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Epigenetic perspectives on systemic autoimmune disease

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ABSTRACT

Autoimmune diseases are characterized by increased reactivity of the immune system towards self-antigens, causing tissue damage. Although their etiology remains largely unknown, genetic, microbial, environmental and psychological factors are recognized as contributing elements. Epigenetic changes, including covalent modifications of the DNA and histones, are critical signaling mediators between the genome and the environment, and thus potent regulators of cellular functions. The most extensively studied epigenetic modifications are Cytosine DNA methylation and histone acetylation and methylation on various residues. These are thought to affect chromatin structure and binding of specific effectors that regulate transcription, replication, and other processes. Recent studies have uncovered significant epigenetic alterations in cells or tissues derived from autoimmune disease patients compared to samples from healthy individuals and have linked them with disease phenotypes. Epigenetic changes in specific genes correlate with upregulated or downregulated transcription. For instance, in many systems, reduced DNA methylation and increased histone acetylation of interferon-inducible genes correlate with their increased expression in autoimmune disease patients. Also, reduced DNA methylation of retroelements has been proposed as an activating mechanism and has been linked with increased immune reactivity, while epigenetic differences on the X chromosome could indicate incomplete dosage compensation and explain to some extent the increased susceptibility of females over males towards the development of most autoimmune diseases. Besides changes in epigenetic modifications, differences in the levels of many enzymes catalyzing the addition or removal of these marks as well as proteins that recognize them and function as effector molecules have also been detected in autoimmune patients. Although the existing knowledge cannot fully explain whether epigenetic alterations cause or follow the increased immune activation, their characterization is very useful for understanding the pathogenetic mechanisms and complements genetic and clinical studies. Furthermore, specific epigenetic marks have the potential to serve as biomarkers for disease status, prognosis, and response to treatment. Finally, epigenetic factors are currently being examined as candidate therapeutic targets.

1. Introduction

Systemic autoimmune diseases are disorders characterized by increased activity of the immune system, which exhibits reactivity towards self-antigens, causing multiple tissue damage. The group encompasses a broad spectrum of complex and multifactorial diseases, varying regarding specific autoreactive immune cells and/or autoantibodies produced, the tissue or organ attacked and the clinical phenotype. The latter can be quite diverse even among patients with the same diagnosis. However, some common features are recognized among systemic autoimmune diseases, related to etiology and pathogenetic mechanisms, which also dictate similar therapeutic approaches.

Three of the most common systemic autoimmune diseases are Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA), and Sjögren's Syndrome (SS), which have also been intensively studied and will be mostly discussed in this review. SLE can affect multiple organs and systems, including the skin, joints, kidneys, lungs and the hematopoietic system and patients usually exhibit anti-nuclear-antibodies (ANA) and a strong type I interferon response [1]. RA is characterized by inflammation and, if untreated, progressive destruction of symmetrical joints. SS is an autoimmune epithelitis, primarily affecting the salivary and lachrymal exocrine glands [2,3]. Cells of the adaptive immune response are critical in autoimmune disease development, even though the contribution of different subtypes may vary among different pathological entities.

The exact causes of autoimmune disease remain poorly understood, but in general, an interplay between genetic and environmental factors has been proposed. Some associations have been observed between

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genetic variants and pathogenic phenotype, however, usually only a subset of carriers develop disorders and even genome-wide association studies have left many unanswered questions. The current view is that, in a predisposed individual, a hormonal, microbial or psychological trigger could initiate loss of immunological tolerance to self-antigens and sustained immunological response, resulting in tissue destruction [4]. In the past decades, epigenetic factors have arisen as critical mediators of the cross-talk between the cellular environment and the genome. Evidence suggests that epigenetic regulation can serve as a fine-tuning or even as a switch on/off mechanism on genes involved in the autoimmune response, affecting quantitative and qualitative disease characteristics, modulating aggressiveness and reversibility that is usually observed in remission. This review focuses on research studying the contribution of epigenetic regulation to the pathogenesis of autoimmune diseases. Specific associations and underlying biological mechanisms are discussed.

2. Epigenetic regulation

In the eukaryotic cell, DNA is packaged into chromatin. The basic repeating unit of chromatin is the nucleosome, consisting of a stretch of about 146 nucleotides wrapped around an octamer of two copies of each of the core histones H2A, H2B, H3, and H4. The term epigenetic refers to all modifications occurring on chromatin without altering the DNA sequence. These include covalent modifications on DNA and the histone molecules, incorporation of histone variants, nucleosome remodeling and small or long non-coding RNA molecules. The first two categories, which are more extensively studied both generally and in the context of autoimmunity as well, will be discussed herein (Fig. 1).

The best-understood DNA modification is methylation of Cytosine, which occurs primarily in the context of CpG dinucleotides. In the human genome, CpG dinucleotides are under-represented. Genomic regions with relatively enriched CpG content are termed CpG islands [5]. These are often located near promoters of genes, especially housekeeping genes, which are ubiquitously expressed in all cell types. Methylation of CpG islands seems to be a mechanism that regulates transcription. In general, Cytosine methylation correlates with transcriptional silencing. Beyond the regulation of individual promoters, DNA methylation is critical in larger-scale chromosomal silencing, as in heterochromatin formation, silencing of repetitive sequences and retrotransposons, as well as X chromosome inactivation. The silencing effect of methylation is thought to occur by inhibition of binding of transcriptional activators as well as recruitment of transcriptional repressors. Covalent addition of the methyl group to the fifth carbon of Cytosine is catalyzed by DNA methyltransferases (DNMTs). In humans, DNMT3A and DNMT3B can function as *de novo* methyltransferases, whereas DNMT1 prefers semi-methylated substrates and is thus considered a maintenance methyltrasferase. This property makes it essential in the transmission of genomic methylation patterns from the parental to the daughter cells during cell division. Maintenance of epigenetic memory is critical for the propagation of cell lineage in replicating cells. For immune cells, particularly memory T cells, epigenetic memory is also essential for their ability to display enhanced functional capacity following repeated exposure to an antigen, which is the basis for immunization.

