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Epigenetic Reprogramming in Naive CD4+ T Cells Favoring T Cell Activation and Non-Th1 Effector T Cell Immune Response as an Early Event in Lupus Flares

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Objective. **Systemic lupus erythematosus (SLE) is a relapsing autoimmune disease that affects multiple organ systems. T cells play an important role in the pathogenesis of lupus; however, early T cell events triggering disease flares are incompletely understood. This study was undertaken to examine DNA methylation in naive CD4+ T cells from lupus patients to determine if epigenetic remodeling in CD4+ T cells is an early event in lupus flares.**

Methods. **A total of 74 lupus patients with an SLE Disease Activity Index score of 0–18 were included. Naive CD4+ T cells were isolated from peripheral blood samples, and DNA was extracted for genome-wide methylation assessment. RNA was also extracted from a subset of patients to determine the relationship between epigenetic changes and transcription activity using RNA sequencing and microRNA arrays.**

Results. **We demonstrated that naive CD4+ T cells in lupus undergo an epigenetic proinflammatory shift, implicating effector T cell responses in lupus flare. This epigenetic landscape change occurs without changes in expression of the corresponding genes, poises naive CD4+ T cells for Th2, Th17, and follicular helper T cell immune responses, and opposes inhibitory transforming growth factor β signaling. Bioinformatics analyses indicate that the epigenetic modulator EZH2 might play an important role in shifting the epigenetic landscape, with increased disease activity in lupus naive CD4+ T cells. Further, the expression of microRNA-26a, which is sensitive to glucose availability and targets EZH2, was negatively correlated with disease activity in lupus patients.**

Conclusion. **An epigenetic landscape shift in naive CD4+ T cells that favors T cell activation and non-Th1 immune responses predates transcription activity and correlates with lupus activity. A role for EZH2 dysregulation in triggering lupus flares warrants further investigation.**

Systemic lupus erythematosus (SLE) represents a heterogeneous disease group characterized by the production of autoantibodies against cellular nuclear components. Lupus is a chronic relapsing disease, characterized by a disease course consisting of intermittent flares alternating with periods of relative disease quiescence in the majority of affected patients.

The etiology of lupus remains incompletely understood. A high concordance rate in monozygotic compared to dizygotic twins, familial aggregation, and differences in disease prevalence between populations suggest a strong etiologic genetic component in lupus. This has been demonstrated with a growing list of confirmed genetic susceptibility loci in lupus (1,2). More recently, a role for inherited epigenetic changes has also been demonstrated (3).

With the exception of epigenetic accessibility of interferon (IFN)–regulated genes in lupus (4) and inherited epigenetic changes that are directly influenced by genetic variants in the germline (3), DNA methylation changes in lupus T cells have been shown to be dynamic. Indeed, CD4+ T cells in lupus patients demonstrate a DNA methylation defect that is more pronounced in patients with active

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disease than in those with inactive disease (5). This is explained by defective ERK signaling and reduced expression of DNA methyltransferase 1 (DNMT-1) (6). Inducing a DNA methylation defect in T cells can result in a lupus-like autoimmune disease in vivo (7,8).

DNA methylation plays an important role in T cell differentiation and activation. Naive CD4+ T cells selectively undergo an epigenetic shift, rendering the *IFNG*, *IL4–IL5–IL13*, and *IL17* loci accessible for transcription upon differentiating into Th1, Th2, and Th17 cells, respectively (9). These key cytokine gene loci are heavily methylated and hence transcriptionally silent in naive CD4+ T cells prior to T cell activation or differentiation (9).

Because DNA methylation changes are generally dynamic and T cells play an important role in the pathogenesis of lupus (10), we sought to determine epigenetic changes in CD4+ T cells that correlate with disease activity in lupus patients. We focused on naive CD4+ T cells to understand the earliest T cell epigenetic changes in lupus flares that predate T cell activation. We performed RNA sequencing in the same cells to determine the relationships between epigenetic changes and transcription activity.

PATIENTS AND METHODS

Patient demographic characteristics and disease activity. Seventy-four female patients who had previously been diagnosed as having lupus were included in this study. All patients fulfilled the American College of Rheumatology classification criteria for SLE (11). The average age of the patients in the study was 41 years (range 18–66 years). An SLE Disease Activity Index (SLEDAI) score (12) was calculated at the clinical visit concurrently with enrollment in the study and blood draw. The SLEDAI scores of patients included in this study ranged from 0 to 18, with a mean of 3 and a median of 2. There was no difference in age between patients with active disease (SLEDAI $\geq$5) and patients with less active disease (SLEDAI $<$5) ($P = 0.21$). Of the 74 patients, 49 were taking hydroxychloroquine, 26 were taking mycophenolate mofetil, 13 were taking azathioprine, and 39 were taking glucocorticoids of various doses at the time of sample collection. One patient was taking methotrexate, and no patients were receiving cyclophosphamide, cyclosporine, leflunomide, tacrolimus, rituximab, belimumab, or intravenous immunoglobulin. Patients were recruited at the Oklahoma Medical Research Foundation, University of Michigan Health System, and Henry Ford Health System. The institutional review boards at the participating institutions approved this study. Written informed consent was obtained from all participants prior to enrollment.

Naive CD4+ T cell isolation and purity. Naive CD4+ T cells were isolated from whole blood by negative selection using a naive CD4+ T cell Isolation Kit II, human according to the recommendations of the manufacturer (Miltenyi Biotec). This kit uses an antibody cocktail to directly label the surface of undesired cells which are then bound to magnetic beads, allowing unlabeled naive CD4+ T cells to be separated. The purity of the isolated populations was confirmed using surface protein staining and flow cytometry as CD3+, CD4+, and CD45RA+ using human fluorescein isothiocyanate–conjugated human anti-CD3, human phycoerythrin (PE)–conjugated anti-CD4, and human Pacific Blue–conjugated anti-CD45RA antibodies (BioLegend). Cell population purity for all samples included in this study was >95%.

