A Longitudinal and Transancestral Analysis of DNA Methylation Patterns in Systemic Lupus Erythematosus

by

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Dedication

To my parents Stephen and D'Etta Coit,

and to the rest of my family

for always supporting me,

believing in me,

and giving me somewhere

I could always come home

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Abstract

Objective: Systemic lupus erythematosus (SLE or lupus) is a complex heterogeneous autoimmune disease that can affect multiple organ systems. Lupus predominantly affects women, with African-American women carrying higher disease burden and poorer outcomes than European-American women. Disruption in the epigenetic landscape of immune cells, including DNA methylation, is a recognized feature of lupus. Global hypomethylation is consistently detected across lymphoid and myeloid immune cells from lupus patients, with the most extensive hypomethylation occurring in type I interferon response genes. Genetic background plays a role in shaping the epigenome of immune cells and in the risk of developing lupus. The aim of this dissertation was to measure the influence of genetics on DNA methylation differences between African-American and European-American lupus patients and those between lupus patients and healthy controls. Further, we sought to measure the variation in DNA methylation longitudinally over time and with disease activity in lupus.

Results: We first analyzed granulocyte DNA methylation over time in a longitudinal cohort of African-American and European-American lupus patients followed for up to four years. Ethnicity-associated differential methylation in lupus granulocytes included genes associated with the type I interferon response and NFκB pathways. Approximately 16% of differentially methylated sites between ethnicities were associated with a nearby (≤ 1kb) genetic variant known as methylation quantitative trait loci (meQTL). An meQTL involving the promoter region of *TREML4*, a regulator of TLR7 response in myeloid cells, was among the strongest meQTL

detected. Genotype-specific mRNA expression of *TREML4* negatively correlated with promoter methylation in this locus. Methylation levels in two CpG sites were associated with changes in disease activity in African-American, but not European-American patients. Hypomethylation in *GALNT18* was associated with the development of lupus nephritis during follow up. The lupus-associated epigenotype in granulocytes was not explained by genetics in lupus patients. To further examine the role of genetics in lupus-associated epigenetic changes, we studied DNA methylation in naïve CD4+ T cells from lupus patients and controls. CpG-associated genes in meQTL of lupus patients showed overlap with lupus risk genes. Some meQTL were shared between lupus granulocytes and naïve CD4+ T cells, including *HLA-DQB1* and *IRF7*. However, confirming the results in lupus granulocytes, less than 1% of differentially methylated sites in lupus T cells were associated with meQTL. We then used a trend deviation analysis to compare disease-associated DNA methylation differences between lupus patients and controls in naïve CD4+ T cells, with methylation correlation patterns in over 16,000 tissue samples. The promoter of the miR-17-92 cluster, which regulates T cell proliferation and differentiation, was hypomethylated in lupus. Expression of two member microRNAs in this cluster were positively correlated with lupus disease activity.

Conclusions: We have identified significant differences in the DNA methylome between African-American and European-American lupus patients, which are in part associated with nearby genetic variants. We have determined that granulocyte DNA methylation is predominantly stable over time and across disease activity levels in both African-American and European-American lupus patients. Furthermore, using DNA methylation profiles in granulocytes and naïve CD4+ T cells, we have determined that the DNA methylation signature of lupus, which is defined by robust hypomethylation of type I interferon genes, has little

association with nearby genetic variants. These findings suggest that non-genetic factors play a predominant role in the DNA methylation signature of lupus.

Chapter 1 Introduction

1.1 Systemic Lupus Erythematosus

1.1.1 Description and Epidemiology

Systemic lupus erythematosus (referred to herein as lupus) is a complex, heterogeneous autoimmune disease that can affect almost every organ system in the body. It is characterized by a loss of self-tolerance, the production of autoantibodies against self-antigens, and inflammatory immune responses towards autoantibody-containing immune complexes leading to tissue and organ damage. Lupus incidence and prevalence are highly variable across geographic regions and populations. Estimated global incidence of lupus ranges from 1.4-8.6 per 100,000 person-years and global prevalence from 13-366.6 per 100,000 people(1). Estimated incidence varies from 3.7-49 per 100,000 person-years and estimated prevalence ranges from 47.9-366.6 per 100,000 people in North America (1-5) and is steadily increasing (3, 4). In North American population studies, lupus incidence and prevalence in African-Americans are more than twice that in European-Americans(1). Incidence and prevalence are estimated to be equal to or even higher in American Indian and Alaskan Native populations compared to African-American populations(5). Lupus prevalence is nine times greater in women than in men(1, 6). Contributions to the sex bias in lupus are postulated to include X chromosome dosage and altered inactivation that can affect a number of immune genes(7-12), and estrogen(13).

Lupus has a significant impact on the survival and life expectancy of affected individuals. A meta-analysis of all-cause mortality over a ten year period found that lupus

patients have an estimated standardized mortality ratio (SMR) of 2.6 compared to the general population with the highest cause-specific rates being infection (SMR 5.0), renal disease (SMR 4.7) and cardiovascular disease (SMR 2.3)(14). Lupus ranks among the top 20 contributing causes of death in US women (2000-2015) after exclusion of 3 common external injuries(15). Increasing disease activity is associated with decreased quality of life and increased direct and indirect costs to patients particularly those who develop lupus nephritis(16-18). Lupus patients are estimated to incur an average combined direct and productivity cost of $20,924 annually, with higher disease activity and worse physical health contributing to higher costs(19). Patients with severe disease and organ damage have standardized unemployment rates of 4.4 and 5.6, respectively, compared to the background population(20). Taken together, a lupus diagnosis can incur a significant cost upon a patient's health, quality of life, and financial stability. Improving tools for lupus detection and monitoring to reduce the occurrence of severe disease manifestations and lupus flares would improve patient outcomes.

1.1.2 SLE Manifestations

The predominant disease course for lupus patients consists of remitting-relapsing periods punctuated by increased disease activity ("flares"), though some patients will maintain a chronically active state or enter prolonged remission(21, 22). Flares are associated with increased risk of organ damage making their management and prediction a major therapeutic goal(23). Newly diagnosed or flaring patients can present with general symptoms that include fever, malaise, arthralgia, and weight changes(24). The appearance of autoantibodies is a hallmark of autoimmune disease and can be detected almost 10 years prior to diagnosis(25). Clinical diagnosis of SLE requires the presence of anti-nuclear antibodies (ANA), though this is not

unique to lupus patients, with an estimated 27% of the healthy adult population carrying a positive ANA titer and 2.5% an elevated ANA titer(26). Further disease manifestations fall into 7 clinical classifications: constitutional, hematologic, neuropsychiatric, mucocutaneous, serosal, musculoskeletal, and renal as well as 3 immunological features: antiphospholipid antibodies, complement proteins, and SLE-specific antibodies(27). Cutaneous manifestations are frequent in lupus patients with between 70%-80% developing skin lesions during their disease course(24). Due to the heterogeneity of lupus, it is useful to measure the relationship of clinical features and demographics to further classify patients. Our work showed that lupus patients in a western Pennsylvania cohort formed three clusters classified by disease manifestations(28). The cluster with the most frequent renal and hematological involvement also included significantly more African-American and male lupus patients than the two other clusters with milder disease characterized by photosensitivity, malar rash, and arthritis(28).

Earlier diagnosis is associated with lower disease activity, tissue damage, fatigue, and increased quality of life(29) making improved diagnostic tools and assays especially important. Indeed, lupus patients within their first year of diagnosis have a higher mortality rate than patients with longer disease durations(30). Manifestations with neuropsychiatric and renal damage have the largest negative impact on 5-year survival of lupus patients(31). An estimated 31%-48% of lupus patients will develop lupus nephritis, many within 5 years of diagnosis(32). Patients who develop renal failure have higher mortality (SMR 4.7) (14). Renal damage can reduce the life span of a lupus patient by up to 23.7 years (33). Demographic factors that increase the risk of developing lupus nephritis include being male, younger, and being of African-American or Hispanic ethnicities(32). Like the difference in lupus incidence and prevalence across ethnicities, disease manifestations also show a bias within subpopulations. African-

American lupus patients are diagnosed at a younger age than European-American patients (34.4 versus 41.9 years, respectively) and have over twice the occurrence of renal disease (40.5% versus 18.8%, respectively) and end-stage renal disease (15.3% versus 4.5%, respectively). African-American and Hispanic patients have greater disease severity with more deleterious outcomes like higher rates of lupus nephritis compared to European-Americans(34, 35). On average, African-American patients die 13 years younger than European-American patients(6). This disparity in mortality is attenuated somewhat by controlling for socioeconomic factors, but not entirely(36).

Therapeutic innovations for lupus have been slow to develop. The complex heterogeneity of lupus patients has been pointed to as a reason for recent trials failing to meet their primary endpoints(37). A current focus of the field is using integrated molecular data and clinical presentation to stratify patients into clinical clusters with varying disease progression and aid in clinical trial design(38-40).

1.2 The Immune System and Pathogenesis of SLE

Lupus is the result of a loss of self-tolerance by the immune system towards self-antigens. Availability of self-antigens and a poorly regulated immune response result in damage in a variety of organs and tissues including the kidneys, skin, joints, and nervous system. Currently there are no definitive conclusions on what exposures promote lupus pathogenesis. Our current understanding points towards a model of interacting pathogenic intrinsic (genetic) and extrinsic (environmental) factors. These will be described further in this section as well as dysregulation of the immune system in lupus.

1.2.1 Genetics

Genetics plays an important role in conferring disease risk in lupus. Twin studies represent a classical model to estimate the contribution of genetics to disease risk. The pairwise concordance rate of lupus for monozygotic twins is estimated to be 14%-57% and ~4% for dizygotic twins(41-43). Lupus has an estimated heritability rate of 44%-66% and confers an increased risk of lupus and other autoimmune diseases in relatives(44-48). Over 100 genetic risk loci for lupus have been identified(49) across multiple functional pathways.

Highly penetrant genetic polymorphisms within a single gene or gene family have been implicated in the development of lupus-like disease. The most striking examples are genetic deficiencies in the complement pathway. People with a complement deficiency in C1 complex genes (C1q, C1r, and C1s) have rates of SLE or SLE-like disease of 50-93%, 75% with C4 deficiency, and 10% with a C2 deficiency(50). Their symptoms include photosensitivity, arthritis, and nephritis(51). The underlying pathogenic mechanisms that lead to lupus-like illness are thought to be related to C1q's role in clearance of apoptotic materials, partially through macrophage-mediated phagocytosis, and ability to suppress interferon alpha (IFNα) production by pDCs(52). People with genetic defects in *DNASE1* and *DNASE1L3* that encode proteins that degrade and help clear extracellular DNA during cell apoptosis also develop lupus-like disease(53, 54).

While these studies shed a light on potential pathogenic pathways, most of lupus patients carry a wide range of disease-associated polymorphisms in multiple genes that confer very small risk individually, but together increase susceptibility. Some genes, like *C4A*, *C4B* and *DNASE1L3* are risk loci in both monogenic and polygenic lupus(55, 56). The polygenic risk score, a sum of allele-specific risk for lupus from a set of SNPs in an individual, has shown that higher risk scores are associated with earlier onset of lupus(57) and greater risk of lupus

nephritis(58). There is less known about how genetic ancestry influences lupus risk as a majority of the genome-wide association studies (GWAS) studies have been performed in European-derived populations. Analyses of trans-ancestral genetic risk have found that many risk loci are shared between ancestry groups in addition to the unique risk loci carried within each population(59, 60).

Like monogenic examples, studies of polygenic risk in lupus highlight the importance of the type I interferon pathway. High type I interferon (IFN) expression tends to cluster in lupus families, with unaffected first degree relatives showing high IFN levels similar to affected patients, suggesting type I IFN is a heritable risk factor for lupus(61). Toll-like receptor (TLR) 7 polymorphisms are suspected to contribute to type I IFN production potentially through copy number variations on the X chromosome(62, 63). Lupus-associated variants in *IRF5* and *IRF7*, which regulate expression of IFNα, have been suggested to contribute to its elevated expression in lupus(64, 65). STAT4 is a transcription factor that regulates lymphoid cell response to cytokine signaling including IFNα and regulates proliferation and differentiation. Lupus-associated polymorphisms in *STAT4* have been suggested to contribute increased sensitivity to IFNα and the presence of dsDNA autoantibodies(66-68). Other examples include SNPs that affect lymphoid cell signaling. Polymorphisms associated with reduced expression of *BLK*, a protein that regulates signaling downstream of the B cell receptor, may promote the development of autoreactive B cells(69). A lupus-associated polymorphism in *PTPN22*, which encodes a phosphatase protein that inhibits TCR signaling, increases the inhibitory activity of PTPN22 which could impact T cell selection and Treg stimulation in lupus patients(70).

Genome-wide association studies (GWAS) have contributed much to our understanding of the inherited risk in lupus patients. Determining the functional impact of risk loci is more difficult as most are in non-coding regions. Fine mapping of risk loci is typically required to better isolate the true causal SNP which is unlikely to be the risk SNP identified by GWAS if they are in high linkage disequilibrium (LD) within a haplotype. Explaining the functional impact of non-coding risk SNPs and how this relates to lupus is of considerable interest to the field. One model for explaining these findings is that risk SNPs alter gene expression, rather than function, and epigenetics provides an intermediary mechanism(71). Expression quantitative trait loci (eQTL) studies have become increasingly popular as a method to identify these associations across the genome. One survey of the functional impact of 39 lupus risk SNPs found that these variants were associated with expression differences at multiple genes, could play a role in exon choice during transcription, and could interact with multiple genes by altering chromatin interactions. This model has been useful for exploring gene-environment interactions. Gene expression of some eQTL in lupus patients are modified by exposure to anti-IL-6 therapy and circulating type I IFN response, likely through disruption of genotype-specific TF binding(72). These eQTL studies reinforce the need to consider patient genetics in treatment and diagnosis. Genomic variants can also be associated with DNA methylation changes as demonstrated by methylation quantitative trait loci (meQTL) studies(73). Potential mechanistic models include risk SNPs overlapping TF binding sites that could alter binding affinity in an allele-specific manner. Some TFs may suppress DNA methylation in the region by inhibiting DNMT activity through site occupancy or recruitment of suppressive co-factors or recruitment of TET proteins(74). Alternatively, DNA methylation in the region can be increased by reducing TF occupancy or direct recruitment of DNMTs(74). Transcription factors that include CpG sites

within their binding motifs may also show differences in affinity for methylated versus unmethylated sites, including TFs with methyl-binding domains. If this occurs in areas near promoters or enhancer regions, changes in the epigenome and chromatin accessibility could promote or suppress expression of nearby genes.

One model for the impact of genetics on epigenetic dysregulation in lupus is the *MECP2/IRAK1* risk locus. The Methyl-CpG-binding Protein 2 (MeCP2) gene *MECP2* is located on the X chromosome and encodes a methyl-binding domain (MBD) protein that can recognize and bind to methylated CpG sites and can itself recruit DNMT1(75). MeCP2 is a bifunctional transcriptional regulator, acting as a transcriptional repressor through recruitment of nucleosomal repressor complex proteins and histone deacetylases(76, 77) and a transcriptional activator through association with activating transcription factor CREB1 at gene promoters(78). MeCP2 provides a mechanistic link between DNA methylation and chromatin remodeling in regulating expression. Candidate gene studies in lupus patients identified, confirmed and fine-mapped polymorphisms in the Xq28 *MECP2/IRAK1* locus(79, 80) with a haplotype-associated differential gene expression signature of interferon-regulated genes in lupus B cells and increased expression of CREB1(81). In lupus patients carrying the *MECP2/IRAK1* risk haplotype, MeCP2 expression is increased in stimulated CD4+ T cells corresponding with a decrease in DNA methylation of HLA and interferon-regulated genes(82). Overexpression of *Mecp2* in mice led to increased serum ANA and increased expression of proinflammatory and interleukin signaling pathways in stimulated CD4+ T cells(82).

1.2.2 Environment

Almost two-thirds of systemic lupus erythematosus patients will have cutaneous involvement(83) and exposure to UV radiation may be an environmental trigger for lupus through UV-induced damage to keratinocytes and production of autoantigens and cytokines(84). UV radiation can induce the production of reactive oxygen species (ROS) in keratinocytes and cause apoptosis(85). Reduced apoptotic body clearance in the skin of lupus patients is thought to increase the availability of autoantigens to the immune system(86). UV exposure has also been observed to increase the translocation of autoantigens to the cell membrane where they can be bound by autoantibodies(87). There is some evidence of UV-associated DNA methylation changes in circulating immune cells(88) including a decrease in LINE-1 methylation of peripheral blood cells, suggestive of global hypomethylation(89).

Occupational and therapeutic chemical exposures are potential environmental triggers for lupus. Hydralazine and procainamide are two compounds with the highest risks of causing drug-induced lupus(90) Hydralazine inhibits ERK signaling pathway and leads to decreased DNMT expression and procainamide directly inhibits DNMT1 activity, both capable of causing global hypomethylation in human T cells (91-93). Occupational exposure to crystalline silica(94, 95) and mercury have been associated with the development of lupus as well(96).

The ability of viral and bacterial infections to elicit an abnormal immune response in genetically susceptible individuals makes them potential etiological factors in lupus(97). The presence of an overactive type I interferon response in lupus patients suggests that viral infections might be an important environmental trigger for lupus. The herpes virus family member Epstein-Barr Virus (EBV) has received a large amount of interest after it was observed that juvenile and adult lupus patients have higher rates of EBV seroconversion compared to the healthy population(98, 99). EBV-infected B cells undergo alterations to the chromatin landscape

around genes associate with autoimmune diseases including lupus(100) and disease-associated polymorphisms may influence EBV protein binding and gene expression in an allele-specific manner contributing to autoimmunity(101). EBV proteins themselves may also serve as molecular mimics of endogenous proteins provoking an immune response that is reactive towards self-antigens(102, 103). Host cell DNA methylation is necessary for controlling EBV latency in the genome and EBV requires host DNA methylation for entering the lytic phase(104). Lupus patients have higher EBV viral loads independent of disease activity and medication use, suggesting that a consequence of the dysfunctional T cell response that contributes to autoimmunity is also inadequate to control EBV infection(105).

While smoking is a major risk factor for chronic conditions including cardiovascular disease and cancer, current smokers have 1.5 higher odds of developing lupus than former and non-smokers(106, 107). For lupus patients, smoking status is a significant risk factor for cardiovascular events(108) and development of end-stage renal disease(109). Smoking is associated with tissue-specific DNA hypomethylation in myeloid cells including neutrophils and macrophages and relatively few changes in lymphoid cells(110). These changes occurred in sites overlapping DNase hypersensitivity sites that occur in inflammatory macrophages, suggesting a proinflammatory DNA methylation state(110).

Folic acid metabolism is necessary to regenerate the pool of *S*-adenosylmethionine used as methyl group donors by DNMT when producing 5-methylcytosine(111). Dietary folate intake has been associated with DNA methylation levels in lymphocytes of post-menopausal women(112) and adenocarcinoma patients(113). Lupus-susceptible C57BL/6xSJL mice carrying an inducible ERK defect in CD4+ T cells on a methyl-donor restricted diet developed hematuria at higher rates and more severe glomerulonephritis than mice fed a regular or methyl-donor

supplemented diet (114). CD4+ T cells from mice fed a methyl-donor restricted diet displayed decreased promoter methylation of *Cd40lg* and increased CD40LG expression. These results suggested that dietary intake of methyl-donor nutrients including folic acid may play an important role in regulating DNA methylation of immune genes of lupus patients.

Maintaining the balance of reactive oxygen species (ROS) and antioxidant production and damage repair because of cellular metabolism is vital to a cell's ability to survive and function properly. UV exposure, smoking and viral infections have all been implicated as sources of oxidative stress(115). Excessive oxidative stress can alter signal transduction pathways and differentiation in T cells potentially contributing to lupus pathogenesis(116). Oxidative stress is also linked to changes in DNA methylation in immune cells. *In vitro* oxidative stress has been shown to reduced ERK pathway signaling in CD4+ T cells and reduce DNMT1 activity concurrent with global hypomethylation and overexpression of lupus-relevant methylation-sensitive genes(117). Oxidative stress can also restrict the size of the methyl-donor pool required by DNMTs(116).

While the contribution of genetics to lupus pathogenesis is important, they do not tell the entire story on their own. Currently, there is an interest in understanding gene-environment interactions in lupus and other autoimmune diseases though few interactions have been explicitly studied so far(118). Understanding environmental risks and their mechanisms of action could be beneficial for improving patient health. DNA methylation has been considered as both a biomarker for environmental exposures as well as a mechanism by which exposures contribute to pathogenesis(119). DNA methylation within healthy individuals seems to be predominantly stable across time(120-122) though monozygotic twin studies have shown that DNA methylation divergence increases over time suggesting that the influence of environment on epigenetic

phenotype increases with age(123, 124). Understanding the effect of environmental factors on disease risk in the context of the genomic background of lupus patients could provide explanations for the "missing heritability" seen in complex polygenic diseases including lupus(125).

1.2.3 The Immune System in Lupus

Lupus is fundamentally a disruption in normal adaptive immune function that supports an environment where self-tolerance is lost, and autoantibodies and autoreactive immune cells can persist and cause tissue damage. Increased cellular apoptosis, potentially due to higher type I IFN, and decreased clearance (complement deficiency and phagocytosis impairment) are thought to be underlying mechanisms in the persistence of autoantibody immune complexes (ICs) in the body. Commonly measured autoantibodies in lupus are directed towards nuclear components including complexes of nucleic acids and nuclear proteins. Sensing of these components by pattern recognition receptor proteins like TLRs in addition to autoantibody binding contributes to the increased production of type I IFN in lupus.

Neutrophils are an important part of the innate immune response that produce microbiocidal granules and have the specialized ability to externalize nuclear material as neutrophil extracellular traps (NETs). NETs form a physical barrier on which microbes can be bound and exposed to antimicrobial proteins, release proinflammatory cytokines, and enhance phagocytosis by macrophages and antigen presenting cells(126). In autoimmune disease, reduced clearance of extracellular autoantigens including NETs is thought to contribute to their persistence and ability to promote enhanced IFNα expression through TLR-mediated recognition by plasmacytoid dendritic cells (pDCs)(127, 128). Recent research has shown that oxidized

mitochondrial DNA released in NETs, potentially enhanced due to oxidative stress, are particularly adept at this(52). Neutrophils express FcγRIIA on their surface giving them the ability to bind autoantibodies in immobilized ICs and upregulate reactive oxygen species (ROS) production in response(129). Lupus patients show an enrichment of a neutrophil gene signature in peripheral blood that correlates with development of lupus nephritis and suggests a disruption in the granulocyte compartment(130-132). In addition, neutrophils from lupus patients undergo apoptosis more readily(133). In lupus, there is also the presence of a subset of pathogenic low-density granulocytes which arise from the neutrophil population. These cells have a higher propensity for NETosis than normal density neutrophils and overexpress antimicrobial granule and alarmin proteins(128). NETs have been found in the skin of lupus patients and kidneys during lupus nephritis(128) and can activate the inflammasome of nearby macrophages, promoting IL-18 expression that can further activate neutrophils(134).

Monocytes and macrophages play an important role as cytokine producers, phagocytes, and antigen presenting cells. Monocyte-derived dendritic cells in lupus patients show increased expression of CD86, likely due to IFNα exposure, enhancing their costimulation and antigen presentation to T cells(135, 136). Macrophages are efficient phagocytes that clear apoptotic cells from the body to reduce the availability of autoantigens. Monocyte-derived macrophages in lupus patients have reduced phagocytic capacity including removing apoptotic material from germinal centers of lupus patients, apoptotic neutrophils, and autoantibody ICs(86, 133, 137-139). Lupus macrophages show altered gene expression upon activation taking on a proinflammatory M1-like profile with an enrichment of IFN signaling and inflammatory gene pathways(140).

T cells show altered responses to cytokine signaling and production, proliferation, differentiation, and regulatory function in lupus. Downregulation of CD3ζ and upregulation of FcRγ in the TCR complex alters the downstream signaling outcome of TCR stimulation leading to changes in calcium flux(141, 142) which activates the nuclear factor of activated T cells (NFAT)transcription factor, altering gene expression profiles of lupus T cells(143). One consequence is overexpression of CD40 ligand (CD40LG) that increases their ability to activate and differentiate B cells(144, 145). These signaling changes can also promote hyperpolarization of T cell mitochondria, promoting T cell necrosis(146). Effector T cell populations are altered in lupus as well. The frequency of peripheral Th17 cell populations increase in lupus patients as Tregs decrease, with the ratio increasing in lupus nephritis patients along with increasing levels of IL-17 which can stimulate antibody production by B cells(147, 148). The T helper cytokine profile of lupus patients also includes reduced IFNγ (Th1 cytokine) and TGF-β1 (Treg cytokine)(149). Impaired production of IL-2 in lupus patients is the result of skewed CRE-binding protein (CREB) and CRE-modulator (CREM) balance in T cells that suppresses IL-2 expression(150). Reduced IL-2 contributes to reduction in activation-induced cell death that allows autoreactive T cells to persist, reduction in Treg differentiation, and cytotoxic CD8+ T cell activity(151). There is an increase in circulating follicular helper T cells in the periphery of lupus patients that correlates with increased IL-21 expression(152, 153). IL-21 promotes antibody production, class switching, and plasma cell differentiation in B cells suggesting that follicular helper T cells promote germinal center reactions and autoantibody production in lupus(154, 155).

Lupus B cells show an exaggerated response to BCR signaling including increased intracellular calcium flux and signaling protein tyrosine phosphorylation(156). This coincides

with alterations to B cell subsets that favor autoreactive B cells due to faulty tolerance checkpoints related to altered signaling thresholds allowing them to escape anergy or death(157, 158). Peripheral B cell populations in lupus patients have an increased frequency of class-switched memory B cells and reduced frequency of naïve B cells(159). Memory B cells have lower activation thresholds allowing autoreactive B cells to persist. Circulating plasmablast levels are highly expanded in lupus patients and have a positive correlation with disease activity in lupus and are associated with anti-dsDNA autoantibody production(160, 161). B cell activating factor (BAFF) is necessary for mature B cell survival in the periphery including the persistence of autoreactive B cells(162). Peripheral expression of BAFF is increased in lupus and can be driven by IFNα(162, 163). Nucleic acids contained in autoantibody ICs can effectively stimulate autoreactive B cells through TLR7/9 signaling(164, 165). Reduced expression of the inhibitory FcγRIIb in lupus patients is thought to lower the threshold of activation for memory B cells and contribute to their aberrant activity(166).

1.2.4 Type I Interferon and SLE

Type I interferons are a class of immunoregulatory cytokines that includes IFNα (13-14 subtypes), IFNβ, IFNε, IFNκ, IFNω, IFNδ, IFNζ, and IFNτ(167). IFN classes are defined by their shared signaling receptor which in type I IFN are interferon alpha receptors (IFNAR) 1 and 2. IFNAR1/IFNAR2 are expressed by almost every tissue in the body including lymphoid and myeloid cells(168). The primary role of type I IFN is marshalling the immune system to mount a response upon viral infection by promoting expression of antiviral genes downstream of receptor signaling that suppress viral replication(169). Type I IFNs are produced through triggering of cytosolic and membrane-bound endosomal pattern recognition receptors, including TLRs, by

microbial components such as nucleic acid motifs and pathogen-associated molecular patterns. Cellular response to acute type I IFN signaling is potent and antiviral, but with chronic exposure it becomes immunosuppressive(167).

Type I IFN can promote maturation of dendritic cells and enhance antigen-presentation by MHC-II and promoting T cell activation(170, 171). Type I IFN induced expression of CCL2 in virally infected tissues draws monocytes from circulation where they differentiate into macrophages and DCs that can stimulate effector T cells(172).

The observation that dsRNA mimics (polyI:C) could stimulate the production of interferon and anti-RNA antibodies along with accelerating the development of autoimmune pathology in a genetically-susceptible lupus-prone murine model (NZB/NZW) was an early indication of the importance of type I interferons in lupus pathogenesis(173). IFNAR-deficient 129Sv/EV mice showed ameliorated development of autoantibodies and glomerulonephritis in response to 2,6,10,14-tetramethylpentadecane treatment to induce lupus-like disease(174). Observations of elevated IFNs in lupus patients that correlate with disease activity and autoantibody presence(175, 176) and the development of lupus-like pathology among patients receiving IFNα therapies(177-179) supported the role of type I IFN in lupus.

TLR activation through ligation of nucleic acids bound in autoantibody immune complexes is thought to be one mechanism by which immune cells produce type I IFN in lupus(180, 181). Plasmacytoid dendritic cells are known to be the most potent producers (though not sole producers) of type I interferon that are critical for the antiviral response(182, 183). They express both TLR7 and TLR9 that can produce IFNα in response to both microbial and endogenous ligands(184). Immune complexes in lupus contain both IgG autoantibodies that can bind Fcγ receptors and nucleic acids that can act as ligands for TLR7 (ssRNA) and TLR9

(unmethylated CpG DNA) acting as an etiological mechanism for the chronic production of type I IFN in lupus patients(185). Material released from necrotic and apoptotic cells when combined with lupus IgG antibodies can stimulate pDCs in a similar manner(185). Oxidized mitochondrial DNA released in NETs are also able to stimulate pDCs to produce type I IFN(127, 186). Increased apoptosis and decreased clearance of apoptotic bodies are thought to contribute to this pathogenic mechanism(86, 187). Neutrophils stimulated by TLR8 ligands show enhanced production of proinflammatory IL-6 in the presence of IFNα(188).

Peripheral mononuclear blood cells of lupus patients have increased expression of IFN-regulated genes (an "interferon signature") that was associated with more severe disease manifestations, though this signature is not universal among lupus patients(189) and can remain consistent within patients over time(190). Juvenile lupus patients show a similar IFN signature in peripheral blood cells that correlates with disease activity and response to glucocorticoid treatment(130). The association of elevated IFNα levels in the serum of lupus patients with circulating autoantibodies was observed to be consistent across African-American, European-American, and Hispanic patients, though IFNα levels were not associated with specific clinical presentations in this cohort(191). A recent review of IFN signature gene expression studies in lupus patients found that expression of a subset of genes (*RSAD2, IFI44, IFI44L, PRKR,* and *IFIT1*) in peripheral blood was associated with African ancestry more so than disease activity supporting the idea that genetics is a significant contributor to this pathogenic process(192).

1.3 DNA Methylation in SLE

1.3.1 DNA Methylation Function

Epigenetic traits are stable heritable phenotypes resulting from changes in a chromosome without altering the underlying DNA sequence(193). These factors regulate gene expression and by extension cellular identity and function (phenotype) by altering the structure and accessibility of genomic DNA to factors necessary for gene transcription. Mammalian DNA methylation is the enzymatic addition of a methyl group donated from *S*-adenosyl-L-methionine to the 5th carbon of the cytosine nucleotide to produce 5-methylcytosine (5mC) (Figure 1-1). This occurs primarily in the context of CpG dinucleotide motifs, and an estimated 70-80% of CpG sites in the human genome are methylated(194).

