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Epigenetics in systemic lupus erythematosus: leading the way for specific therapeutic agents

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Abstract

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder of an unclearly determined etiology. Past studies, both epidemiological and biological, have implicated epigenetic influences in disease etiology and pathogenesis. Epigenetics describes changes in gene expression not linked to alterations in the underlying genomic sequence, and is most often typified by three modifications: methylation of DNA, addition of various side chains to histone groups and transcriptional regulation via short ncRNA sequences. The purpose of this article is to review the most important advances that link epigenetic changes to lupus. The contribution of DNA methylation changes to lupus pathogenesis is discussed. These include the role of apoptotic DNA, ultraviolet radiation, endogenous retroviruses, dietary contributions and aging. Hypomethylation of specific genes overexpressed in lupus T cells such as *ITGAL* (CD11a), *CD40LG* (CD40L), *TNFSF7* (CD70), *KIR2DL4* and *PRF1* (perforin), and *CD5* in lupus B cells seem to play an important role. Moreover, histone modifications such as increased global H4 acetylation in monocytes are highly associated with SLE. ncRNAs, especially miR-21, miR-148a and miR-126, control other elements of epigenetic regulation; particularly, transcription of the maintenance DNA methylation enzyme DNMT1. Epigenetic contributions to SLE etiology have been well established, but much is still unknown. Epigenome-wide studies coupled with functional analysis of the epigenomic changes discovered will uncover novel pathways important in disease pathogenesis. Epigenetic therapies for SLE may be feasible in the future, particularly if they are designed to target specific regions within the genome.

Keywords

DNA methylation; epigenetics; histone modification; lupus; miRNA; SLE; T cells

Systemic lupus erythematosus (SLE) is a chronic relapsing multisystem autoimmune disorder, characterized by nuclear-targeted autoantibody production, of incompletely described etiology. It afflicts both sexes, although a significant female predominance of patients is seen. In the USA, lupus is significantly more common among African-American,

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Hispanic and Asian than Caucasian women [1]. Geography also plays a role: a recent epidemiological survey pegged the lowest global incidence of lupus in Iceland and Japan, and highest in the USA and France [2], perhaps reflecting genetic makeup or environmental factors.

Discordance in inheritance noted during early twin studies conclusively demonstrated that the disorder is not entirely genetic in origin, and the predisposition of certain groups suggests the contribution of environmental factors [3]. Epigenetics is the biological science describing heritable changes in gene-expression patterns not coincident with alterations in the underlying DNA sequence that allow biological systems to affect transcription in response to a variety of environmental stimuli. There are three modifications most frequently implicated in epigenetic control: DNA methylation, histone modifications and RNAi (Figure 1).

Studies of epigenetic aberrations in lupus patients have been quite revealing, including a recent analysis demonstrating a high association between changes in DNA methylation patterns and twin discordance in lupus [4]. Could they one day offer novel diagnostic or therapeutic avenues as well? The field could certainly stand to benefit. Until recently, only three drugs have been US FDA-approved for the treatment of lupus: aspirin, prednisone and the antimalarial drug hydroxychloroquine. A monoclonal antibody targeting the B-cell activator of the TNF family (BAFF), belimumab, has recently been approved for the treatment of lupus [5].

We therefore present this article with two principles in mind: first, to offer an introduction to epigenetics and highlight what we consider the most important and interesting discoveries in lupus epigenetics to date; and second, to briefly introduce approaches to epigenetic therapies that may one day hold the key to long-term improvement in the lives of SLE patients.

DNA methylation

Methylation of DNA most frequently occurs at the fifth carbon of the pyrimidine ring in cytosine residues. Although this methylation has traditionally been described for cytosines located in cytosine–guanine dinucleotides (CG pairs), more recent studies implicate other motifs, although these appear to be restricted to transcriptional regulation of embryonic and induced pluripotent stem cells [6]. CG pairs are found ubiquitously throughout the genome; however, their transcriptionally repressive activity is generally ascribed to regions within the promoter of the gene in question, particularly when located between 1000 bp and 800 bp upstream of the transcription start site [7]. Genes wherein cytosines of this region exhibit frequent methylation tend to be transcriptionally repressed, whereas genes whose promoters are hypomethylated are accessible for transcription. Although DNA methylation was initially thought to be a stable modification with only passive removal of methyl groups possible, a growing body of evidence supports the notion that DNA methylation and demethylation occur actively and with regularity. In fact, active DNA methylation appears to play a pivotal role in a variety of cellular responses to environmental stimuli, including hypoxia [8], hormonal signaling [9], viral latency and reactivation [10], and X-chromosome inactivation [11]. Furthermore, one may see both pathologic, aberrant hypo- and hyper-methylation of various genes in the same cell, epitomized in neoplastic cells [12]. *In vivo*, DNA methylation patterns are established by the activity of the *de novo* DNA methyltransferase enzymes DNMT3A and DNMT3B. DNMT2 functions to methylate RNA. DNMT1 is the maintenance DNA methylation enzyme, recognizing hemimethylated cytosines during the S phase of the cell cycle and copying these marks onto the nascent strand during DNA synthesis.

Historical perspective of DNA methylation in SLE

Early studies of SLE epigenetics demonstrated that CD4⁺ T cells treated with DNA methylation inhibitors (specifically, 5-azacytidine [5-azaC]), lose antigen selectivity and respond to the presentation of self antigens [13]. Similarly, injecting CD4⁺ T cells that have been chemically demethylated into syngeneic mice causes a lupus-like syndrome [14,15]. The observation that drugs causing DNA demethylation cause autoimmunity led to an interesting corollary: perhaps medications previously known to be associated with the development of drug-induced lupus function via inhibition of methylation? Of particular interest were procainamide and hydralazine, which produce positive antinuclear antibodies (ANA) titers in a majority of patients taking them, and known to produce a lupus-like syndrome in a genetically-predisposed subset of patients [16]. Both were found to inhibit DNA methylation and produce murine lupus, although via distinct mechanisms: whereas hydralazine was found to be an ERK pathway inhibitor by blocking signaling through PKC δ , procainamide was shown to be a competitive antagonist of the maintenance DNA methyltransferase DNMT1 [17–19]. Furthermore, one of the most commonly-used lupus-prone mouse strains, MRL lpr , was found to exhibit the same reduction in CD4⁺ T-cell Dnmt1 levels with subsequent demethylation and overexpression of some genes known to be demethylated and overexpressed in human lupus T cells, such as *TNFSF7* (CD70) [20]. A transgenic mouse expressing a dominant-negative MEK in T cells, thereby inhibiting T cell ERK pathway signaling, showed similar downregulation of Dnmt1, increased expression of methylation sensitive genes such as *Itgal* and *Tnfsf7*, and an induction of anti-dsDNA autoantibodies [21].

