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DNA methylation as a transcriptional regulator of the immune system

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Abstract

DNA methylation is a dynamic epigenetic modification with a prominent role in determining mammalian cell development, lineage identity and transcriptional regulation. Primarily linked to gene silencing, novel technologies have expanded the ability to measure DNA methylation on a genome-wide scale and uncover context-dependent regulatory roles. The immune system is a prototypic model for studying how DNA methylation patterning modulates cell type- and stimulus-specific transcriptional programs. Preservation of host defense and organ homeostasis depends on fine-tuned epigenetic mechanisms controlling myeloid and lymphoid cell differentiation and function, which shape innate and adaptive immune responses. Dysregulation of these processes can lead to human immune system pathology as seen in blood malignancies, infections and autoimmune diseases. Identification of distinct epigenotypes linked to pathogenesis carries the potential to validate therapeutic targets in disease prevention and management.

Keywords

DNA methylation; immune system; epigenetics; transcription; hematopoiesis; leukemia; autoimmunity; DNA methyltransferase inhibitors

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Introduction

Initially coined by embryologist Conrad Waddington, *epigenetics* refers to the study of heritable changes in gene function that do not alter the DNA sequence yet give rise to distinct phenotypes during development [1]. Fine-tuned epigenetic mechanisms reshape the topology of the genome to influence gene expression and regulate cell development, differentiation and phenotypic plasticity in response to environmental cues. Epigenetic phenomena include non-coding RNAs along with DNA methylation and post-transcriptional modifications of nucleosomal histones that alter chromatin accessibility and recruitment of the transcriptional machinery to modulate gene expression [2]. Because of their inherently plastic and cue-sensing nature, epigenetic mechanisms play a prominent role in governing the transcriptional programs of the immune system in health and disease.

DNA methylation is a widely studied chromatin modification given its pivotal role in gene silencing, X-chromosome inactivation, genomic stability and imprinting [3]. In mammalian cells, methylation occurs almost exclusively at the fifth carbon position of cytosines in the context of CpG dinucleotides (5' – cytosine – phosphate – guanine – 3'). Although prokaryotes classically display non-CG methylation (mCH, in which H is any non-guanine residue), a recent study revealed the presence of non-canonical (non-CG) methylation in multiple human tissues [4]. There are over 28 million CpG dinucleotides in the human genome, and 60–80% display methylation in any given cell. Conversely, CpG dinucleotide-enriched regions known as CpG islands, mostly located near gene promoter sequences, are predominantly hypomethylated, linking *de novo* methylation marks to gene expression [5].

DNA methylation patterns are established through the function of a family of DNA methyltransferases (DNMTs) that catalyze the covalent addition of a methyl group to the 5-carbon of the substrate base cytosine. The product of the DNA methyltransferase reaction is 5-methylcytosine (5mC). In mammals, DNMT3A and DNMT3B catalyze *de novo* methylation, and the maintenance DNA methyltransferase DNMT1, which binds hemi-methylated DNA during cell division, copies the parental strand CpG methylation pattern to the daughter strand [6]. A group of methylated-DNA binding proteins, including methyl-CpG-binding domain proteins, zinc finger proteins and certain transcription factors, act as readers of methylation marks to link DNA methylation and gene expression changes [7, 8]. Alterations to the DNA methylation maintenance machinery over successive rounds of cell division can result in passive DNA demethylation. Additionally, active DNA demethylation plays an important role during development, cell differentiation and function. The ten-eleven translocation (TET) family of enzymes bears primary responsibility for active DNA demethylation [9]. Fig 1 outlines the biochemistry of DNA methylation dynamics.

In order to better understand the influence of DNA methylation on gene expression (the transcriptome), numerous technologies have sought to accurately profile DNA methylation on a genome-wide scale (Table 1). Because DNA methylation marks are erased by molecular biology techniques used for whole-genome DNA sequencing, such as PCR amplification and cloning of DNA fragments into bacterial vectors, different tools were developed in order to identify methylated DNA through endonuclease digestion, immunoprecipitation or bisulfite conversion [10]. Integration of these tools with array-based

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and next-generation sequencing technologies allows for genome-scale DNA methylation profiling.

The immune system is responsible for host defense, a primordial function of living organisms, with the goal of preserving tissue and organismal homeostasis. Fine-tuned immunological mechanisms allow the host to develop a nearly unlimited repertoire to respond to a variety of pathogens and environmental cues while providing a long-lasting memory to fight recurrent triggers (immunological memory) and at the same time mitigate damage to its own tissues (as seen in cancer and autoimmunity). Two distinct branches divide the immune response into innate and adaptive immunity. Innate immunity exhibits germline inheritance and corresponds to a fast-paced, non-specific response against pathogens coordinated by myeloid phagocytes, toll-like receptors and the complement system. Adaptive immunity is a more sophisticated, highly specific and long-lasting response orchestrated by B and T lymphocytes that relies on antigen recognition through a very large set of lymphocyte-specific receptors [11]. Epigenetic modifications, including DNA methylation, tightly regulate immune system development, differentiation and function (Fig 2).

In this review, we summarize the role distinct DNA methylation patterns play in regulating the transcriptomic landscape of the immune system. We will examine how DNA methylation shapes early immune cell development and differentiation, immune function in host defense mechanisms and immune dysregulation exhibited during blood malignancies and autoimmune diseases. Our review concludes with an overview of epigenetic therapeutic approaches in malignant immune system disorders.

DNA methylation patterning during immune system progenitor development, differentiation and lineage commitment

Hematopoiesis constitutes an ideal system to study the intricate gene regulatory networks controlling multi-lineage cellular differentiation, identity and function. During the early 20th century, Alexander Maximov introduced the unitarian theory of hematopoiesis, in which distinct subpopulations of mature blood cells are generated from a single precursor cell known as the hematopoietic stem cell [12]. Hematopoietic stem cells are long-lived progenitors that display self-renewal capacity as well as the ability to differentiate into intermediate progenitors through a stepwise fate restriction process that ultimately confers lineage identity. DNA methylation regulates self-renewal and differentiation processes in the hematopoietic system and is specific to individual cell types that retain epigenetic memory of their lineage trajectory. Analysis of cell type-specific DNA methylation patterns therefore allows hierarchical reconstruction of a cell type's developmental history [13].

Early studies established a pivotal role for *de novo* DNA methylation in enhancing hematopoietic stem cell self-renewal capacity without altering hematopoietic cell differentiation [14, 15]. Experimental models using hypomorphic *Dnmt1* mice and hematopoietic stem cells revealed that constitutive maintenance DNA methylation is not only required for self-renewal capacity of the hematopoietic stem cell pool, but also plays a crucial role in regulating myeloerythroid versus lymphoid lineage differentiation.

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Transcriptomic analysis of hypomethylated murine hematopoietic stem cells showed increased expression of signature genes encoding for lineage-specific transcription factors such as *Gata1, Id2* and *Cebpa,* which are involved in myeloerythroid differentiation [16, 17]. Array-based analysis of genome-wide DNA methylation patterns in hematopoietic progenitors revealed marked epigenetic plasticity during myeloid and lymphoid differentiation. Hematopoietic progenitor cell differentiation from multipotent progenitors (MPP) into common lymphoid and myeloid progenitors (CLP and CMP) involved DNA methylation of CMP-specific genomic regions. Specifically, lymphoid cells demonstrated increased DNA methylation at several key regulatory transcription factors of the myeloid lineage, such as *Gata2, Tal1* and *Lmo2* [18]. Moreover, in an *in vitro* assay in which MPP were treated with the hypomethylating agent 5-aza-2'deoxycytidine (decitabine), the overall percentage of CMP increased at the expense of CLP, further confirming that reduced methylation favors myeloid as opposed to lymphoid differentiation [19].