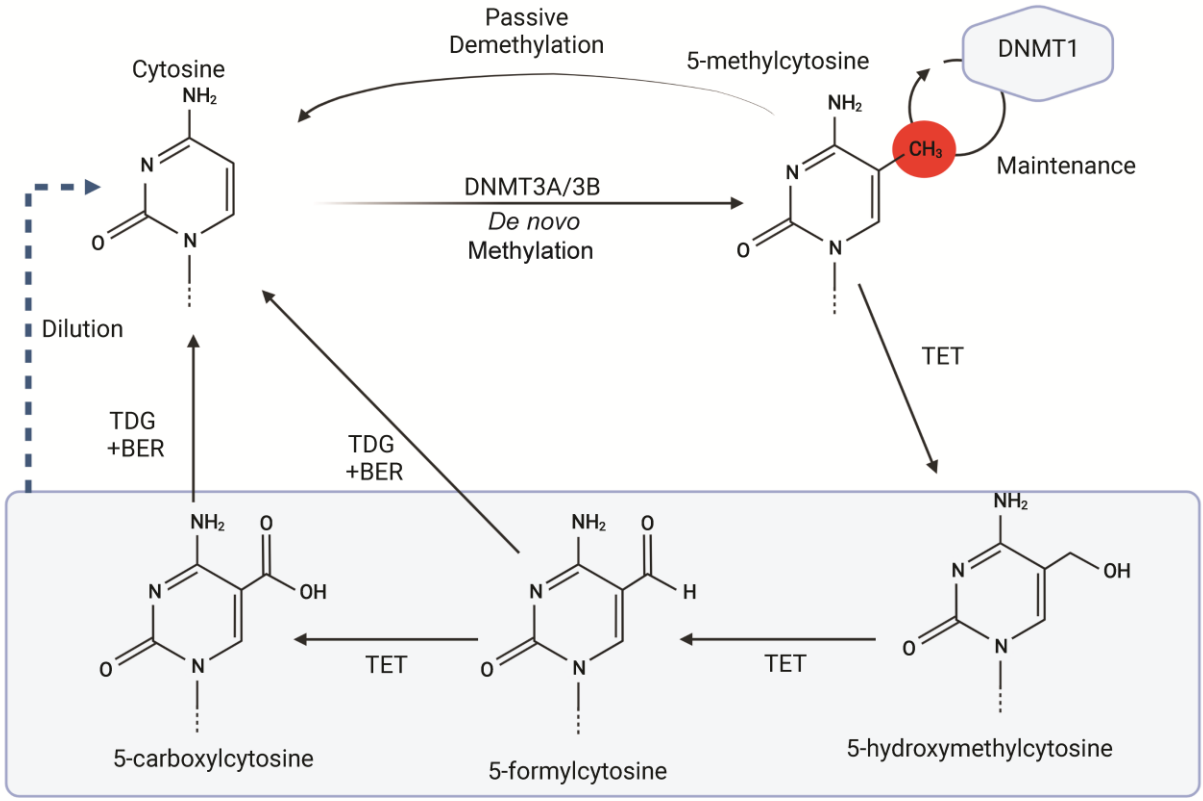

Figure 1-1: Diagram of cytosine methylation in the cell. DNA methylation of cytosine nucleotides in cytosine-guanine dinucleotide residues is catalyzed by DNA methyltransferase (DNMT) enzymes that are indispensable for development. De novo methylation is primarily established during embryogenesis by DNMT3A and DNMT3B. DNMT1 maintains methylation marks on newly synthesized DNA strands through recognition of hemimethylated sequences. DNA methylation can be lost through passive demethylation by reduction in DNMT activity and successive replication. Active demethylation that does not require DNA replication occurs through successive oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), then 5-formylcyotsine (5fC), and finally 5-carboxylcytosine (5caC) by ten-eleven translocation (TET) methylcytosine dioxygenases. Thymine DNA glycosylase (TDG) and base excision repair mechanisms replace 5fC and 5caC with cytosine nucleotides. 5hmC, 5fC, and 5caC can additionally be replaced with unmodified cytosine by passive dilution through replication. Created with BioRender.com.

The presence of chemically modified DNA nucleotides in the mammalian genome was first described in 1948 by Hotchkiss(195). The role of these modifications as a heritable signature that can regulate gene expression in mammals was postulated but not confirmed until the late 1970's(196, 197) followed by the discovery that *in vitro* methylated DNA sequences will maintain their methylation pattern across cellular generations after being inserted into mouse cells(198). The sequencing and cloning of the first eukaryotic DNA methyltransferase (DNMT) enzyme gene *Dnmt1* in mice followed(199). The DNMT family includes four members: DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMT function in the cell can be categorized

by their function maintaining (DNMT1) methylation in somatic cells or *de novo* (DNMT3A, DNMT3B, and DNMT3L) methylation of germline cells during development(197, 200). The *de novo* methylation activity of DNMT3 proteins is independent of DNMT1 activity(201, 202). During gametogenesis and later during preimplantation embryogenesis, the genome is systematically demethylated, followed by re-establishment of methylation patterns on maternal and paternal chromosomes by *de novo* methyltransferases DNMT3A and DNMT3B(203, 204). DNMT3L lacks a catalytic domain for direct methylation activity, but stimulates DNMT3A activity during *de novo* methylation(205). DNMT1 shows a preference for hemi-methylated DNA sequences over unmethylated and localizes to DNA replication foci to catalyze DNA methylation of newly formed strands during DNA replication in the cell's S phase(206, 207). The importance of DNMT activity development is demonstrated by the embryonic lethality of recessive mutations in mice and human embryonic stem cells(208-210) (Figure 1.1).

Most CpG dinucleotides in mammalian genomes are found unmethylated and clustered in regions termed CpG islands (CGIs). These regions can be hundreds of base pairs long with an average CpG density of about 18% compared to the >1% across the entire genome (211). The increased CpG density of CGIs is hypothesized to be the result of selective hypomethylation of the germline sequence to avoid transition mutation of 5mC to thymine bases through spontaneous deamination first to uracil and then subsequent activity by thymine DNA glycosylase (TDG) and base excision repair (BER) machinery(212, 213). This process is thought to explain the relative sparsity of CpG sites in the rest of the genome across evolutionary time (214, 215). Approximately 45,000 CGIs are found in the haploid human genome (215) and 72% of gene promoters are estimated to be associated with them(216). DNA methylation of gene promoters CGIs is generally associated with the presence of condensed chromatin and repressive

histone marks and ultimately gene silencing(217, 218). Tissue-specific DNA methylation occurs in CpG island "shores" located about 2kb distant from islands and CpG-poor promoters both of which have lower comparative CpG density and show the same relationship with gene expression(219, 220). In contrast, gene body DNA methylation is typically associated with genomic accessibility though the relationship is non-monotonic(221). Possible mechanisms underlying this observation are the suppression of intergenic transcription and increasing the stability of transcript elongation and a side-effect of competition between transcriptional and methylating enzymes for access to accessible chromatin(221).

While the density of promoter DNA methylation has a positive correlation with promoter silencing, it is not necessarily the direct mechanism by which genes are suppressed. One model of gene repression requires the ability of transcription factors to differentiate between methylated states. About 22% of surveyed human transcription factors have decreased binding to motifs including 5mC compared to unmethylated cytosines(222). Another model is the recruitment of chromatin remodeling machinery by DNMT1 or by proteins containing methyl-binding domains (MBDs) that show affinity for 5mC and can recruit repressive nucleosome remodeling proteins like histone deacetylases(223-225).

Epigenetic patterns in cells must be maintained with rigidity to prevent regression to a pluripotent state, but labile enough to allow cells to transition into new identities as seen in hematopoietic cells where gene promoter methylation is observed to fluctuate in a lineage-specific manner as cells develop(226). For decades it was assumed that DNA methylation was lost passively when DNMT1 failed to replicate methylation marks as DNA was replicated. The recent discovery of ten-eleven translocation (TETs) proteins provided a mechanism for active demethylation within the cell that could occur without the DNA replication required of passive

demethylation(227). TET1, TET2, and TET3 comprise a family of methylcytosine dioxygenases that catalyze the iterative steps of oxidative demethylation. 5-methylcyotsine residues are first oxidized to 5-hydroxymethylcytosine (5hmC), then 5-formylcytosine (5fC), and finally 5-carboxylcytosine (5caC). Any of these three stages can then be passively diluted through DNA replication, while a combination of TDG and BER machinery can actively replace 5fC and 5caC with cytosine residues independently of DNA replication (228). 5hmC is a stable epigenetic mark independent of 5mC that is positively correlated with gene expression and RNA polymerase II occupation of gene promoters. 5mC and 5hmC are indistinguishable using the bisulfite conversion method of measuring DNA methylation which is the gold standard and is widely used in epigenome-wide association studies (EWAS) performed by microarray and sequencing. This has left the contribution of 5hmC, and TET activity underappreciated. TET function is necessary for the proper development of myeloid and lymphoid cells, regulating vital proliferative functions and cellular identity(229). TET activity is necessary for proper antibody class switch recombination and affinity maturation in B cells, expression of transcription factors that maintain T cell lineages and prevent proliferation of autoreactive cells, and proper myeloid lineage differentiation and gene expression(230). Increased 5hmC in 131 gene promoters of CD4+ T cells of lupus patients has previously been reported enriched in genes from calcium, mTOR, and MAP kinase signaling pathways that positively correlated with expression(231).

In eukaryotic genomes, DNA methylation fulfills several important functions: repression of transposable elements that provides genomic stability, imprinting of inherited parental genomic sequences during development, X chromosome inactivation, and regulation of gene expression. An estimated 45% of the human genome is comprised of transposable elements (TEs), predominantly LINE-1 sequences, that are heavily methylated in somatic cells to maintain

genomic stability by preventing deleterious insertion events(232). Genome-wide hypomethylation during germ cell development is the typical period in which TEs can become active, but TE activation in somatic cells is also possible as the result of defective repression by the DNA methylation maintenance machinery (233). The ability of TEs to disrupt genomic stability has generated interest in their role in oncogenesis and autoimmunity. Human endogenous retroviral elements (HERVs), a type of TE, have been found to be overexpressed in lupus patients, and DNMT1 inhibition *in vitro* can induce HERV transcript expression in healthy cells(234).

Genomic imprinting occurs when gene transcription comes primarily or solely from one parental chromosome while the other is epigenetically suppressed. This process is vital for proper development, and imprinted loci are resistant to the epigenetic reprogramming that occurs during embryogenesis. X chromosome inactivation (XCI) is the random epigenetic silencing of one of two X chromosomes inherited by female progeny facilitated by the X-inactive specific transcript (*XIST*) non-coding RNA that coordinates with epigenetic machinery including histone modifiers and DNMTs(235, 236). XCI allows for dosage compensation of X-linked genes. As lupus is predominantly seen in women and several immunomodulatory genes are located on the X chromosome including *CD40LG* and *TLR7*, improper XCI has been considered as a contributing factor to the disease(237). CGI promoter methylation correlated with expression status of genes that have undergone XCI and about 70% of genes have the same XCI status across tissues based on DNA methylation profiling of their promoters, though some show XCI escape in a tissue-dependent manner(238). Escape of X-linked immunomodulatory genes are thought to contribute to the development of autoimmunity(237, 239, 240).

1.3.2 DNA Methylation and the Immune System

DNA methylation is an important epigenetic regulator of immune system function. DNMT1 in necessary for early T cell survival, development, and effector gene expression(241). Naïve CD4+ T cells are by necessity plastic, and epigenetic remodeling including DNA methylation helps to stabilize the expression of transcription factors and cytokines in response to differentiating signals(242). DNMT1 and DNMT3a are necessary for regulating T cell differentiation into effector subsets(243). For example, DNMT3 is required to epigenetically silence the IFNγ locus in naïve CD4+ T cells after differentiation into Th2, Th17, and induced regulatory T cells(244). Th2 cells require DNMT function to suppress IFNγ and upregulated IL-4 expression during differentiation(245). The DNA methylation profile of Th17 cells more closely matches that of naïve CD4+ T cells than Th1 cells providing an explanation for the plasticity seen in Th17 subsets that can adopt a non-classical Th1 or Th2 phenotypes(246). Natural Tregs have stable expression of Foxp3, their major TF. Naïve and active CD4+ T cells and TGFβ-induced Tregs that arise from peripheral CD4+ T cells fail to stably express Foxp3 due to DNMT activity and DNA methylation-mediated suppression of the Foxp3 locus(247).

Naïve B cells maintain a hypermethylated state outside of lineage-reinforcing genes like the transcription factor PAX5 that promote cellular identity until encountering activating signals and undergoing DNA demethylation as they differentiate into germinal center B cells(248, 249). DNA methylation profiles of GC and plasma B cells show enrichment for transcription factor binding sites distinct from naïve B cells, suggesting a lowered epigenetic threshold for reactivation upon subsequent challenge by antigen(250).

Compared to lymphoid genomes, myeloid genomes are hypomethylated as they progress through developmental stages from hematopoietic progenitors(251), with neutrophils showing

the most pervasive hypomethylation(226). Expression of neutrophilic-granule genes like myeloperoxidase and elastase increased as DNA methylation decreased at each stage of maturation to the promyelocyte stage. Expression dropped with differentiation to primary neutrophils while DNA methylation remain unchanged, suggesting non-epigenetic factors suppressed gene expression at epigenetically persistent sites allowing for rapid response to infectious signals(252). DNA methylation changes favoring transcription factor binding and enhancer sites occur in monocytes as they differentiate into macrophages(253). This less stringent epigenetic environment is thought to reflect the necessity of the innate immune cells to respond quicky to a wide variety of immune insults.

1.3.3 Lupus-Associated DNA Methylation

Disease-associated alterations in DNA methylation of immune cells is a recognized feature of lupus (Figure 1.2). Early observations showed that procainamide and hydralazine, both etiological agents of drug-induced lupus, were associated with inhibition of DNMT activity and induction of CD4+ T cell autoreactivity, similar to CD4+ T cells treated with the DNA methylation inhibitor 5-azacytidine(254, 255). These autoreactive CD4+ T cells showed hypomethylation of autoimmune-related genes including *ITGAL*, *TNFSF7*, and *CD40LG* (256, 257) similar to lupus CD4+ T cells that exhibited global hypomethylation(258). The ability of *in vitro* demethylated CD4+ T cells to induce lupus-like disease including glomerulonephritis and autoantibodies when injected into syngeneic mice provided more evidence for a mechanistic link between DNA methylation changes and autoreactivity(259, 260).

The advent of microarray technology allowed for EWAS measuring site-specific DNA methylation levels from tens to hundreds of thousands of CpG sites simultaneously. Early studies found that monozygotic twins discordant for lupus show shifts in leukocyte DNA methylation in

genes associated with immune response and cytokine production, and global hypomethylation in the affected twin providing a potential mechanism for explaining non-genetic factors that contribute to lupus pathogenesis(261).

EWAS studies of lupus CD4+ T cells showed a predominant hypomethylation compared to healthy controls(262-264). This hypomethylation included enrichment of genes involved in the type I interferon response present across naïve and mature CD4+ T cell subsets including memory and regulatory T cells(262-264). Naïve CD4+ T cells reflected an epigenetically poised state where the hypomethylation was observed but without an associated increase in gene expression(264). Lupus manifestations carried their own unique naïve CD4+ T cell DNA methylation signatures. Profiles of patients with discoid rash were distinct from patients with malar rash or neither cutaneous manifestation. Hypomethylated regions were unique to each group, but antigen processing and presentation genes were shared between them all(265). Genes encoding regulatory proteins for tissue hypoxia response (*HIF3A*) and type I interferon expression and response (*IRF7* and *IFI44*) have decreased DNA methylation in patients that develop lupus nephritis when compared to lupus patients that do not(266, 267). Increasing lupus disease activity is associated with DNA methylation changes in naive CD4+ T cells that potentially favor differentiation towards Th2 and Th17 differentiation upon activation(268). Genes with a positive correlation between DNA methylation and disease activity were enriched in binding sites for the transcription factor enhancer of zeste homolog 2 (EZH2)(268). Genes with a negative correlation between DNA methylation and disease activity were depleted in EZH2 binding sites suggesting the EZH2 maybe be an important regulatory factor upstream of the epigenetic shift observed in lupus CD4+ T cells(268). EZH2 forms part of the catalytic function of the polycomb repressive complex 2 that can interact with DNMTs to increase DNA

methylation and repress gene expression(269). CD4+ T cells, B cells, monocytes, and neutrophils from lupus patients show greater expression of EZH2 compared to controls(270, 271). Inhibition of EZH2 increased survival and decreased circulating anti-double stranded DNA autoantibodies and glomerulonephritis in lupus-prone MRL/*lpr* mice(271).

Overexpression of EZH2 in CD4+ T cells decreased DNA methylation of leukocyte adhesion and migration genes including the junctional adhesion molecule A (JAM-A). EZH2 overexpression in CD4+ T cells also increases expression of JAM-A(270). JAM-A expression is higher in the CD4+ T cells of lupus patients than those in healthy controls(270). Blocking of JAM-A in EZH2-overexpressing CD4+ T cells and lupus CD4+ T cells reduces their ability to adhere to vascular endothelial cells(270). EZH2 activity can be influenced by environmental factors and cellular metabolism. Oxidative stress increases EZH2 expression in CD4+ T cells(272). Oxidative stress also increases mTORC1 activity and subsequently glycolysis and cellular metabolism in CD4+ T cells(272). Inhibiting mTORC1 or glycolysis downregulates EZH2 as well, suggesting that environmental stressors like reactive oxygen species can influence the activity of the key epigenetic regulatory factor EZH2 through mTORC1 activity and contributing to the shift in DNA methylation seen in lupus CD4+ T cells.

A subset of demethylated T cells is expanded in lupus patients and increases in size with disease activity. This subset is characterized by overexpression of methylation sensitive genes including *ITGAL*, *CD40LG*, and killer Ig-like receptors (KIR), and is identified as CD4+CD28+KIR+CD11a^{hi} T cells(273). The methylation and expression profiles of primary of CD4+KIR+CD11a^{hi} T cells isolated from lupus patients compared to autologous CD4+CD28+KIR-CD11a^{low} T cells recapitulated what was seen in CD4+KIR+CD11a^{hi} T cells generated *in vitro* with DNA methylation inhibitors(274). Hypomethylated and overexpressed

genes in this subset were enriched in pathways related to graft versus host disease, inflammatory response to bacterial infection, and chemotactic immune response(274).

Trans-tissue methylation analysis of CD4+ T cells, B cells, neutrophils, and monocytes found that while each tissue carries a unique DNA methylation signature, there is enrichment for hypomethylated genes in interferon-mediated immunity across all cell types(263, 275). In contrast to the findings that type I interferon serum concentrations sometimes vary with disease activity, hypomethylation of interferon genes did not change between quiescent and active disease (263, 264).

In addition to IFN-regulated genes, lupus CD8+ T cells have extensive hypomethylation of *HLA-DRB1*, which was overexpressed in the presence of type I IFN(276). *HLA-DRB1* expression was found to be and dependent on STAT1 and CIITA which are upregulated in response to IFNα(276). These HLA-DRB1+CD8+ T cells can activate autologous naive CD4+ T cells *in vitro* and could contribute to disease pathogenesis(276).

A proinflammatory CD4-CD8- T cell subset ("double negative (DN) T cells") is significantly expanded in the peripheral blood of lupus patients(277). DN T cells can produce IL-17, IFNγ and IL-4, and infiltrate the kidneys in lupus nephritis patients(277, 278). Compared to CD4+ and CD8+ T cells, DN T cells show extensive hypomethylation of proinflammatory genes and an associated reduction in DNMT1, DNMT3A, and DNMT3B expression(279). CD4 and CD8 gene loci were specifically hypermethylated likely contributing to their repression in DN T cells. Hypomethylated genes in DN T cells were enriched in cell adhesion and communication functional categories and proinflammatory cytokine genes including IL-17, IFNγ, and BLYS which is a potent stimulator of B cells(279). T cell-specific delivery of 5-azacytidine to lupus-prone MRL/*lpr* mice suppressed disease pathology potentially by upregulating Foxp3 expression

in CD4+ T cells, supporting the differentiation of regulatory T cells and preventing the conversion of CD8+ T cells to a pathogenic double-negative phenotype(280). Future studies of targeted delivery of drugs regulating DNA methylation within cells may provide new therapeutic options for lupus.

Transposable elements in the genome of lupus patients are shown to be hypomethylated in CD4+ and CD8+ T cells, B cells, and neutrophils, reflecting an overall global hypomethylation. In T cells and B cells, LINE-1 and HERV hypomethylation is associated with disease activity(281, 282). LINE-1-containing genes are also overexpressed in lupus neutrophils suggesting that these transposable elements may alter the expression of apoptosis and immune response genes(283).

Recent studies of the relationship between DNA methylation and genetics in lupus has been through the identification of methylation quantitative trait loci (meQTL). MeQTL combines both genome-wide DNA methylation and genotyping data to measure associations between DNA methylation and nearby polymorphisms. The first of such studies in peripheral blood of lupus patients found significant meQTL enriched in genes associated with transcription factor activity and leukocyte activation and overlapping active enhancer regions in T and B cells(73). MeQTL also included associations with genetic susceptibility loci for lupus, such as *IRF7*, suggesting that genetic risk loci might mediate pathogenic effects in lupus via altering DNA methylation(73). One study of a multi-ethnic lupus cohort found that DNA methylation differences associated with patient clustering by clinical criteria were partially associated with meQTL and potentially mediated the SNP-cluster association(284).

Ethnicity-associated genetic and epigenetic differences have received increasing attention in the field lately to explain in part the observed differences in disease activity and manifestations between populations. Genetic ancestry explains approximately 60%-75% of the DNA methylation variance in the healthy population suggesting the remaining variance is due to personal environmental and non-genetic factors(285, 286). PBMCs and naïve CD4+ T cells isolated from African-American patients with lupus show an increased hypomethylation of the interferon-regulated gene signature and apoptosis-related genes, respectively, when compared to European-American patients(287, 288).

DNA methylation studies have the potential for the discovery of new disease biomarkers. Two CpG sites associated with the interferon-regulated gene *IFI44L* that are hypomethylated in lupus patients were found to be highly sensitive and specific for a diagnosis of lupus when compared to rheumatoid arthritis and primary Sjogren's syndrome patients and healthy controls in a Chinese cohort(289). Sensitivity and specificity for these sites was slightly lower in a European cohort(289). *IFI44L* methylation status was not influenced by type I interferon exposure *in vitro*(290).

1.4 Rationale

Decades of research have demonstrated that DNA methylation alterations in the immune cells of lupus patients reflect a disease-associated process influenced by both genetic and environmental factors (Figure 1-2). Currently, DNA methylation in lupus patients consist of cross-sectional studies that are unable to evaluate DNA methylation changes over time within patients. Expression profiles of peripheral blood cells in lupus patients change with disease activity over time and it is not hard to imagine that DNA methylation could potentially follow a similar

course. This led us to propose a longitudinal analysis of DNA methylation in a cohort of lupus patients followed for up to four years. Using this dataset, we wanted to address the association of DNA methylation in neutrophils over time and disease activity in lupus patients. We also wanted to evaluate the potential for genetic-DNA methylation interaction in lupus patients by performing meQTL analysis. The deliberate inclusion of African-American and European-American lupus patients allowed us to address the contribution of genetic ancestry to the observed DNA methylation changes. We applied a similar analysis to DNA methylation data we derived from naïve CD4+ T cells collected from a cross-sectional, case-control cohort to tease apart patient-specific genetic contributions to the DNA methylation signature that defines lupus.

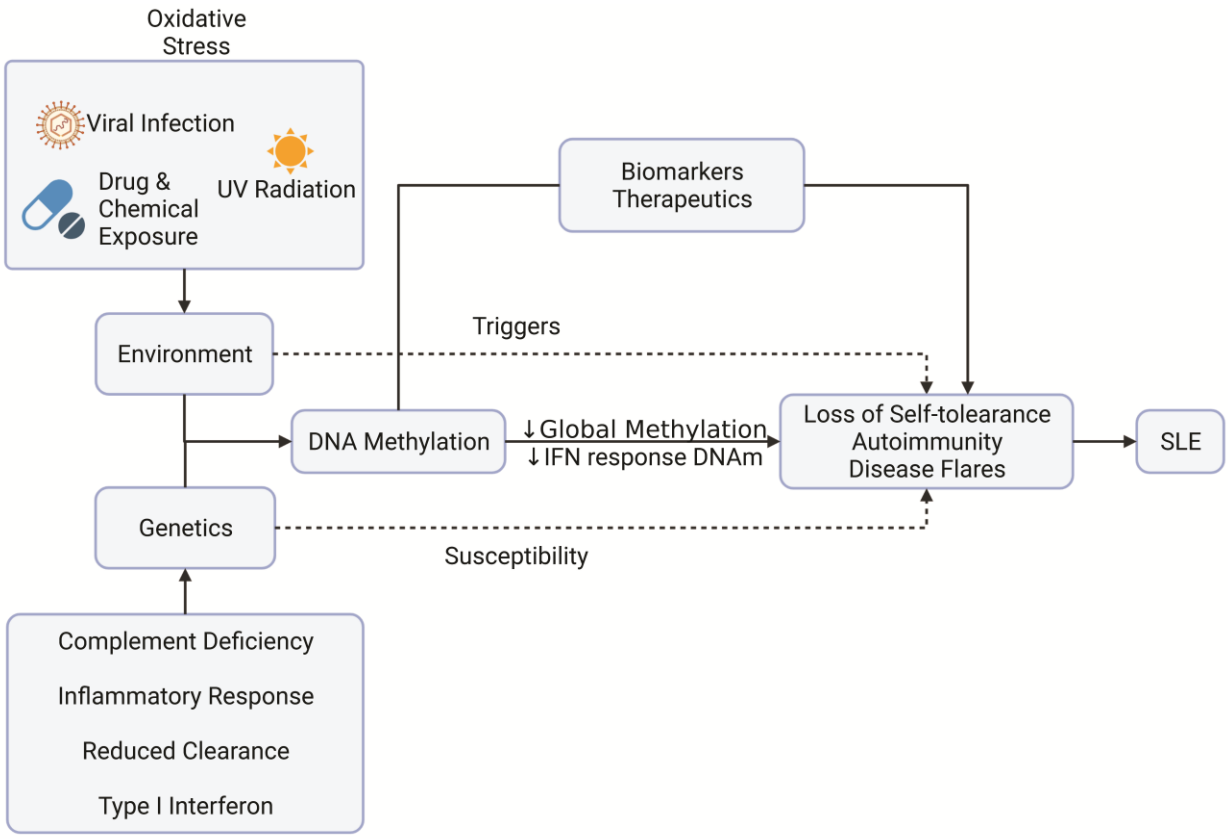

Figure 1-2: DNA methylation is the interface of genetic and environmental factors in lupus. In lupus, environmental factors such as UV radiation and viral infection are potential triggers for loss of self-tolerance and the development of autoimmunity or disease flares. Genetic background in genes regulating the inflammatory and type I interferon responses of the immune system can make individuals more susceptible to developing autoimmunity. Environmental exposures and genetics can both influence DNA methylation. Reductions in global methylation and specifically IFN-response genes are a characteristic of lupus immune cells. These could contribute to loss of self-tolerance and the development of lupus as well as contribute to disease flares. DNA methylation can serve as a potential biomarker for monitoring disease activity or a therapeutic target. Created with BioRender.com.

Chapter 2 A Longitudinal and Transancestral Analysis of DNA Methylation Patterns and Disease Activity in Systemic Lupus Erythematosus Neutrophils

2.1 Abstract

Objective: Epigenetic dysregulation is implicated in the pathogenesis of lupus. We performed a longitudinal analysis of DNA methylation in lupus patients and assessed epigenetic changes over time and across disease activity status. Combining genetic and epigenetic analyses, we also examined ancestry-specific DNA methylation and DNA methylation changes influenced by genetic variants across the genome.

Methods: A total of 54 female lupus patients, including 32 European-American and 22 African-American, were followed for up to 4 years. Blood samples were obtained at routine follow up visits and during disease flares, with a total of 229 samples collected. Disease activity at each blood draw was determined by SLEDAI. Granulocytes were isolated and DNA extracted. Genotyping was performed using the Infinium Global Screening Array v2.0, and genome-wide DNA methylation was assessed at each time-point using the Infinium MethylationEPIC array. Ancestry-specific DNA methylation changes and methylation quantitative trait loci (meQTL) were identified. A linear mixed effects model was implemented to identify DNA methylation alterations that vary with disease activity and the development of lupus nephritis during follow up.

Results: We identified 487 hypomethylated and 420 hypermethylated CpG sites in African-American compared to European-American lupus patients, annotated to 391 and 316 unique genes, respectively. Differentially methylated genes include type I interferon-response

genes such as *IRF7* and *IFI44*, and genes related to the NFκB pathway. After adjusting for age, medications, and genetic background, DNA methylation levels in 142 (15.7%) differentially methylated sites were found to be allele-specific and influenced by at least one genetic variant located within 1kb. *TREML4*, which plays a vital role in toll-like receptor signaling, was hypomethylated in African-American patients and demonstrated a strong *cis*-meQTL association (r^2=0.91). The associated genetic variant (rs9369265) significantly differs in allele frequencies between African-American and European-Americans and is located within an active enhancer region in neutrophils and modifies *TREML4* expression. *In vitro* patch methylation experiments confirmed the regulatory effects of *TREML4* methylation upon gene expression. Experiments to assess the functional effects of *TREML4* overexpression in human neutrophils are underway in our laboratory. Interestingly, the DNA methylome was highly stable across disease activity levels and over time. Two sites cg26104306 (*SNX18*; FDR-adjusted P-value = 3.38 x 10-2) and cg06708913 (FDR-adjusted P-value = 3.43 x 10-2) were associated with changing disease activity levels in African-American patients. Demethylation of a CpG site located within *GALNT18* was associated with the development of active lupus nephritis.

Conclusion: Lupus granulocytes demonstrate significant differences in DNA methylation patterns between African-American and European-American patients. DNA methylation profiles in lupus patients are influenced by ancestry-specific genetic variants and are highly stable over time independent of disease activity levels. Progressive demethylation in *SNX18* was observed with increasing disease activity in granulocytes from African-American lupus patients, and demethylation in *GALNT18* was associated with the development of lupus nephritis in our cohort during follow up.

2.2 Introduction

Systemic lupus erythematosus (SLE or lupus) is an autoimmune disease of incompletely understood etiology. Genetic, epigenetic, and environmental factors are thought to play key roles in the immune dysregulation underlying the development of the disease(291). Lupus is characterized by the production of autoantibodies to nuclear antigens, and a remitting-relapsing disease course that can target multiple organ systems(292). Frequent disease flares and prolonged periods of active disease are associated with a more deleterious outcome in lupus patients and a higher risk of tissue and organ damage(293).