DNA and RNA extraction. Genomic DNA was extracted from isolated naive CD4+ T cell populations using the DNeasy Blood and Tissue Kit (Qiagen). Total RNA was extracted simultaneously by lysing cells in TRIzol reagent (Thermo Fisher Scientific) and then purified using Direct-zol RNA MiniPrep Kit (Zymo Research) according to the manufacturer's instructions.

DNA methylation assessment and analysis. Five hundred nanograms of genomic DNA was bisulfite converted with an EZ DNA Methylation kit (Zymo Research) and used as input in an Infinium HumanMethylation450 BeadChip kit (Illumina). Initial processing of DNA methylation data was conducted using GenomeStudio Methylation Module software (Illumina), as previously described (13). All samples passed array quality control measures prior to analysis. Probe fluorescence intensity values were normalized for all samples. An average methylation value was calculated for each probe, represented as a beta value for all samples, and used for subsequent analyses.

Bioinformatics analyses of DNA methylation data. Methylation data were preprocessed using methylumi v2.16.0 R package (14). Potential confounding factors were examined, and the race effect was removed using the ComBat function from sva v.3.18.0 R package (15). Beta values for each methylation site were median-averaged for each level of SLEDAI score, and correlation was assessed using Pearson's correlation coefficient. Adjusting for medication use had a negligible effect on beta values, with results almost identical to unadjusted data, and the correlations with SLEDAI scores were also almost identical to the unadjusted analysis. Methylation sites that correlated with the SLEDAI score at a P value of less than 0.01 were selected. Annotation of the locations of methylation sites was performed using ChIPseeker v.1.7.4 R package (16). Mapping between methylation sites and genes was performed using an annotation file provided by the manufacturer. Functional enrichment analysis in Gene Ontology categories and KEGG canonical pathways was performed using GOStats v.2.36.0 R package (17).

Chromosome-centric and transcription factor binding sites enrichment analyses were performed using GenomeRunner (18). Briefly, for chromosome-centric enrichment analysis, genomic locations (hg19) of methylation sites that positively or negatively correlated with SLEDAI scores were tested for enrichment within each chromosome using Fisher's exact test. For transcription factor binding site enrichment analysis, genomic locations of 161 transcription factors were extracted from the *wgEncodeRegTfbsClusteredV3* supertrack from the UCSC genomic database (19). Genomic locations of all methylation sites on the 450K array were used as the reference point to calculate the statistical significance of all genomic feature enrichments identified. All reported P values were corrected for multiple comparisons using the false discovery rate (20).

A literature analysis was conducted using IRIDESCENT (21), which uses a thesaurus of concepts (genes, diseases, phenotypes, chemicals, ontology categories, etc.) to identify terms associated with these concepts within Medline records. Co-mentions of terms are summed, with sentence co-mentions receiving a weight of 0.8 and abstract co-mentions receiving a

