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Genome-Wide DNA Methylation Patterns in Naive CD4+ T Cells From Patients With Primary Sjögren's Syndrome

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Objective. **Primary Sjögren's syndrome (SS) is a systemic autoimmune disease with incompletely understood etiology. This study was undertaken to investigate the role of epigenetic dysregulation in the pathogenesis of primary SS.**

Methods. **A genome-wide DNA methylation study was performed in naive CD4+ T cells from 11 patients with primary SS compared to age-, sex-, and ethnicity-matched healthy controls. Cytosine methylation was quantified using the Illumina Infinium HumanMethylation 450 BeadChip array, and the data were validated using bisulfite sequencing.**

Results. **Genome-wide analyses identified 553 hypomethylated CpG sites and 200 hypermethylated CpG sites in naive CD4+ T cells from patients with primary SS as compared to healthy controls, representing 311 hypomethylated and 115 hypermethylated gene regions. The hypomethylated genes in patients with primary SS included *LTA* (encoding lymphotoxin α). Other relevant genes, such as *CD247*, *TNFRSF25*, *PTPRC*, *GSTM1*, and *PDCD1*, were also hypomethylated. The interferon signature pathway was represented by hypomethylation of *STAT1*, *IFI44L*, *USP18*, and *IFITM1*. A group of genes encoding members of the solute carrier proteins were differentially methylated. In addition, the transcription factor gene *RUNX1* was hypermethylated in patients with primary SS, suggesting a possible connection to lymphoma predisposition. Gene ontology (GO) analysis of hypomethylated genes demonstrated enrichment of genes involved in lymphocyte activation and immune response. GO terms for hypermethylated genes included antigen processing and presentation.**

Conclusion. **This is the first epigenome-wide DNA methylation study in patients with primary SS. These findings highlight a role for DNA methylation in primary SS and identify disease-associated DNA methylation changes in several genes and pathways in naive CD4+ T cells from patients with primary SS that may be involved in the pathogenesis of this disease.**

Primary Sjögren's syndrome (SS) is a complex autoimmune disease characterized by production of autoantibodies against RNP particles (Ro/SSA and La/SSB) and muscarinic acetylcholine receptor antigens, dysfunction of water transport processes, and lymphocytic infiltration of exocrine glands resulting in glandular atrophy and dysfunction (1,2). Although xerostomia (dry mouth) and xerophthalmia (dry eyes) are the main clinical features of primary SS (3), the full spectrum of the disease encompasses involvement of different organ systems and predisposition to lymphoproliferative disease (4). Primary SS predominantly affects women, with a female-to-male distribution of 9 to 1 (5).

The etiology of primary SS is incompletely understood, but a growing body of evidence suggests that diseases with certain clinical features similar to those of

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primary SS, such as systemic lupus erythematosus (SLE), have common epigenetic factors that may contribute to the pathogenesis of autoimmunity (6). Furthermore, recent evidence suggests that global DNA methylation is reduced in salivary gland epithelial cells from patients with primary SS (7). DNA methylation is considered the core epigenetic mechanism that regulates gene expression, by altering transcriptional accessibility of regulatory regions within gene sequences (8). In a recent study, we characterized the DNA methylation changes in naive CD4+ T cells from patients with lupus, revealing that changes in DNA methylation occurred prior to the differentiation and activation of T cells, and demonstrating that interferon (IFN)–regulated genes in naive CD4+ T cells from patients with lupus are epigenetically poised for transcription (9). In the present study, we performed a genome-wide DNA methylation study in naive CD4+ T cells from patients with primary SS and healthy control subjects. We also validated our results using bisulfite sequencing of selected differentially methylated loci. The findings demonstrated a pattern of differential methylation in key genes and disease-associated pathways pertinent to the pathogenesis of primary SS.

Table 1. Demographic characteristics and medication use of the study participants*

Subject/age/sex	Ethnicity	Medication
Patients with primary SS		
1/50/female	European American	Methotrexate
2/51/female	European American	Hydroxychloroquine
3/30/female	European American	
4/23/female	European American	Hydroxychloroquine
5/62/female	European American	Azathioprine
6/32/female	European American	
7/35/female	European American	
8/60/female	European American	
9/58/female	European American	
10/35/female	European American	Hydroxychloroquine
11/69/female	European American	Hydroxychloroquine
Healthy controls		
12/52/female	European American	
13/47/female	European American	
14/34/female	European American	
15/23/female	European American	
16/58/female	European American	
17/36/female	European American	
18/36/female	European American	
19/55/female	European American	
20/58/female	European American	
21/38/female	European American	
22/64/female	European American	

* The mean age of the patients with primary Sjögren's syndrome (SS) and healthy controls was 45.9 years and 45.5 years, respectively (P = 0.95).