Lupus is associated with changes in gene expression, including prominent type I interferon and neutrophil gene signatures in the peripheral blood(131, 189, 294, 295). Further, increased disease activity in lupus is associated with transcriptional profiles implicating different innate and adaptive peripheral immune cells in individual patients followed longitudinally (131). Notably, progression to active nephritis in lupus patients was associated with gradual enrichment in neutrophil transcripts(131). Indeed, a prominent role for neutrophils in the pathogenesis of lupus is being more clearly elucidated(296).

DNA methylation, an epigenetic mechanism that regulates gene expression, is altered in the immune cells of lupus patients and is potentially influenced by both environmental and genetic factors(297). DNA methylation defects in lupus are suggested to promote an overactive immune response when exposed to inflammatory signals like autoantibody-autoantigen complexes or endogenous nucleic acids(266, 268, 275). Methylation quantitative trait loci (meQTL) are genetic polymorphisms that are associated with DNA methylation either directly through alteration of CpG dinucleotides or at a distance through an intermediary process. MeQTL identified in prior lupus studies show enrichment for lupus susceptibility genes and type

I interferon response genes suggesting that altering DNA methylation levels at specific loci could be a potential mechanism by which risk alleles contribute to disease susceptibility in lupus(73, 82, 284). Lupus susceptibility is significantly higher in patients of non-European ancestry, who are also more likely to develop more severe disease even after accounting for the influence of social and environmental factors(298). Thus, meQTL analysis provides a potential approach to better understand the mechanisms underlying the observed differences in disease manifestations and outcomes in lupus patients of different ancestries.

Recent work investigating DNA methylation changes in lupus and associated downstream effects and underlying upstream regulatory mechanisms have resulted in significant insights into the pathogenesis of lupus and the identification of novel therapeutic targets for the disease(271). Cell-type specific precision delivery systems to modify the epigenome are promising novel approaches in the treatment of autoimmune diseases including lupus (280). Further, DNA methylation changes have been suggested as diagnostic markers and markers that can potentially predict specific disease manifestations in lupus(265, 266, 289, 299). However, DNA methylation studies in lupus to date have been cross-sectional, and longitudinal studies investigating epigenetic changes in patients with lupus over time have not been reported.

We have previously demonstrated robust demethylation of interferon-regulated genes in lupus neutrophils compared to normal healthy controls(275). In this study, we investigate neutrophil DNA methylation changes over time and across disease activity levels in a cohort of lupus patients followed longitudinally for up to about 4 years. Moreover, we sought to increase our understanding of how DNA methylation is impacted by the genetic background. We compared DNA methylation patterns between African-American and European-American lupus patients, performed meQTL analyses in lupus neutrophils, and identified CpG sites that show

methylation changes correlating with disease activity and the development of lupus nephritis across the course of the disease.

2.3 Methods

2.3.1 Study Participants and Demographics

54 female lupus patients were recruited from the University of Michigan Health System and Henry Ford Health System for this study (see Table B-1). Our cohort included 32 patients of European-American ancestry and 22 patients of African-American ancestry. Patients were followed over a 43 month period. The patients selected for this study had at least one change in disease activity as measured by the systemic lupus erythematosus disease activity index (SLEDAI) score across all timepoints. This resulted in a total of 229 timepoints across all patients (4 median timepoints per patient; range: 2-11 timepoints). The mean age of patients at the initial visit was 41.0±13.1 years (mean±sd; range: 19-70 years). The mean SLEDAI score of patients was 3.9±3.9 (mean±sd; range: 0-20) at their initial visit and 4.0±3.7 (mean±sd; range: 0-20) across all timepoints. All patients in this study fulfilled the American College of Rheumatology classification criteria for systemic lupus erythematosus(300).

DNA methylation data from normal healthy control neutrophils (n= 5 and 6 African-American, and European-American, respectively) generated using the Illumina Infinium HumanMethylation450 array and previously reported were also used(275) (GEO accession GSE65097).

2.3.2 DNA Isolation

Whole blood was collected from each patient at each time point during clinic visits in vials containing EDTA. Granulocyte fractions were isolated using density centrifugation with Ficoll-Histopaque (GE Healthcare, Chicago, IL, USA). Genomic DNA was isolated from the enriched granulocyte layer using either phenol-chloroform extraction or Qiagen DNEasy Blood and Tissue kit (Qiagen, Germantown, MD, USA), or following the removal of red blood cells using dextran (Sigma-Aldrich, St. Lois, MS, USA) and hypotonic lysis(301). DNA was eluted in water and quantified using Qubit DNA fluorescence quantification assays (Thermo-Fisher, Waltham, MA, USA).

2.3.3 DNA Methylation Measurement

350ng of DNA from each sample was bisulfite converted using the EZ-96 DNA Methylation Kit (Zymo, Irvine, CA, USA) following the manufacturer's instructions. Samples were hybridized to the Infinium MethylationEPIC array (Illumina, San Diego, CA, USA) to assess site-specific DNA methylation of over 850,000 methylation sites across the genome. Samples were randomized across all arrays to minimize batch effects. Sample hybridization and array scanning were performed at the University of Michigan Advanced Genomics Core.

2.3.4 DNA Methylation Quality Control and Analysis

DNA methylation data analysis was performed in the R statistical computing environment (v.3.6.3)(302). Raw .idat files were generated for each sample and read into the R package *minfi* (v.1.32.0) for quality control and downstream analysis(303, 304). Probes with less than three beads and zero intensity values across all samples were removed according to best practices as

implemented by the *DNAmArray* package (v.0.1.1)(305). Then, background signal and dye bias were corrected followed by normalization of signal intensities using functional normalization in the *preprocessFunnorm.DNAmArray* function(305, 306). This method uses the first three principal component values calculated from signal intensities of control probes present on all array spots to correct for technical variation. Probes with detection P-values < 0.01 were removed as were probes that returned signal intensities in fewer than 98% of samples. Signal intensities were then converted to M-values with a maximum bound of ±16. M-values were used for all regression testing and converted to beta values (0-100% methylation scale) using *minfi* for reporting.

We masked any probes with potential technical issues if the probe met any one of the following criteria described by *Zhou, Laird & Shen (2017)*(307): A unique probe sequence of less than 30bp, mapping to multiple sites in the genome, polymorphisms that cause a color channel switching in type I probes, inconsistencies in specified reporter color channel and extension base, mapping to the Y chromosome, and/or having a polymorphism within 5bp of the 3' end of the probe with a minor allele frequency (MAF) > 1% with exception of CpG-SNPs with C>T polymorphisms which we retained for analysis. Batch correction was performed using the *ComBat* function in the *sva* (v.3.34.0) package(308).

We implemented a mixed correspondence analysis with the *PCAmixdata* package (v.3.1) to calculate eigenvalues using patient medication data for prednisone, hydroxychloroquine, azathioprine, mycophenolate mofetil, and cyclophosphamide(309). The top four components accounted for a cumulative 88.4% of variability in the medication data. Each component value was used as an independent variable in regression analysis to adjust for medication usage across individuals.

Cell type-specific DNA methylation profiles were used to assess enrichment of neutrophils in our DNA samples(310). Of 73 CpG sites previously identified to accurately discriminate between neutrophils and other cell types in peripheral blood (namely CD4+ T cells, CD8+ T cells, B cells, natural killer cells, and monocytes) methylation levels in 71 sites passed our quality control measures in our dataset. DNA methylation levels in these sites were very highly correlated in our DNA samples with DNA isolated from neutrophils (r = 0.996, see Figure C-1).

2.3.5 Genotyping and Methylation Quantitative Trait Loci (meQTL) Analysis

Genotyping data were generated using Infinium Global Screening Array-24 v2.0 (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Stringent quality controls (QC) were applied before analyses using *PLINK* (v.1.9)(311). Single nucleotide polymorphisms (SNPs) with a genotyping call rate < 98%, MAF < 5%, and deviating from Hardy-Weinberg equilibrium (HWE; P-value < 1E-03) were filtered out. Samples were removed if they had a genotyping call rate < 95%. Sex chromosomes were not analyzed. About 100,000 independent SNPs were pruned and used to perform principal component analysis (PCA) with *Eigensoft* (v.6.1.4) software(312). Genotyping data of a single African-American lupus patient was removed at the QC step due to failing quality measures. All meQTL analyses presented in this chapter are obtained from the methylation and genotyping profiles of n = 21 African-American and n = 32 European-American lupus patients.

2.3.6 Functional Enrichment Analysis

Gene annotation of CpG probes was done using GENCODE v22 (hg38) annotations from a manifest file produced by *Zhou, Laird & Shen (2017)*(307). Gene network analysis of differentially methylated genes was done using Ingenuity Pathway Analysis (Qiagen, Germantown, MD, USA). ToppGene Suite was used for functional gene ontology enrichment analysis(313). Molecular Function and Biological Process Gene Ontologies and KEGG Pathways were selected for enrichment. P-values were derived using a hypergeometric probability mass function and a Benjamini-Hochberg FDR-adjusted P-value cutoff of < 0.05 was used as a threshold of significance. Ontologies and pathways had to have a minimum membership of 3 genes and maximum of 2000 genes to be included.

Interferon-regulated genes were identified using the differentially methylated gene set as input for Interferome (v.2.01)(314) limiting results to genes with an expression fold change of 1.5 or greater between type I interferon-treated and untreated samples using datasets derived from peripheral whole blood.

2.3.7 In vitro TREML4 *Promoter Patch Methylation*

We used an *in vitro* patch methylation assay to examine the role of DNA methylation in regulating the expression of *TREML4*. To accomplish this, we used the pCpGfree-promoter-Lucia plasmid (InvivoGen, San Diego, CA, USA) which lacks CpG sites to prevent any DNA methylation occurring outside of the inserted sequences. Chemically competent GT115 *E. coli* (InvivoGen, San Diego, CA, USA) were transfected with 1ug of plasmid and grown according to manufacturer's direction. Isolated colonies were grown in 3mL of Fast-Media Zeo TB (InvivoGen, San Diego, CA, USA) for 7h at 37°C while shaking at 250rpm, then allowed to continue growing overnight in the same conditions in an additional 200mL of Fast-Media Zeo

TB (InvivoGen, San Diego, CA, USA). Bacterial cells were spun at 2000xg to pellet and frozen at -20℃. Plasmid was isolated using the QIAGEN Plasmid Maxi Kit (Qiagen, Hilden, Germany) according to manufacturer's direction.

The *TREML4* promoter sequence (chr6:41228103-41228452; hg38) oligonucleotide was synthesized (Integrated DNA Technologies, Coralville, IA, USA) using the sequence available from the GRCh38 build of the human genome (see Appendix B). Restriction digest sequences for SbfI (5'- TAAGCACCTGCAGG-3') was added to the 5' end of the *TREML4* sequence and SpeI (5'- ACTAGTTAAGCA-3') to the 3' end with the addition of a 5'-TAAGCA-3' sequence to enhance digestion. The promoter sequence included five CpG sites available for methylation. The *TREML4* promoter and plasmid backbone were prepared using separate restriction digest reactions with SpfI-HF and SpeI-HF enzymes using NEBuffer CutSmart (New England Biolabs, Ipswich, MA, USA) using 500ng of plasmid DNA and 100ng of promoter DNA, respectively. A ligation reaction using T4 ligase (New England Biolabs, Ipswich, MA, USA) and a 7:1 mass ratio of *TREML4* insert:plasmid backbone was performed to produce a complete plasmid. Restriction digest with SbfI-HF and HindIII-HF (New England Biolabs, Ipswich, MA, USA) was used to remove the native EF-1α promoter from the pCpGfree vector followed by quick blunting and T4 ligation (Quick Blunting Kit; New England Biolabs, Ipswich, MA, USA) using purified plasmid according to manufacturer's direction. Colony PCR screening was used to identify colonies with properly inserted plasmid using the One*Taq* Quick-Load Master Mix (New England Biolabs, Ipswich, MA, USA) according to manufacturer's direction. EF-1α removal and *TREML4* promoter insertion were verified using Sanger sequencing of isolated plasmid with the manufacturer's suggested PCR primers (Forward: 5'-GTACCAGTTTTATTGTTTTTAGTGGTAGTG-3'; Reverse: 5'-

GCCATGTGCTCTCTGCCCACTGAG-3'; Integrated DNA Technologies, Coralville, IA, USA).

In vitro methylation of the *TREML4* promoter plasmid was performed using the CpG methyltransferase M.SssI (New England Biolabs, Ipswich, MA, USA) using 1ug of plasmid DNA as input according to manufacturer's direction. Restriction digest with AciI and SacI-HF (New England Biolabs, Ipswich, MA, USA) and agarose gel electrophoresis was used to confirm CpG methylation. Only reactions showing complete methylation were selected for use in *in vitro* patch methylation assay. A mock control reaction lacking the addition of M.SssI was performed simultaneously. Agarose gel electrophoresis and gel extraction was performed to isolate purified DNA from restriction enzyme digests, M.SssI reactions, and PCR products using the QIAquick PCR & Gel Cleanup kit (Qiagen, Hilden, Germany). All Sanger sequencing was performed by Azenta Life Sciences (Pittsburgh, PA, USA).

HEK-293 cells were cultured using DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ThermoFisher Scientific, Waltham, MA, USA). Opaque white 96-well plates were seeded with 20,000 HEK-293 cells/well in 75uL of DMEM media overnight. 25ng of the desired plasmid (methylated or mock methylated control) and 1ng of pGL4.73[*hRluc*/TK] (Promega, Madison, WI, USA) was mixed with Fugene 6 (Promega, Madison, WI, USA) diluted in Opti-MEM Reduced Serum media (ThermoFisher Scientific, Waltham, MA, USA) using a 3:1 ratio of Fugene 6 volume:DNA mass (ug) per well. Wells with no DNA added were included as control for background signal. Transfected HEK-293 cells were incubated at 37℃ for 48h. Lucia luciferase is secreted and chemiluminescence can be measured using culture media. The assay was performed using the QUANTI-Luc Gold detection medium (InvivoGen, San Diego, CA, USA). Briefly, 10uL of supernatant was removed from each well

and transferred to the complementary well on an opaque white 96-well plate and 50uL of QUANTI-Luc Gold medium added according to manufacturer's direction. *Renilla* luciferase chemiluminescence was measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) by adding the detection medium to the original well plate containing HEK-293 cells according to manufacturer's direction.

Luciferase measurements were recorded using the Synergy HTX MultiMode luminometer (BioTek Instruments, Winooski, VT, USA) with a channel gain of 135. Each assay included ten replicate wells per group (methylated plasmid, mock methylated plasmid, and no DNA control) and was repeated in two independent experiments. Signals from the two plasmid groups (in both Lucia and *Renilla* plates) were normalized to their respective background control wells within each plate before the final Lucia/*Renilla* value being calculated per well.

2.3.8 PLB-985 Culture and Differentiation

PLB-985 are a myeloblast cell line and a subclone of the HL-60 acute myeloid leukemia cell line that can be induced to differentiate into a neutrophil-like form *in vitro* using dimethyl sulfoxide (DMSO)(315, 316). PLB-985 cells (ACC139, LOT:10 20.12.2019) were obtained from the Leibniz-Institut DSMZ (Braunschweig, Germany). PLB cells were cultured in RPMI 1640 (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum, 100U/mL penicillin, 100ug/mL streptomycin, and 1% 200mM L-glutamine solution (ThermoFisher Scientific, Waltham, MA, USA). Cells were seeded in T25 flasks at a density of 0.5E6 - 0.8E6 cells/mL at 5% CO_2/37℃ and split every 3 days. PLB-985 cells were differentiated by the addition of 1.3% DMSO solution to culture medium for three to six days depending on the required assay at a starting density of 1E6 cells/mL. Differentiation media were

replaced with fresh media on day three for cells differentiated over six days. Differentiation was monitored by CD11b expression measured using flow cytometry with anti-human CD11b-PECy7 (ICRF44; Biolegend, San Diego, CA, USA).

2.3.9 TLR7 Stimulation of PLB-985 cells

We wanted to identify gene expression changes in PLB-985 cells in response to TLR7 stimulation. PLB-985 cells were seeded at a density of 2.5E6 cells/mL with the addition of 1.3% DMSO and incubated for 4 days. In addition, one well received 5ug/mL of imiquimod (InvivoGen, San Diego, CA, USA) at day 0. Each group included two replicate wells. Dead cells were removed using the Dead Cell Removal Kit (Miltenyi Biotec, Westphalia, Germany) according to manufacturer's direction. Cells were lysed in TRIzol Reagent (ThermoFisher Scientific, Waltham, MA, USA) and frozen at -80℃. RNA was isolated using the Direct-zol Miniprep Kit (Zymo Research, Irvine, CA, USA). RNA concentration was measured using the Qubit Broad Range Assay (ThermoFisher Scientific, Waltham, MA, USA).

We used the nCounter Myeloid Innate Immunity Panel (NanoString, Seattle, WA, USA) to measure expression of 770 myeloid-relevant genes. RNA samples were hybridized to arrays at the University of Pittsburgh Genomics Research Core. Sample quality control and data analysis was done using the nSolver Analysis Software (v4.0) using the NS_HS_MYELOID v2.0 codeset (NanoString, Seattle, WA, USA). All samples passed recommended software default quality control for array imaging, probe binding, and internal positive control probe signal. Sample expression signal data passed recommended software default quality control for positive internal control probe detection. Array background signal was compensated for by setting the minimum signal threshold to two standard deviations from the average of the internal negative control

probes and adjusting any probe signal within that range to the threshold. Sample normalization was performed by first normalizing to internal positive control probes to adjust for technical variation, then adjusting for a set of internal housekeeping genes within each sample to adjust for sample input variation.

2.3.10 TREML4 *Transfection of PLB-985 Cells*

We wanted to explore the role of *TREML4* overexpression in neutrophil-like PLB-985 cells receiving TLR7 stimulation. A made-to-order *TREML4* open reading frame clone (RC211020; OriGene, Rockville, MD, USA) was inserted into a pCMV6-A-GFP backbone (PS100026; OriGene, Rockville, MD, USA) under the control of a CMV6 promoter. An empty backbone plasmid was generated to act as a control using restriction digest with MluI-HF and AsiSI-HF enzymes (New England Biolabs, Ipswich, MA, USA) followed by blunting and T4 ligation. 100ng of plasmid was used to transform NEB 10-beta competent *E. coli* (New England Biolabs, Ipswich, MA, USA) according to manufacturer's direction. Plasmid was isolated from bacterial cells using the EndoFree Maxi kit (Qiagen, Germantown, MD, USA).

Plasmid was inserted into PLB-985 cells using electroporation. Each transfection consisted of 5E6 PLB-985 cells cultured with 1.3% DMSO for 3 days to induce differentiation. Differentiated cells were combined with 100uL of nucleofection solution and 0.2ug of either *TREML4* or empty CMV6 plasmid. A mock transfection using water was included as a control. Transfection of PLB-985 cells was performed using the Nucleofector IIb device (Lonza, Basel, Switzerland) using the A-20 program. Cells were then transferred to 2mL of prewarmed culture media and allowed to incubate for 6 hours then washed in fresh culture media and allowed to incubate for an additional 13 hours. Cells were lysed in TRIzol Reagent (ThermoFisher

Scientific, Waltham, MA, USA) and frozen at -80°C. RNA was isolated using the Direct-zol Miniprep Kit (Zymo Research, Irvine, CA, USA). RNA concentration was measured using the Qubit Broad Range Assay (ThermoFisher Scientific, Waltham, MA, USA). Each experiment consisted of PLB-985 cells cultured in 1.3% DMSO for 3 days then transfected with the CMV6-*TREML4* plasmid or an empty backbone. One well in each group was treated with 5ug/mL of imiquimod for 6 hours prior to lysis. These experiments were repeated four independent times. 1ug of RNA was used as input to generate cDNA using the Verso cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, US) with RT Enhancer to remove any contaminating double-stranded DNA. We measured the expression of *CCL2* (Forward: 5'-CAGCCAGATGCAATCAATGCC-3'; Reverse: 5'-TGGAATCCTGAACCCACTTCT-3'), *IL1B* (Forward: 5'-CCACAGACCTTCCAGGAGAATG-3'; Reverse: 5'-GTGCAGTTCAGTGATCGTACAGG-3'), *TREML4* (Forward: 5'-GAAGTCTCATTACACAATCTGG-3'; Reverse: 5'-GAAGAGTCCACATAGGAGAC-3'), and *ACTB* expression (Forward: 5'- GTCAGGCAGCTCGTAGCTCT-3'; Reverse: 5'-GCCATGTACGTTGCTATCCA-3') using a primer concentration of 10uM and the PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer's direction. Each sample included four technical replicates per plate. Delta Cq values for each sample were calculated by subtracting the average beta actin Cq value from the average Cq values for each gene from. Delta-delta Cq values (ddCq) for each treatment sample were then calculated by subtracting the delta Cq of the control sample (differentiated PLB-985 cells mock transfected without DNA or imiquimod) from the delta Cq of each respective sample. DdCq values were used for statistical testing and transformed 2^{-ddCq} values for figures.

2.3.11 NETosis Assay

We used immunofluorescence microscopy to evaluate the ability of PLB-985 cells to undergo NETosis. PLB-985 cells were differentiated in 1.3% DMSO for six days. This time point was chosen based on a prior study of NETosis in PLB-985 cells(317). 1E5 cells were seeded onto a poly-L-lysine coated coverslip and allowed to adhere for 20 minutes. Adhered cells were incubated with either 200nM or 400nM of phorbol myristate acetate (PMA) diluted in RPMI 1640, 200nM L-glutamine, and 1% FBS serum for 5 hours. Cells were incubated in media lacking PMA as a treatment control. Cells were fixed with 4% paraformaldehyde for 10 minutes then blocked overnight in PBS with 10% FBS (blocking buffer). Fixed cells were stained with anti-human neutrophil elastase rabbit polyclonal antibody diluted 1:100 in blocking buffer (Millipore Sigma, Burlington, MA, USA), followed by staining with anti-rabbit Goat F(ab')2 IgG FITC-conjugated secondary antibody (Southern Biotech, Birmingham, AL, USA) diluted 1:200 in blocking buffer. DNA was stained using Hoescht 33342 diluted 1:500 in blocking buffer. Immunofluorescence micrographs were taken using the STELLARIS 5 confocal microscope (Leica Microsystems, Wetzlar, Germany) using a 60x oil immersion lens.

2.3.12 Statistical Analysis

We used probe-wise linear regressions to detect CpG sites in our cohort that show methylation difference between African-American and European-American patients using the *limma* (v.3.42.2) package(318). Patient age and the top four medication components were adjusted for in each regression and an empirical Bayes moderated t-statistic and P-value calculated for each probe. CpG sites were significantly differentially methylated if they had a Benjamini-Hochberg FDR-adjusted P-value < 0.05 and were differentially methylated by at least 10% between African-American and European-American patients.

Methylation M-values from the initial time point samples (n = 53), sample genotypes (n = 53), sample age, the top four medication components, and top ten genotype principal components were used to build a linear model for detecting meQTL using *MatrixEQTL* (v.2.3) in R(319). *Cis*-meQTL were defined as CpG sites with methylation values associated with a SNP within a conservative 1000bp of the CpG dinucleotide. We used a Benjamini-Hochberg FDR-adjusted P-value cutoff of < 0.05 for significant associations.

Analysis of the association with SLEDAI score and DNA methylation in our longitudinal cohort (n = 93 African-American & 136 European-American patient samples) was performed by fitting a linear mixed model using the *lmerTest* (v.3.1-2)(320) and *MuMIn* (v.1.43.17) packages in R(321). A regression model was fit in a probe-wise manner for all samples in each ancestry group to allow detection of ancestry-associated sites. Regression models were adjusted for age at sample collection as a fixed effect and SLEDAI score as the variable of interest. Repeated samples were grouped by patient which was accounted for as a random effect in the model. CpG methylation and SLEDAI score had a statistically significant association if they had a Benjamini-Hochberg FDR-adjusted P-value < 0.05 and a suggestive association with a Benjamini-Hochberg FDR-adjusted P-value < 0.10. The impact of adjusting for medication components was determined by comparing the fit of the previously specified mixed effect regression model with an extended model that includes additional fixed effects for the top four medication components of each lupus patient's timepoint. A chi-square difference test for nested models was applied using the *anova* function in R to determine if model fit was improved. A P-value < 0.05 was considered statistically significant and indicates that the larger model has improved data fit.

Longitudinal analysis of nephritis in our cohort was performed by fitting a linear mixed model as above to each probe using methylation profiles for n = 11 lupus patients (n = 7 African-

American and n = 4 European-American) at a timepoint with active nephritis (as defined by SLEDAI) and the nearest preceding or receding timepoint without nephritis after adjusting for the top four medication components, age, and ancestry as fixed effects. Sample pairs were included as a random effect. A Benjamini-Hochberg FDR-adjusted P-value threshold of < 0.05 was used to identify statistically significant associations.

Two-group testing of mean ages between ancestry groups was done using a t-test. SLEDAI criteria and medication differences were compared using Fisher's exact test. Comparing allelic proportions between ancestry groups was done using a two-proportion z-test. All P-values were two-tailed and a significance threshold of P-value < 0.05 was used.

An unpaired *t* test with Welch's correction was performed to evaluate the difference in background-corrected Lucia luciferase signal normalized to background-corrected *Renilla* luciferase signal between *in vitro* methylated and mock methylated *TREML4* promoter pCpGfree plasmids. Results were considered significant if the P-value < 0.05. An unpaired *t* test was used to compare gene-wise expression counts between differentiated PLB-985 cells treated with imiquimod and untreated control cells. P-value adjustment for multiple testing was done using the Benjamini-Yekutieli false discovery rate method. Statistical testing was performed using the nSolver Analysis Software (v4.0) with the NS_HS_MYELOID v2.0 codeset (NanoString, Seattle, WA, USA). A paired *t* test was used to compare the average ddCq expression of *TREML4* normalized to a mock transfected control between differentiated PLB-985 cells transfected with a *TREML4* expression plasmid and the empty control plasmid. For repeated measures, one-way ANOVA with Geisser-Greenhouse correction was used to compare average ddCq expression of *IL1B* and *CCL2* in differentiated PLB-985 cells transfected with either a *TREML4* expression plasmid or the empty control plasmid and treated with imiquimod compared

to untreated cells. Comparisons between imiquimod treated and untreated controls were made within each plasmid group and between plasmid groups receiving imiquimod treatment. P-value correction for multiple comparisons was performed using Sidak's multiple comparison testing correction. Results were considered significant if the P-value < 0.05. All statistical testing for *TREML4* promoter methylation and *TREML4*, *IL1B*, and *CCL2* expression experiments were performed using Prism (v9.3.0) (GraphPad Software, San Diego, CA, USA).

2.4 Results

2.4.1 Longitudinal analysis of DNA methylation over time and across disease activity levels in lupus patients

The Infinium MethylationEPIC array measures the methylation status of 866,836 methylation sites across the genome including 863,904 CpG and 2932 CNG sites (C, cytosine; N, any nucleotide; G, guanine)(322). After QC and technical probe masking, a total of 745,477 (86.0%) sites were retained for analysis. Heterogeneity in disease manifestations, patient genetic background, and the environment are all factors that complicate the understanding of lupus pathogenesis. Using repeated sampling of lupus patients followed longitudinally, we can account for these factors and detect novel changes in DNA methylation that are associated with disease activity over time. We followed a total of 54 lupus patients for up to 43 months and assessed genome-wide DNA methylation levels in neutrophils in a total of 229 patient samples. Our cohort included 22 African-American and 32 European-American lupus patients followed across 93 and 136 timepoints, respectively. We assessed correlation between DNA methylation changes in individual methylation sites across the genome with disease activity as measured by Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores in each ancestry group. After

removing CpG-SNP probes with a minor allele frequency > 1% to avoid a bias due to intra-ancestral allele frequency differences, we analyzed a total of 733,192 (84.6%) methylation sites. In the African-American cohort we identified a total of eight CpG sites that met our suggestive FDR-adjusted P-value < 0.1 (Figure 2-1A, Table 2-1). Two sites cg26104306 (*SNX18*; FDR-adjusted P-value = 3.38E-02) and cg06708913 (FDR-adjusted P-value = 3.43E-02) were significantly associated with changing disease activity levels in our cohort (Figure 2-1B and 2-1C).

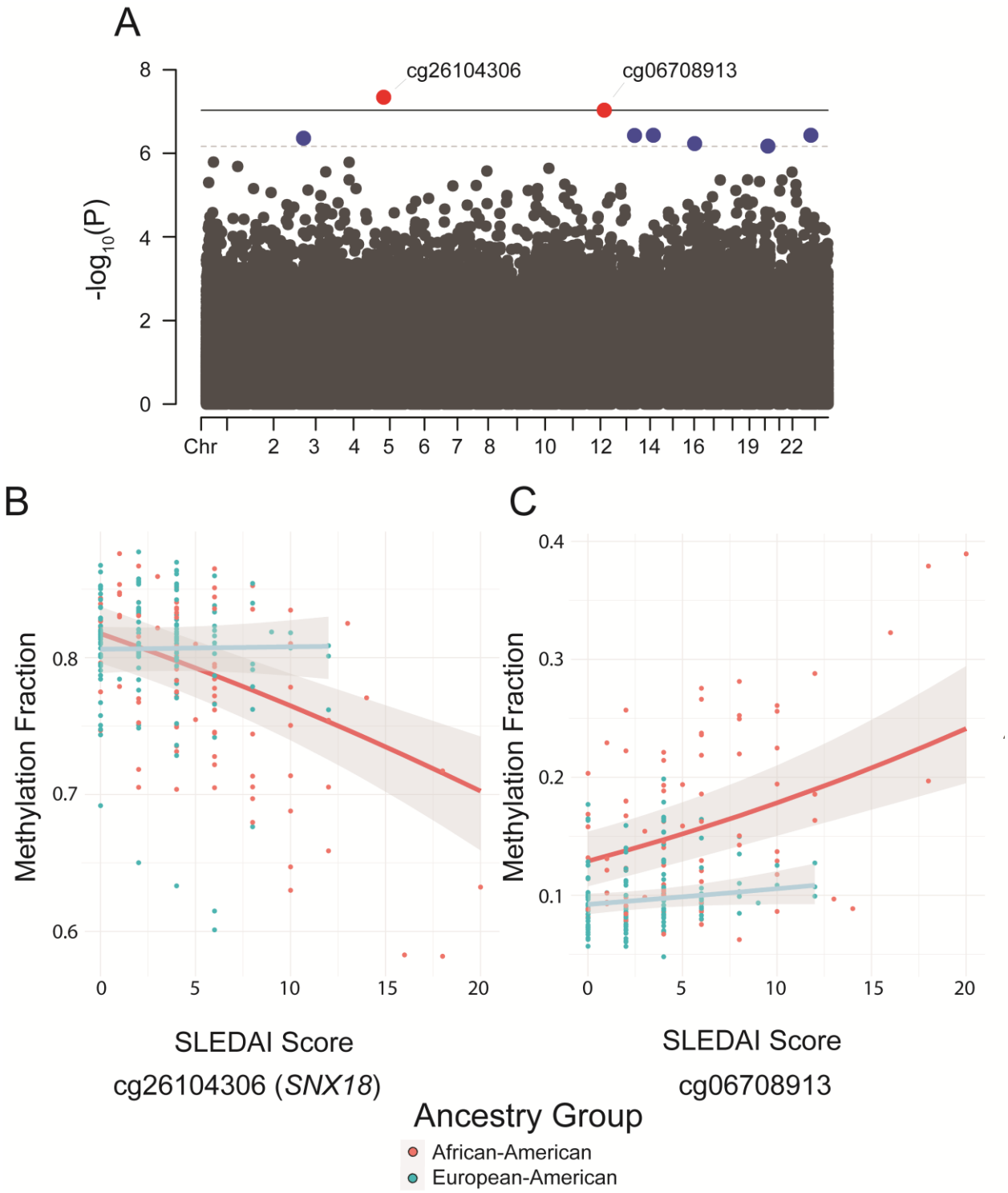

Figure 2-1: The relationship between DNA methylation changes and disease activity in a longitudinal cohort of lupus patients. (A) A Manhattan plot depicting the significance of correlation between methylation levels of CpG sites and disease activity as measured using SLEDAI scores in African-American lupus patients (n = 93 samples). The red dots are CpG sites that meet the threshold for significance of FDR-adjusted P-value < 0.05 (bold line) and the blue dots are CpG sites that meet the suggestive threshold of FDR-adjusted P-value < 0.10 (dashed line). (B) Methylation status of cg26104306 (P = 4.61E-08; FDR-adjusted P = 3.38E-02) and (C) cg08708913 (P = 9.35E-08; FDR-adjusted P = 3.43E-02) across SLEDAI scores for African-American (n = 93 samples; red dots/line) and European-American (n = 136 samples; blue dots/line) lupus patients.