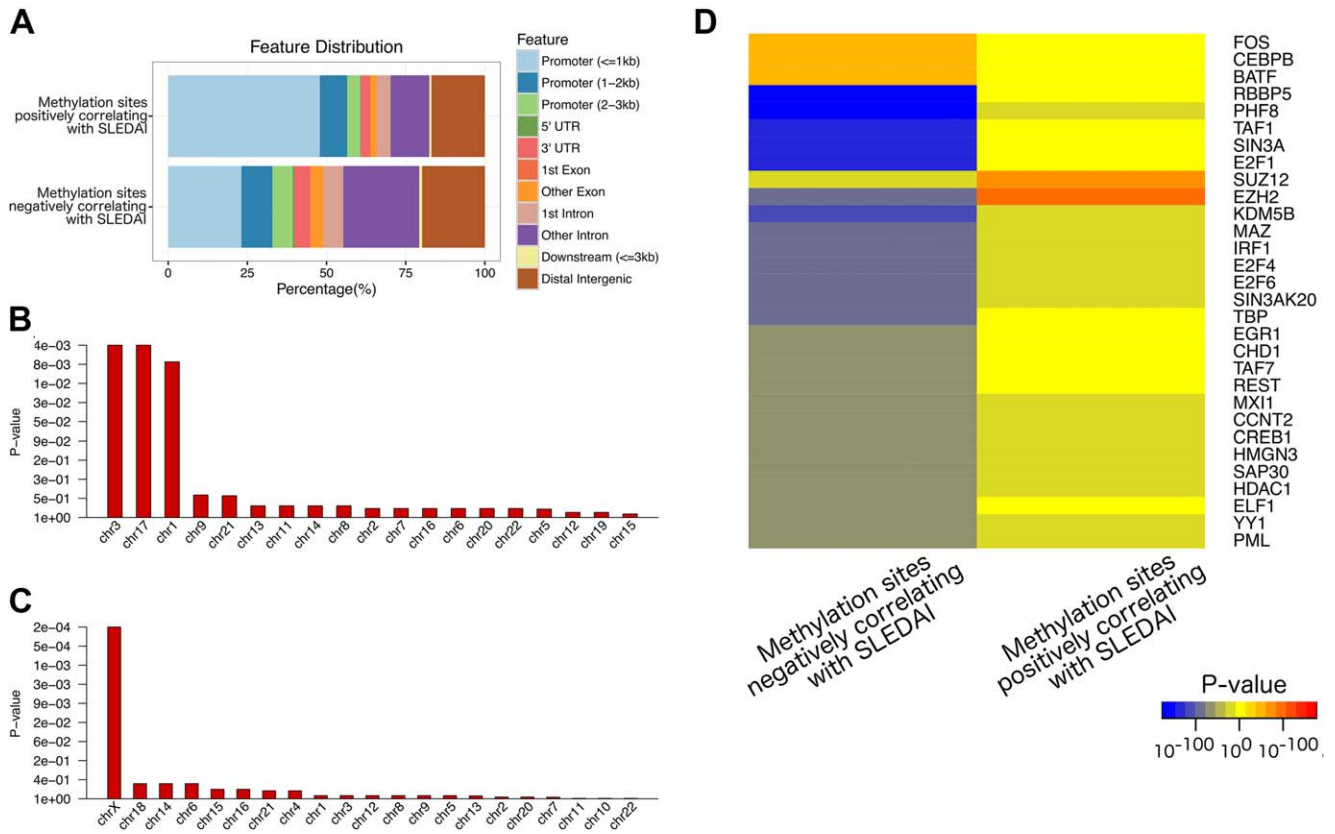

Figure 1. **A,** Annotation of the locations of methylation sites that positively correlate with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores (top) and negatively correlate with SLEDAI scores (bottom) in lupus patients relative to transcription start sites and gene structures. 5′-UTR = 5′-untranslated region. **B** and **C**, Enrichment, determined by chromosome analysis, of DNA methylation sites negatively correlated (**B**) and positively correlated (**C**) with disease activity as measured by SLEDAI scores in lupus patients. False discovery rate–corrected enrichment *P* values are shown on the y-axis; chromosome numbers, arranged in order of most to least enrichment, are shown on the x-axis. **D**, Heatmap of transcription factor binding sites (shown on the y-axis) enriched in DNA methylation sites negatively and positively correlated with SLEDAI score (shown on the x-axis). Color gradient indicates the significance of depletion (blue) and enrichment (red). The top 30 transcription factors that were most differentially enriched between the 2 sets of methylation sites are shown.

weight of 0.5. Weights are based on previous studies of the probability that a co-mention refers to a nontrivial relationship. The top 100 loci with the strongest correlations with SLEDAI were then analyzed, with 66 of the positively correlated loci being proximal to a gene and 69 of the negatively correlated loci being proximal to a gene. Gene lists along with associated concepts were analyzed with IRIDESCENT for their connectedness. Unconnected genes are not shown in order to reduce clutter.

RNA sequencing and analysis. A subset of 12 of the patients enrolled in the study had total RNA extracted for messenger RNA (mRNA) sequencing. These patients had an average age of 42 years and mean and median SLEDAI scores of 3 and 2.5, respectively. A sequencing library was created from purified total RNA using a TruSeq Stranded mRNA library kit (Illumina), and 100-bp, single-end mRNA reads were sequenced on a HiSeq 2500 sequencer (Illumina). Sequenced reads were cleaned using Trimmomatic 0.32 preprocessing workflow and then mapped to the human genome (hg19) with SHRiMP (version 2.2.3) (22,23). Aligned reads for each gene within each sample were scaled to the number of fragments in each sample library as counts per million (CPM) for gene i of sample j using the equation $CPM_{ij} = \text{Aligned counts}_i/\text{Total fragments}_j \times 10^6$. The CPM values were used for subsequent analyses. Correlation analysis with SLEDAI scores was assessed using Pearson's correlation coefficient and median-averaged expression values at each SLEDAI score level.

MicroRNA microarray analysis. MicroRNA expression levels in a subset of lupus patients (n = 16) were assessed using an Affymetrix miRNA 4.1 Array Strip. This array covers >2,000 premature and 2,500 mature human miRNA sequences. Total RNA was isolated from naive CD4+ T cells from lupus patients as described above. RNA sequences were first polyadenylated, then ligated to a biotin-labeled oligomer using a FlashTag Biotin HSR RNA Labeling Kit (Affymetrix). Biotin-labeled sequences were hybridized to array probes, washed, and stained with streptavidin–PE. Biotin–streptavidin–PE fluorescence was measured and used for analysis in Affymetrix