Removal of the methyl mark from DNA can occur either passively or actively (Fig. 2). Passive demethylation is achieved if cell division is not followed by maintenance demethylation. On the other hand, several proteins are thought to be implicated in the active removal of the methyl group from Cytosine. These include the Activation Induced Deaminase (AID), with known function in somatic hypermutation and immunoglobulin class-switch recombination, DNA glycosylases, a member of the methyl-CpG binding domain protein family (MBD2), the p53-effector gene Growth Arrest and DNA Damage induced GADD45A, and enzymes of the ten-eleven-translocation (TET) family [6]. The latter catalyze the oxidation of methylated Cytosine, generating hydroxyl-methyl-Cytosine (hmC), which is an intermediate in the demethylation reaction. More recently, evidence supports that hmC may have additional biological functions besides serving as a demethylation intermediate.

Histones can be subject to a large repertoire of covalent post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitination and other [7]. These modifications affect histone-histone and histone-DNA interactions, generating more open or compact local chromatin structures. Furthermore, specifically modified histones serve as docking sites for the recruitment of protein complexes regulating all chromatin-templated processes, such as replication, transcription and DNA repair. The functional output of histone modifications depends on multiple factors, including the type and degree of the modification, which residue is modified, the genomic location, the presence of additional modifications in the locus and other. Cross-talk between histone and DNA modifications has also been described in many cases, with some promoting others and displaying synergy and others inhibiting or mutually excluding each other. In combination, the totality of epigenetic marks at a genomic location will generate an open and accessible chromatin environment or a closed configuration, restrictive to transcription, repair or additional factors. Histone modifications are reversible and, in most cases, enzymes catalyzing their addition or removal are known. Acetylation of histones is catalyzed by histone acetyltransferases (HATs) and deacetylation by histone deacetylases (HDACs). Similarly, a growing list of histone methyltransferases and demethylases, kinases and phosphatases, ubiquitin ligases and deubiquitinases has been identified and their biological role, which often displays cell-type specificity, is a subject of intense research focus.

3. Epigenetic mechanisms regulating immune cell differentiation and function

Cell differentiation, maintenance of cell identity and homeostasis rely on accurate orchestration of gene expression, which is regulated by multiple mechanisms, including chromatin modifications. The immune system serves as a prototypical model for understanding how epigenetic patterns are shaped in a hierarchical, stepwise differentiation process, through which different cell types arise from common progenitors. Subsequently, the dynamic regulation of epigenetic patterns also regulates the function of these cell types and their response to various stimuli. The examples provided below illustrate the role of epigenetic regulation in immune cell biology, which explains how deviations could result in disorders like autoimmunity.

Extensive DNA methylation changes are involved in the differentiation and maturation of T cells to cluster of differentiation 4 and/or 8 (CD4, CD8) positive T cells [8]. Deletion of Dnmt1 results in a significant reduction of methylation in $CD4^+CD8^+$ thymocytes as well as in naïve $CD4^+$ and $CD8^+$ T cells, and increased expression of cytokine genes [9]. TET-mediated demethylation is also important in T cell development and 5-hmC enrichment is observed at multiple active cell-type-specific enhancers and key transcription factor encoding genes (like *ThPOK*, *Gata 3*, and *Runx 3*) [10–12]. DNA methylation and histone modifications also regulate responses of peripheral T lymphocytes to antigenic stimulation by controlling the expression of immune-related genes, in a cell-type-specific manner. For instance, demethylation of genes encoding for the transcription factors c-JUN and Nuclear factor of activated T cells (NFATc1) as well as the effectors perforin (PRF1) and granzyme B (GZMB) is required for $CD8^+$ T cell function [13,14]. Moreover, DNA methylation regulates expression of several genes in memory T cells compared to naïve cells, like interferon gamma (*IFNG*) and interleukin 2 (*IL2*) in memory $CD8^+$ T cells and Chemokine receptor 6 (*CCR6*), RAR-related orphan receptor C (*RORC*) and genes encoding ligands for P-selectin and E-selectin in memory $CD4^+$ T cells compared to naïve cells [15,16]. Besides effector molecule expression, DNA methylation also regulates the expression of critical factors for immune cell survival and proliferation, such as the cell death-related