The advent of next-generation sequencing technologies such as whole-genome bisulfite sequencing made possible a more comprehensive and unsupervised analysis of the DNA methylome at single-CpG dinucleotide resolution. Implementation of this novel approach to the study of hematopoietic stem cell differentiation uncovered that functional and dynamic methylation changes were located outside of CpG-dense regions, overlapping with *cis*-regulatory sites (such as enhancers and promoters) and inversely correlating with gene expression profiles [20]. For example, DNA methylation affects nucleosome dynamics and binding of CCCTC-binding factor (CTCF), a DNA-binding protein with insulator properties best known for its function as a linker between nuclear architecture and gene expression [21, 22]. Nucleosome positioning is widely inconsistent across the genome with the exception of chromatin surrounding CTCF-binding sites. Therefore, analysis of these sites has the potential to understand the oscillatory relationship between DNA methylation and nucleosome positioning (i.e., peaks of DNA methylation correlating to valleys of nucleosome density). Recently, investigators have shown that during B and T lymphocyte development, CTCF-binding sites demonstrate an increased oscillatory methylation pattern as opposed to a less divergent pattern during myeloid differentiation, indicating a dissimilar usage of DNA methylation between immune cell lineages [23].

The adaptive immune system is mainly comprised of B and T lymphocytes. The expression of specific surface receptors allows these cells to recognize, differentiate, proliferate and acquire lineage-specific effector functions in order to respond to diverse pathogens, tumors and environmental cues. Hematopoietic progenitors of the T cell lineage enter the thymus as *double-negative* cells ($CD4^-CD8^-$, DN), differentiate into *double-positive* cells ($CD4^+CD8^+$, DP) and ultimately, through sequential maturation stages, become either naïve $CD4^+$ or $CD8^+$ T cells as they egress from the thymus. Lee and colleagues showed that Cre-mediated deletion of *Dnmt1* in DN thymocytes led to a significant reduction of DP thymocytes and peripheral naïve $CD4^+$ and $CD8^+$ T cells, demonstrating a key role for methylation in thymopoiesis [24]. Interestingly, *Dnmt1* deletion at the DP stage caused a significant increase in cytokine production by naïve T cells, suggesting that methylation prevents premature activation of differentiation and functional programs that could lead to immune pathology. Global methylation analysis during human intrathymic and peripheral T cell differentiation revealed an increased frequency of TET-mediated demethylation events often

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associated with gene expression programs controlling lymphopoiesis [25, 26]. Investigators demonstrated 5hmC enrichment at active cell-specific enhancers and genes encoding key lineage-specifying transcription factors (for example, *ThPOK*, *Gata3* and *Runx3*) at distinct maturation stages, positively linking DNA methylation kinetics with transcriptional regulatory networks during T cell development [27]. Taken together, these studies demonstrate a functional role for active loss of DNA methylation during early thymic and peripheral T cell development, which determines their capacity to further differentiate and acquire lineage-specific functions.

Upon antigenic stimulation, naïve $CD4^+$ T cells differentiate into distinct helper T (Th) cells whose phenotypic characterization is defined by the expression of several signature cytokines (for example, IL-2, IL-10, IFN-γ and IL-17)—a process also regulated by active DNA demethylation along with histone modifications [28]. These differentiated cells go on to orchestrate immune function through enhancement of B cell-mediated antibody production, macrophage and $CD8^+$ T cell effector function as well as maintenance of immunological memory and self-tolerance.

Cytotoxic $CD8^+$ T cells are essential for clearance of intracellular pathogens and tumor cells. After stimulation of naïve $CD8^+$ T cells with lymphocytic choriomeningitis virus, Scharer and colleagues demonstrated that differentially methylated regions were enriched for transcription factors that regulated effector cytolytic functions in $CD8^+$ T cells, suggesting that methylation patterns are not only key for T cell differentiation but also for the establishment of cellular immunity [29]. After successful clearance of a pathogen, most effector T cells undergo programmed cell death, except for a persistent fraction of memory T cells. This subset of long-lived lymphocytes is able to regain effector function after recognition of the pathogen that initially triggered their activation and therefore are important in maintaining long-term immunity as conferred after vaccination [30]. Interestingly, methylation profiling of terminal effector versus memory precursor $CD8^+$ T cell subsets showed that the subset of cells that give rise to memory cells acquired repressive *de novo* DNA methylation at genes expressed by naïve cells. Effector genes became demethylated, further demonstrating that these cells are poised and capable of initiating an effective cellular immune response without needing further differentiation [31].

Non-canonical DNA methylation has been described in pluripotent stem cell development and differentiation and has been found in multiple human tissues during development. In this context, mCH is anti-correlated with gene expression and decreases during cell differentiation [4, 32]. Shuyler and colleagues described prominent levels of mCH in myeloid leukemia and naïve T cells compared to lymphoid neoplasms and myeloid cells with a subsequent disappearance of this mark with T cell lineage development. Moreover, lymphoid neoplasms showed reduced levels of CpG methylation in comparison with myeloid malignancies. These observations have important clinical relevance, given that demethylating agents commonly used to treat myeloid leukemias primarily target canonical (CG context) DNA methylation and could potentially demonstrate divergent therapeutic efficacy in myeloid neoplasms as opposed to lymphoid-derived malignancies [23].

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Overall, studying mammalian blood formation offers the opportunity to identify the different layers of dynamic epigenetic modifications, including DNA methylation along with its respective functional genomic annotations, which control cell fate decisions, renewal capacity and identity. Isolation and methylation analysis of cellular intermediates targeted by blood malignancy-related mutations could provide links between epigenotypes and disease development, as well as novel therapeutic targets for drug discovery.

Dysregulated DNA methylation in immune system and hematological malignancies

Disruption of DNA methylation has long been associated with the pathobiology of cancers of the immune system and hematopoietic progenitors. Investigators initially reported global DNA hypomethylation in multiple cancer cell lines and tumors when compared with normal tissues [33, 34]. In contrast, hypermethylation of CpG islands at the promoter regions of tumor-suppressor genes has also been linked tightly to tumorigenesis [35]. Because epigenetic modifications are pivotal in determining cell-fate decisions during hematopoiesis, investigators predicted that alterations of this gene regulatory machinery would play a key role in the development of hematological malignancies.