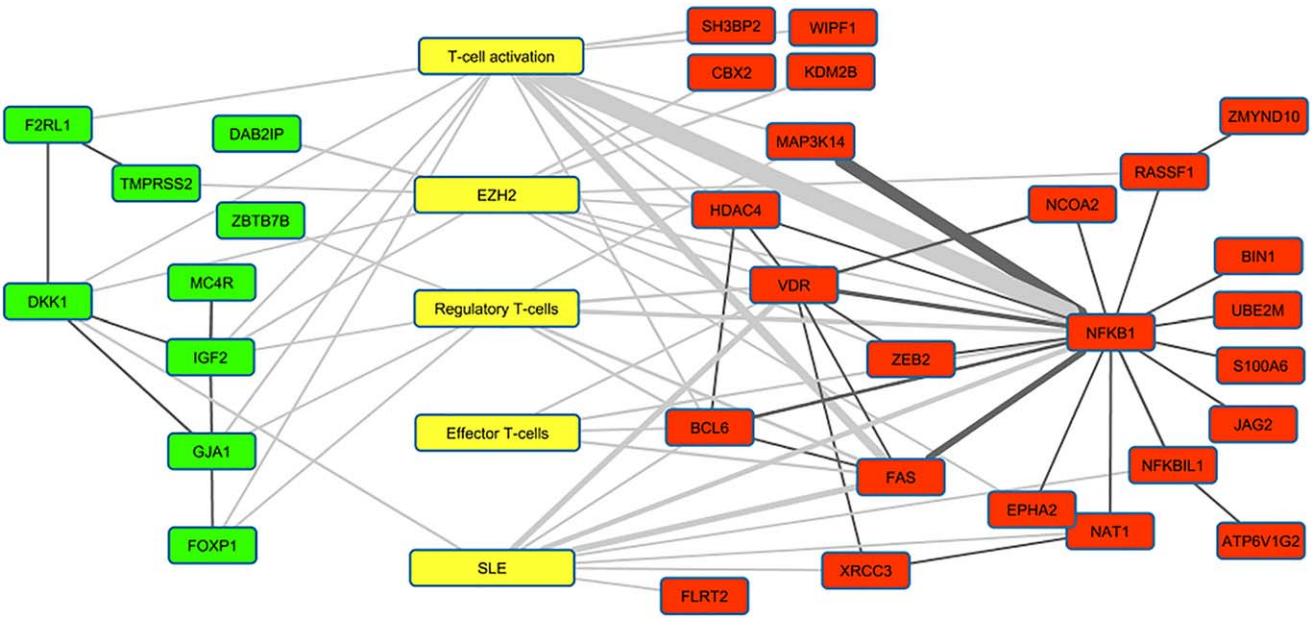

Figure 2. Findings of a literature analysis of how concepts (yellow) are connected to genes whose methylation status is positively correlated with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores (green) or negatively correlated with SLEDAI scores (red). The top 100 positively and negatively correlated methylation sites were included in this analysis. The existence of a line indicates that the 2 nodes appear together in Medline titles or abstracts at least 3 times, while the thickness of the line correlates with frequency of the 2 terms being co-mentioned. Gray lines show links between concepts and genes; black lines show links between genes.

Expression Console & Transcriptome Analysis Console 2.0 software. All samples passed signal intensity, polyadenylation, and ligation quality control measures. Signal intensities were background adjusted and normalized, and a $\log_2$-transformed expression value for each probe set was calculated using a robust multiarray average model (24). These expression values were used for subsequent analyses. Correlation between microRNA-101 (miR-101) and miR-26a expression and SLEDAI scores was assessed using Pearson's correlation coefficient and median-averaged expression values at each SLEDAI score level.

RESULTS

We identified 4,839 methylation sites that were negatively correlated and 1,568 methylation sites that were positively correlated with disease activity in lupus, as measured by SLEDAI score. The distribution of these methylation sites relative to transcription start sites and gene structures within the genome is depicted in Figure 1A. Compared to all methylation sites included on the 450K methylation array, methylation sites that negatively correlated with disease activity in lupus were enriched on chromosomes 3, 17, and 1 ($P = 3.50 \times 10^{-3}$, 2.98×10^{-3}, and 6.67×10^{-3}, respectively). Methylation sites that correlated positively with disease activity were enriched on chromosome X ($P = 2.15 \times 10^{-4}$) (Figures 1B and C).

Transcription factor binding site analysis revealed that methylation sites positively correlated with disease activity in lupus are most significantly enriched for binding sites of 2 repressive transcription factors, EZH2 ($P = 1.06 \times 10^{-100}$) and SUZ12 ($P = 3.24 \times 10^{-74}$), which are both members of the polycomb repressive complex 2 (PRC2). DNA methylation sites with methylation levels negatively correlated with disease activity were enriched in binding sites for BATF, FOS, CEBPB, JUN, and STAT3 ($P = 1.21 \times 10^{-53}$, 2.44×10^{-53}, 4.63×10^{-50}, 6.36×10^{-45}, and 7.10×10^{-38}, respectively). In contrast to positively correlated methylation sites, methylation sites that demonstrate reduced methylation level with disease activity are significantly depleted in EZH2 binding sites ($P = 5.80 \times 10^{-102}$) (Figure 1D). This suggests that the key transcription regulator EZH2, which possesses repressive activity, might be playing a key role in the T cell epigenetic conformational changes that predispose to disease flare in lupus. Indeed, literature mining analysis (21) independently identified an association between genes nearest the highest SLEDAI-correlated methylation sites and EZH2. The negatively correlated genes were highly associated ($P < 0.0001$ by 2-tailed chi-square test with Yates' correction), whereas the positively correlated genes did not quite reach significance ($P < 0.07$). This analysis also demonstrates a proinflammatory epigenetic landscape change in naive CD4+ T cells as the disease becomes more active (Figure 2).

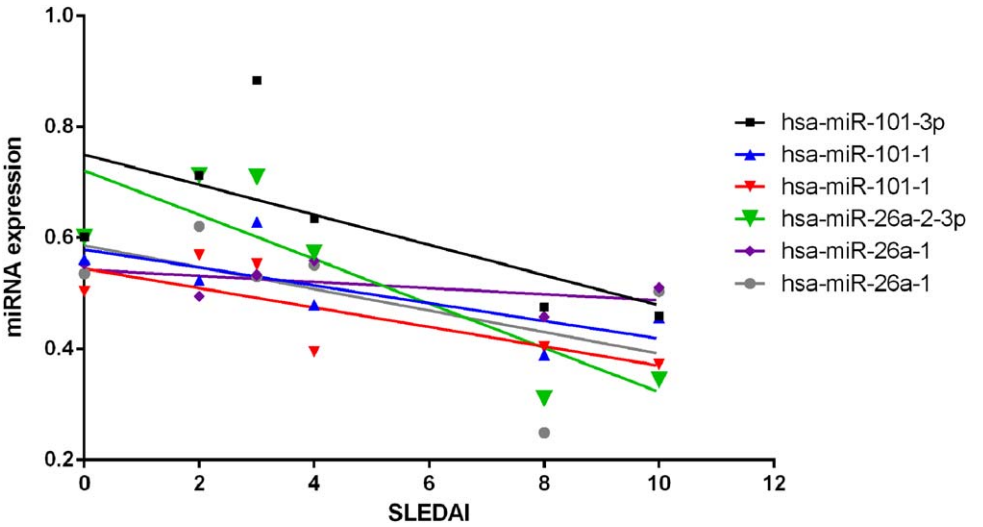

Figure 3. Expression levels of microRNA-101 (miR-101) and miR-26a in naive CD4+ T cells from lupus patients. Probes that show Pearson's correlations (r) with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores of $\geq|0.50|$ are depicted. The negative correlation between miR-26a-2-3p levels and SLEDAI scores was statistically significant ($r = -0.86$, $P = 0.028$). Symbols show median expression values for miR-101 and miR-26a probes for each SLEDAI score. Two probe sets each for miR-101-1 and miR-26a-1 were included.