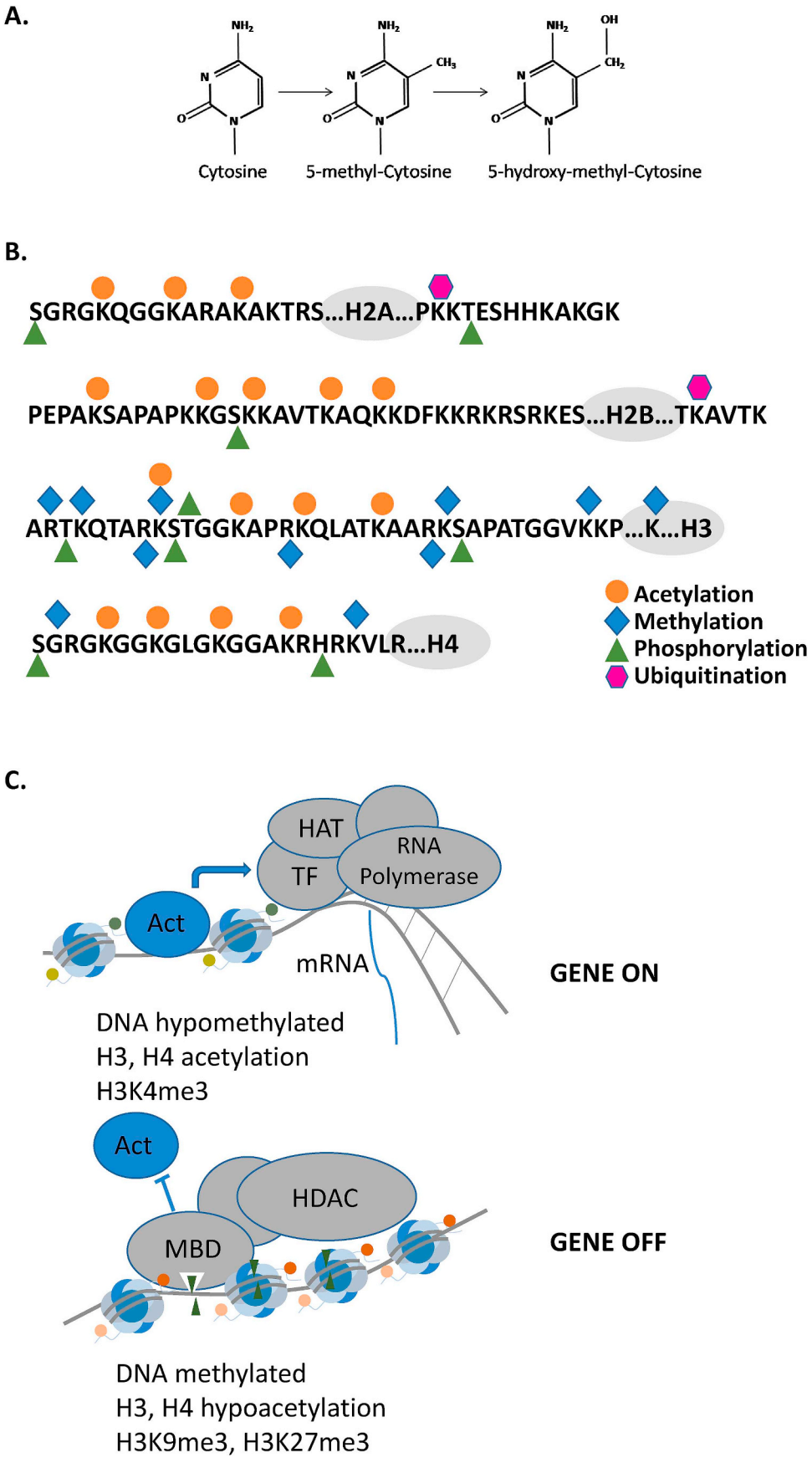

Fig. 1. Examples of (a) DNA and (b) histone modifications, as well as (c) a schematic model of how they are linked to transcriptional activity. Act: Activator, TF: Transcription factor, MBD: Methyl-CpG-binding domain.

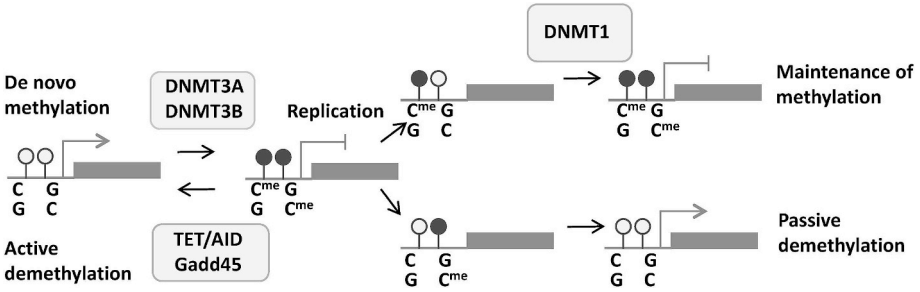

Fig. 2. Model of active and passive DNA demethylation. Maintenance of methylation keeps the locus repressed (flat-ended line) while demethylation correlates with transcriptional activation (angled arrow).

gene *Noxa* in $CD4^+$ memory cells and the programmed cell death 1 (*Pdcd1*) in $CD8^+$ T cells [17,18].

Epigenetic marks play an important role in memory formation in immune cells, which gives them the ability to respond more robustly to an antigen that they have been previously exposed to, although the exact mechanisms shaping immunological memory are only partially understood. For instance, H3K9 acetylation in the promoters of the effector molecules eomesodermin (*EOMES*), *PRF1* and *GZMB* was increased in memory $CD8^+$ T cells compared to naïve $CD8^+$ T cells [19]. Interestingly, although expression levels of EOMES and PRF1 are higher in $CD8^+$ memory compared to naïve cells both at resting state and upon activation, it was only after activation that $CD8^+$ memory cells expressed higher levels of GZMB compared to naïve $CD8^+$ T cells. Moreover, DNA demethylation occurs in the *IFNG* and *IL2* loci upon activation in memory but not in naïve $CD8^+$ T cells [20]. The above findings suggest that chromatin marks, such as DNA methylation and histone acetylation, could account for maintaining a chromatin environment poised for re-activation of effector genes, producing an enhanced response. Since epigenetic modifications can be transmitted from the parental to the daughter cells, they could provide a molecular basis for memory preserved through cell division.

Recently an interesting example of epigenetic regulation of the immune response came from studying the effect of gp96 on antigen-presenting cells (APCs). Gp96 is an intracellular chaperone that binds various endogenous peptides released from damaged cells and can function as an immunogen via its receptor cluster of differentiation 91 (CD91), expressed on the surface of dendritic cells (DCs). Experiments using mice immunized with gp96 revealed that the antigen can induce differential responses depending on the administered dose. At low doses, gp96 preferentially engages conventional dendritic cells (cDCs) while at high doses it engages plasmacytoid dendritic cells (pDCs) [21]. In the first case, it primes a T helper type 1 (Th1)/Cytotoxic T lymphocyte (CTL) immune response, while in the second case it primes regulatory T cell- (Treg) mediated immunosuppression. The divergent responses depend on global cell-type specific changes in DNA methylation, leading to differential gene expression profiles. Methylome remodeling depends on DNMT1 nuclear architecture, which is mediated by the nuclear factor κB (NFκB), a transcription factor with important immune functions. Given that CD91 is also expressed in other cell types, including the salivary gland epithelial cells, the repertoire of responses could be even broader [22].