Accumulation of somatic mutations leading to clonal expansion of progenitor cells in the aging population can drive cancer evolution [36]. Identification of recurrent mutations, many of which affect the epigenetic machinery, are usually present years before the clinical onset of cancer. Known pre-malignant states include monoclonal gammopathy of unknown significance in multiple myeloma [37], monoclonal B cell lymphocytosis in chronic lymphocytic leukemia [38], and more recently clonal hematopoiesis of indeterminate potential (CHIP) in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) [39]. Interestingly, the most common mutations found in CHIP are in the *DNMT3A* locus [36, 40, 41]. *DNMT3A* mutations have also been observed in patients with MDS and myeloproliferative neoplasms [42, 43] and have been linked to secondary AML, suggesting these mutations might represent an early event in leukemic transformation [44]. With the use of an unsupervised deep sequencing approach, tumor sampling of adult patients with AML uncovered that close to 20% of study subjects carried *DNMT3A* mutations, which were associated with an unfavorable prognosis [45, 46]. DNA methylation profiling identified distinct clusters of AML patients associated with specific mutations. Samples with *DNMT3A, MLL* fusions, *NMP1* and *FLT3* mutations were linked to extensive loss of DNA methylation, while samples with isocitrate dehydrogenase 1 and 2 mutations (*IDH1* and *IDH2*) demonstrated a substantial gain of methylation marks (Table 2). Collectively, these data show that profiling of the DNA methylome is a powerful tool for clinical stratification and development of targeted therapeutic strategies [47–49].

Active DNA demethylation has a well-established role in the maintenance of hematopoietic homeostasis, differentiation of granulomonocytic progenitors and leukemogenesis [48, 50–54]. In fact, the discovery of active DNA demethylation by the TET family of enzymes was first described through the cloning of *TET1* as a fusion partner of *MLL* (mixed lineage leukemia gene on 11q23, which frequently harbors cytogenetic abnormalities related to

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AML) in AML patients with t(10;11) (q22;q23) translocations [55–57]. Deletion of *TET2* in the hematopoietic compartment can lead to the development of distinct myeloid disorders including chronic myelomonocytic leukemia, MDS, myeloproliferative neoplasms and AML [58–60]. Additionally, metabolic changes can lead to oncogenic disruption of the active demethylation machinery. Isocitrate dehydrogenase 1 and 2 (IDH 1 and IDH 2) are metabolic enzymes that convert isocitrate to α-ketoglutarate (α-KG) during the Krebs cycle. DNA sequencing in AML patients identified different IDH mutations that result in neomorphic enzymatic activity leading to conversion of α-KG into 2-hydroxyglutarate (2-HG) [61–63]. Subsequent studies demonstrated that 2-HG is an oncometabolite that exerts its oncogenic effect through competitive inhibition of α-KG-dependent TET proteins, thereby phenocopying a *TET2* mutation methylation profile [64, 65].

Altogether, the past decade has seen discoveries into the role played by epigenetic modifier-related mutations in the development of myeloid malignancies. These revelations have come in contrast to the prior long-held dogma that the pathogenesis of myeloid malignancies was the result of class I and class II mutations, which are associated with dysregulated cell growth (activation of signaling pathways, such as signal transducer and activators of transcription) and impaired cellular differentiation (altered expression of key transcription factors), respectively. This paradigm shift has resulted in the development of novel therapeutic agents targeting epigenetic modifiers such as ivosidenib (an IDH1 inhibitor), which has recently demonstrated clinical efficacy with durable and complete remission in patients with refractory AML [66].

DNA methylation dynamics as a modulator of immune system function and host-pathogen interactions

The mammalian immune system is comprised of two distinct branches: innate and adaptive immunity. Along with the epithelium, myeloid cells of the innate immune branch constitute the first line of host defense against invading pathogens. Macrophages are specialized phagocytes that orchestrate this initial immune response through antigen presentation, cytokine release and pathogen clearance. The interplay of transcriptional and epigenetic mechanisms tightly regulates macrophage identity, function and differentiation. Earlier studies have mainly focused on the interaction of pioneer, lineage-specifying tissue factors such as PU.1 with cell-specific enhancers, as they modulate and reshape the chromatin landscape during macrophage development and activation [67–71]. The combination of the well-known chemical stability of DNA methylation as an epigenetic mark and the fast-changing nature of innate immune responses initially limited the study of DNA methylation in innate myeloid immune cells. However, Pacis and colleagues showed that *Mycobacterium tuberculosis* infection of dendritic cells induces rapid loss of DNA methylation within 24 hours at distal enhancers that activate master immune transcription factors (including Nuclear Factor-kB and members of the Interferon Regulatory Factor family), suggesting an important role for DNA methylation in regulating innate immune responses [72]. More recently, Wallner and colleagues demonstrated that a gene regulatory network controlling macrophage structure and phagocytosis undergoes TET-mediated demethylation during monocyte-to-macrophage differentiation [73]. Furthermore, both *de novo* and maintenance

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DNA methylation can modulate macrophage polarization in murine models of chronic inflammatory diseases, such as obesity and atherosclerosis [74, 75]. Overall these data show that epigenetic modifications not only exert a pivotal role during immune cell *development* but also modulate functional immune programs during disease, offering an opportunity for the development of targeted therapies.

Epigenetic landscape of host-pathogen interactions

The interaction of infectious pathogens with host cells is a dynamic relationship that carries clinical relevance as we aim to target the host response to infection. In response to infection, the host can undergo epigenetic reprogramming, provoking a drastic change of gene expression and phenotype leading to worsening or *de novo* human pathology [76]. The initial immune response that a host mounts to overcome a particular pathogen must be tightly balanced in order to ensure an effective defense accompanied by minimal tissue damage. Although immunological memory has traditionally been ascribed to the adaptive immune system, the characterization of *trained immunity* and *endotoxin tolerance* as innate-type memory responses displayed by innate immune cells has challenged this long-held dogma [77]. Trained immunity explains how the initial priming of innate immune cells can lead to an enhanced response upon re-stimulation. Lipopolysaccharide (LPS)-mediated macrophage stimulation expands the enhancer landscape governing gene expression, which then confers an epigenetic footprint that strengthens immune responses upon secondary challenges [71]. Conversely, endotoxin tolerance consists of a partially hypo-responsive state displayed by macrophages after recurrent LPS exposures aimed at mitigating tissue damage during ongoing inflammation (e.g., sepsis). Tolerized macrophages undergo functional reprogramming through distinct patterning of histone modifications resulting in an immunocompromised state that can portend a high risk for secondary nosocomial infections in septic patients [78, 79]. Although immunologic memory can be useful to overcome and tolerate infections, it can also have deleterious consequences. For example, rewiring of the monocytic epigenetic landscape leading to induction of trained immunity and endotoxin tolerance has been associated with the development of autoimmune diseases and chronic bacterial colonization in cystic fibrosis patients, respectively [80, 81].

Epigenetic reprogramming of host gene expression profiles in response to infection can also lead to pathogen persistence and dissemination, dynamically affecting host-pathogen interactions. In a study by Masaki and colleagues, infection of adult Schwann cells with *Mycobacterium leprae* resulted in their differentiation into a pluripotent stem cell state, followed by promoter demethylation and upregulation of several genes controlling epithelial-to-mesenchymal transition programs. Reprogrammed cells then go on to differentiate into skeletal and smooth muscle cells and form granuloma-like structures that promote dissemination of infection [82]. Finally, pathogen-induced abnormal cellular reprogramming has been causally linked to oncogenesis. *Helicobacter pylori* infection induces aberrant DNA methylation in human gastric mucosa at promoter regions of methylated genes found in gastric carcinoma cells [83]. Similarly, promoter hypermethylation and subsequent down-regulation of the tumor-suppressor gene *PTEN* is associated with Epstein Barr virus-related gastric cancer [84]. Altogether, these studies demonstrate the potential for epigenetic therapeutic interventions in the management of

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infectious diseases. Antibiotic resistance is a global health problem that mandates development of novel drugs, and modulation of both pathogen-induced epigenetic modifications could represent viable therapeutic targets.