PATIENTS AND METHODS

Patients and controls. We studied 11 patients with primary SS and 11 healthy control subjects. Patients and controls were matched for age (within 5 years), sex, and ethnicity. The demographic features and medications of the study participants are shown in Table 1. Classification of primary SS was based on the American-European Consensus Group 2002 revised criteria (10). The clinical features of the patients with primary SS included in this study are shown in Table 2. Disease manifestations in the patients were extensively characterized by a team of oral, ocular, and rheumatology specialists through the Oklahoma Medical Research Foundation (OMRF) Sjögren's Research Clinic. Controls were recruited at the OMRF and the University of Michigan. The institutional review boards at the OMRF and the University of Michigan approved this study. All study participants signed a written informed consent form prior to participation.

Isolation and purity of naive CD4+ T cells. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples obtained from patients and controls using density-gradient centrifugation (Ficoll-Paque; GE Healthcare Life Sciences). Naive CD4+ T cells were separated from PBMCs using a naive CD4+ T Cell Isolation Kit II (Miltenyi Biotec) that allows for indirect isolation of naive CD4+ T cells, or by sorting the CD3+CD4+CD45RA+ naive T cell population on a FACSAria instrument (BD Biosciences). The purity of the naive CD4+ T cells was confirmed by flow cytometry, using fluorochrome-conjugated antibodies against CD4 and CD45RA. The purity of the isolated naive CD4+ T cells was consistently >95%, regardless of the method used. DNA was isolated using the DNeasy kit (Qiagen), as described in the manufacturer's protocol.

DNA methylation studies and array validation. Genome-wide DNA methylation in naive CD4+ T cells from the patients with primary SS and healthy controls included in this study was assessed using the Illumina Infinium HumanMethylation450 BeadChip array, which allows for the interrogation of >485,000 methylation sites within the entire genome. This array covers 99% of RefSeq genes, with an average of 17 CpG sites per gene across the promoter region, 5′-untranslated region (5′-UTR), first exon, gene body, and 3′-UTR. It also covers 96% of CpG islands. Non-CpG methylated sites recently identified in human stem cells are also covered, as well as microRNA promoter regions.

Validation of the array data was performed using bisulfite DNA sequencing in selected hypermethylated and hypomethylated gene regions. The primers were as follows: for *HDAC4*, forward 5′-TGGTTTTATTTTTTGTAGTTAAAAA-3′ and reverse 5′-ATAAAACCTCTATACCTCACTCAAC-3′; for *SLC38A4*, forward 5′-TTTGGATTTTTTAATTAAGTTGT-TA-3′ and reverse 5′-TCTACAATATTAATACTCCTACAA-ACC-3′; for *DUSP22*, forward 5′-TTATTTGTTTTTTTAGG-GTAGGGAG-3′ and reverse 5′-AATCTCCAAATCCCCCT-TTAAC-3′; for *GSTM1*, forward 5′-GTTAGGATTTGG-TTGGTGTTTTAAG-3′ and reverse 5′-ATCCCAATACCC-CAATATCATAAAC-3′; for *RUFY1*, forward 5′-GTAGG-AGAGGTTTTGAGTTGGATT-3′ and reverse 5′-TCCTC-CATCATCTAACACTTAAAAA-3′; for *NAPRT1*, forward

Table 2. Clinical characteristics of the patients with primary SS (n = 11)*

Dry eyes, no. (%)	11 (100)
Dry mouth, no. (%)	11 (100)
Schirmer's test for eye dryness	
Abnormal findings, no. (%)	6 (55)
Mean response, mm	10.8
Abnormal lissamine green ocular staining score, no. (%)	9 (82)
Abnormal WUSF, no. (%)	5 (45)
Positive for anti-Ro/SSA, no. (%)	6 (55)
Positive for anti-La/SSB, no. (%)	6 (55)
MSG biopsy findings consistent with primary SS, no. (%)	10 (91)
Mean focus score	4.5

* The focus score is the number of mononuclear cell infiltrates containing at least 50 inflammatory cells in a 4-mm^2 glandular section. SS = Sjögren's syndrome; WUSF = whole unstimulated salivary flow; MSG = minor salivary gland.