Among immune-related genes, some key transcription factors attract special interest, as they hold hierarchically higher positions, regulating the expression of a large number of genes, including other transcription factors. They are thought to function as master regulators of certain gene expression programs and defects in their activity are detrimental. One such example is the autoimmune regulator (AIRE), a transcription factor expressed in medullary thymic epithelial cells (mTECs). These cells play a significant role in immune tolerance, the ability to distinguish self from foreign antigens, by ectopically expressing all self-antigens, including those that are highly tissue-specific, and presenting them to T cells. T cells that react to antigens they would normally encounter once released to the periphery undergo negative selection. Expression of antigen encoding genes in mTECs is thought to rely on AIRE. Regulation of the *AIRE* gene depends on complex epigenetic mechanisms. In cells that do not express AIRE, its genetic locus is hypermethylated and insulated by the global chromatin organizer CCCTC-binding factor (CTCF) [23]. Transcriptional activation of *AIRE* in mTECs requires the eviction of CTCF and TET-enzyme-mediated DNA demethylation on the promoter and downstream elements, facilitated by the concerted action of several factors, including Interferon Regulatory Factor 8 (IRF8), T-box transcription factor TBX21 and Transcription Factor 7 (TCF7), which render the locus accessible to the polymerase. The ability of AIRE to regulate its targets has been proposed to be modulated by its plant homeodomain (PHD) finger, which mediates its binding to H3K4 hypomethylated chromatin regions, thereby recruiting it to weakly transcribed loci [24]. In mice, deletion of *Aire* or mutation of its PHD finger have been linked with autoimmune symptoms in some strains [24]. The above illustrate that a complex interplay of epigenetic changes is critical for the regulation of this master immune regulator and its transcriptional targets.

Another cell population critically implicated in maintaining immune tolerance and restraining immune response towards both self and foreign antigens are the regulatory Treg cells, a distinct lineage of $CD4^+$ T cells. The development and function of Tregs depend on the expression of forkhead box P3 (FOXP3), a winged-helix family transcription factor, which serves as the master switch of their gene expression program. The gene encoding FOXP3 is located on the X chromosome and its expression during Treg differentiation is regulated by CpG islands located in its upstream enhancer as well as in three conserved noncoding sequence (CNS) elements in the first intron [25]. The methylation status of these elements is regulated by the opposing function of DNMTs and TET enzymes and determines its transcriptional activity [26,27]. The latter proteins, specifically Tet 2 and Tet 3 are critical for stable expression of Foxp3 in Tregs and maintenance of their identity. Upon inflammation, Interleukin 6 (IL-6) and Signal transducer and activator of transcription 3- (STAT3) mediated induction of DNMTs is thought to challenge Foxp3 expression and is counteracted by the function of the zinc finger transcription factor Blimp1, which serves to maintain demethylated and active Foxp3. In this way, Blimp1 is critical to preserve Treg stability even under severe inflammation, which poses a threat to their identity and function [28].

4. Epigenetic alterations in autoimmune disease

4.1. - DNA methylation

A connection between autoimmunity and epigenetic regulation was established when it was observed that the DNA of SLE patients is hypomethylated and the degree of hypomethylation correlates with disease activity. Treatment with DNA methylation inhibitors, such as 5-azacytidine (5-azaC), can render antigen-specific cloned T cells autoreactive, responding to self major histocompatibility complex determinants (human leukocyte antigen HLA-D molecules) in the absence

of relevant antigens [29]. Moreover, adoptive transfer of autoreactive T cells obtained by exposure to demethylating agents can induce SLE-like symptoms in mice, resembling the human disease [30]. Subsequent studies revealed that procainamide, a major substance responsible for drug-induced lupus, is a strong DNA methylation inhibitor [31]. In agreement with the observed hypomethylation, the gene encoding DNMT1 has been reported to be reduced in SLE peripheral blood mononuclear cells (PBMCs) compared to controls, while the growth arrest and DNA damage-induced 45a Gadd45-alpha protein expression, which is implicated in demethylation, was found increased [32,33].

Changes in global DNA methylation levels have also been observed in epithelial cells of the minor salivary gland (MSG) in SS patients. These cells comprise one of the primary autoimmune targets in this disorder and have a central role in its pathogenesis. Immunohistochemical analysis in minor salivary gland tissue revealed a global reduction of 5-methyl cytosine (5 mC) in SS patients compared with sicca control patients, whose glands don't display lymphocytic infiltration [34]. Hypomethylation in minor salivary glands was correlated with anti-La/SSB positivity in sera. Methylation of the promoter of the gene encoding Sjögren Syndrome Type B Antigen (SSB), one of the main autoantigens in SS, is also reduced in SS patient MSG epithelial cells compared to controls. Reduced methylation correlated with increased expression levels of SSB mRNA and protein and peripheral autoantibody positivity indicating a possible connection. This observation probably explains the abundance of autoantigen within the tissue lesion and, eventually, the continuation and sustainability of the autoimmune response. It is noteworthy that global hypomethylation compared to controls is maintained even in SS patient-derived salivary gland epithelial cells (SGECs) in culture compared to controls, as assessed by an enzyme-linked immunosorbent assay (ELISA) utilizing an anti-5mC antibody, suggesting that the observed epigenetic alterations are relatively stable and maintained even after dissociation of cells from the tissue and ex vivo culture [35]. Although no differences were noticed by ELISA in peripheral T cell or B cell global methylation levels, B cells infiltrating the salivary gland tissue are thought to contribute to the observed epithelial cell hypomethylation. This is supported by the observation that salivary gland biopsies with abundant B cells exhibit even more pronounced hypomethylation, whereas treatment with the anti-CD20 B cell depleting agent Rituximab associates with an increase in patient MSG methylation levels [35].

In RA, global reduction in DNA methylation has been reported in synovial fibroblasts (SFs), a cell type with a prominent implication in the disease [36]. Immunohistochemical analysis using an antibody specifically recognizing anti-5mC revealed generalized genomic hypomethylation in synovial tissue sections from RA compared to patients with Osteoarthritis (OA), which is a non-inflammatory joint disease. Similar to the SS epithelial cell cultures, SFs isolated from synovial tissue and cultured ex vivo showed significantly reduced anti-5mC staining intensity compared to OA SFs. The differences remained significant even after exposure to proinflammatory cytokines, which generally increased anti-5mC content, indicating that epigenetic differences could be associated with altered physiological responses of the cells. Consistent with reduced methylation, the levels of DNMT1 were lower in RA than OA synovial tissue and treatment with the DNMT1 inhibitor 5-azaC caused normal SFs to resemble RA SFs, further supporting functional implications of the altered methylation pattern.

