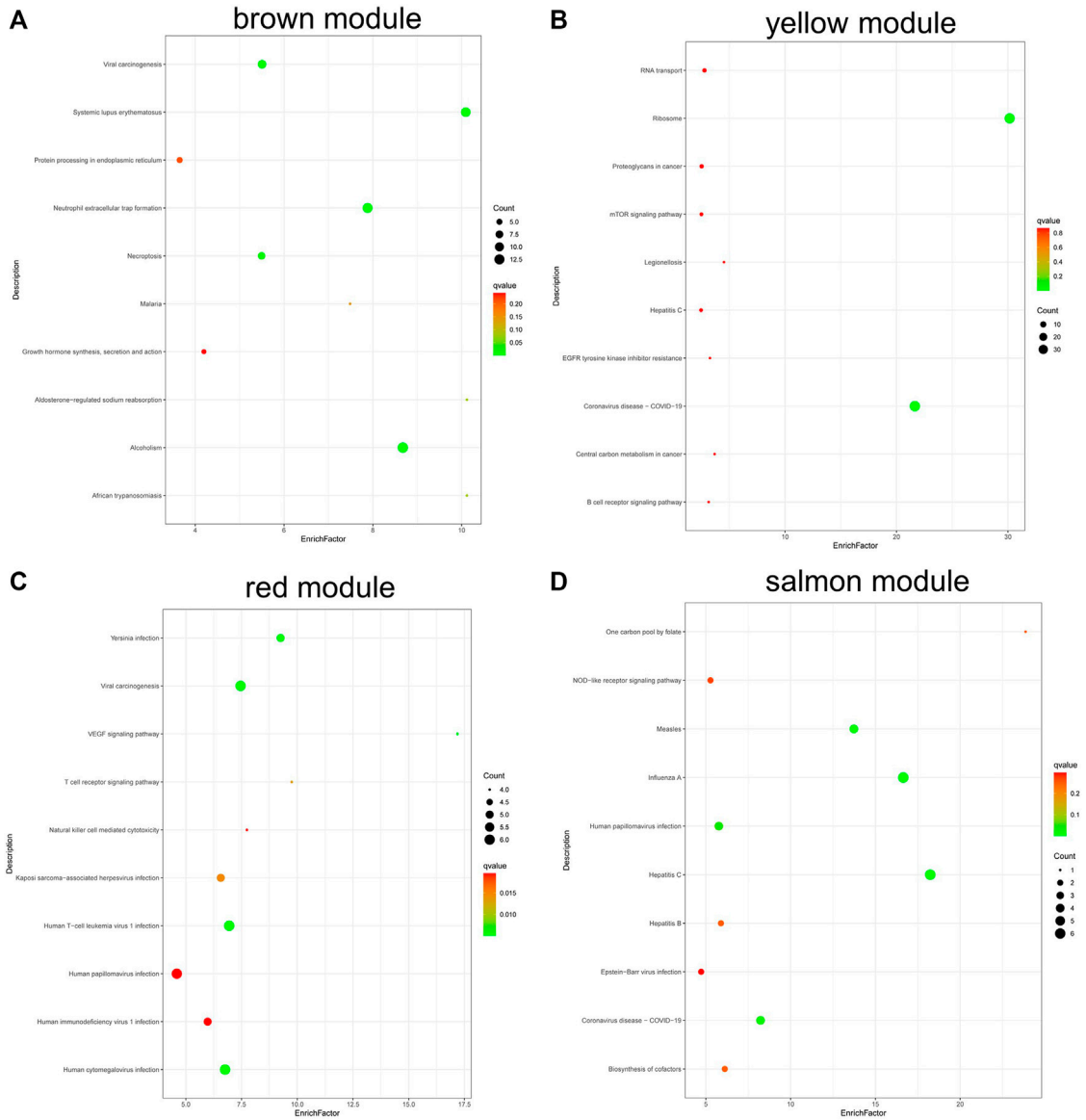


**FIGURE 8 |** KEGG pathway analysis of genes in coexpression modules associated with relapsed GD. **(A)** Brown module; **(B)** Yellow module; **(C)** Red module; **(D)** Salmon module.

methylation, calcineurin-NFAT signaling cascade, and calcineurin-mediated signaling; genes in salmon module were mainly enriched in type I interferon signaling pathway, response to virus, etc. The genes in yellow module were enriched in viral transcription, viral gene expression, etc. These findings suggest that multiple biological processes are involved in the pathogenesis of relapsed GD.

Among the identified hub genes, we found the expression of three lncRNAs (NONHSAT093153.2, NONHSAT118924.2, and NONHSAT209004.1) and four mRNAs (RPL8, OAS2, NFAT5, and DROSHA) were significantly downregulated in the relapsed GD patients, suggesting that these genes are involved in the occurrence of recurrent GD.

NONHSAT093153.2, NONHSAT118924.2, and NONHSAT209004.1 were firstly investigated in the relapsed GD patients. RPL8, a member of ribosomal proteins, is a component of the 60S ribosomal subunit in eucaryotic cells (Sun et al., 2015). It has been reported that RPL8 was related to multiple sclerosis (MS) and was a potential biomarker of MS(Chen et al., 2021b). RPL8 has not been reported in GD, and our study suggests that RPL8 was significantly decreased in GD and is worthy of further study. NFAT5 is a member of the Rel family of transcriptional factors (Lopez-Rodriguez et al., 1999). Recent emerging studies have reported the role of NFAT5 in the development and activation of macrophages and T cells (Lee et al., 2019). NFAT5 can induce the activation of pathogenic pro-inflammatory macrophages and pathogenic Th17 cells (Choi et al., 2016; Aramburu and López-Rodríguez, 2019). Numerous studies found that increased expression of NFAT5 was involved in

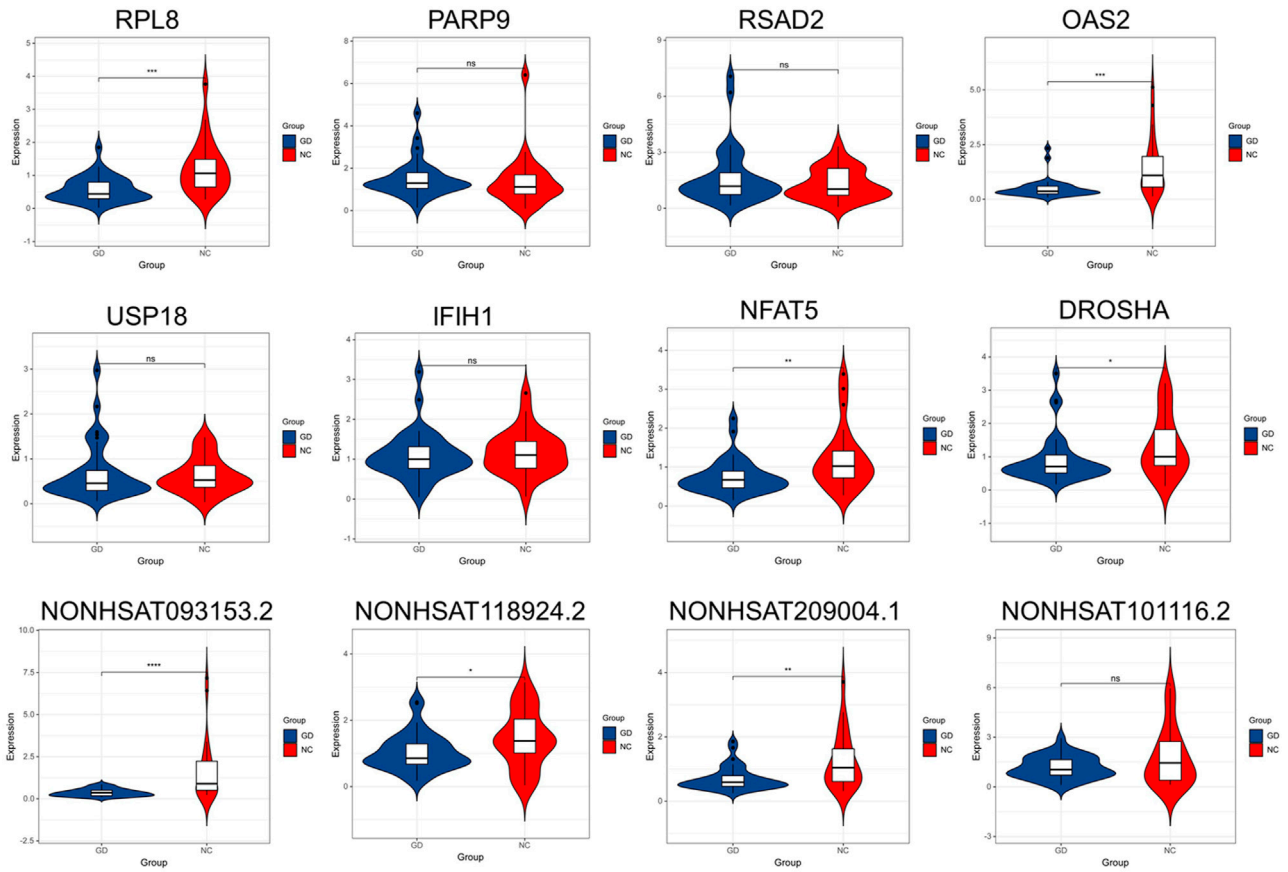


**FIGURE 9 |** Validation of key lncRNAs and mRNAs by qRT-PCR. The expression of RPL8, OAS2, NFAT5, DROSHA, NONHSAT093153.2, NONHSAT118924.2, and NONHSAT209004.1 was significantly decreased in GD group ($p < 0.001$, $p < 0.001$, $p < 0.01$, $p < 0.05$, $p < 0.001$, $p < 0.05$, and $p < 0.01$, respectively). No significant difference was found in the expression level of PARP9, RSAD2, OAS2, USP18 A, and NONHSAT101116.2 between GD and normal control (NC) groups. $*p < 0.05$, $**p < 0.01$, *** or **** $p < 0.001$, ns, not significant.

inflammatory and autoimmune diseases (Choi et al., 2017; Choi et al., 2018). OAS2 is a potential new sensitive biomarker, which can predict the activity and severity of psoriasis, and can evaluate the clinical treatment efficacy (Zhou et al., 2020). OAS2 can also be considered as biomarker gene for systemic lupus erythematosus (SLE) diagnosis (Fang et al., 2021). OAS family genes including OAS2 were revealed to be closely related to lupus nephritis (Cao et al., 2020). Drosha is RNase III enzyme necessary for most miRNA biogenesis. Study has found that the Drosha polymorphism was associated with GD development (Saeki et al., 2016). Our results showed that Drosha expression was significantly decreased in relapsed GD patients.

Although IFIH1, RSAD2, and PARP9 were found to be associated with a variety of autoimmune or inflammatory disease development, such as SLE, RA, Sjögren's syndrome (SS), type 1 diabetes (T1D), and AITD (Frommer and Kahaly, 2021; Zedan et al., 2021), we did not find that these genes were differentially expressed between the recurrent GD group and the normal group. Ubiquitin-specific peptidase 18 (USP18) plays a crucial role in the development of Th17 cells and can regulate the differentiation and function of Treg cells (Yang et al., 2021). In our study, no significant difference was found in the USP18 expression level between relapsed GD group and NC.

The present study also has some limitations. Firstly, we did not further explore the molecular mechanism of the hub genes in relapsed GD. Secondly, the number of samples we recruited to verify gene expression was too small, because blood samples from patients with recurrent GD are very difficult to collect. Thus, the hub gene expression and the potential role of them in GD still need to be further investigated and validated in more samples.

In summary, this study is the first to explore the coexpression gene networks including lncRNAs and mRNAs related to relapsed GD through WGCNA analysis with large sample size. Our study mainly finds involvement of the key gene co-expression modules, functional biological pathways, and hub genes in the development of relapsed GD. Although the potential mechanism of functional pathways and hub genes in relapsed GD still needs to be further investigated, these initial and innovative findings provide new insights into the pathogenesis of relapsed GD undoubtedly.

## 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: NCBI [accession: PRJNA763124].

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Zhoupu Hospital. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

QY, ZS, BW, and JZ contributed for the experimental planning; QY and ZS performed the experiments; QY and ZS analyzed the data; QY wrote the main manuscript; RS and JZ collected samples; JZ revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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# Genetics, Epigenetics, Cellular Immunology, and Gut Microbiota: Emerging Links With Graves' Disease

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Graves' disease (GD) is a well-known organ-specific autoimmune disease characterized by hyperthyroidism, goiter, and exophthalmos. The incidence of GD is approximately 2.0–3.0% in China and 0.5–2.0% in Western countries. Due to the complex pathogenesis and etiology of GD, current treatment methods have great side effects that seriously endanger human health. Therefore, it is particularly important to understand the pathogenesis of GD. Various studies have shown that genetics, epigenetics, cellular immunology, and gut microbiota are all involved in the development of GD. Genetically, *CD25* gene and *VDR* gene polymorphisms are involved in the development of GD by increasing the ratio of Th17/Treg cells. Epigenetically, miR-23a-3p and lncRNA-MEG3 lead to Th17/Treg imbalance and participate in the progression of GD. Moreover, commensal microbe deletion can disrupt Th17/Treg balance and participate in the occurrence of GD. The imbalance of Th17/Treg cells induced by genetics, epigenetics, and gut microbiota plays a vital role in the pathogenesis of GD. Therefore, this article reviews the role of genetics, epigenetics, cellular immunology, and gut microbiota in the pathogenic mechanism of GD. This may lead to the development of novel therapeutic strategies and providing promising therapeutic targets.

**Keywords: graves' disease, pathogenesis, genetics, epigenetics, cellular immunology, gut microbiota**

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## INTRODUCTION

Graves' disease (GD), also known as toxic diffuse goiter, is one of the most common autoimmune thyroid diseases (AITDs). It is the main cause of hyperthyroidism, and hyperthyroidism syndrome, and the main clinical characteristics include varying degrees of goiter and exophthalmos (Wemeau et al., 2018). GD is believed to represent the autoimmune process of the thyroid, in which irritant autoantibodies combined with the thyroid-stimulating hormone receptor (TSHR) and activate thyroid function, leading to hyperthyroidism (Prabhakar et al., 2003). GD tends to occur in adult females aged 30–50, and the risk in women is six times higher than in men, with an annual morbidity of 20–50 cases per 100,000 people (Smith and Hegedus, 2016). The immunological characteristics of GD are the presence of thyroid-stimulating hormone receptor antibodies (TRAbs), thyroid peroxidase antibodies (TPOAbs), thyroglobulin antibodies (TgAbs), and other autoantibodies in the serum, leading to hyperthyroidism and diffuse thyroid enlargement (McLachlan and Rapoport, 2014).

GD is a T cell-mediated organ-specific autoimmune disorder. The infiltration of T lymphocytes in GD directly destroys the thyroid. Moreover, it can also stimulate B cells to differentiate into plasma

cells that secrete antibodies (Li et al., 2019). Study has found that an increased number of $CD4^+$ T cells and plasma cells were observed in GD patients (Ben-Skowronek et al., 2013). Naïve $CD4^+$ T cells can differentiate into T helper 17 (Th17) and regulatory T cells (Tregs) when stimulated by specific antigens (Borst et al., 2018). A number of studies have shown that Th17/Tregs balance is vital to the pathogenesis of GD. Besides GD, Th17/Tregs balance may be also involved in the etiology of other autoimmune diseases, including rheumatoid arthritis (RA), psoriasis, multiple sclerosis, inflammatory bowel disease (Lee, 2018). Therefore, immune system alterations play an important role in the etiology of GD. In addition, genetics, epigenetics, and gut microbiota are also vital to the pathogenesis of GD.

Currently, three treatments are widely applied in clinical practice: radioiodine (RAI) therapy, antithyroid drugs (ATDs), and thyroidectomy. However, each method has side effects (Hu et al., 2020). Hence, the development of effective and specific treatments that can etiologically treat the disease is essential. Exploring the pathogenesis of GD may help to identify drug targets for novel therapies.

This article reviews the pathogenesis of GD from the aspects of genetics, epigenetics, cellular immunology, and gut microbiota. The aim is to provide a comprehensive overview of the basic principles to inform further development and clinical study of new drugs targeting the pathogenesis of GD.

# CELLULAR IMMUNOLOGICAL MECHANISMS OF GD

## Th1/Th2 Balance in GD

Stimulation by interleukin-12 (IL-12), interferon-$\gamma$ (IFN-$\gamma$), and IL-2 and expression of the transcription factor T-bet induce naïve $CD4^+$ T cells to differentiate into Th1 cells. This subset mainly synthesizes IL-1, IL-2, IFN-$\gamma$, and transforming growth factor beta (TGF-β), mainly through interactions with macrophages and other T lymphocytes. The presence of IL-4 suppresses the differentiation of naïve $CD4^+$ T cells into Th1 cells and promotes the production of Th2 lymphocytes. GATA3 transcription factors are also implicated in Th2 development; this subset mainly synthesizes IL-4, IL-5, IL-6, IL-10, and IL-13. Th2 cells mainly interact with B and plasma cells, resulting in an increased production of antibodies and thereby mediating the humoral immune response (Eshaghkhani et al., 2016; Ramos-Levi and Marazuela, 2016).

Compared with controls, *T-bet* and *IFN-*$\gamma$ mRNA levels in peripheral blood mononuclear cells (PBMCs) were prominently upregulated in GD patients, whereas *GATA3* and *IL-4* mRNA expression levels were downregulated. In addition, a significant decrease in plasma IL-4 levels was observed in GD patients, while IFN-$\gamma$ levels were higher in patients than in controls. These results suggest a Th1/Th2 imbalance in GD, which may be related to the pathogenesis of the disease (Eshaghkhani et al., 2016). Another study showed that the serum levels of IL-2 and IL-10 were elevated, while IFN-$\gamma$ levels were lower in patients than in controls. IL-4 levels were not significantly different between patients and controls (Esfahanian et al., 2013). However, the levels of IFN-$\gamma$ and IL-4 are discordant with the results of Eshaghkhani et al., and may be attributable to different sample types. In mouse models, IL-10 and IFN-$\gamma$ expression levels were significantly increased (Ueki et al., 2011).

Taken together, the abnormal expression of Th1/Th2 cells and the abnormal secretion of related cytokines disrupts of Th1/Th2 balance. However, their expression in GD remains controversial, and additional studies on the role of Th1/Th2 balance in GD are warranted.

## Th17/Treg Balance in GD

Th17 cells are a subset of $CD4^+$ T cells, primarily characterized by the generation of IL-17A, IL-17F, IL-21, and IL-22. The interaction of IL-1β, IL-6, and IL-23, the expression of the transcription factor retinoid-related orphan receptor gamma t (RORγt), and the activation of the STAT3 intracellular pathway play vital roles in the differentiation of Th17 cells (Tan et al., 2019). Treg lymphocytes are a heterogeneous population of lymphocytes characterized by their immunosuppressive function (Shao et al., 2018). Tregs act as negative regulatory cells through the synthesis of IL-10 and TGF-β (Tan et al., 2019). In recent years, the close relationship between Tregs and Th17 cells in GD has been explored in several studies (Li et al., 2016; Qin et al., 2017).

In Th17 cells and Tregs in PBMCs isolated from the peripheral blood of GD patients, the percentage of $CD4^+IL17^+$ T cells was significantly increased in GD patients compared with controls. In addition, the percentage of $CD4^+FoxP3^+$ Tregs was significantly reduced in GD patients in comparison to controls (Zhang et al., 2019). Analysis of Th17/Tregs ratio in the peripheral blood from GD patients indicated markedly lower ratios of $CD4^+IL17^+/CD4^+CD25^+CD127^-$ and $CD4^+IL17^+/CD4^+CD25^+CD127^-FoxP3^+$ than in the controls. In untreated GD patients, a statistically significant positive correlation in $CD4^+IL17^+/CD4^+CD25^+CD127^-$, $CD4^+IL17^+/CD4^+CD25^+CD127^-FoxP3^+$ T cells, and TRAb levels was observed. A positive association was also reported between the proportion of $CD4^+IL\text{-}17^+$ T cells and thyroid-stimulating antibody (TSAb) levels (Bossowski et al., 2016). In a mouse model of GD, the number of $CD4^+CD25^+Foxp3^+$Treg cells decreased and the expression of IL-17 mRNA increased, but the $CD4^+IL\text{-}17^+$T cell subpopulation did not significantly differ from the controls (Yuan et al., 2017). Treg cells and the balance of Th17/Treg might be associated with the pathogenesis of GD, yet Th17 cells have not been shown to promote GD (Zhou et al., 2012). IL-17 and TGF-β were all upregulated in GD patients and mouse models (Yuan et al., 2017; Han et al., 2020).

In summary, inhibition of Treg cells disrupts the Th17/Treg balance, which ultimately leads to the occurrence and development of GD. In addition, Th17/Treg balance is vital to the pathogenesis of other autoimmune diseases (Noack and Miossec, 2014).

## Tfh Cells in GD

Follicular helper T (Tfh) cells have been identified as a new subgroup of effector helper T cells, which are vital to regulating the development of antigen-specific B cell immunity. Tfh cell

differentiation is strictly regulated by the specific transcription factor Bcl-6, which is characteristically expressed in Tfh cells (Chen J et al., 2015).

An increasing body of evidence indicates that Tfh cells are significantly decreased in GD, which suggests that Tfh cells play an essential role in the pathogenesis of GD (Chen J et al., 2015; Wang et al., 2019). The proportions of effectors circulating Tfh (cTfh) and cTfh cell subsets (Tfh1, Tfh2, and Tfh17) in the peripheral blood of GD patients have been investigated. The proportion of effector cTfh cells and the Tfh2 subset were found to be increased in GD patients. Furthermore, a positive correlation between circulating Tfh2 (or PD-$1^+$ Tfh) cells and serum TPOAb levels in GD patients has been reported (Liu et al., 2018). Tfhs and relevant factors in GD thyroid tissues were upregulated and protein expression levels of Tfh-related factors were also higher in GD thyroid tissues than in normal tissues (Zhang et al., 2015). These results imply that Tfh participates in the pathogenesis of GD.

These studies have revealed the abnormal expression of Tfh cells and related cytokines in GD patients. It shows that Tfh cells play a crucial role in the pathogenic mechanisms of GD and are thus potential therapeutic targets for GD. However, identification of the exact roles of Tfh cells in the pathogenesis of GD requires further investigation.

## Breg Cells in GD

Breg cells are immunosuppressive cells that support immune tolerance. Through the production of IL-10, IL-35, and TGF-β, Breg cells inhibit immunopathology and prohibit the expansion of pathogenic T cells and other pro-inflammatory lymphocytes (Rosser and Mauri, 2015).

