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Hypomethylation of *STAT1* and *HLA-DRB1* is associated with type-I interferon-dependent *HLA-DRB1* expression in lupus CD8+ T cells

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Abstract

Objective—We examined genome-wide DNA methylation changes in CD8+ T cells from patients with lupus and controls and investigated the functional relevance of some of these changes in lupus.

Methods—Genome-wide DNA methylation of lupus and age, sex and ethnicity-matched control CD8+ T cells was measured using the Infinium MethylationEPIC arrays. Measurement of relevant cell subsets was performed via flow cytometry. Gene expression was quantified by qPCR. Inhibiting *STAT1* and *CIITA* was performed using fludarabine and *CIITA* siRNA, respectively.

Results—Lupus CD8+ T cells had 188 hypomethylated CpG sites compared with healthy matched controls. Among the most hypomethylated were sites associated with *HLA-DRB1*. Genes involved in the type-I interferon response, including *STAT1*, were also found to be hypomethylated. IFN α upregulated *HLA-DRB1* expression on lupus but not control CD8+ T cells. Lupus and control CD8+ T cells significantly increased *STAT1* mRNA levels after treatment with IFN α . The expression of *CIITA*, a key interferon/*STAT1* dependent MHC-class II regulator, is

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data sharing statement Raw and processed DNA methylation data from patients and controls have been deposited in Gene Expression Omnibus (GEO accession number GSE123003)

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induced by IFN α in lupus CD8+ T cells, but not healthy controls. CIITA knockdown and STAT1 inhibition experiments revealed that HLA-DRB1 expression in lupus CD8+ T cells is dependent on CIITA and STAT1 signalling. Coincubation of naïve CD4+ T cells with IFN α -treated CD8+ T cells led to CD4+ T cell activation, determined by increased expression of CD69 and cytokine production, in patients with lupus but not in healthy controls. This can be blocked by neutralising antibodies targeting HLA-DR.

Conclusions—Lupus CD8+ T cells are epigenetically primed to respond to type-I interferon. We describe an HLA-DRB1+ Cd8+ T cell subset that can be induced by IFN α in patients with lupus. A possible pathogenic role for CD8+ T cells in lupus that is dependent on a high type-I interferon environment and epigenetic priming warrants further characterisation.

Introduction

Systemic lupus erythematosus (SLE) is a chronic relapsing autoimmune disease characterised by the production of autoantibodies and multiple organ involvement. The aetiology of lupus is incompletely understood; however, heightened interest in changes specific to the DNA methylome of lupus immune cells is emerging.^{1–8} Previous work examining differential DNA methylation in the T lymphocytes of patients with lupus has primarily been performed with CD4+ T cells.⁹ As lymphocytes are heavily involved in both the regulation and initiation of the immune response, investigation of DNA methylation changes in additional immunological cell types is of potential interest to further elucidate unknown components of lupus pathogenesis.

While the epigenetic landscape of CD8+ T cells in lupus has yet to be described, regulatory and functional changes of CD8+ T cells in lupus have been previously examined. Among total CD8+ T cells, patients with lupus with active disease have increased proportion of naïve CD8+ T cells and reduced proportion of effector CD8+ T cells.^{10,11} Effector CD8+ T cells in patients with lupus have reduced effector functionality through altered cytokine production, diminished suppressor function and decreased cytotoxic T cell activity.^{10,12} The cytokine profiles of lupus CD8+ T cells have been found to favour increased IL-12 and decreased IL-6 production, thus resulting in dysregulation of the stimulatory and inhibitory roles of CD8+ T cells, respectively.¹² In addition, CD8+ T cells in patients with lupus are characterised by reduced expression of signalling lymphocytic activation molecule family member 7, which is a type I transmembrane glycoprotein receptor that promotes effector CD8+ T cell function.¹⁰ In contrast, Blanco *et al* reported that patients with systemic lupus erythematosus disease activity index (SLEDAI) scores of seven or greater had a diminished naïve CD8+ T cell population and an elevated effector CD8+ T cell population.¹³ CD8+ T cells are critical in blocking viral infections, which might trigger disease activation in lupus by increased type-I interferon production. Indeed, a pathogenic role for Epstein Barr virus infection in inducing lupus has been suggested, and linked to increased type-I interferon production, and more recently to genetic susceptibility in lupus.^{14–18} As the role of CD8+ T cells in lupus remains incompletely understood and is likely dependent on currently unknown mechanisms, further examination of CD8+ T cell epigenetic changes in lupus could provide beneficial insight into this enigmatic disease.

In this study, we investigated genome-wide DNA methylation changes in CD8+ T cells of patients with lupus compared with age, sex and ethnicity matched healthy controls. Functional annotation analysis of genes hypomethylated in lupus CD8+ T cells, followed by functional studies, suggest that lupus CD8+ T cells are epigenetically primed to respond to interferon (IFN) α and overexpress HLA-DRB1.