Beyond global changes in DNA methylation levels, recent technologies aim to characterize and decipher the totality of epigenetic modifications within the cell, termed the epigenome. Such approaches have allowed mapping of the differentially methylated regions, which can provide information on the genes and pathways affected. Methodologies include the use of arrays targeting a large number (hundreds of thousands) of CpG sites across the genome, covering gene promoters, 5′ untranslated regions (5′UTR), first exons, gene bodies and 3′ untranslated regions (3′UTRs), as well as CpG islands and their surrounding shores (immediately flanking CpG islands) and shelves (immediately flanking CpG shores). Application of this technology in different cell types of autoimmune disease relevance has uncovered regions across the genome that were differentially methylated between patients and controls. Information on differential methylation on promoters or other regulatory elements becomes more relevant when accompanied by changes in gene expression. In combination, datasets have uncovered genes and pathways that are subject to methylation-dependent regulation and implicated in disease pathogenesis.

Epigenome-wide methylation analysis in peripheral blood mononuclear cells identified 41 differentially methylated sites between SLE patients and controls, the majority of which (85%) were hypomethylated in patients [37]. The differentially methylated sites were associated with 30 genes, including the interferon related genes MX dynamin-like GTPase 1 MX1, IFN-induced protein 44-like (*IFI44L*), Poly-ADP-Ribose Polymerase Family Member 9 (*PARP9*), IFN-induced protein with tetratricopeptide (*IFIT1*), and Interferon regulatory factor 7 (*IRF7*) among others. These were hypomethylated and their expression was increased in patient versus control cells. Aberrant hypomethylation of interferon-related genes, including some of the above and others, like signal transducer and activator of transcription 1 (*STAT-1*), bone marrow stromal cell antigen 2 (*BST2*) and tripartite-motif-containing 22 (*TRIM22*), was also observed when the analysis was performed in lupus $CD4^+$ T cells [38]. Hypomethylation of a number of loci, including genes encoding cytokines, Killer-cell immunoglobulin-like receptor (*KIR*), perforin and the Reducing Regulatory factor X1 (*RFX1*) in SLE $CD4^+$ T cells leads to their overexpression, which results in autoreactivity and aberrant inflammatory responses in multiple immune cell types. In contrast, hypomethylation of other loci, like the gene encoding the nuclear factor interleukin-3-regulated protein (*NFIL3*), could be indicative of compensatory mechanisms to downregulate autoimmune responses in SLE patients (Table 1).

Comparison of genome-wide methylation profiles between SS patient and control-derived peripheral $CD4^+$ T cells and $CD19^+$ B cells identified a large number of loci displaying differential methylation between the two groups, without changes in global methylation levels [39]. Differences were more pronounced in B cells, with similar numbers of hypo and hypermethylated loci. Cells derived from patients with active disease showed more significant changes. Some of the differentially methylated loci map near genes associated with increased disease risk, like the major histocompatibility complex/human leukocyte antigen (MHC/HLA) region, Interferon regulatory factor 5 (*IRF5*), *BLK*, Signal Transducer And Activator Of Transcription 4 (*STAT4*), Interleukin 12A (*IL12A*), TNFAIP3 Interacting Protein 1 (*TNIP1*) and C-X-C Motif Chemokine Receptor 5 (*CXCR5*), suggesting that genetic and epigenetic alterations could result in aberrant expression of these genes and be implicated in SS pathogenesis. Other differentially methylated genes of potential interest for their implication in the disease are RUNX Family Transcription Factor 3 (*RUNX3*), TNF Alpha Induced Protein 8 (*TNFAIP8*), IKAROS family zinc finger 1 (*IKZF1*), Solute Carrier Family 15 Member 4 (*SLC15A4*), Growth Factor Receptor Bound Protein 2 (*GRB2*), MicroRNA 21 (*MIR21*), Interleukin 21 Receptor (*IL21R*), and TNF Receptor Associated Factor 5 (*TRAF5*), supporting an implication of the interferon response, the innate immune system, B cell activation and T cell/natural killer (NK) cell activation pathways in SS pathogenesis. A similar analysis was performed in long term cultures of SGECs [40]. The comparison identified a large number of differentially methylated loci between patient and control-derived SGECs, corresponding to 2560 genes. About one-fifth of these genes contained two or more differentially methylated sites. Pathway analysis highlighted the significant representation of interferon-regulated genes and genes implicated in the calcium pathway (mainly hypomethylated) as well as the Wnt pathway (mainly hypermethylated).

DNA methylation patterns were also evaluated in RA Fiboblast-like-Synoviocytes (FLS) and compared with FLS derived from individuals with OA [41–43]. RA FLS exhibit a distinct methylome signature than RA FLS and normal FLS, with differentially methylated genes that are

Table 1
Epigenetic alterations that have been observed in different cell types and tissues in autoimmune diseases.

Disease	cell type/tissue	gene/promoter/element	alteration	Reference
SLE	PBMC	MX1, PARP9, IFIT1, IRF7, IFI44L, IFNGR2, MMP14, LCN2, 18S, 28S rDNA	reduced DNA methylation	[37, 70, 77]
	T cells	KIR3DL1	reduced DNA methylation	[78]
	$CD4^+$ T cells	STAT1, BST2, TRIM22, KIR, NFIL3, RFX1, FOXP3, ITGAL,CD70, CD40LG, PRF1, IFN-induced genes	reduced DNA methylation	[14, 38, 78–83]
	$CD4^+$ T cells	IL2, IL17	increased DNA methylation	[57]
	$CD19^+$ B	CD5	reduced DNA methylation	[84]
	Monocytes	IRF1 binding sites	increased H3K4me3	[85]
SS	$CD4^+$ T cells	CD70, LTA, CD247, TNFRSF25, PTPRC, GSTM1, PDCD1, STAT1, IFI44L, USP18, IFITM1, IFN-induced genes	reduced DNA methylation	[86–87]
	$CD4^+$ T cells	FOXP3, RUNX1	increased DNA methylation	[87–88]
	minor salivary glands	OAS2, IFN-induced genes	reduced DNA methylation	[39]
	minor salivary glands	BP230	increased DNA methylation	[89]
	Salivary gland epithelial cells	LINE1, IFN-induced genes	reduced DNA methylation	[40, 50]
	whole blood, $CD19^+$ B cells	IFN-induced genes	reduced DNA methylation	[39]
RA	PBMC	IL6, PDCD1	reduced DNA methylation	[90–91]
	$CD4^+$ T cells	FOXP3 enhancer, IFN-induced genes	reduced DNA methylation	[83, 92]
	Fibroblast-like synoviocytes	LBH enhancer	reduced DNA methylation, increased H3K4me1	[93]
	Fibroblast-like synoviocytes	CXCL10, IL6	Increased H4ac	[94–95]
	Synovial fibroblasts	SFRP1	reduced H3K4me3, increased H3K27me3	[96]
	Synovial Fibroblasts	LINE1	reduced DNA methylation	[36]
APS	whole blood	F3	increased DNA methylation	[45]
	whole blood	IL8	reduced DNA methylation	[45]