Patients with idiopathic systemic lupus have similar changes in DNA methylation. CD4⁺ T cells isolated from active lupus patients demonstrate global DNA hypomethylation secondary to decreased DNMT1 levels [22,23]. Upon further examination, SLE patients were also found to have a PKC δ - and ERK-pathway signaling defects in T cells which explains reduced DNMT1 expression [19]. The reduction in DNMT1 level has been shown to correlate with lupus disease activity scores [22].

General topics in SLE DNA methylation

Hypomethylated apoptotic DNA—There is substantial evidence that apoptotic DNA plays a key role in the pathogenesis of lupus. Lupus patients are less able to clear apoptotic cells than their disease-free counter-parts [24–25]. The DNA-anti-DNA antigen-antibody complex isolated from SLE patients is conveniently precisely the correct size to have resulted from extracellular breakdown of apoptotic chromatin [26]. Injection of DNA extracted from *ex vivo* stimulated lymphocytes into BALB/c mice is capable of inducing an autoimmune disease closely resembling human lupus, including production of anti-dsDNA antibodies and lupus nephritis [27]. The induction of this lupus-like syndrome is entirely dependent upon the methylation level of apoptotic DNA. Naturally demethylated apoptotic DNA, upon remethylation via treatment with DNA methylase, is robustly impaired in its ability to induce anti-dsDNA antibody production upon injection. Conversely, demethylation of either necrotic or normal DNA via treatment with 5-azaC before extraction greatly potentiates its ability to stimulate an autoimmune response, producing up to 70% of the peak antibody production and proteinuria seen with apoptotic DNA [28]. This selective response to hypomethylated DNA is likely due to signaling through Toll-like receptor 9, which evolved as part of the innate immune system to recognize methyl-deficient bacterial genome sequences and stimulate immune response through cytokine signaling and type-I interferon [29]. Emphasizing the importance of methylation marks in this process, DNA isolated from antigen-antibody complexes in this model system reveal a high frequency of CG-rich sequences [28]. Since DNA demethylation is known to be associated with SLE, and greater levels of DNA demethylation may be correlated to disease activity, increased cellular

apoptosis and release of apoptotic DNA may act as a pathogenic positive feedback loop; further stimulating autoimmunity and leading to additional demethylation.

Ultraviolet light exposure—Studies indicate that exposure to ultraviolet radiation, particularly UVB, can induce expression and secretion of various interleukins including IL-1 and TNF- α in keratinocytes, mast cells, and Langerhans cells, and may function to recruit dendritic and T cells [30]. Compared to baseline, peripheral blood mononuclear cells (PBMCs) originating from lupus patients exhibit significant reduction in global DNA methylation levels upon exposure to both moderate (50 mJ/cm²) and high doses (100 mJ/cm²) of UVB radiation. Interestingly, healthy controls only demonstrate significant reduction in methylation levels following high-dose UVB radiation exposure. SLE patients, furthermore, have a more profound decrease in global methylation than do controls. Importantly, this effect is independent of DNMT1 levels, which remain stable after ultraviolet exposure [31]. These observations suggest that lupus photosensitivity may be due, in part, to epigenetic phenomena.

Endogenous retrovirus—Endogenous retroviruses and retroviral elements are ubiquitous components of vertebrate genetic code, making up as much as 40% of the human genome [32]. Several lines of evidence implicate viruses and viral DNA sequences in the pathogenesis of SLE [33]. First, viral contributions have been linked to pathogenesis in lupus mouse models [34–36]. In addition, antibodies and antigens for retroviral components are found in organs and serum of patients with SLE [37–45]. Furthermore, viron-like tubuloreticular structures exist in endothelial cells and lymphocytes of lupus patients with increased type I interferons [46]. Finally, viral components derived from endogenous retroviruses induce autoimmunity similar to SLE in model systems [47,48]. Retroviruses, in particular, have been implicated via detection of p30 gag protein in renal glomeruli and reactivity to p30 gag antigen in lupus patients [49]. Retroviral elements may contribute to autoimmunity in either of two ways: either by encoding autoantigens directly or by influencing the expression of genes involved in the host immune response and tolerance.

Autoantigens encoded by endogenous retroviruses have been extensively documented; HRES-1 and ERV-3 antigenicity is a common finding in SLE, having been previously reported in 21–52 and 32% of lupus patients, respectively. In addition, the Sm D antigen, a highly specific SLE marker, is mimicked by Epstein–Barr virus nuclear antigen-1 [50]. Expression of endogenous retroviral sequences is regulated, at least in part, by DNA methylation. Expression of HERV-E4-1 in PBMCs from lupus patients is greatly increased when compared with controls. Expression may be further increased with the addition of the DNA methylation inhibitor 5-azaC and greatly decreased with the addition of steroid hormone [45]. Moreover, the HERV-E4-1 clone has sequence homology to the Ro/SS-A antigen, antibodies against which are frequently associated with the autoimmune disease Sjögren’s syndrome [51].