The proportion of IL-10-producing B (B10) cells in the peripheral blood of GD patients was decreased and negatively correlated with TRAb levels. In addition, mouse disease models have generated similar results (Ji et al., 2020). These findings indicate that B10 cells suppress autoantibody production, and their abundance is decreased in GD patients. With the onset of GD, the proportion of $CD19^+CD1d^{hi}CD5^+$ Breg cells in the PBMCs of Chinese patients is reduced (Qin et al., 2017). In addition, a conspicuous decrease in the number of $CD19^+CD24^{hi}CD27^+IL\text{-}10^+$ and $CD19^+IL\text{-}10^+$ B lymphocytes has been observed in untreated GD patients compared with controls (Rydzewska et al., 2018b).

In conclusion, Th1/Th2 balance, Th17/Treg balance, Tfh cells, and Breg cells contribute to the occurrence and development of GD. However, their precise roles in disease pathogenesis remain unclear. Additional studies on the cellular immunological mechanisms underlying GD to elucidate their interactions and provide new therapeutic strategies for GD are necessary.

# THE GENETIC PATHOGENESIS OF GD

## The Association Between *HLA*-Related Gene Polymorphisms and GD Susceptibility

*HLA* is located on chromosome 6p21.3, has a total length about 4,000 kb, and contains a set of closely related genes, which are expressed on the cell surface as HLA-I and HLA-II molecules. These genes play important roles in antigen presentation to $CD4^+$ T and $CD8^+$ T cells (Xiaoheng et al., 2017). *HLA*-A, -B, -C, and -DRA are overexpressed in GD patients, with *HLA*-C showing the most significant upregulated expression (Yin et al., 2014).

*HLA*-I genes have been identified to be significantly associated with GD. In Iran, people with *HLA*-A*68 (case/control: 80/180, $p = 0.004$, OR = 4.23) and -B*08 (case/control: 80/180, $p = 0.030$, OR = 3.72) genes were susceptible to GD, while *HLA*-A*33 (case/control: 80/180, $p = 0.011$) appeared to play a protective role (Mehraji et al., 2017). A meta-analysis on the correlation between GD and *HLA*-B*46 in Asian populations revealed that *HLA*-B*46 was associated with an increased risk of GD (Li et al., 2013). In addition, the coexistence of *HLA*-B*46 (case/control: 73/159, $p < 2.4 \times 10^{-8}$, OR = 5.455) and -Cw*01 (case/control: 73/159, $p < 0.00016$, OR = 2.208) may be a genetic marker of early-onset AITD in Koreans. Moreover, the *HLA*-A*02 (case/control: 41/159, $p < 0.014$, OR = 1.905) was shown to confer susceptibility to GD, whereas -Cw*07 (case/control: 41/159, $p < 0.001$, OR = 0.144) played a protective role (Cho et al., 2011).

Among the *HLA*-II genes, the allelic frequency of *HLA*-DRB1*07 (case/control: 41/159, $p < 0.015$, OR = 0.128), -DQB1*0201 (case/control: 80/180, $p = 0.040$, OR = 0.50) and -DQA1*0201 (case/control: 80/180, $p = 0.045$, OR = 0.37) was found to be significantly lower among GD patients, whereas the frequency of *HLA*-DRB1*08 (case/control: 41/159, $p < 5.6 \times 10^{-5}$, OR = 3.436) was higher in GD patients than in controls (Cho et al., 2011; Mehraji et al., 2017). In a Romanian population, *HLA*-DRB1*03 (case/control: 77/445, $p < 0.0001$, OR = 3.29) and -DRB1*11 (case/control: 77/445, $p = 0.045$, OR = 1.70) were identified as the potential main susceptibility alleles of *HLA*-DRB1 related to GD. In addition, GD patients with *HLA*-DRB1*03/11 had a higher FT4/TT3 ratio and anti-TgAb levels, but *HLA*-DRB1*01 (case/control: 77/445, $p = 0.007$, OR = 0.20) and -DRB1*15 (case/control: 77/445, $p = 0.038$, OR = 0.42) were likely to have a protective function (Martin et al., 2014). Some *HLA* alleles were also associated with clinical features in GD patients, *HLA*-DRB1*1301 and -DQB1*0603 carriers more frequently develop larger goiters (Park et al., 2005).

In conclusion, *HLA* is the most diverse gene in the human genome, and its variation may be associated with the occurrence of GD or play a protective role. Hence, individuals carrying certain *HLA* alleles are susceptible to GD.

## The Association Between Non-*HLA* Gene Polymorphisms and GD Susceptibility

### Association of T Cell-Related Gene Polymorphisms with GD Susceptibility

#### CTLA*4 Gene Polymorphisms in GD*

The *CTLA*4 gene is located on human chromosome 2q33 and has a total length of approximately 8.44 kb. It contains four exons and encodes 233 amino acids, generating two CTLA4 protein isoforms, namely, full-length CTLA4 (flCTLA4) and soluble CTLA4 (sCTLA4) (Fang et al., 2015). FlCTLA4 is expressed on the surface of the cellular membrane of activated T cells to play an immunosuppressive role. SCTLA4 may interfere with the

coordinated stimulation signal and inhibit T cell proliferation (Patel et al., 2016).

The correlation between *CTLA*4 and the incidence of GD has been confirmed in multiple populations (Xiaoheng et al., 2017). In the Chinese Han population, the synergistic interactions of the *CTLA*4 SNPs, including rs231775 (case/control: 260/248, $p$ = 0.002, OR = 1.521), rs231779 and rs3087243 (case/control: 260/248, $p$ = 0.007, OR = 1.615), were associated with a significant increase in the risk of GD (Chen et al., 2018). Similarly, *CTLA*4/rs3087243 is also significantly associated with GD susceptibility in Kashmiri (case/control: 135/150, $p < 0.001$, OR = 2.21) as well as Brazilian (case/control: 282/308, $p < 0.0001$, OR = 2.593) populations (Shehjar et al., 2020; Bufalo et al., 2021). In addition, a significant association between *CTLA*4/rs231775 (case/control: 135/150, $p < 0.001$, OR = 1.85) and GD development has been reported in a Kashmiri population (Shehjar et al., 2020). Therefore, *CTLA4* polymorphisms are correlated to GD susceptibility. In patients with ophthalmopathy, the frequency of the rs231775 G allele was higher than in those without ophthalmopathy, suggesting that GD patients with *CTLA*4/rs231775 were more likely to develop ocular symptoms (Esteghamati et al., 2009).

### PTPN*22 Gene Polymorphisms in GD*

*PTPN*22 is located on chromosome 1p13.3-13.1, consists of 16 exons, and encodes the 110-kDa lymphoid protein tyrosine phosphatase (LYP). LYP plays a negative regulatory role in T cell signaling (Xiaoheng et al., 2017).

With respect to the association between *PTPN*22 and GD susceptibility, the most studied SNP is rs2476601. The SNP rs2476601 is located in the N-terminal proline-rich motif and results in the substitution of arginine (Arg) with tryptophan (Trp) at codon 620 (Zhebrun et al., 2011). A significant association between *PTPN*22/rs2476601 (case/control: 171/200, $p < 0.05$, OR = 4.23) polymorphism and GD has been identified in a population from Northwest Russia and in Caucasians (Zhebrun et al., 2011; Luo et al., 2012). Moreover, female teenagers with GD in Poland have also exhibited a similar correlation, and allele A (case/control: 142/160, $p$ = 0.009, OR = 2.13) was found to be an important risk factor (Rydzewska et al., 2018a). However, Bufalo et al. reported the opposite findings in Brazil (Bufalo et al., 2021). On the other hand, C allele of PTPN22/rs3789604 may be associated with an increased risk of liver damage in GD patients (Zhang et al., 2017).

### FoxP*3 Gene Polymorphisms in GD*

The *FoxP*3 gene, which is located on the X chromosome, consists of 11 exons and encodes 431 amino acids. *FoxP*3 plays an essential role in the development and function of T cells, as well as differentiation into natural Tregs (Shehjar et al., 2018).

In a Caucasian population, the *FoxP*3 rs3761549 polymorphism was suggested to be involved in GD development in women (case/control: 109/75, $p$ = 0.03, OR = 2.174) (Bossowski et al., 2014). In a Kashmiri population, the AC genotype (case/control: 135/150, $p < 0.001$, OR = 3.48) of rs3761548 and the TT genotype (case/control: 135/150, $p < 0.001$, OR = 5.62) of rs3761549 were considered risk factors of GD development (Shehjar et al., 2018). Similarly, in the Chinese Han population, allele A and genotype AA of rs3761548 were correlated with susceptibility to GD (Zheng et al., 2015).

### CD*25 Gene Polymorphisms in GD*

*CD*25, also known as *IL-2RA*, is located on chromosome 10p15.1 and has a length of 60 kb. The expression of *CD*25 is a pivotal feature and phenotypic marker of Tregs (Brand et al., 2007).

It has been reported that *CD*25/rs2104286 (case/control: 650/1300; AA genotype: $p = 8.772 \times 10^{-6}$, OR = 1.636; A allele: $p$ = 0.004, OR = 1.322) confers to GD susceptibility in the Chinese Han population (Du et al., 2021). The minor allele A (case/control: 1474/1609, $p$ = 0.00017, OR = 1.43) of rs41295061 and the homozygous AA genotype (case/control: 1474/1609, $p$ = 0.0053, OR = 1.54) of rs11594656 conferred susceptibility to GD in a Russian population (Chistiakov et al., 2011).

In summary, the expression in T cell-related genes may affect the differentiation and function of T cell subsets, and polymorphisms may lead to an imbalance of T cell subsets, thereby contributing to the occurrence of GD.

## The Association Between B Cell Related Gene Polymorphisms and GD Susceptibility

### IKZF*3 Gene Polymorphisms in GD*

The *IKZF*3 gene is found on chromosome 17q12-q21.1 in humans, contains nine exons, and is 104 kb in length. IKZF3 is a crucial transcription factor that inhibits the proliferation and differentiation of B cells (Li et al., 2018).

Li et al. were the first to report the relationship between *IKZF*3 polymorphisms and GD. They found that the association between the minor alleles of rs2941522 (case/control: 604/814, $p$ = 0.02, OR = 1.21), rs907091 (case/control: 604/814, $p$ = 0.006, OR = 1.25), rs1453559 (case/control: 604/814, $p$ = 0.007, OR = 1.25), rs12150079 (case/control: 604/814, $p$ = 0.006, OR = 1.29), and rs2872507 (case/control: 604/814, $p$ = 0.004, OR = 1.27) and GD was significant (Li et al., 2018). More functional studies are needed to determine the contribution of *IKZF*3 polymorphisms to GD pathogenesis.

### BAFF *Gene Polymorphisms in GD*

*BAFF* is located on chromosome 13q33, and plays an essential role in regulating the maturation, proliferation, differentiation and survival of B cells (Lin et al., 2016a).

Lin et al. found that serum BAFF levels in Chinese GD patients were higher than those of healthy controls. Further research showed that serum BAFF levels were significantly correlated with TSHRAb and anti-TPOAb levels only in women with active GD (Lin et al., 2016a). Lin et al. also studied SNPs in *BAFF* and showed that the G allele (case/control: 223/243, $p$ = 0.009, OR = 0.70) of rs2893321 may decrease the risk of GD in women (Lin et al., 2016b). Lane et al. found that the frequency of another SNP (rs4000607) (case/control: 444/447, $p$ = 0.019, OR = 1.80) was significantly different between GD patients and controls (Lane et al., 2019).

### CD*40 Gene Polymorphisms in GD*

*CD*40 is located on chromosome 20q12-q13 and encodes a co-stimulatory receptor protein that plays an important role in B

lymphocyte differentiation and antibody secretion. It is a member of the tumor necrosis factor receptor superfamily that has been associated with the pathogenesis of multiple autoimmune diseases (Wang D. et al., 2017).

With respect to the association between *CD*40 and GD susceptibility, rs1883832 is the most widely studied SNP. The CC genotype and the C allele with rs1883832 were associated with GD in the Japanese (case/control: 61/42; CC genotype: $p = 0.041$, OR = 2.438; C allele: $p = 0.031$, OR = 1.972) and Chinese populations (case/control: 196/122; CC genotype: $p = 0.003$, OR = 2.043; C allele: $p = 0.008$, OR = 1.57) (Inoue et al., 2012; Wang D. et al., 2017). Nevertheless, there was no significant association between *CD*40/rs1883832 and GD in the Brazilian and Pakistani populations (Mustafa et al., 2018; Bufalo et al., 2021). In addition, the G allele (case/control: 196/122, $p = 0.001$, OR = 5.472) of C64610G have been described as susceptibility factors for GD (Wang D. et al., 2017).

### BACH*2* *Gene Polymorphisms in GD*

*BACH*2 is located on chromosome 6q15, and encodes the BACH2 protein, which regulates the transformation of B cells into plasma cells. *BACH*2 is expressed in all PBMC isoforms, with the highest expression in B cells. A genome-wide association study (GWAS) via three stages verified that *BACH*2 is associated with GD in the Chinese Han population. *BACH*2/rs2474619 (case/control: 8882/9431, $p = 3.28 \times 10^{-8}$, OR = 1.13) is significantly associated with GD. However, there was no significant association between the rs2474619 genotypes and the level of *BACH2* gene expression (Liu et al., 2014).

### FAM*167*A-BLK *Gene Polymorphisms in GD*

*FAM*167*A-BLK* is located on chromosome 8p23.1. The function of the FAM167A protein, also known as c8orf13, is unclear. BLK encodes a Src kinase that functions as a B cell signal transducer, is mainly expressed in B cells, and may influence the proliferation and differentiation of B cells. Song et al. found that the GG genotype of rs2618431 (case/control: 624/797, $p = 0.04$, OR = 1.246) may contribute to susceptibility to GD (Song et al., 2018).

In summary, B lymphocytes are the main effectors of antigen presentation and autoantibody production. B cell-related gene polymorphisms affect B cells, which are involved in the occurrence of GD.

## Association Between Thyroid Hormone Related Gene Polymorphisms and GD Susceptibility

### TSHR *Gene Polymorphisms in GD*

*TSHR* is located on chromosome 14q31 and encodes TSHR (Rydzewska et al., 2018a), a 764 amino acid polypeptide synthesized by a G protein-coupled receptor. It undergoes post-translational cleavage, producing TSHR A and B chains. The extracellular A subunit, which is shed, leads to the production of auto-antigens, facilitating the activation of non-self-tolerant $CD4^+$ T cells. This ultimately results in the generation of stimulating antibodies (Hesarghatta Shyamasunder and Abraham, 2017).

The frequencies of the GG genotype (case/control: 180/111, $p = 0.000105$, OR = 2.602) and the G allele (case/control: 180/111, $p = 0.0008$, OR = 1.853) of rs4411444, the AA genotype (case/control: 180/111, $p = 0.0228$, OR = 1.740) of rs2300519, and the AA genotype (case/control: 180/111, $p = 0.0052$, OR = 1.984) and the A allele (case/control: 180/111, $p = 0.0158$, OR = 1.548) of rs179247 were higher in GD patients than in controls. The frequencies of these genotypes and alleles, as well as the rs2300519 A allele (case/control: 62/48, $p = 0.0243$, OR = 1.967) and the rs4903961 GG genotype (case/control: 62/48, $p = 0.0147$, OR = 2.588) and G allele (case/control: 62/48, $p = 0.0166$, OR = 2.061), were higher in patients with intractable GD than in controls and patients in GD remission (Fujii et al., 2017; Rydzewska et al., 2018a). In addition, rs179247 allele A was found significantly more frequently in GD patients without oculopathy than in patients with oculopathy, indicating that this allele is associated with a lower risk of GD in patients with oculopathy (Jurecka-Lubieniecka et al., 2014).

### TG *Gene Polymorphisms in GD*

*TG*, which is located on chromosome 8q24, encodes a 660-kDa glycoprotein that supports the generation of thyroid hormones (Xuan et al., 2019). A significant relationship between rs2069550 (case/control: 436/316, $p = 0.01$, OR = 1.49) of the *TG* gene and GD has been reported (Gu et al., 2010).

In the Chinese Han population, rs2294025 (case/control: 9757/10626, $p = 1.52 \times 10^{-9}$, OR = 1.16) and rs7005834 (case/control: 9757/1062, $p = 1.62 \times 10^{-7}$, OR = 1.16) are two independent loci associated with susceptibility to GD (Xuan et al., 2019). Also, the frequency of the TT genotype (case/control: 131/89, $p = 0.0283$, OR = 0.484) of rs2703013 was significantly lower in GD patients than in controls. The distribution of the rs2958692 T allele (case/control: 50/40, $p = 0.0055$, OR = 2.382) was significantly different between patients with intractable GD and those with GD in remission (Mizuma et al., 2017).

In summary, thyroid hormone-related gene polymorphisms lead to the abnormal expression of related proteins. In addition, the production of autoantibodies leads to thyroid hormone secretion disorders, which are involved in the pathogenesis of GD.

## The Association Between Apoptosis Related Gene Polymorphisms and GD Susceptibility

Apoptosis as a regulatory mechanism can remove excess unnecessary cells such as autoreactive lymphocytes. A lack of this ability causes the proliferation of cells that react with their own antigen, in turn leading to autoimmune diseases, including GD (Bossowski et al., 2008).

### VDR *Gene Polymorphisms in GD*

*VDR* is located at chromosome 12q12-12q14 and consists of eight coding exons and 3 alternative 5′-noncoding exons spanning more than 75 kb of DNA (Meng et al., 2015). VDR is a nuclear receptor that controls the transcription of regulatory genes that are related to calcium metabolism and immune responses (Inoue et al., 2014).

Allele A (case/control: 417/301, $p = 0.041$, OR = 1.278) of rs7975232 has been associated with GD in the Chinese Han population (Meng et al., 2015). Similarly, the frequency of the C

allele of *VDR*/rs7975232 was higher in patients with AITD (case/control: 255/76, $p = 0.037$, OR = 1.514), particularly GD patients (case/control: 139/76, $p = 0.0349$, OR = 1.594), than in controls (Inoue et al., 2014). Moreover, the A allele (case/control: 650/1209, $p = 2.62 \times 10^{-2}$, OR = 1.20) and AA genotype (case/control: 650/1209, $p = 3.45 \times 10^{-4}$, OR = 1.87) of rs7975232 were associated with a significant increase in susceptibility to GD. In GD patients with ophthalmopathy, the frequency of the rs7975232 AA genotype was higher than in those without ophthalmopathy, suggesting that GD patients with *VDR*/rs7975232 are more likely to develop ocular symptoms (Zhou et al., 2021). The frequency of the TT genotype of rs731236 was associated with the higher expression of VDR (Inoue et al., 2014). A meta-analysis found that the TT genotype (case/control: 2380/2235, $p = 0.025$, OR = 1.42) of rs731236 was associated with an increased risk of GD, but rs1544410 and rs7975232 of *VDR* were not different between GD patients and controls (Veneti et al., 2019).

### FAS *Gene Polymorphisms in GD*

The *FAS* gene is located on chromosome 10q23. The receptor-ligand combination FAS-FAS ligand (FASL) is a chief mediator of apoptosis in many main physiologic processes. FAS and FASL both exist as membrane-bound and soluble types. The level of soluble FAS in subjects with the AA genotype of *FAS* -670 A/G was markedly higher than in those carrying the GG genotype (Mahfoudh et al., 2007).

There were no significant differences in rs2234767, rs1800682, and rs763110 between controls and AITD patients. However, serum soluble FASL levels in GD patients were significantly higher than in controls (Inoue et al., 2016).

### Bcl-*2 Gene Polymorphisms in GD*

*Bcl*-2 is an anti-apoptosis factor, and the upregulated expression of Bcl-2 facilitates cell survival. In the *Bcl*-2/rs1800477 polymorphism, the frequency of the G allele (case/control: 264/79, $p = 0.011$, OR = 2.630), which is related to stronger anti-apoptotic function than the A allele, was markedly higher in AITD patients than in controls. No association between the polymorphism of the rs2279115 site and GD susceptibility has been observed (Inoue et al., 2016).

### TNFR*1 and* TNFR*2 Gene Polymorphisms in GD*

The *TNFR*1 and *TNFR*2 genes, which encode TNF-RI and TNF-RII have been mapped to chromosomes 12p13 and 1p36, respectively (Glossop et al., 2005). TNF-RI possesses a death domain which can transduce the signal for cell death. While no differences in the genotype and allele frequency of *TNFR*1 rs2234649 have been observed, the G allele (case/control: 160/87, $p = 0.038$, OR = 1.827) of *TNFR*2 rs1061622 is associated with an increased risk of GD (Inoue et al., 2016).

### RNASET*2 Gene Polymorphisms in GD*

*RNASET*2 is located on chromosome 6q27 and is the only RNase T2 family member in humans. *RNASET*2 plays a role in the initiation of human dendritic cells for the Th2 polarization of $CD4^+$ T cells (Chen X J et al., 2015). There was a significant association between the risk allele G (case/control: 701/938, $p = 0.005$, OR = 1.225) of rs9355610 and a decrease in *RNASET*2 mRNA levels in a Chinese population (Chen et al., 2015). This association was also identified in the previous GWAS of GD (case/control: 5530/5026, $p = 6.85 \times 10^{-10}$, OR = 1.19) (Chu et al., 2011).

Genetic factors also play an important role in other autoimmune diseases. *HLA-DRB*1/rs13192471 confers a genetic predisposition to RA in a northeast Indian population (Das et al., 2018). *FoxP*3, *VDR*, *PTPN*22, *CTLA*4, and *CD*40 gene polymorphisms were associated with susceptibility to autoimmune diseases including RA, systemic lupus erythematosus, alopecia areata, and psoriasis (Wu et al., 2016; Bin Huraib et al., 2018; Hashemi et al., 2018; Moravvej et al., 2018; Fouad et al., 2019). With the extensive development of GWAS, many GD susceptibility genes have been identified that partly explain the clinical manifestations and pathogenesis of GD (**Supplementary Table S1**). However, due to differences in region, ethnicity, sample size, and genotyping methods, studies on the correlation between gene polymorphisms and GD susceptibility have generated inconsistent results. Therefore, it is necessary to increase sample sizes to elucidate the relationships between gene polymorphisms and GD susceptibility which can also provide reference for the identification of the susceptibility genes of other autoimmune diseases.