EZH2 expression in T cells has recently been shown to be inhibited by glucose restriction, via increased expression of miR-101 and miR-26a, which target EZH2 (25). Since CD4+ T cells in lupus patients demonstrate enhanced glycolysis (26), we hypothesized that EZH2 dysregulation upon lupus flares might be mediated by changes in miR-101 or miR-26a expression. We measured the expression of miR-101 and miR-26a species in a subset of lupus patients and found an overall negative correlation between expression of these microRNAs and disease activity in lupus, and a significant negative correlation between miR-26a-2-3p levels in naive CD4+ T cells and disease activity ($r = -0.86$; $P = 0.028$) (Figure 3) (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39720/abstract). This microRNA expression pattern is consistent with increased activity of EZH2 in naive CD4+ T cells with lupus flares, predicted by our bioinformatics analyses.

To better understand the nature of the epigenetic change in naive CD4+ T cells that occurs upon lupus flare, we performed a functional enrichment analysis for genes that demonstrate reduced methylation (negatively correlated) and increased methylation (positively correlated) with disease activity. Genes with methylation levels that negatively correlated with SLEDAI scores in lupus were represented in several immune-related pathways, including asthma, allograft rejection, autoimmune thyroid disease, and graft-versus-host disease. These pathways seem to have several cytokines in common, including the Th2 cell cytokine genes *IL4*, *IL5*, and *IL13*, as well as several HLA class II genes (Table 1). Genes with methylation levels that positively correlated with SLEDAI scores were enriched in several pathways, most notably the inhibitory transforming growth factor β (TGFβ) signaling pathway (Table 1).

To further characterize epigenetic changes that might influence T cell differentiation in naive CD4+ T cells upon increased disease activity, we performed a gene ontology analysis focused on cytokine-related ontologies that are enriched in genes with methylation levels negatively and positively correlated with disease activity (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39720/abstract). These analyses showed significant enrichment in cytokine-related ontologies in negatively correlated genes, with a pattern most consistent with an epigenetic shift toward a Th2 and possibly Th17 response, suggested by reduced methylation in key cytokine genes such as *IL4*, *IL5*, *IL13*, *IL12B*, and *IL17F* when disease activity was increased in lupus. Indeed, the gene ontology for Th2 cytokine production was the most enriched ontology among genes that we are hypomethylated with disease activity (odds ratio 17.5; $P = 2.04 \times 10^{-2}$). Other inflammatory T cell cytokine genes that showed reduced methylation levels with disease activity include *IL9* and *IL22*. In contrast, genes with methylation levels that increased with disease activity include inhibitory cytokine genes such as TGFβ-related genes.

Table 1. Pathway analysis for genes in naive CD4+ T cells with DNA methylation changes that correlated negatively or positively with disease activity in lupus*