Diet—A recent study by Li *et al.* sought to identify whether changes in dietary intake with subsequent reduction in availability of methylation-related micronutrients could alter the epigenetic milieu of PBMCs. In order to faithfully copy DNA methylation from a parent cell to its daughter cell, the maintenance methylation enzyme DNMT1 requires methyl group donors; specifically, *S*-adenosylmethionine (SAM), to perform the following reaction, producing *S*-adenosylhomocysteine (SAH):



In vitro, restriction of methionine or a leftward shift of the above equation (away from methylation of cytosine) via supplementation with homocysteine was able to produce functional hypomethylation. In particular, reduced methylation of the *KIR* gene family and *TNFSF7* was shown, along with significantly increased expression of these genes. However, these alterations were only noted in T cells from healthy adults aged 50 years or older but not in younger adults. T-cell DNMT1 levels decline with age [52]. These studies indicate that T cells become hypersensitive to their environmental micronutrient levels as they age; and, when these methyl donors are restricted, methylation-sensitive genes may be upregulated. DNA methylation is globally reduced in lupus T cells similar to T cells in aging [53]. Therefore, it is likely that reduced dietary methyl donor levels or elevated homocysteine levels would result in more hypomethylation and overexpression of methylation sensitive genes, similar to T cells from older individuals. This raises the possibility that dietary supplementation with methyl donors or efforts to reduce homocysteine levels may be of therapeutic benefit in lupus patients [52].

Aging—The delayed onset nature of lupus and other autoimmune diseases may at least partly be explained by differences in methylation encountered with aged DNA, as alluded to above. First described in 2000, the phenomenon known as ‘inflammaging’ describes the loss of protective immunity and increased low-grade chronic inflammation that accompany aging [54]. Interestingly, titers of ANA increase considerably with age [55].

Considering the global DNA hypomethylation pattern noted in lupus patients’ DNA and delayed peak of disease activity, one would expect to find similar epigenetic changes with aging. However, diverse patterns including both increased and decreased DNA methylation are seen among distinct tissues with aging [56]. Interestingly, a recent study demonstrated a link between immunogenicity of whole genomic DNA, upon introduction into dendritic cells *in vitro*, and the age of the donor patient. Delivery of DNA from aged (65–90 years old) compared with young (20–35 years old) subjects into PBMCs stimulated a significantly greater secretion of an inflammatory cytokine (IFN- α) and costimulatory markers CD80 and CD86. The DNA used was, as would be expected, significantly hypomethylated in aged patients compared with younger controls [57].

An interesting corollary is that young identical twins have nearly indistinguishable DNA methylated patterns in PBMCs, contrasted with aging twins in their 50s and 60s, which display widespread epigenetic differences, known as ‘epigenetic drift’, affecting both DNA methylation and histone acetylation patterns [58]. These data provide interesting clues into monozygotic twin discordance noted among lupus patients. Indeed, as noted earlier, distinct DNA methylation patterns are seen among monozygotic discordant SLE twins that may, at least in part, explain their divergent disease status [4].

Specific topics in SLE DNA methylation

We shall now highlight some of the more promising individual genes whose DNA methylation status has been highly associated with the development and/or progression of lupus, either in human patients or mouse models, segregated by immune cell of origin. Also included is a brief summary of studies recently completed by our group interrogating DNA methylation at a genome-wide level.

T lymphocytes

ITGAL (CD11a): CD11a is an integrin involved in costimulation and cellular adhesion, the product of the *ITGAL* gene. CD11a dimerizes with CD18 to form leukocyte function-associated antigen 1 (LFA-1), and is expressed on the surface of a number of cell types, including T cells. On CD4⁺ T cell, LFA-1 binds to ICAM-1, among other adhesion

molecules expressed on antigen-presenting cells, providing a critical primary contact mediating T cell-antigen-presenting cell interactions and helping to stabilize the binding of the T cell receptor with MHC class II-antigen complex. It is postulated that the overexpression of CD11a on SLE T cells serves to strengthen the initial T cell-antigen-presenting cell interaction, therefore lowering the threshold for T cell stimulation in the presence of a low affinity interaction between the T-cell receptor and MHC-antigen complex, such as when the antigen presented via the MHC molecule is a self antigen [59]. The importance of CD11a in immune regulation is perhaps best evidenced by leukocyte adhesion deficiency syndrome, a disorder characterized by lack of functional CD11a, which results in increased susceptibility to infection and muting of systemic inflammatory responses [60]. CD11a is overexpressed in T cells taken from active lupus patients by approximately 40% [61].

Seminal studies by Lu *et al.* have demonstrated the importance of CD11a overexpression in lupus and its regulation via DNA methylation. First, overexpression of CD11a from CD4⁺ T cells in patients with active lupus is statistically correlated with systemic lupus erythematosus disease activity index (SLEDAI) score ($p = 0.026$). Second, demethylation of the promoter regulatory region of *ITGAL* (CD11a) is seen in active lupus patients compared with inactive patients and normal controls ($p = 0.010$). Furthermore, the extent of demethylation correlated with SLEDAI score. Treatment of T-cell cultures *in vitro* with the DNA methylation inhibitor 5-azaC caused a similar pattern of hypomethylation and overexpression of CD11a, as did procainamide, a drug known to produce drug-induced lupus *in vivo* [62].

Overexpression via CD11a transfection into cloned murine Th2 cells is sufficient to induce autoreactivity *in vivo* as evidenced by anti-dsDNA antibody production, immune-complex glomerulonephritis and pulmonary alveolitis following adoptive transfer into syngeneic mice [63]. Mice engineered to reproduce the lupus-associated ERK pathway signaling blockade via an inducible dominant-negative MEK transgene demonstrate reduced expression of the maintenance DNA methylation enzyme Dnmt1 and similar upregulation CD11a, along with autoantibody production and differential expression of interferon-regulated genes [64].