Epigenetic regulation of autoimmune diseases

There are over 80 different known autoimmune diseases collectively affecting 7% of the United States population [85]. Despite the high prevalence of autoimmune diseases, there are still significant gaps in our understanding of the pathobiology and causative factors of these diseases. Thus far, we know that autoimmunity ensues when a genetically predisposed host is challenged by distinct environmental factors resulting in a dysre**gulated immune response and loss of self-tolerance. The epigenome connects these environmental** cues with gene expression patterns during immune cell development and maintenance and provides the necessary plasticity required to respond to cellular stressors throughout the lifespan. Therefore, researchers have recently shifted their focus onto the analysis of differential epigenetic patterns in autoimmune diseases, as epigenetic phenomena may explain the molecular mechanisms underpinning disease development, progression and phenotypic variability in patients with similar genetic backgrounds.

The study of monozygotic twins presents an opportunity to uncover the role played by environmental epigenetic modifications in the susceptibility to autoimmune diseases, as they likely explain how identical genotypes lead to distinct phenotypes. Javierre and colleagues performed a global DNA methylation analysis in identical twins discordant for systemic lupus erythematosus (SLE) and found a significant reduction in the overall DNA methylation content in the SLE-affected twin. Furthermore, gene ontology-based functional enrichment analysis demonstrated differential gene expression profiles related to immune function and molecular pathways linked to the pathogenesis of SLE [86].

The majority of autoimmune disease-affected patients are females, with female:male ratios ranging from 3:1 for rheumatoid arthritis (RA) to 9:1 for SLE, suggesting a potential contribution of X-chromosome-located genes to the pathogenesis of autoimmune diseases [87]. Since epigenetic mechanisms (prominently DNA methylation) control X-chromosome inactivation, it is unsurprising that new-onset epigenetic modifications can result in differential expression of genes encoded on the X-chromosome. For example, the X-chromosome-encoded gene *CD40L* is a B cell costimulatory transmembrane protein that shows increased expression on the surface of activated $CD4^+$ T cells from patients with SLE [88]. Two independent studies found that DNA demethylation of *CD40L* regulatory elements leads to overexpression of this gene in the $CD4^+$ T cells of SLE and systemic sclerosis patients [89, 90], thereby contributing to the overproduction of auto-antibodies that drive disease pathogenesis.

B cells and regulatory T cells: drivers and mitigators of autoimmunity

Epigenetic modifications modulate B cell maturation and production of autoreactive antibodies by B cell-derived plasma cells. Upon antigenic stimulation and T cell-mediated activation (i.e., *CD40L* costimulatory signal) mature naïve B cells migrate to secondary lymphoid organs and differentiate into germinal center B cells. Marked activation-induced

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demethylation and increased heterogeneity of methylation patterning of germinal center B cells accompanies this transition [91, 92]. Through initiation of somatic hypermutation and class switch recombination, activation-induced cytidine deaminase generates a diversified repertoire of high-affinity antibodies that modulate adaptive immune responses. After egressing germinal centers, B cells undergo further differentiation into either antibody-producing plasma cells or long-lived memory B cells capable of mounting a more efficient response to subsequent challenges. DNA methylation profiling comparing naïve versus memory B cells showed differentially methylated regions associated with key genes regulating their immune function (for example, *RUNX3, RELA* and *PAX5),* revealing that DNA methylation reprogramming poises memory B cells to exhibit a more sustained and enhanced recall response compared to naïve B cells [93].

Drug-induced lupus erythematosus is an autoimmune disorder triggered by the chronic use of certain medications with DNA methyltransferase inhibitor (DNMTI) characteristics, including hydralazine and procainamide. Mazari and colleagues showed that passive transfer of hydralazine-treated B cells into syngenic mice leads to increased detection of autoreactive antibodies through disruption of receptor editing and B cell tolerance, demonstrating a causal role for the loss of B cell DNA methylation and the development of autoimmunity [94]. The association of dysregulated B cell methylation and autoimmunity is not only limited to SLE. In a genome-wide DNA methylation study of patients with primary Sjögren's Syndrome, investigators demonstrated that DNA methylation alterations were more prevalent in genetic risk loci of B cells when compared with T cells. Moreover, methylation alterations in B cells were enriched for pathways involved in inflammation, interferon signaling and positively correlated with disease activity [95]. These studies demonstrate that epigenetic regulation of B cell function is a key mechanism driving the development and severity of autoimmune disease (Fig 3).

Immunomodulatory regulatory T (Treg) cells constitute a subset of $CD4^+$ T cells with a key role in mitigation of autoimmunity and maintenance of immune homeostasis. The master transcription factor of Treg cells is Forkhead box P3 (*FOXP3*), a member of the fork-winged helix family exclusively expressed at stable levels in Treg cells and responsible for their differentiation and repressive function [96]. Early after its discovery, investigators demonstrated that *FOXP3* mutations result in human neonatal onset of immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome, implicating Treg cell dysfunction as a cause of severe autoimmunity [97, 98]. Induction and stabilization of *FOXP3* expression is under tight epigenetic regulation. Specifically, both the *FOXP3* promoter and one of its supporting conserved noncoding DNA sequences (CNS), CNS2, display hypomethylation in Treg cells and relative hypermethylation in conventional T cells, linking DNA methylation to T cell lineage commitment and function [99–103].

Treg cells exert their repressive function on effector T cells through expression of inhibitory checkpoint molecules, such as cytotoxic T lymphocyte antigen 4 (CTLA-4), which is involved in cell contact-mediated suppression. Impairment of T cell proliferation through secretion of inhibitory cytokines, such as IL-10 and transforming growth factor-β (TGF-β) constitutes another immunosuppressive effect of Treg cells [104]. Compared with patients with inactive SLE, isolated Treg cells from patients with active SLE showed a significant

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decrease in their ability to suppress $CD4^+$ effector T cell proliferation and cytokine secretion. *FOXP3* message and protein from patients with active SLE also showed significant reductions [105, 106]. Moreover, several groups of researchers described a global reduction of Treg cells that inversely correlated with SLE activity [107, 108]. Interestingly, glucocorticoid therapy increased Treg cell numbers in these patients [109]. Similarly, in a mixed pool of patients with either limited or diffuse systemic sclerosis, the number of Treg cells along with levels of serum IL-10 and TGF-β were significantly lower when compared with healthy controls [110]. Moreover, Baraut and colleagues showed that in patients with diffuse systemic sclerosis, the decrease of both Treg cell overall numbers and Treg cell-mediated suppressive capacity were rescued after autologous hematopoietic stem cell transplantation [111]. Collectively, these studies showed that impaired suppressive function of Treg cells promotes the development of autoimmunity (Fig 3). Interventions that reconstitute the Treg cell pool (e.g., through adoptive transfer or IL-2 therapy) or modulate epigenetic modifications that translate into enhancement of Treg cell number and suppressive function could provide keys to preventing and managing these disorders.