5′-TATGGTGGTTTGGTAGAGGTTAGTG-3′ and reverse 5′-ACTAATCTATCCTCCACCCTTTCC-3′; and for *SLC9A1*, forward 5′-GTTTTTTTATTTAGAGAGGGGTA-GG-3′ and reverse 5′-AACCAAAAAAAACTACAACTAA-ACC-3′. We used a Bio-Rad T100 Thermocycler with the following protocol: 1 cycle at 94°C for 5 minutes, followed by 40 cycles (94°C for 45 seconds, annealing temperature for each primer set for 45 seconds, 72°C for 90 seconds), and then 74°C for 10 minutes. The annealing temperatures for *HDAC4, SLC38A, DUSP22, GSTM1, RUFY1, NAPRT1,* and *SLC9A1* were 54°C, 57°C, 53°C, 54°C, 56°C, 56°C, and 53°C, respectively.

We confirmed the presence of polymerase chain reaction (PCR) product with the use of 1.6% agarose gel electrophoresis. DNA was purified using a QIAquick PCR Purification Kit (Qiagen), as specified by the manufacturer. Bisulfite-treated DNA was sequenced using Sanger sequencing. The DNA methylation level on each CpG following bisulfite sequencing was quantified using the Epigenomics ESME software package.

Statistical and bioinformatics analyses. DNA methylation studies were performed using the GenomeStudio methylation analysis package (Illumina), as previously described (8). The average level of DNA methylation (β value) on each CpG site was compared between patients with primary SS and healthy controls. To identify differentially methylated CpG sites between patients and controls, we used 3 data-filtering criteria: 1) CpG site with a mean difference in the DNA methylation level of at least 1.2-fold, 2) differential methylation score of $\geq$22 ($P \leq 0.01$), after adjustment for multiple testing using a false discovery rate (FDR) of 5%, and 3) exclusion of CpG sites assessed by probes with a genetic variant located within 10 bp of the 3′ end of the probe.

To systematically highlight the most overrepresented biologic terms among the differentially methylated genes, we performed gene ontology (GO), network, and pathway analyses using the DAVID bioinformatics database (version 6.7) (11). In pathway and GO analyses, we used an Expression Analysis Systematic Explorer (EASE) score threshold of <0.1 for detection of gene enrichment. The EASE score represents a modified *P* value determined by Fisher's exact test, which is considered to represent the significance of gene-term enrichment. In addition, we used a fold enrichment threshold of 1.5, with an FDR of <10% to correct for multiple testing (12).

RESULTS

We evaluated DNA methylation changes in naive CD4+ T cells from patients with primary SS and age-, sex-, and ethnicity-matched healthy controls. We identified 753 differentially methylated CpG sites in naive CD4+ T cells from patients with primary SS (Table 3 and Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38264/abstract). Two hundred sites were hypermethylated and 553 were hypomethylated in patients with primary SS as compared to healthy controls. A total of 426 differentially methylated unique genes were identified in naive CD4+ T cells from patients with primary SS, with the majority of the genes (311 [75%]) being hypomethylated. Bisulfite DNA sequencing was used to validate the DNA methylation array results by studying a group of hypermethylated (4 genes, 7 loci) and hypomethylated (3 genes, 3 loci) CpG sites. The mean Pearson's correlation coefficient (r^2 value) for the correlation between the Illumina Infinium HumanMethylation450 array data and the bisulfite sequencing data was 0.803 (see Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38264/abstract).

Although the purity of the naive CD4+ T cells was consistently >95%, we wanted to ensure that the differential methylation patterns identified between patients and controls in our study were not influenced by potential differences in the naive CD4+ T cell activation status, which could have been induced by the different isolation procedures used. We therefore examined the DNA methylation levels of a region in the *IL2* promoter–enhancer sequence that is known to readily demethylate upon T cell activation. Demethylation of

Table 3. Summary of the differentially methylated CpG sites and genes in naive CD4+ T cells from patients with primary Sjögren's syndrome as compared to healthy controls

	Increased methylation	Decreased methylation	Total
No. of CpG sites	200	553	753
Fold change			
Range	1.20, 5.20	1.20, 5.22	
Mean	1.65	1.60	
Differential score			
Range	22.0, 342.0	−22.0, −339.8	
Mean	+95.6	−66.1	
No. of genes	115	311	426