CD40LG (CD40L): CD40L, a type II transmembrane protein encoded on the X chromosome, is a member of the TNF superfamily primarily expressed on the surface of CD4⁺ T cells, but also found on a variety of other cellular surfaces and solubilized in serum [65,66]. It functions as a costimulator, binding to its complement, CD40 [66]. CD40L has specific actions in certain cell types: when presented to macrophages, it functions in concert with IFN- γ signaling to upregulate CD40 and TNF receptor synthesis and recruitment to the cell surface; when stimulating B cells, it causes activation of resting cells and, in concert with concomitantly produced IL-4, results in antibody isotype switching and terminal differentiation of B cell to antibody-producing plasma cell; finally, presentation to endothelial cells results in activation and production of reactive oxygen species (ROS) and increased expression of cellular adhesion molecules, including ICAM-1, VCAM-1, and E-selectin, thereby promoting leukocyte recruitment [67]

Elevations in soluble CD40L levels are found in human lupus patients and lupus mouse models [68–70]. Binding of CD40 on B-cells with CD40L on T cells reduces the threshold of B cell activation [71]. Even at predisease ages, lupus-prone New Zealand black \times New Zealand white F1 mice have naive CD4⁺ T cells with fully formed CD40L expressed on their cell surface. These T cells, when challenged by mitogen, show a brisk immunization response with subsequent production of anti-dsDNA antibodies. Pretreatment with anti-CD40L abrogated this effect and suppressed production of anti-dsDNA antibodies [72]. CD40L expression is partially controlled by DNA methylation of its promoter. T cells from

active lupus patients are known to have reduced global levels of DNA methylation, and treatment of human T cells with DNA methylation inhibitors including 5-azaC, procainamide, or ERK pathway inhibitors such as PD98059 and hydralazine cause demethylation of the *CD40LG* promoter and subsequent increase in its expression and induction of T-cell autoreactivity *in vitro* [73]. Although disease-free men and women have approximately equal expression of CD40L on CD4⁺ T cells, female lupus patients have roughly twice the level of CD40L mRNA compared with male lupus patients; likely secondary to its location on the X chromosome. Interestingly, female lupus patients exhibit demethylation of promoter sequences of *CD40LG* on the inactive X indicating impaired functioning of DNA methylation machinery, typically attributed to the maintenance of the Barr body [74]. As a consequence, reactivation of genes typically suppressed on the inactive X chromosomes of female lupus patients, via aberrant maintenance of established DNA methylation patterns or active demethylation, could arguably contribute to the female predominance of SLE.

TNFSF7 (CD70): CD70, also known as CD27 ligand and encoded by *TNFSF7*, is a member of the TNF family typically expressed on activated CD4⁺ and CD8⁺ T cells and early B-cell progenitors. It functions as a vital costimulatory molecule; CD70/CD27 inhibition via anti-CD27 monoclonal antibody is sufficient to mitigate pokeweed mitogen-driven B-cell IgG production *in vitro* [75]. Conversely, overexpression of CD70 in human CD4⁺ T cells *in vitro* stimulates IgG synthesis in B cells [76]. *In vitro*, CD70-transfected fibroblasts augment T-cell proliferation [77]. Furthermore, CD70 promotes cytotoxic CD8⁺ T-cell production *in vitro* [78]. In addition, the CD70/CD27 interaction prolongs survival of CD3-stimulated T cells *in vivo* when compared with CD27 knockout mice [79]. A CD70 transgenic MRL lpr mouse model displays effector-type T-cell expansion and differentiation, greatly increasing circulating IFN- γ levels. Interestingly, these effects appear to be independent of the IL-2 signaling pathway, as expression of IL-2 and its receptor are not altered in these mice [75].

Transcription of CD70 is epigenetically regulated. *TNFSF7* promoter demethylation is concomitant with increased expression in human T cells treated with both traditional DNA demethylation inhibitors (i.e., 5-azaC), as well as medications implicated in drug-induced lupus, including hydralazine and procainamide [80]. CD4⁺ T cells from lupus patients exhibit similar demethylation patterns [81], as do MRL lpr lupus-prone mice as they develop autoimmunity [20]. The afore-mentioned inducible B6.dnMEK mouse model, which reduces maintenance DNA methylation via reduction in Dnmt1 expression, also causes similar overexpression of CD70 [64]. Of note, a strikingly similar pattern of demethylation and overexpression of CD70 has been recently identified in other autoimmune diseases, including Sjögren's syndrome [82], subacute cutaneous lupus [83] and rheumatoid arthritis [84].

KIR gene family: The KIR is a rapidly evolving polymorphic family most highly associated with natural killer (NK) cells but also found on CD4⁺ and CD8⁺ T cells, particularly on senescent CD4⁺ CD28⁻ T cells known to overexpress a variety of proinflammatory cytokines. They are highly associated with acute coronary syndrome and aging [85–87]. KIRs' primary function is to aid in discrimination between self and nonself through interaction with MHC class I molecules of target cells; certain KIR interactions are known to repress the cytolytic activity of NK cells, whereas others stimulate lysis and killing. In addition, certain KIR subtypes stimulate further internal signaling culminating in the upregulation of cytokine expression [88]. KIRs play a pivotal role in control of the immune response, and altered transcription levels of these genes may be associated with autoimmunity. KIR is greatly overexpressed in lupus CD4⁺ T cells compared with controls, and this overexpression correlates with lupus disease activity [89]. The promoter regions of most *KIR* genes share >91% sequence similarity [90], suggesting that they likely share

promoter regulatory mechanisms. Their expression is regulated by DNA methylation: treatment with demethylating agents causes significant increase in transcription, and the promoter region of the stimulatory *KIR2DL4* is significantly demethylated in T cells isolated from lupus patients compared with controls [91]. *KIR2DL4*, when crosslinked, stimulates IFN- γ production, a pivotal cytokine in the inflammatory response of SLE [92].

PRF1: Perforin (PRF1) is a cytolytic protein contained in granules of CD8⁺ T and NK cells. It functions in concert with the serine protease granzyme B to insert itself into target cells' plasma membrane, forming a pore and subsequently lysing the cell [93]. Although normally expressed at high levels only in CD8⁺ cytotoxic lymphocytes (CTLs), PRF1 has been shown to be over-expressed in lupus patients' CD4⁺ T cells, and these cells exhibit the same cytolytic characteristics normally ascribed to CD8⁺ lymphocytes, functioning in a peptide-specific and MHC class II restricted manner [94]. Overexpression in both CD4⁺ and CD8⁺ lymphocytes has been shown to contribute to SLE pathogenesis, and this overexpression is driven by demethylation of CG sites in the promoter region of the *PRF1* gene. Lupus disease activity scores are highly correlated with both CD4⁺ and CD8⁺ PRF1 expression levels *in vivo* [95–97]. Furthermore, demethylation and overexpression of PRF1 in CD4⁺ T cells has been associated with subacute cutaneous lupus erythematosus, a distinct disease entity generally affecting a younger subset of patients, without many of the common visceral manifestations associated with SLE [98]. These studies point to the importance of epigenetic dysregulation of PRF1 in the pathogenesis not only of SLE but other lupus-like autoimmune diseases, particularly the cutaneous manifestations thereof.