Table 2-1: Correlation of DNA methylation and disease activity in a cohort of African-American lupus patients (n = 22) across 93 samples after adjusting for age using a mixed effects model.

African-American Patients (n = 22; 93 timepoints)						
Probe ID	**Genes**	**Location (hg38)**	**SLEDAI Score Coefficient**	**F (Satterthwaite)[#]**	**P-value**	**FDR-adjusted P-Values[*]**
cg26104306	*SNX18*	chr5:54517014	-0.046	36.57	4.61E-08	**3.38E-02**
cg06708913	-	chr12:89880778	0.055	34.77	9.35E-08	**3.43E-02**
cg24682077	*FGD1*	chrX:54496205	-0.026	30.33	3.72E-07	5.35E-02
cg15563677	*ELMSAN1;RP5-1021I20.1*	chr14:73788514	-0.039	31.15	3.73E-07	5.35E-02
cg26138978	*EFNB2*	chr13:106530743	-0.038	30.14	3.77E-07	5.35E-02
cg17038326	-	chr3:27614709	-0.049	30.64	4.38E-07	5.35E-02
cg22284518	-	chr16:49351267	-0.016	29.63	5.90E-07	6.18E-02
cg00465267	*NFATC2*	chr20:51497407	0.038	29.28	6.76E-07	6.19E-02

[#]F-value (Satterthwaite) is the F-value for the F-test conducted by lmerTest using the Satterthwaite method for denominator degrees of freedom.
[*]All sites represented here met our suggestive significance threshold of $P < 0.10$ and bolded P-values meet our significance threshold of $P < 0.05$.

Cg26104306 shows stark demethylation with increasing disease activity compared to our European-American patients which showed very little methylation change across time and disease activity. Similarly, cg06708913 shows a much higher rate of increasing methylation with disease activity in African-American patients relative to European-American patients. The inclusion of the top four medication components as fixed effects did not improve the fit of our model for these two CpG sites (cg26104306 Chi-square P-value = 0.25 and cg06708913 Chi-square P-value = 0.83). Our European-American sample cohort analysis did not identify any CpG-SLEDAI score associations at either P-value threshold. Importantly, these data suggest that DNA methylation patterns defining lupus patients are largely stable over time and across disease activity.

We next performed an analysis in a subset of patients who developed active lupus nephritis at any time point during our study and in whom a sample from at least one time point without evidence of lupus nephritis is available. After adjusting for medications, age, and ancestry we identified a single CpG site with a statistically significant relationship between DNA methylation levels and active nephritis in n = 11 lupus patients. DNA methylation levels in cg16204559, which is located within the gene *GALNT18*, are significantly reduced during active nephritis in lupus patients (Figure 2-2) (see Table A-1: “Supplementary Table 1”).

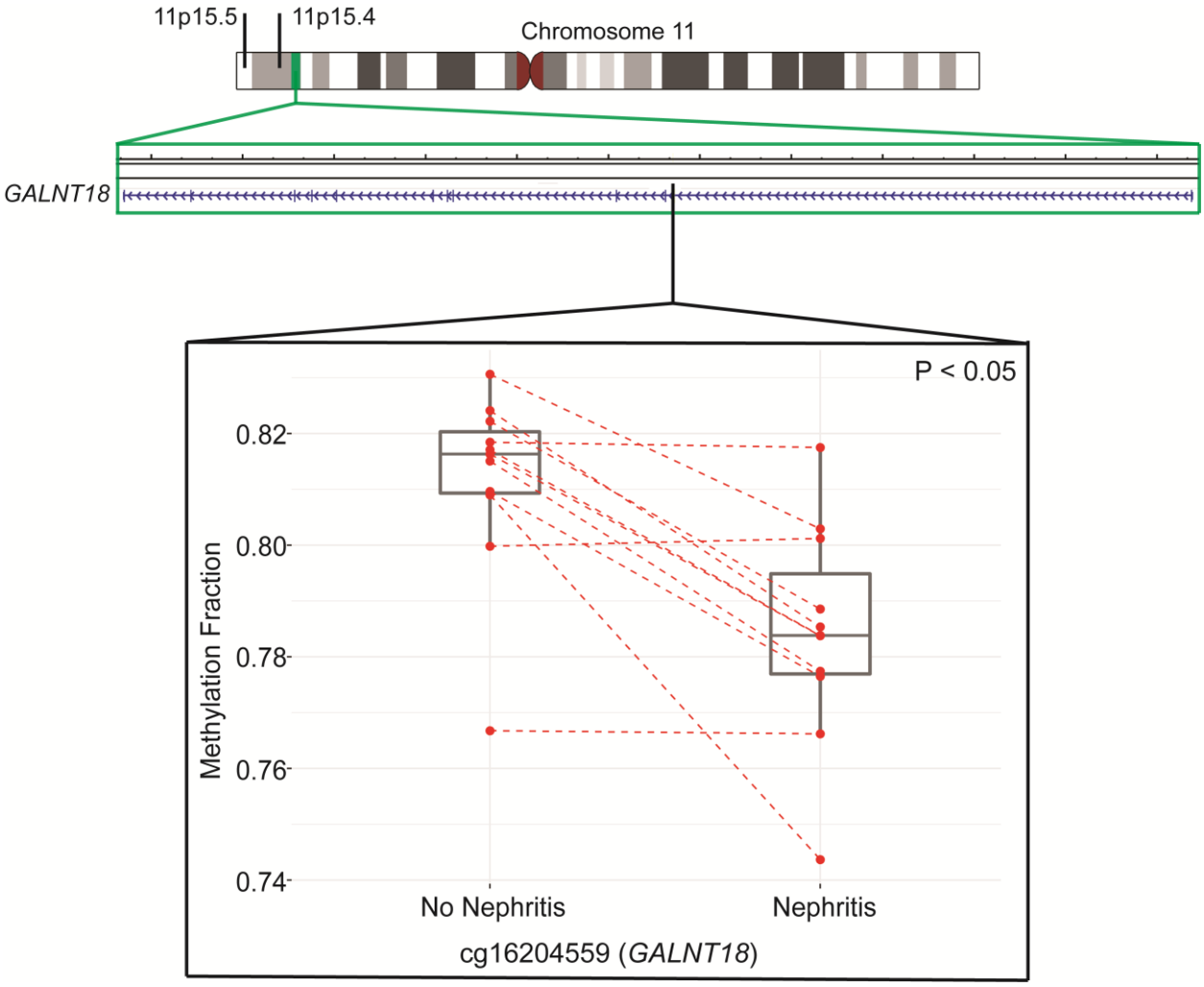

Figure 2-2: Cg16204559 (*GALNT18*) is hypomethylated upon development of lupus nephritis. Ideogram of chromosome 11 showing the location of 11p15.5 and 11p15.4 cytobands. Cg16204559 (black line) is within the body of *GALNT18* located in the 11p15.4 region (green box). Methylation profiles for n = 11 lupus patients (red dots (n = 7 African-American and n = 4 European-American) at a timepoint with active nephritis and the nearest preceding or receding timepoint without nephritis were compared after adjusting for medications, age, and ancestry group using a linear mixed effects model. Cg16204559 (*GALNT18*) was significantly demethylated (FDR-adjusted P-value = 0.048) with the occurrence of nephritis. Mean β Nephritis: 78.4% (25^{th} Percentile: 80.9%, 75^{th} Percentile: 82.0%) and Mean β Non-nephritis: 81.2% (25^{th} Percentile: 77.7%, 75^{th} Percentile: 79.5%). Whiskers extend to the maximum value within 1.5 times the interquartile range on either end of the group. Points beyond the whiskers are outliers.

2.4.2 DNA methylation differences in neutrophils of African-American and European-American lupus patients

We then performed a differential DNA methylation analysis comparing lupus patients with African-American (n = 22) and European-American (n = 32) ancestry after adjusting for medication use and age. A multidimensional plot of the 5000 most variable CpG sites in these patients showed that methylation patterns tended to cluster by patient ancestry group (Figure 2-3A). African-American lupus patients in our cohort had more active disease compared to European-American lupus patients (SLEDAI 5.2±4.5 vs 2.9±3.2, respectively; P-value (t-test) = 0.03). Medication use at the initial timepoint were not significantly different between the ancestry groups. Alopecia was the only SLEDAI criteria showing a significant difference in frequency between African-American and European-American patients (18.2% vs. 0%, respectively; P-value (Fisher's exact test) = 0.02) (see Table B-1). We identified 907 differentially methylated CpG sites using an FDR-adjusted P-value threshold of < 0.05 and a differential methylation between ancestry groups of at least 10% (Figure 2-3B) (see Table A-1: "Supplementary Table 3").

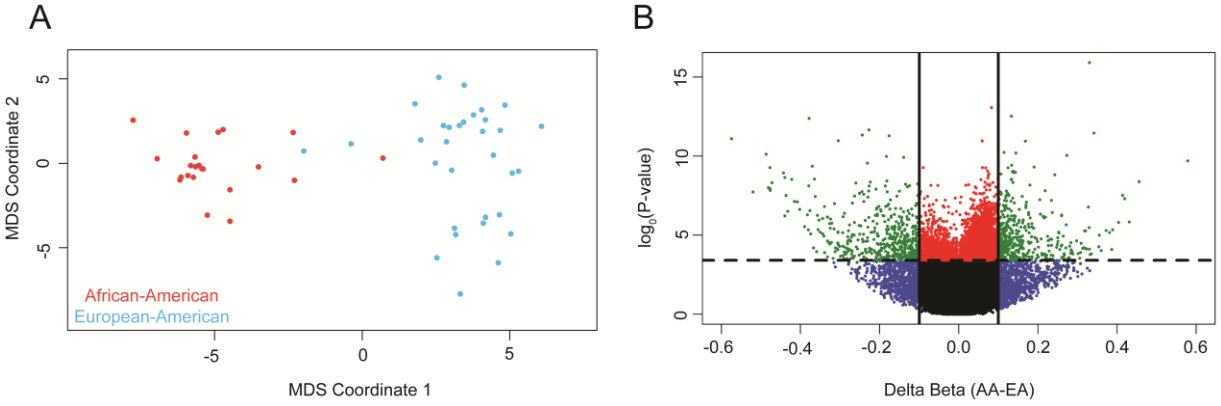

Figure 2-3: Neutrophils of African-American and European-American lupus patients show DNA methylation differences associated with ancestry. (A) Multidimensional scaling plot of top 5000 most variable CpG sites in African-American (n = 22; green circles) and European-American (n = 32; orange circles) lupus patients at initial sample collection. (B) Volcano plot of differentially methylated CpG sites between African-American (n = 22) and European-American (n = 32) lupus patients at initial sample collection. Each dot represents a CpG site (n = 745,477). Significantly differentially methylated sites (green) are differentially methylated by at least 10% between ancestry groups and with an FDR-adjusted P-value < 0.05 (n = 907).

487 (53.7%) of these sites were hypomethylated in African-American compared to European-American lupus patients and 420 (46.3%) were hypermethylated (Figure 2-4A).

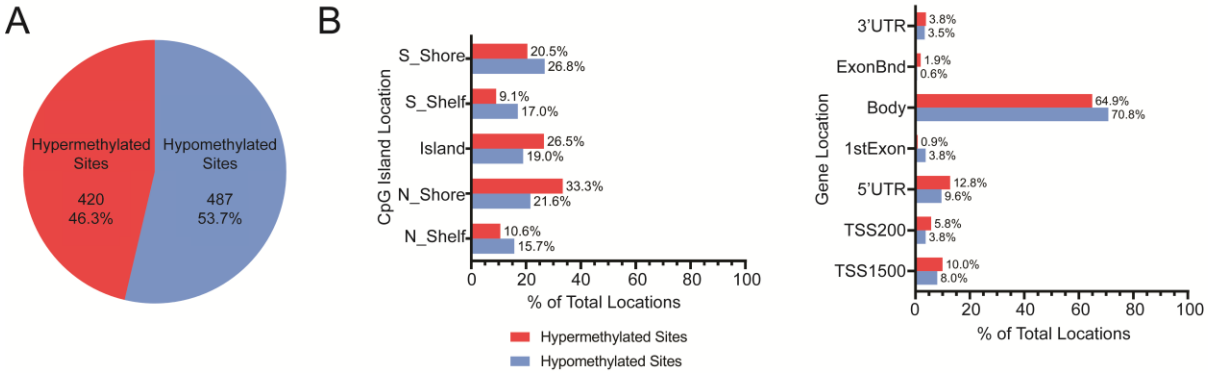

Figure 2-4: Differential Methylation of Neutrophils in African-American and European-American lupus patients. (A) Pie chart (left) showing the percent of sites hypermethylated (n = 420 (46.3%)) and hypomethylated (n = 487 (53.7%)) in African-American lupus patients compared to European-American controls. (B) Barcharts showing the distribution of hypermethylated (red) and hypomethylated (blue) sites annotated to locations with CpG islands and genes (middle and left, respectively). S_Shore: South Shore; S_Shelf: South Shelf; N_Shore: North Shore; N_Shelf: North Shelf. 3'UTR: 3' Untranslated Region; ExonBnd: Exon Boundary; 5'UTR: 5' Untranslated Region; TSS200: 200bp upstream of Transcription Start Site; TSS1500: 1500bp upstream of Transcription Start Site.

Hypomethylated and hypermethylated sites showed similar distributions across CpG island and gene locations (Figure 2-4B).

DNA methylation levels among differentially methylated sites differed by 16.5% on average (SD:8.2%; range:10.0% - 57.9%) between ancestry groups. The hypomethylated and hypermethylated sites were associated with 391 and 316 genes, respectively. Hypomethylated genes showed enrichment for gene ontologies for granulocyte differentiation (GO:0030852; FDR-adjusted P-value = 2.23E-02 & GO:0030853; FDR-adjusted P-value = 3.20E-02), cell adhesion (GO:0007155; FDR-adjusted P-value = 1.26E-02 & GO:1903037; FDR-adjusted P-value = 3.41E-02), and TLR signaling pathways (GO:0002224; FDR-adjusted P-value = 3.41E-02 & GO:0034121; FDR-adjusted P-value = 4.46E-02) (see Table A-1: "Supplementary Table

4”). Hypermethylated genes were enriched for fewer ontologies representing primarily Rho guanine nucleotide exchange factor (GEF) protein activity (GO:0005089; FDR-adjusted P-value = 7.76E-03) and ATP binding (GO:0005524; FDR-adjusted P-value = 2.80E-02) (see Table A-1: “Supplementary Table 5”).

To estimate the proportion of CpG sites differentially methylated between African-American and European-American lupus patients that are influenced by ancestral genetic differences, we performed differential DNA methylation analysis comparing neutrophils isolated from normal healthy African-American and European-American controls, using publicly available DNA methylation data generated using the Illumina HumanMethylation450 array. Of 437 sites differentially methylated between African-American and European-American lupus patients that were included on the HumanMethylation450 array, 185 CpG sites (42.3%) were also differentially methylated between African-American and European-American healthy controls (see Table A-1: “Supplementary Table 6”). Indeed, among these methylation sites, DNA methylation differences between the two ancestries in controls and lupus patients showed a high degree of correlation (r = 0.872; Pearson's product-moment correlation t = 24.077, DF = 183, P-value < 2.2E-16). These data indicate that at least a proportion of differential methylation identified between African-Americans and European-American lupus patients can be explained by differences in the ancestral genetic background between the populations.

2.4.3 Methylation quantitative trait loci (meQTL) analysis

We next identified associations between DNA methylation and genotype in our cross-sectional cohort of lupus patients (n = 53) after controlling for age, medications, and genetic

background. *Cis*-meQTL in our cohort were defined using a conservative range of 1000bp to focus on localized effects (Figure 2-5) (see Table A-1: "Supplementary Table 7").

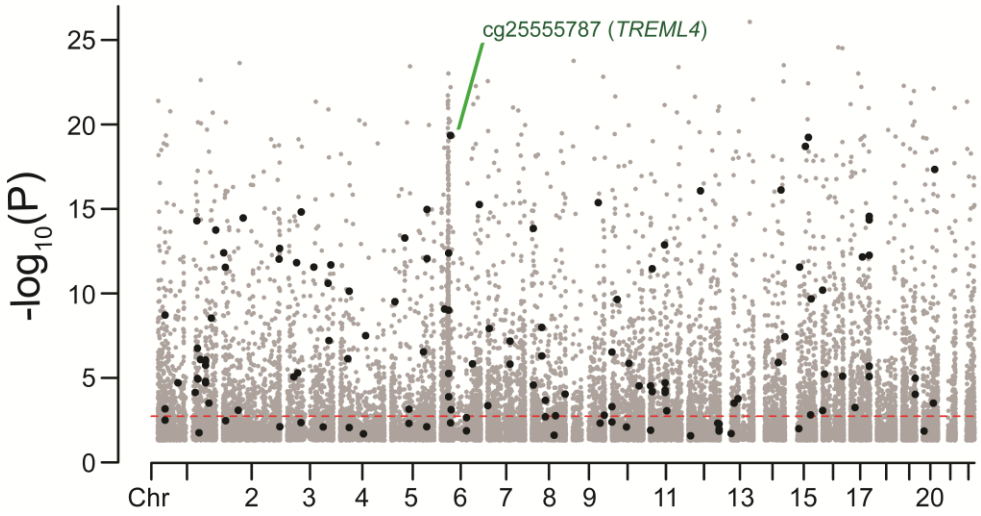

Figure 2-5: Methylation quantitative trait loci (*cis*-meQTL) analysis in lupus neutrophils. A Manhattan plot showing CpG sites (black and gray dots) in *cis*-meQTL pairs identified in our lupus cohort. Black dots represent CpG sites in non–CpG-SNP *cis*-meQTL pairs that had a significantly different average methylation between African American and European American patients (FDR-adjusted $P < 0.05$). The red dashed line represents an approximate FDR-adjusted *P* value threshold of 0.05 for all *cis*-meQTL across the entire genome. An meQTL associated with *TREML4* was among the most significant meQTL effects detected.

We identified a total of 8855 pairs of CpG sites and SNPs with an FDR-adjusted P-value < 0.05. These meQTL pairs represented 7614 (86.0%) unique methylation sites and 7094 (80.1%) unique polymorphisms. 7269 (82.1%) of meQTL did not contain CpG-SNPs. Functional enrichment analysis of the 3871 unique genes associated with the CpG sites revealed numerous ontologies and pathways. The most significantly enriched included ontologies and pathways for cell and biological adhesion (GO:0007155; FDR-adjusted P-value = 6.43E-20, GO:0022610; FDR-adjusted P-value = 6.43E-20, & KEGG:83069; FDR-adjusted P-value = 5.29E-04) and calcium ion binding and signaling pathways (GO:0005509; FDR-adjusted P-value = 1.46E-07 &

KEGG:83050; FDR-adjusted P-value = 9.58E-04) (Figure 2-6) (see Table A-1: "Supplementary Table 8").

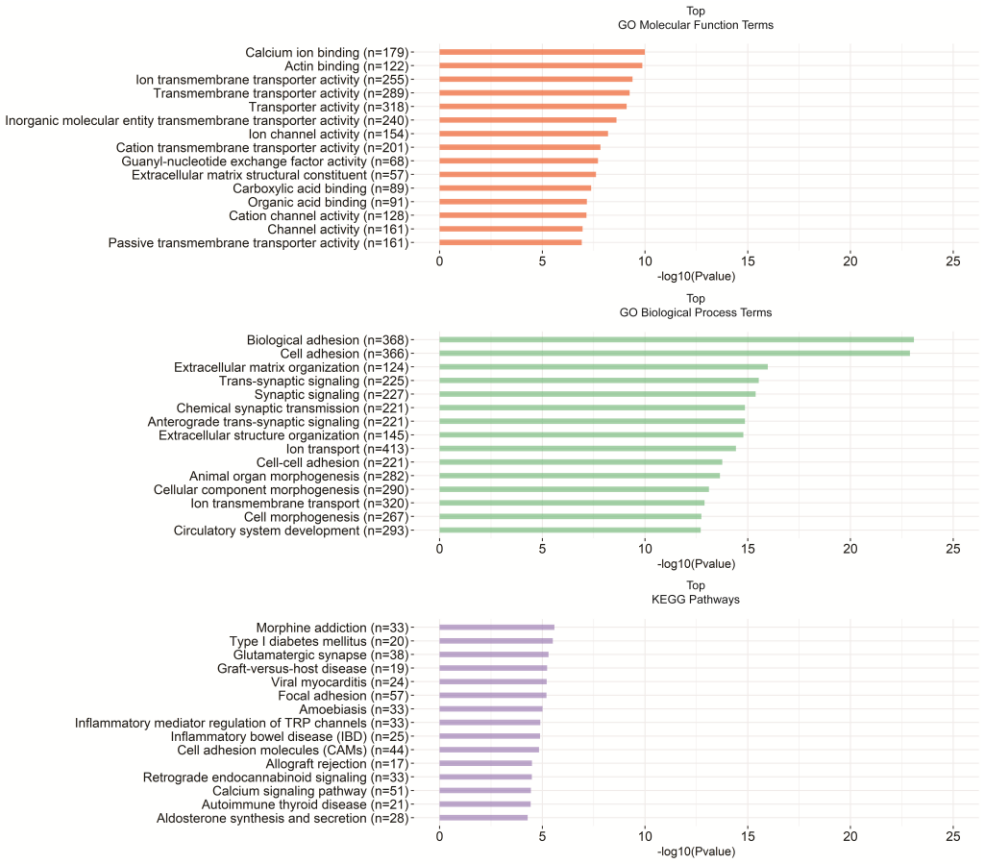

Figure 2-6: Enrichment of gene ontologies and pathways among annotated genes associated with CpG sites with *cis*-meQTL effects in lupus neutrophils. Barcharts show the most significant molecular function (orange) and biological process (green) gene ontology terms, and KEGG pathways (purple) by $-\log_{10}$ (*P* value). All terms have an FDR-adjusted $P < 0.05$.

The meQTL revealed in our study are, at least in part, responsible for a proportion of the observed DNA methylation differences between African-American and European-American patients. Indeed, of the 907 differentially methylated CpG sites in our cohort, 142 (15.7%) were also meQTL (see Table A-1: "Supplementary Table 9"). These included sites associated with *IL16* (cg02810829; $\Delta\beta = -0.23$) and an meQTL associated with the triggering receptor expressed on myeloid cells (TREM)-like 4 gene *TREML4* (cg25555787; $\Delta\beta = -0.20$). Cg25555787 had one of the strongest meQTL associations in this study (rs9369265 meQTL $r^2 = 0.91$) (Figure 2-7).

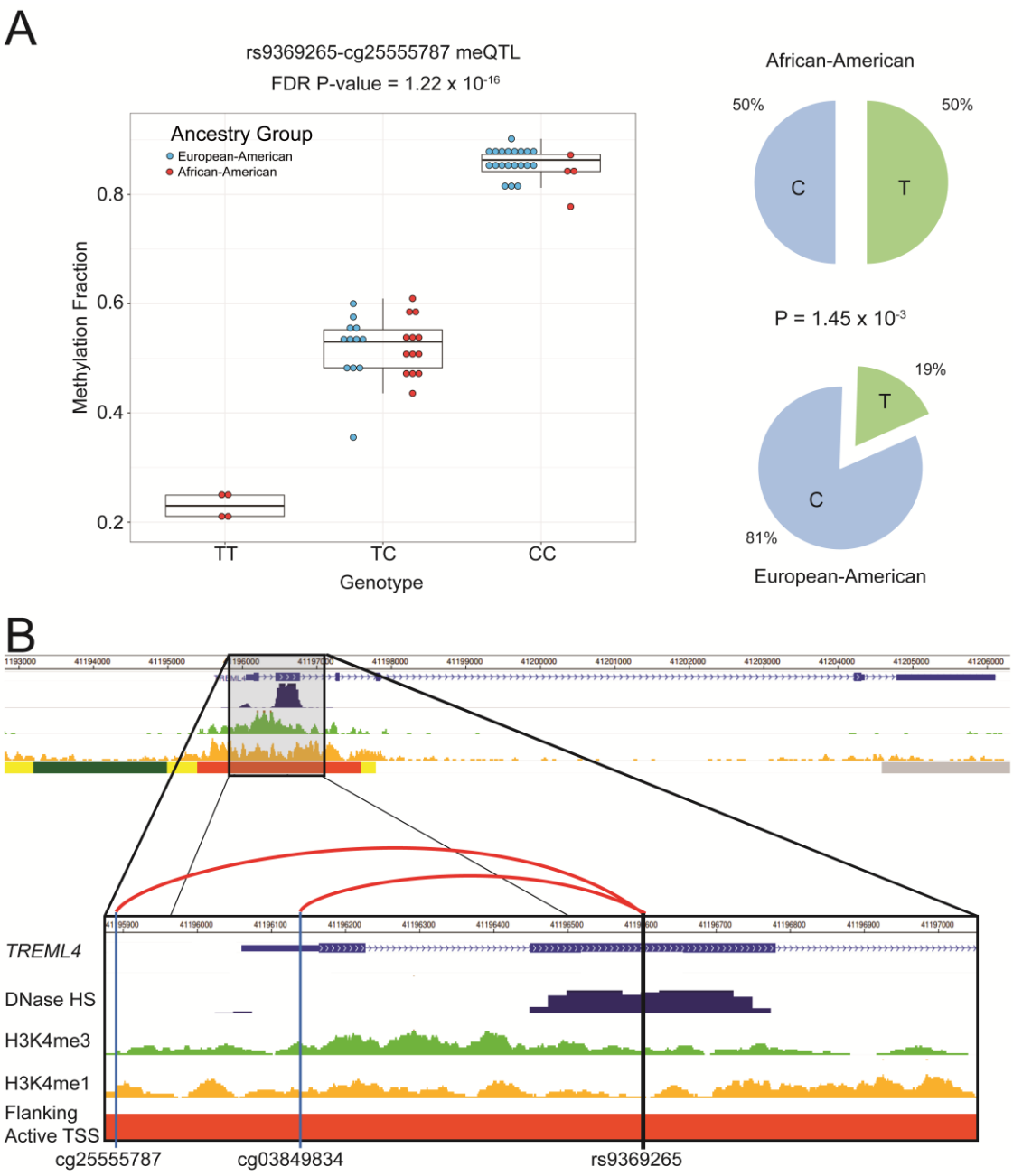

Figure 2-7: MeQTL involving the SNP rs9369265 within *TREML4* in lupus patients. (A) Rs9369265 is significantly associated with the methylation status of cg25555787 (FDR-adjusted *P* value = 1.22×10^{-16}). Mean β for genotype TT = 23% (25th percentile = 21.2%, 75th percentile = 25.0%, *n* = 4), mean β for genotype CT = 52.0% (25th percentile = 48.3%, 75th percentile = 55.2%, *n* = 25), and mean β for genotype CC = 85.6% (25th percentile = 84.2%, 75th percentile = 87.3%, *n* = 24). Whiskers extend to the maximum value within 1.5 times the IQR on either end of the group. Points beyond the whiskers are outliers. The minor allele frequency of rs9369265 significantly differed between European American (*n* = 32) and African American (*n* = 21) lupus patients ($P = 1.45 \times 10^{-3}$), with the T allele associated with lower DNA methylation. Comparing allelic proportions between ancestry groups was done using a 2-proportion *z* test. All *P* values were 2 tailed, and a significance threshold of $P < 0.05$ was used. (B) Rs9369265 is an exonic SNP in *TREML4* and is significantly associated with the methylation status of 2 CpG sites upstream of the transcription start site of *TREML4* (cg25555787 and cg03849834) (hg19). This region has epigenetic marks including DNase hypersensitivity (DNase HS), histone 3 lysine 4 mono- (H3K4me1) and –tri-methylation (H3K4me3) and is labeled as an enhancer region for *TREML4* (Flanking Active TSS; orange bar) in primary human neutrophils. Data for **B** were generated using the WashU Epigenome Browser (https://epigenomegateway.wustl.edu/) using ENCODE and Epigenome Roadmap ChromHMM data tracks from peripheral primary human neutrophils (E030).

We identified 1586 (17.9%) meQTL that were tagged as including CpG-SNPs and comprised many of the strongest methylation-genotype associations (see Table A-1: "Supplementary Table 7").

2.4.4 The contribution of methylation quantitative trait loci to differential methylation, genetic susceptibility, and the type I interferon response in lupus

We compared the CpG sites associated with the meQTL pairs to our previously published data on differential DNA methylation between lupus patients and age-, sex-, and ethnicity-matched healthy controls(275). Twenty-two of the 273 (8.06%) differentially methylated sites we previously identified in the neutrophils of lupus patients and controls were associated with an meQTL in the neutrophils of lupus patients. Comparing methylation site-associated genes in meQTL pairs with previously identified lupus susceptibility loci from GWAS(59, 323-326), we identified 79 meQTL pairs (8.7%) in 28 lupus susceptibility genes (see Table A-1: "Supplementary Table 10"). These included interferon regulatory factors *IRF7* and *IRF8* and *STAT4* which are involved in the type I interferon response. To identify type I interferon regulated genes that are associated with meQTL in our cohort, we compared our meQTL-associated genes with the genes included in the Interferome (v.2.01) database(314). 64 of the 3871 unique genes (1.7%) associated with methylation sites in meQTL were identified as type I interferon regulated genes (see Table A-1: "Supplementary Table 11").