METHODS

Patients and controls

A total of 61 patients with lupus (mean $\pm$ SEM age: 42.1 $\pm$ 1.4; median age: 42 and age range: 20–66 years) and 46 healthy controls (mean $\pm$ SEM age: 43.7 $\pm$ 1.7; median age: 40 and age range: 23–65 years) participated in this study. All patients with lupus fulfilled the American College of Rheumatology classification criteria for SLE.¹⁹ The mean SLEDAI score for patients with lupus involved in this study was 3.41 with a median of 4 (range: 0–12). Patients with lupus on cyclophosphamide or methotrexate were excluded from participating in the study as these drugs cause changes in cell surface expression of activation markers in lymphocyte subsets and altered epigenetic patterns, respectively.^{20–22} All participants signed informed consent approved by the Institutional Review Board of the University of Michigan.

Sample collection and DNA extraction from isolated CD8+ T cells

For DNA methylation studies, eight patients with lupus and eight healthy controls recruited from the University of Michigan rheumatology clinics were matched by age ($\pm$ 5 years), sex and ethnicity (online supplementary table 1). Peripheral blood mononuclear cells (PBMCs) were extracted from whole blood via Ficoll-Paque PLUS density centrifugation (GE Healthcare Bio-Sciences). Total T cells were isolated using negative selection by magnetic bead separation with the Human Pan T cell Isolation II kit (Miltenyi Biotec). Flow cytometry sorting was used to isolate CD3+ CD56-TCR $\alpha\beta$ + CD8+ T cells using the following fluorophore-conjugated antibodies: APC anti-human CD56 (clone: HCD56), FITC anti-human CD8a (clone: RPA-T8), Pacific Blue anti-human CD3 (clone: HIT3a) and PE anti-human alpha/beta-TCR (clone: IP26) (BioLegend), as previously described.²³ DNA was extracted from CD8+ T cells using the DNeasy Blood and Tissue kit (Qiagen) and bisulfite converted with the EZ DNA Methylation kit (Zymo Research) for DNA methylation analysis. It is worth noting that TCR $\alpha\beta$ +CD8+T cells constituted ~99% of CD8+ T cells in the peripheral blood of patients with lupus or controls.

Differential DNA methylation and gene functional annotation analysis of CD8+ T cells in patients with lupus

Methylation status of CD8+ T cells was measured by hybridising bisulfite-converted DNA to the Infinium MethylationEPIC Bead-Chip array (Illumina). Methylation β values were calculated from raw intensity values using GenomeStudio Methylation module software (Illumina) after subtracting background, normalisation to control probes, error correction using the Illumina custom model and correction for false discovery rate. Group comparison and statistical significance were determined using

$$\beta = \beta_{SLE} - \beta_{Control} \text{ and } DiffScore = 10 \times \operatorname{sgn}(\beta_{SLE} - \beta_{Control}) \times \log_{10}(P), \text{ respectively.}$$

Probes were filtered using the following criteria: $|\Delta\beta| \geq 0.10$, $|\text{DiffScore}| \geq 22$ (equivalent to an FDR-adjusted $p < 0.01$), detection $p < 0.05$ and no single nucleotide polymorphism (SNP) within 10 bp of the 3' end of the probe. Gene annotations for hypermethylated and hypomethylated probes were used as input for gene function annotation analysis using DAVID.²⁴ Categories were restricted to molecular function and biological process gene ontologies and KEGG pathways and only annotations with a minimum gene number of 2, a modified Fisher's exact $p < 0.1$ and FDR 5% were considered. Raw and processed DNA methylation data from patients and controls have been deposited in Gene Expression Omnibus (GEO accession number GSE123003).

IFN- α treatment and expression of HLA-DRB1 on CD8+ T cells

PBMCs were stimulated overnight in cell culture wells that were precoated with 10 $\mu\text{g/mL}$ of anti-human CD3 (Clone: UCHT1) and 2.5 $\mu\text{g/mL}$ of soluble anti-human CD28 (Clone: 28.2) (BD Biosciences) in T cell media (RPMI media with 10% fetal bovine serum and 2 mM L-glutamine) in the presence of 1000 U/mL IFN α 2B (Sigma-Aldrich). Culture media were then replaced and these cells were cultured for an additional 4 days with IFN α as this condition has been shown to produce peak HLA-DR expression on T cells.²⁵ At the end of the incubation, PBMCs were stained with fluorochrome-conjugated antibodies for the cell-surface markers CD3, CD8a and HLA-DRB1. The detailed flow cytometry procedure and analysis is described below.