Epigenome-wide association studies in autoimmune diseases

Genome-wide association studies (GWAS) have been fundamental in identifying single-nucleotide polymorphisms (SNPs) associated with human diseases, and over 300 loci have been associated with autoimmune diseases [112]. However, the vast majority of SNPs do not alter protein-coding sequences, limiting our ability to elucidate their molecular function in disease pathogenesis. Moreover, the effect of environmental factors on disease initiation, progression and phenotypic variation are not included in GWAS. In order to circumvent this limitation and integrate the effect of environmental exposures with known genetic variations to uncover inter-individual variation in common diseases, investigators have focused on the analysis of epigenome-wide association studies (EWAS) [113]. EWAS aim to quantify distinct epigenetic modifications, such as DNA methylation, in order to derive changes in the epigenetic landscape with causality of a specific pathology or trait. Moreover, the identification of SNPs with differentially methylated positions, known as methylation quantitative trait loci (meQTLs), permits the integration of GWAS and EWAS as a means to reveal how distinct genotypes can drive gene expression through epigenetic modifications [114, 115]. Recently, Imgenber-Kreuz and colleagues uncovered several SLE-associated meQTLs, which included *PTPRC* (encodes CD45), *MHC-class II, UHRF1BP1, IRF5, IRF7, IKZF3* and *UBE2L3*. These findings suggested that some SLE-associated genetic variants exert their effect on disease phenotype through DNA methylation variance [116]. The combined study of genetic and epigenetic modifications offers a comprehensive tool to analyze the mechanisms underlying disease initiation, progression, severity and therapeutic response profile, which could ultimately lead to the development of novel preventive and therapeutic interventions.

Limitations of epidemiological epigenetic studies must be understood in order to enhance their clinical value. Whole blood has been the tissue of choice for most EWAS, and therefore cellular heterogeneity has been a known limitation hindering the interpretation of these studies. Another complicating factor for EWAS interpretation is whether epigenetic modifications drive causality or are the result of the disease (reverse causality). After cell-

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type proportion adjustment and use of mediation analysis in order to exclude associations likely related to reverse causality, investigators reported nine differentially methylated positions in the major histocompatibility cluster that strongly correlated with genetic risk for RA, providing a potential marker for disease development [117]. One of the key effector cells in the pathogenesis of RA are fibroblast-like synoviocytes, which contribute to joint destruction through the production of pro-inflammatory cytokines and proteases. DNA methylation analysis of isolated fibroblast-like synoviocytes from RA and osteoarthritis patients revealed significant hypomethylation and a simultaneous increase in the expression of genes involved in inflammation, chemotaxis and extracellular matrix regulation among RA patients, highlighting the potential of EWAS in accurately differentiating different pathologies [118]. Besides the potential for discovery of novel mechanisms involved in disease pathogenesis, identification of biomarkers, and characterization of phenotypic variation in disease, EWAS could also enable the recognition of selective therapies that benefit individual patients. Based on this premise, in a cohort of prospectively-followed RA patients, close to 20 differentially methylated positions were found to be associated with response to disease modifying anti-rheumatic agents with the strongest associations found for the *ADAMTSL2* and *BTN3A2* loci [119]. Altogether, these data validate the need to integrate EWAS findings with GWAS in order to identify high-risk patients based on their genetic background and cumulative environmental exposures. In patients with established disease, these studies could provide insight into the active determinants of therapeutic response, allowing clinicians to modify pharmacotherapy.

Targeting the epigenetic landscape in the treatment of human disease

With an understanding of how epigenetic modifications can drive immune system pathology, there is now a focus on translational applications to modify the epigenome for treatment of human disease. The most well established therapies alter DNA methylation and histone acetylation. The DNMTIs, 5-azacytadine and 5-aza-2'-deoxycytidine (decitabine), were originally developed in the 1960s as cytotoxic agents [120], but it was nearly 20 years before their effects on DNA methylation were discovered [121] (Table 3). Initial studies with these agents were unsuccessful, as conventional dose escalation strategies caused toxicities and poor tolerability [122]. With improved understanding of their mechanism-of-action, researchers discovered that nanomolar doses could achieve effective inhibition of DNA methylation while also improving tolerability [123].

As discussed previously, multiple hematologic malignancies are associated with dysregulation of DNA methylation and were therefore initial targets for epigenetic therapies. Myelodysplastic syndrome represents a heterogeneous group of hematologic disorders derived from abnormal progenitor cells that result in hypoproliferative bone marrow and place patients at risk for transformation into various forms of acute leukemia. 5-azacytidine was first studied in patients with MDS, as it was known *in vitro* to cause immature cell differentiation, especially of malignant cells such as promyelocytes [124, 125]. In the first clinical application of DNMTIs to treat MDS, study participants who received 5-azacytidine had improved response rates, less time to transformation to acute leukemia, and prolonged survival (18 months vs 11 months, p=0.03) when compared with supportive therapy alone [126]. In phase II trials, decitabine showed similar clinical outcomes [127]. DNMTI therapy

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relies on integration into DNA through multiple cell cycles, and thus studies have reported improvements in survival with longer treatment courses [128].

Following establishment of DNMTI-based therapies as effective with high tolerability in hematologic malignancies, investigators evaluated their efficacy in solid organ malignancies. Non-small cell lung carcinoma [129] and ovarian cancer [130, 131] have shown response to treatment with DNMTIs, although response is highly variable between individuals. The greatest potential of these therapies appears to be in combination with cytotoxic agents, with which DNMTIs appear to sensitize tumors and increase efficacy of conventional cytotoxic agents, even in those patients who have not responded to these therapies previously [132]. Recently, epigenetic therapies have shown promise when used synergistically with novel immunotherapies. Immune checkpoint inhibitors, such as CTLA-4 inhibitors and programmed death-1 (PD-1) inhibitors, have potential for increased efficacy when used with epigenetic modifiers [133]. In melanoma, 5-azacytidine induces specific double stranded RNA production, utilized in host viral defense mechanisms, which upregulates transcription of interferon-β and sensitizes malignant cells to CTLA-4 inhibitors [134]. A similar mechanism has also been shown in colon cancer [135], raising the possibility that this mechanism could be effective against multiple different malignancies. Clinical trial NCT01928576 is currently underway to investigate the efficacy of combined PD-1 inhibition with both 5-azacytidine and a histone deacetylase (HDAC) inhibitor (entinostat) in non-small-cell lung carcinoma.

The first next-generation DNMTI, guadecitabine, may increase the efficacy of hypomethylating agents in the treatment of cancer. Guadecitabine's novel structure makes it resistant to degradation by the enzyme cytidine deaminase, which prolongs *in vivo* exposure [136, 137]. Early phase I studies demonstrated the efficacy and safety when used to treat AML [138], with a phase III study currently ongoing (NCT02920008). Outside of AML, there are also multiple preliminary trials evaluating the efficacy of this novel hypomethylating agent in solid tumors such as hepatocellular carcinoma and ovarian cancer.

Conclusions and future directions

A single genome gives rise to the myriad cellular phenotypes that constitute the progenitors and effectors of the immune system. The cellular transcriptional machinery is sensitive to DNA methylation and results in gene expression profiles that confer different cellular functions while maintaining cellular identity. These epigenetic modifications link a cell's heritable and developmental history with their functional programs in the context of dynamic changes from environmental input, aging and stochasticity. The National Institutes of Health Roadmap Epigenomics Mapping Consortium and the International Human Epigenome Consortium have spearheaded recent efforts to map DNA methylation and other epigenetic marks at a genome-wide scale across different species and human tissues. Through development of novel molecular techniques and bioinformatic approaches allowing single-cell analysis, investigators have been able to uncover how cellular heterogeneity can drive immune system development and cancer progression. Equipped with well-annotated databases, these highly dimensional sequencing and machine-learning approaches promise to provide similar insights into the role of DNA methylation in regulating host defense and

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autoimmunity. Currently available hypomethylating agents lead to global (even if non-uniform) epigenetic changes. The use of CRISPR-based DNA methylation editing technologies offers the potential for locus-specific methylation editing [139], which could add specificity to epigenetic therapies. Integration of DNA information and DNA modifications (genome and epigenome) with RNA information and RNA modifications (transcriptome and epitranscriptome) will provide a better understanding of the regulatory mechanisms underpinning complex immune system disorders. These datasets could then inform the design of epigenetic pharmacotherapies that target blood malignancies, promote homeostasis in microbial host defense, and mitigate autoimmunity.