critical to cell trafficking, inflammation, and cell–extracellular matrix interactions, joint architecture and inflammation. Examples include the cartilage-specific antigen Chitinase 3 like 1 (*CHI3L1*), also known as *gp39*, which is the target for autoimmunity in RA, *STAT3*, a key signaling protein activated in RA and associated with interleukin (IL)-6 function, TNF signaling (*TRAF2*), IL-1 regulation (*caspase 1*) and purinoreceptors, several collagen proteins, proteases (*ADAM12*) and protease inhibitors (*TIMP2*), and genes implicated in movement, adhesion, and trafficking. The relevance of these genes to RA pathology underscores the link between their aberrant methylation and disease pathogenesis.

A methylome analysis was recently performed in patients suffering from the autoimmune thrombophilia Antiphospholipid Syndrome (APS) [44]. The syndrome is characterized by recurrent thromboembolism and/or pregnancy morbidity in the presence of Antiphospholipid antibodies. The analysis was performed in neutrophils and identified a number of differentially methylated CpG sites in patients compared to healthy controls, with 17 of them hypomethylated and 25 hypermethylated in the disease group. Among these, there were known genetic risk loci for SLE (*ETS1*) and other autoimmune diseases (Protein Tyrosine Phosphatase Non-Receptor Type 2, *PTPN2*). The hypomethylated category was also enriched in genes involved in pregnancy, providing a possible connection with the pregnancy morbidity occurring in APS patients. Using a different approach, the methylation status of two key mediators in APS, Coagulation Factor III (*F3*) and interleukin 8 (*IL8*) in whole blood DNA was also found to differ significantly between APS patients and healthy donors, in a manner correlating with clinical parameters [45]. In an ex vivo model mimicking APS, stimulation of monocytes induced dynamic changes in methylation in the above loci, while in human umbilical vein endothelial cell cultures, the treatment also caused transcriptional upregulation of several epigenetic factors including Methyl-CpG Binding Protein 2 (*MECP2*), *DNMT3B*, *TET1*, *HDAC9* and the AT-Rich Interactive Domain-Containing Protein 5B (*ARID5B*). The above data combined illustrate how epigenetic changes could be involved in transcriptional regulation of key factors implicated in APS.

4.2. - Methylation of retroelements

Among genomic regions regulated by methylation, a large fraction corresponds to retroelements. These are genetic elements that have the ability to spread in the eukaryotic genome via replication. Termination codons and methylation sites normally make these sequences inactive. Though their functions remain poorly understood, retroelements including non-long terminal repeat (non-LTR) retrotransposons and endogenous retroviruses (ERVs), seem to be important for cellular homeostasis, as their deregulation has been linked with disease. This connection has also been observed in the context of autoimmunity and

several mechanisms have been proposed to explain it, including transcriptional interference with other genes, competition for TF binding, expression of proteins or insertional mutagenesis [46,47].

An example is the human endogenous retrovirus (HERV), which is found in the human genome in multiple copies and has been linked with SLE pathogenesis [48,49]. Supporting evidence includes increased transcript levels of HERV sequences in SLE patients compared with normal controls, as well as detection of antibodies against HERV in about 50% of SLE patients but not in controls. Moreover, a synthetic HERV clone derived peptide can induce $CD4^+$ T cell activation. The effect is amplified by 5-azaC treatment, which enhances transcription of the synthetic HERV clone in PBMCs from healthy individuals, suggesting that it is regulated by DNA methylation. Reduced methylation of HERV could increase its transcription and is thought to contribute to autoimmunity by promoting the production of endogenous HERV antigens.

Another example of retroelements linked with autoimmunity are the Long Interspersed Nuclear Elements (LINEs). Full-length LINEs are 6 Kb, but they become shorter and often inactive with age. Transcriptional repression of LINEs depends on the methylation of critical CpG dinucleotides within their promoter. Loss of methylation can cause transcriptional activation of LINEs containing active promoters. Demethylation and increased expression of LINE1 have been described in MSG tissue from SS patients as well as in lupus nephritis kidneys compared to controls [50,51]. LINE1 expression correlates with type I IFN expression suggesting a role of LINE transcripts in the activation of innate immune response. In addition, a positive correlation was noted between LINE1 and DNMT3B, DNMT1 and methyl CpG binding protein 2 (MeCP2) transcripts in both SS MSG and lupus renal tissues. The observed upregulation possibly comprises a compensatory mechanism to silence these elements, though future research should further examine this. LINE1 retrotransposable elements were also found to be expressed in RA synovial tissues and cultured RA SF, by in situ hybridization using riboprobes to ORF2/L1 and immunohistochemistry using antibodies to the ORF1/L1-related p40 protein [52]. Treatment with 5-azaC induced LINE1 expression in SFs in a time and dose-dependent manner. The above data suggest that LINE1 induction could be implicated in SF activation in RA.