2.4.5 **TREML4** *promoter regulation and the role of* **TREML4** *in TLR7 response in the PLB-985 neutrophil-like cell line*

We used an *in vitro* patch methylation assay to examine the role of DNA methylation in regulating gene expression in the *TREML4* promoter. *In vitro* methylation of the CpG sites in the *TREML4* promoter decreased Lucia luminescence significantly after 48 hours of culture in HEK-293 cells (replicate 1: P-value = 0.0005 & replicate 2: P-value = 0.0209) (Figure 2-8).

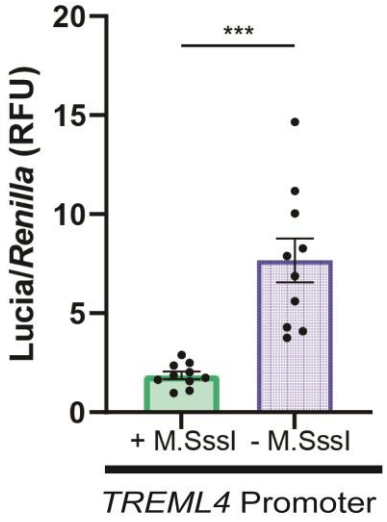

Figure 2-8: In vitro DNA methylation impairs gene expression downstream of the *TREML4* promoter. The *TREML4* promoter region was inserted upstream of a Lucia luciferase gene in a pCpGfree-Lucia backbone. The pCpGfree-Lucia backbone lacks CpG sites outside of the inserted sequence. CpG methyltransferase M.SssI was used to methylate five CpG sites in the *TREML4* promoter sequence. *In vitro* methylation reduced Lucia luciferase expression in HEK-293 cells compared to cells transfected with a mock methylated control plasmid (P-value = 0.0005). *Renilla* luciferase was included as a transfection control. Lucia luciferase and *Renilla* signals were read on separate plates and each normalized to background signal within their respective plates to control wells mock transfected without DNA. Lucia was then normalized to *Renilla* signal to account for transfection efficiency. Each group included ten replicates. This figure represents one of two independent replicated experiments using the same plasmids. Error bars represent standard error of the mean. *** = P-value ≤ 0.001. RFU: relative fluorescence units.

The role of *TREML4* in the neutrophil response to TLR7 stimulation was examined using a PLB-985 myeloblast cell line. We wanted to identify candidate genes for measuring PLB-985 cell response to TLR7 stimulation. We measured over 700 genes in DMSO-differentiated cells treated with imiquimod for four days using a myeloid immune expression panel. Two genes, *CCL2* (t = -19.3, P-value = 2.70E-3) and *IL1B* (t = -9.76, P-value = 0.03) had a significant increase in expression in imiquimod treated cells compared to untreated control cells (see Figure

C-2). These genes were chosen as reporters for TLR7 stimulation in our PLB-985 cell line in further experiments. *IL1B* encodes the proinflammatory IL-1 beta cytokine that can be produced by mature neutrophils(327) and circulating leukocytes treated with imiquimod(328). *CCL2* encodes the c-c motif chemokine ligand 2 (CCL2) which is a chemotactic cytokine that draws neutrophils to inflammatory sites and can also be produced by neutrophils when exposed to TLR agonists(329, 330)

We overexpressed *TREML4* in PLB-985 cells using a CMV6 promoter-driven plasmid. *TREML4* expression was significantly increased in transfected cells compared to cells transfected with plasmid lacking the *TREML4* open reading frame (t = 6.31, P-value = 1.00E-4) (Figure 2-9A). Expression of *IL1B* significantly increased with imiquimod treatment in differentiated PLB-985 cells carrying the empty plasmid (Adjusted P-value = 0.024), but not when compared to imiquimod-treated cells transfected with the *TREML4* expression plasmid (Figure 2-9B). *CCL2* expression showed an increasing trend of expression with imiquimod treatment in cells carrying the *TREML4* expression plasmid or the empty plasmid, but the expression difference between the imiquimod-treated *TREML4* expression plasmid and empty plasmid was not significant (Figure 2-9C).

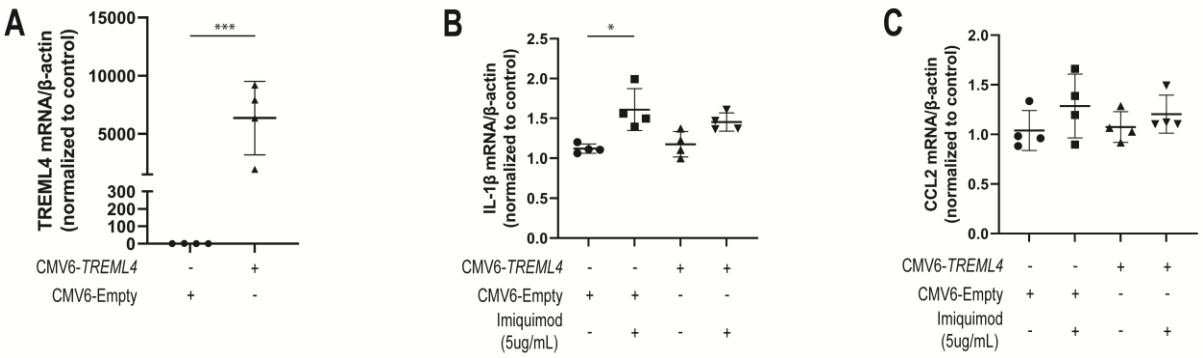

Figure 2-9: Response to TLR7 stimulation in differentiated PLB-985 cells overexpressing *TREML4*. (A) *TREML4* expression is significantly increased in differentiated PLB-985 cells transfected with a CMV6-TREML4 plasmid compared to the empty control plasmid (t = 24.76, P-value = 1.00E-4). (B) *IL1B* expression increased significantly with imiquimod treatment in cells receiving the empty plasmid (t = 6.31, Adjusted P-value = 0.024), but not in cells overexpressing *TREML4 (*t = 2.99, Adjusted P-value = 0.165) or when comparing imiquimod-treated cells transfected with the *TREML4* or empty plasmid (t =2.26, Adjusted P-value = 0.292). (C) *CCL2* expression was not significantly different with imiquimod treatment in cells transfected with the empty plasmid (t = 2.96, Adjusted P-value = 0.168), the *TREML4* expression plasmids (t = 3.03, Adjusted P-value =0.159) 0.159), or when comparing treated cells transfected with the empty or *TREML4* expression plasmid (t = 0.54, Adjusted P-value = 0.949). PLB-985 cells were differentiated for three days using 1.3% DMSO prior to transfection with specified plasmids. Imiquimod treatment was for 6 hours prior to RNA collection. Error bars represent standard deviation. n = 4 independent experimental replicates per group. * = P-value ≤ 0.05. *** = P-value ≤ 0.001.

NETosis is an important function of neutrophils and can be induced downstream of TLR signaling(331). We wanted to determine if NETosis can be induced in DMSO-differentiated PLB-985 cells as previously reported(332). PMA-induced NETosis of PLB-985 cells after six days of differentiation was unsuccessful (see Figure C-3). Nuclear morphology showed no difference between control cells and cells treated with 200nM and 400nM PMA for five hours.

2.5 Discussion

Neutrophils are the most numerous cells in circulating blood and are early responders to inflammatory events throughout the body. They play an important role in entering sites of infection to identify pathogens through a variety of receptors, destroying pathogens, and secreting inflammatory signals to mobilize the immune system in response(333). Their primary methods of destroying pathogens include phagocytosis, production of reactive oxygen species, release of granules containing antimicrobial enzymes, and the release of NETs which physically

bind and expose pathogens to antimicrobial proteins(333). In lupus, neutrophils display several abnormal phenotypes including enhanced apoptosis, increased NETosis after type I interferon priming, and impaired phagocytosis(334). Our prior work has found that lupus neutrophils display a DNA methylation signature common to other immune cell types, primarily demethylation of type I interferon response genes(275).

We interrogated the DNA methylome of neutrophils in a cohort of lupus patients followed longitudinally for about four years across 229 timepoints to assess DNA methylation changes over time and across different levels of disease activity. We showed that the DNA methylome is largely stable over time and across disease activity in lupus patients. We identified two CpG sites (cg26104306 and cg06708913) with DNA methylation levels that significantly correlated with disease activity. These correlations were detected in African-American but not European-American lupus patients. Cg26104306 lies 745bp upstream of the transcription start site of the gene *SNX18* which encodes the sorting nexin 18 protein SNX18. It is located on the 5' north shore of a CpG island (chr5:54517549-54519476 (hg38)) that overlaps the *SNX18* promoter region. Methylated CpG islands are typically indications of silenced gene promoters in somatic cells and hypomethylation suggests disease-associated disruption in this silencing. SNX18 localizes to the plasma membrane of cells and plays a functional role in endocytosis and autophagosome formation in cells(335, 336). Cg06708913 overlapped a long non-coding RNA AC009522.1 and is proximal to an enhancer-like region denoted by transcriptionally permissive DNase hypersensitivity and increased H3K27ac modifications(337). One CpG site that reached suggestive significance for correlation with disease activity in African-American lupus patients, cg24682077 (FDR-adjusted P-values = 5.35E-02), is associated with FYVE, RhoGEF And PH domain containing 1 gene *FGD1*. FGD1 interacts with Rho GTPase Cdc42 which regulates

neutrophil motility in response to extracellular signals(338). Cg24682077 is located 39bp downstream of the transcription start site of *FGD1* and within a promoter-associated CpG island.

The small number of CpG sites that change methylation levels with disease activity in our longitudinal study suggests that DNA methylation levels are stable in lupus neutrophils over time and across different disease activity levels. An inception study of lupus patients across time is necessary to detect DNA methylation biomarkers that indicate the onset of disease. A larger cohort size may bring more of these sites beyond the significance threshold or reveal novel associations in other ancestry groups. These associations will require replication to be confirmed but serve as indicators that novel disease-associated loci can be detected in longitudinal data from lupus patients. They also demonstrate that accounting for genetic ancestry in lupus studies can reveal novel associations.

Lupus nephritis is one of the most severe manifestations of lupus that can lead to chronic kidney damage and renal failure. We compared two timepoints from lupus patients with samples collected with and without nephritis in the same patient and adjusted DNA methylation changes for medication use, age, and ethnicity. A single methylation site, cg16204559, passed our FDR significance threshold corrected for multiple testing. Cg16204559 (chr11:11451256-11451258 (hg38)) is in the 11p15.4 cytoband within an intron of the gene *GALNT18* which encodes the polypeptide N-acetylgalactosaminyltransferase 18 protein. 11p15.4 is adjacent to 11p15.5 which has previously been identified as the location of the Systemic Lupus Erythematosus Nephritis 3 (*SLEN3*) locus. This locus is near the p telomere of chromosome 11 and was identified as a susceptibility locus for lupus using genetic linkage in multiplexed pedigrees of African-American ancestry that included lupus patients with nephritis(339). Understanding the biological role of this demethylation in lupus nephritis will require further investigation. Our study reveals

the value of using longitudinal epigenetic studies to identify novel DNA methylation changes that could provide insight for specific disease manifestations. Precision medicine approaches in lupus, enabling epigenetic modification in specific cell types and possibly in key specific genetic loci in the near future are very promising(280).

Ancestry-associated DNA methylation differences and meQTL analyses showed a significant enrichment in Rho GEF pathways. GEFs are proteins that catalyze the cycling of GDP/GTP binding in Rho GTPases which results in their activation(340, 341). Rho GTPase activity regulates neutrophil function by controlling cytoskeletal arrangements in response to activation of signaling pathways(342). They regulate reactive oxygen species production, endothelial adhesion and transmigration, and production of neutrophil extracellular traps (NETs)(342). We used network analysis to further characterize ancestry-specific differential DNA methylation in lupus. One such network was centered around the transcription factor complex nuclear factor kappa B (NFκB) (see Figure C-4). NFκB is activated through degradation of inhibitory proteins in response to inflammatory signaling, such as TLR engagement, where it then translocates to the nucleus(343). There it coordinates the expression of proinflammatory gene programs in neutrophils that delay apoptosis, promotes production of proinflammatory cytokines, increases cell adhesion, and NETosis when cells are sufficiently activated(344). Resting human neutrophils tightly regulate NFκB activation through high levels of nuclear IκBα that is rapidly degraded upon proinflammatory stimulation(345). The gene *IKBKB* (cg20242624; $\Delta\beta$ = -0.11) encodes the inhibitor of NFκB kinase subunit beta protein (IKKβ) which is part of the IκB kinase (IKK) complex which is required for activation and nuclear translocation of NFκB by phosphorylation of the NFκB inhibitory subunit IκBα(346). *BCL10* (cg17322118; $\Delta\beta$ = -0.18) encodes the B-cell lymphoma/leukemia 10 protein BCL10 which is also an activator of

NFκB through ubiquitination of the IKK subunit protein IKKγ(347). Hypomethylation of these genes in neutrophils may reflect an increased response to inflammatory stimuli that promotes tissue invasion and inflammatory damage. Differentially methylated genes involved in regulating the type I interferon response were also present (see Figure C-5). In particular, *IRF7* (cg08926253; $\Delta\beta$ = -0.14 & cg22016995; $\Delta\beta$ = 0.13) was significantly hypomethylated similar to what we previously observed in naïve CD4+ T cells of African-American lupus patients and the neutrophils of lupus patients compared to healthy controls(275, 288). *IFI44*, also a type I interferon response gene was also significantly hypomethylated in African-American patients (cg01079652; $\Delta\beta$ = -0.23). We compared differentially hypomethylated genes in African-American lupus patients to the Interferome (v.2.01) database(314) to identify other type I interferon-regulated genes. Of interest, the cytokine gene *IL16* was hypomethylated in African-American patients (cg02810829; $\Delta\beta$ = -0.23) and had a modest association in an meQTL pair (rs35130261 meQTL r^2 = 0.68; ΔMAF = 0.33). IL-16 is a chemoattractant cytokine that induces infiltration of T cells, macrophages, and eosinophils into sites of inflammation, and promotes pro-inflammatory cytokine release by monocytes in vitro(348). It also promotes IL-2 receptor expression on the surface of CD4+ T cells, enhancing IL-2 activity(349) and the migration and expansion of regulatory T cells in sites of inflammation(350). A recent study observed that neutrophils produce and store inactive pro-IL-16 in the cytosol which is released and activated by caspase-3 upon secondary necrosis(351). Increased circulating IL-16 levels in lupus patients is associated with more severe disease(352, 353), and primary neutrophils of lupus patients more readily undergo apoptosis and increased secondary necrosis with reduced clearance of apoptotic material(354). This suggests that hypomethylation of *IL16* (in part related to meQTL) could

promote an exaggerated inflammatory response upon neutrophil secondary necrosis in lupus patients.

A demonstration of the mechanism underlying meQTL associations can be seen in two of the strongest meQTL pairs, cg25555787 (*TREML4*; rs9369265 meQTL $r^2 = 0.91$; $\Delta MAF = 0.31$) and cg03849834 (*TREML4*; rs9369265 meQTL $r^2 = 0.81$; $\Delta MAF = 0.31$). Functionally, TREML4 has previously been identified as playing an important role in modulating the response to TLR7 signaling when bound to single-stranded RNA and TLR9 binding to unmethylated CpG-DNA(355). Rs9369265 lies in the second exon of *TREML4* within an active region flanking the transcription starts site of *TREML4* in neutrophils. Rs9369265 genotype is also significantly associated with the expression of *TREML4* in whole blood (Gene-Tissue Expression Portal P-value = 1.2E-163), with the C allele associated with reduced expression and increased DNA methylation in our data. The presence of H3K4me3 peaks in this region and DNase accessibility suggest this is an important regulatory region for controlling *TREML4* expression as a promoter. A reduction in DNA methylation corresponds with an increase in H3K4me3 and promoter activity(356). *In vitro* patch methylation of the *TREML4* promoter showed that increasing DNA methylation significantly reduces the promoter's ability to induce expression. This suggests that DNA methylation is a mediating factor between the genotype and expression of *TREML4* we reported in this study.

The ligand for *TREML4* is unknown, but it readily binds to dead and dying cells(357). Reduced clearance of necrotic material in lupus patients might provide more stimulation to TREML4 and TLRs, promoting the exaggerated type I interferon response seen in lupus patients and contributing to the development of renal disease in lupus(358). Indeed, it has been observed that lupus-prone MRL*/lpr* mice have higher survival, produce fewer dsDNA autoantibodies, and

develop less renal damage when *Treml4* is knocked out(355). Neutrophils from *Treml4*$^{-/-}$ mice show reduced expression of *Cxcl2* which is a potent neutrophil chemoattractant, but unimpaired motility and phagocytosis(355). The higher frequency of the T allele in our African-American lupus patients that correlates with increased *TREML4* expression suggests potential for a more robust response to TLR stimulation. This is supported by the observation of increased expression of the proinflammatory cytokines interferon alpha and TNF alpha in the whole blood of female African-American lupus patients compared to female European-American patients(359). We investigated the ability of TREML4 to modify TLR7 response in neutrophils. Because primary neutrophils have a half-life of about six to eight hours they are difficult to manipulate *in vitro*(360). We utilized a transfectable neutrophil-like cell line (PLB-985)(316) in which we successfully overexpressed *TREML4* and measured the response of two genes, *IL1B* and *CCL2*, to TLR7 stimulation using imiquimod in the presence of *TREML4* overexpression. There was no significant difference in *IL1B* and *CCL2* expression with imiquimod treatment in the presence of *TREML4*. *TREML4* expression in DMSO-differentiated PLB-985 cells is lower than in primary human neutrophils as are *MYD88* and *TLR7*, raising the possibility that these cells are not able to respond efficiently to TLR7-stimulation in the presence of TREML4 and are not a proper model for this purpose(315). *TREML4* is expressed in other myeloid cell types including dendritic cells and macrophages that could potentially share the meQTL relationship we observed with neutrophils, making them interesting potential models as well(355). TLR7 can induce NETosis through reactive oxygen species production in viral infections suggesting an important role for TREML4 in the antiviral response as well(361, 362). We were also unable to induce NETosis in this cell line after six days of differentiation with DMSO and could not address the role of TREML4 in NETosis.

The mechanisms underlying the association between genotype and DNA methylation status will require further investigation. Potential mechanisms could include an inherited haplotype tagged by rs9369265 that promotes or suppresses transcription factor accessibility and binding, which is reflected in a repressive epigenetic state represented by increased DNA methylation correlating with reduced gene expression. This effect could also extend to other myeloid cells that express *TREML4* including macrophages and dendritic cells which contribute to the proinflammatory response(355).

We compared the DNA methylation profiles of neutrophils from a small cohort of healthy African-American and European-American female controls to determine if the observed differences in lupus patients were unique to the disease or shared by healthy ancestral populations. Of methylation sites that were assessed in both patients and healthy controls, 42.3% of the differentially methylated sites between African-American and European-American lupus patients overlapped with differentially methylated sites in healthy control neutrophils between the two populations. Further, 30 (48.3%) of the 62 CpG sites included on both the Infinium MethylationEPIC array EPIC and Infinium HumanMethylation450 arrays that are in an meQTL pair and differentially methylated in patient neutrophils were also differentially methylated in control neutrophils, between the two ethnicities. Taken together, ancestry-associated methylation variability in lupus patients include both genetically-determined methylation differences and methylation changes that might be related to non-genetic factors. Additional work is required to differentiate benign ancestry-associated epigenetic variability from epigenetic changes that might contribute to the pathogenesis of lupus or to differences in disease presentation and progression between populations.

Our current study was focused on epigenetic evaluation of neutrophils isolated from lupus patients, given the increasingly recognized role of neutrophil dysfunction in lupus. Future longitudinal studies in other cell types involved in the pathogenesis of lupus are likely to provide additional insights. For example, a prominent role for T cell aberrancies in the pathogenesis of lupus is well established(363). Investigating T cell DNA methylation changes over time in patients with lupus is warranted.

In summary, we have analyzed the association of DNA methylation with disease activity across time in the neutrophils of lupus patients. We demonstrate that the DNA methylome is at least in part determined by genetic variants in lupus patients and is largely stable over time and across disease activity levels in a longitudinal multi-ethnic lupus cohort. We identified two CpG sites unique to patients of African-American ancestry with methylation levels associated with disease activity in lupus. We also identified a single CpG site with an association among patients who developed active lupus nephritis. Using genome-wide DNA methylation and genotyping data we characterized ancestry-associated DNA methylation changes in lupus neutrophils and identified meQTL effects throughout the genome. Two genes, *TREML4* and *IL16* contained meQTL and were also significantly hypomethylated in African-American lupus patients. Both genes play roles in promoting inflammatory response to TLR signaling and infiltration of peripheral immune cells into tissue.

Chapter 3 Hypomethylation of MiR-17-92 Cluster in Lupus T cells and No Significant Role for Genetic Factors in the Lupus-associated DNA Methylation Signature

3.1 Abstract

Objective: Epigenetic dysregulation plays an important role in the pathogenesis of lupus, a systemic autoimmune disease characterized by autoantibody production. Lupus T cells demonstrate aberrant DNA methylation patterns dominated by hypomethylation of interferon-regulated genes. The objective of this study was to identify additional disease-associated DNA methylation changes in naïve CD4+ T cells from an extended cohort of lupus patients and determine the genetic contribution to epigenetic changes characteristic of lupus.

Methods: Genome-wide DNA methylation was assessed in naïve CD4+ T cells isolated from a cohort of 74 lupus patients and 74 age-, sex-, and race-matched healthy controls. We applied a trend deviation analysis approach, comparing methylation data in our cohort to methylation data from over 16,500 samples to characterize lupus-associated DNA methylation patterns. Methylation quantitative trait loci (meQTL) analysis was used to determine genetic contribution to lupus-associated DNA methylation changes.

Results: In addition to the previously reported epigenetic signature in interferon-regulated genes, we observed hypomethylation of the promoter regions of microRNA (miRNA) genes in the miR-17-92 cluster in lupus patients. Members of this miRNA cluster play an important role in regulating T cell proliferation and differentiation. Expression of two miRNAs within this cluster, miR-19b1 and miR-18a, showed a significant positive correlation with disease activity in lupus patients. meQTL were identified by integrating genome-wide DNA

methylation profiles with genotyping data in lupus patients and controls. Patient meQTL show overlap with genetic risk loci for lupus. However, less than 1% of differentially methylated CpG sites in lupus patients were associated with an meQTL, suggesting minimal genetic contribution to lupus-associated epigenotypes.

Conclusion: The lupus defining epigenetic signature, characterized by robust hypomethylation of interferon-regulated genes, does not appear to be determined by genetic factors. Hypomethylation of the miR-17-92 cluster that plays an important role in T cell activation is a novel epigenetic locus for lupus.

3.2 Introduction

Systemic lupus erythematosus (lupus or SLE) is a heterogeneous autoimmune disease of incompletely understood etiology. The disease is characterized by a loss of immunotolerance and the development of autoantibodies against nuclear antigens. Severe manifestations of lupus have significant impact on quality of life and can lead to organ damage and mortality in affected patients, particularly among patients of non-European genetic ancestry(364, 365). Genetic risk contributes to the development of lupus, but the estimated heritability of lupus is ~30%(49, 59, 366). Indeed, monozygotic twin studies in lupus suggest a substantial non-genetic contribution to the etiology of lupus(367). Environmental exposures across the lifespan that can directly impact epigenetic regulation and cellular function are suggested to be involved in the pathogenesis of lupus(124, 261).

DNA methylation is an epigenetic mechanism that regulates gene expression through the enzyme-mediated addition of a methyl group to the cytosine bases in the genome. DNA methylation is heritable across cell generations and can promote gene silencing, making it an important component in regulating the plasticity of immune cell identity and function(241).

Early work demonstrated that adoptive transfer of CD4+ T cells treated ex vivo with DNA methyltransferase (DNMT) inhibitors was sufficient to cause lupus-like disease in mice(260) mimicking the DNA methylation inhibition in patients with drug-induced lupus(254). Since then, other studies have observed that CD4+ T cells of lupus patients show a distinct shift in global DNA methylation compared to healthy individuals, potentially in part due to defective MEK/ERK signaling, suppressing DNMT1 activity in CD4+ T cells, and leading to hypomethylation and overexpression of costimulatory genes(258, 368-371).

We have previously observed a robust hypomethylation signature in interferon-regulated genes defining lupus patients(262, 264). Our initial findings in CD4+ T cells were subsequently confirmed and expanded to other cell types by our group and others(275, 299, 372). In CD4+ T cells, we observed hypomethylation in interferon-regulated genes at the naïve CD4+ T cell stage, preceding transcriptional activity. This epigenetic "poising" or "priming" of interferon-regulated genes was independent of disease activity(264). The genetic contribution to this lupus-associated epigenotype is currently unknown.

Methylation quantitative trait loci (meQTL) are genetic polymorphisms that are associated with the methylation state of CpG sites either through direct nucleotide change within the CpG dinucleotide or intermediary mechanisms. Prior studies of lupus patients show an enrichment of meQTL associated with type I interferon genes, genetic risk loci, and specific clinical manifestations in whole blood and neutrophils(73, 284, 373). Furthermore, our previous work suggests that meQTL might at least in part explain differences in DNA methylation between African-American and European-American lupus patients(373).

Herein, we evaluated genome-wide DNA methylation data in naïve CD4+ T cells from a large cohort of lupus patients compared to matched healthy controls. We integrated DNA

methylation and genotyping data to better understand the influence of genetic factors upon the DNA methylation changes observed in lupus.

3.3 Methods

3.3.1 Study Participants and Demographics

74 female lupus patients and 74 female healthy age (± 5 years), race, and sex-matched controls were recruited as previously described(267, 288) (see Table C-1). All patients fulfilled the American College of Rheumatology (ACR) classification criteria for SLE(300). Institutional review boards at our participating institutions approved this study. All participants signed a written informed consent prior to participation.

3.3.2 Sample collection

DNA isolation, and data generation. Genomic DNA samples for this study were collected from naïve CD4+ T cells as previously described(264). Briefly, magnetic beads and negative selection was used to isolate naïve CD4+ T cells from whole blood samples collected from lupus patients and controls. Genomic DNA was directly isolated from collected cells and bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). The Illumina HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA) was used to measure DNA methylation levels at over 485,000 methylation sites across the genome.

3.3.3 Epigenome-wide association study

Epigenome-wide association study (EWAS) for identifying associations between specific CpG sites and disease status was performed using *GLINT*(374, 375). Covariates for age, race, and technical batch were included for the analysis prior to other preprocessing. No outliers beyond four standard deviations were detected in the first two components of PCA space, all 148 samples were included in the analysis. Reference-less cell type composition correction was performed using *ReFACTor*, with six components used in the downstream analysis to account for any cell-type heterogeneity in the samples. An additional covariate was included to account for effects of genetic admixture using the *EPISTRUCTURE* algorithm included in GLINT. Cell-type composition covariate components generated by *ReFACTor* were included at this step to reduce bias from potential cell-type heterogeneity, and polymorphic CpG sites were excluded from this step and the EWAS. Using the initial age, race, and technical batch covariates, along six *ReFACTor* components and one *EPISTRUCTURE* component, logistic regression for disease status was performed across all CpG sites, excluding the polymorphic and unreliable cross-reactive probes previously described in the literature, as well as CpG sites with low variance (standard deviation <0.01) (376, 377).

3.3.4 Differential DNA methylation analysis of gene promoters

Raw .idat files were used to generate methylation beta value profiles across all samples using GenomeStudio (Illumina, San Diego, CA, USA) after background subtraction and normalizing to internal control probes. Missing probe values were imputed using *sklearn.impute.KNNImputer*, and *ComBat* was used to correct for batch effects associated with technician sample preparation(378-380). Ensembl gene loci for hg19 were downloaded using *PyEnsembl*(381). For each gene, loci for 1500 base pairs upstream of the transcription start

site(382) to the TSS were mapped to the overlapping CpG probes using *PyBedtools*, and the mean of the associated probes for each gene was used as the representative methylation value for the resulting 20,437 mapped genes(383). Differential methylation analysis comparing patients and controls was performed on the mean TSS1500 methylation using *limma*, and false discovery rate adjustment using the Benjamini-Hochberg method was used to correct P-values for multiple testing. Gene Ontology Enrichment for Biological Process terms was performed on the differentially methylated gene list using *Enrichr* with the mapped promoter gene list used as the background(318, 384).

3.3.5 Trend deviation analysis

DNA methylation data derived using the Illumina 450k methylation array from 23,415 samples were downloaded from Gene Expression Omnibus (GEO)(385). To reduce batch effects, samples from experiments with less than 50 samples were omitted, and the resulting samples were quantile normalized(386). A matrix of pairwise Pearson's correlation values for DNA methylation levels was computed across TSS1500 gene promoters in 16,541 samples across 37 tissues to create a multi-tissue correlation network (see Figure D-1). The differentially methylated genes in lupus naïve CD4+ T cells were clustered by their correlation in global signature created from the GEO data. Hierarchical clustering was performed using *Scipy's* hierarchical linkage. KEGG enrichment analysis was performed using *Enrichr*(387), and P-values were reported after false-discovery rate adjustment.

The goal of a trend deviation analysis is to detect correlation patterns among differentially methylated genes in large DNA methylation datasets. A correlation in methylation among a set of differentially methylated genes between patients and controls suggests a trend is

being observed, reinforcing the significance and robustness of the differential DNA methylation detected between patients and controls.

3.3.6 Genotyping

Genomic DNA isolated from naïve CD4+ T cells was used as input for the Infinium Global Screening Array-24 v2.0 (Illumina, San Diego, CA, USA). Single nucleotide polymorphisms (SNPs) with a genotyping call rate < 98%, minor allele frequencies (MAF) < 5%, and deviating from Hardy-Weinberg equilibrium (HWE; P-value < 1E-3) were filtered out. Samples were removed if they had a genotyping call rate < 95%. Sex chromosomes were not analyzed. About 100,000 independent SNPs were pruned and used to perform principal component analysis (PCA) with *Eigensoft* (v.6.1.4) software(312). Genotyping data were analyzed using *PLINK* (v.1.9)(311). Genotype profiles were generated for n = 63 patients and n = 68 controls.

3.3.7 Methylation Quantitative Trait Loci (meQTL) Analysis

Raw .idat files were used to generate methylation profiles using *minfi* (v.1.32.0)(303, 304) and to check median intensity values and reported sex in the R statistical computing environment (v.3.6.3)(302). Probes with less than three beads and zero intensity values across all samples were removed using the *DNAmArray* package (v.0.1.1)(305). Background signal and dye bias were corrected, followed by normalization of signal intensities using functional normalization in the *preprocessFunnorm.DNAmArray* function(305, 306) using the first three principal component values calculated from signal intensities of control probes present on all

array spots to correct for technical variation. Any probe with a detection P-value < 0.01 or returned signal intensities in fewer than 98% of samples was removed. This resulted in a total of 476,767 probes used for further analysis. Signal intensities were then converted to M-values with a maximum bound of ±16. M-values were used for meQTL analysis and converted to beta values (0-100% methylation scale) using *minfi* for reporting.