Pathway	OR	*P* (FDR adjusted)	Genes
Genes with negative correlation			
Asthma	8.49	2.67×10^{-6}	*IL13, IL4, IL5, HLA–DMB, HLA–DPA1, HLA–DRB1, HLA–DQA2, HLA–DMA, FCER1G, RNASE3, FCER1A, IL9, HLA–DQA1, HLA–DPB1, HLA–DRB5, IL3, CD40*
Allograft rejection	5.81	1.69×10^{-5}	*IL12B, IL4, IL5, HLA–DMB, HLA–DPA1, HLA–DRB1, HLA–DQA2, HLA–A, CD86, HLA–DMA, HLA–DQA1, PRF1, HLA–DPB1, HLA–DRB5, GZMB, FAS, CD40, CD80*
Autoimmune thyroid disease	4.11	2.94×10^{-4}	*IL4, IL5, HLA–DMB, HLA–DPA1, HLA–DRB1, HLA–DQA2, HLA–A, CD86, TPO, HLA–DMA, HLA–DQA1, PRF1, HLA–DPB1, HLA–DRB5, GZMB, FAS, CD40, CD80*
Graft-versus-host disease	4.17	4.98×10^{-4}	*HLA–DMB, HLA–DPA1, HLA–DRB1, HLA–DQA2, HLA–A, CD86, IL6, HLA–DMA, KLRD1, HLA–DQA1, PRF1, HLA–DPB1, HLA–DRB5, GZMB, FAS, CD80*
Type 1 diabetes mellitus	3.88	4.98×10^{-4}	*IL12B, HLA–DMB, HLA–DPA1, HLA–DRB1, HLA–DQA2, PTPRN2, HLA–A, CD86, HLA–DMA, CPE, HLA–DQA1, PRF1, HLA–DPB1, HLA–DRB5, GZMB, FAS, CD80*
Regulation of actin cytoskeleton	1.93	5.47×10^{-4}	*DOCK1, VCL, TIAM2, CHRM2, FGFR2, LIMK2, FGF11, ITGB2, MYLK, CRK, ARHGEF6, FGF1, PIP5K1C, PIK3CD, ITGAD, ITGAX, FGF12, ITGB4, PDGFA, FGFR4, PIK3R1, DIAPH1, GNA12, FGF21, ITGAE, ITGB1, MYL7, MYL9, PXN, CHRM3, CYFIP1, VAV2, FGFR1, FGFR3, ITGA3, PIP4K2A, PDGFRB, MYLK2, ITGA11, BAIAP2, MYH9, NCKAP1L, ACTN4, MYH10, EGFR, GSN, ARHGEF7, PPP1CA, IQGAP2, SSH1, VAV3, PDGFD, DIAPH3, PIK3CB*
Genes with positive correlation			
Pathways in cancer	2.39	7.62×10^{-4}	*TPM3, ARNT2, WNT10A, SMAD3, TCF7, CTNNA2, FGF12, KITLG, PIK3CD, CBL, ETS1, HDAC1, PDGFRA, BMP4, LAMA3, AXIN2, GLI3, TGFBR2, COL4A6, FGF13, BCR, FGF4, BIRC2, VEGFB, RASSF5, APC2, APC, TGFB2, MMP2, PLCG1, FGFR2, TGFBR1*
Neuroactive ligand–receptor interaction	2.31	2.80×10^{-3}	*GALR1, MTNR1A, MC4R, GRM6, CHRM2, F2RL1, P2RX1, ADRA2A, GRM7, KISS1R, NPY1R, OPRM1, TSHR, NTSR2, GABRA5, GABRB2, HCRTR2, CHRNA3, GABRG2, CRHR1, GABBR2, GRIN1, PTGFR, PTGDR, S1PR1, MCHR1*
TGFβ signaling pathway	3.15	1.03×10^{-2}	*SMAD3, PPP2R1B, SMURF2, BMP4, TGFBR2, BMPR1A, ID4, ID3, PITX2, TGFB2, TGFBR1*
Colorectal cancer	3.53	1.03×10^{-2}	*SMAD3, TCF7, PIK3CD, AXIN2, TGFBR2, APC2, APC, TGFB2, TGFBR1*
Glycosaminoglycan biosynthesis–chondroitin sulfate	6.07	1.03×10^{-2}	*B3GAT1, CHST11, DSE, CHSY1, CHST13*
Phosphatidylinositol signaling system	3.06	1.03×10^{-2}	*DGKZ, INPP5A, ITPR3, PIK3CD, PIP4K2A, ITPKB, DGKA, PLCB1, INPP4A, PLCG1*

* OR = odds ratio; FDR = false discovery rate; TGFβ = transforming growth factor β.

We next examined DNA methylation changes in key CD4+ T cell subset transcription factors. Our data showed reduced methylation with disease activity in the genes encoding PU.1, retinoic acid receptor–related orphan nuclear receptor γt (RORγt), and BCL-6, which promote Th9, Th17, and follicular helper T (Tfh) cell responses, respectively. Genes encoding critical Tfh cell molecules, such as inducible costimulator (ICOS) and CXCR5, were also hypomethylated with increased disease activity in lupus, while no methylation change with disease activity was observed in *IL21*. Genes encoding the Th1 and Th2 transcription factors T-bet and GATA-3, respectively, showed hypermethylation with disease activity in lupus (Figure 4).

To determine whether this "epigenetic shift" toward an activated T cell phenotype precedes T cell activation and differentiation, we performed RNA sequencing in a subset of naive CD4+ T cells included in this study. The majority of genes that showed DNA methylation changes negatively or positively correlated with disease activity in lupus demonstrated no significant change in gene expression levels with disease activity. This observation was not unexpected and is consistent with the naive phenotype of the examined T cells and also indicates that epigenetic changes that favor T cell activation predate gene expression changes. This was shown for the Th2 cytokine genes and the transcription factor NF-κB in Figure 5. Therefore, our data suggest that a proinflammatory

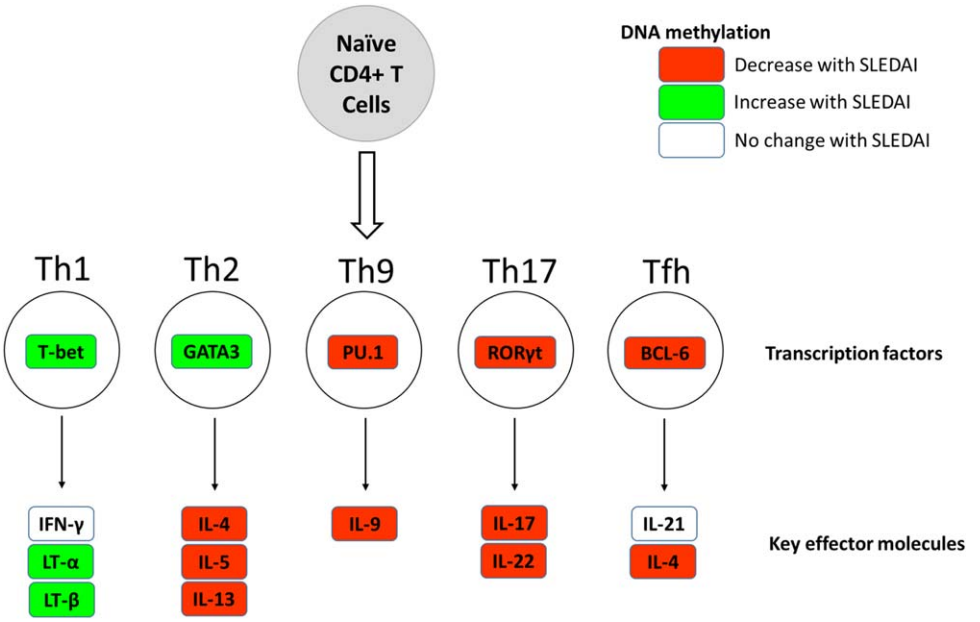

Figure 4. Schematic representation of changes in DNA methylation with changes in disease activity, measured by Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), in lupus. Changes in methylation are shown for key effector CD4+ T cell subset genes in naive CD4+ T cells from lupus patients. Tfh = follicular helper T cell; RORγt = retinoic acid receptor–related orphan nuclear receptor γt; IFNγ = interferon-γ; LTα = lymphotoxin α; IL-4 = interleukin-4.