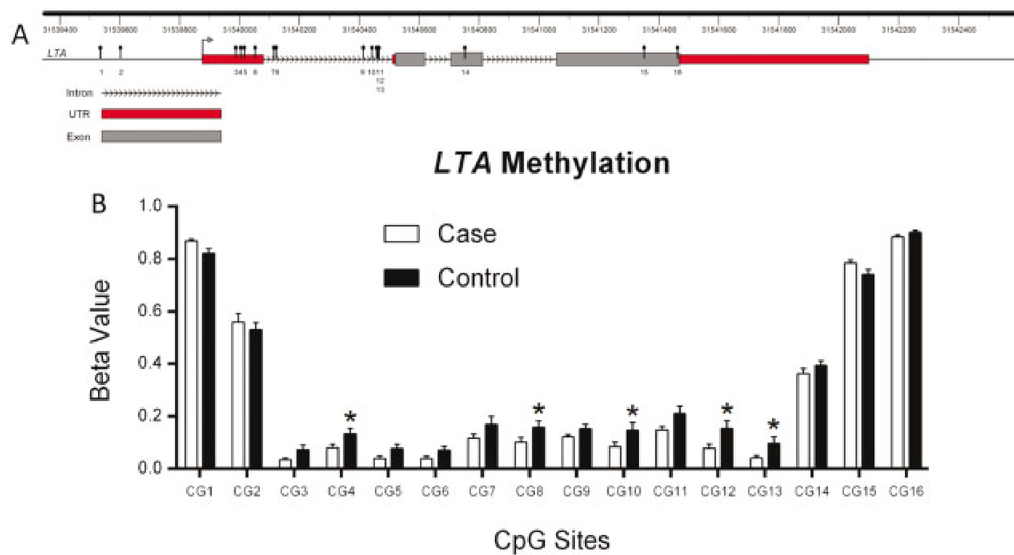

Figure 1. DNA methylation of the *LTA* gene (encoding lymphotoxin α) in naive CD4+ T cells from patients with primary Sjögren's syndrome and healthy controls. **A,** Schematic of *LTA* DNA methylation (31,539,814–31,542,537 bp on chromosome 6; GRCh37/hg19), depicting the location of the CpG sites evaluated. UTR = untranslated region. **B,** DNA methylation fractions (β values) across the CpG sites in *LTA*, evaluated in naive CD4+ T cells from patients with primary Sjögren's syndrome and healthy controls. **Asterisks** indicate the 5 hypomethylated CpG sites in the *LTA* gene region in cases compared to controls. Values are the mean ± SD.

the promoter–enhancer region of *IL2* is a sensitive indicator of CD4+ T cell activation (13,14) and is a prerequisite for *IL2* transcription (15). We detected high DNA methylation levels in the *IL2* promoter–enhancer region, as expected in naive CD4+ T cells, and there was no difference between patients and controls (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38264/abstract).

Furthermore, we examined the DNA methylation levels in genetic loci known to demethylate in specific antigen–experienced and regulatory CD4+ T cell subsets, including *IFNG* (Th1 cells), *IL4, IL5*, and *IL13* (Th2 cells), *IL17* (*IL17A*) and *IL17F* (Th17 cells), and *FOXP3* (regulatory T cells). Again, we observed that the DNA methylation levels were consistently high across these genetic loci, and there was no difference between patients and controls (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38264/abstract).