We removed any probe for meeting any of the following technical criteria: A unique probe sequence of less than 30bp, mapping to multiple sites in the genome, polymorphisms that cause a color channel switching in type I probes, inconsistencies in specified reporter color channel and extension base, mapping to the Y chromosome, and/or having a polymorphism within 5bp of the 3' end of the probe with a minor allele frequency > 1% with the exception of CpG-SNPs with C>T polymorphisms which were retained(307). Batch correction for chip ID was performed using the *ComBat* function in the *sva* (v.3.34.0) package(308). After technical filtering, there were a total of 421,214 probes used for meQTL analysis.

We implemented a mixed correspondence analysis with the *PCAmixdata* package (v.3.1)(309) to calculate eigenvalues using patient medication data for prednisone, hydroxychloroquine, azathioprine, mycophenolate mofetil, and cyclophosphamide. The top four components accounted for a cumulative 89.3% of variability in the medication data. Each component value was used as an independent variable in regression analysis to adjust for medication usage across individuals. MeQTL association analysis was performed in patients and controls separately using methylation M-value profiles and corresponding sample genotypes. Age, the top four medication components, and top ten genotype principal components were included as covariates to build a linear model for detecting meQTL using *MatrixEQTL* (v.2.3)(319). *Cis*-meQTL were defined as CpG sites with methylation values associated with a

SNP within a conservative 1000bp of the CpG dinucleotide. We used a Benjamini-Hochberg FDR-adjusted P-value cutoff of < 0.05 as a threshold for significant associations. The above EWAS results were compared with the meQTL results to determine overlap of lupus-associated differentially methylated CpG sites and those CpG sites in an meQTL.

3.3.8 Functional Enrichment Analysis

ToppGene Suite was used for functional gene ontology enrichment analysis(313) of Molecular Function and Biological Process Gene Ontologies and KEGG Pathways in meQTL loci. P-values were derived using a hypergeometric probability mass function, and a Benjamini-Hochberg FDR–adjusted P-value cutoff of < 0.05 was used as a threshold of significance. A minimum membership of 3 genes and maximum of 2000 genes in each term was used as a threshold for inclusion. IFN-regulated genes were identified using the set of genes associated with the CpG site in each meQTL as input using Interferome (v.2.01)(314). The type I interferon response genes were defined as genes with an expression fold change of 1.5 or greater between type I interferon-treated and untreated samples using gene expression datasets from all available CD4+ T cell experiments in the Interferome database.

HaploReg (v4.1)(388) was used to identify genetic variants in linkage disequilibrium (LD, $r^2 \geq 0.80$ in Europeans) with previously described lupus genetic susceptibility loci(389) that overlap with SNPs identified in our meQTL analysis.

3.3.9 MicroRNA Expression Microarray

MicroRNA (miRNA) expression was measured in naïve CD4+ T cells from a subset of lupus patients and healthy matched controls (n = 16). Cells were immediately lysed with TRIzol Reagent (ThermoFisher Scientific, NY, USA) followed by storage at -80C. Total RNA was isolated using the Direct-zol RNA MiniPrep Kit (Zymo Research, CA, USA) following the manufacturer's directions. The Affymetrix miRNA 4.1 Array Strip (Affymetrix, CA, USA) was used to measure expression of over 2,000 premature and 2,500 mature human miRNA sequences. RNA sequences were polyadenylated and ligated to a biotin-labeled oligomer using the FlashTag Biotin HSR RNA Labeling Kit (Affymetrix, CA, USA). Biotin-labeled sequences were hybridized to array probes and washed then stained with streptavidin-PE. The Affymetrix Expression Console & Transcriptome Analysis Console 2.0 software (Affymetrix, CA, USA) was used to analyze biotin/streptavidin-PE fluorescence measurements. All samples passed signal intensity, polyadenylation, and ligation quality controls. Signal intensities were background adjusted and normalized. Log2-transformed expression values for each probeset was calculated using a robust multi-array average model(390). The Pearson r correlation coefficient for median expression values of probes for miR-17, miR-18a, miR-19a, miR-19b1, and miR-20a and patient SLEDAI score were calculated using GraphPad Prism (v9.3.0) (GraphPad Software, CA, USA).

3.4 Results

3.4.1 Differential methylation of gene promoters in naïve CD4+ T cells isolated from lupus patients.

A comparison of DNA methylation profiles from circulating naïve CD4+ T cells isolated from 74 lupus patients and 74 age, sex and race matched healthy controls revealed a total of

2,627 CpGs out of 334,337 total CpG sites included in the EWAS with a significant differential methylation. Significant hypomethylation in interferon-regulated genes was observed, consistent with previous reports (Table 3-1) (see Table A-2: “Supplementary Table 2”).

Table 3-1: Top 10 most hypomethylated and hypermethylated CpG sites in naïve CD4+ T cells in lupus patients compared to healthy controls matched by age, sex, and ethnicity. Δβ is the difference in average methylation (β) between lupus patients and controls. CpG sites shown in this table had an FDR-adjusted P-value < 0.05.

Probe ID	Average β Lupus	Average β Controls	Δβ	Probe Location (hg19)	P-value	FDR-adjusted P-value	UCSC RefGene Name	Relation to UCSC CpG Island
cg21549285	0.346	0.651	-0.305	chr21:42799141	1.84E-07	0.00154454	*MX1*	S_Shore
cg14392283	0.596	0.900	-0.304	chr8:144103587	3.47E-05	0.01477556	*LY6E*	N_Shelf
cg00959259	0.335	0.596	-0.261	chr3:122281975	1.93E-07	0.00154454	*DTX3L;PARP9*	N_Shore
cg03607951	0.469	0.696	-0.227	chr1:79085586	3.30E-06	0.00436127	*IFI44L*	
cg08122652	0.522	0.746	-0.224	chr3:122281939	6.02E-07	0.00211873	*DTX3L;PARP9*	N_Shore
cg05552874	0.566	0.785	-0.219	chr10:91153143	1.53E-05	0.01003301	*IFIT1*	
cg05696877	0.297	0.513	-0.216	chr1:79088769	1.06E-08	0.00154454	*IFI44L*	
cg06981309	0.417	0.627	-0.210	chr3:146260954	4.66E-07	0.00202278	*PLSCR1*	N_Shore
cg15065340	0.489	0.698	-0.209	chr3:195632915	4.29E-07	0.00195458	*TNK2*	N_Shelf
cg01028142	0.643	0.848	-0.204	chr2:7004578	2.81E-05	0.01340079	*CMPK2*	N_Shore
cg17593958	0.279	0.170	0.109	chr20:62199034	2.22E-06	0.00360447	*PRIC285*	N_Shore
cg01890417	0.495	0.385	0.110	chr1:91488275	4.80E-06	0.00521362	*ZNF644*	S_Shore
cg19863426	0.331	0.221	0.110	chr10:124138853	7.20E-06	0.00638871	*PLEKHA1*	S_Shelf
cg09844573	0.243	0.128	0.115	chr20:62199190	6.93E-07	0.00224857	*PRIC285*	N_Shore
cg15529432	0.542	0.425	0.117	chr5:16615750	8.83E-05	0.02394923	*FAM134B*	N_Shore
cg10555744	0.389	0.270	0.120	chr1:25946258	2.53E-05	0.01277911	*MAN1C1*	S_Shore
cg00492070	0.598	0.478	0.120	chr3:107810716	2.64E-06	0.00389560	*CD47*	S_Shore
cg14011789	0.374	0.248	0.126	chr17:75452044	8.95E-06	0.00726884	*SEPT9*	
cg05617307	0.425	0.298	0.127	chr10:121413182	1.25E-05	0.00887401	*BAG3*	S_Shore
cg15262954	0.331	0.115	0.216	chr20:62198872	2.95E-06	0.00413747	*PRIC285*	Island

Average promoter methylation for each gene was calculated by including all CpG sites on the array within 1500bp of the associated gene's transcription start site (TSS). A total of 51 genes showed a significant difference in average promoter methylation between lupus patients and controls (17 hypomethylated and 34 hypermethylated in patients compared to controls) (Table 3-2) (Figure 3-1). Biological Process Gene Ontology enrichment analysis of differentially methylated promoter regions did not show significant enrichment compared to the background of all gene promoters after adjusting for multiple testing (see Table A-2: "Supplementary Table 2").

Table 3-2: Genes with differentially methylated promoter regions in naive CD4+ T cells of lupus patients compared to healthy controls. FDR correction was performed using the Benjamini-Hochberg method with an FDR-adjusted P value threshold of < 0.05. Δβ: methylation difference in median methylation value of CpG sites within 1500bp upstream of the associated gene's transcription start site (TSS1500) between lupus patients and healthy controls**.**

Gene	Δβ	-log$_{10}$ (FDR-adjusted P-value)	*t*-statistic
IFI44L	-0.177	infinity	-10.757
DTX3L	-0.130	infinity	-11.566
BST2	-0.089	11.323	-9.285
RABGAP1L	-0.088	9.165	-8.421
BCL2L14	-0.086	5.520	-6.908
MIR19B1	-0.059	3.169	-5.846
IFI44	-0.059	2.057	-5.304
MIR20A	-0.055	3.088	-5.807
MIR17	-0.054	6.882	-7.487
MIR18A	-0.051	6.537	-7.342
MIR19A	-0.049	4.771	-6.579
IKZF4	-0.048	3.289	-5.902
MX1	-0.046	10.624	-9.004
TRIM34	-0.045	2.184	-5.367
ODF3B	-0.034	1.712	-5.128
GNG2	-0.033	2.138	-5.344
FAM177B	-0.025	1.897	-5.223
MZF1	0.008	1.493	5.014
SSBP4	0.015	1.344	4.934
ATP6V0D1	0.018	2.594	5.569
DCUN1D1	0.025	2.068	5.309
C14orf93	0.025	1.922	5.236
TIPARP	0.026	2.069	5.310
LMBRD1	0.027	2.211	5.381
HAVCR2	0.027	2.574	5.560
KIAA1949	0.030	3.158	5.841
GPD2	0.032	1.953	5.251
CNTF	0.033	1.705	5.124
CD47	0.034	4.259	6.350
ARHGAP9	0.036	3.339	5.926
IL27RA	0.036	1.367	4.946
RAP1A	0.036	2.573	5.559
LAMA3	0.037	1.445	4.988
ABI3	0.037	1.436	4.983

Table 3-2 (continued)

Gene	Δβ	-log$_{10}$ (FDR-adjusted P-value)	*t*-statistic
FAM102A	0.038	3.161	5.842
CXCR5	0.039	1.439	4.985
DPEP2	0.040	1.889	5.219
DYRK2	0.041	3.924	6.197
TMEM71	0.044	2.757	5.649
ADORA2A	0.046	2.234	5.392
SEPT9	0.047	2.036	5.293
PSMB4	0.052	2.935	5.734
TOM1	0.055	5.415	6.862
PRIC285	0.057	9.934	8.729
LTB	0.062	2.036	5.293
MIR1205	0.067	1.698	5.121
ACER3	0.073	2.612	5.578
BCL9L	0.079	4.034	6.248
MDS2	0.080	3.149	5.836
SNORA5B	0.083	1.712	5.128
PTPRCAP	0.091	3.620	6.057

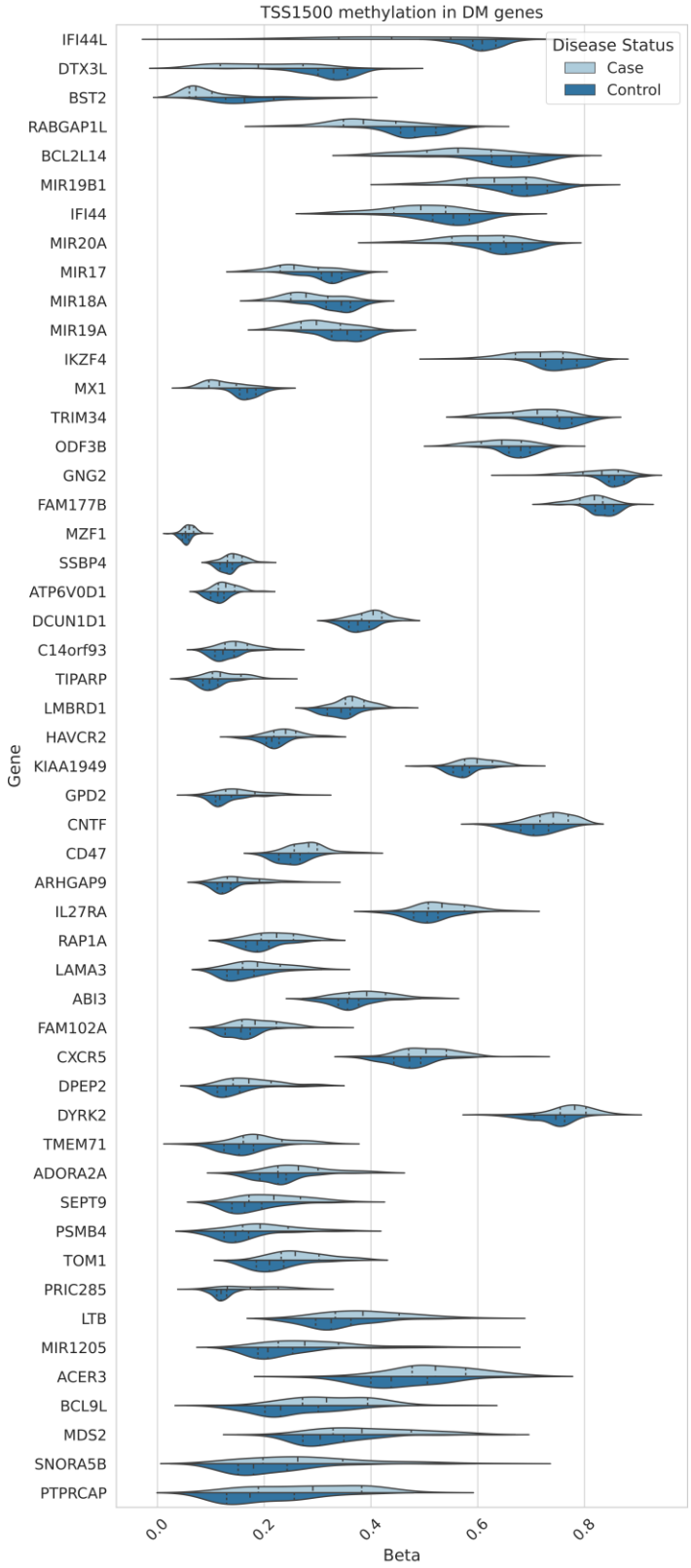

Figure 3-1: Distribution of average CpG methylation levels within 1500bp of the TSS for the respective genes differentially methylated in naïve CD4+ T cells of lupus patients compared to healthy controls. Beta is the fraction of methylated CpG sites to total CpG sites.

The pairwise correlation of the 51 gene promoters identified above was calculated across a collection of 16,541 samples from 37 tissues available in GEO. Hierarchical clustering of correlations showed that 21 out of the 51 gene promoters were highly correlated. KEGG Pathway enrichment analysis showed a significant enrichment for three pathways among the 21 correlated gene promoters: “microRNAs in cancer” (P-value = 3.86E-04), “cytokine-cytokine receptor interaction” (P-value = 4.34E-02), and “rheumatoid arthritis” (P-value = 4.34E-02) (Figure 3-2) (Table 3-3).

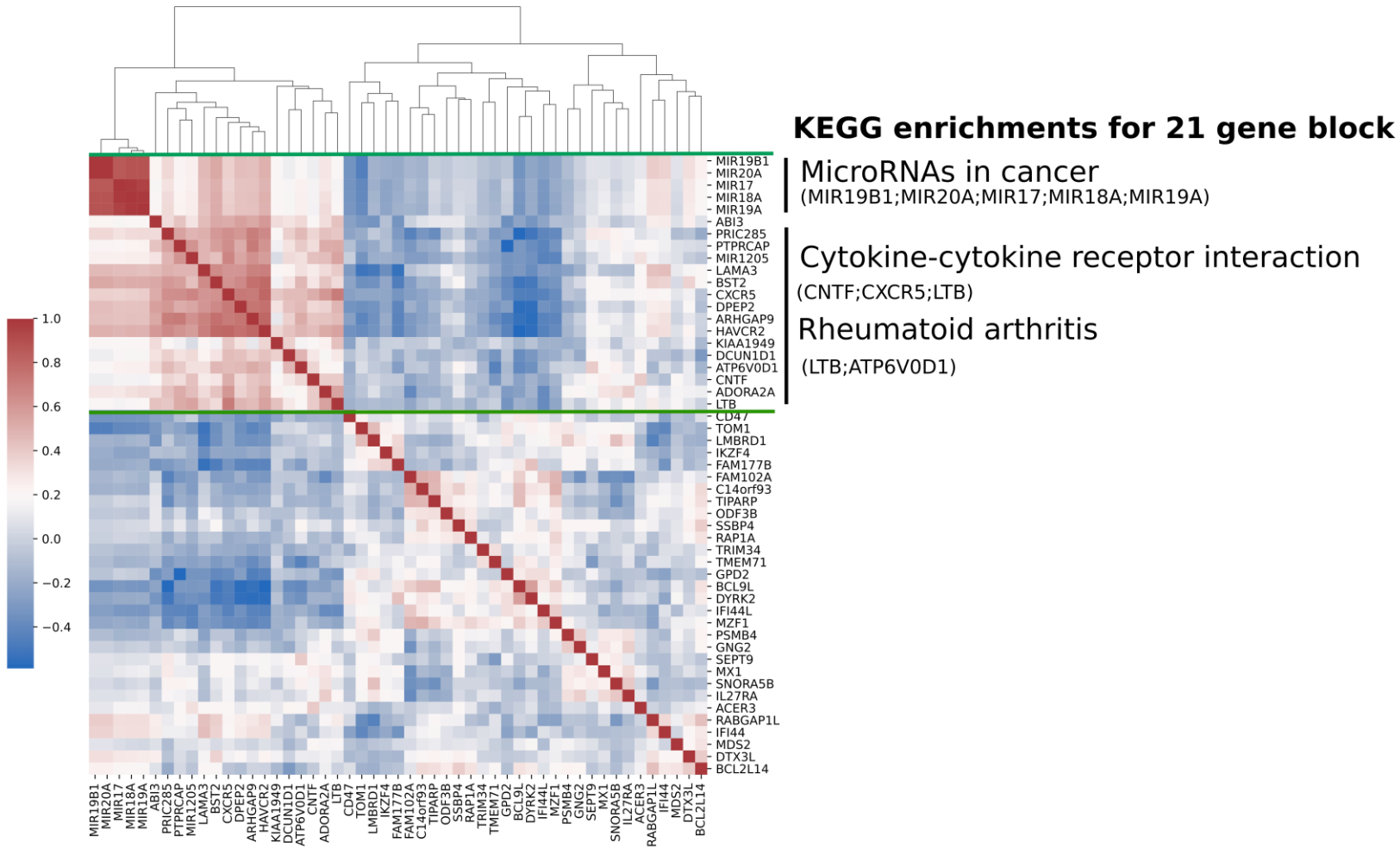

Figure 3-2: Heatmap of hierarchical clustering of pairwise Pearson correlation coefficient values of 51 differentially methylated gene promoters (TSS1500) in global tissue signature derived from 16,541 samples. Range from +1 (red) to -1 (blue), represent a greater to lower correlation in global tissue, respectively. KEGG pathways are significantly enriched (FDR-adjusted P-value < 0.05) in a block of 21 genes (green bars).

Table 3-3: KEGG Pathway gene enrichment of 21 gene promoters highly correlated with each other in multi-tissue DNA methylation data constructed from 16,541 samples available through GEO. FDR: False Discovery Rate-adjusted P-value. OR: Odds Ratio.

Pathway (KEGG_2019_Human)	O**verlap**	**P-value**	**FDR**	**OR**	**Score**	**Genes**
MicroRNAs in cancer	5/299	1.21E-05	3.86E-04	20.92	236.95	*MIR19B1;MIR20A;MIR17;MIR18A;MIR19A*
Cytokine-cytokine receptor interaction	3/294	3.44E-03	0.043	11.28	63.97	*CNTF;CXCR5;LTB*
Rheumatoid arthritis	2/91	4.06E-03	0.043	23.52	129.51	*LTB;ATP6V0D1*

The microRNAs in cancer" pathway included genes encoding miR-17, miR-18a, miR-19a, miR-19b1, and miR-20a. Four of seven CpG sites used to calculate the average promoter methylation (TS1500) in this locus showed a significant reduction in median methylation in lupus patients compared to healthy controls (Figure 3-3A). These sites: cg17799287 (Δβ= -5.5%; P-value = 2.05E-03), cg07641807 (Δβ = -4.4%; P-value = 1.71E-02), cg23665802 (Δβ= -5.8%; P-value = 1.19E-02), and cg02297838 (Δβ= -4.9%; P-value = 3.48E-02) were all hypomethylated in lupus patients compared to healthy controls and overlapped with enhancers and regions flanking TSSs in peripheral naïve CD4+ T cells using data collected from the Epigenome Roadmap(391) and visualization using the WashU Epigenome Browser(392). We examined expression levels of the microRNAs included in the "microRNAs in cancer" pathway (miR-17, miR-18a, miR-19a, miR-19b1, and miR-20a) in naïve CD4+ T cells of a subset of our lupus patients (n = 16) and healthy matched controls (n = 16). We did not observe a difference in expression between patients and control. However, two miRNAs, miR-18a-5p and miR-19b1-5p, showed a significant positive correlation (hsa-miR-18a-5a P-value = 0.038 & hsa-miR-19b1-5p P-value = 0.042) between median expression level and SLEDAI scores in lupus patients (Figure 3-3B) (see Table C-2).

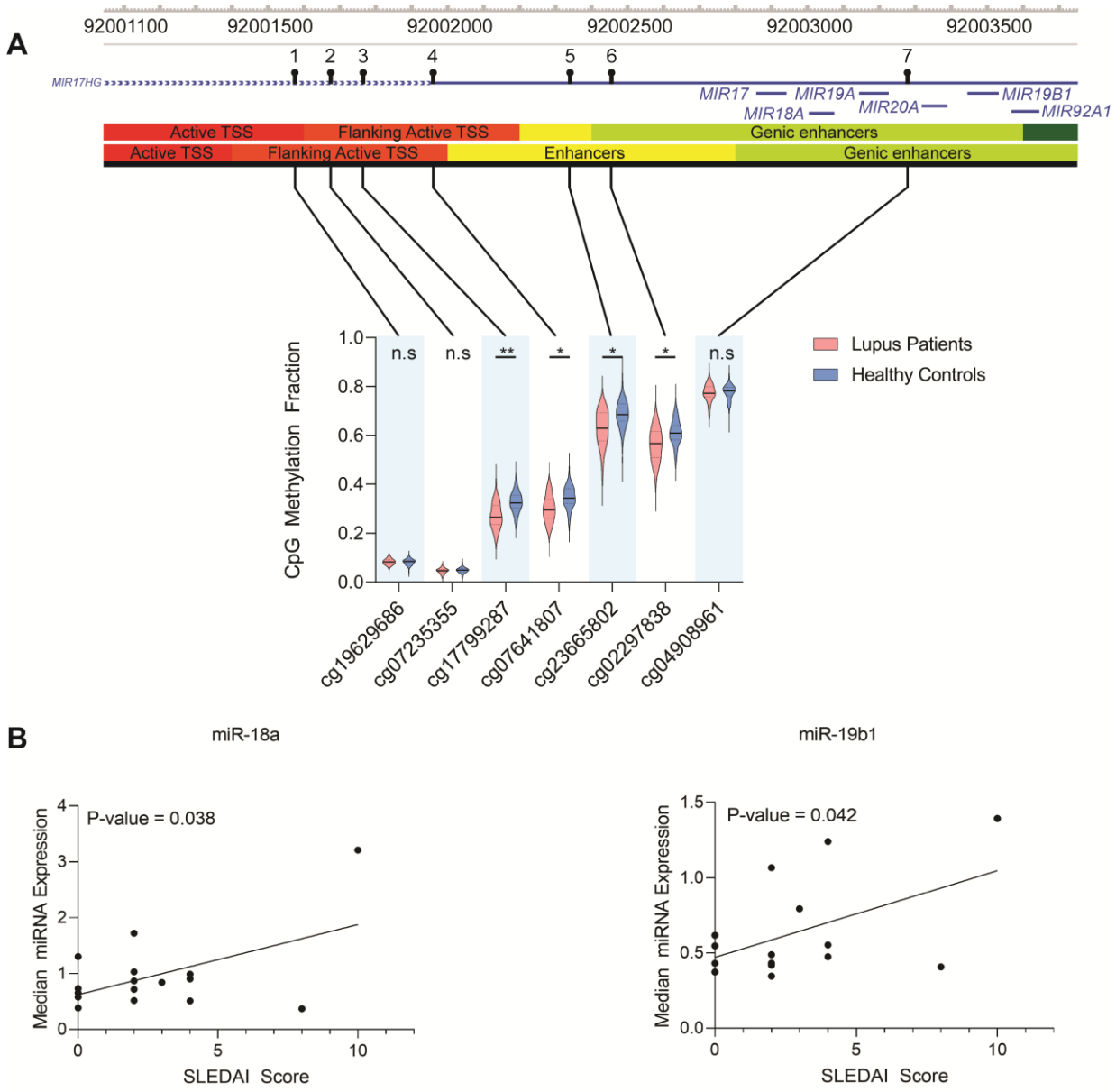

Figure 3-3: miR-17-92 promoter hypomethylation and gene expression association with disease activity in lupus naive CD4+ T cells. (A) Violin plots of the seven CG probes in lupus patients and healthy controls used to calculate the average promoter methylation (TSS1500) for the miR-17-92 cluster. The solid black bar represents the median value and the dashed lines the first and third quartiles. Genomic visualization and annotation are from WashU Epigenome Browser using AuxillaryHMM tracks from peripheral naïve CD4+ T cell tissues (E038 and E039, top and bottom tracks, respectively). For P-values: n.s. = not significant, * = P < 0.05, ** = P < 0.01. (B) Correlation of median miRNA expression in naïve CD4+ T cells of a subset (n = 16) of lupus patients with SLEDAI score. Hsa-miR-18a-5p and hsa-miR-19b1-5p had a Pearson correlation (r) of 0.52 (P-value = 0.038) and 0.51 (P-value = 0.042), respectively.

3.4.2 Naïve CD4+ T cell methylation quantitative trait loci (meQTL) in lupus patients.

Global genotype profiles were generated in a subset of patients and controls and compared with global DNA methylation profiles to identify CpG sites with allele-specific methylation associations. There was no significant difference in the average age between the patient (n = 63) and control (n = 68) subsets (patient average age = 41.6; patient age SD = 12.8; control average age = 40.8; control age SD = 12.5; t-test statistic = 0.3811; two-tailed P-value = 0.7038). Allele-specific DNA methylation associations were measured as meQTL where the CpG site was within 1000bp of the measured SNP separately in patients and controls. After adjusting for age, genetic background, and medication use in patients, we identified 5,785 meQTL present in the naïve CD4+ T cells of lupus patients with an FDR-adjusted P-value < 0.05 (see Table A-2: "Supplementary Table 5"). These meQTL represented 4,649 (80.4%) unique CpG sites and 4,120 (71.2%) unique polymorphisms. Of the 4,791 meQTL with a CpG-associated gene annotation, 2,356 (49.2%) were unique.

A linear model adjusting for age and genetic background was fit to controls separately. We identified a total of 7,331 meQTL with an FDR-adjusted P-value < 0.05 in controls (see Table A-2: "Supplementary Table 6"). These meQTL represented 5,885 (80.3%) unique CpG sites and 5,138 (70.1%) unique polymorphisms. Of the 6,061 meQTL with a CpG-associated gene annotation, 2,846 (47.0%) were unique.

We compared meQTL in lupus patients and healthy controls with the 2,627 CpG sites differentially methylated between the two groups. Of these, we identified 17 (0.6%) and 34 (1.3%) unique CpG sites with a significant change in DNA methylation in lupus patients and healthy controls, respectively (Figure 3-4A and 3-4B). We examined the overlap of meQTL in

lupus patients and healthy controls and identified a total of 3,957 meQTL (68.4% of lupus patient meQTL and 54.0% of healthy control meQTL) shared between both patients and control meQTL sets (see Table A-2: "Supplementary Table 7"). This shared set of meQTL contained 8 (0.3% of differentially methylated CpG sites) unique CpG sites that we identified as differentially methylated between lupus patients and controls (Figure 3-4C).

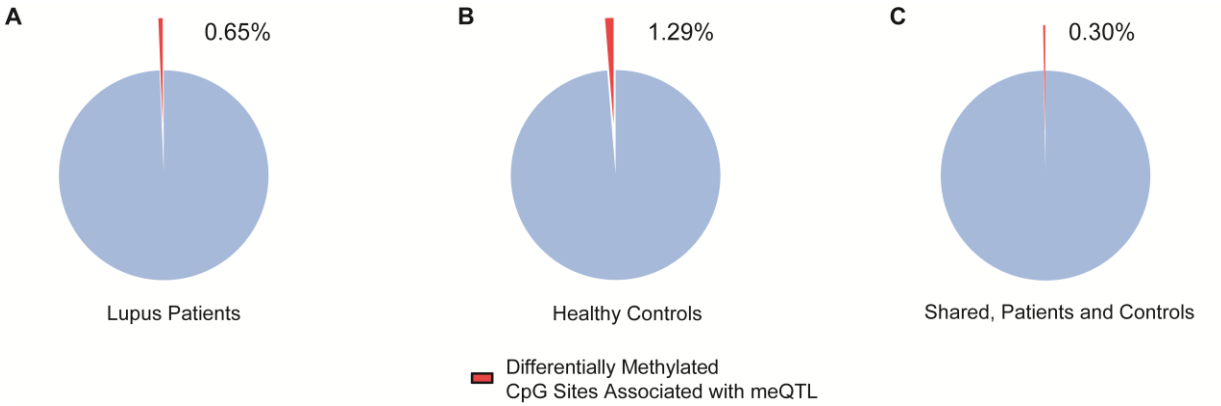

Figure 3-4: Percentage of CpG sites differentially methylated between lupus patients and controls associated with meQTL in naïve CD4+ T cells. Using the list of differentially methylated CpG sites, we calculated the proportion associated with the list of *cis*-meQTL identified separately in (A) lupus patients, (B) healthy controls, and (C) the subset of meQTL shared between lupus patients and healthy controls.

Functional enrichment analysis was performed using genes associated with CpG sites in our meQTL shared between patients and controls. This revealed multiple ontologies and pathways for cell adhesion ("cell-cell adhesion"; P-value = 1.04E-12, "biological adhesion"; P-value = 6.80E-12, and "cell adhesion"; P-value = 8.25E-12, "Cell adhesion molecules (CAMs)"; P-value = 2.25E-06), transporter associated with antigen processing (TAP) proteins and antigen presentation ("TAP binding"; P-values = 1.59E-7, "peptide antigen binding"; P-value = 4.40E-5), immune disorder pathways ("Type I diabetes mellitus; P-value = 1.92E-8, and "Graft-versus-host disease"; P-value = 4.38E-7) (see Table A-2: "Supplementary Table 8").