Coincubation of IFN- α treated CD8+ T cells with naïve CD4+ T cells

After extraction from whole blood through Ficoll-Paque PLUS density centrifugation (GE Healthcare Bio-Sciences), PBMCs were divided into three portions. The first portion was cultured in T cell media for 5 days without any stimulation and treatment for naïve CD4+ T cell isolation using the Naïve CD4+ T Cell Isolation Kit II (Miltenyi Biotec), which allows for isolating untouched naïve CD4+ T cells. The second portion was stimulated with anti-human CD3 and anti-human CD28 in the presence of 1000 U/mL IFN- α 2B (Sigma-Aldrich) overnight in T cell media. These cells were then cultured for an additional 4 days with IFN α . The last portion of the PBMCs was similarly stimulated overnight with anti-human CD3 and anti-human CD28, and cultured for a total of 5 days, however, without IFN α treatment. Under these culturing conditions, the per cent cell death (based on trypan blue staining) of the PBMCs was 7.97 ± 2.38 , 9.53 ± 4.55 and 9.22 ± 5.51 , in the portion for naïve CD4+ T cell isolation, IFN α -treated PBMCs and non-IFN α -treated PBMCs, respectively. At the end of the culture period, CD8+ T cells were isolated via the CD8+ T Cell Isolation Kit (Miltenyi Biotec), which isolates untouched TCR $\alpha\beta$ + CD8+ T cells. To examine whether IFN α -treated CD8+ T cells can stimulate naïve CD4+ T cells, the isolated CD8+ T cells with or without IFN α treatment were incubated with naïve CD4+ T cells for 20–22 hours in a 1:1 ratio. The cells were then stained with fluorochrome-conjugated antibodies for CD3, CD4 and CD69 at a density of 2×10^4 cells per 1 μL of 1X phosphate-buffered saline (PBS). The percentage of CD4+ T cells bearing CD69, an early activation marker, was measured by flow cytometry for the CD3+ CD4+ T cells of both coincubation groups, as described below. For cytokine analysis, coculture of naïve CD4+ T cells and CD8+ T cells was carried out in T cell media containing 0.1% FBS. Cytokines were measured using Bio-Plex Pro Human Cytokine 17-Plex Assay which includes MIP-1 β , IL-6,

IFN γ , IL-5, GM-CSF, TNF, IL-2, IL-1 β , IL-13, IL-4, MCP-1, IL-8, IL-10, G-CSF, IL-7, IL-12 and IL-17. Cytokine levels were normalised to the total protein content and presented as pg/mg protein.

Coincubation of IFN α treated CD8+ T cells with naïve CD4+ T cells in the presence of anti-HLA-dR blocking antibody

PBMCs were isolated and divided into two groups. The first group was incubated in T cell media for 5 days and naïve CD4 + T cells were isolated. PBMCs in the second group were activated overnight with anti-human CD3 and anti-human CD28 and treated with 1000 U/mL IFN α overnight. The media for the PBMCs were then replaced and the cells were treated with IFN α for an additional 4 days. At the end of the incubation period, untouched TCR $\alpha\beta$ + CD8+ T cells were isolated using the CD8+ T Cell Isolation Kit (Miltenyi Biotec) and split evenly into three groups: Anti-HLA-DR, isotype control and no antibody. Prior to use, the Fc portions of the LEAF Purified anti-human HLA-DR Antibody and LEAF Purified Mouse IgG2a, κ Isotype Ctrl Antibody were degraded using the Pierce F(ab')₂ Preparation Kit (Thermo Fisher Scientific) to diminish non-specific binding of the Fc portion. The CD8+ T cells were treated with either 20 ug/mL of LEAF Purified anti-human HLA-DR Antibody (Clone: L243) or 20 ug/mL of LEAF Purified Mouse IgG2a, κ Isotype Ctrl Antibody (Clone: MOPC-173) (BioLegend) at 37°C for 1 hour, while the CD8+ T cells in the no antibody group were left untreated. The cells were then washed with PBS twice to remove any residual IgG. The CD8+ T cells from the three groups were then coincubated with naïve CD4+ T cells overnight. At the end, cells were stained with fluorochrome-conjugated antibodies for CD3, CD4 and CD69. The percentage of CD4+ T cells expressing CD69, an early activation marker, was measured by flow cytometry for the CD3+ CD4+ T cells for all coincubation groups.