The hypomethylated genes detected in naive CD4+ T cells from patients with primary SS included *LTA* (encoding lymphotoxin α [LTα]), which is involved in the LTβ receptor (LTβR) signaling pathway, activation of follicular dendritic cells, and expression of IFNα. We identified 5 differentially methylated CpG sites in *LTA*, located at the 5′-UTR within 200 bp upstream of the transcriptional start site (hereafter referred to as TSS 200) and in the first exon (Figure 1). The mean differential methylation score (Diff. score) of the 5 CpG sites was −35.4 (range −22.3 to −53.8; mean fold change 1.82). Other relevant hypomethylated genes included *CD247,* which encodes for T cell receptor (TCR) ζ-chain (2 CpG sites located in the body of the gene, with Diff. scores of −23.9 and −32.3, respectively; mean fold change 1.96), *TNFRSF25* (1 CpG site located 1,500 bp upstream of the TSS [TSS 1,500], with a Diff. score of −23.2; mean fold change 1.59), *PTPRC* (2 CpG sites in the body of the gene and in TSS 1,500, with Diff. scores of −49.7 and −75.5, respectively; mean fold change 1.96), *GSTM1* (2 CpG sites located in TSS 200, with Diff. scores of −31.4 and −48.8, respectively; mean fold change 1.40), and *PDCD1* (1 CpG site located in TSS 1,500, with a Diff. score of −46.9; mean fold change 2.63). The type I IFN pathway, which plays a major role in the pathogenesis of primary SS (16), was represented by hypomethylation of *STAT1* (2 CpG sites located in the 5′-UTR, with Diff. scores of −23.9 and −79.2, respectively; mean fold change 1.41), *IFI44L* (1 CpG site located in the 5′-UTR, with a Diff. score of −24.1; mean fold change 1.22), *IFITM1* (1 CpG site located in TSS 1,500, with a Diff. score of −35.8; mean fold change 1.75), and *USP18* (1 CpG site located in the 5′-UTR, with a Diff. score of −65.1; mean fold change 1.40).

Table 4. Pathway analysis of the differentially methylated genes in naive CD4+ T cells from patients with primary Sjögren's syndrome as compared to healthy controls*

Category	Term	Molecule	*P*	Fold enrichment	FDR
KEGG_PATHWAY	Type 1 diabetes mellitus	PTPRN2; CD28; LTA; HLA–A; HLA–B; HLA–C; HLA–F; HLA–DMA; HLA–DRB1; HLA–DRB4; HLA–DRB5	4.59×10^{-6}	9.005	0.005
KEGG_PATHWAY	Allograft rejection	CD28; HLA–A; HLA–B; HLA–C; HLA–F; HLA–DMA; HLA–DRB1; HLA–DRB4; HLA–DRB5	1.66×10^{-4}	8.171	0.191
KEGG_PATHWAY	Viral myocarditis	MYH13; CD28; CASP9; HLA–A; HLA–B; HLA–C; HLA–F; HLA–DMA; HLA–DRB1; HLA–DRB4; HLA–DRB5	2.34×10^{-4}	5.327	0.268
KEGG_PATHWAY	Graft-versus-host disease	CD28; HLA–A; HLA–B; HLA–C; HLA–F; HLA–DMA; HLA–DRB1; HLA–DRB4; HLA–DRB5	2.63×10^{-4}	7.543	0.301
KEGG_PATHWAY	Autoimmune thyroid disease	CD28; HLA–A; HLA–B; HLA–C; HLA–F; HLA–DMA; HLA–DRB1; HLA–DRB4; HLA–DRB5	1.15×10^{-3}	5.768	1.315
KEGG_PATHWAY	Antigen processing and presentation	LTA; HLA–A; HLA–B; HLA–C; HLA–F; HLA–DMA; HLA–DRB1; HLA–DRB4; HLA–DRB5; TAP2	3.22×10^{-3}	4.051	3.637
KEGG_PATHWAY	Cell adhesion molecules	NCAM1; SELL; PDCD1; CD28; HLA–A; HLA–B; HLA–C; HLA–F; HLA–DMA; HLA–DRB1; HLA–DRB4; HLA–DRB5	3.73×10^{-3}	3.184	4.201

* FDR = false discovery rate; KEGG = Kyoto Encyclopedia of Genes and Genomes.

Table 5. GO analysis of hypomethylated and hypermethylated genes in naive CD4+ T cells from patients with primary Sjögren's syndrome*