There were 1,828 meQTL detected only in lupus patients but not in controls. These were enriched in gene ontologies and pathways related to tissue growth and development ("animal

organ morphogenesis"; P-value = 8.44E-10, "urogenital system development", P-value = 1.05E-07) and gene silencing ("negative regulation of gene silencing by miRNA"; P-value = 2.54E-6, "negative regulation of posttranscriptional gene silencing"; P-value = 5.41E-6) (see Table A-2: "Supplementary Table 9").

We compared our list of meQTL in lupus patients to lupus susceptibility loci previously identified from GWAS(59, 323-326). We found 41 meQTL that contained CpG site-associated genes that overlapped with 20 lupus risk loci (see Table A-2: "Supplementary Table 10"). This included interferon regulatory factor genes *IRF5* and *IRF7*. We found three meQTL in naïve CD4+ T cells that included, or were in high LD ($r^2 \geq 0.80$) with, a known lupus genetic risk variant (Table 3-4)(389). We applied the same analysis to meQTL results we obtained from our lupus granulocyte study. We found meQTL associated with lupus risk variants in *CFB* (rs170942) and *IRF7* (rs1131665) in both naïve CD4+ T cells and granulocytes from lupus patients. In addition, an meQTL associated with the *TMEM86B-PTPRH* locus was observed in naïve CD4+ T cells. When we compared the lupus risk allele with DNA methylation levels, we found that the presence of the risk allele at rs1270942 (*CFB*) is associated with increased DNA methylation of cg16505946. The presence of the risk allele at rs1131665 (*IRF7*) (Figure 3-7) and rs56154925 (*TMEM86B-PTPRH*) was associated with decreased DNA methylation of cg16486109 and cg01414877, respectively. The direction of the risk allele-DNA methylation association in the *CFB* and *IRF7* meQTL was the same in both naïve CD4+ T cells and granulocytes.

Table 3-4: MeQTL in naive CD4+ T cells and granulocytes of lupus patients that include a known lupus risk variant.

Lupus Naïve CD4+ T cell meQTL

CpG Site	meQTL SNP	Lupus Risk SNP[#]	Risk SNP-associated Gene	Lupus Risk Allele	Direction of CpG methylation associated with risk allele
cg16505946	rs558702	rs1270942	*CFB*	C	↑
cg16486109	rs1131665	rs1131665	*IRF7*	A	↓
cg01414877	rs56154925	rs56154925	*TMEM86B-PTPRH*	C	↓

[#]rs558702 and rs 1270942 have an LD $r^2 \geq 0.80$.

Lupus Granulocyte meQTL

CpG Site	meQTL SNP	Lupus Risk SNP[#]	Risk SNP-associated Gene	Lupus Risk Allele	Direction of CpG methylation associated with risk allele
cg16505946	rs558702	rs1270942	*CFB*	C	↑
cg16486109	rs1131665	rs1131665	*IRF7*	A	↓

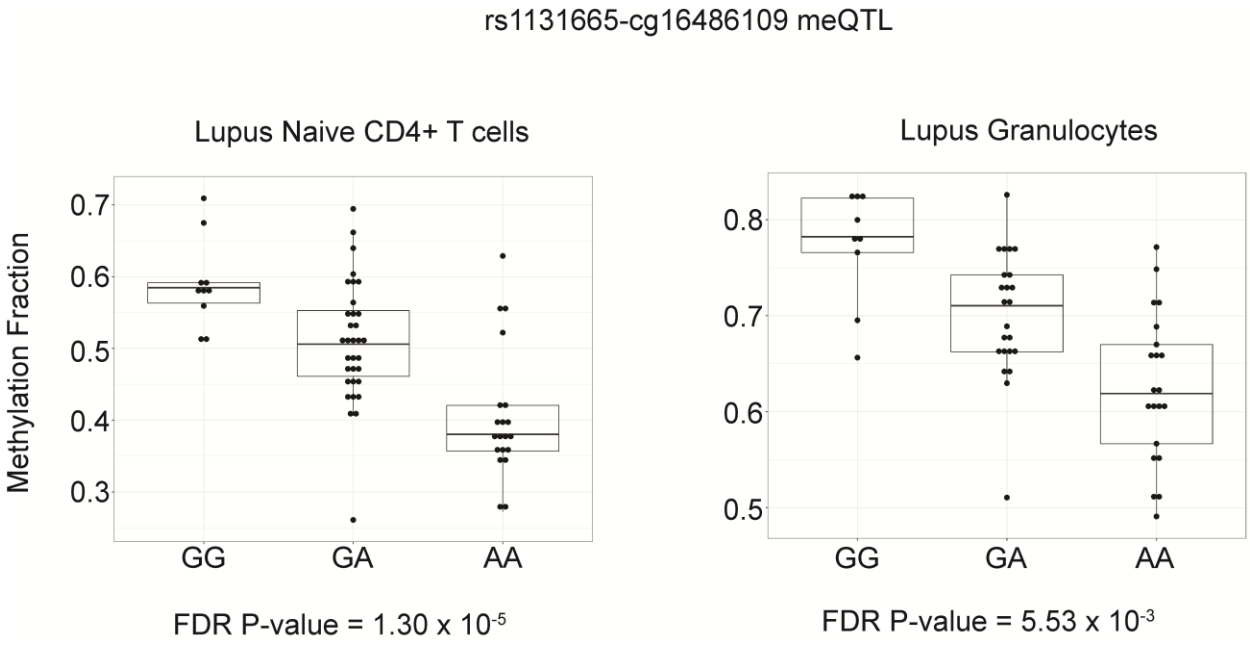

Figure 3-5: The presence of the lupus risk allele at rs1131665 has a significant negative correlation with DNA methylation of cg16486109 located in *IRF7*.

We examined the overlap of our meQTL-associated genes identified in lupus patient naïve CD4+ T cells and genes that respond to type I interferon treatment in CD4+ T cells to better understand the association between patient genetics and type I interferon-response gene

methylation differences in lupus. A total of 101 unique type I interferon-response genes were meQTL loci in our data (see Table A-2: “Supplementary Table 11”).

3.5 Discussion

We generated genome-wide DNA methylation data in naïve CD4+ T cells from a large cohort of lupus patients and matched healthy controls. Implementing an innovative trend deviation analysis, we identified a cluster of microRNAs (miRNAs) (miR-17, miR-18a, miR-19a, miR-19b1, miR-20a) among differentially methylated loci in lupus patients. Promoter methylation analysis revealed significant hypomethylation in this microRNA cluster in lupus patients compared to controls. Trend deviation analysis suggested a coordinated, disease-associated change in promoter methylation for these miRNAs. Indeed, the expression of miR-18a and miR-19b1 included within this cluster positively correlated with disease activity, as measured using SLEDAI score, in our lupus patients.

MiRNAs play an important role in post-transcriptional gene regulation by targeting specific complementary gene transcripts for degradation(393). Peripheral blood cells in lupus patients show altered expression of miRNAs(394). Some deregulated miRNAs in lupus target DNA methyltransferase 1 (DNMT1), and as a result, contribute to altered DNA methylation patterns in lupus CD4+ T cells(395-397). MiR-17, miR-18a, and miR-20a form the “miR-17 family” while miR-19a and miR-19b1 form the “miR-19 family”, which are grouped by sequence homology and encoded in a single polycistronic miRNA gene called the “miR-17-92 cluster”. This cluster has been well-studied as an oncogene and an immune regulator(398). Average promoter methylation of miR-17, miR-18a, miR-19a, miR-19b1, and miR-20a was reduced by ~5% in lupus patients compared to controls, which has not been previously described

in immune cells of lupus patients. Enterovirus 71 infection has been observed to suppress miR-17-92 cluster expression by increasing DNMT-mediated promoter methylation(399), and chemical inhibition of DNMT1 activity in bleomycin-induced lung fibrosis mouse model increases miR-17-92 cluster expression in lung fibroblasts(400). This suggests that miR-17-92 cluster promoter methylation plays an important role in regulating the expression of its members.

MiR-17-92 cluster genes play a vital role in regulating T cell activities including proliferation and differentiation. Overexpression of miR-17-92 cluster genes promotes lymphoproliferative disease and autoimmunity in mice by targeting critical immunotolerance regulators Bim and PTEN(401). Conditional knock out of miR-17-92 cluster in a murine model of chronic graft-versus-host disease (cGVHD) reduced disease-associated T cell infiltration and IgG deposition in the skin(402). In cGVHD mice, miR-17-92 cluster expression in CD4+ T cells supports Th1, Th17, and Tfh cell differentiation. Loss of miR-17-92 cluster expression leads to a corresponding reduction in Tfh-dependent germinal center formation and plasma cell differentiation(402). MiR-17, miR-18a, miR-19a, and miR-20a are overexpressed in splenic T cells of MRL/*lpr* mice(403). Similarly, miR-17, miR-17a, miR-18a, miR-19a, miR-19b1, and miR-20a are overexpressed in circulating CD4+ T cells of lupus patients(404). MiR-19b1 expression, specifically, has a significant positive correlation with disease activity as measured by SLEDAI score(404). MiR-17 and miR-20 are downregulated in circulating PBMCs(405), B cells(406), as well as circulating free miRNAs(407) of lupus patients compared to healthy controls, suggesting tissue-specific and miR-specific expression patterns. Of the miR-17-92 cluster miRNAs identified as differentially methylated in our analysis, only miR-18a and miR-19b1 showed a significant positive correlation between median expression and disease activity in naïve CD4+ T cells of lupus patients, consistent with these prior observations. MiR-19b1

promotes proliferation of mature CD4+ T cells, Th1 differentiation and IFN-γ production, and suppresses inducible Treg differentiation(408). MiR-18a expression increases rapidly early on in CD4+ T cell activation(409, 410), and suppresses Th17 cell differentiation through direct targeting of critical Th17 transcription factor transcripts including Smad4, Hif1a, and Rora in human CD4+ T cells in vitro and in vivo murine airway inflammation models(409). We did not observe a difference in the expression of members in the miR-17-92 cluster between lupus patients and controls in naïve CD4+ T cells, likely because these miRNAs are upregulated upon T cell activation. Evidence for hypomethylation in lupus in naïve CD4+ T cells suggests epigenetic priming of this locus, similar what we previously observed in interferon-regulated gene loci in lupus(264). Further study is needed to determine if altered DNA methylation at these miRNA promoter sites is associated with expression changes in miRNAs that play a role in T cell development and lupus pathogenesis and their potential use as a biomarker for monitoring disease activity.

We used analysis of meQTL to identify allele-specific DNA methylation associations across the genome of naïve CD4+ T cells from lupus patients and healthy controls. Our primary objective was to understand to what extent are DNA methylation changes associated with lupus (the lupus-defining epigenetic profile), explained by genetic factors. We found that < 1% of differentially methylated sites in lupus patients compared to healthy controls were associated with a *cis*-meQTL. This suggests that almost all the DNA methylation alterations observed in lupus are not associated with local allelic differences in the genome, suggesting a greater contribution from non-genetic and possibly environmental factors to epigenetic dysregulation in lupus. A previous study of meQTL in whole blood of lupus patients found that a majority of meQTL were shared between patients and controls(73). We observed 68% of meQTL in lupus

patients and 54% of meQTL in healthy controls were shared by both groups, supporting this observation. Gene ontology and pathway analysis of the meQTL-associated genes unique to lupus patients were enriched for KEGG pathways related to type I diabetes, viral myocarditis, and graft-versus-host disease all of which were primarily driven by the presence of meQTL in HLA genes. Gene ontologies were related to the development of various tissues without an apparent relationship to disease pathogenesis. Of note, we did not observe meQTL effect involving the miR-17-92 cluster.

Our prior analysis of neutrophils from a cohort of lupus patients identified overlap in meQTL genes and lupus genetic risk loci(373). MeQTL pairs including *ARID5B* (cg13344587-rs10821936), *HLA-DQB1* (cg13047157-rs9274477), and *IRF7* (cg16486109-GSA-rs1131665) were found in both neutrophils and naïve CD4+ T cells from lupus patients. Risk loci genes unique to naïve CD4+ T cell meQTL included *CD80* (cg06300880-GSA-rs3915166), *TYK2* (cg06622468-rs280501), *IKBKE* (cg22577136-GSA-rs17020312), and *CTLA4* (cg05092371-GSA-rs16840252, cg05092371-rs4553808). Naïve CD4+ T cell-specific meQTL risk loci genes are all related to signal response and activation in CD4+ T cells compared to the more general DNA repair and type I interferon signaling seen in the shared meQTL risk loci genes. Disease-relevant meQTL show tissue-specific patterns which should be considered when teasing apart their potential impact.

We identified three meQTL that include SNPs previously identified as lupus genetic risk variants. One meQTL is in the complement factor B gene *CFB* (cg16505946-rs558702) where the risk allele is associated with increased DNA methylation of the nearby CpG site. Complement factor B (CFB) combines with C3 to form the C3 convertase after cleavage by complement factor D as part of the alternative complement pathway. Complement pathway

defects have long been studied as a model of monogenic lupus and contribute to increased risk of lupus in the general population(389). We identified an additional meQTL that included a known lupus risk variant in *IRF7* (cg16486109-rs1131665). Rs1131665 is a missense variant in the inhibitory domain of IRF7 (Q412R). This lupus-associated amino acid change was demonstrated to enhance IRF7-induced expression response in a luciferase reporter assay(411). This same risk allele is also associated with decreased DNA methylation of cg16486109. Though the relative DNA methylation fractions are different between naïve CD4+ T cells and granulocytes of lupus patients, the direction of the allele-specific DNA methylation is the same. This suggests that the observed meQTL effect may be present in other lymphoid and myeloid tissues, potentially including plasmacytoid dendritic cells, which are major producers of type I interferons. This is the first time a direct association between a lupus risk allele and local hypomethylation of a CpG site in *IRF7* has been described in lupus. This observation provides new insights regarding possible biological mechanisms underlying pathogenic consequences of lupus-associated genetic polymorphisms.

In summary, we investigated genome-wide DNA methylation changes in naïve CD4+ T cells from an extended cohort of lupus patients and controls, and using a methylation trend deviation analysis method, we showed promoter hypomethylation of the miR-17-92 cluster that has a significant regulatory function in T cells growth, function, and differentiation. Combining genome-wide DNA methylation and genotyping data, we were able to determine genetic contribution to the lupus-defining epigenotype. We identified allele-specific DNA methylation associations involving lupus genetic risk loci, including *CFB* and *IRF7*, which could contribute to our knowledge of how these variants confer risk. Our data indicate that epigenetic changes characteristic of lupus are not under direct genetic influence. This suggests a more important role

for non-genetic factors in the epigenetic dysregulation observed in lupus patients, including the robust demethylation of interferon-regulated genes.

Chapter 4 Conclusions and Future Directions

DNA methylation and genetics influence the pathogenesis of lupus and the cellular mechanisms underlying the disease. In these chapters, we explored DNA methylation changes within lupus granulocytes and naïve CD4+ T cells and the influence of genetics on DNA methylation changes. To accomplish this, we generated genome-wide data of the DNA methylome and genotypes to address the role of genetics in regulating DNA methylation differences between lupus patients and controls and within lupus patients. In chapter two, we analyzed DNA methylation in lupus patients over time and across disease activity levels, and how genetic factors contribute to the DNA methylation differences between African-American and European-American lupus patients. In chapter three, we utilized DNA methylation and genotyping data from naïve CD4+ T cells of lupus patients and matched healthy controls to answer how much of the observed DNA methylation associated with lupus is determined by genetic factors.

We expand upon our current understanding of the role of epigenetic dysregulation in lupus by measuring DNA methylation changes over time and across disease activity in lupus patients followed longitudinally. From this analysis, we provided a major finding that the DNA methylome of lupus is predominantly stable over time. Early studies of longitudinal methylation showed that global DNA methylation within individuals changes over time, at least on the order of decades, and that these changes had the greatest similarities within families, suggesting a genetic influence on the shape of the epigenome(412). The stability of the DNA methylome

suggests that disease-associated epigenetic shifts we see in lupus could be set earlier in the disease process or perhaps outside of the periphery in the bone marrow during immune cell development. This has implications for utilizing the DNA methylation signature for disease monitoring in lupus patients. Larger cohorts and fine-mapping of the methylome across multiple cell types in longitudinal cohorts would be needed to confirm our findings. Comparing the DNA methylome of immune progenitor cells in the bone marrow would also be of interest to determine if these epigenetic changes develop early in the disease process in lupus. A large prospective lupus cohort with uniform sampling of immune cells longitudinally would be the most beneficial next step towards generating a more complete picture of the lupus DNA methylome over time. Combining this with environmental exposure data prior to points where the DNA methylome acquires the type I interferon hypomethylation signature could reveal environmental factors that are contributing to lupus and resulting in associated DNA methylation changes.

Our differential DNA methylation data were integrated with meQTL analysis to tease out what race-specific epigenetic effects might be associated with genetic determinants. Approximately 16% of the differential methylation observed between African-American and European-American lupus patients is associated with a nearby SNP. Forty-two percent of the differential DNA methylation we observed in granulocytes between African-American and European-American lupus patients was also present between African-American and European-American healthy individuals. Similarly, a prior study we performed in T cells found that a majority of lupus-related genes that were differentially methylated between African-American and European-American healthy controls were also differentially methylated between the two races in lupus patients(288). The sites that could likely yield the most interesting discoveries are

those that are only differentially methylated between races in lupus patients and not in controls. CpG sites that are differentially methylated between races in both patients and controls will require more scrutiny and likely contribute to a greater lupus risk in African-Americans. Those differentially methylated sites that are not associated with meQTL could reveal more about how social and environmental factors influence methylation and disease risk. The 42% of the race-associated DNA methylation signature that is shared between patients and controls in African-Americans compared to European-Americans is potentially already present prior to the onset of lupus. Patients would likely have a partial epigenotype similar to that of the healthy background population, and perturbations (like environmental exposures) set them on a different trajectory towards a stable disease epigenotype. Race and ethnicity are social constructs that simplify complex factors like genetic ancestry and shared environments, and the role of non-genetic factors should not be discounted. A study comparing DNA methylation between subgroups of Latino individuals found that genetic ancestry explained 75% of the DNA methylation variation between subgroups and suggested that the remaining 25% was attributable to environmental and social factors(285). In addition, the study demonstrated that even environmentally-influenced DNA methylation changes can interact with genetic ancestry, underlining the importance of taking genetics into account when performing studies on DNA methylation in racially and ethnically diverse cohorts(285).

We found that our ancestry-associated differential methylation in lupus granulocytes included genes in both the NFκB and interferon response pathways like *IRF7*, suggesting that these pathways are influenced in part by genetic ancestry in both the healthy and lupus population. African ancestry is associated with a stronger inflammatory response to bacterial infection by macrophages, more effective suppression of bacterial replication, and enrichment

for genes associated with autoimmune diseases compared to European ancestry(413). Seventy-five percent of the ancestry-associated infection response gene signature was associated with a *cis*-eQTL(413). These inherited differences in inflammatory genes may confer an increased risk for autoimmunity throughout life including changes in DNA methylation regulation in healthy individuals, and the contribution of other genetic and non-genetic factors are the final push required for a person to develop lupus. It is likely that other inflammatory states, similar to lupus, may overlap with similar pathways and include DNA methylation as an intermediary factor in the eQLT relationship. More work is needed to tease apart the full extent of how genetic ancestry and race/ethnicity-related environmental factors might predispose some individuals to lupus and not others. Changes in DNA methylation may provide a mechanistic explanation for how lupus-associated genetic polymorphisms confer increased disease risk in patients. We identified meQTL in the naïve CD4+ T cells and granulocytes of lupus patients that included, or were in high LD with, a lupus risk variant. This further extends our knowledge about how genetic-epigenetic interaction might be mechanistically involved in explaining genetic risk in autoimmunity. A better understanding of lupus genetic risk loci and the epigenetic environment surrounding them, such as our findings that *IRF7* is associated with an meQTL and is differentially methylated between races, will likely yield the most relevant discoveries in the future.

We show that approximately 1% to 8% of differential methylation between lupus patients and controls was attributable to local *cis*-meQTL, in naïve CD4+ T cells and granulocytes, respectively. This result suggests that much of the DNA methylation difference we detect between lupus patients and controls is not controlled by local genetic effects. One explanation is that consistent hypomethylation in type I IFN response genes and others in lupus patients may

not include meQTL because they are being coordinated by key upstream transcription factors. A deleterious SNP at a key regulatory factor could induce a widespread change in promoter DNA methylation and gene expression in genes that are not associated with an meQTL. *IRF7* may fit this model. *IRF7* has an meQTL (cg16486109-rs1131665) that is present in the granulocytes of lupus patients and naïve CD4+ T cells of both lupus patients and controls. When comparing lupus patients and controls, there is a distinct hypomethylation of *IRF7* that is in line with the overall interferon response signature. The *IRF7* lupus risk alleles may predispose individuals in the population towards developing lupus potentially through DNA methylation changes as an meQTL. Indeed, the SNP rs1131665 in *IRF7* is associated with susceptibility to lupus in multiple populations(411). The allele that is associated with increased lupus risk is also associated with decreased methylation of cg16486109 in the granulocytes and naïve CD4+ T cells of lupus patients. The genetic risk in combination with other triggering factors (additional genetic risk loci or environmental) subsequently induces a stable hypomethylation of the *IRF7* locus. Upon exposure to an inflammatory stimulus, perhaps oxidative stress from a viral infection or ICs resulting from increased NETs, IRF7 promotes the expression of type I IFNs and downstream hypomethylation of IFN response genes. This type of model may be better captured as a *trans*-meQTL where the variant of the regulatory factor is associated with the methylation of target genes which may range across the entire genome. A more thorough analysis of the *IRF7* locus and its genetic-epigenetic associations in lupus are required to support this putative model.

We show that granulocyte DNA methylation is predominantly stable within individuals across time and disease activity. Prior studies of inter-individual DNA methylation in granulocytes found the least variability among gene promoters while gene bodies carried the most variation(414). The most variable gene promoters were enriched for PU.1 (encoded by

SPI1) binding sites, a major transcription factor that regulates neutrophil development(414). Notably, the *TREML4* promoter region we identified as containing an meQTL, and that regulates gene expression in response to DNA methylation, overlaps with a PU.1 binding site in HL-60 cells(415). PU.1 is a pioneering transcription factor for neutrophils, one that can coordinate the binding of complexes that regulate the activation or suppression of genes(416, 417). PU.1 can interact directly with DNMT3A, DNMT3B, and TET2 during myeloid development and differentiation, suggesting that it plays a role in regulating DNA methylation around its binding sites(418, 419). The *TREML4* meQTL we have identified could serve as model for how genetics can directly modify a disease-relevant immune gene (*TREML4*) through disruption of transcription factor binding and promoter function that is reflected in, or potentially mediated by, DNA methylation. Measuring *TREML4* promoter-enhancer interactions could reveal more about this relationship. Our experiments overexpressing *TREML4* in PLB-985 cells did not modify the TLR7 response. This could be due to the differentiated PLB-985 phenotype being a poor model for primary neutrophil function. Other myeloid tissues may provide better models for testing the importance of this *TREML4* meQTL *in vitro*. Plasmacytoid dendritic cells are proficient producers of type I interferons in response to TLR7 signaling during viral infection. There is a positive correlation between TLR7-mediated type I IFN production by pDCs and disease activity in lupus patients(420). These cells would yield valuable information about the relationship of the *TREML4* and *IRF7* meQTL we identified in these chapters with type I interferon signaling.

A study analyzing the association of the meQTL-SNP and methylation site and *TREML4* expression in lupus immune cells would be an ideal next step. This would require identifying lupus patients with the different genotypes in the identified meQTL-SNP and measuring *TREML4* expression and promoter methylation in granulocytes. Phenotypic changes in these

cells related to TLR7 signaling, such as increased type I interferon production and propensity for NETosis, could also be measured using the same cells.

Our study does have limitations. The under-representation of severe lupus flares in our population does limit our ability to detect longitudinal DNA methylation changes that might be more apparent in a larger cohort. The 1000bp window we selected to define the *cis*-meQTL relationship in our study is relatively narrow leading to an under-reporting of *cis*-meQTL associations though other studies have found that the significance of the meQTL association increases with decreasing distance between the SNP and CpG site(421). We elected to use a narrow meQTL window to focus on more direct genetic determinants of DNA methylation changes in our studies. One study using a 6kbp window to define *cis*-meQTL estimated a median distance of 76bp for meQTL effects, and that 87% of CpG sites were within 3kbp of the associated SNP in lymphoblastoid cells(422). Another shortcoming is that the Infinium arrays only capture a small proportion of the approximately 28 million CpG sites across the genome. Increasing the density of the DNA methylome assessment in lupus patients could reveal additional information.

The findings presented in this thesis have broadened our understanding of the associations of genetics and DNA methylation in lupus patients. Epigenetic mechanisms provide an interesting avenue for biomarker and treatment development. Directly targeting the DNA methylome through DNMT inhibition has traditionally required the use of nucleoside analogs like 5-azacytidine, though this leads to global demethylation and gene expression changes which can also lead to T cell dysfunction and autoimmunity(423, 424). Using these drugs in a tissue-specific manner can yield potential therapeutic results as has been demonstrated by targeted delivery to CD4+ T cells ameliorating lupus-like symptoms in MRL/*lpr* mice(280). Maturation of DNA methylation editing technologies may also provide a method for directly modifying the

methylation status of genes. Fusions of dead Cas9 and either DNMT or TET domains allow for selective methylation or demethylation, respectively. Cas9-targeted mutation of promoter CpG sites or selective promoter demethylation with dCas9-TET1 fusions can induce expression of genes with methylation-sensitive promoters(425). Another potential therapeutic model includes selective hypermethylation of CTCF binding sites using dCas9-DNMT3a that disrupt the CTCF-mediated looping required for enhancer-promoter activity and a subsequent reduction in gene expression(426). dCas9-DNMT-induced methylation could be applied to reverse the hypomethylation of the miR-17-92 promoter in lupus T cells to potentially suppress T cell activity during lupus flares. Further exploration of meQTL associations in lupus risk genes could provide more information on the biological mechanisms underlying the conferred risk. Mendelian randomization can measure the causal relationship of GWAS risk loci and DNA methylation on the occurrence of lupus and could further refine the meQTL we have identified and their relationship to genetic risk(427, 428). Furthermore, integration of gene expression with DNA methylation and genotyping data can further refine the causal effect of genotype on gene expression through mediation by DNA methylation.

Peripheral blood may not capture DNA methylation changes occurring in the tissue-infiltrating subset of immune cells in lupus patients. Comparing tissue-infiltrating cells of lupus patients with those in the periphery may reveal DNA methylation changes that contribute to the infiltrating phenotype reflecting the unique expression signatures found within myeloid and lymphoid cells in the kidneys of lupus patients(429). Advancements in single-cell DNA methylation techniques provide an avenue for exploring the DNA methylome in a cell-specific manner. This not only allows for analysis of rare cell subsets, such as tissue-infiltrating immune cells, but also overcomes the limitation of microarray and high-throughput sequencing that can

only provide DNA methylation averages at the population level that are influenced by cell population differences. Integrating single-cell DNA methylation data with other genomic approaches, such as gene expression or chromatin accessibility, and genotyping information, would give a clearer picture of how DNA methylation is related to genetic regulation in lupus immune cells.

This work has furthered our understanding of the role of genetics on DNA methylation in lupus patients. For the first time, we demonstrated the predominant longitudinal stability of the lupus epigenotype across time and disease activity status. Our analysis found nephritis-associated demethylation of a CpG site in *GALNT18* that could provide a useful biomarker. We also highlight the importance of considering race/ethnicity and genetic ancestry when performing DNA methylation studies. We found the methylation level of two CpG sites significantly associated with disease activity, but only in African-American not European-American lupus patients. We found approximately 16% of the differential methylation signature between African-American and European-American lupus patients to be associated with a nearby genetic polymorphisms as meQTL. *TREML4* provides an example of an inflammatory response gene with a race-associated meQTL where DNA methylation of the meQTL-associated gene promoter can impact gene expression and may contribute to disparity in disease severity. We compared DNA methylation of naïve CD4+ T cells between lupus patients and controls to address the contribution of genetics to the lupus epigenotype. Using a trend deviation analysis, we found that the promoter of the miR-17-92 cluster is hypomethylated in lupus patients, and that the expression of two members of this miRNA cluster positively correlated with disease activity. We found that approximately 1% to 8% of lupus-associated methylation differences are in a *cis*-meQTL. This suggested that there is little association between the robust hypomethylation of

type I interferon response genes characteristic of lupus and nearby genetic variants. A predominant role for non-genetic factors in the lupus DNA methylation signature is suggested. In addition, we found a subset of meQTL associated with known lupus genetic susceptibility loci that may provide insights into how genetics can confer disease risk through DNA methylation changes.

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Appendices

Appendix A Supplementary Data Resources

Chapter 2

Portions of this chapter have been previously published here:
Coit P, Ortiz-Fernandez L, Lewis EE, McCune WJ, Maksimowicz-McKinnon K, Sawalha AH. A longitudinal and transancestral analysis of DNA methylation patterns and disease activity in lupus patients. *JCI Insight*. 2020 Nov 19;5(22):e143654.

Supplementary tables can be found here: https://doi.org/10.1172/jci.insight.143654.

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Chapter 3

This chapter is available as a preprint manuscript in MedRxiv:
Coit P, Roopnarinesingh X, Ortiz-Fernandez L, Maksimowicz-McKinnon K, Lewis EE, Merrill JT, McCune WJ, Wren JD, Sawalha AH. Hypomethylation of miR-17-92 cluster in lupus T cells and no significant role for genetic factors in the lupus-associated DNA methylation signature. *medRxiv* 2022.02.21.22271293.

Supplementary tables can be found here:
https://www.medrxiv.org/content/10.1101/2022.02.21.22271293v1.supplementary-material

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Table A-1: Supplementary data tables for Chapter 2.