## EPIGENETIC PATHOGENESIS OF GD

### The Role of DNA Methylation in GD

DNA methylation is a typical epigenetic modification (Wang B. et al., 2017). The methylation reaction is catalyzed by a cluster of enzymes, including DNA methyltransferases (DNMTs) (Picascia et al., 2015). The DNMT family includes DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (Nawrocki et al., 2017). Among these, three enzymes are known to have DNMT activity: DNMT3a and DNMT3b are responsible for *de novo* methylation, while DNMT1 is required for maintaining DNA methylation (Jeltsch and Jurkowska., 2014). Compared to controls, the relative mRNA level of *DNMT*1 was significantly reduced in GD patients (Cai et al., 2015). Global hypomethylation and downregulation of *DNMT*1 mRNA expression in $CD3^+$ T and $CD19^+$ B lymphocytes in patients with newly diagnosed GD have also been observed, while there were no significant differences in DNMT3a and DNMT3b expression between GD patients and controls (Guo et al., 2018).

A genome-wide analysis of DNA methylation identified that 365 and 3322 CpG sites in $CD4^+$ and $CD8^+$ T cells are respectively differentially methylated in GD patients in comparison with controls. In addition, hypermethylation in the first intron of the *TSHR* gene has been associated with GD (Limbach et al., 2016). However, these findings are discordant with the results of Guo et al. on global hypomethylation patterns in $CD3^+$ T and $CD19^+$ B lymphocytes with GD patients, and may be attributable to different types of cells. Another study found a negative association between global methylation in B cells and serum TPOAb (Guo et al., 2018).

A statistically significant difference in methylation status intercellular adhesion molecule 1 (ICAM1) between GD patients and controls was observed, and there was a significant association between decreased *ICAM*1 methylation and exophthalmos in GD patients (Shalaby et al., 2019). Another study showed that DNA methylation decreased and DNA hydroxymethylation increased at the promoter of *ICAM*1, and this change was correlated with *ICAM*1 mRNA expression (Liu et al., 2017). Morita et al. assessed the methylation status of six CpG sites in the *TNFA* promoter region and found that the methylation level of the -72 CpG was notably higher in GD patients than in controls (Morita et al., 2018). Kyrgios et al. reported that *IL-2RA* gene promoter methylation status significantly differed among GD patients and controls, and the level of *IL-2RA* promoter DNA methylation was negatively correlated to anti-TPO and anti-TSI (Kyrgios et al., 2020).

Based on the above information, it can be hypothesized that abnormal DNA methylation is involved in the pathogenesis of GD. Nevertheless, it is still necessary to explore the abnormal DNA methylation of specific genes to provide new targets for the treatment of GD.

## The Role of Histone Modifications in GD

Chromatin is a dynamic structure that helps encapsulate the entire eukaryotic genome into the nucleus, as well as regulates DNA-related metabolic processes, including DNA transcription, recombination, repair, and replication. The basic unit of chromatin is composed of about 146 base pairs of DNA wrapped around a histone octamer (Li et al., 2007; Venkatesh and Workman, 2015; Martire and Banaszynski, 2020). Compared with GD patients carrying no chromosomal abnormalities, those with Turner- or Down-syndrome exhibited a prominent higher rate of transition from the previous Hashimoto's hypothyroidism to Graves' hyperthyroidism. This implies that chromosomal abnormalities and the coexistence of Hashimoto's thyroiditis may increase the risk of developing GD. In addition, the dynamics of chromatin structure are strictly regulated by a variety of mechanisms including histone modification (Aversa et al., 2014). Histone modifications include acetylation, phosphorylation, methylation, ubiquitylation and SUMOylation (Bannister and Kouzarides, 2011). Mounting evidence has shown that abnormal histone modification profiles contribute to an imbalance in immune response, which in turn leads to the development of various autoimmune diseases (Araki and Mimura, 2017). In general, high levels of acetylation and trimethylation of the Lys-4 residue of histone 3 (H3K4me3) were detected in the promoter regions of transcriptionally active genes in individuals with GD (Stefan et al., 2011).

Genome-wide analysis of DNA methylation in $CD4^+$ and $CD8^+$ T cells revealed that hypermethylation was related to reduced levels of H3K4me3 and H3K27ac marks in several T cell signaling genes. However, these results were discordant to the findings of Guo et al. on global hypomethylation in $CD3^+$ T and $CD19^+$ B lymphocytes of GD patients, which may be attributed to the use of different types of cells. Regions with lower H3K4me3 or H3K27ac signals were related to hypermethylated CpGs and areas with strong H3K4me3 or H3K27ac signals overlapping with hypomethylated CpGs. In addition, gene ontology and pathway analysis have suggested reduced H3K4me3 and H3K27ac signals in genes involved in T cell activation (Limbach et al., 2016). In PBMCs, global histone H3K9 methylation was significantly downregulated in GD patients compared with controls. Global H3K4 methylation was decreased in GD patients, but this difference was not statistically significant (Yan et al., 2019).

Yan et al. reported that the mRNA expression of histone deacetylases HDAC1 and HDAC2 in the PBMCs of GD patients was significantly higher than that of controls. This indicates that histone acetylation modifications are abnormal in PBMCs of GD patients (Yan et al., 2015).

In summary, histone expression is associated with methylation status and histone modifications of the genes that may regulate its expression. It is obvious that histone modifications influence the occurrence of GD. However, studies on the role of histone modification in GD are limited, and more investigations are needed to elucidate its role in GD.

## The Role of miRNAs in GD

MicroRNAs are a class of small non-coding RNAs that negatively control gene expression by directly combining with the 3′ untranslated region of their mRNA targets (Mehta and Baltimore, 2016). MicroRNAs play a crucial regulatory role in Toll-like receptor signaling, resulting in the activation of the NF-κB, IRF, and AP-1 transcription factors, which regulate the expression of pro-inflammatory cytokines (Wu et al., 2015).

The effects of miRNAs in GD are described below. MiR-181d was found to be upregulated in GD patients and miR-346 was significantly downregulated. Furthermore, miR-346 regulates $CD4^+CXCR5^+$ T cells by acting on Bcl-6, a positive regulator of Tfh cells (Chen J et al., 2015). The expression level of miR-146a in plasma was significantly reduced in AITD patients in comparison with controls. However, the expression level of miR-146a in PBMCs was increased. The reason of discordant expression levels in miR-146a in plasma and PBMCs might be that miR-146a inhibits the release of miR-146a from PBMC into plasma, and thus inducing the excessive inflammation in the thyroid of AITD patients. Thus miR-146a may be related to the occurrence of AITD (Otsu et al., 2017). MiR-155 enhances Treg and Th17 cells differentiation by targeting SOCS1 (Yao et al., 2012). However, another study showed that overexpression of miR-155 results in increased Th1 differentiation. This result is complementary to the observed bias toward Th2 differentiation in $CD4^+$ T cells lacking miR-155, thereby contributing to the regulation of Th1/Th2 balance (Banerjee et al., 2010). The expression of miR-154, miR-376b, and miR-431 was suppressed in the PBMCs of initial GD patients (Liu et al., 2012). In comparison to GD patients in remission, circulating miR-23b-5p and miR-92a-39 levels were reduced in intractable GD patients, whereas let-7g-3p and miR-339-5p were increased (Hiratsuka et al., 2016). The serum levels of miR-16, miR-22, miR-375, and miR-451 were increased in GD patients compared with controls (Yamada et al., 2014). MiR-23a-3p levels were significantly downregulated in GD patients. Animal

experiments have demonstrated that miR-23a-3p overexpression strengthens Tregs function *in vivo* (Zhang et al., 2019). Yao et al. discovered five miRNAs that were differentially expressed between GD patients and controls. There were four upregulated miRNAs (has-miR-122-5p, 16-1-3p, 221-3p, and 762) and one downregulated miRNA (has-miR-144-3p) (Yao et al., 2019). Qin et al. reported that miR-22 and miR-183 were overexpressed in GD patients, but miR-101, miR-197, and miR-660 levels were downregulated in the thyroid tissue of GD patients (Qin et al., 2015).

Martínez-Hernández et al. indicated that miRNAs are associated with autoimmune antibody levels in GD. They found that miR-let7d-5p was negatively correlated to TPOAb levels, while miR-21-5p and miR-96-5p were positively correlated with TPOAb, TgAb, and TRAb levels. MiR-142-3p and miR-301a-3p were only positively associated with TRAb levels, and miR-6503-3p was correlated with TPOAb levels (Martinez-Hernandez et al., 2018).

The above studies indicated that miRNAs participate in the regulation of gene and autoimmune antibody expression, ultimately contributing to the occurrence and development of GD and may hence serve as biomarkers for the diagnosis, treatment, and prognosis of GD.

### The Role of lncRNAs in GD

Long non-coding RNAs (LncRNAs) are non-coding RNAs with lengths of >200 nt that are transcribed by RNA polymerase II. Most lncRNAs are transcribed from the 5′ end to 3′ end of genes, but lncRNAs do not encode proteins (Ponting et al., 2009).

LncRNAs have a wide range of effects and are involved in transcriptional regulation, post-transcriptional regulation, and participate in chromatin remodeling and protein transport (Quinn and Chang, 2016). LncRNAs can be used as miRNA sponges that control the expression of target genes in various physiopathological processes (Zhang et al., 2018). Qiu et al. found that lncRNA-MEG3 may serve as a competing endogenous RNA that regulates RORγt expression via miR-17, thus affecting Th17/Treg balance. This indicates that the lncRNA/miRNA axis plays an important role in the etiology of GD (Qiu et al., 2019). The level of the RNA transcript Heg in monocytes is negatively correlated to TRAb levels in untreated GD patients (Christensen et al., 2008). However, Christensen et al. found that a decrease in TRAb levels in treated GD patients was not correlated to changes in Heg RNA levels (Christensen et al., 2011). These results suggest that Heg is related to the pathogenesis of GD.

Studies on the role of epigenetic factors in the development of GD have improved our understanding of the pathogenic mechanisms of GD (**Supplementary Table S2**). In parallel, epigenetics also play a pivotal role in other autoimmune diseases (Long et al., 2016; Karagianni and Tzioufas, 2019). Understanding the role of epigenetics in GD allows the generation of new ideas on the pathogenesis of other autoimmune diseases. Therefore, further studies are needed to: 1) elucidate the relationship between epigenetic factors and GD, 2) explore the causes and mechanisms of epigenetic changes, and 3) identify effective molecular targets for the diagnosis, treatment and prognosis of GD.

## THE GUT MICROBIOTA AND GD

The human microbiome is an essential but largely ignored part of the human genome (Grice and Segre, 2012). The collection of bacteria, archaea, and eukarya colonizing the human gastrointestinal (GI) tract is called the "gut microbiota" which has coevolved with the host for thousands of years to form a complex and mutually beneficial relationship. The microbiota brings many benefits to the host, through a series of physiological functions. Microbial composition disruption is known as dysbiosis. With the development of increasingly sophisticated methods for analyzing and characterizing complex ecosystems, the role of microbiota in a variety of intestinal and extraintestinal diseases has become increasingly clear (Thursby and Juge, 2017).

An analysis of the relative richness and diversity has indicated that the diversity of intestinal bacteria in GD patients was lower than in controls. The microbiota in GD patients mainly included four phyla (Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria). One study showed that in GD patients, the abundance of Bacteroidetes and Proteobacteria was higher and the level of Firmicutes was lower than in controls (Ishaq et al., 2018). Moreover, another study revealed that the relative abundance of Bacteroidetes and Actinobacteria increased and Firmicutes decreased in GD patients compared with healthy controls (Chang et al., 2021). In the Han population of Southwest China, GD patients showed a significantly higher abundance of Firmicutes, Proteobacteria, and Actinobacillus. Additionally, the proportion of Firmicutes was significantly higher in GD patients than in controls, while the proportion of Bacteroidetes was significantly lower in GD patients than in controls (Yang et al., 2019). However, another study on the Han population of Shanghai China found that the proportion of Firmicutes in GD patients was significantly reduced, while the proportion of Bacteroidetes was significantly increased (Jiang et al., 2021). The reason for these opposite results may be that two studies collected samples from different regions and had small sample sizes (case/control: the former 15/15; the latter 46/59).

In the genus level, the abundance of *Lactobacillus* is higher in GD in several studies, which suggests *Lactobacillus* may be important to the pathogenesis of GD. The abundance of the genera *Oribacterium*, *Lactobacillus*, *Aggregatibacter*, and *Mogibacterium* was significantly higher in GD patients than in controls (case/control: 15/15, $p < 0.05$) (Yang et al., 2019). Furthermore, GD patients have higher counts of *Bacteroides* and *Lactobacillus* (case/control: 45/59, $p < 0.05$) (Jiang et al., 2021). Chen et al. found that the relative abundance of *Lactobacillus*, *Veillonella* and *Streptococcus* were significantly higher in GD (case/control: 15/14, $p < 0.05$). Moreover, *Synergistetes* and *Phascolarctobacterium* were negatively correlated to TRAb, which suggests that *Synergistetes* and *Phascolarctobacterium* play a protective role in GD by protecting the thyroid gland (Chen et al., 2021). Changes in gut microbiota composition have also been reported in other

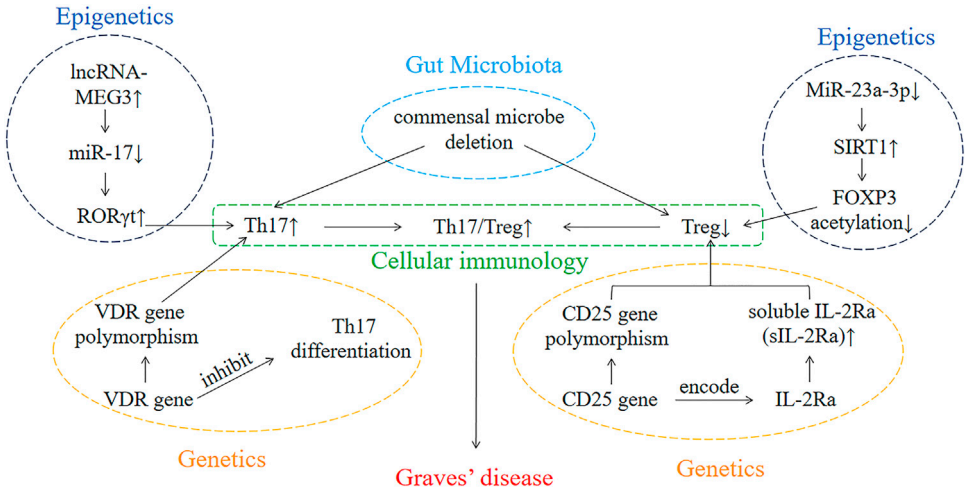


**FIGURE 1 |** The interaction of genetics, epigenetics, cellular immunology, and gut microbiota involved in the development of GD.

autoimmune diseases and generally involve Proteobacteria, Actinobacteria, and *Lactobacillus* (Xu et al., 2019).

Some specific intestinal bacteria also play a crucial role in the development of GD. One study showed that the abundance of the genus *Prevotella* was significantly higher in GD patients (Ishaq et al., 2018). The incidence of AITD was significantly greater in patients with *Helicobacter pylori* infection (Arslan et al., 2015). Similarly *Yersinia enterocolitica* infection may play a role in the etiology of GD in Turkey (Corapcioglu et al., 2002).

The gut microbiota have functions to regulate Th17 cells (Duan and Kasper, 2011; Kim et al., 2016). Similarly, the presence of gut microbiota can increase the induction of Tregs, and the butyrate produced by gut microbiota enhances Tregs differentiation and maturation (Furusawa et al., 2013). Propionic acid is an important metabolite of *Bacteroides fragilis* (*B. fragilis*). Su et al. found that *B. fragilis* regulates Th17/Treg balance via the propionic acid-mediated pathway (Su et al., 2020). We can infer that a disruption of the gut microbiota result in abnormal secretion of Th17 and Tregs, which leads to the development of GD.

Gut microbiota disorders may induce the abnormal expression of Th17 and Treg cells, which in turn influence the occurrence and development of GD. Therefore, in-depth studies on gut microbiota disorders may provide new approaches for the treatment and prevention of this disease (**Supplementary Table S3**).

## FUTURE PERSPECTIVES AND TREATMENT OF GD

Recently, Chen et al. observed that Tregs dysregulation and the expansion of pathogenic T cell clones might be involved in the long-lasting phase of GD via upregulating chemotaxis or inflammation response, and proposed a treatment with ATDs combined therapies, especially those aimed at improving Tregs frequencies or targeting specific expanded pathogenic TCR clones (Chen et al., 2021).

ATDs combination therapy provides new ideas and methods for the treatment of GD. In particular, targeting the Th17/Tregs balance may be a new approach for treating GD in the future.

## CONCLUSION

Overall, genetics, epigenetics, cellular immunology, and gut microbiota play crucial roles in the pathogenesis of GD (**Figure 1**). In parallel, these factors also influence the occurrence and development of other autoimmune diseases. Consequently, if we elucidated the pathogenesis of GD, then it will also provide new ideas in the study of the pathogenesis of other autoimmune diseases and facilitate in the development of methods for the treatment of other autoimmune diseases.

GD is an organ-specific autoimmune disease, in which the T cell immune imbalance of patients not only participates in the occurrence and development of the disease, but also affects the function of the thyroid gland. However, no studies have explained why the changes of PBMC only affect the thyroid gland, and further studies should focus on this issue.

In this Review, we found that the similarities between GD patients and mouse models (**Supplementary Figure S1**). This discovery helps us to have a clearer comprehension of GD, thereby providing a direction for future research.

Although we have some understanding of the pathogenesis of GD through the Review, our comprehension of the etiology of GD is still limited. In the future, we need to further investigate the factors mentioned above in order to find potential targets for GD therapy.

## AUTHOR CONTRIBUTIONS

HY conceived the idea. FZ drafted the manuscript and searched the literatures. XW, GT, WW, GZ, XM, DT, LW, XS, and HY participated in the manuscript preparation and revised it critically

for important intellectual content. Final approval of the version was submitted by all authors.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.794912/full#supplementary-material

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REVIEW
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# The Potential Role of Genetics, Environmental Factors, and Gut Dysbiosis in the Aberrant Non-Coding RNA Expression to Mediate Inflammation and Osteoclastogenic/Osteogenic Differentiation in Ankylosing Spondylitis

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Ankylosing spondylitis (AS) or radiographic axial spondyloarthritis is a chronic immune-mediated rheumatic disorder characterized by the inflammation in the axial skeleton, peripheral joints, and soft tissues (enthesis, fascia, and ligament). In addition, the extra-skeletal complications including anterior uveitis, interstitial lung diseases and aortitis are found. The pathogenesis of AS implicates an intricate interaction among HLA (HLA-B27) and non-HLA loci [endoplasmic reticulum aminopeptidase 1 (*ERAP1*), and interleukin-23 receptor (*IL23R*), gut dysbiosis, immune plasticity, and numerous environmental factors (infections, heavy metals, stress, cigarette smoking, etc.) The latter multiple non-genetic factors may exert a powerful stress on epigenetic regulations. These epigenetic regulations of gene expression contain DNA methylation/demethylation, histone modifications and aberrant non-coding RNAs (ncRNAs) expression, leading to inflammation and immune dysfunctions. In the present review, we shall discuss these contributory factors that are involved in AS pathogenesis, especially the aberrant ncRNA expression and its effects on the proinflammatory cytokine productions (TNF-α, IL-17 and IL-23), T cell skewing to Th1/Th17, and osteoclastogenic/osteogenic differentiation. Finally, some potential investigatory approaches are raised for solving the puzzles in AS pathogenesis.

**Keywords: ankylosing spondylitis, HLA-B27, endoplasmic reticulum aminopeptidase, gut dysbiosis, IL-23/IL-17 axis, enthesitis, circular RNA, osteogenesis/osteoclastogenesis**

## INTRODUCTION

Ankylosing spondylitis (AS) and allied diseases, also known as radiographic axial spondyloarthritis (r-AxSpA), are a set of common immune-mediated inflammatory arthritides mainly affecting axial skeleton, particularly the sacroiliac joints, non-synovial spinal joints and enthesis (the connection between tendon and bone). Chronic pain with ankylosis of the spine and disability are the characteristics of AS/r-AxSpA. It is estimated that 0.5% of the world population are affected by AS, rendering it an important health-care and socioeconomic issue. Although the exact etiology and pathogenesis of AS/r-AxSpA remain obscure, genetic predisposition by human leukocyte antigen (HLA)-B27 subtypes is known to have a strong association with the disease. However, other genetic loci of non-major histocompatibility complex (MHC) including endoplasmic reticulum aminopeptidase (*ERAP1*) and interleukin 23 receptor (*IL-23R*), gut microbiome, local immune-metabolomics in gastrointestinal (GI) tract and joints, as well as T cell plasticity may also be implicated in its pathogenesis (Voruganti and Bowness, 2020; Hwang et al., 2021). In addition to musculoskeletal manifestations, the extra-articular complications such as anterior uveitis (AU), interstitial pulmonary fibrosis, osteoporosis, syndesmophyte formation, and cardiovascular diseases (e.g., aortitis) may also occur (El Maghraoui, 2011; Stolwijk et al., 2015; Redeker et al., 2020). On the other hand, the effects of some environmental factors have been recognized through studying the relationships of different urinary tract infections and the contaminating inorganic compounds in the urine of AS patients. Shiue (2015) have reported significantly higher urine concentrations of cadmium, antimony, tungsten, uranium, and trimethylarsine in patients with AS. In a prospective cohort study, Zeboulon-Ktorza et al. (2013) have demonstrated a moderate statistical significance

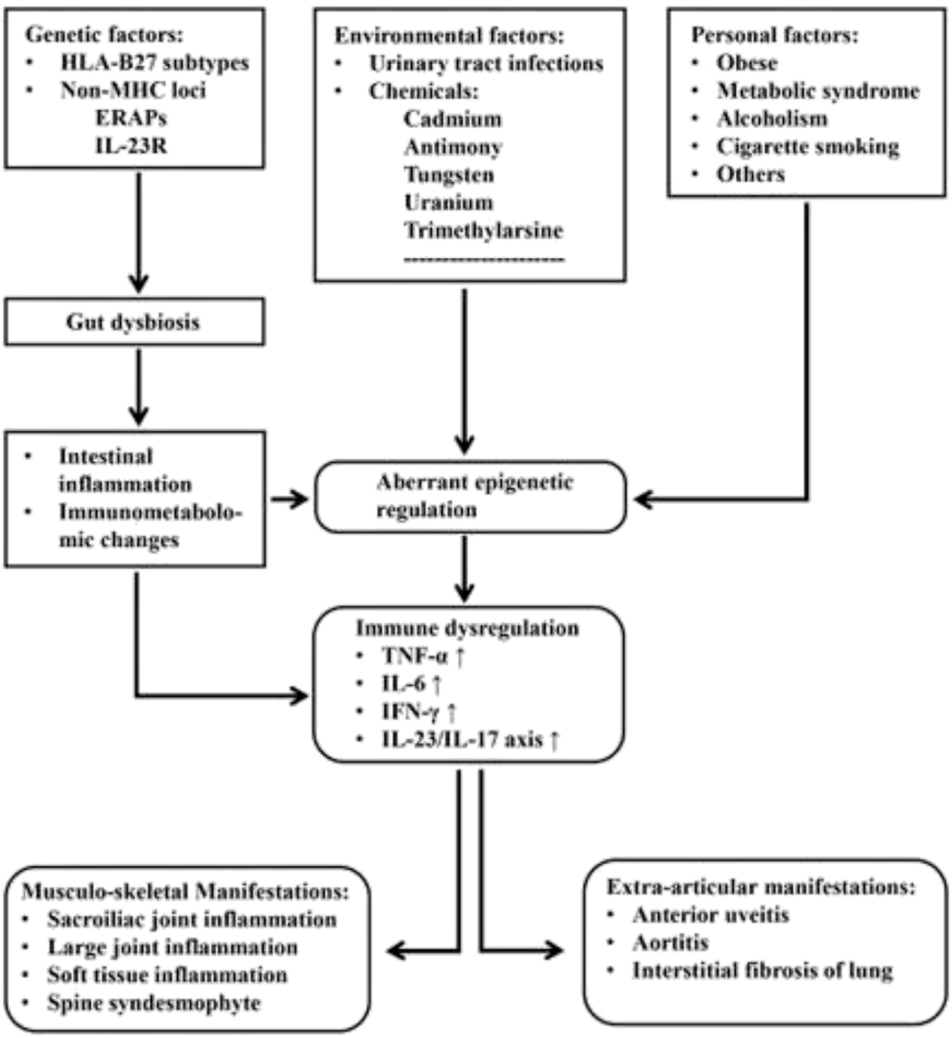


**FIGURE 1 |** A model depicting the possible pathogenetic elements in patients with ankylosing spondylitis: Many genetic, environmental and personal factors are involved in the induction of gut dysbiosis and aberrant epigenetic regulation. Subsequently, these induced pathological changes may lead to intestinal inflammation, immunometabolomic alterations and immune dysfunction. Finally, the musculo-skeletal and extra-articular manifestations ensue.

between vaccination and an elevation of Bath ankylosing spondylitis disease activity index (BASDAI) in AS. In spite of these factors, the investigations on the prevalence and influence of multi-morbidities in the disease activity of patients with AS revealed no direct cause-effect relationship between environmental factors and aberrant epigenetic regulation. Fitzgerald et al. (2020) have found that only the individual factors of metabolic syndrome are associated with more severe disease. Based on these backgrounds, we'll discuss consecutively the possible molecular bases underlying the development of AS from viewpoints of genetics, gut dysbiosis, aberrant epigenetic regulation, and immune dysregulation to further understand the immunopathogenesis of AS. A proposed multifactorial co-morbid model in causing AS is depicted in **Figure 1**.