Category	GO term	GO identification no.	Gene	*P*	Fold enrichment	FDR
Hypomethylated genes						
GOTERM_BP_FAT	Lymphocyte activation	0046649	BCL11B; CD3G; CD28; CD7; CHD7; FOXP1; HDAC4; ITPKB; JMJD6; RHOH; SLC11A1; SKAP2; UNC13D	1.10×10^{-4}	3.859	0.185
GOTERM_BP_FAT	Leukocyte differentiation	0002521	BCL11B; CEBPE; CD28; CHD7; FOXP1; HDAC4; ITPKB; JMJD6; RHOH	1.42×10^{-3}	4.168	2.361
GOTERM_BP_FAT	Immune response	0006955	BTLA; CD28; CD300A; CD7; FAIM3; ST6GAL1; C4BPB; FOXP1; IFI44L; IL36G; IL16; LY86; LTA; HLA–A; HLA–B; HLA–H; HLA–DRB5; PDCD1; SLC11A1; TCF12; TCF7; UNC13D; ETS1	2.20×10^{-3}	1.969	3.646
GOTERM_BP_FAT	Chromatin organization	0006325	BCOR; CREBBP; DNMT3A; H2AFV; SATB1; SMARCB1; APBB1; CHD7; HDAC4; JMJD6; KDM2B; RBBP7; TSPY4; TSPY1; TSPY2; TSPY3; TSPY7P; TLK1	3.90×10^{-3}	2.344	6.356
GOTERM_BP_FAT	T cell differentiation	0030217	BCL11B; CD28; CHD7; ITPKB; JMJD6; RHOH	4.20×10^{-3}	5.453	6.855
GOTERM_BP_FAT	Homophilic cell adhesion	0007156	PCDHAC2; PCDHA2; PCDHA3; PCDHA4; PCDHA5; PCDHA7; PCDHA8; PCDHA9	5.80×10^{-3}	3.608	9.360
GOTERM_BP_FAT	L-amino acid transport	0015807	CACNA1A; HTT; SLC11A1; SLC7A8	6.10×10^{-3}	10.274	9.687
Hypermethylated genes						
GOTERM_BP_FAT	Antigen processing and presentation	0019882	TAP2; HLA–B; HLA–C; HLA–F; HLA–H; HLA–DMA; HLA–DRB4; HLA–DRB5	6.89×10^{-6}	14.817	0.010

* Statistically significant gene ontology (GO) terms are shown. FDR = false discovery rate; GOTERM_BP_FAT = gene ontology term biologic process.

A group of genes encoding members of the solute carrier proteins, which are membrane transport proteins that are important for maintenance of cell function, were hypomethylated, including *SLC11A1*, *SLC11A2*, *SLC22A23*, *SLC25A25*, *SLC25A3*, *SLC25A33*, and *SLC6A20*, whereas *SLC9A1*, which is important for the maintenance of pH homeostasis, was hypermethylated in patients with primary SS as compared to healthy controls. In addition, the transcription factor *RUNX1* was hypermethylated in patients with primary SS. Supplementary Table 1 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38264/abstract) provides a summary of all CpG sites that were differentially methylated in patients with primary SS in our study.

Next, we used DAVID software (11) to facilitate the systematic identification and grouping of differentially methylated genes into biologic networks. Canonical pathway analysis identified type 1 diabetes mellitus ($P = 4.59 \times 10^{-6}$), allograft rejection ($P = 1.66 \times 10^{-4}$), viral myocarditis ($P = 2.34 \times 10^{-4}$), graft-versus-host disease ($P = 2.63 \times 10^{-4}$), autoimmune thyroid disease ($P = 1.15 \times 10^{-3}$), antigen processing and presentation ($P = 3.22 \times 10^{-3}$), and cell adhesion molecules ($P = 3.73 \times 10^{-3}$) as the most significant pathways unifying the differentially methylated genes in naive CD4+ T cells from patients with primary SS (Table 4).

GO analysis of hypomethylated genes demonstrated enrichment of genes involved in lymphocyte activation ($P = 1.10 \times 10^{-4}$), leukocyte differentiation ($P = 1.42 \times 10^{-3}$), immune response ($P = 2.20 \times 10^{-3}$), chromatin organization ($P = 3.90 \times 10^{-3}$), T cell differentiation ($P = 4.20 \times 10^{-3}$), homophilic cell adhesion ($P = 5.80 \times 10^{-3}$), and L-amino acid transport ($P = 6.10 \times 10^{-3}$). GO terms for hypermethylated genes included antigen processing and presentation ontology ($P = 6.89 \times 10^{-6}$) (Table 5).

DISCUSSION

In this study, we performed an unbiased genome-wide DNA methylation analysis of naive CD4+ T cells from patients with primary SS, and thereby identified a set of differentially methylated genes and involved pathways in patients with primary SS as compared to matched healthy controls. The molecular basis of primary SS is not well characterized; however, cumulative evidence indicates that activation of the LTβR pathway plays an integral role in the pathogenesis of primary SS (17). We identified 5 hypomethylated CpG sites in the *LTA* gene in naive CD4+ T cells from patients with primary SS as compared to healthy controls.