4.3. - DNA hydroxymethylation

The oxidative derivative of (5-mC), 5-hmC is a DNA modification that lately attracts increasing interest. 5-hmC is generated by the action of TET proteins, including TET1, 2, and 3, as an intermediate in the demethylation reaction of 5 mC, however, its functions could extend beyond this role. Recent studies indicate that its deregulation could also be linked to autoimmunity. Specifically, increased 5-hmC levels were detected in $CD4^+$ T cell DNA of SLE patients compared to controls, along with an increased expression of the TET2 and TET3 enzymes [53]. In addition, a substantial number (2748) of genes showed increased promoter hydroxymethylation in the SLE CD4 + T cells over controls, including genes implicated in critical pathways, like the neurotrophin, WNT, Mitogen-activated protein kinases (MAPK), calcium and the mechanistic target of rapamycin (mTOR) signaling pathways. In a fraction of the promoters showing increased hydroxymethylation, including some with immune-relevant functions, upregulated gene expression was also observed, suggesting that this mark could be implicated in their regulation. The levels of 5-hmC were also reported to be increased in MSG inflammatory and epithelial cells from SS patients compared to controls, while the latter also displayed increased TET2 levels, which was also proposed to be regulated by cytokines [54]. Genome-wide technologies and functional studies are expected to address the role of 5-hmC in autoimmunity in the years to come.

4.4. - Histone modifications

Besides changes in DNA methylation, alterations on histone modifications have also been identified in autoimmune disease models. This comes as no surprise since factors recognizing methylated DNA are often components of protein complexes with other chromatin-modifying and remodeling activities. Both global and locus-specific alterations with functional implications have been reported in autoimmune diseases.

Although the effects of histone modifications often depend on the genomic context, some modifications have been generally linked with transcriptional activation or repression. For instance, H3K9ac and H3K27ac are normally associated with active promoters and enhancers, H3K4me3 is also found in active promoters and H3K4me1 in enhancers, while H3K36me3 in transcribed gene bodies. In contrast, H3K9me3 and H3K27me3 are usually considered silent marks. Thus, it is expected that differential gene expression patterns in autoimmune cell types would be accompanied by changes in some of the above histone modifications. This has been reported for several examples. For instance, in SLE monocytes, acetylation of the tumor necrosis factor (*TNF*) promoter correlates with increased maturation and cytokine expression, while in SLE T cells, histone acetylation and methylation regulate interleukin 17 (*IL17*) gene expression [55–57]. Moreover, a global reduction of histone acetylation and H3K9 methylation was reported for SLE $CD4^+$ T cells, indicating broad changes in the chromatin landscape, which affect the expression of multiple genes [58].

Analysis of histone modifications in splenocytes from the MRL-lpr/lpr lupus mouse model using stable isotope labeling followed by mass spectrometry revealed global hypoacetylation in H3 and H4 as well as increased histone methylation (excluding H3K4) compared to control mice [59]. Interestingly, novel histone modifications, including H3 K18 methylation, H4 K31 methylation, and H4 K31 acetylation, were reported at different levels in the two mouse strains. Their implications in the pathogenesis and relevance to the human disease are certainly worthy of further examination.

5. X-chromosome inactivation

In humans, females seem to be more resistant to bacterial and viral infections compared to males and have a known ability to produce higher titers of serum IgM and antibodies. However, stronger immune reactivity may not always provide an advantage and could make women more susceptible to autoimmunity. Most autoimmune diseases show a female bias. Though the underlying mechanisms remain elusive, this sex discordance is thought to be partially due to a dosage effect of the X-chromosome. Consistent with this view, an increased number of X chromosomes in a karyotype, as observed in XXX and XXY individuals, is associated with increased risk for SLE and SS, while XO individuals, e.g. Turner syndrome patients, have reduced risk for SLE [60–62]. A possible explanation for the link between X chromosome dosage and autoimmunity could have to do with the fact that multiple immune-related genes map on the X chromosome. Thus, aberrant expression of these genes resulting from X chromosome aneuploidy could disrupt normal immune physiology and result in autoimmunity.

In humans, X-inactivation in females is a mechanism of dosage compensation between sexes. Inactivation of either the maternal or the paternal X chromosome is thought to occur stochastically starting from early embryonic development. One of the key factors implicated in this process is the long non-coding RNA X Inactive Specific Transcript (XIST), which is expressed at random from the X chromosome that will be inactivated [63,64]. The Xist RNA serves as a scaffold to recruit chromatin-modifying enzymes in cis and induce DNA hypermethylation and silencing histone marks, like H3K27me3, H3K9me2/3, H4K20me1, and ubiquitinated H2A, generating a nucleation center for the formation of heterochromatin. This transcriptionally repressive chromatin environment ensures dosage compensation of X-linked genes, most of

which become transcriptionally inactive. A small fraction of X genes escape this inactivation, but even those are thought to be expressed at lower levels from the inactive X compared to its active homolog.

X inactivation is thought to be stable and maintained with high fidelity by XIST across mitotic divisions for most female somatic cells. An exception to this general rule has been reported for developing B and T cells, where an interesting temporal pattern of XIST localization and inactive X chromosome H3K27 methylation has been observed [65–68]. XIST RNA and H3K27me3 marks are detectable on the inactive X in hematopoietic stem cells and common lymphoid progenitors. At the pro-B stage, the XIST signal is gradually lost, followed by a gradual loss of heterochromatin marks during B cell differentiation. During T cell differentiation in the thymus, XIST and H3K27 marks gradually disappear from the inactive X and remain undetectable in peripheral T cells. Cytological examination has revealed that in vitro activation of mature B and T cells induces these marks to reappear on the inactive X. The transient loss of repression is thought to generate a euchromatic conformation of the inactive X of female lymphocytes, permissive to transcription of genes that would normally be monoallelically expressed, leading to increased expression of X-linked genes compared to male lymphocytes.

In SLE patients, the X inactivation pattern seems to be affected, characterized by aberrant XIST localization, leading to upregulation of X-linked genes, a phenomenon also observed in T cells derived from female mice of the NZB/W F1 SLE animal model. Whole transcriptome analysis revealed that 143 X-linked transcripts display differential expression between peripheral T cells from SLE patients and healthy donors [67]. Interestingly, the differences became more pronounced with increased disease activity index. The levels of XIST did not seem to differ between patients and controls, however, a reduction of some other genes encoding proteins interacting with it and implicated in X inactivation, like DNMT1, were reduced. Overexpressed genes included genes normally subject to X inactivation and functioning in metabolism, cell cycle, and splicing. Thus, defective X inactivation in SLE patients could impact several important cellular processes, contributing to the disease phenotype. Examples of critical immunoregulatory genes located on X which are found to be upregulated in SLE patients compared to controls include the Treg lineage-specific marker FOXP3 and the CD40 ligand (CD40L). The latter is a T cell transmembrane protein, overexpressed upon activation and implicated in the stimulation of B cells towards antibody-and auto-antibody production.