## PROPOSED MOLECULAR PATHOGENETIC MECHANISMS FOR AS

At least five hypotheses to account for the molecular pathogenesis in AS patients have been proposed: 1) arthritogenic peptide stimulation, 2) unfolding protein response, 3) HLA-B*27 homodimer formation, 4) dysfunction of endoplasmic reticulum aminopeptidase (ERAP), and 5) gut inflammation caused by microbiota dysbiosis (Sharip and Kunz, 2020). We'll first discuss in detail the link of genetics (HLA subtypes and non-HLA loci) with these hypotheses in the next section.

## IMPLICATION OF GENETICS IN THE AS PATHOGENESIS

HLA-B27 positivity is present in 85–95% of patients with AS/r-AxSpA in different ethnicities in the world (Jamalyaria et al., 2017; Reveille, et al., 2019). However, only 5% of HLA-B27 (+) individuals in the general population have AS/r-AxSpA or undifferentiated spondyloarthropathy (USpA) (Akkoc and Khan, 2005). Nevertheless, HLA-B27 is still considered to be an important genetic factor highly associated with the development of AS. Six mechanisms have been suggested for the disease association; (A) The presentation of an arthritogenic peptide (Faham et al., 2017) to $CD8^+$ T lymphocyte enriched in the inflamed joint (Gracey et al., 2020); (B) The presence of subtype HLA-B27 heavy chain, B*27:02, with a greater tendency to fold erroneously and accumulate in endoplasmic reticulum (ER)-derived vesicles. This may lead to a response to an unfolded peptide that can activate intracellular biochemical events and upregulate proinflammatory cytokines such as interferon-γ (IFN-γ), IL-17 and IL-23 (Jeanty et al., 2014; Jah et al., 2020; Navid et al., 2021); (C) A striking tendency of HLA-B27 heavy chains to adhere with each other, forming homodimers. These homodimers on the cell surface can be recognized by killer cell immunoglobulin-like receptor (KIR) and leukocyte immunoglobulin-like receptor (LILR) on natural killer (NK) cells (Lim Kam Sian et al., 2019); (D) HLA-B27 bearing individuals show impaired intracellular killing of pathogenic microorganisms that can lead to the persistence of intracellular bacterial pathogens and consequently stimulating proinflammatory cytokine production (Sahlberg et al., 2012); (E) Trimolecular complex (B27 heavy chain, α2 microglobulin and peptide) of HLA-B27 itself, free heavy chain, or homodimers of HLA-B27 may be recognized as neoantigens by the T cell receptor on $CD_4^+$ T lymphocytes in activating autoimmune responses (Boyle et al., 2001), (F) HLA-B27 bearing individuals generate an altered intestinal microbiome to increase HLA-B27 subtype expression involved in the immunopathogenesis of AS. These six potential effects conferred by HLA-27 subtypes are illustrated in **Figure 2**.

In addition to the HLA-B27 subtypes, genome-wide association studies (GWAS) have also identified more than 100 non-HLA-B27 loci associated with AS. These loci include machinery for antigen presentation (*ERAP*1 and *ERAP*2), some loci in Th17 cells (encoding *IL-6R*, *IL-23R*, *TYK1* and *STAT3*) and others in macrophages and T cells (encoding *IL-7R*, *CSF2*, *RUNX3* and *GPR65*) (Hanson and Brown, 2017; Kavadichanda et al., 2021; Wordsworth et al., 2021).

While over 90% of AS patients have an HLA-B*27 haplotype, only around 5% of individuals bearing HLA-B*27 develop AS. This implies a presence of additional risk factors to facilitate the disease development. It has been reported that strong epistatic gene-gene interactions between *HLA-B27* and specific *ERAP1* variants may work (Reveille, 2012). In addition, one of the major functions of ERAP1 is to trim endogenous peptides before their binding onto MHC-class I molecules. The epistatic interactions between *ERAP1* and *HLA* allele may be involved in AS pathogenesis (Evans et al., 2011; Cortes et al., 2015). Furthermore, some reports have unveiled that ERAP1 can suppress both innate and adaptive immune responses (Goto et al., 2011; Aldhaman et al., 2013; Aldhamen et al., 2015). In experiments to evaluate bone morphogenesis of the axial skeletons in *ERAP1* −/− mice, the authors have found that *ERAP1* −/− mice can serve as a useful model for AS to observe spinal ankyloses, osteoporosis and inflammation. In addition, it can reduce both Tγ1-like regulatory T cells and tolerogenic dendritic cells (Pepelyayeva et al., 2018), which are important for Tγ1 differentiation and function (Wakkach et al., 2003). Finally, inflammation in the spine happens. The two ERAP genes, *ERAP1* and *ERAP2*, are ubiquitous, zinc-dependent, and multifunctional, playing a role in several HLA-class I-mediated diseases in addition to AS (McGonagle et al., 2015; de Castro et al., 2016; Vitulano et al., 2017; Hansen et al., 2018). However, it is quite interesting that only functional polymorphisms of *ERAP1* affect AS risk in HLA-B27 bearing individuals (Evans et al., 2011). *ERAP2* appears independent from HLA-B27 (Robinson et al., 2015). Recently, authors discovered that a single nucleotide polymorphism (SNP), *rs75862629*, in the *ERAP2* promoter region can influence the *ERAP2* expression that can be counteracted by a higher expression of *ERAP1* (Paladini et al., 2018). Furthermore, this SNP was found capable of modulating simultaneously the expression of both *ERAP1* an *ERAP2* and protecting hosts from AS in HLA-B27-positive individuals in Sardinia populations (Paladini et al., 2019). In contrast, a significant association between *ERAP1* polymorphisms, *rs30187* and *rs27037*, conferred an increased risk for AS in

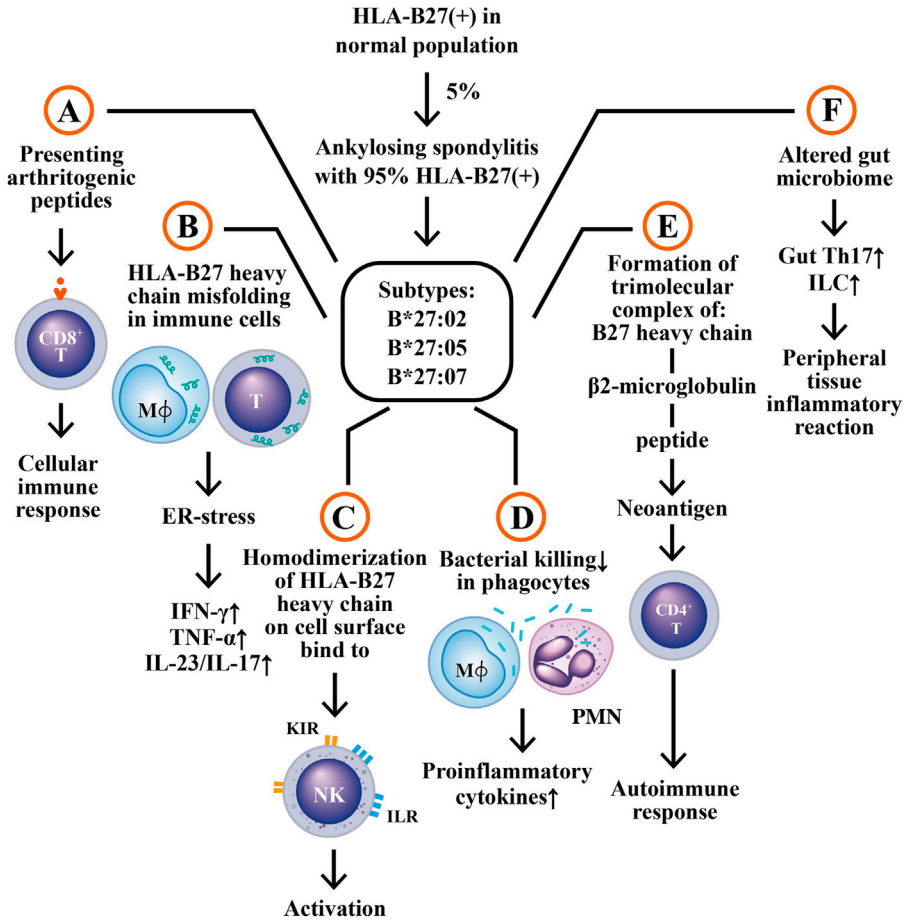


**FIGURE 2 |** The six potential mechanisms by which HLA-B27 subtypes may be involved in AS pathogenesis. **(A)** The presentation of arthritogenic peptides from endogenous or intestinal microbes to HLA-B27-expressing $CD_8^+$ T cells to elicit cellular immune responses; **(B)** Misfolding of HLA-B27 subtype heavy chain in macrophages and T cells to induce endoplasmic reticulum stress (ER stress) and subsequent production of immune- and inflammation-related cytokines; **(C)** Homodimerization of HLA-B27 heavy chains, binding to surface KIR and ILR molecules to activate NK cells; **(D)** Defective bacterial killing activity of the HLA-B27 (+) phagocytes to enhance pro-inflammatory cytokine productions; **(E)** Formation of a trimolecular complex of "B27 heavy chain + β2 microglobulin + peptide" as neoantigen to stimulate autoimmune responses of $CD4^+$ T cell; **(F)** Altered gut microbiome in HLA-B27 (+) patients to enhance the generation of Th17 and innate like T cells (ILC) in the alimentary tract, which then migrate to peripheral tissues to induce inflammatory reactions. KIR, kill cell immunoglobulin like receptor; ILR, immunoglobulin like receptor; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin. Mϕ, macrophage.

East Asian population (Jiang et al., 2018). A meta-analysis of *ERAP1* gene polymorphism unveiled that SNPs *rs27044* and *rs30187* are significantly associated with AS susceptibility in Caucasians rather than Asians (Gao et al., 2020). Silencing of *ERAP1* suppresses HLA-B27-free heavy chain expression and Th17 responses in AS (Chen L. et al., 2016; Nakamura et al., 2021). Moreover, the effects of *ERAP1* polymorphisms on the proinflammatory and anti-inflammatory cytokine expressions in AS patients have also been investigated. It was found that T allele of *rs30187* and C allele of *rs2287987* were associated with risk of HLA-B27-positive AS development by significant overexpression of proinflammatory cytokines (IL-17A, IL-17F, IL-23, TNF-α and IFN-γ) and under-expression of anti-inflammatory cytokines (IL-10 and TGF-β) in PBMC of HLA-B27 (+) AS patients (Babaie et al., 2020)

On the other hand, the studies exploring the association between *TNF* polymorphisms and AS remain inconclusive. A meta-analysis has revealed that the A allele in *TNF*-238 and *TNF*-308, the C allele in *TNF*-1031, the T-allele in *TNF*-850 and *rs769138* are significantly associated with AS susceptibility in the total population (Hu et al., 2021). In addition, *IL-12B* gene polymorphism (Ivanova et al., 2019), *IL-23R* SNPs and *IL-10*-819 polymorphism (Xia et al., 2018) are associated with AS pathogenesis.

In short summary, the aminopeptidases, ERAP1 and ERAP2, trim the peptides to a length suitable for fitting into the groove of MHC class I molecules for protection from viral infection. The epistatic interactions between HLA-B27 peptide repertoires can determine the innate immunological function of HLA-B27 such as antigen presentation to T cells. However, the process also produces a by-product, an intracellular misfolded HLA-B27, or an HLA-B27 homodimer on the cell surface, which can elicit ER stress responses, autophagic engulfment, or innate immune responses. These aberrant interplays between HLA-B27 and

ERAP1/ERAP2 result in a deviation of the physiological function from defending against infections to pathological induction of spondyloarthritis (Vitulano et al., 2017). Moreover, the accumulation of misfolded HLA-B27 heavy chain along with β2-microglobulin and ER chaperones (calnexin, calreticululin, BiP, 94kD glucose-regulated protein) into ER-derived vesicles is different from the peptide-loading complex. These abnormal behaviors may become the unique features of HLA-B27 subtypes predisposing to AS (Jah et al., 2020).

## ROLES OF HAZARD HLA ALLELE-ASSOCIATED GUT DYSBIOSIS AND ITS METABOLITES IN THE PATHOGENESIS OF AS

Sufficient evidence has demonstrated the pathogenic roles of gut microbiome in inflammatory arthritides including SpA. Human gut is colonized with bacteria, viruses, and fungi, which actively interact with each others (Li M. et al., 2019). More than 100 trillion bacteria reside in the mammalian gut to establish a symbiotic relationship. This intimate association can influence many aspects of the host's metabolism, physiology and immunity. Accordingly, intestinal dysbiosis may play an important role in the development of AS by altering intestinal permeability, stimulating immune responses, and exerting molecular mimicry (Yang et al., 2016).

To determine whether AS patients' guts carry a distinct microbial signature from that in the healthy individuals, 16S ribosomal RNA sequences of microbiome in the terminal ileum were analyzed. A higher abundance of five families of bacteria (*Lachnospiraceae*, *Rumincoccaceae*, *Rikenellaceae*, *Porphyromonadaceae*, and *Bacteroidaceae*) were reported (Costello et al., 2015). In addition, a quantitative metagenomic study based on the shotgun sequencing of the gut microbial DNA in Chinese AS patients has revealed the increases in *Prevotella melaninogenica*, *Prevotella copei*, and *Prevotella* spp. C561, but a decrease in *Bacteroides* spp. In addition, *Bifidobacterium* genus, which is commonly used in probiotics, was found accumulated in AS patients (Wen et al., 2017). An amplicon gene sequencing study on 16S ribosomal RNA disclosed the genus *Dializer* as a microbial marker for disease activity in SpA (Tito et al., 2017). Recently, by using real-time polymerase chain reaction (PCR) to analyze intestinal microbiota in stool samples, investigators have found that the total quantity of bacteria is decreased in patients with AS. The intestinal dysbiosis is associated with a more severe articular disease as evidenced by the findings that *Bifidobacterium* and *Lactobacillus* were increased in active AS patients (Cardoneanu et al., 2021). Besides, gut microbiota and mycobiota in AS patients were detected by 16S rRNA gene and ITS2-based DNA sequencing. It revealed that *Proteobacteria* was increased and *Bacteroides* was decreased. This abnormality was resulted from enrichment of *Escherichia-Shigella*, *Veillonella*, *Lachnospiraceae* NK4A136 group, and reduction of *Prevotella* strain 9, *Megamona*, and *Fusobacterium*. On the other hand, the mycobiota in alimentary tract of AS patients, as shown by 16S rRNA gene analyses, exhibited higher expression of *Ascomycota* (especially the class of *Dothudeomycetes*) and lower expression of *Basidiomycota*, mainly because of a decrease in *Agaricales* (Li X. et al., 2019). These results may imply that decreased ITS2/16s biodiversity ratios and altered bacterial-fungal inter-kingdom networks somewhat contribute to the pathogenesis of AS. The gut dysbiosis in AS may cause bowel inflammation as reflected by increased fecal calprotectin levels (Klinberg et al., 2019).

Since HLA-B27-associated SpA is relevant to an altered gut microbiota and bowel inflammation, it is interesting to search for the cause-effect relationships among HLA-B27, gut dysbiosis and host/bacterial metabolites. Sufficient evidence indicates HLA alleles (HLA-B27 and HLA-DRB1) can affect gut microbiota as reported in rat models of SpA and RA (Asquith et al., 2017; Gill et al., 2018) as well as human AS and RA (Asquith et al., 2019; Xu and Yin, 2019). The HLA-B27-induced gut dysbiosis and zonulin upregulation may change microbiota-derived metabolites and antigens. These changes can further alter vascular barrier of gut epithelium (Ciccia et al., 2017) and deregulate intestinal immune system to activate IL-23/IL-17, IFN, TNF-α and IL-1 expression (Gill et al., 2018) in AS patients. In rat model of SpA, both microbial and host metabolites are altered. These metabolites include amino acid, carbohydrate, xenobiotics and medium-chain fatty acid. Thus, upregulation of histidine, tyrosine, supermidine, N-acetylmuramate and glycerate can be found in HLA-B27/β2m rats. The HLA-B27 presentation is also associated with altered host expression of microbial metabolite receptor genes such as *FFAR2*, *FFAR3* and *NIACR1* (Asquith et al., 2017). The studies on fecal signatures of AS patients with either gender have further revealed differences in steroid metabolites (He et al., 2019). These experiments have shown that male-specific fecal signatures include cholestan-3-ol, tocopherol, stigmastan-3,5-diene, cholest-3-ene, cholest-4-en-6-one and 1-heptatriacotanol. In contrast, the female-specific fecal signatures are ergost-5-en-3-ol acetate and D-myo-inositol. These results may indicate gender-attributed fecal signature differences between males and females, reflecting AS gender features. Metagenome-wide association study on the alterations in the gut composition has disclosed that AS subjects harbor more bacterial species associated with carbohydrate metabolism and glycan biosynthesis in their feces than normal individuals. They also express bacterial profiles with less liability to degrade xenobiotics biologically or to synthesize and transport vitamins (Huang et al., 2020). To understand thoroughly the molecular mechanisms linking intestinal microbial dysbiosis, intestinal inflammation and Th17 immunity in patients with AS, Berlingberg et al. (2021) conducted LC-MS-based metabolomic screening and shotgun metagenomic measurements in paired colon biopsies and fecal specimens. The authors also showed significant alterations in metabolites in tryptophan pathway that can increase indole-3-acetate (IAA) and indole-3-acetaldehyde (I3Ald) in AxSpA. The shotgun metagenomics confirmed abundance of numerous enzymes involved in tryptophan metabolism such as indole pyruvate decarboxylase. These enzymes can enhance significantly the generation of IAA and I3Ald to facilitate tryptophan synthesis. Tryptophan and its metabolites in this particular gut microbiome may disturb immune functions and further expedite the development of

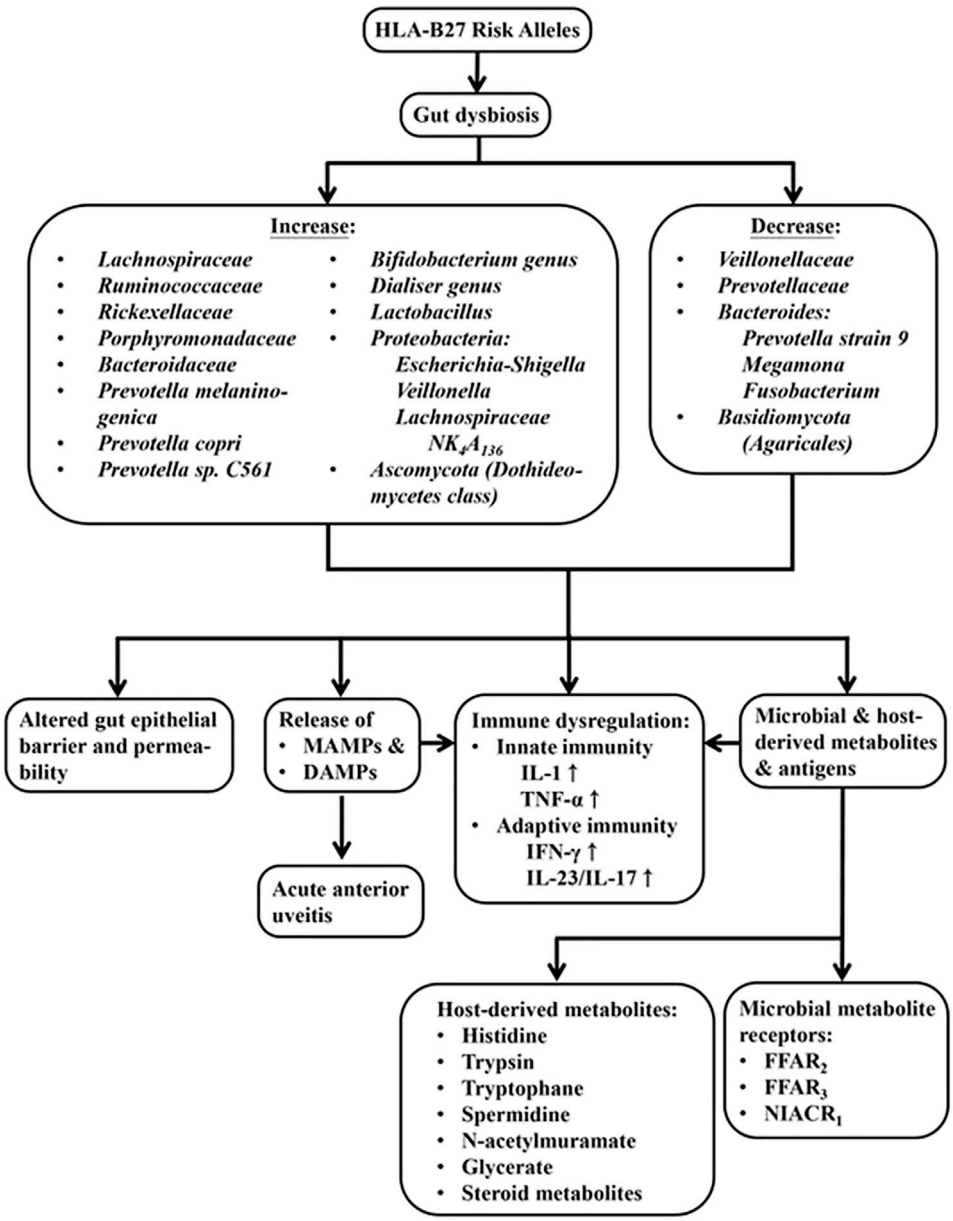


**FIGURE 3 |** The effect of HLA-B27 risk alleles on gut dysbiosis, ensuing microbial and host metabolomic changes and immune dysregulation in the host. The dysbiosis may result in the alteration of gut epithelial barrier and its permeability, release of microbe-associated molecular pattern (MAMPs), damage-associated molecular patterns (DAMPs) from hosts, and microbial as well as host-derived metabolites. All of these molecules can elicit abnormal innate and adaptive immune responses in patients with AS.