Flow cytometry analysis

PBMCs were stained with fluorochrome-conjugated antibodies at a density of 2×10^4 cells per 1 μ L of $1 \times$ PBS for 30 min on ice. The following fluorochrome-conjugated antibodies were used for flow cytometry antibody staining: FITC anti-human CD8a (clone: RPA-T8), APC anti-human CD69 (clone: FN50), APC/Cy7 anti-human CD4 (clone: RPA-T4), Pacific Blue anti-human CD3 (clone: UCHT1) and PE anti-human HLA-DRB1 (clone: NFDL D2). All fluorochrome-conjugated antibodies were purchased from BioLegend. PBMCs were fixed with 500 μ L of Biolegend Fixation Buffer per 10^6 cells for 20 min at ambient temperature. The iCyt Synergy SY3200 Cell Sorter (Sony Biotechnology Inc.) was used in conjunction with WinList 9.0.1 software (Verity Software House) for flow cytometry sample processing and analysis, respectively. PBMCs were gated to select for CD3+ CD8+ HLA-DRB1+ T cells (online supplementary figure 1). The median fluorescence intensity (MFI) of PE anti-human HLA-DRB1 was measured for the CD3+ CD8+ T cell population and normalised to the background PE anti-human HLA-DRB1 MFI of the unstained PBMC population. The normalised MFI (nMFI) was determined by the following equation:

$$nMFI = \frac{PE\ MFI_{sample}}{PE\ MFI_{unstained\ control}}.$$

²⁶ FlowJo version 10 was used to generate MFI histograms (FlowJo LLC).

STAT1 inhibition and CIITA knockdown

To examine the effect of STAT1 on IFN α -induced CIITA expression, CD8+ T cells isolated from lupus PBMCs were activated overnight with anti-human CD3 and anti-human CD28 while treated with 50 μ M of the STAT1 inhibitor fludarabine for 48 hours in the presence or absence of IFN α . Protein lysate was collected and the expression of phosphorylated STAT1 (Cell Signalling), STAT1 (Cell Signalling), HLA-DRB1 (Novus Biologicals) and CIITA (Santa Cruz Biotechnology) was measured by Western blotting. β -actin (Sigma-Aldrich) was used as a loading control. Densitometry was analysed using ImageJ.²⁷ In a separate experiment, isolated CD8+ T cells from patients with lupus were transfected with control or CIITA siRNAs (400 nM, both from Dharmacon) using the Amaxa 4D- Nucleofactor system (Lonza). After transfection, CD8+ T cells were allowed to rest for 5 hours before replacing the culture media with fresh T cell media. The cells were then stimulated overnight with anti-CD3 and anti-CD28 antibodies. After stimulation, the transfected CD8+ T cells were treated with or without IFN α for an additional 48 hours before RNA were collected. The mRNA levels of CIITA and HLA-DRB1 were measured by qPCR.

Quantitative real-time PCR

RNA was isolated with the Direct-zol RNA MiniPrep kit according to the manufacturer's instructions (Zymo Research). The Verso cDNA Synthesis Kit (Thermo Fisher Scientific) was used along with reverse transcriptase PCR to produce the cDNA necessary for quantitative real-time PCR (qPCR). qPCR was done with Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) and used along with human KiCqStart SYBR Green primers to measure mRNA expression of *STAT1*, *CIITA* (both from Sigma-Aldrich) and *HLA-DRB1* (Qiagen). All gene targets were run in duplicates and normalised to β -actin. qPCR was run using either a ViiA 7 Real-Time PCR system or the ABI 7900HT instrument.

Statistical analysis

Graphpad Prism 7.0 was used for statistical analysis and creation of graphs. For comparing sample groups from the same subjects but with different cell culture treatments the paired, non-parametric Wilcoxon Test was used. For tests comparing the same treatment conditions but with different subjects the unpaired, non-parametric, Mann-Whitney Test was used. For correlation analysis, a Pearson correlation coefficient was used. For all statistical tests, a significance cut-off of $p < 0.05$ was used.

Results

DNA methylation changes in lupus CD8+ T cells

In contrast to the reported global hypomethylation in lupus T cells²⁸ and naïve CD4+ T cells,²⁹ DNA methylation analysis of lupus CD8+ T cells revealed significant hypermethylation of 943 loci and hypomethylation of 188 loci compared with matched control CD8+ T cells (online supplementary table 2). Our data, however, agree with what was observed recently in lupus B cells, which showed predominant hypermethylation changes compared with controls.⁸ This discrepancy might be due to differences in metabolic control in different T cell subsets and B cells as well as the availability of cofactors

necessary for the DNA methylation machinery.³⁰ The most hypomethylated CpG site in lupus CD8+ T cells was found within the major histocompatibility complex class II gene *HLA-DRB1* (cg11404906; $\beta = -0.333$) (table 1). Four other loci were hypomethylated in *HLA-DRB1*, two of which were also among the most hypomethylated (cg09949906; $\beta = -0.270$ & cg09139047; $\beta = -0.247$). These loci are in or around exon 2 of *HLA-DRB1* and a DNase I hypersensitivity cluster, suggesting a regulatory function in this genetic region (figure 1). We did not observe correlation between DNA methylation levels in *HLA-DRB1* and disease activity in patients with lupus, as measured by SLEDAI scores, although our sample size was not powered to examine such a correlation (data not shown). The most hypermethylated site in lupus CD8+ T cells was not associated with a gene (cg01005486; $\beta = 0.325$), but loci associated with *HLA-DRB5* (cg22635523; $\beta = 0.239$) and *HLA-DRB6* (cg06559318; $\beta = 0.207$ & cg20083297; $\beta = 0.189$) did have significantly higher methylation levels in lupus CD8+ T cells (table 1).