B lymphocytes

IL-6 & CD5: B cells of the B1 subtype, characterized by the expression of CD5, are associated with lupus. They express B-cell receptors (BCRs) that react to autoantigen [99], have a longer lifespan compared with their conventional B2 counterparts, and enter into the cell cycle less readily [100]. In human cells, the concentration of membrane-bound CD5 is controlled by three mechanisms: internalization of the transmembrane protein, shedding into the extracellular space, and expression of the two isoforms, E1A and E1B, via regulation of transcription [101]. Whereas E1A is the full-length, transmembrane-directed protein, E1B is a truncated isotype that fails to translocate following translation, remaining cytoplasmic. The membrane density of CD5 E1A on B1 cells is significantly reduced in lupus patients compared with similar cells in controls, and the intracellular E1B levels significantly elevated [101]. Interestingly, the regulatory element of a human endogenous retrovirus (HERV) is located 5' of the *CD5* gene, and the E1B isotype is indeed a fusion protein containing this element (the 5' LTR) and the truncated CD5 protein [102]. The 5' LTR motif is a commonly occurring, DNA methylation-controlled regulatory element directing transcription of a variety of retroviruses and retroviral elements. Correspondingly, transcriptional levels of the E1B isotype of CD5 are highly inversely correlated with the level of the maintenance DNA methylation enzyme DNMT1 [103]. In lupus patients' B cells, this LTR is significantly hypomethylated compared with controls. Typically, the activation of B-cell receptors causes an induction of DNMT1 expression and the subsequent methylation of the E1B isotype. However, overproduction of IL-6 by lupus B cells leads to cell cycle arrest at late G1 phase and prevents the induction of DNMT1, resulting in increased expression of the E2B isotype of CD5 in these cells [104]. The induction of other methylation-sensitive genes by IL-6-mediated DNA methylation disruption has not yet been carefully studied.

Genome-wide methylation studies: A quite interesting study was performed by Javierre *et al.* in 2010, comparing cytosine methylation levels of 1505 cytosine residues among 807 genes in monozygotic twin pairs discordant for lupus. DNA was isolated from the total white

blood cell population. The study reported 49 genes that were differentially methylated between lupus patients and their healthy monozygotic siblings. These genes corresponded to enriched functional ontologies including ‘defense response’, ‘cell activation’, ‘immune response’, ‘cell proliferation’ and ‘cytokine production’. In addition, the array confirmed results seen in previous studies regarding a widespread hypomethylation state in lupus patients [4].

A genome-wide survey of DNA methylation in lupus CD4⁺ T cells was recently completed by our group, comparing methylation levels of 27,578 CG sites spanning approximately 14,495 genes in lupus patients compared with age- and sex-matched controls using an Illumina Infinium™ microarray [105]. We identified 236 hypomethylated and 105 hypermethylated CG sites in lupus CD4⁺ T cells compared with healthy normal controls. Hypomethylated genes included matrix metalloproteinase 9 (*MMP9*) which functions to break down connective tissue basement membranes during remodeling processes and has been previously linked to Sjögren’s syndrome and the cartilage destruction seen in rheumatoid arthritis [106,107]. Antibodies against a second hypomethylated gene identified, *PDGFRA*, are detected in 36% of lupus patients with active disease (and only 10% of inactive lupus patients) and linked to the development of hemolytic anemia [108]. *CD9*, which functions as a T-cell coactivator [109] was also hypomethylated, as was *BST2* (Tetherin), which prevents release of viral particles in infected cells by ‘tethering’ them to the surface [110]. Interestingly, *CD9* was also identified as differentially hypomethylated in the lupus twins study mentioned previously [4]. Hypermethylated ontologies and genes included ‘folate biosynthesis’, represented by hypermethylation of *FOLH1* and *GGH*, which suggest dysregulation of reactions leading to methylation substrates, namely SAM. A number of transcription factors were also hypermethylated, including *RUNX3*, which interacts directly with the promoter sequence of the aforementioned *ITGAL* (CD11a) [111,112]. Protein–protein interaction maps revealed a variety of ontologies, including apoptosis, and the novel transcription factor HNF4a was identified as a regulatory hub affecting a number of both hyper- and hypo-methylated gene. A particular strength of genome-wide studies such as this is the possibility of identifying candidate methylation biomarkers that may correlate with disease activity; we found five such genes. Three displayed strong positive correlation and two showed strong negative correlation with disease activity; which, if validated, may be used in combination with clinical data for predicting lupus activity in the future.

Role of RFX1: RFX1 binds to promoter regions of target genes and recruits a variety of repressive epigenetic factors, including histone deacetylase (HDAC)-1 and the maintenance DNA methyltransferase DNMT1, consequently suppressing target genes [113]. In lupus T cells, downregulation of RFX1 is at least partially responsible for the demethylation of *ITGAL* (CD11a) and *TNFSF7* (CD70) via reduction in DNMT1 and HDAC1 recruitment. Trimethylation of the histone residue H3K9 also appears to be regulated by RFX1 levels, and this may further predispose to chromatin structure permissiveness for CD11a and CD70 expression [114].

Histone modification

Histones are a group of highly conserved protein structures with critical functions in DNA strand stability, replication and transcriptional control. Their contribution to the etiology of lupus has been realized, and continues to be an important topic of research. Various post-translational modifications to the amino acid side chains of the histone octamer contribute to both conformational changes and direct interaction with transcriptional machinery to alter gene expression, including acetylation, methylation, ubiquitination, sumoylation,

deimination and poly(ADP)-ribosylation [115], although not all have been associated with SLE.