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Abbreviations:

(5' – cytosine – phosphate – guanine – 3')
CpG dinucleotides

(mCH)
non-CG methylation

(DNMT)
DNA methyltransferase

(5mC)
5-methylcytosine

(TET)
ten-eleven translocation

(5hmC)
5-hydroxymethylcytosine

(5fC)
5-formylcytosine

(5caC)
5-carboxylcytosine

(TDG)
thymine DNA glycosylase

(BER)
base excision repair

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(AID/APOBEC)
activation-induced deaminase/apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like; proteins

(UHRF)
ubiquitin-like, containing PHD and RING finger domains

(5mU)
5-methyluracil

(5hmU)
5-hydroxymethyluracil

(AP)
apyrimidinic

(MeDIP)
methyl-DNA immunoprecipitation

(ChIP)
chromatin immunoprecipitation

(MBD)
methyl-CpG binding domain

(RRBS)
reduced representation bisulfite sequencing

(WGBS)
whole-genome bisulfite sequencing

(MPP)
multipotent progenitors

(CLP)
common lymphoid progenitors

(CMP)
common myeloid progenitors

(CTCF)
CCCTC-binding factor

(CHIP)
clonal hematopoiesis of indeterminate potential

(MDS)
myelodysplastic syndrome

(AML)

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acute myeloid leukemia

(NMP1)
nucleophosmin 1

(*MLL*)
mixed lineage leukemia

(IDH 1 and IDH 2)
isocitrate dehydrogenase 1 and 2

(α-KG)
α-ketoglutarate

(2-HG)
2-hydroxyglutarate

(CD4-CD8-, DN)
double-negative cells

($CD4^+CD8^+$, DP)
double-positive cells

(Th)
helper T

(LPS)
lipopolysaccharide

(Dam)
DNA adenine methyltransferases

(SLE)
systemic lupus erythematosus

(RA)
rheumatoid arthritis

(DNMTI)
DNA methyltransferase inhibitor

(Treg cells)
regulatory T cells

(*FOXP3*)
Forkhead box P3

(IPEX)
immune dysregulation, polyendocrinopathy, enteropathy X-linked

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(CNS)
conserved noncoding DNA sequences

(CTLA-4)
cytotoxic T lymphocyte antigen 4

(TGF-β)
transforming growth factor-β

(GWAS)
genome-wide association studies

(SNPs)
single-nucleotide polymorphisms

(EWAS)
epigenome-wide association studies

(meQTLs)
methylation quantitative trait loci

(PD-1)
programmed death-1

(HDAC)
histone deacetylase

(NSCLC)
non-small cell lung cancer

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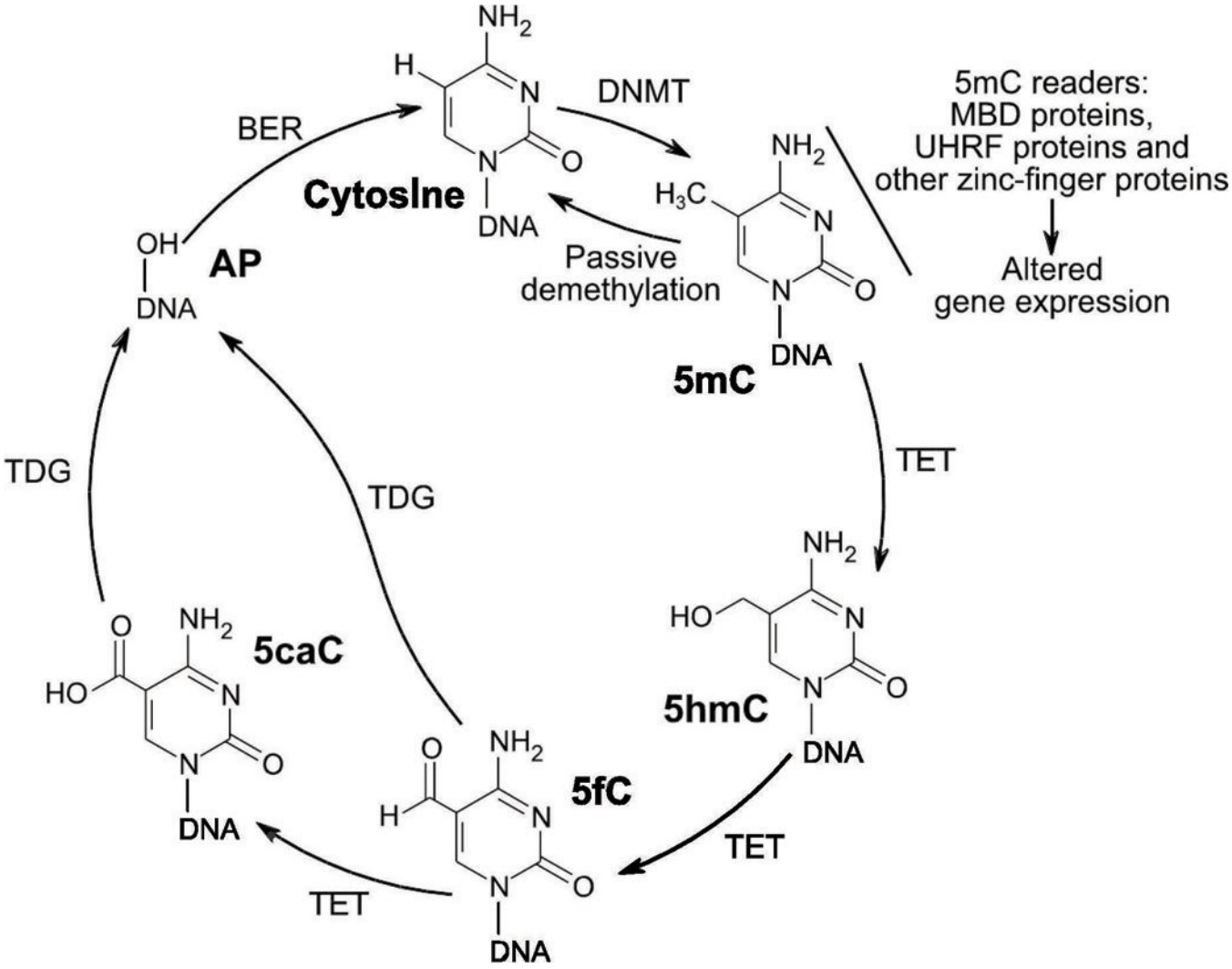

Figure 1. DNA methylation chemistry.