Genes involved in the type I interferon response were also found to be hypomethylated in lupus CD8+ T cells including the transcription factor *STAT1* (cg00676801; $\beta = -0.150$ & cg14951497; $\beta = -0.138$) and *MX1* (cg21549285; $\beta = -0.239$ and cg22862003; $\beta = -0.122$) (table 1 and online supplementary table 2), both type I interferon-related genes that have previously been identified as being hypomethylated in several immune cell types in patients with lupus, including CD4+ T cells.¹ This was supported by gene enrichment analysis, which revealed a predominance of gene ontology terms for interferon response and signalling: ‘interferon-gamma-mediated signalling pathway’ (GO:0060333; $p = 1.41E-05$), ‘defense response to virus’ (GO:0051607; $p = 7.56E-04$) and ‘type I interferon signalling pathway’ (GO:0060337; $p = 2.72E-03$) (online supplementary table 3). Antigen presentation was also a major theme that included ‘peptide antigen binding’ (GO:0042605; $p = 2.17E-04$) and ‘antigen processing and presentation’ (GO:0019882; $p = 1.76E-03$) terms. KEGG pathways enriched in hypomethylated genes showed that ‘Cell adhesion molecules (CAMs)’ (KEGG:hsa04514; $p = 6.73E-05$) was the most significantly enriched pathway among hypomethylated genes (online supplementary table 3). Several disease pathways were also enriched including autoimmune diseases like rheumatoid arthritis (KEGG:hsa05323; $p = 9.51E-04$) and SLE (KEGG:hsa05322; $p = 4.44E-03$) as well as viral infections (‘Influenza A’ KEGG:hsa05164; $p = 1.68E-03$ and ‘Herpes simplex infection’ KEGG:hsa05168; $p = 2.10E-03$). A pathway including glutathione *S*-transferase genes (GST) (‘Drug metabolism – cytochrome P450’ KEGG:hsa00982; $p = 4.76E-03$) was also enriched (online supplementary table 3). GSTs catalyse the conjugation of reduced glutathione to electrophilic compounds like xenobiotics and increase their solubility and excretion from the cell and reduce the toxicity of reactive oxygen species in the cell.³¹ The hypermethylated genes displayed broader functional annotation groups and did not show dominance of enriched pathways by HLA genes that were seen among hypomethylated genes (online supplementary table 3).

IFN α induces HLA-DRB1 expression on Lupus CD8+ T cells

HLA-DRB1 was robustly hypomethylated in lupus CD8+ T cells. We also observed hypomethylation in *STAT1* and the interferon-signalling pathway, which regulate MHC class II expression. Therefore, we sought to study the functional implications of these epigenetic

changes by investigating the expression of HLA-DRB1 and the role of type-I interferon response in the CD8+ T cells of patients with lupus. Indeed, we found that both patients and controls possess a CD8+ HLA-DRB1+ T cell subset; however, the subset's response to IFN α differs in patients with lupus. In lupus CD8+ T cells treated with IFN α , the expression of HLA-DRB1 was significantly elevated compared with untreated cells ($p = 0.02$). In addition, when lupus CD8+ T cells were treated with IFN α , they expressed significantly more HLA-DRB1 on their surface than IFN α treated healthy control cells ($p = 0.04$, figure 2A,B). Similarly, HLA-DRB1 expression, as measured by nMFI, was increased with IFN α treatment in lupus CD8+ T cells ($p = 0.04$), but not in healthy controls ($p = 0.79$, figure 2C,D). These data suggest that hypomethylation of *HLA-DRB1* epigenetically primes lupus CD8+ T cells for type-I interferon dependent HLA-DRB1 overexpression.

STAT1 and CIITA are elevated in IFN α -treated lupus CD8+ T cells

We observed hypomethylation of loci associated with STAT1 in lupus CD8+ T cells (online supplementary table 2). STAT1 and CIITA are both important regulators in the MHC-II expression pathway (online supplementary figure 2). For these reasons, we evaluated the expression of *CIITA* and *STAT1* mRNA in lupus CD8+ T cells with and without IFN α treatment. *STAT1* expression was elevated following IFN α treatment in both patients with lupus ($p = 0.02$) and controls ($p = 0.02$) (figure 3A). The expression of *CIITA* was induced by IFN α stimulation in lupus CD8+ T cells ($p = 0.03$), however, not in healthy controls (figure 3B). These findings suggest a type-I IFN dependent mechanism for the increased expression of HLA-DRB1 on lupus CD8+ T cells possibly mediated through *STAT1* and *CIITA* in a high type-I IFN environment.