Lymphotoxins (LTα, LTβ) and their receptors are part of the tumor necrosis factor superfamily (18). Soluble LTα promotes production of IFNs and multiple chemokines that are important activating signals to the immune cells (19). Moreover, LTα forms a heterodimer with LTβ (LTα1β2), which in turn binds to LTβR (20). Activation of the LTβR pathway is crucial for the development, organization, and maintenance of lymphoid structures (21) and for modulating the expression of chemokines and adhesion molecules that aid in trafficking of lymphocytes and follicular dendritic cell activation (22).

Furthermore, studies in murine models have highlighted an interesting role of LTα in the pathogenesis of Sjögren's-like disease. LTα was found to be overexpressed in the salivary gland secretions and sera of IL14α-transgenic (TG) mice, a murine model of SS (23). The IL14α-TG mouse reproduces the clinical and immunologic changes characteristic of SS (24). Interestingly, IL14α-TG mice with deletion of the *LTA* gene did not develop disease (23). In another murine model of SS using NOD mice, Gatumu and colleagues (22) showed that blocking the LTβR pathway resulted in ablation of the lymphoid organization in the NOD mouse salivary glands and an improvement in salivary gland function (22). In another study, blocking of LTβR reduced the size of leukocyte infiltrates in the lacrimal glands, and improved tear production and corneal integrity (25). Additional evidence that LTα has a role in SS comes from the observation that LTα is overexpressed in the salivary glands and sera of patients with SS (23). Indeed, a clinical trial to evaluate the use of an LTβR fusion protein (baminercept) in the treatment of patients with primary SS is currently in progress. Our results suggest that epigenetic factors may play a role in LTα overexpression in patients with primary SS, as cytosine demethylation predisposes patients to transcriptionally permissive chromatin architecture in this locus.

Patients with primary SS have an activated type I IFN response (16,26), as has been demonstrated by observations of increased type I IFN activity and an "IFN signature" in PBMCs (27), saliva (28), and minor salivary gland biopsy tissue (29,30) from patients with primary SS. Several of the hypomethylated genes that were identified herein in naive CD4+ T cells from patients with primary SS were IFN-regulated genes (*STAT1*, *IFI44L*, *IFITM1*, and *USP18*). The extent of hypomethylation of IFN-regulated genes seems to be less robust in patients with primary SS in comparison to

patients with SLE (9), in whom we have recently identified and reproduced 21 hypomethylated IFN-regulated genes in naive CD4+ T cells.

In this study, we identified several differentially methylated genes that are important in activation of the immune system. *CD247* encodes for the TCR ζ-chain, is important for signal transduction upon antigen stimulation (31), and was hypomethylated in naive CD4+ T cells in patients with primary SS. Other pertinent genes included *PTPRC,* which encodes protein tyrosine phosphatase, receptor type C (also known as CD45 antigen or B220). PTPRC is a signaling molecule that is essential for T and B cell activation, cellular differentiation, and oncogenic transformation (32). *TNFRSF25*, which plays a role in lymphocyte homeostasis and in apoptosis, was also hypomethylated in patients with primary SS. Furthermore, we detected hypomethylation of *GSTM1*, which encodes a cytoplasmic glutathione S-transferase that is important in detoxification of electrophilic compounds, including carcinogens and environmental toxins (33). Of particular interest, *GSTM1* was proposed as a genetic risk locus for primary SS in a previous study (34), which might suggest that this gene has a role in genetic–epigenetic interactions in the pathogenesis of primary SS. Indeed, we have previously demonstrated a genetic association between primary SS and variants in *MECP2* (encoding methyl-CpG binding protein 2), a key transcriptional regulator with a critical role in DNA methylation (35). Among the transcription factors, we identified *RUNX1* as a hypermethylated gene in the present study. RUNX-1 regulates the differentiation of hematopoietic stem cells into mature cells (36) and has been linked to cancer predisposition (37,38). These findings suggest that *RUNX1* has a possible connection to lymphoma predisposition in primary SS.