Increased localized acetylation of histones directs a shift from heterochromatin to euchromatin, thus allowing transcriptional machinery access to the underlying DNA structure. Treatment of lupus-prone mice with inhibitors of HDAC has been shown to reduce disease activity, perhaps by increased expression of genes involved in apoptosis [116]. Offering further support, hyperacetylation of histones has been shown to lead to DNA damage via double strand breaks [117]. However, it appears that increased histone acetylation is a double-edged sword. The lupus mouse model-derived and lupus patient-derived autoantibody KM-2 reacts against apoptotically derived histones [118], and these antibodies show greatly enhanced reactivity against acetylated histone residues. Shifts in histone acetylation patterns were further attributed to changes in expression of HDAC and histone acetyltransferase (HAT) activity. Acetylated histone autoantibody reactivity has also been shown to be important in disease progression; particularly, H2BK12 acetylation autoantibodies have been isolated from predisease MRL lpr mice, with later generalization of the immune response to unmodified H2B [119].

Triacetylated histone H4 is capable of modulating disease in MRL lpr mice, enhancing mortality, proteinuria, skin lesions and glomerular deposition of antibody-antigen complexes, whereas the unmodified protein had no such effects [120]. Interestingly, hyperacetylated histone administration to dendritic cells *in vitro* increases expression of the proinflammatory cytokines IL-6 and TNF- α , and these dendritic cells activate syngeneic T cells [121]. Approximately 60% of lupus patients have autoantibodies that react to the monoubiquitinated form of histone H2A (UH2A) [122]. Interestingly, 80% of lupus patients have autoantibodies directed to ubiquitin in general, compared with less than 10% of normal controls [123]. UH2A glomerular deposits have been described in renal biopsies from lupus patients with nephritis [124].

Global histone H4 acetylation was investigated by Zhang *et al.* in 2010. In this study, monocytes were isolated from SLE patients and controls, and H4 acetylation levels determined by chromatin immunoprecipitation (ChIP)-chip analysis, with concordance to gene-expression data analyzed. Network and transcription-factor relationships were explored for differentially acetylated genes. The results were impressive: histone H4 acetylation levels of 179 genes were significantly increased in SLE patients' monocytes compared with controls (out of 11,321 genes analyzed). Network analysis revealed three consistently implicated molecules (IFN- α , NF- κ B and MAP kinases) and five particularly enriched ontologies, including macrophage activation, cell proliferation, CNS toxicity and antiviral immunity. Interestingly, treatment of disease-free monocytes with IFN- α resulted in H4ac changes and upregulation of genes similar to those seen in lupus patients' monocytes. These data demonstrate the importance of histone H4 changes in downstream regulation of genes implicated in SLE [125].

NcRNA

Increasing attention has recently been paid to ncRNAs and their contribution to post-transcriptional silencing of target genes. MiRNAs are typically 22-nucleotides-long ncRNAs that affect gene expression by binding to complementary sites on the 3'-untranslated region of target mRNAs. This causes degradation via the RNase III enzyme Dicer as part of the RNA-induced silencing complex (RISC) [126]. Interestingly, miRNAs may affect DNA methylation by targeting methylase enzymes directly. For example, in lung cancer and acute myeloid leukemia, the miR-29 family inactivates the *de novo* methyltransferases DNMT3A and DNMT3B, leading to global hypomethylation [127,128]. Computational genomics

studies place miRNA-coding sequences at approximately 3% of the entire genome, and they regulate at least 30% of human mRNAs [129].

Although several studies have shown association between lupus and miRNA expression patterns, two recent papers are of particular importance. In the first, a screen of 585 mouse miRNAs examined in lupus-prone MRL lpr mice identified two miRNAs with greatly increased expression in CD4⁺ T cells, miR-21 and miR-148a. When transfected into the human Jurkat T-cell line, these two miRNAs were shown to greatly reduce DNMT1 levels [130]. MiR-21 reduced DNMT1 mRNA by 58%, whereas miR-148a reduced DNMT1 post-transcriptionally. Further inquiry indicated that miR-21 indirectly regulated DNMT1 expression through 3'-untranslated region pairing of RASGRP1, thereby suppressing signaling through the Ras-MAPK pathway, previously linked in multiple studies to reduction in DNMT1 levels and subsequent lupus-like disease. These findings were confirmed with *RASGRP1* knockdown experiments, with similar depression of the Ras-MAPK pathway and DNMT1 expression. Interestingly, miR-148a exerts its suppressive action through binding of translation-ready DNMT1 transcripts [130]. Furthermore, miR-21 and miR-148a-transfected primary CD4⁺ T cells exhibit overexpression of the lupus-related and methylation-sensitive genes CD70 and CD11a in a fashion similar to that seen in lupus patients. CD4⁺ T cells were then isolated from active lupus patients and treated with hairpin inhibitors of these two miRNAs. DNMT1 levels were increased and CD70 and CD11a mRNA levels were reduced significantly [130].

A more recent study demonstrates overexpression of miR-126 in CD4⁺ T cells from lupus patients [131]. With a series of elegant experiments, Zhao *et al.* demonstrate that miR-126 directly inhibits DNMT1 by targeting its 3'-untranslated region. They further demonstrate that overexpressing miR-126 in normal CD4⁺ T cells results in reduced DNMT1 protein expression, and hypomethylation and overexpression of CD11a and CD70, similar to lupus CD4⁺ T cells [131].

A report by Dai *et al.* identified a common lupus-associated miRNA expression pattern consisting of the same 15 miRNAs in splenic lymphocytes of three lupus-prone mouse models, NZBW_{F1}/J, MRL lpr , and congenic B6 lpr . Of particular importance, with the exception of one, these miRNAs were not significantly upregulated in these mice at a young age when overt disease is not yet present. Further experiments demonstrated that this upregulation was independent of lymphocyte stimulation, further indicating that changes in the expression of miRNAs are strongly associated with aging and development of disease in these mouse models [129].

Towards an epigenetic therapy for SLE

With all of the previously mentioned associations in mind, how may we now begin to construct a framework for an epigenetic therapeutic approach for lupus? First, and possibly most straightforward, one may consider those genes known to be hypomethylated and upregulated. These genes may be targeted with a number of gene-specific approaches. Unfortunately, this is not always successful. For example, an immunosuppressant monoclonal antibody designed by Genentech, efalizumab (Raptiva®), targeted CD11a and was marketed as a treatment for psoriasis before being withdrawn from the market in 2009 following an association with bacterial sepsis, viral meningitis and progressive multifocal leukoencephalopathy [132].