AxSpA. Moreover, gut microbiome may evolve together with the human hosts and provide them with a myriad of molecules such as microbe-associated molecular patterns (MAMPs) to augment intestinal inflammatory processes. In association with damage-associated molecular patterns (DAMPs), the inflammatory processes may be triggered further in the already ongoing pathological status such as HLA-B27-associated acute anterior uveitis (Rosenbaum and Asquith, 2018; Lu Yang et al., 2021). These data support that the invisible "essential organs" can communicate with each other and "talk" across with host immune cells in healthy individuals as well as in AS patients. The effects of HLA-B27 risk alleles on gut microbiota and metabolomic changes in patients with AS are depicted in **Figure 3**.

## IMMUNE DYSFUNCTIONS IN PATIENTS WITH AS

Many studies have shown that various immune-related cells, *via* their secreting cytokines and molecules, may play crucial roles in

AS pathogenesis (Mei et al., 2011; Liu et al., 2015; Madej et al., 2015; Sveaas et al., 2015; Wang et al., 2016; Yang et al., 2016; Wang et al., 2018). These data indicate that both innate and adaptive immune cells are involved in AS pathogenesis. The innate immune cells include dendritic cells, macrophages and natural killer cells. The adaptive immune cells include helper T, Treg, $CD8^+$T, and B cells (Rezaiemanesh, et al., 2018). Much evidence has demonstrated that certain phenotypes of resting and activated macrophages expressing scavenger receptor, CD163, can link between immune alterations of the gut and synovial inflammation in AS (Baeten et al., 2002; Slobodin et al., 2012; Talpin et al., 2014; Wright et al., 2016; Rezaiemanesh et al., 2017). The abnormal polarization of macrophages induced by IL-4 was found in AS patients (Lin et al., 2015). Besides, the studies on immune dysfunctions of T cell subpopulations are prosperous. These results revealed increased frequency of Th2 (Yang et al., 2004) and Th17 (Jandus et al., 2008; Shen et al., 2009; Xueyi et al., 2013), abnormal form of HLA-B27 expression on $CD4^+$ T cells (Boyle et al., 2004), defective function of $CD24^+CD38^+$ regulatory B cells (Chen M. et al., 2016), and expansion of $CD4^+CD28^{high}$ Treg cells (Ciccia et al., 2010). Recently, two molecules on T lymphocytes, T cell immunoglobulin and mucin-domain-containing molecule 3 (Tim-3) and programmed death-1 (PD-1) for negative regulation of immune responses, attracted investigators to focus on AS pathogenesis (Zhou et al., 2015). PD-1 expression in T cells was reported to inversely relate to the spinal radiologic changes in Taiwanese patients with AS (Chen et al., 2011). Tim-3 polymorphism could result in down-regulation of the expression of itself and be involved in AS susceptibility (Wang et al., 2014). Furthermore, other investigations revealed a decreased expression of PD-1 on $CD8^+$ T cells in AS patients. The deficiency may activate immune responses in AS patients (Duan et al., 2017). More recent investigations have disclosed that $Tim\text{-}3^+CD8^+$ and $PD\text{-}1^+CD8^+$ T cells can produce more IL-10 than other subsets.

Another studies have revealed that either low percentage (Wu et al., 2011; Zhao et al., 2011; Xueyi et al., 2013) or functional impairment (Guo et al., 2016; Wang et al., 2018) in $CD4^+$ Treg may be present in AS patients. On the contrary, meta-analyses have unveiled that only the proportions of $CD4^+CD25^+FOXP3^+$ Treg, $CD4^+CD25^{high}CD127^{high}$ cells, or $CD4^+CD25^+CD127^{low}$ cells in peripheral blood of AS patients are significantly decreased (Lai et al., 2019; Li M. et al., 2020).

Several studies have been published in recent years demonstrating the pivotal role of gut-microbiota and IL-23/IL-17 axis in the AS pathogenesis (Jandus et al., 2008; Milanez et al., 2016; Mei et al., 2011; Zeng et al., 2011; Appel et al., 2011; Singh, et al., 2011; Babaie et al., 2018). Enthesis inflammation (enthesitis) was demonstrated to be IL-23-dependent (Sherlock et al., 2012; Benham et al., 2014) and likewise IL-17-dependent (Shabgah et al., 2017). Moreover, investigations from the blood samples of AS patients revealed the abundance of Th17 (Shen et al., 2009; Zhang et al., 2012), Th22 (Zhang et al., 2012) and γ/δ T cells (Kenna et al., 2012) with high levels of IL-17 in the circulation (Wendling et al., 2007; Mei et al., 2011; Lin et al., 2015). The innate lymphoid cells (ILCs) can stimulate inflammation in the gut with respect to AS. $NKp44^+$ ILC3 cells were found expanded in the intestine, synovial fluid, bone marrow and peripheral blood of patients with AS to produce IL-17, IL-22 (Ciccia et al., 2015), and granulocyte-macrophage colony-stimulating factor (GM-CSF) but not IL-17A in the inflamed joints (Blijdorp et al., 2019). The intraepithelial lymphocytes (IELs) are another T cell subpopulation within the intestinal epithelium in close contact with bacteria that can be affected by intestinal microbiota. The total number of IELs is significantly decreased in AS due to a decrease in TCR $\beta^+$ IELs. These IELs can secrete increased amount of IL-1β, IL-17A and IFN-γ in Crohn's disease and significantly enhance the amount of TNF-α in AS (Regner et al., 2018). All of these results may suggest a correlation between altered microbiota and IEL function in AS.

The pathogenesis of AS is characterized by a predilection of adaptive immunity toward IL-23/IL-17 axis with the presence of a polarization stimulator for Th17 response. As a result, the IL-17 and TNF-α production are enhanced. However, failure of IL-23 blockade in the treatment of spinal polyenthesitis but not

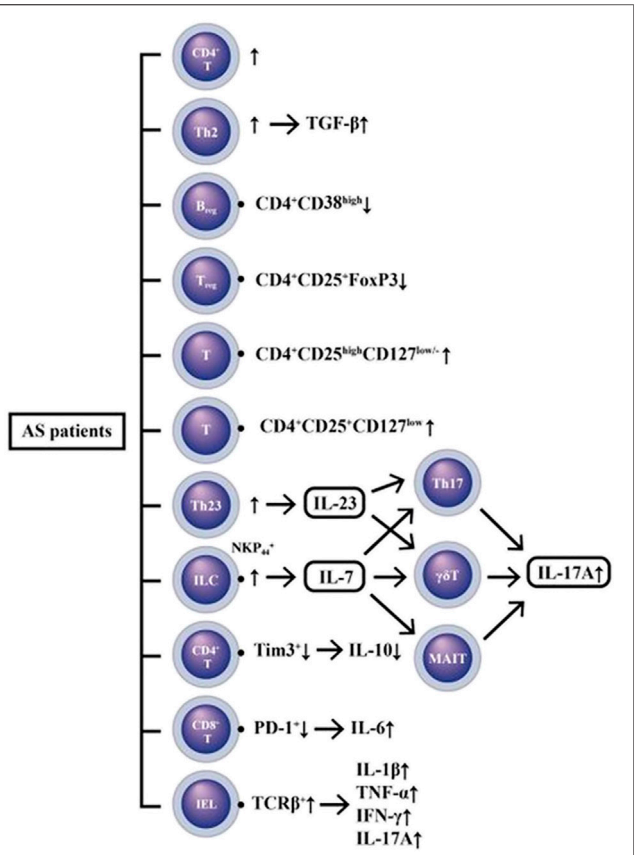


**FIGURE 4 |** A diversity of immune cell dysfunctions with excessive innate (IL-1β, IL-6, TNF-α) and adaptive (IFN-γ and IL-17A) cytokine production in patients with AS. IL-7 released from gut innate-like lymphoid cells (ILC) can stimulate Th17 γ/δT and MAIT (mucosa-associated invariant T) to produce IL-17A. In addition, the intra-epithelial lymphocytes (IELs) are the T cells within intestinal epithelium which can also excrete IL-17A and other proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ) to mediate tissue inflammation. Furthermore, both regulatory T cell (Treg) and regulatory B cell (Breg) hypofunctions may also participate in the immune dyregulation in AS patients.

peripheral enthesitis has ever been encountered (McGonagle et al., 2021). Thus, the importance of IL-23 pathway in AS pathogenesis awaits further evaluations. A recent study has even demonstrated high levels of IL-7 mRNA and peptide in the peripheral type SpA (Rihl et al., 2008). IL-7 belongs to a hematopoietin cytokine family with a molecular weight of 17.4 kDa (Sutherland et al., 1989). It can stimulate Th17 cells, innate immune cells like γ/δ T cells (Michel et al., 2012) and mucosa-associated invariant T (MAIT) cells (Tang et al., 2013) to produce proinflammatory cytokines including IL-17. In addition, these innate-like T cells rather than Th17 cells have been proved to be the main source of IL-17A (Venken and Elewaut, 2015; Debusschere et al., 2016). It appears that IL-7 is more important than IL-23 in the polarization of type 17 (IL-17) signature because IL-7 receptor is present in the key cells of innate immunity that are essential for the polarization of type 3 (IL-3) response and SpA (Gonçalves and Duarte, 2019). The immune dysfunctions in patients with AS are illustrated in **Figure 4**.

## PATHOGENESIS OF ENTHESITIS IN AS PATIENTS

Enthesis is regarded as the region where tendon attaches to bone. In broad sense, the entheseal tissues include fibrocartilage, bursa, fat pad, deeper fascia, adjacent trabecular bone networks and enthesis. These tissues play an anchorage between mobile organs and stress resistance (Benjamin and McGonagle, 2009). It has been recognized that enthesitis become the primary pathological process underlying SpA-associated skeletal inflammation (Watad et al., 2018). Normal entheseal tissues contain group 3 NK$p44^+$ ILCs, γ/δ T cells, conventional $CD4^+$ and $CD8^+$ T cells, and cells of myeloid lineage. These cells are capable of producing prostaglandins, different growth factors and proinflammatory cytokines (TNF-α and IL-17) for physiological tissue repair and homeostasis (Reinhardt et al., 2016; Cuthbert et al., 2017; Cuthbert et al., 2019; Watad et al., 2020; Russel et al., 2021). In pathological condition, the IL-23-dependent γ/δ T cells can produce IL-17 that accumulates in the enthesis, aortic valve and ciliary body to cause the extra-skeletal manifestations in patient with AS (Reinhardt et al., 2016; Bridgewood et al., 2020).

Altered microbiota associated with abnormal immune responses to commensal micro-organisms may also contribute to the occurrence of enthesitis-related arthritis (Stoll et al., 2014). The "danger signals"from exogenous intestinal microbial adjuvants or pathogen-associated molecular patterns (PAMPs) can destroy "self-molecules" within the cells. Alternatively, the damage-associated molecular patterns (DAMPs) from highly biomechanically stressed entheses can disturb "fine tuning" of cytokine production in homeostatic entheseal tissues. The net-effect of these processes may serve as key drives for the onset, evolution, sustenance, flare-up, and eventual outcomes of r-AxSpA (Sharif et al., 2020). The cellular and molecular bases for enthesitis in AS patients are illustrated in **Figure 5**.

## PATHOGENESIS OF OSTEOPOROSIS AND OSTEOGENESIS IN AS PATIENTS

A mystery of skeletal damage in patients with AS is the consequence of bone destruction followed by the new bone formation. The inflammation-induced osteoporosis in the spine and peripheral bones is quite common in the early stage of AS. It can cause trabecular bone weakness and lead to an increase in spinal fracture rate (Davey-Ranasinghe and Deodhar, 2013). Enhanced expression of IL-17 in the serum and synovial fluid has been reported implicating in the bone loss of AS patients (Akgöl et al., 2014). However, another puzzle of proinflammatory cytokine, IL-17 family, effecting on the bone metabolism, arises since IL-17A can enhance both bone loss and osteogenesis.

Investigations have unraveled that IL-17 can activate osteoclasts (OCs) to express RANK ligand (RANKL), which then reciprocally stimulates OCs themselves by RNAK-RANKL interaction and induces bone absorption (Page and Miossec, 2005; Miossec, 2009). IL-17A-stimulated miR214 expression in OCs is an important inhibitor for bone formation in AS patients (Wang et al., 2013; Zhao et al., 2015; Liu et al., 2020). Nevertheless, adding of exogenous IL-17A into cultured normal primary bone-derived cells (BdCs) promotes OC activity and differentiation as evidenced by increased alkaline phosphatase (ALP) activity through JAK2/STAT3 pathway (Jo et al., 2018a; Wang et al., 2018).

On the other hand, abnormal bone remodeling with excessive new bone formation can cause syndesmophytes or even "bamboo spine" to limit the spinal motility in AS patients. Many studies have revealed that higher bone morphogenetic proteins (BMP), lower Dickkopf-1 (DKK-1) levels (Liao et al., 2018), and increased ALP activity (Jo et al., 2019) in serum are parallel to the accelerated osteogenesis in the BdCs derived from AS patients (AS-BdCs) (Jo et al., 2018b; Kang et al., 2018). For investigating the relevance of ALP to the regulation of osteoblast (OB) differentiation in AS patients, ALP was inhibited in AS-BdC culture. A remarkable suppression of the master transcriptional factor in OB, RUNX2, was observed. This implies that RUNX2 can regulate promoter activity of ALP through a positive ALP-RUNX2 feedback mechanism (Jo et al., 2019). To further identify the role of HLA-B27 in syndesmophyte formation in AS, the mesenchymal stem cells (MSCs) obtained from enthesis of AS patients were studied. The results demonstrated that HLA-B27-mediated activation of the SXBP1/RARB/TNAP (tissue non-specific alkaline phosphatase) axis is essential in the development of syndesmophyte in AS patients (Liu C.-H. et al., 2019). Furthermore, the expression of miR-146a is up-regulated and *DKK1* is down-regulated respectively in capsular tissue of the hip in AS patients. Therefore, a negative correlation was displayed between the expressions of miR-146a and *DKK1*. Further investigations disclosed that miR-146a could inhibit *DKK1* expression by directly targeting 3′-UTR region of *DKK1* (Di et al., 2018). In addition, the level of miR-17-5p is significantly elevated in fibroblasts and ligament tissues, which is assumed to be targeting the 3′-UTR of ankylosis protein homolog (ANKH). This may subsequently increase osteogenesis in AS patients. Down-regulation of miR-17-5p slowed AS progression *via*

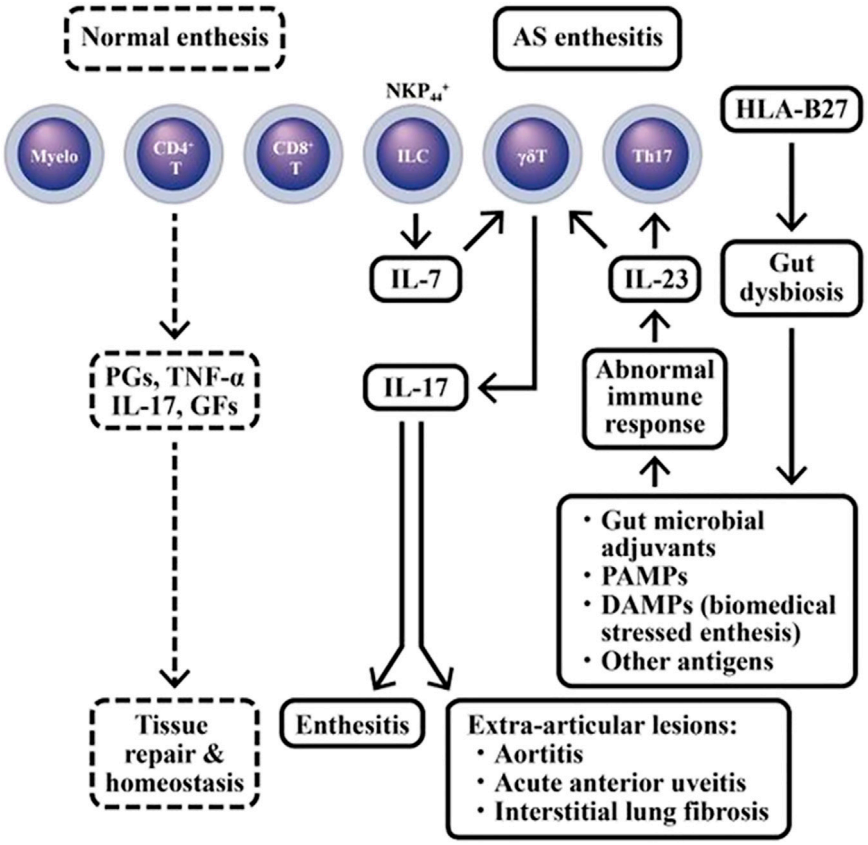


**FIGURE 5 |** Dissection of immune-related cells in normal enthesis and AS enthesitis. In normal enthesis, many different cell populations may release physiological amount of prostaglandins (PGs), TNF-α, IL-17 and various growth factor (GFs) to maintain tissue repair and homeostasis in these biomedical stressed-entheses. However, in HLA-B27 risk allele (+) AS patients, the aberrant IL-17 production can cause inflammation in enthesis (enthesitis) as well as extra-articular manifestations such as aortitis, acute anterior uveitis, and interstitial lung fibrosis. PAMP, pathogen-associated molecular pattern; DAMP, damage-associated molecular pattern; NKP44, Natural cytotoxicity triggering receptor 2 or CD336.

regulation of DKK1 and VEGF. These findings have verified the role of miR-17-5p-ANKH axis in regulating heterotropic ossification in AS patients (Qin et al., 2019).

The transgenic SpA-associated and non-SpA-associated HLA-B27 subtypes in *Drosophila* revealed an antagonistic interaction of HLA-B27 against activin receptor-like kinase-2 (ALK2). This antagonistic interaction may exert inhibitory effects on TGF-β1BMP signaling pathway at the cross-road between inflammation and ossification and become a putative mechanism for HLA-B27-mediated SpA development (Grandon et al., 2019). The molecular basis underlying the dilemma in inflammation-mediated bone metabolism in AS patients is shown in **Figure 6**.