To further confirm the involvement of STAT1 in IFN α -mediated CIITA and HLA-DRB1 expression in lupus CD8+ T cells, STAT1 was blocked using an inhibitor (fludarabine) and the expression of CIITA and HLA-DRB1 was examined. As shown in figure 3C and online supplementary figure 3, IFN α significantly increased CIITA, phosphorylated STAT1 and STAT1, while it increased HLA-DRB1 expression slightly. Comparing the levels of IFN α -induced HLA-DRB1 in this experiment and what was presented in figure 2, it appears that the effect of IFN α on HLA-DRB1 in CD8+ T cells relies on the presence of other cell types, as PBMCs and isolated CD8+ T cells were used in figures 2 and 3C, respectively. We also observed a positive correlation between STAT1 and HLA-DRB1 levels in these lupus CD8+ T cells (online supplementary figure 4) while no correlation was found between CIITA and HLA-DRB1. As expected, the addition of fludarabine downregulated both phosphorylated STAT1 and total STAT1. This subsequently led to downregulation of CIITA and HLA-DRB1. These data suggest a critical role of STAT1 in IFN α -HLA-DRB1 pathway. In a separate experiment, we knocked down CIITA in lupus CD8+ T cells in the presence of IFN α and examined whether this affected the ability of IFN α to induce HLA-DRB1 expression. Indeed, CIITA knockdown resulted in downregulation of HLA-DRB1 in lupus CD8+ T cells (figure 3D). A $68.7\% \pm 16.2\%$ knockdown of CIITA led to a $58.2\% \pm 28.4\%$ reduction in HLA-DRB1 in the presence of IFN α .

Lupus CD8+ T cells activate autologous naïve CD4+ T cells in a type-I interferon and HLA-DR regulated manner

We showed that *HLA-DRB1* and *STAT1* are hypomethylated in lupus CD8+ T cells, which primes for increased HLA-DRB1 cell surface expression in the presence of IFN α . To demonstrate if HLA-DRB1 overexpression on lupus CD8+ T cells has functional consequences, we examined whether lupus CD8+ T cells have the ability to stimulate autologous naïve CD4+ T cells. IFN α treatment increased the ability of lupus CD8+ T cells to induce CD69 expression, which is an early marker for T cell stimulation, on autologous naïve CD4+ T cells ($p = 0.002$), while it had non-significant minimal effect on CD8+ T cells from healthy controls (figure 4A,B). This difference in response resulted in significant elevation of the percentage of stimulated CD4+ T cells after incubation with IFN α -treated CD8+ T cells from patients with lupus compared with those isolated from healthy controls ($p = 0.04$). Similarly, significant elevation of CD69 expression, as measured by nMFI, on CD4+ T cells after cocubation with IFN α -treated lupus CD8+ T cells was also observed compared with IFN α -treated control CD8+ T cells ($p=0.0004$; Online supplementary figure 5). To further examine the effect of CD8+ T cells on naïve CD4+ T cell activation, we measured cytokine production in the CD4+/CD8+ T cell coculture. Of the 17 cytokines measured, only 5, including TNF, IFN γ , IL-8, MIP-1 β and MCP1, were above detection limit (figure 4C). Elevated levels of cytokines were observed in lupus samples. IFN α -pretreatment of CD8+ T cells was unable to increase cytokine production in CD4+/CD8+ T cell coculture in healthy controls except for IFN γ , while IFN α increased TNF, MIP-1 β and MCP1 production significantly in lupus T cells. All of these cytokines have been implicated in lupus pathogenesis.^{32–36}

To determine if activation of naïve CD4+ T cells in the coculture experiments is dependent on HLA-DRB1 expression on IFN α -treated CD8 + T cells, we performed blocking experiments using neutralising antibodies to HLA-DR. The increase in CD3+ CD4+ CD69+ T cells as a result of cocubation with IFN α -treated lupus CD8+ T cells was inhibited when CD8 + T cells were pretreated with anti-HLA-DR blocking antibody (figure 4D,E, $p=0.03$). These data suggest that IFN α -treated lupus CD8+ T cells are capable of stimulating autologous naïve CD4+ T cells in a manner dependent on induced HLA-DRB1 expression.