A second, and perhaps more tantalizing approach, involves epigenetic modification as a means of therapy. Here again, we must consider two paths. One could certainly imagine a T-lymphocyte or CD4⁺ specific transfection strategy using lentiviral vectors encoding a methylase (perhaps DNMT1) under control of a strong promoter to raise global methylation

levels. One must consider, however, the possibility of inducing a global hypermethylated state. Similar states have been extensively described in the development and progression of a number of leukemias and lymphomas, precisely the same cells to be targeted by a lupus therapeutic [133–135]. Site-directed DNA remethylation via fusion proteins consisting of methylase domains linked to site-specific DNA binding domains would remedy many of these issues, provided they had minimal off-target effects.

More difficult would be site-directed DNA demethylation, as we have yet to definitively identify specific catalytic enzymes that function to remove methyl groups from cytosine residues *in vivo*, although a few likely candidates have recently been identified. Specifically, plant studies identified a family of DNA glycosylases including Demeter and Demeter-like genes and the related glycosylase ROS1, although these appear to be without mammalian analogues [136,137]. Vertebrate studies have identified a coordinated demethylation approach involving the 5-methyl-cytosine deaminase activation-induced (cytidine) deaminase and mismatch-specific thymine glycosylase Mbd4 [138]. Selection of single-gene targets would be critical and must be based on careful analysis of the most influential methylation-associated disease-causing pathways. A therapeutic strategy based on an attempt to restore all known associated methylation derangements would be technically challenging. Particular care must also be taken to ensure that off-target methylation does not occur.

Histone modifications in a site-specific manner would likely prove difficult as they are *trans*-acting elements, although altering global levels may be more feasible. Generally speaking, histone acetylation is maintained via the activity of HDAC and HAT enzymes. Studies examining treatment of SLE with HDAC inhibitors have shown some promise [139]. In chronic obstructive pulmonary disease, where significant deficiencies in histone acetyltransferase predominate, low-dose theophylline has been shown to upregulate HAT activity and, when combined with corticosteroids, resulted in increased histone acetylation targeted to transcriptionally active inflammatory genes [140–144].

NcRNA is a relatively new field, but one that holds great promise. The introduction of small, noncoding miRNAs via cell-specific transfection or transduction could directly affect transcription of important lupus-associated genes both by modulating expression of single genes or more globally by targeting DNA methylation machinery via upregulating DNMT1, as mentioned earlier [130].

Conclusion

The epigenetic contribution to etiology and disease progression in lupus is unquestionable (Table 1), although the field is still in its infancy and a complete understanding of global epigenetic aberrations in this highly complex and heterogeneous disease is still lacking. No doubt, the exponential increase in speed and decrease in cost of DNA sequencing in the coming years will allow ever more accurate and robust conclusions to be drawn regarding epigenetic disease associations. The inevitability of targeting the epigenome of lupus to provide a durable therapeutic response is enticing, and will be no less than a revolution in treatment of this debilitating disease.

Future perspective

The future for increasing our understanding of the etiological contributions of epigenetics to lupus looks bright indeed. It is becoming increasingly clear that epigenetic modifications influencing disease states are not isolated events; therefore, changes must be examined in an increasingly genome-wide fashion. Over the next 5–10 years, one can expect further sequencing experiments which examine the entire epigenome with increasing coverage and

precision, with concomitant genomic and proteomic studies that may provide a more global functional perspective in specific cells important to lupus etiology. As our knowledge regarding the context and genesis of these changes becomes clearer, we also look forward to seeing the first specific epigenetic therapies for lupus be developed with the understanding that they must be carefully targeted to achieve success in this highly complex disease.

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Executive summary

- Differences in lupus presentation based on sex, geography, race and age, as well as discordance noted among twin studies point to a nongenetic environmental contribution to disease etiology.
- Epigenetics refers to changes in gene expression not related to mutation in the underlying DNA sequence, and includes DNA methylation, histone modification and ncRNA expression.

DNA methylation: general topics

- Early studies demonstrated a hypomethylated state in lupus T cells: treatment with methylation inhibitors or medications implicated in drug-induced lupus (which, as it turns out, are themselves methylation inhibitors) produce a distinctly lupus-like phenotype in both human cells and mouse models.
- Apoptotic DNA is hypomethylated, and stimulates an increased immune response through TLR9 signaling, culminating in anti-dsDNA production and lupus nephritis in mice.
- UV light exposure can induce significant reductions in global DNA methylation levels in human peripheral blood mononuclear cells, which is exaggerated in peripheral blood mononuclear cells from lupus patients.
- Endogenous retroviruses may be reactivated via the hypomethylated state present in lupus patients, and many retroviral elements mimic autoantigen and may induce an autoantibody response.
- *In vitro* restriction of methylation-related micronutrients, including methionine, resulted in reduced methylation of certain lupus-related genes. This suggests that dietary restriction or supplementation of methylation-related micronutrients may affect the epigenetic state of lupus-related genes.
- Whole genomic DNA isolated from aged (65–90 year olds) compared with young (20–35 year olds) individuals induces a significantly increased immune response, perhaps secondary to aging-related methylation changes.

DNA methylation: specific topics

- *ITGAL* (CD11a) is a cellular adhesion integrin that is hypomethylated and overexpressed in lupus T cells. This hypomethylation is proportional to disease activity as measured by SLEDAI score. Overexpression of CD11a into murine Th2 cells is sufficient to produce autoimmunity, including anti-dsDNA production, glomerulonephritis and pulmonary alveolitis.
- *CD40LG* is a type II transmembrane, TNF superfamily gene which functions as a costimulatory molecule, and is hypomethylated and overexpressed in lupus patients' T cells.
- *TNFSF7* (CD70) is a costimulatory molecule expressed on activated CD4⁺ and CD8⁺ T cells. It is hypomethylated and overexpressed in both lupus patients and lupus mouse models, including *MRL/lpr* mice. A CD70 transgenic mouse model displays greatly increased circulating IFN- γ levels.
- *KIR* family of genes function in self-versus nonself-discrimination, most often associated with natural killer cells but also found on CD4⁺ and CD8⁺ T cells. KIR is greatly overexpressed in lupus CD4⁺ T cells compared to controls, and

this overexpression correlates with lupus disease activity. KIR expression is controlled by DNA methylation.