DNA methyltransferases (DNMT) use S-adenosylmethionine as a methyl donor to catalyze the addition of a methyl group to the 5-carbon position of cytosine, resulting in 5-methylcytosine (5mC). 5mC can be read by multiple nuclear proteins that lead to changes in gene expression, including methyl-CpG-binding domain (MBD) proteins; ubiquitin-like, containing PHD and RING finger domains (UHRF) proteins; and other zinc-finger proteins. Demethylation of 5mC back to cytosine can occur passively during cell division. Active demethylation can occur via the ten-eleven translocation (TET) enzymes, which use oxygen, 2-oxoglutarate, and ferrous iron to catalyze the conversion of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). Each reaction generates an oxidized ferric iron, succinate, and carbon dioxide. G/T-mismatch-specific thymine DNA glycosylase (TDG) can then excise 5fC and 5caC, resulting in an apyrimidinic (AP) site. These AP sites can then undergo base excision repair (BER), which replaces cytosine at that position. Not shown are other demethylation pathways including deamination of 5mC or 5hmC by activation-induced deaminase/apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (AID/APOBEC) family members to form

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5-methyluracil (5mU) or 5-hydroxymethyluracil (5hmU), respectively, which can be catalyzed to cytosine via the TDG/BER pathway.

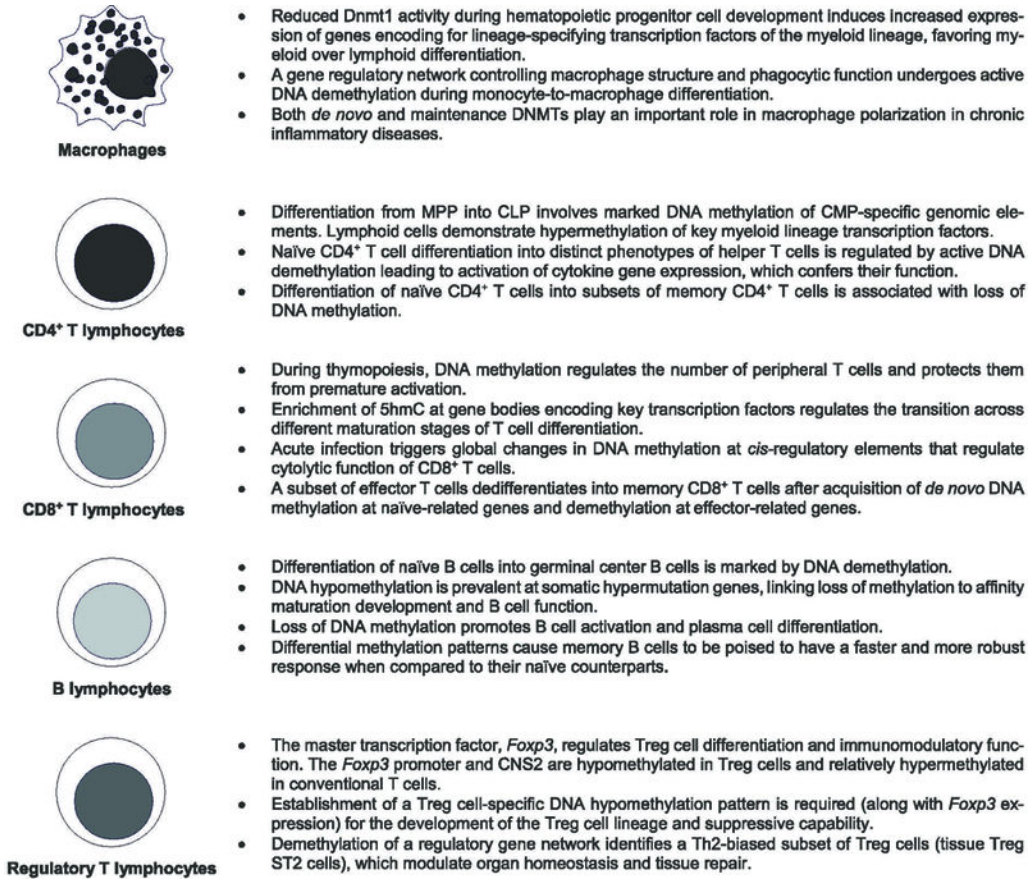

Figure 2.

DNA methylation state during innate and adaptive immune cell development, differentiation and function. Epigenetic modifications, including DNA methylation, modulate the self-renewal capacity of the hematopoietic stem cell pool throughout the lifespan and tightly regulate the hierarchical differentiation process of immune cells. DNA methylation patterning is specific to immune cell types during distinct maturation stages and correlate with a cell's gene regulatory and functional programs. DNA methyltransferase (DNMT), multipotent progenitors (MPP), common lymphoid progenitors (CLP), common myeloid progenitors (CMP), 5-hydroxymethylcytosine (5hmC), conserved noncoding sequence 2 (CNS2).

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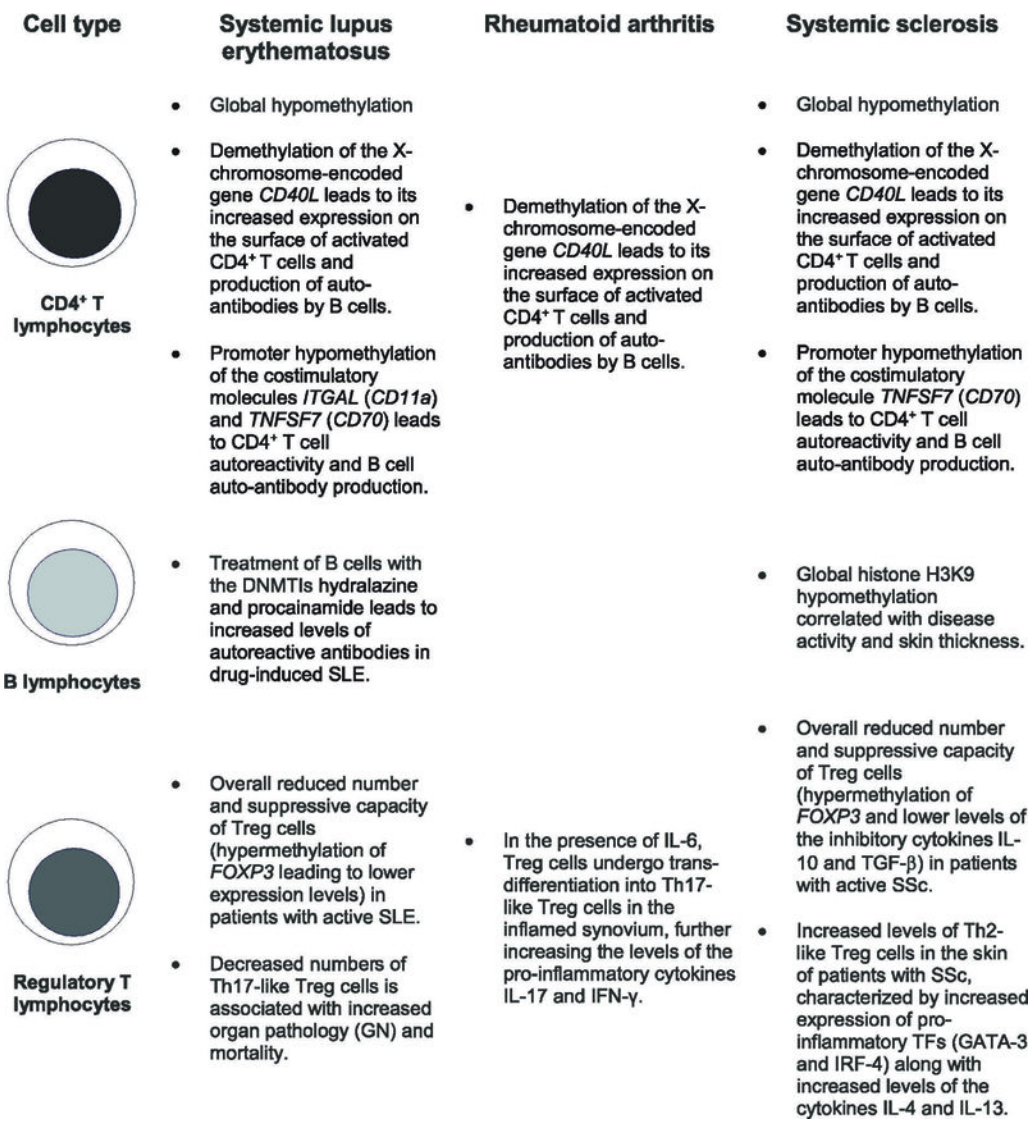