Discussion

We performed a DNA methylation analysis in lupus CD8+ T cells and identified hypomethylation in CpG sites associated with *HLA-DRB1* and *STAT1* compared with healthy matched controls. Because Interferon/STAT1 signalling induces MHC class II expression and type-I interferon production is increased in patients with lupus, we hypothesised that demethylation in *HLA-DRB1* and *STAT1* might be associated with HLA-DRB1 expression on lupus CD8+ T cells, especially in the presence of type-I interferon. Indeed, our data demonstrate HLA-DRB1 cell surface expression in a subset of CD8+ T cells that is expanded in lupus compared with healthy controls and that HLA-DRB1 expression can be induced in the presence of type-I interferon in lupus but not control CD8+ T cells. These data suggest that epigenetic priming facilitates a type-I interferon-dependent over-expression of HLA-DRB1 in lupus CD8+ T cells. We further show that lupus CD8+ T

cells treated with type-I interferon can activate autologous naïve CD4+ T cells and that this activation is abrogated by preincubating lupus CD8+ T cells with HLA-DR blocking antibodies.

Both *HLA-DRB1* and *STAT1*, which are hypomethylated in lupus CD8+ T cells, are two possible downstream gene targets of the JAK-STAT signalling cascade initiated by type-I IFN signalling (online supplementary figure 2).^{37–39} IFN α can upregulate the surface expression of HLA-DRB1 by first binding to an IFN α receptor on the cell surface and initiate the JAK-STAT signalling cascade that produces STAT1-STAT1 (STAT1–1) homodimers and STAT1-STAT2 (STAT1–2) heterodimers.^{40,41} STAT1–1 homodimers and STAT1–2 heterodimers translocate to the nucleus where they bind to the gamma-activated sequence (GAS) and allow for the expression of the class II transactivator (CIITA) transcription factor; CIITA binds to the MHC-II enhanceosome which upregulates MHC-II expression.^{37–39} The canonical route taken for STAT dimerisation during a type-I IFN response is a STAT1–2 heterodimer binding to ISGF3, translocating to the nucleus and binding to the interferon stimulated response element to initiate the antiviral response.⁴⁰ However, as STAT1 homodimers binding to GAS are the typical path necessary for MHC-II expression, it is important to note that the type-I IFN response can also produce STAT1 homodimers and STAT1–2 heterodimers that can bind to GAS.^{37,41,42} In this study, we indeed demonstrated that type-I IFN stimulates *STAT1* expression in CD8+ T cells to a similar extent in both healthy controls and patients with lupus. However, the response in *CIITA* expression after type-I IFN treatment is what sets the controls and patients apart; *CIITA* was significantly elevated after IFN α treatment in lupus CD8+ T cells but not in healthy controls. This might potentially explain the higher HLA-DRB1 expression on lupus CD8+ T cells.

We also identified a subset of cell adhesion molecules differentially methylated in lupus CD8+ T cells. *SELL* encodes the cell adhesion molecule L-selectin (Lymphocyte-selectin) and was hypomethylated at a single locus in our analysis (cg06816239; $\beta = -0.15$), which was not identified in other genome-wide DNA methylation studies in lupus.⁹ L-selectin is vital to the ability of activated CD8+ T cells to traffic to tissues infected by viruses.⁴³ Our analysis also revealed that a single CpG site in the 3' untranslated region of *SELPLG* was hypermethylated in lupus CD8+ T cells (cg02520593; $\beta = 0.17$). PSGL-1 (encoded by *SELPLG*) is a ligand for the P-selectin that plays a role in T cell tissue infiltration.⁴⁴ PSGL-1 expression is associated with reduced TCR and IL-2 stimulatory activity in murine CD8+ T cells, leading to decreased cell survival and tumour cell killing.⁴⁴ Despite the enrichment in cell adhesion molecules that was observed from the DNA methylation profiles, we did not observe any significant differences in cell adhesion between control and lupus CD8+ T cells in a T cell-endothelial cell assay (data not shown).

Previous studies investigating the role of CD8 + T cells in lupus have primarily focused on T cell cytotoxicity, autoantibody production and cellular markers indicative of disease flares. Most reports showed defective cytotoxic responses in lupus CD8+ T cells, which has been suggested as a possible mechanism contributing to increased infection rates in patients with lupus.^{10,12} In addition, the conversion of CD8+ T cells into double-negative T cells has been suggested to compromise cytotoxicity of CD8+ T cells in lupus.^{45,46} Other studies, however,

suggest that patients with higher disease activity may have increased cytotoxic T cell activation and elevated proportion of CD 8+ T cells expressing granzyme B, perforin and HLA-DR.¹³⁴⁷ In our study, we did not detect DNA methylation changes in the gene loci encoding for perforin and granzyme B. In addition, other cytotoxic gene loci encoding for granzyme A, granzyme H, granzyme K, granzyme M and the genes encoding transcription factors eomesodermin (Eomes) and T-bet, which play key roles in CD 8+ T cell differentiation, were also not differentially methylated between patients with lupus and controls. A recent study by Li *et al* using targeted delivery of a DNA methyltransferase inhibitor 5-azacytidine into CD4+ or CD8+ T cells points to the importance of cell type-specific modulation of DNA methylation in lupus pathogenesis and also emphasises the necessity of the presence of functional cytotoxic T cells in this disease.⁴⁸