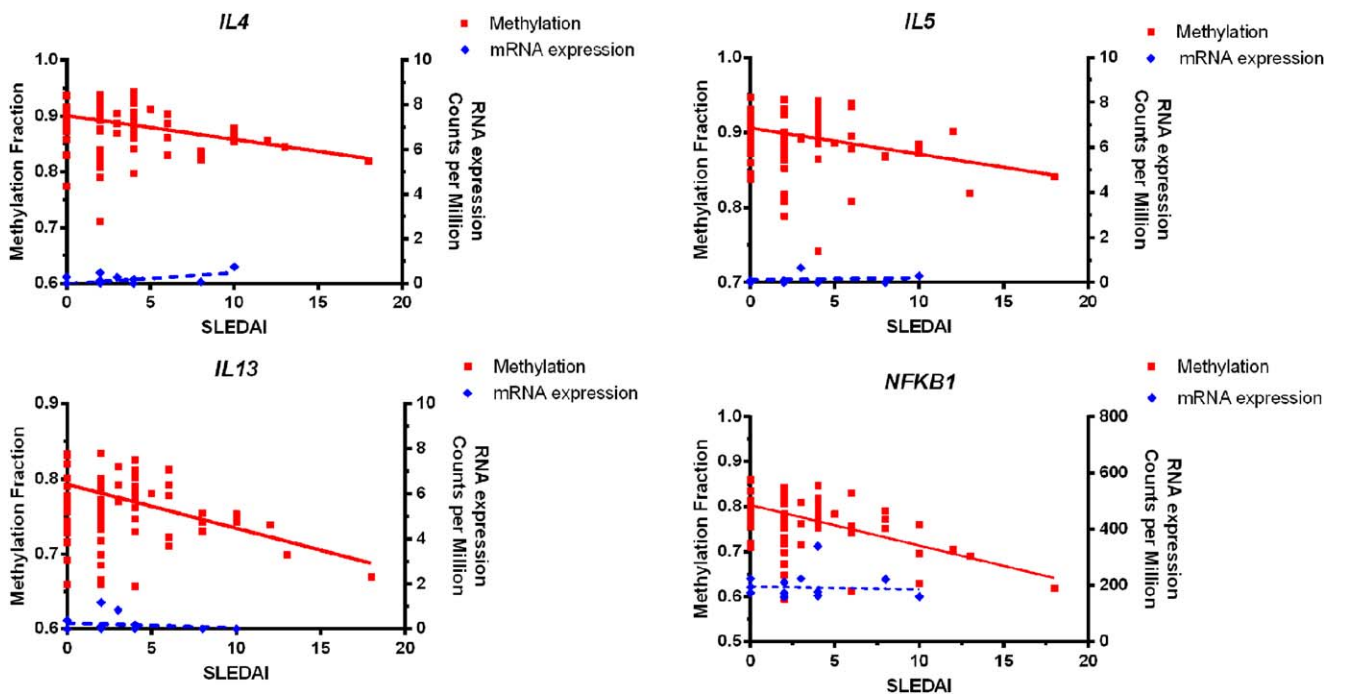

Figure 5. Correlations between disease activity in lupus patients and DNA methylation in Th2 cytokine genes (*IL4*, *IL5*, and *IL13*) and *NFKB1* in naive CD4+ T cells. Red shows negative correlation between disease activity and DNA methylation ($r = -0.74$, $P = 0.0095$ for *IL4*; $r = -0.74$, $P = 0.0092$ for *IL5*; $r = -0.83$, $P = 0.0017$ for *IL13;* and $r = -0.94$, $P = 1.36 \times 10^{-5}$ for *NFKB1*); blue shows lack of correlation between disease activity and mRNA levels measured using RNA sequencing ($P = 0.15$ for *IL4*, $P = 0.81$ for *IL5*, $P = 0.63$ for *IL13*, and $P = 0.78$ for *NFKB1*). Symbols represent individual patients. Reduced methylation levels with disease flares in these genes precede active transcription, as evidenced by the lack of correlation between mRNA levels and disease activity.

epigenomic remodeling in naive CD4+ T cells is an early event occurring with increased lupus disease activity.

DISCUSSION

In this study, we demonstrated that epigenetic landscape remodeling in naive CD4+ T cells is associated with disease activity in lupus patients. With increased disease activity, as reflected by higher SLEDAI scores, naive CD4+ T cells undergo an epigenetic shift that predisposes to an active T cell phenotype, which is evident at the naive CD4+ T cell stage before T cell differentiation and activation. Indeed, by examining naive CD4+ T cells, we can predict early changes in T cell composition that accompany disease flares. Arguably, these compositional changes might be triggering or at least contributing to the initiating events resulting in a lupus flare.

The nature of the epigenetic shift detected suggests hypomethylation of genes involved in T cell activation and differentiation and hypermethylation in inhibitory signaling pathways such as TGFβ. Genes that demonstrated reduced methylation with increased disease activity in naive CD4+ T cells from lupus patients include Th2 signature genes, such as *IL4*, *IL5*, and *IL13*. Th2 cytokine gene hypomethylation was observed despite a paradoxical increase in methylation of GATA-3, suggesting that epigenetic poising of the Th2 cytokine gene locus with lupus flares is independent of the methylation status of GATA-3. The negative correlation between DNA methylation in *IL12B*, *IL17F,* and *RORC* (encoding RORγt) and disease activity in lupus suggests that a Th17 response might also be an early event in lupus flares. Similarly, the negative correlation between disease activity and DNA methylation in the genes encoding BCL-6, ICOS, and CXCR5 and between disease activity and DNA methylation in the genes encoding PU.1 and IL-9 suggests a role for Tfh cell and Th9 responses, respectively, in lupus flares.