The link between X dosage and autoimmune disease is also illustrated by the fact that four of the six known X chromosome SLE susceptibility loci map on genes escaping inactivation. These include the gene encoding toll-like receptor 7 (*TLR7*), which has been causatively linked with SLE, as well as the *CXorf21*, which encodes for a small protein colocalizing with TLR7 in B cells [69]. CXorf21 is induced upon IFNα stimulation with significantly higher magnitude in female compared to male B cells. Since the interferon pathway is implicated in SLE pathogenesis, the above findings provide some mechanistic understanding of the sexual dimorphism in autoimmunity.

6. Twin studies

Monozygotic twins provide a model to investigate the contribution of epigenetic factors to autoimmunity. The rationale for these studies is that since genetic variation is minimized, phenotypic disparities could be associated with epigenetic differences between the siblings. For most autoimmune disorders, discordance rates between monozygotic twins exceed 50%, supporting that genetic predisposition can only partially explain disease development, which agrees with the fact that most cases are sporadic rather than familial.

Comparison of DNA methylation between twins discordant for SLE revealed significant differences. Consistent with previous knowledge, the patients displayed reduced global methylation in DNA derived from their white blood cells, as well as reduced levels of DNMT1 and DNMT3B mRNA compared to their healthy twin [70]. Genome-wide analysis using a bead array interrogating methylation status of CpG containing promoters revealed a large number of genes exhibiting significant differences of DNA methylation, among which many could be relevant to SLE pathogenesis. Examples include the interferon gamma receptor 2 (*IFGNR2*) and interferon-stimulated genes, the colony-stimulating factor 3 receptor (*CSF3R*), matrix metalloproteinases, the common marker for lupus nephritis Lipocalin 2 (*LCN2*), and CD9 [70]. The ribosomal genes *18S* and *28S* were also found to be hypomethylated in patients compared to their healthy siblings, an observation that could be linked to the presence of autoantibodies against ribosomal RNA and proteins in SLE patients, although this connection remains to be examined.

A similar analysis in twins discordant for RA identified genomic locations displaying significant differences in DNA methylation variability between the siblings rather than differences in methylation levels [71]. The study was performed using whole blood DNA. In RA twins, hypervariable loci were mostly enriched in the 3′UTR and gene bodies, while in their healthy siblings, hypervariable loci were mostly enriched in gene bodies. Among the top-ranked differentially hypervariable loci were binding sites for RUNX3, which has been implicated in RA pathogenesis. These data suggest that the regulation of RUNX3 target gene transcription could be modified by CpG methylation. Other loci exhibiting differentially variable methylation between RA and healthy twins are mapped on stress-response genes, genes implicated in K63-Ubiquitination, which plays a role in the oxidative stress response, as well as the low-density lipoprotein receptor activity, which has been proposed as a potential biomarker of anti-TNF treatment response in RA patients. Functional studies could further address whether differentially variable methylation of certain loci also partially explains heterogeneity in disease presentation or response to therapies among RA patients.

7. Summary and future perspectives

Epigenetic mechanisms are critical in ensuring normal immune cell function and their deregulation is linked with autoimmunity. The connection may be causative in some cases, while in others the changes can be a result of the pathologic processes or even provide a compensatory response of the organism to resist damage. Deciphering the mechanistic role of epigenetic changes in disease development is challenging, and would require functional studies in animal models. However, most animal models are not adequate to reproduce the human pathology and can only partially address specific questions. The complexity of epigenetic regulation is further increased by the fact that the effects could be cell type or cell state-specific. When accessible, studies of the affected cells or tissues usually prove to be most informative. On the other hand, findings derived from peripheral lymphocytes could have diagnostic utility. To this end, epigenetic marks arise as attractive biomarkers for disease diagnosis, monitoring and even prediction and assessment of therapeutic response. The potential of epigenetic modifications to serve as biomarkers is further supported by the finding that several epigenetic alterations occur in disease risk loci. Integrating epigenetic data with transcriptomic analyses and genetic associations may provide a better picture of their functional implications.

Elucidation of the cross-talk of epigenetic factors with signaling pathways and other environmental parameters will also be useful in protection from damage. Given that many of the enzymes catalyzing chromatin modifications use metabolites as co-factors, the interplay between epigenetic regulation and metabolism could have significant effects. Current research also investigates the potential of histone and chromatin-modifying enzymes and proteins that recognize and bind to chromatin modifications as potential therapeutic targets. For instance, DNMT inhibitors have been proposed as a means to promote FOXP3 expression in Tregs expanded ex vivo for cellular therapies post

allogeneic stem cell transplantation [72]. This approach is based on the methylation-sensitive regulation of FOXP3 as discussed above. However, experiments in mice showed that conditional deletion of *Dnmt1* in Tregs causes death attributed to autoimmunity, and support that Dnmt1 is required for conversion of conventional T cells into Foxp3+ Tregs [15]. Other studies have proposed the use of inhibitors of the deacetylase Sirtuin 1 as potentiators of the suppressive function of Tregs as well as suppressors of T helper 17 (Th17) cell differentiation, which could be beneficial in the therapy of autoimmune disorders [73,74]. In an experimental model of autoimmune encephalitis, administration of a selective inhibitor of the lysine-specific demethylase JMJD3 ameliorated the phenotype by affecting dendritic cells, which showed reduced expression of costimulatory molecules and proinflammatory cytokines and increased expression of tolerogenic molecules [75]. Finally, besides enzymes catalyzing epigenetic modifications, proteins that recognize these marks may also serve as therapeutic targets. An example derives from bromodomain-containing proteins, which recognize acetylated lysine on histone tails. An inhibitor of these proved efficacious in mouse models of autoimmunity, by suppressing differentiation and activation of Th17 cells [76]. Modern technologies of ex vivo treatment of immune cells could allow for safe clinical applications of the above therapies, minimizing off-target effects on other cell types.

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