Figure 3.

Epigenetic patterns of immune cell subsets in the pathogenesis of autoimmune diseases. Cell-specific methylation changes are linked to immune cell differentiation and function during the development of autoimmune processes. Epigenetic marks can identify subjects at increased risk for organ damage, disease progression and overall mortality in these human pathologies. Systemic lupus erythematosus (SLE), systemic sclerosis (SSc), regulatory T cells (Treg cells), glomerulonephritis (GN), TFs (transcription factors).

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Table 1.
Principal methods for genome-wide DNA methylation analysis.

There is an extensive variety of DNA methylation profiling techniques available to survey the whole genome. Selection of a specific method requires appropriate understanding of their respective advantages and disadvantages. The type and quantity of available sample combined with the desired depth of genome coverage and resolution provides guidance to select an approach. Methyl-DNA immunoprecipitation (MeDIP), chromatin immunoprecipitation (ChIP), methyl-CpG binding domain (MBD), reduced representation bisulfite sequencing (RRBS), whole-genome bisulfite sequencing (WGBS).

Method (ref)	Description	Advantages	Disadvantages
MeDIP [140]	Utilizes specific antibodies capable of immuno-capturing single-stranded methylated cytosines followed by analysis through tiling arrays (MeDIP-ChIP) or next-generation sequencing (MeDIP-seq).	Increade Sensitivity for identifying regions of low CpG density (gene bodies and shores) when compared to MBD	Similar to MBD. this method has a strong bias toward identification of hypermethylated DNA regions and lacks single-CpG dinucleotide resolution
MBD [141]	Affinity-based capture method using beads coated with the DNA-binding protein MBD, which specifically binds double-stranded methylated CpGs. This is followed by a salt fractionation step that allows DNA methylation density assessment and fragment separation. Analysis is then performed through profiling with tiling microarrays (MBD-ChIP) or next-generation sequencing (MBD-seq).	Cost-efficient method Increased sensitivity for identifying regions of high CpG density when compared to MeDIP. Ionic strength modulates MBD affinity for methylated DNA therefore, changes in salt elution permit isolation of DNA based on methylation density	Due to enrichment technique for identification of methylated DNA regions followed by their subsequent amplification and sequencing, there is a bias for analyzing densely methylated regions over hypomethylated regions. Lack of power to discern DNA methylation changes at single-CpG dinucleotide resolution.
WGBS [142]	Treatment of genomic DNA with sodium bisulfite selectively deaminates unmethylated cytosine residues into uracil at a much faster rate than methylated cytosines. Uracil bases are then converted to thymidine bases after PCR amplification while unmethylated cytosines remain unchanged. The resulting library undergoes next-generation sequencing.	Cost-efficient method Identifies nearly every cytosine residue on a genome-wide scale, making this method the gold standard in DNA methylome analysis. A modification to the bisulfite sequencing chemistry also permits assessment of 5hmC marks.	Genome-wide sequencing requirements result in high costs. DNA degradation due to bisulfite conversion. Captures the majority, but not all CpG islands and promoters
RRBS [103,143]	Combines the use of restriction enzyme digestion (e.g., MspI) to select for CpG-enriched regions followed by bisulfite conversion and next-generation sequencing	Reduces sequence redundancy resulting from bisulfite treatment by selecting specific regions for sequencing Requires low amounts of DNA, which translates into fewer reads needed for accurate sequencing, thereby reducing cost	Limited coverage of other genomic regions (e.g., distal regulatory elements and shores). DNA degradation due to bisulfite conversion.

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Table 2.

DNA methylation profiling of common genetic mutations associated with acute myeloid leukemia.

DNA methylation patterning identifies specific methylation signatures in genes associated with the development of AML. Since epigenetic mechanisms are linked to gene regulation in leukemogenesis, they constitute a novel tool to enhance our understanding of AML pathogenesis, classification, and development of targeted therapies. DNA methyltransferase 3A (DNMT3A), ten-eleven translocation 2 (TET2), isocitrate dehydrogenase 1 and 2 (IDH 1 and IDH 2), nucleophosmin 1 (NMP1), mixed lineage leukemia (*MLL*), acute myeloid leukemia (AML), α-ketoglutarate (α-KG), 2-hydroxyglutarate (2-HG).

Genetic mutation	DNA menthylation pattern	Oncogenic mechanism in AML (ref)
DNMT3A	Primarily hypomethylated	Mutation of the DNMTA3 residue Arg882 (R882) leads to potentiation of aberrant stemness genes linked to AML development [144].
TET2	Hypermethylation	Loss of TET2 leads to a genome-wide enhancer hypermethylation state including downregulation of putative tumor suppressor genes linked to AML development [145].
IDH 1 and 2	Marked hypermethylation	Hypermethylation leads to loss of IDH enzymatic activity and α-KG production accompanied by an increase in the oncometabolite 2-HG [61].
NMP1	Primarily hypomethylated	Unclear mechanism. NMP1 is known to be important for nucleolar integrity (ribosome biogenesis) and function (DNA-repair processes). Involved in regulating activity of tumor suppressor genes (e.g., p53) [146]
MLL-fusion proteins	Marked hypomethylation	Gain of function of MLL fusion proteins leads to increased levels of MEIS1 and HOXA gene expression which have been linked to leukemogenesis [147].

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Table 3.

DNA methyltransferase inhibitors.

5-azacitadine is a ribonucleotide that can be incorporated into RNA as well as DNA. Guadacitabine is a dinucleotide followed by deoxyguanosine, which makes it less prone to breakdown by cytidine deaminase [136]. DNA methyltransferase (DNMT), myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), non-small cell lung cancer (NCSLC).

Therapeutic Agent	Mechanism Of Action	Clinical Indications (ref)
5-azacytadine **5-aza-2'-deoxycytidine (decitabine)**	Cytosine analogs that incorporate into DNA and • at low doses, covalently bind DNMT resulting in hypomethylation	- MDS [126] - AML [148] - NSCLC [129] - ovarian cancer [130] - AML [149] - MDS [127] - ovarian cancer [150]
2'-deoxy 5-azacytidylyl-(3'→5')-2'deoxyguanosine (guadacitabine)	• at high doses, halts DNA replication resulting in direct cytotoxicity	- AML [138]