- Perforin (PRF1) is a cytolytic protein contained in granules of CD8⁺ T cells. Overexpression in CD4⁺ and CD8⁺ T cells contributes to lupus pathogenesis, and is driven by demethylation of its promoter. This epigenetic activation is also seen in subcutaneous lupus erythematosus, a distinct disease entity.
- Differential expression of an isoform of CD5 which retards cell cycling of B1-type B cells (E2B), is driven by demethylation of a human endogenous retrovirus located upstream of the *CD5* promoter, and is increased in lupus patients' B cells. Overproduction of IL-6 by lupus B cells leads to cell cycle arrest in late G1 phase, prevents induction of the maintenance DNA methyltransferase DNMT1, and subsequent increased expression of E2B.

DNA methylation: genome-wide studies

- A genome-wide study of twins discordant for lupus reported 49 differentially-methylated genes, reiterating the hypomethylated state noted in earlier studies, and revealing enriched functional ontologies including 'defense response', 'cell activation', 'immune response', 'cell proliferation' and 'cytokine production'.
- The largest genome-wide study to date examined 27,578 CG sites spanning 14,495 genes across the genome, and found 236 hypo- and 105 hyper-methylated CG sites in lupus CD4⁺ T cells. Novel ontologies and differentially methylated genes were identified. Five genes were found to be highly correlated with lupus disease activity.

Role of RFX1

- RFX1 binds to promoter regions of target genes and recruits a variety of repressive epigenetic factors. Downregulation of *RFX1* has been linked to demethylation of *ITGAL* and *TNFSF7* via reduction in DNMT1 and histone deacetylase 1 recruitment.

Histone modifications

- Antibodies targeted to acetylated histones are found in lupus patients and mouse models, and is considered to be important in disease progression.
- Triacetylation of histone H4 is capable of modulating disease in *MRL/lpr* lupus-prone mice. Administration of acetylated H4 increases expression of proinflammatory cytokines *in vitro*.
- Global histone H4 acetylation is significantly increased in lupus. Acetylation levels of 179 genes (of 11,321 analyzed) were noted to be increased. Consistently implicated molecules included IFN- α , NF- κ B and MAP kinases.

NcRNA

- MiRNAs miR-21 and miR-148a are overexpressed in lupus-prone *MRL/lpr* mice and lupus patients' T cells. When these two were transfected into primary T cells *in vitro*, they greatly reduced DNMT1 levels.
- MiR-126 was recently shown to be overexpressed in CD4⁺ T cells from lupus patients, and also inhibits DNMT1 by targeting its 3'-untranslated region. *In vitro* transfection of this micro RNA induced decreased DNMT1 activity and overexpression of methylation-sensitive CD11a and CD70 were noted, similar to lupus CD4⁺ T cells.

Towards an epigenetic therapy for SLE

- Epigenetic studies may allow for novel therapies for SLE in two ways: first, by identifying novel targets for pharmacotherapy; and secondly, by laying the groundwork for epigenetically-targeted therapy that would seek to correct the underlying epigenetic aberrations seen in lupus.

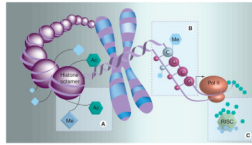


Figure 1. Three major epigenetic alterations seen among lupus patients
(A) Increase in histone acetylation leading to local relaxation of chromatin structure, (B) hypomethylation of cytosines in cytosine–guanosine dinucleotides, leading to increased transcription of methylation-sensitive genes, and (C) alteration in post-transcriptional regulation of genes (including those involved in DNA methylation) at the tRNA level via increased miRNA expression and activation of the RISC.
Ac: Acetyl; Me: Methyl; RISC: RNA-induced silencing complex.

Table 1

Summary of the key epigenetic alterations identified in systemic lupus erythematosus.

Category	Target/gene	Alteration in SLE	Function/consequence
DNA methylation, specific genes	<i>ITGAL</i> (CD11a)	Hypomethylation	Cellular adhesion
	<i>CD40LG</i> (CD40L)	Hypomethylation	Costimulation
	<i>TNFSF7</i> (CD70)	Hypomethylation	Costimulation
	<i>KIR</i> family	Hypomethylation	Discrimination of self versus nonself
	<i>PRF1</i> (Perforin)	Hypomethylation	Cytolysis
DNA methylation, general topics	Apoptotic DNA	Hypomethylation	Stimulation of autoimmunity through TLR signaling
	Aged DNA	Hypomethylation	Increased immunogenicity
	UV light exposure	Hypomethylation	Unknown
	Endogenous retroviruses	Hypomethylation	Upregulation of autoantigens
	Diet (restriction of methionine)	Hypomethylation	Upregulation of methylation-sensitive genes <i>in vitro</i>
DNA methylation, genome-wide studies	<i>RUNX3</i>	Hypermethylation	T-cell maturation
	<i>MMP9</i>	Hypomethylation	Cellular basement membrane breakdown
	<i>BST2</i> (Tetherin)	Hypomethylation	Viral capsid tether to cell membrane
	CD9	Hypomethylation	T-cell activation
	Various folate metabolism-related genes	Hypermethylation	? altered folate metabolism and methylation reactions
Methylation modulator	<i>RFX1</i>	Reduced expression	Demethylation of <i>ITGAL</i> (CD11a), <i>TNFSF7</i> (CD70), reduction in trimethylation of histone H3K9
Histone acetylation	Histone H4	Increased acetylation	Increased expression of proinflammatory cytokines
	miR-21	Increased expression	Reduction in DNMT1 expression
	miR-148a	Increased expression	Reduction in DNMT1 expression
NcRNA	miR-126	Increased expression	Reduction in DNMT1 expression

SLE: Systemic lupus erythematosus; TLR: Toll-like receptor.