In addition to the above mentioned factors contributing to AS pathogenesis, it is believed that the environmental factors might affect the hereditable epigenetic regulation of the down-stream gene expression in developing human diseases. These may include infectious, autoimmune/inflammatory, or neoplastic diseases. Recently, (Ghafouri-Fard et al., 2021a), have unveiled the interaction between ncRNAs and Toll-like receptors (TLRs) in transducing both MyD88-dependent and TRIF-dependent signaling cascades to induce human inflammatory and autoimmune disorders. Furthermore, the same group have found in the literature that both miRs and lncRNAs can regulate bone development processes including osteogenesis. Both intramembranous and endochondrial ossification of osteogenesis were observed (Ghafouri-Fard et al., 2021b). miRs were found to exert their actions through both Wnt/β-catenin and TGF-β/BMP pathways whereas lncRNAs worked as molecular sponge for binding miRs to directly affect these pathways and osteogenic transcription factors. The examples include MALAT1/miR-30, MALAT1/miR-214, LEF1-AS1/miR-24-3P, MCF2L-AS1/miR-33a, MSC-AS1/miR-140-5P, and KCNQ1OT1/miR-214. It is quite interesting that nuclear factor-kappa B (NF-κB) represents a group of inducible transcription factors (TFs) to regulate gene expression implicated in the immune responses. NF-κB can functionally interact with ncRNAs to construct an intricate NF-κB-miRs-lncRNAs network in regulating down-stream gene expression in different aspects. This type of network interactions among miR-146a/b, MALAT1, NKILA and NF-κB have been reported in the pathogenesis of some inflammatory conditions (Ghafouri-Fard et al., 2021c). Collectively, these data can provide some clues to support the interactions among environmental factors, gut

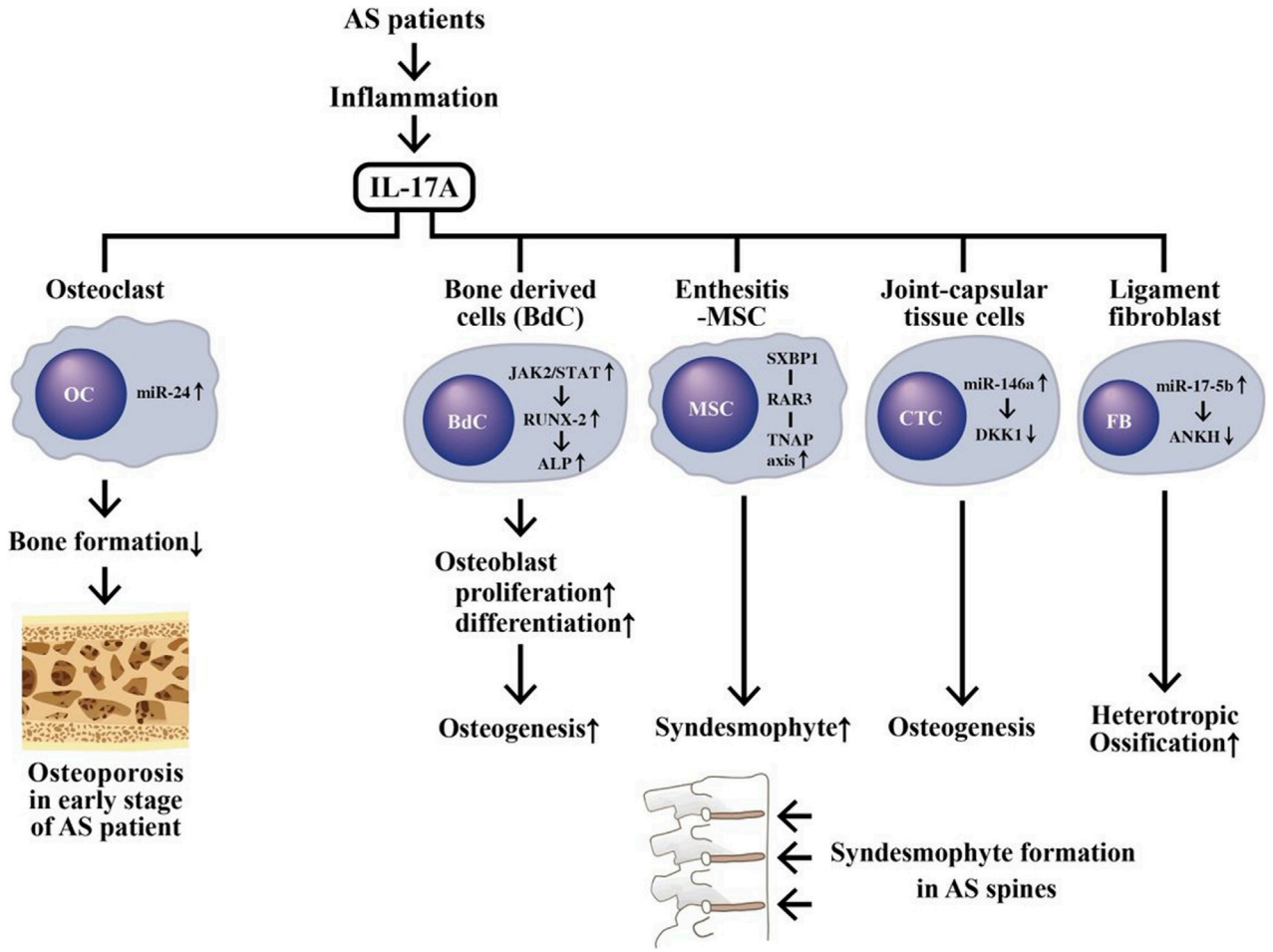


**FIGURE 6 |** The aberrant IL-17A production during inflammation in AS patients causing a coexistence of osteoporosis in early stage of disease and new bone formation (syndesmophyte) in late stage of disease. IL-17A can activate osteoclasts (OCs) *via* stimulating miR-24 expression to suppress bone formation and subsequently result in osteoporosis. On the other hand, IL-17A can also stimulate Wnt/β-catenin, ALP/TNAP and abnormal ncRNAs expression to impede DKK1 pathways in bone-derived cells (BdC), enthesitis-derived mesenchymal stem cells (MSCs), connective tissue cells (CTCs) and ligament fibroblasts (FB). These activated cells can then induce osteogenesis and syndesmophyte formation. JAK, Janus kinase; STAT, signal transducer and activator of transcription; ANKH, Progressive ankylosis protein homolog (ANK ilosis H omolog).

dysbiosis, aberrant ncRNA expression and inflammation/autoimmunity in the development of AS. We are going to discuss in detail the aberrant epigenetic regulation in AS pathogenesis in the next section.

## ABERRANT EPIGENETIC REGULATION IN AS

Epigenetics is a study on the heritable changes in gene expression which takes place without an alteration in DNA sequence but with modulations of chromatin-associated molecules caused by environmental factors. These environmental factors include dietary nutrition, lifestyle, exercise/physical activity, drugs/toxins and other miscellaneous contributory factors (Abdul et al., 2017; Heinbockel and Csoka, 2018; Martin and Fry, 2018). In general, epigenetic study may include DNA methylation, histone acetylation/deacetylation and circular RNA (cRNA) regulation (Berlingberg and Kuhn, 2020).

DNA methylation is an epigenetic modification with addition of methyl groups to cysteine or adenine residue to control gene transcription (Dor and Cedar, 2018). Many factors can affect DNA methylation such as age, sex, smoking, medications, alcohol and nutrition-diet (Whyte et al., 2019). DNA methyltransferase 1 (DNMT1) is an enzyme that regulates methylation of cytosine residues. Decreased DNMT1 expression can increase gene expression. Previous studies have shown that the expression level of DNMT1 in AS patients is significantly down-regulated which is associated with hypermethylation of the promoter region of *DNMT1* (Aslani et al., 2016). These results suggest that the dysregulation of DNMT1 expression *via* altered methylation level of the other target genes may contribute to AS pathogenesis. Recently, the genome-wide DNA methylation profile analysis identified many altered DNA methylation sites in the peripheral

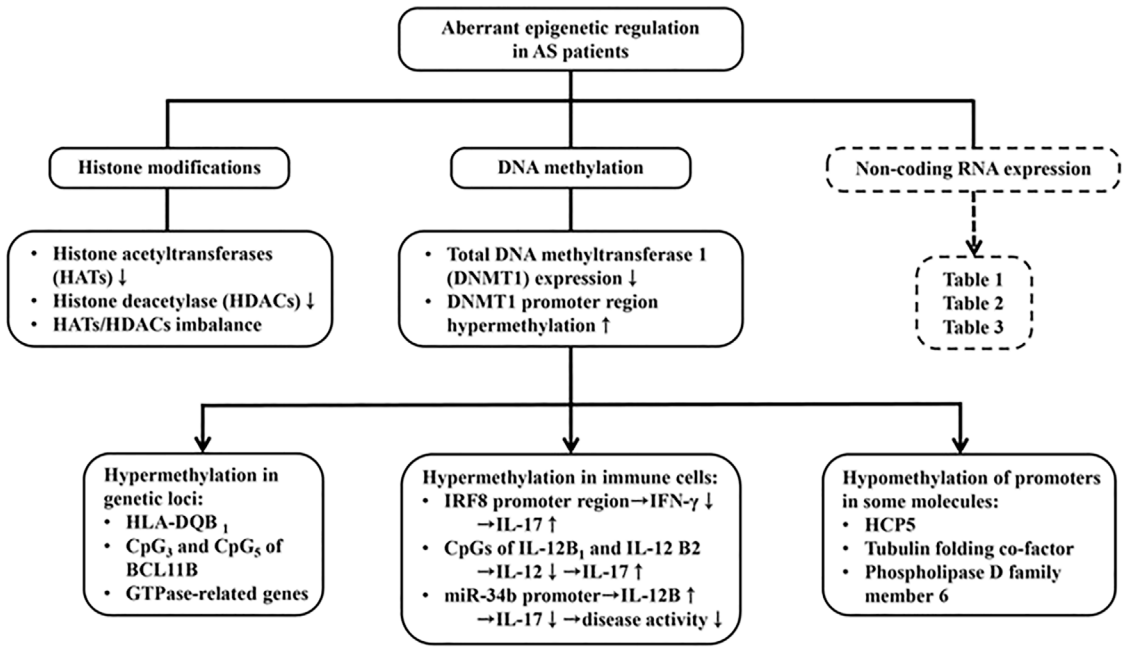


**FIGURE 7 |** The contribution of aberrant epigenetic regulations in AS pathogenesis including histone modiciations, DNA methylation and ncRNA expression. Decreased both HATs and HDACs expression, and therefore imbalanced HATs/HDACs ratio are found in AS. Moreover, hypermethylation of HLA-DQB1, CpG3 and CpG5 isolates of BCLIIB (B cell chronic lymphocytic leukemia IIB) and GTPase-related genes are found. In contrast, DNA methyltransferase (DNMT1) promoter region hypermethylation was found in AS patients. This can cause hypermethylation in the promoter region of IFN-γ and IL-12 and conversely enhance IL-17 production. On the contrary, hypermehtylation of miR-34b promoter increase IL-12 production to suppress IL-17 expression. The abnormal ncRNA expressions in the intracellular and extracellular parts with their modes of action are presented in **Tables 1–3**.

blood mononuclear cells (PBMCs) of AS patients. These sites include hypermethylation of *HLA-DQB1* (Hao et al., 2017), hypermethylation of CpG3 and CpG5 in B-cell chronic lymphocytic leukemia/lymphoma 11B (*BCL11B*) (Karami et al., 2017), and hypermethylation of GTPase-related genes (Coit et al., 2019). However, HLA-B27 bearing patients with AS were found to have some hypomethylated DNA promoters in HCP5 tubulin folding cofactor A (*TBCA*) and phospholipase D family member 6 (*PLD6*) (Coit et al., 2019).

To elucidate the epigenetic regulation of immune dysfunctions in AS, the DNA methylation profile of blood cells was analyzed. Hypermethylation of the promoter in interferon regulatory factor 8 (*IRF8*) (Chen et al., 2019) and 2CpG islands of IL-12B (*IL12B-1* and *IL12B-2*) (Zhang et al., 2019) were found. Both IFN-γ and IL-12 are crucial cytokines in suppressing Th17 cell proliferation and differentiation, which contribute in consequence to reduce severity of AS. Further investigations disclosed that hypermethylated miR-34b promoter leads to upregulation of miR-34b, thus inhibiting the IL-12B gene expression and alleviating disease activity of AS (Meng et al., 2021).

On the other hand, histone modification allows activation (euchromatin) and deactivation (heterochomatin) of chromatin by two enzymes, histone acetyltransferase (HATs) and histone deacetylase (HDACs) (Allis and Jenuwein, 2016). In PBMC study, decrease in and imbalance between HAT and HDAC activities were present in AS patients, compared to the healthy controls (Toussirot et al., 2013). The aberrant DNA methylation and histone modifications in PBMC are depicted in **Figure 7**.

## The Characteristics of Non-Coding RNAs and Their Roles in the Pathogenesis of and Clinical Applications in AS Patients

ncRNAs are single-stranded RNAs composed of microRNAs (miRs, with 20-24 nucleotides) and long non-coding RNAs (lncRNAs, with more than 24 nucleotides and less than 300 nucleotides). They regulate gene expression and therefore are involved in physiological and pathophysiological processes. They exist in the cells, extracellular fluid and cell-derived exosomes in a stable form. In addition, lncRNAs can act as sponge for modulating miR functions. Accordingly, ncRNA expression profiles can serve as biomarkers for disease activity, pathogenesis, prognosis and therapeutic monitoring of the diseases (Mohammadi et al., 2018; Berlingberg and Kuhn, 2020; Motta et al., 2020). Besides their molecular stability, the characteristic tissue specificity, easy obtainability from different biological fluids (plasma, saliva, urine, synovial fluid and other tissue fluid), and powerful discrimination render ncRNA profiling a useful tool in studying autoimmune, inflammatory and neoplastic diseases (Park et al., 2009; Pauley et al., 2009). Therefore, we will discuss in detail the contribution of ncRNAs to the deranged T cell responses, inflammation, altered bone homeostasis and monitoring of disease activity in AS.

**TABLE 1 |** Intracellular ncRNA expressions, their modes of action and clinical applications in the immune cells of patients with AS.

| Cell/Body fluid | ncRNA | Mode of action | Biomarker | References |
|---|---|---|---|---|
| T cell | miR-16↑<br>miR-221↑<br>lncRNA let-7i↑ | IFN-γ↑ | Disease activity in lumbar spine | Lai et al. (2013) |
| PBMC | miR-29↑ | DKK-1↑<br>β-catenin↑<br>RUNX2↑<br>GSK-3β↓ | Disease activity<br>Diagnostic biomarker for new bone formation | Huang et al. (2014)<br>Huang et al. (2017) |
| $CD_{14}^{+}$ Mϕ | miR-361-3p↓<br>miR-223-3p↓<br>miR-484↓<br>miR-16-5p↓ | AS pathogenesis | | Fogel et al. (2019) |
| $CD_{4}^{+}$ T | miR-16-1-3p↑<br>miR-28-5p↑<br>miR-199a-5p↑<br>miR-126-3p↑ | | | |
| PBMC | miR-17-5p↑<br>miR-27a↑<br>miR-29a↑<br>miR-126-3p↑ | | Diagnostic biomarker | Li et al. (2019a) |
| PBMC | miR-335-5p<br>miR-27a<br>miR-218 | | New bone formation | Yang et al. (2019) |
| PBMC/Serum* | miR-495↓ | $PDCD_{10}$↑<br>β-catenin↑<br>TGF-$β_1$↑ | New bone formation | Ni and Leng, (2020) |

*Mϕ, macrophage; PBMC, peripheral blood mononuclear cell; CD, cluster of differentiation; IFN, interferon; RUNX, Runt related transcriptional factor; PDCD, programmed cell death protein; GSK, glycogen synthase kinase; TGF, transforming growth factor; DKK, Dickkopf related protein. * also appearing in serum.*

Lai et al. (2013) discovered that three ncRNAs, miR-16, miR-221 and let-7i, were over-expressed in T cells from AS patients (AS-T). TLR-4 has been confirmed to be the target molecule of let-7i in AS-T cells. In addition, increased expression of let-7i enhanced IFN-γ production in AS patients. miR-221 and let-7i were also associated with disease activity of lumbar spine (as calculated by BASRI) in AS. Huang et al. (2014) found that significant higher expression of miR-29 in PBMCs of AS patients, although not correlated to disease activity, could be used as a useful diagnostic biomarker in new bone formation. The same authors also found that the mRNA levels of miR-29a, *DKK-1*, β-catenin and *RUNX2* were significantly higher whereas that of *GSK-3b* was significantly lower in AS patients (Huang et al., 2017; Huang et al., 2019). These data imply that miR-29 might become a useful marker for new bone formation in AS patients as evidenced by its ability to regulate *DKK-1* in *Wnt* signaling pathway.

Fogel et al. (2019) investigated the miR expression in both $CD14^{+}$ monocytes and $CD4^{+}$ T lymphocytes from AS patients. The group found downregulated miR-361-3p, miR-223-3p, miR-384, and miR-16-5p in monocytes and upregulated miR-16-1-3p, miR-28-5p, miR-199a-5p, and miR-126-3p in T lymphocytes that might contribute to AS pathophysiology. Li X et al. (2019) reported elevated expression of miR-17-5p, miR-27a, miR-29a and miR-126-3p in PBMCs of axial SpA, which might be regarded as useful diagnostic markers in AS. Furthermore, Yang et al. (2019) provided evidence that miR-335-5p, miR-27a and miR-218 would predispose syndesmophyte formation in AS patients. Recently, Ni and Leng, 2020 have found that miR-495 in PBMC, whole blood, and serum is downregulated because its promoter region is highly methylated in AS patients. Besides, the miR-495 expression is negatively associated with programmed cell death protein 10, *PDCD10*. This may indicate *PDCD10* expression can be targeted by miR-495 in AS. Bioinformatic analyses and signaling pathway studies have revealed that miR-495 can down-regulate β-catenin and TGF-β1. The intracellular ncRNA expressions in immune cells (PBMCs, T cells or monocytes), their modes of action and clinical applications in AS are summarized in **Table 1**.

The extracellular ncRNAs may include those in circulatory tissue fluid (serum, plasma, etc.) and tissue-derived (exosomes) ones. Qian et al. (2016) unraveled that serum miR-146a and miR-155 were significantly upregulated in AS. Moreover, the serum level of miR-155 is associated with disease activity and the severity of thoracolumbar kyphosis secondary to AS. Prajzlerová et al. (2017) found that miR-625-3p can reflect disease activity in AS with spinal involvement. Moreover, miR-29a-3p, miR-146a-5p and miR-222-3p are involved in extracellular matrix formation and inflammation, and are associated with spinal changes and disease activity (BASDAI) in AS patients. These dysregulated miRs are also suggestive of their potential as biomarkers for disease progression. Perez-Sanchez et al. (2018) unclosed that higher expression levels of miR-146a-5p, miR-125a-5p, miR-151a-3p and miR-22-3p and lower expression levels of miR-150-5p and miR-451a were present in the AS plasma. Bioinformatic analysis has revealed that these six miRs target proinflammatory and bone remodeling genes. Besides, miR-125a-5p, miR-151a-3p, miR-150-5p and

**TABLE 2 |** Extracellular (serum, plasma) ncRNA expressions, their modes of action and clinical applications in patients with AS.

| Source | ncRNA | Mode of action | Biomarker | References |
|---|---|---|---|---|
| Serum | miR-146a↑<br>miR-155↑ | | Disease activity and kyphosis | Qian et al. (2016) |
| | miR-625-3p | | Disease activity and<br>Spine involvement | Prajzlerová et al. (2017) |
| Serum | miR-29a-3p<br>miR-146a-5p<br>miR-222-3p | Extracellular matrix formation and inflammation | Disease activity and disease progression | |
| Plasma | miR-146a-3p↑<br>miR-125a-5p↑<br>miR-151a-3p↑<br>miR-22-3p↑<br>miR-150-5p↓<br>miR-451a↓ | Target inflammatory and bone remodeling | Diagnostic and new bone formation | Perez-Sanchez et al. (2018) |
| Plasma (Mexico) | lncRNA let-7↑<br>miR-16↓ | MMP-1↑, CRP↑ | Diagnostic<br>Disease activity | Reyes-Loyola et al. (2019) |
| Plasma | lncRNA $MEG_3$↓<br>miR-146a↑ | IL-1β↑, IL-6↑<br>TNF-α↑ | Disease activity | Li et al. (2020b) |
| Plasma (Egypt) | miR-125a↑<br>miR-451a↓ | Structural damage | Diagnostic, prognostic and disease burden | Fotoh et al. (2020) |

*IL, interleukin; MMP, matrix metalloprotein; CRP, C-reactive protein; TNF, tumor necrosis factor.*

miR-451a expression are related to the presence of syndesmophytes in AS. Accordingly, these six plasma miR signature can become gorgeous non-invasive biomarkers for AS diagnosis. Recently, Reyes-Loyola et al. (2019) assessed plasma levels of ncRNAs in Mexican AS patients. They found plasma lnc let-7 was higher in patients and might serve as a diagnostic biomarker in Mexican AS patients. On the other hand, plasma miR-16 level is inversely correlated to ASDAS-CRP score and MMP-1 level, thus, serving as disease activity marker. Li Y et al. (2020) have further found that plasma lncRNA MEG3 is downregulated and negatively correlated to the levels of IL-1β, IL-6 and TNF-α in AS patients, and can block the inflammatory response of the immune cells in AS patients. Conversely, plasma miR-146a was upregulated and positively correlated to the proinflammatory IL-1β, IL-6 and TNF-α. The authors also clarified that over expression of miR-146a could revert the inhibitory effect of abnormal MEG3 expression on inflammatory cytokines. These data imply that lncRNA MEG3 plays an anti-inflammatory role *via* targeting miR-146a and thus can provide a new potential therapeutic role for AS treatment. In further elucidation of the molecular mechanism for the therapeutic potential of lncRNA MEG3, Ma et al. (2020) found that the expression levels of *MEG3* and sclerostin (*SOST*) are decreased but lncRNA let-7 is increased in AS patients. Their results confirmed that *MEG3* can interact with (sponge) let-7i in AS fibroblast and promotes *SOST* expression to restrain the progression of AS. This would provide a new treatment modality in AS. Fotoh et al. (2020) discovered a higher expression of miR-125a and a lower expression of miR-451a in the plasma from active Egyptian AS patients. Interestingly, both miRs were able to distinguish AS patients with a structural damage and could be used as sensitive diagnostic, prognostic and disease burden biomarkers for AS patients. The extracellular (serum or plasma) ncRNA expression, their modes of action and clinical application in AS patients are listed in **Table 2**.