In contrast, no epigenetic changes were detected in or near the gene encoding IFNγ, which is the key Th1 cytokine gene that is typically demethylated upon Th1 differentiation. In addition, genes that encode other Th1 response molecules, such as lymphotoxin α (LTα), LTβ, and the Th1 key transcription factor T-bet, showed increased methylation with disease activity, and therefore are predicted to be epigenetically silenced with lupus flares. These data suggest that a non-Th1 response is the likely early T cell event occurring with increased disease activity in lupus and underscores the role of T cell–dependent B cell activation and plasma cell expansion in lupus flares.

The relative importance of Th2, Th17, or Tfh cell differentiation as an early event in disease flare in lupus patients remains unclear and is also complicated by the possibility of a transient role of these T cell responses in initiating disease relapse. Once a flare is established, it is possible that multiple other immune signaling and activating pathways become involved, which might or might not mask a predicted role for non-Th1 immune responses suggested by our data. These epigenetic changes occur without corresponding transcriptional changes, indicating that a change in the epigenetic landscape in CD4+ T cells is an early event in lupus flares. The master proinflammatory cytokine regulator gene encoding NF-κB is also progressively demethylated in lupus CD4+ T cells with increased disease activity. In contrast, inhibitory pathways such as the TGFβ signaling pathway are more methylated in active disease. Taken together, these data suggest that an epigenetic remodeling event shifting naive CD4+ T cells toward activation and non-Th1 effector T cell differentiation and away from Treg cell differentiation could predispose to lupus flares.

To understand possible upstream events that might contribute to this epigenetic shift in naive CD4+ T cells during disease flare, we determined whether common transcription factors might be involved in regulating genes with methylation levels that correlated positively or negatively with disease activity in lupus. We found a robust enrichment of binding sites of the transcription factor EZH2 in methylation sites positively correlated with disease activity. Interestingly, methylation sites that were negatively correlated with disease activity were depleted of EZH2 binding sites. EZH2 is the catalytic member of PRC2. It encodes a histone methyltransferase, which methylates lysine 27 in histone H3, resulting in transcriptional repression, and it can also recruit DNA methyltransferases including DNMT-1, DNMT-3A, and DNMT-3B (27,28). PRC2 and EZH2 play an important role in X chromosome inactivation (29,30), which might explain the relative enrichment of methylation sites that positively correlated with disease activity in lupus on the X chromosome.

EZH2 plays an essential role in CD4+ T cell plasticity and differentiation. EZH2 has been implicated in the transcriptional silencing of Th2 cytokine genes, while EZH2 deficiency was shown to reduce Th1 differentiation by reducing the expression of T-bet and STAT-4 (31,32). However, other studies have shown that under Th1, Th2, and Th17 differentiation conditions, EZH2 deficiency enhances IFNγ, IL-13, and IL-17 production, respectively (33). Further, EZH2 is critical in maintaining Treg cell function during T cell activation and differentiation, and EZH2 deficiency has been demonstrated to reduce Treg cell differentiation (33). Interestingly, EZH2-deficient Treg cells as well as effector T cells demonstrate functional defects. Effector T cells deficient in EZH2 failed to

expand in culture and to induce autoimmunity in a colitis model, and failed to protect against *Toxoplasma gondii* infection, which is known to elicit a Th1 response (33). In contrast, EZH2 deficiency enhanced Th2 responses in vivo in a mouse model of asthma (34). Taken together, these data suggest a complex, sometimes contradictory, but crucial role for EZH2 in T cell responses. Indeed, recent studies suggest that EZH2+ T cells are polyfunctional, expressing multiple effector cytokines, and that EZH2 expression is increased with T cell activation and enhances T cell survival and effector function by inhibiting Notch signaling repressors (25).

Recent evidence suggests that EZH2 expression is sensitive to glucose availability. Glucose restriction suppresses EZH2 expression by inducing the expression of 2 microRNAs that target EZH2 (miR-101 and miR-26a) (25). Consistent with our model of lupus flares, we found a negative correlation between miR-26a expression and disease activity in lupus patients. This suggests that reduced microRNA regulation of EZH2, possibly mediated by increased glycolysis and glucose availability, might result in a transient increase in EZH2 activity, leading to epigenetic reprograming in T cells favoring T cell activation. This model is consistent with the findings of recent studies showing enhanced glycolysis in CD4+ T cells from lupus patients and lupus mouse models (26). It should be noted that we did not observe a correlation between EZH2 mRNA expression levels and disease activity in naive CD4+ T cells in lupus (data not shown). This might be explained by the sometimes transient nature of gene expression changes, which in this case could have been missed since the epigenetic changes presumably resulting from EZH2 have already been established in naive CD4+ T cells.

In summary, we provide evidence of epigenetic changes in CD4+ T cells in lupus that suggests a role of T cell activation and non-Th1 functions as early contributing immune responses in lupus flares. These epigenetic changes might be mediated by EZH2, which is an epigenetic regulator that is sensitive to glucose availability and plays an important role in T cell plasticity, activation, and differentiation. Further studies into the direct role of EZH2 in lupus progression and flares are warranted.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sawalha had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Sawalha.

Acquisition of data. Coit, Merrill, McCune, Maksimowicz-McKinnon, Sawalha.

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