Table ID	Table Title in Publication
Supplementary Table 1	Top 50 differentially methylated sites in a longitudinal subset of n = 11 lupus patients (n = 7 African-American and n = 4 European-American) with nephritis and the nearest preceding or receding timepoint without nephritis using a mixed effects model.
Supplementary Table 3	DNA methylation sites differentially methylated between African-American (n = 22) and European-American (n = 32) lupus patients at initial timepoint.
Supplementary Table 4	Gene ontology and pathway analysis of genes hypomethylated in African-American lupus patients compared to European-American lupus patients ($\Delta\beta$ of < -10% and FDR-adjusted P-value < 0.05).
Supplementary Table 5	Gene ontology and pathway analysis of genes hypermethylated in African-American lupus patients compared to European-American lupus patients ($\Delta\beta$ of > 10% and FDR-adjusted P-value < 0.05).
Supplementary Table 6	DNA methylation sites differentially methylated in neutrophils isolated from healthy African-American (n=5) and European-American (n=6) controls (Infinium HumanMetylation 450 BeadChip array) and overlap with sites differentially methylated between ancestry groups in the lupus cohort.
Supplementary Table 7	*Cis*-meQTLs in cross-sectional analysis of n = 53 (n = 21 African-American and n = 32 European-American) lupus patients.
Supplementary Table 8	Gene ontology and pathway analysis of genes annotated to CpG sites in all *cis*-meQTL pairs genome-wide.
Supplementary Table 9	DNA methylation sites differentially methylated between all African-American (n = 22) and European-American (n = 32) lupus patients and associated with a SNP within 1000bp as a *cis*-meQTL.
Supplementary Table 10	*Cis*-meQTL pairs that have CpG-associated genes that include a previously identified lupus susceptibility locus.
Supplementary Table 11	Type I interferon-regulated genes containing a methylation sites differentially methylated between n = 54 African-American and European-American lupus patients.

Table A-2: Supplementary data tables for Chapter 3.

Table ID	Table Title in Publication
Supplementary Table 2	Differentially methylated CpG sites in naïve CD4+ T cells in lupus patients compared to healthy controls matched by age, sex, and ethnicity.
Supplementary Table 3	Top 10 most enriched Biological Process Gene Ontology terms in genes with differentially methylated promoter regions in naive CD4+ T cells of lupus patients compared to healthy controls.
Supplementary Table 5	*Cis*-meQTLs in naive CD4+ T cells of lupus patients (n = 63).
Supplementary Table 6	*Cis*-meQTLs in naive CD4+ T cells of healthy controls (n = 68).
Supplementary Table 7	*Cis*-meQTL present in both lupus patients and healthy controls.
Supplementary Table 8	Gene set enrichment analysis of genes associated with meQTL that are shared in naïve CD4+ T cells of lupus patients and healthy controls.
Supplementary Table 9	Gene set enrichment analysis of genes associated with meQTL that are unique to naïve CD4+ T cells of lupus patients.
Supplementary Table 10	MeQTL with a CpG-associated gene that are also genetic risk loci for lupus.
Supplementary Table 11	Type I interferon-regulated genes associated with a CpG site in an meQTL identified in naive CD4+ T cells of lupus patients.

Appendix B Chapter 2 Supplementary Information and Tables

TREML4 Promoter sequence: 5'-
TAAGCACCTGCAGGCTGGCTGGAAGGCATAAAGCAGCAGACATGGGGAGAAGTTCA
GTGAAAGGCGGCAGAGCAACACAGCAGGGCTGAGGTGCCCAGGCCCAGAGACAGA
CCCTGTAGCTGGTGGCCCCTTTCAAGGCCTGGCTGCCCCTCCTCCCCTGGTCTGCAG
AGCCCGCCCTCCCAGGCCGACCATTTCCCCAGCAAGACATTGACTTCCTCAATTTTA
CCAGCTGAGAGACACTGGGAACCTGGGGCAGAATCAGACCCAGCGTCTGACTCCTC
CTGAGAGGGCTCCCTTTTTTCTCCTCTCCTCCGCTGTCAGAAACAGATCTGGGCTGGA
ATGGCCTGGGGTGGGGTCCACACCTACTAGTTAAGCA-3'

Table B-1: Disease activity criteria and medications in a cohort of female European-American and African-American lupus patients at initial timepoint (n=54).

	European-American (n = 32)	African-American (n = 22)	P-value[#]
Age (years)(mean±SD)	40.9±13.0	40.1±14.0	0.8303
SLEDAI Criteria (%)			
Average SLEDAI Score (mean±SD)	2.9±3.2	5.2±4.5	**0.0325**
Seizure	0 (0%)	0 (0%)	-
Psychosis	0 (0%)	0 (0%)	-
Organic Brain Syndrome	0 (0%)	0 (0%)	-
Visual Disturbance	0 (0%)	0 (0%)	-
Cranial Nerve Disorder	0 (0%)	0 (0%)	-
Lupus Headache	0 (0%)	0 (0%)	-
Cerebrovascular Accidents	0 (0%)	0 (0%)	-
Vasculitis	0 (0%)	0 (0%)	-
Arthritis	5 (15.6%)	5 (22.7%)	0.7230
Myositis	0 (0%)	0 (0%)	-
Urinary Casts	0 (0%)	1 (4.5%)	0.4074
Hematuria	0 (0%)	1 (4.5%)	0.4074
Proteinuria	2 (6.3%)	4 (18.2%)	0.2111
Pyuria	1 (3.1%)	0 (0%)	1.0000
Rash	11 (34.4%)	8 (36.4%)	1.0000
Alopecia	0 (0%)	4 (18.2%)	**0.0231**
Mucosal Ulcers	3 (9.4%)	1 (4.5%)	0.6377
Pleurisy	1 (3.1%)	1 (4.5%)	1.0000
Pericarditis	0 (0%)	1 (4.5%)	0.4074
Low Complement	7 (21.9%)	10 (45.4%)	0.0814
Increased DNA Binding	8 (25%)	10 (45.5%)	0.1480
Fever	0 (0%)	0 (0%)	-
Thrombocytopenia	0 (0%)	0 (0%)	-
Leukopenia	1 (3.1%)	3 (13.6%)	0.2927
Medications			
Prednisone	21 (65.6%)	17 (77.3%)	0.5451
Hydroxychloroquine	26 (81.2%)	15 (68.2%)	0.3383
Cyclophosphamide	0 (0%)	0 (0%)	-
Azathioprine	3 (9.4%)	2 (9.1%)	1.0000
Methotrexate	1 (3.1%)	2 (9.1%)	0.5601
Cyclosporin	1 (3.1%)	0 (0%)	1.0000
Tacrolimus	0 (0%)	0 (0%)	-
Mycophenolate Mofetil	10 (31.3%)	5 (22.7%)	0.5509
Leflunomide	1 (3.1%)	0 (0%)	1.0000
IV Ig	0 (0%)	0 (0%)	-
Rituximab	0 (0%)	0 (0%)	-
Belimumab	1 (3.1%)	3 (13.6%)	0.2927

[#]Age and SLEDAI score P-values were calculated using a t test; all other P-values were calculated using a Fisher's exact test. All P-values are two-tailed. Bold P-values are $P < 0.05$.

Appendix C Chapter 2 Supplementary Figures

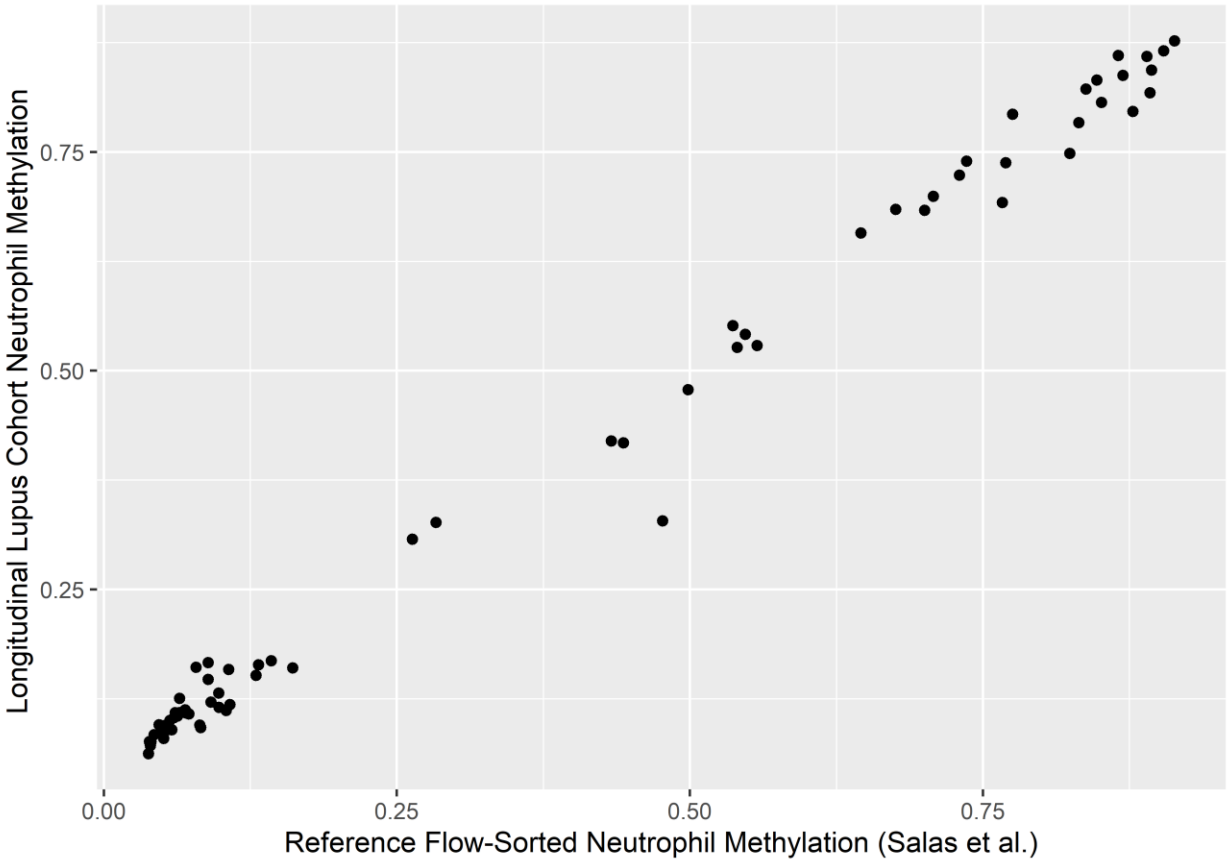

Figure C-1: DNA methylation of lupus patient samples is highly correlated with neutrophil-specific probes. Dot plot showing correlation of mean DNA methylation for 71 neutrophil-specific probes from purified cell populations in Salas *et al*(310) and mean DNA methylation of the same probes in our longitudinal lupus cohort (n = 229). Pearson's product-moment correlation test had a $r = 0.996$ and P-value < 2.2E-16.

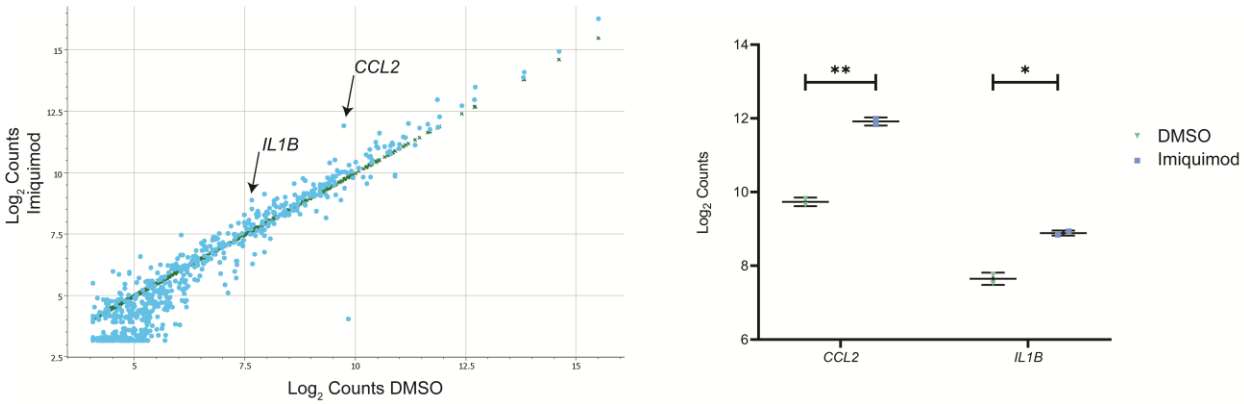

Figure C-2: CCL2 and IL1B expression increases in PLB-985 cells in response to TLR7 stimulation. PLB-985 cells were differentiated for four days using 1.3% DMSO. Treatment included the addition of 5ug/mL of imiquimod at day zero. Gene expression was measured on the nCounter Myeloid Innate Immunity Panel. *CCL2* (t = -19.3; P- value = 2.70E-3; FDR-adjusted P-value = 1) and *IL1B* (t = -9.76; P- value = 0.03; FDR-adjusted P-value = 1) were significantly increased with imiquimod treatment. * = P-value ≤ 0.05, ** = P-value ≤ 0.01. n = 2 independent replicate wells per group. Error bars on right panel represent standard deviation.

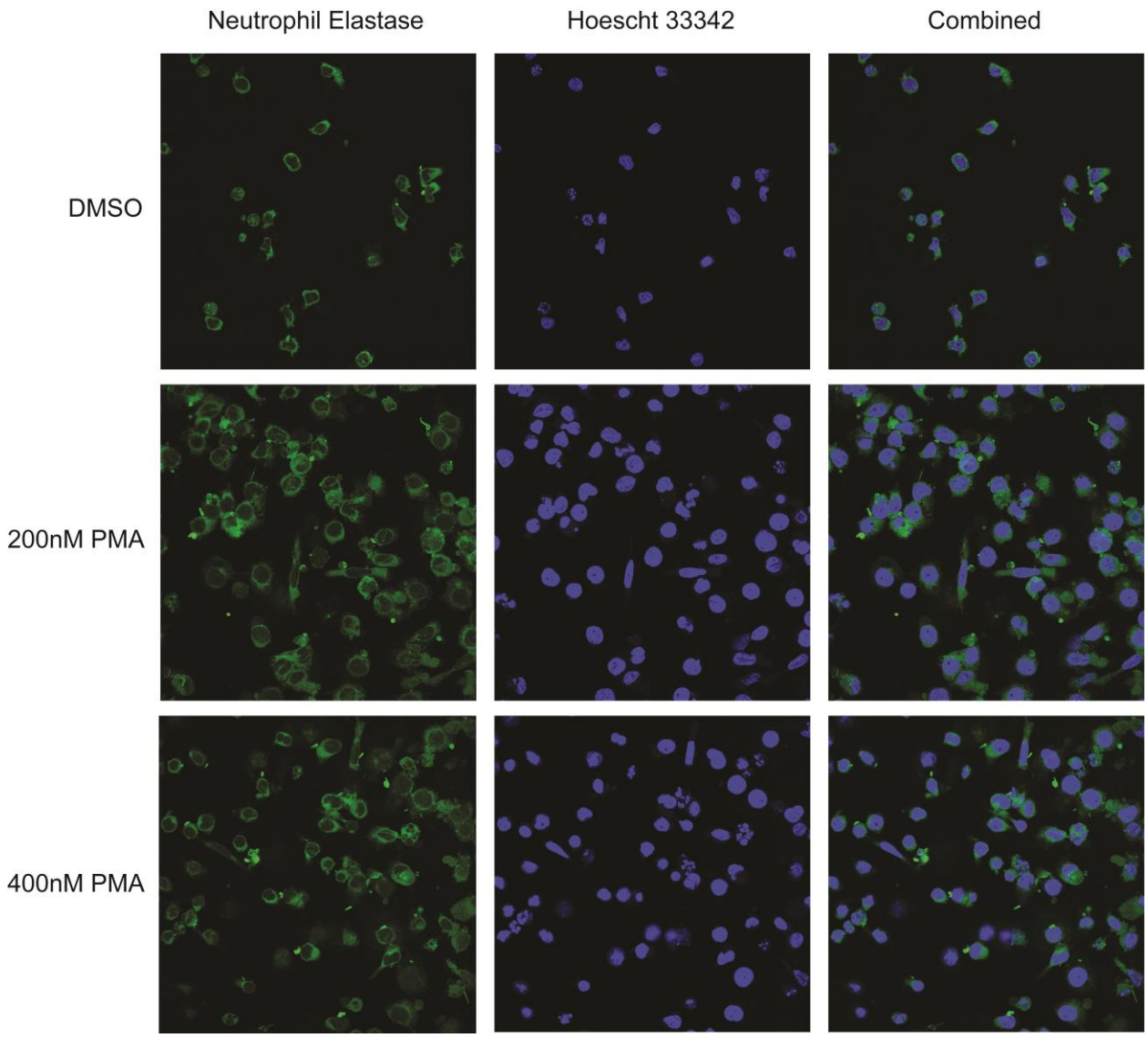

Figure C-3: PLB-985 NETosis Assay. PLB-985 cells were differentiated with 1.3% DMSO for six days. Cells were adhered to a poly-L-lysine coated coverslip and exposed to either 200nM or 400nM PMA for five hours followed by immediate fixation with 10% paraformaldehyde. Untreated cells were included as a control (DMSO). Fixed cells were stained with anti-human neutrophil elastase rabbit polyclonal antibody (1:100) and anti-rabbit Goat F(ab')2 IgG FITC-conjugated secondary antibody (1:200). DNA was stained using Hoescht 33342 (1:500) in blocking buffer. All images were taken using a 60x objective oil immersion lens on a Leica STELLARIS 5 fluorescence microscope.

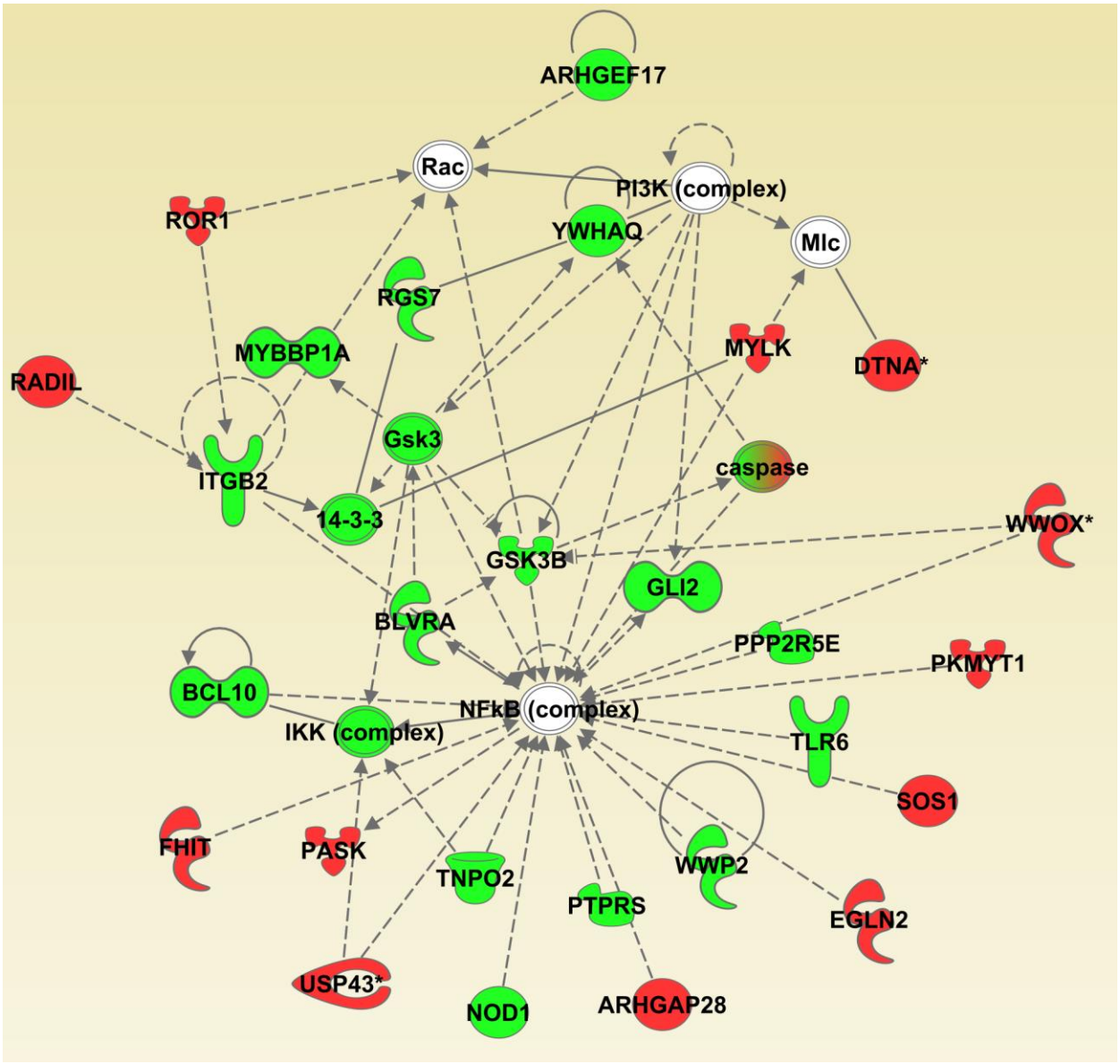

Figure C-4: NFκB-associated gene network of differentially methylated sites between African-American and European-American lupus patients. Networks of genes with hypomethylated (green shapes) and hypermethylated (red shapes) CpG sites in African-American compared to European-American lupus patients. White shapes represent genes included in the network by IPA through their relationship to the input genes. Green-red shapes included both hypo- and hypermethylated CpG sites. Dashed and solid lines represent indirect and direct interactions, respectively.

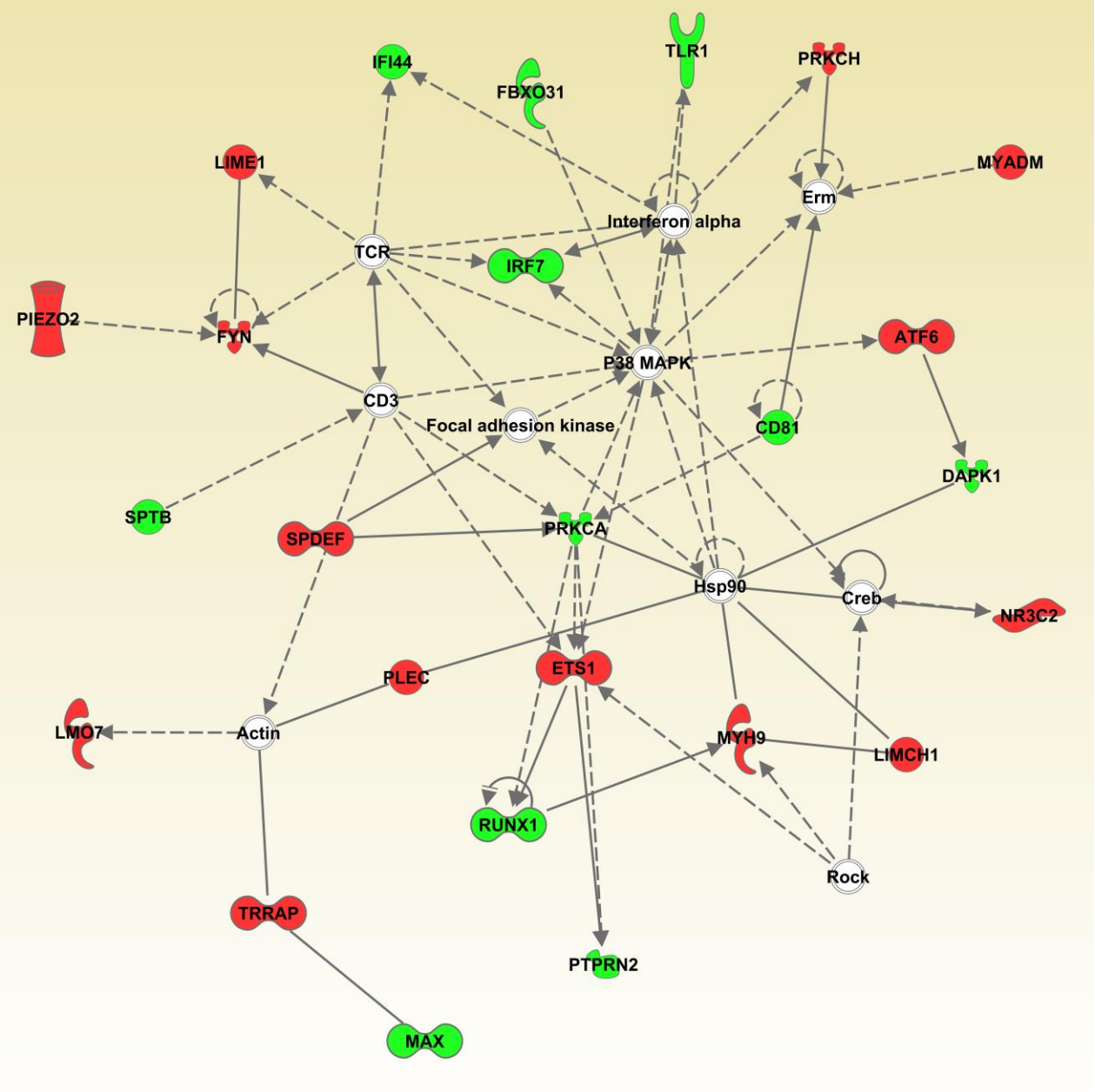

Figure C-5: Type I interferon-associated gene network of differentially methylated sites between African-American and European-American lupus patients. Networks of genes with hypomethylated (green shapes) and hypermethylated (red shapes) CpG sites in African-American compared to European-American lupus patients. White shapes represent genes included in the network by IPA through their relationship to the input genes. Dashed and solid lines represent indirect and direct interactions, respectively.

Appendix D Chapter 3 Supplementary Information and Tables

Table D-1: Demographics of lupus patients and healthy controls. All study participants were female and matched by age and ethnicity.

	Cases (n = 74)	**Controls (n = 74)**
Age (years)(mean±SD)	40.9±12.7	40.8±12.4
SLEDAI Score (mean)(range)	3.2 (0-18)	-
Race/Ethnicity (%)		
White	94 (63.5%)	
Black	44 (29.7%)	
Asian	6 (4.5%)	
Hispanic	2 (1.4%)	
Indian/Arabic	2 (1.4%)	
Medications (%)		
Prednisone	38 (51.4%)	-
Hydroxychloroquine	49 (66.2%)	-
Cyclophosphamide	0 (0%)	-
Azathioprine	13 (17.6%)	-
Methotrexate	1 (1.4%)	-
Cyclosporin	0 (0%)	-
Tacrolimus	0 (0%)	-
Mycophenolate Mofetil	26 (35.1%)	-
Leflunomide	0 (0%)	-
IV Ig	0 (0%)	-
Rituximab	0 (0%)	-
Belimumab	0 (0%)	-

Table D-2: Correlation of miR-17, miR-18a, miR-19a, miR-19b1, and miR-20a median gene expression in naïve CD4+ T cells with SLEDAI score in lupus patients (n = 16). Bolded rows represent miRNA's with a significant P-value.

MiRNA	Pearson correlation coefficient *r*	P-value
hsa-miR-17-5p	0.2652	0.3209
hsa-miR-17-3p	-0.000007643	>0.9999
hsa-miR-18a-5p	**0.5221**	**0.038**
hsa-miR-18a-3p	0.135	0.6182
hsa-miR-19a-5p	-0.3902	0.1351
hsa-miR-19a-3p	-0.1216	0.6538
hsa-miR-19b1-5p	**0.5125**	**0.0424**
hsa-miR-19b-3p	0.1919	0.4765
hsa-miR-20a-5p	0.1243	0.6464
hsa-miR-20a-3p	0.06115	0.822

Appendix E Chapter 3 Supplementary Figures

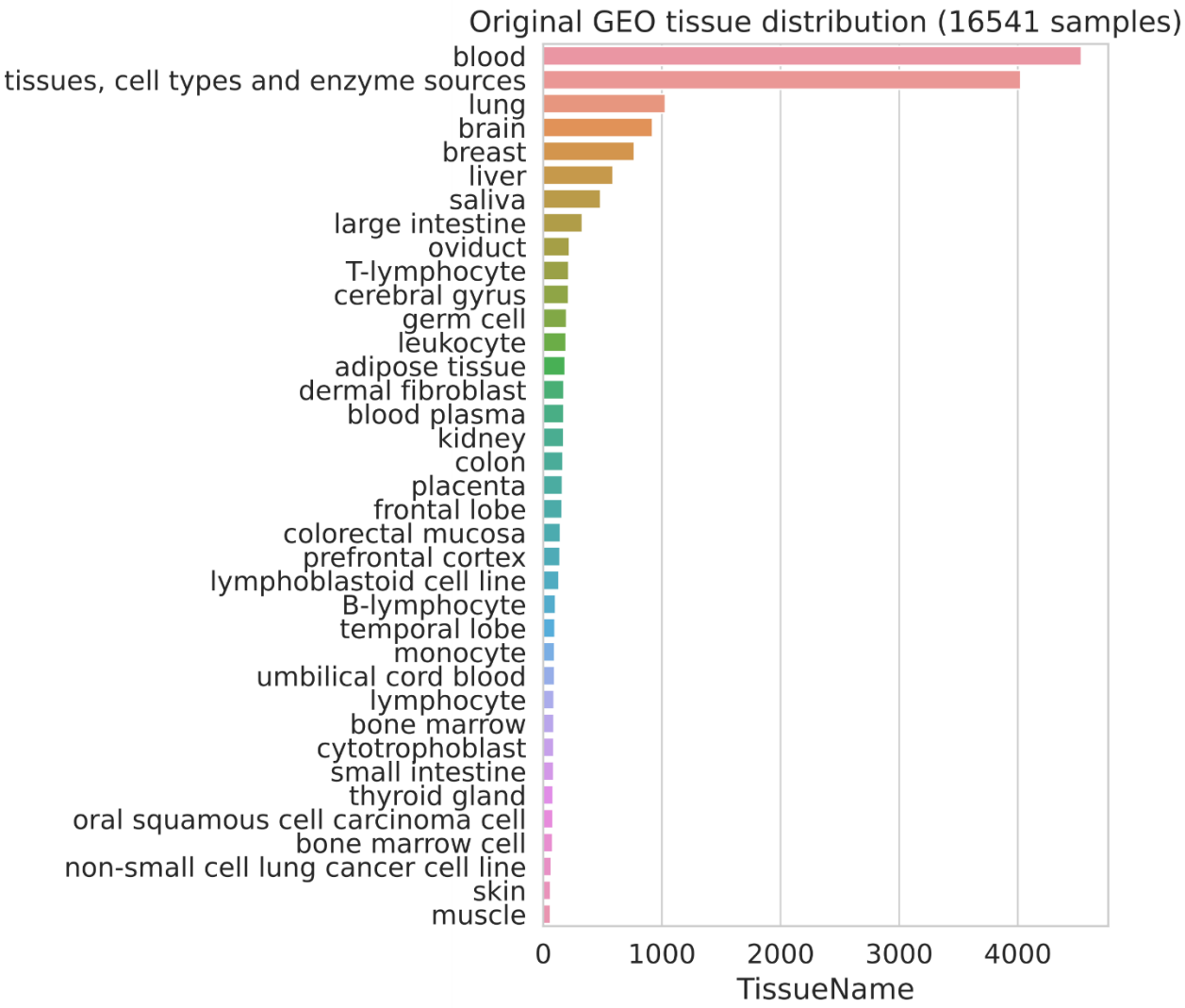

Figure E-1: Sample distribution of 16,541 Infinium HumanMethylation450 array samples across 37 tissues sourced from the Gene Expression Omnibus (GEO). These samples were used to develop a multi-tissue correlation network used for trend deviation analysis.