In summary, these aberrant soluble extracellular ncRNAs expression in AS patients can be divided into three categories in clinical practice as shown below;

1) Biomarkers for disease diagnosis: lncRNA let-7, miR-146a-3P, miR-125a-5P, miR-151a-3P, miR-22-3P, miR-150-5P, and miR-451a.
2) Biomarkers for disease activity: miR-146a, miR-155, miR-625-3P, miR-29a-3P, miR-146a-5P, miR-222-3P, and lncRNA-MEG.
3) Biomarkers for both diagnosis and disease activity: miR-16a, miR-146a, miR-125a, and miR-451a

## The Role of ncRNAs in Enthesitis and Ligament Inflammation

Enthesis is the characteristic sites where pathological processes occur in AS patients, causing enthesitis. To reflect ossifications more realistically in the ligaments of AS patients, the epigenetic regulation of the cultured ligament-derived fibroblasts were analyzed for osteogenic differentiation. Zhang et al. (2017) compared miR, lncRNA and mRNA profiles in hip joint ligament tissues from AS patients. The authors identified that miR-17-5p and miR-27b-3p could increase the potentials of osteogenic differentiation in ligament fibroblasts of the hip joint. Tang et al. (2018) isolated ligament fibroblasts from AS patients and induced them to differentiate into osteoblast (OB). During osteogenic differentiation, miR-124, β-catenin, osteorix and *RUNX2* expression gradually increased, while that of GSK-3β gradually declined. Zhao et al. (2020) have demonstrated that miR-204-5p can negatively regulate *NOTCH* 2 expression in osteogenic differentiation in the ligament fibroblasts derived from AS patients *in vitro*. These results may provide a

**TABLE 3 |** Intracellular expressions of ncRNAs in ligament-derived fibroblast, osteoblasts, bone marrow-derived mesenchymal stem cells (MSCs) and human fibroblast-like synovial cells (HFLSs) from AS patients.

| Source | ncRNA | Mode of action | References |
|---|---|---|---|
| Fibroblast | miR-17-5p↑<br>miR-27b-3p↑ | Osteogenic differentiation↑ | Zhang et al. (2017) |
| Fibroblast | miR-124↑ | β-catenin↑<br>Osterix↑<br>RUNX2↑<br>GSK-3β↓ | Tang et al. (2018) |
| Fibroblast | miR-204-5p↑ | Notch 2 expression↓<br>Ostoegenic differentiation↑ | Zhao et al. (2020) |
| Murine AS osteoblast | miR-96↑ | IL-6↑<br>TNF-α↑<br>IL-10↑<br>*Wnt* signaling↑<br>ALP↑<br>Calcium↑<br>Osteoblast viability↑<br>*SOST*↑<br>New bone formation↑ | Ma et al. (2019) |
| MSC | miR-4284↓ | Osteoclastogenesis↓<br>Osteogenesis↑<br>CXCL5↑ | Liu W et al. (2019) |
| HFLS | miR-495↑<br>DVL-2↓ | Inflammation↓<br>IL-1↓<br>IL-6↓<br>TNF-α↓<br>Osteoprotegerin↑<br>Wnt/β-catenin/*RUNX2*↑<br>RANKL↑ | Du et al. (2019) |

*RUNX, Runt related transcriptional factor; TNF, tumor necrosis factor; IL, interleukin; ALP, alkaline phosphotase;* Wnt*, wingless and Int-1;* SOST*, sclerostin gene; CXCL, ligand for cysteine-X-cysteine chemokine; RANKL, Receptor activator of nuclear factor kappa-B ligand; GSK, glycogen synthase kinase. DVL, segment polarity protein dishevelled homolog.*

therapeutic basis for the effective treatment for patients with AS. In the OBs isolated from murine model of AS, Ma et al. (2019) found that miR-96 expressed at a high level while sclerostin (*SOST*) expressed at a low level. Actually, miR-96 was observed to target and negatively regulate *SOST*. Furthermore, the over-expressed miR-96 activated the *Wnt* signaling pathway and increased proinflammatory cytokines (IL-6, TNF-α, and IL-10), ALP activity, calcium nodule formation and OB viability. These results indicated that the overexpression of miR-96 can enhance OB differentiation and subsequent bone formation in AS mice *via Wnt* signaling pathway. In contrast (Liu W. et al., 2019), showed that mesenchymal stem cells (MSCs) derived from AS patients exhibited a strong capacity to inhibit osteoclastogenesis and secreted more CXCL5. Further studies showed that down-regulation of miR-4284 in AS-MSCs resulted in increased CXCL5, indicating that osteoclastogenesis may be markedly suppressed *via* miR- 4284/CXCL5 axis. By experiments with human fibroblast-like synovial cells (HFLSs) isolated from AS tissues, Du et al. (2019) showed that miR-495 and dishevelled 2 (DVL-2) molecule were negatively correlated with each other in AS. Both molecules can inhibit inflammation by down-regulating proinflammatory cytokines, IL-1, IL-6 and TNF-α and facilitate bone differentiation by up-regulating osteoprotegerin (OPG) and RANKL levels in HFLS. Besides, miR-495 and siRNA, si-DVL-2, enhanced expression of *wnt3a*, *RUNX2* and β-catenin and reduced β-catenin phosphorylation. Collectively, miR-495 depresses inflammatory responses and promotes bone differentiation of HFLSs *via* Wnt/β-catenin/RUNX-2 pathway by targeting DVL-2.

In addition to miRs and lncRNAs, circular RNAs (circRNAs) are a particular class of endogenous ncRNAs with a covalently closed circular structure (Hsu and Coca-Prados, 1979; Hansen et al., 2013; Memczak et al., 2013). Different from the linear RNAs, circRNAs lack free 3′-end poly A tail and 5′-end cap which prevent them from being degraded by nucleic acid endonuclease (Qu et al., 2015). Accordingly, the closed circular structure of circRNAs makes them extremely stable in the extracellular milieu and able to regulate the expression of target miRs by their sponge effects in human diseases (SanterBar and Thum, 2019; Wang et al., 2019). Kou et al. (2020) analyzed the circRNA expression profile of the spinal ligament tissue in AS patients. The authors found 57 circRNAs were up-regulated and 66 were down-regulated which were mainly involved in the regulation of biological processes of peptidyl-serine phosphorylation and immune system relevant to AS pathogenesis. In addition, the circRNA-miR interactions may provide new clues for understanding the mechanisms, diagnosis and potential molecular targets for the treatment in AS patients.

Another interesting findings by Tabrizi et al. (2017) were from the investigations on expression levels of the maturing microprocessor complex of miR in PBMCs from AS patients. It is believed that major enzymes responsible for miR maturation are Dicer, Drosha, and Drosha assistant DGCR8. Their results revealed that both Dicer and DGCR8 mRNA expression were

down-regulated whereas Drosha mRNA expression was not influenced in AS. These data suggest that the down-regulated miR maturation components may probably contribute to the pathogenesis of AS. The intracellular expression of ncRNAs in ligament-derived fibroblasts of AS patients and their modes of action are summarized in **Table 3**.

## CONCLUSION AND PERSPECTIVES

Genetic and environmental factors intriguingly interact with each other in affecting epigenetic modifications in patients with AS. More than 100 genes have been identified to contribute to AS susceptibility. Among them, HLA-B27 subtypes, polymorphic ERAP, and IL-23R mutation seem to be significantly associated. It is also conceivable that the microtrauma in the entheses may trigger the onset of AS. Besides, the arthritogenic peptides (misfolded HLA-B27 antigen and HLA-B27 homodimer), gut dysbiosis, abnormal intestinal metabolomic products, and immune plasticity can also induce soft tissue, articular and extra-articular inflammation. The inflammation-induced osteoporosis, the subsequent osteogenesis, and finally the new bone formation may cause skeletal disability. Extra-musculoskeletal manifestations of AS include mainly anterior uveitis, aortitis and interstitial fibrosis of the upper lungs. The florid immune dysfunctions of innate and adaptive immune responses resulted from the abnormal IL-23/IL-17 axis is paramount crucial. Nevertheless, the aberrant presentations of up-stream epigenetic regulatory mechanisms are the culprits of similar importance for the abnormal immune regulation in AS. That is to say, these abnormalities of ncRNAs (i.e., miRs, lncRNAs and circRNAs) and microRNA maturing microprocessor complex (Dicer and Drosha) in PBMCs, serum/plasma and tissues play a crucial role in disrupting innate and adaptive immune responses implicated in multiple pathological processes in patients with AS. Although, the molecular mechanisms for these characteristic pathogenic events including enthesitis, osteoporosis, osteogenesis, anterior uveitis, aotitis and interstitial lung fibrosis have been discussed in detail above, the real causes of AS pathogenesis remain elusive. Some perspective investigations helpful for the understanding of this complicated epigenetic regulation are suggested:

1) A molecular basis for the induction of gut dysbiosis by HLA-B27 subtypes with its subsequent abnormal epigenetic regulation and immune dysregulation should be clarified.
2) Clinically applicable serum ncRNAs as biomarkers for measuring disease activity and assessing therapeutic response in AS patients should be identified on the level of high sensitivity and specificity, compared to the nonspecific serum CRP and stool calprotectin currently available.
3) The role of aberrant ncRNA expression with subsequent abnormal immune responses in stressful enthesis induced by denatured hyaluronan-I needs to be explored.
4) Molecular and cellular bases for the absence of rheumatoid factors in AS patients that may be relevant to deranged ncRNA expression need to be unveiled
5) The production of anti-CD74 autoantibody with its immunopathogical roles in AS patients that may be relevant to aberrant ncRNA expression should also be delineated.

## AUTHOR CONTRIBUTIONS

C-LY and C-YT supervised the writing of the manuscript. C-YT and H-TL prepared the manuscript and wrote the draft together. T-HL and S-CH prepared the figure drafts. C-YS, C-HW, H-TL, C-HL, C-CL, Y-MK, Y-SS, K-JL, and C-TC actively participated in the discussions and suggestions for the manuscript. All authors have read and agreed the final version of the manuscript.

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# Genome-Wide DNA Methylation Profiling in CD8 T-Cells and Gamma Delta T-Cells of Asian Indian Patients With Takayasu Arteritis

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**Background:** Takayasu's Arteritis (TA) is a chronic inflammatory disease that affects aorta and its main branches at their origin. Genetic, pathological and functional studies have shown that CD8 and Gamma delta (γ/δ) T-lymphocytes are involved in inflammatory processes in affected regions of arteries causing vascular damage. The molecular function of these lymphocytes remains unclear and currently no epigenetic studies are available in TA. We primarily performed genome wide methylation analysis in CD8 T cells and γδ T cells of patients with TA and compared with healthy controls.

**Methods:** We recruited 12 subjects in each group namely TA patient and healthy controls. Blood samples were collected after obtaining informed written consent. CD8 T cells and γδ T cells were separated from whole blood. DNA extracted from these cells and were subjected to bisulfite treatment. Finally, bisulfite treated DNA was loaded in Infinium Methylation EPIC array. Bioinformatics analysis was used to identify differential methylation regions which were then mapped to genes.

**Results:** Interleukin (IL)-32 and Lymphotoxin-A were genes significantly hypomethylated in CD8 T-cells. Anti-inflammatory cytokine genes, *IL-10*, *IL-1RN* and *IL-27* were hypomethylated in γδ T cells of TA patients as compared to healthy controls. Gene enrichment analysis using Gene Ontology (GO) database and Kyoto Encyclopaedia of Genes and Genomes (KEGG) identified that genes involved in T-cell receptor signalling pathways were hypomethylated in CD8 T-cells and hypermethylated in γδ T cells of TA patients.

**Conclusion:** CD8 T-cells might play a major role in immunopathogenesis of inflammation in TA, whereas γδ T cells may play a regulatory role.

**Keywords: DNA methylation, epigenetics, large vessel vasculitis, Takayasu's arteritis, Interleukin-32**

- This maiden genome-wide DNA-methylation study in TA revealed hypomethylation of genes, *IL-32* and *LTA* in CD8+T-cells
- Anti-inflammatory cytokine genes *IL-10*, *IL-1RN* and *IL-27* were hypomethylated in γδ+ T-cells of TA patients
- Genes involved in T-cell receptor signalling pathways were hypomethylated in CD8 T-cells of TA patients

## INTRODUCTION

Takayasu's Arteritis (TA) is an idiopathic chronic inflammatory disease that affects the aorta and its main branches at origin. TA is predominantly seen in women of reproductive age group and the onset is before the age of 40 in majority of cases. Aetiology of the TA is unknown. Pathophysiology of TA involves infiltration of leukocytes in vascular tissues involving all layers of large arteries. It is characterized by adventitial thickening, focal leukocyte infiltration of tunica media and intimal hyperplasia.

CD8 T-cells are in excess both in peripheral blood and inflamed vessels of patients with TA compared to giant cell arteritis (GCA) (Kurata et al., 2019; Matsumoto et al., 2019). In fact, even after treatment with biologic disease-modifying antirheumatic drugs (DMARDs), CD8 T-cells were not lowered in TA. This is in contrast to Th1, Th17, and Tfh cells, all of which are shown to be reduced in number after such therapy. High levels of CD8 T-cells are also reported to be associated with relapse in TA (Matsumoto et al., 2019). Both HLA-DR expressing CD8 and $CD4^+$ T cells were increased in peripheral blood of patients with TA (Nityanand et al., 1997). Several other studies have shown that $CD8^+$ T cells are involved in pathogenesis of TA by secreting specific cytokines and chemokines (Uppal and Verma, 2003; Régnier et al., 2020).

**TABLE 1 |** Clinical details of patients with TA.

| Parameter | *n* = 12 |
|---|---|
| Gender (Male: Female) | 2:10 |
| Median age in years (range) | 26 (18–39) |
| Median duration of symptoms in months (range) | 18 (0–48) |
| Angiographic types n(%) | |
| Type I | 1 (8.3%) |
| Type IIb | 1 (8.3%) |
| Type III | 1 (8.3%) |
| Type IV | 1 (8.3%) |
| Type V | 8 (66.6%) |
| Median ESR in mm/1st hour (range) | 42.8 (6–75) |
| Median CRP mg/dl (range) | 31.6 (3–87) |
| Median ITAS 2010 (range) | 8.6 (0–17) |
| Median ITAS -CRP(range) | 10.4 (2–20) |
| Median DEI.Tak (range) | 9.5 (4–13) |
| Treatment details | N (%) |
| Treatment naïve | 8 (66.7%) |
| Glucocorticoids | 2 (16.7%) |
| Defaulters of treatment | 2 (16.7%) |
| Biological DMARDs | Nil |

γ/δ T cells represent 1-5% of peripheral blood T cells. Seko et al. found that γ/δ T cells contribute around 30% of leukocytes infiltrating aortic tissues of TA (Seko et al., 1996). Aortic tissues responding to unknown stimulus express 65 kDa heat-shock protein, which in turn induce expression of MHC- I–related chain A (MICA) on the surface of vascular smooth muscle cell (VSMC). MICA on VSMC is recognized by NKG2D receptors in γ/δ T cells and $CD8^+$ T cells, which secrete cell granules containing perforin and interferon resulting in initiation of vascular inflammation (Arnaud et al., 2011). Though the above mentioned published data demonstrated involvement of CD8 T cells and γ/δ T cells in TA, their pathogenic roles are not yet fully understood. The current study aimed to explore genome wide DNA methylation changes in CD8 T cells and γδ T cells of patients with TA in comparison with healthy individuals as controls.

## METHODS

### Patients and Controls

Twelve patients satisfying ACR 1990 criteria for TA were recruited from Rheumatology clinics of Christian Medical College, Vellore (Arend et al., 1990). Age and sex matched healthy subjects also recruited as controls for the study. Participants were recruited between September 2015 and January 2016 after obtaining written informed consent. The

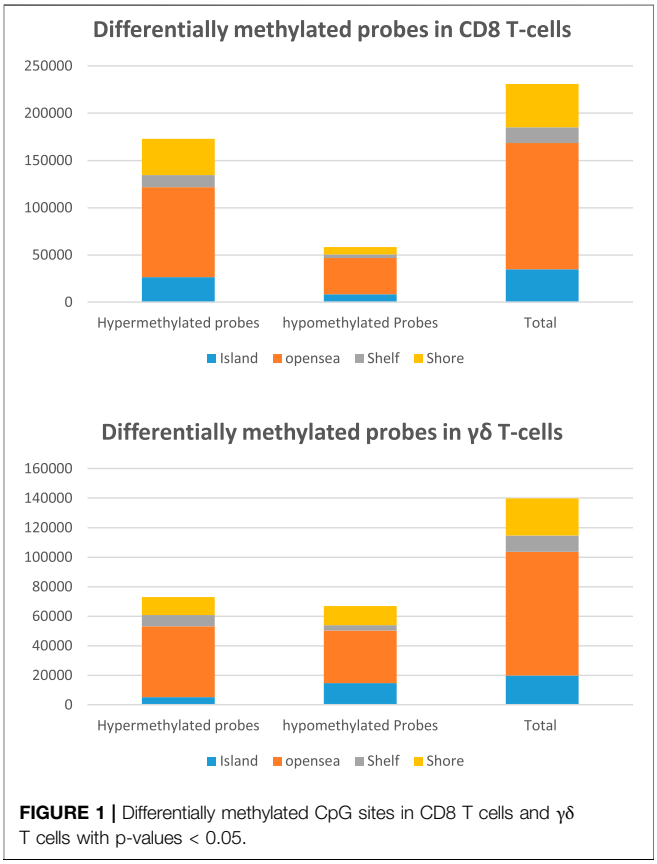


**FIGURE 1 |** Differentially methylated CpG sites in CD8 T cells and γδ T cells with p-values < 0.05.

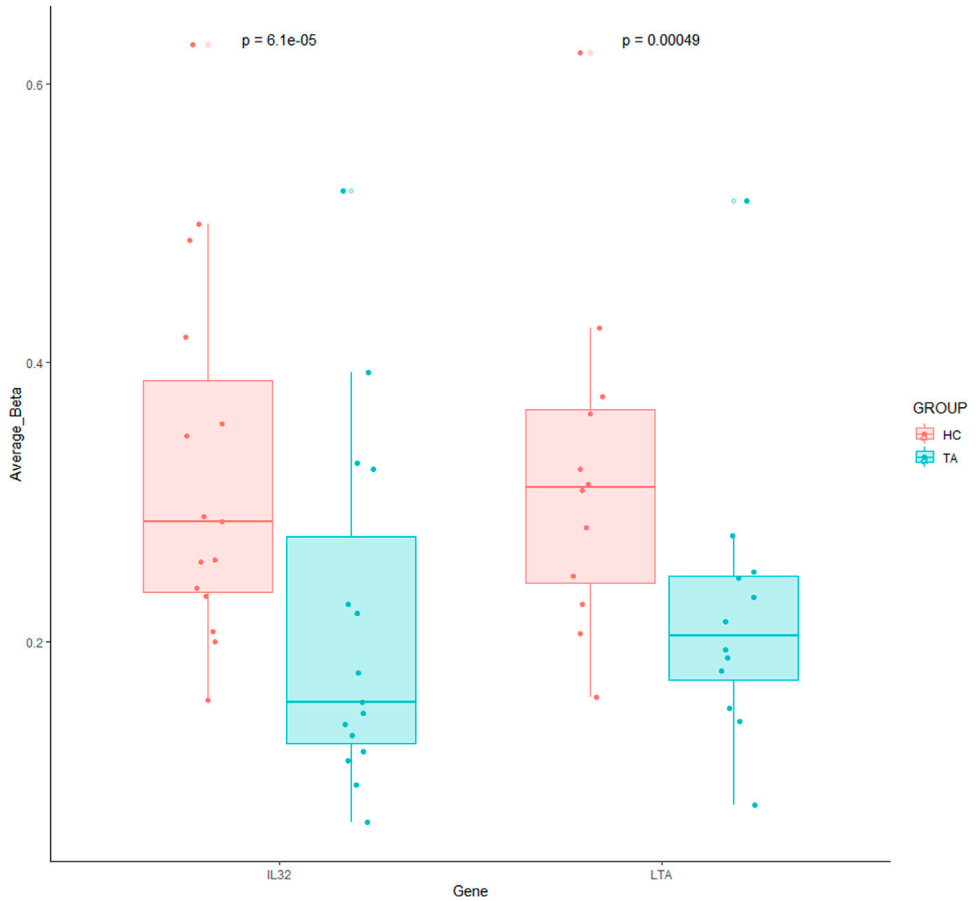


**FIGURE 2 |** Significantly hypomethylated genes in CD8 T-cells of patients with TA. Each dot indicates each CpG probe measured within this gene. A Wilcoxon matched paired test was used to compare between the groups against each CpG probe.

study followed the tenets of the Declaration of Helsinki and was approved by the Institutional review board of Christian Medical College, Vellore.

## Cell Separation, DNA Extraction, and Bisulfite Conversion

Twenty ml of whole blood were collected from each participant in anticoagulated vacutainer tubes. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation using Ficoll-PaqueTM Plus (Catalogue no. 1033378, GE Healthcare). CD8 and γδ T cells were separated from PBMC using magnetic labelling based separation methods. CD8 microbeads (Catalogue no. 130-045-201, Miltenyi Biotec, CA, USA) was used for separation of CD8 T cells by negative selection method, followed by use of Anti-TCR γδ microbead kit (catalog no. 130-050-701, Miltenyi Biotec, CA, USA) for separation of γδ T cells. DNA was extracted from these cells using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. DNA samples were stored at −80°C until processing of methylation profiling was done.

## DNA Methylation Profiling

Genomic DNA samples of both CD8 and γδ T cells from each participant were subjected to bisulfite treatment using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). Infinium Methylation EPIC arrays (Illumina, San Diego, CA) were used to assess the genome-wide DNA methylation levels. This chip array allows for the interrogation of over 850,000 methylation sites within the entire genome covering CpG islands, genes, and enhancers, DNase hypersensitive sites and miRNA promoter regions. All array handling, sample hybridization, and array scanning were performed at a commercial service provider lab (M/s. Medgenome labs Pvt Ltd., Bengaluru, India). The service provider was blinded to the source of the samples. Raw idat and sample annotation files were received from a service provider for bioinformatics analysis.

## Bioinformatics Analysis for Methylation Data

Data from methylation chip array were analysed in R software ChAMP package (Morris et al., 2014). Raw idat files and sample

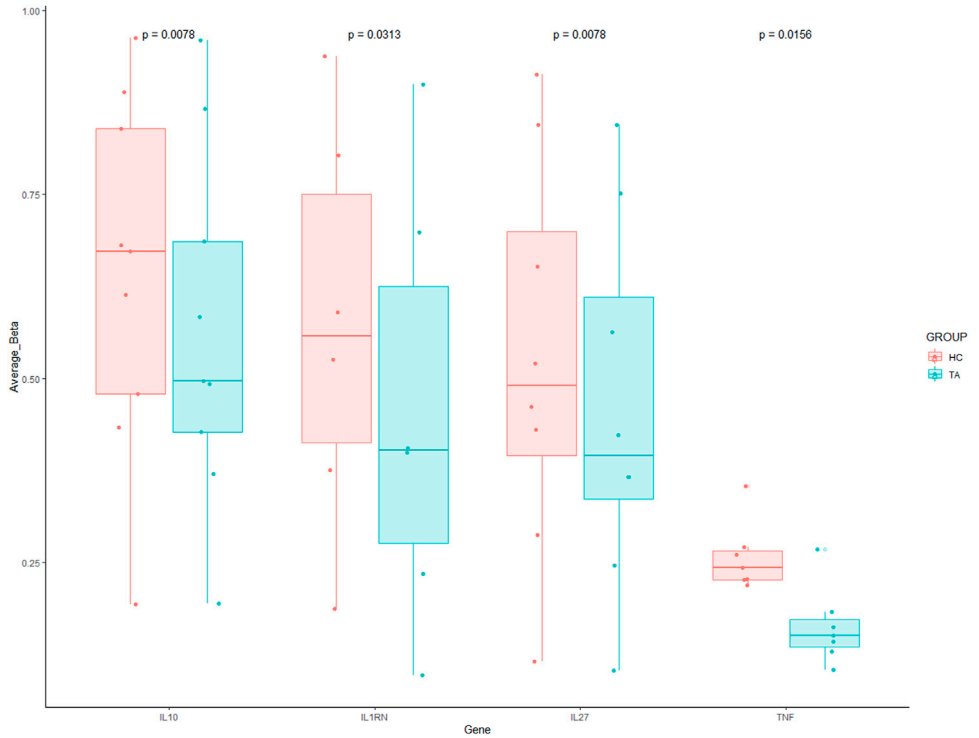


**FIGURE 3 |** Significantly hypomethylated genes in γδ T cells of patients with TA. Each dot indicates each CpG probe measured within this gene. A Wilcoxon matched paired test was used to compare between the groups against each CpG probe.

annotation were uploaded in ChAMP using champ.import function. Default filter function was used to remove low quality probes. This includes removal of each probe having *p*-value above 0.01, non-CpG probes, all SNP-related probes and all probes located on chromosome X or Y. Normalisation of data was done for adjustment of bias in the type-II probe (**Supplementary Figure S1** for before and after normalisation of probes). Samples were run in chip array on different batches. For removal of the batch effect, correction was performed in ChAMP package. Differential methylation probes (DMP) and Differential methylation region (DMR) analysis were performed between TA and healthy controls. DMR analysis were performed separately in the DMRcate package from the Bioconductor platform in R, as this function within the ChAMP pipeline was not functional (Peters et al., 2015). Beta ($\beta$) value is the ratio of methylated intensity and the overall intensity values. Normalised Beta values of each probe were extracted from ChAMP pipeline and loaded in DMRcate package to identify quantitative alteration in DNA methylation levels between cases and controls.