It is generally accepted that defects in membrane water channel proteins contribute to the exocrinopathy in patients with primary SS (39). Our study identified several differentially methylated genes encoding members of the solute carrier proteins. The genes *SLC11A1*, *SLC11A2*, *SLC22A23*, *SLC25A25*, *SLC25A3*, *SLC25A33*, and *SLC6A20* were hypomethylated, whereas *SLC9A1*, which is expressed in the kidneys and plays an important role in the maintenance of pH homeostasis, was hypermethylated in patients with primary SS as compared to healthy controls. Mutations in solute carrier proteins have been previously linked to diseases associated with acid-base disturbances, such as Bartter and Gitelman syndromes (40). We hypothesize that methylation changes in these proteins may explain some of the pathologic aspects of primary SS–like defects in molecular water transport, dysfunction of the exocrine glands, and perhaps distal renal tubular acidosis in some patients with primary SS (41).

We used the DAVID database to facilitate the systematic identification and grouping of differentially methylated genes into biologic networks. Of particular interest, there was enrichment of ontologies involved in both the adaptive and the innate immune systems, underscoring involvement of the 2 arms of immunity in the pathogenesis of primary SS (Table 5).

Two cell-separation methods were used to isolate naive CD4+ T cells from patients and controls, albeit with consistent and equal cell purity (>95%). We further confirmed the absence of T cell activation or differentiation in the study samples with the use of epigenetic immunophenotyping, in which we examined the methylation status of *IL2*, *IFNG*, *IL4*, *IL5*, *IL13*, *IL17*, *IL17F*, and *FOXP3*. It is important to note that identification of the phenotypic specificity of cells is critical to accurately and reliably interpret differential methylation data in autoimmune diseases, while avoiding any perceived differences that could be related to altered cell constituents.

DNA methylation is tightly linked to chromatin accessibility, and gene expression requires both chromatin accessibility and appropriate transcription factors. Therefore, some, but not all, of the differences in DNA methylation identified in the present study can be expected to be associated with expression differences. Examining DNA methylation at a genome-wide level provides another level for discovering fundamental differences that might be pathogenic in the disease process at the chromatin level, which might or might not be linked directly to expression differences. For example, we recently observed that naive CD4+ T cells from lupus patients had widespread hypomethylation of IFN-regulated genes, before an expression difference could be detected (9). Interestingly, to our knowledge, there has not yet been a gene expression study of naive CD4+ T cells from patients with primary SS, and therefore we do not know which genes are differentially expressed in naive CD4+ T cells in this disease. Therefore, whether some of the methylation changes detected in this first DNA methylation study in patients with primary SS are reflective of a difference in the active (or potential) gene expression state remains to be seen.

The recent advances in epigenetics and the emergence of epigenome-wide association studies have resulted in the successful identification of numerous intriguing associations between epigenomic perturbations and human disease. However, this field is still in its

infancy and the issue of "causality" related to the detected epigenetic changes will require careful experimental examination. The issue of causality inference is even more complicated in patients with primary SS, for several reasons. First, understanding of the genetic predisposition to primary SS, which might influence epigenetic variations, is incomplete. Second, the environmental factors involved in the pathogenesis of primary SS remain, by and large, uncharacterized. Third, our study was designed as a retrospective case–control study; alternative study designs would be longitudinal studies of monozygotic twins discordant for primary SS or prospective studies to evaluate epigenetic perturbations prior to the onset of the disease, both of which are study designs that are cumbersome for obvious reasons. Nevertheless, such studies are warranted in the future to move this field forward (42).

Integrating emerging genomic data from patients with primary SS with epigenomic profiling might provide an avenue to discover mechanistic and pathogenic pathways to this disease. For example, we suspect that some genetic susceptibility variants that will be discovered in patients with primary SS might influence some of the methylation differences observed. A simultaneous genomic–epigenomic analysis should therefore focus on allele-specific methylation changes induced by genetic variants associated with primary SS. These approaches will help to identify novel mechanisms and therapeutic targets for this disease, and might enhance our understanding of the functional consequences of some of the genetic susceptibility loci in primary SS.

In summary, we identified, for the first time, DNA methylation changes in naive CD4+ T cells from patients with primary SS. These data indicate that abnormal DNA methylation exists in CD4+ T cells, even before the activation and differentiation of T cells, in patients with primary SS. Therefore, our findings emphasize the potential role of DNA methylation changes in the pathogenesis of primary SS. Our study demonstrates differential methylation of *LTA*, type I IFN–regulated genes, and solute carrier proteins, in addition to other key genes and pathways involved in the pathogenesis of primary SS. Future studies to replicate and determine the functional consequences of the methylation changes observed upon pathophysiologic development of the disease are warranted.

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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.

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