Gene enrichment analysis were performed for genes significant in DMR using cluster Profiler package in R with *p*-value cutoff of <0.05(Yu et al., 2012). Significant genes from DMR were used after converting gene symbols to entrez gene id in the online tool DAVID (https://david.ncifcrf.gov). Gene Ontology (GO) enrichment analysis was performed to identify over-represented GO terms with combined domains of biological processes, molecular function and cellular components. KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathway gene set enrichment (GSE) analysis was performed by employing a hypergeometric test within cluster Profiler package. Significant gene enrichment were visualised by network and pathway based plots using enrichplot and pathview packages respectively in R Bioconductor tool.

# RESULTS

## TA Patient and Control Characteristics

Demographic and clinical details of TA patients are provided in **Table 1**. Median age of healthy controls was 23 (15-48) years and Female: male ratio was 10:2. Both age and sex ratio were matched for cases and controls.

## Differential Methylated CpG Sites in TA

Total 850 K probes were available in the chip array. After quality control and filtering of probes nearly 700 K probes were available for analysis for both CD8 T cells and γδ T cells to examine differential methylation CpG probes between TA and healthy control. Number of significantly hypermethylated and hypomethylated CpG sites for CD8 T cells and γδ T cells are depicted in **Figure 1**.

## Differential Methylated Regions in TA

Nearly 34000 regions were differentially methylated in CD8 T cells between TA and controls which comprised 221665 CpG sites (probes) with a threshold FDR <0.05, including

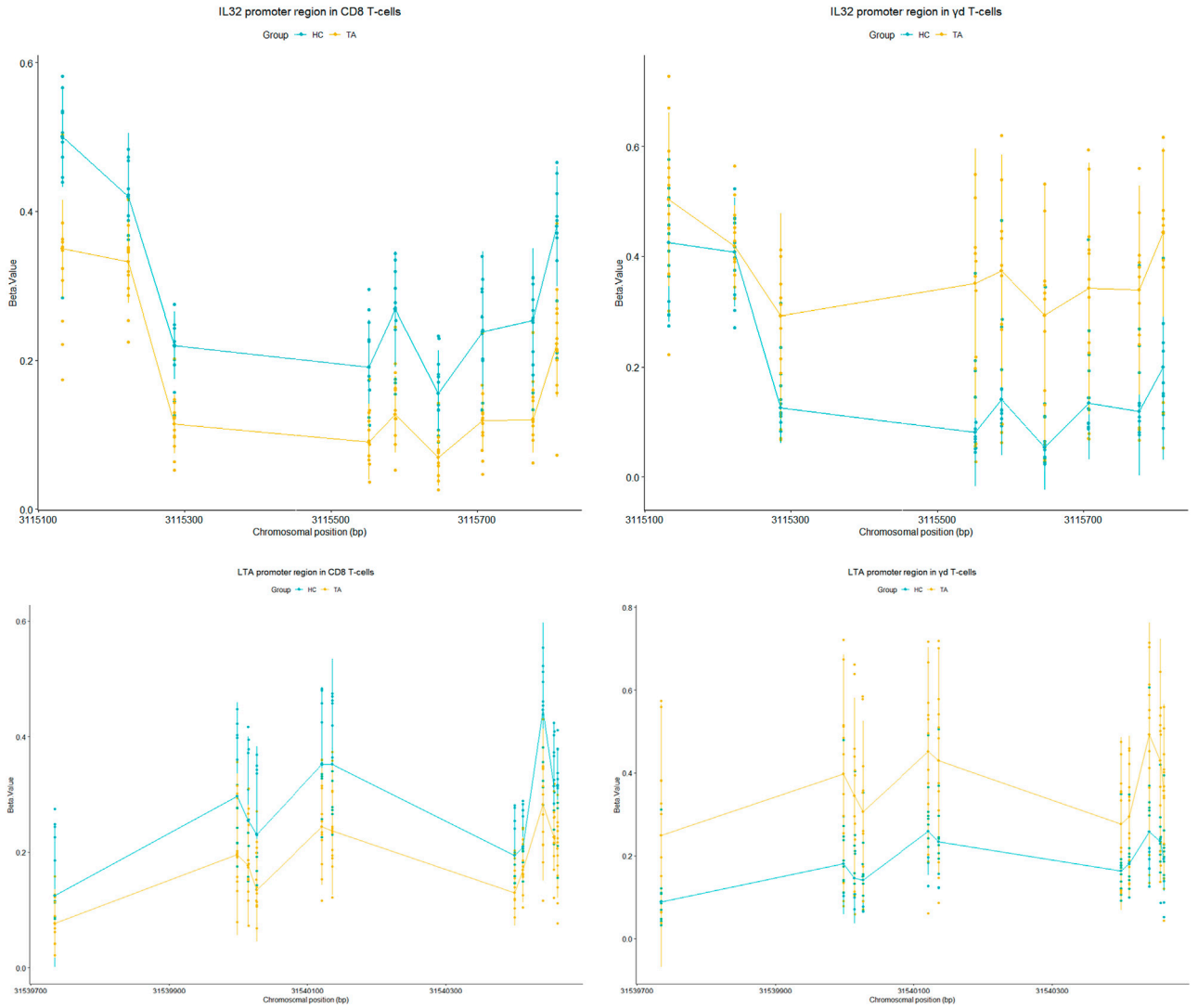


**FIGURE 4 |** Tukey plot showing significantly differentially methylated regions (CpG sites) in promoter region of *IL-32* (chr 16: 3114847–3115809) and *LTA* (chr 6: 31539539- 31541349) genes in CD8 T cells and γδ T cells of TA as well as healthy controls. Both genes were hypomethylated in CD8 T cells and hypermethylated in γδ T cells of TA as compared to healthy controls.

27243 hypermethylated regions and 7104 hypomethylated regions. In γδ T cells, 24088 regions were differentially methylated comprising of 115427 CpG sites, which included 10834 hypermethylated regions and 13255 hypomethylated regions. Top 20 genes containing hypermethylated and hypomethylated regions for CD8 T cells and γδ T cells were listed in **Supplementary Tables S1, S2** respectively.

Hypomethylated regions are associated with increased gene expression, especially in pro-inflammatory cytokine genes, which are of interest in inflammatory diseases. In CD8 T-cells, Interleukin-32 (*IL-32*) and Lymphotoxin -Alpha (*LTA*) were significantly hypomethylated genes in our TA patients compared to healthy controls (**Figure 2**). *TNF-α*, *IL-10* and *IL-27* genes were significantly hypomethylated in γδ T cells of our TA patients (**Figure 3**).

We, therefore, observed differential methylation of regions between CD8 and γδ T cells in patients with TA. *LTA* and *IL-32* were significantly hypomethylated in CD8 T-cells, whereas these genes were hypermethylated in γδ T cells on TA patients (**Figure 4**). This shows cell specific epigenetic changes in patients with TA. Cell specific methylation pattern is better visualised in heatmap plot (**Figure 5**). *BCL6* and *IL21R* are also hypomethylated in CD8 T-cells but hypermethylated in γδ T cells. Inversely, *CCRL2* and *CIITA* genes are hypomethylated in γδ T-cells while hypermethylated in CD8 T-cells in TA patients.

Our study also revealed hypomethylation of genes involved in transcription factors namely, *TBX21* and *EOMES* in CD8 T cells and γδ T cells (**Figure 5**), However, significant hypomethylation for *TBX21* was found only in CD8 T cells and the same for

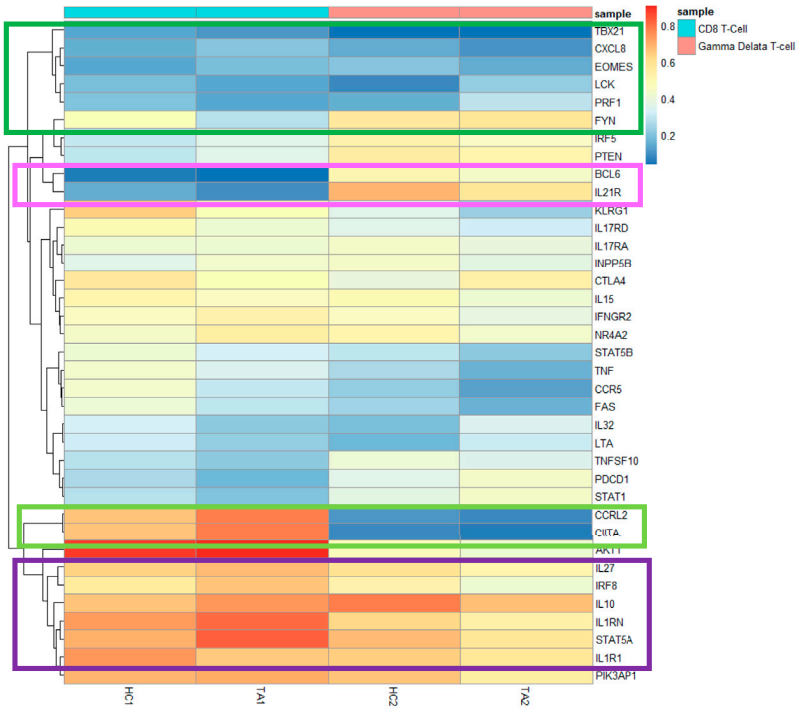


**FIGURE 5 |** Heatmap plot showing hypomethylated genes in CD8 T cells and γδ T cells of TA patients as compared with healthy controls. *BCL6* and *IL21R* were hypomethylated genes in only CD8 T-cells (marked in pink colour box). *CCRL2* and *CIITA* genes were hypomethylated in only γδ T-cells (marked in light green colour box).

*EOMES* was documented only in γδ T cells of our patients with TA. Genes of proteins involved in T-cell receptor (TCR) signalling and perforin-1 were hypomethylated in CD8 T cells at higher levels than γδ T cells. In contrast, genes of anti-inflammatory cytokines, *IL-10*, *IL-1RN*, *IL27* and transcription factor *STAT5A* were hypomethylated at lower levels in CD8 T cells compared to γδ T cells of patients with TA (**Figure 5**).

## DMR Associated Functional Pathways in TA

Having established sets of genes from DMR, we identified function pathways using GO resources and KEGG database. A list of significant pathways in the GO and KEGG database for CD8 T cells and γδ T cells were mentioned in **Supplementary Tables S3, S4** respectively.

Gene ontology (GO) analyses in CD8 T cells revealed neutrophil mediated immunity, neutrophil activation, neutrophil degranulation, and lymphocyte degranulation pathways were hypermethylated in TA, whereas ribosome structure, viral transcription and viral gene expression were hypomethylated (**Supplementary Figure S2**). These findings were confirmed in KEGG enrichment analysis that genes involved in ribosome and T-cell receptor signalling were hypomethylated (**Supplementary Figure S2**) in CD8 T cells of our TA patients.

GO analyses in γδ T cells of TA showed opposite patterns compared to CD8 T-cells. Myeloid cell activation, neutrophil activation, neutrophil degranulation, lymphocyte degranulation were hypomethylated and TCR signalling pathway, antigen-receptor mediated signalling, T-cell activation, T-cell differentiation and adaptive immune response pathways were hypermethylated (**Supplementary Figure S3**). Again, this findings was confirmed in KEGG GSE analysis that TCR signalling pathway, Th17 differentiation and antigen processing and presentation were hypermethylated in γδ T cells of patients with TA (**Supplementary Figure S3**).

Genes involved in statistically significant pathways identified by KEGG analysis for both CD8 T cells and γδ T cells were visualised in network plot (**Figure 6**). Hypomethylated and hypermethylated genes involved in TCR signalling were visualised in pathway plot (**Supplementary Figure S4**).

## DISCUSSION

To our knowledge, this is the first study describing the methylation changes in CD8 T cells and γδ T cells of patients with TA in comparison with healthy controls. Our study showed *IL-32* and *LTA* genes were significantly hypomethylated in CD8 T-cells from patients with TA. Also *TNF-α*, *IL-10* and *IL-27* genes were significantly hypomethylated in γδ T cells of TA patients. Another important finding is that genes involved in T-cell receptor signalling were hypomethylated in CD8-T cells from patients with TA.

In our study, the promoter region of *IL-32* gene is significantly hypomethylated in patients with TA compared to healthy controls.

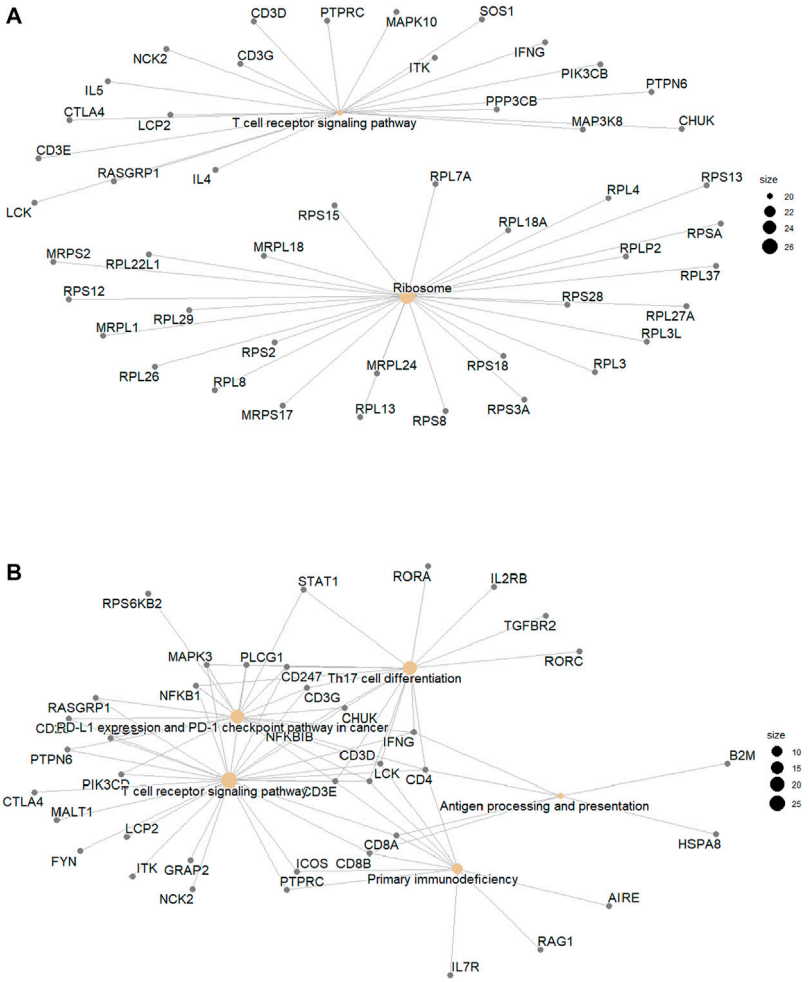


**FIGURE 6 | (A)**. Hypomethylated genes in CD8 T cells and **(B)**. Hypermethylated Genes in γδ T cells by KEGG pathways.

Increased *IL-32* expression and serum levels has been reported in patients of GCA and Anti-Neutrophilic Cytoplasmic Autoantibody (ANCA) associated vasculitis(Ciccia et al., 2011; Bae et al., 2012; Krajewska Wojciechowska et al., 2019). In GCA, *IL-32* expression is found in inflammed vessels and it co-localises with Th1 lymphocytes (Ciccia et al., 2011). *IL-32* expressed by CD8 T-cells was reported to be associated with history of Polymyalgia Rheumatica (PMR) and abnormal neutrophil count in patients with GCA (De Smit et al., 2018). *IL-32* induces dendritic cells to secrete the chemokine *RANTES* (also known as CCL5), which in turn recruits activated T-cells expressing *CCR5* (**Figure 5**) to inflammatory sites and thereby causes vascular dysfunction (Son et al., 2014; Mikolajczyk et al., 2016). Again, in our study *CCR5* is also hypomethylated in CD8 T-cells of TA. This shows IL-32 might contribute to activation and recruitment of CD8 T-cells in TA.

In the present study, another important cytokine gene LTA encoding lymphotoxin-A, previously known as TNF-beta is significantly hypomethylated in CD8 T cells of patients with TA. An earlier study in GCA showed lymphotoxin expression localised with formation of tertiary lymphoid organs (TLOs) in inflamed arteries (Ciccia et al., 2017). *LTA* gene is shown to be hypomethylated in CD4 T-lymphocytes of patients with primary Sjögren's syndrome (pSS) (Altorok et al., 2014). Again, TLOs were the source of autoreactive lymphocytes in inflamed regions of salivary glands of pSS (Asam et al., 2021). Can this suggest that LTA secreted by CD8 T-cells may be involved in formation of TLOs in inflamed arteries of patients with TA?

In Bechet's disease, another vasculitis involving large vessels in vast majority of them, γδ T cells are shown to secrete TNF-α and CXCL8 causing activation signal and recruitment of neutrophils and monocytes to sites of infection and inflammation (Hasan et al., 2015). This is similar to the findings in our study showing hypomethylation of *TNF-α* and *CXCL8* genes in γδ T cells of TA. In addition, anti-inflammatory cytokines *IL-10*, *IL-1RN* and *IL-27* genes were also hypomethylated. This is again similar to previous findings in patients with BD that reported γδ T cells predominantly as regulatory in nature and secrete lower levels of inflammatory cytokines (Parlakgul et al., 2013).

Seko et al. showed that aortic tissues express 65-kD heat-shock protein (HSP-65), which was recognised by infiltrating killer

lymphocytes resulting in secretion of perforin, which led to vascular cell injury of Takayasu's arteritis (Seko et al., 1994). Chauhan et al. demonstrated this cytotoxic function of lymphocytes to be mediated by interaction of Fas and FasL as well as with the help of secretion of IFN-γ (Chauhan et al., 2006; Chauhan et al., 2007). Our findings concur with the findings of that study as perforin-1(*PRF1*), *Fas* and *IFN-γ* genes were significantly hypomethylated in CD8 T-cells, but not in γδ T cells of TA (**Figure 5**). In addition, we didn't find any hypomethylation of *HSP65* gene; rather it was noted in genes of *HSPA1A*, *HSPA1L*, both expressing 70-kd heat-shock protein (data not shown). Thus CD8 T-cells might be more cytotoxic in nature and involved in vascular cell injury in patients of TA.

On the other hand, Transcription factor T-bet encoded by gene *TBX21* is required for differentiation of effector CD8 T-cells producing INF-γ following encounter with self-antigens (Jackson et al., 2014). But, Eomes expression in γδ T cells leads to differentiation of Th1-like lymphocytes producing IFN-γ (Lino et al., 2017). Thus hypomethylation of *TBX21* and *EOMES* in CD8 T-cells and γδ T cells might be attributed to IFN-γ secreting Th1 like subsets in patients with TA; however, it is difficult to conclude this point at this stage and further confirmation by future studies may be needed. *CTLA4* and *IL-21R* were also hypomethylated genes in patients with TA, which are markers of regulatory and follicular T-cells subsets. This shows that different subsets exist amongst CD8 lymphocytes in TA.

Gene enrichment analysis in CD8 T-cells of TA patients showed hypomethylation of genes involved in T-cell receptor signalling. McKinney et al. demonstrated that TCR signalling is most pronounced in effector-memory ($T_{EM}$) subset of CD8 T-cells in patients with ANCA vasculitis (McKinney et al., 2010). *LCK* and *PRF-1* genes were significantly hypomethylated in CD8 T-cells, whereas these genes were hypermethylated in γδ T cells of TA in this study. LCK is a tyrosine kinase essential for downstream signalling of activated T-lymphocytes. In CD8 T-cells, absence of LCK results in reduced perforin mediate killing, thereby affect its cytotoxic function (Milstein et al., 2011). This shows that activated CD8 T-cells of patients with TA have higher cytotoxic ability as compared to healthy controls.

In the current study, genes involved in TCR signalling pathway and Th17 differentiation were hypermethylated in γδ T cells of TA. Evidence from literature suggest that γδ T cells can produce IL-17 in response to IL-1β and IL-23. Activation of TCR in γδ T cells leads to differentiation of IL-17 producing cells (Akitsu and Iwakura, 2018). This suggests that γδ T cells might not be the source of IL-17 in patients with TA as reported earlier (Misra et al., 2016).

This study is not without limitations. We didn't check purity of CD8 T cells and γδ T cells after separation of these cells from PBMC. However, as per the brochure of the magnetic separation kit used in our study, it is expected to achieve greater than 97% purity. Another important limitation of our study: we didn't perform validation assays such as combined bisulfite restriction analyses or pyrosequencing to confirm the findings of the present study.

Strength of our study is the novelty of being the first ever report on genome wide methylation profiling in CD8 T cells and γδ T cells in patients with TA. Measurement of the expression levels of IL-32 and LTA in CD8 T-cells as well as TNF-α, IL-10 and IL-27 in γδ T cells from patients with TA using flow cytometry analysis may be used in future studies, to explore if these cytokines can be used as diagnostic or prognostic biomarkers.

## CONCLUSION

Our study showed that *IL-32* and *LTA* were significantly hypomethylated in CD8 T-cells and anti-inflammatory cytokine genes *IL-10*, *IL-27* and *IL-1RN* were significantly hypomethylated in γδ T cells of TA. Genes involved in TCR signalling pathway and ribosome were also significantly hypomethylated in CD8 T-cells. Genes involved in TCR signalling pathway and Th17 differentiation, on the contrary, were hypermethylated in γδ T cells from patients with TA. Overall evidence from this study, and in the light of the published literature, emphasises that CD8 T-cells are likely to be more crucially involved in pathogenesis of TA, rather than γδ T cells.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the corresponding author on request.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional review board, Christian Medical College, Vellore. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

Design of the study, formal analysis, funding acquisition, acquisition of data and original draft—JK. Acquisition of data and recruitment of patients—HM, RG, and DD. Design of the study, supervision, review and editing—DD, RHS, and SD. Original concept and hypothesis—DD.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.843413/full#supplementary-material

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