In summary, we describe DNA methylation changes in lupus CD8+ T cells at a genome-wide level and provide evidence that CD8+ T cells in lupus are epigenetically primed for HLA-DRB1 overexpression in response to type-I IFN. It should be noted that clinical and molecular disease heterogeneity as well as differences in therapeutic experiences between patients are important factors that can potentially impact DNA methylation changes in this disease. CD8+ HLA-DRB1+ T cell subset is expanded in patients with lupus compared with healthy controls and can be induced by IFN α in lupus but not healthy control CD8+ T cells. In patients with lupus, CD8+ HLA-DRB1+ T cells can stimulate autologous naïve CD4+ T cells in a manner dependent on HLA-DRB1 expression. Our data link a higher type-I interferon environment in patients with lupus to a pathogenic CD8+ T cell subset, which can be potentially targeted by blocking type-I interferon response. These findings provide novel insights into possible pathogenic roles for CD8+ T cells in lupus and suggest an interaction between high type-I interferon environment in patients with lupus with epigenetic dysregulation in CD8+ T cells. Additional studies to evaluate if lupus-associated genetic variants within the HLA region influence DNA methylation patterns in the *HLA-DRB1* locus, as has been recently reported in monocytes, are warranted.⁴⁹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key messages

- ▶ Lupus CD8+ T cells are characterised by significant methylation changes compared with normal CD8+ T cells. The *HLA-DRB1* genetic locus is hypomethylated in lupus CD8+ T cells.
- ▶ We describe an HLA-DRB1+ CD8+ T cell subset that can be induced by IFN α in patients with lupus.
- ▶ CD8+ T cells in patients with lupus are epigenetically primed for HLA-DRB1 cell surface expression in type-I interferon environment.
- ▶ IFN α -induced HLA-DRB1 expression in lupus CD8+ T cells is dependent on CIITA and STAT1 signalling.
- ▶ HLA-DRB1+ CD8+ T cells in lupus can stimulate autologous naïve CD4+ T cells in a manner dependent on HLA-DRB1 expression, providing a novel pathogenic role for CD8+ T cells in lupus.

Figure 1.

Diagram depicting the *HLA-DRB1* gene and the positions of the five CpG sites within *HLA-DRB1* hypomethylated in lupus CD8⁺ T cells compared with normal healthy controls (each CpG site is shown with a red bar). The hypomethylated region overlaps with a known DNase hypersensitivity cluster, suggesting a regulatory potential. ‘HLA-DRB1’ and ‘DNase clusters’ tracks were retrieved from UCSC Genome Browser. DNase clusters represent regions of DNase I hypersensitivity as described in the ‘DNaseI hypersensitivity clusters in 125 cell types from ENCODE (V3)’ track.⁵⁰ Lower panel shows DNA methylation fractions on each of the CpG sites in patients and controls (mean±SEM). *P<0.01 after correction for multiple testing for all probes on the array.

Figure 2.

IFN α significantly increased HLA-DRB1 expression on lupus CD8+ T cells but not in healthy controls. (A) The percentage of CD8+ T cells expressing HLA-DRB1 in patients with lupus and healthy controls with and without IFN α , and a representative dot plot (B). nMFI data (C), and (D) representative histograms (MFI) from a patient with lupus and a healthy control with and without IFN α , showing increased HLA-DRB1 expression with IFN α on the cell surface of lupus CD8+ T cells. Results are presented as mean $\pm$ SEM, and $p < 0.05$ was considered significant. N=number of subjects. nMFI, normalised median fluorescence intensity.

Figure 3.

STAT1 and *CIITA* mRNA expression in CD8⁺ T cells. (A) IFN α treatment significantly increased *STAT1* mRNA expression in CD8⁺ T cells from both patients with lupus and healthy controls. (B) IFN α treatment significantly increased *CIITA* mRNA expression in CD8⁺ T cells from patients with lupus but not healthy controls. (C) The effect of IFN α on *CIITA*, *STAT1* and *HLA-DRB1* can be blocked by fludarabine (Flud), a *STAT1* inhibitor in CD8⁺ T cells from patients with lupus. Figure shown here is representative of seven patients. (D) *CIITA* knockdown in CD8⁺ T cells isolated from patients with lupus resulted in downregulation of IFN α -induced *HLA-DRB1* expression. Results are presented as mean $\pm$ SEM, and p<0.05 was considered significant. N=number of subjects.

Figure 4.

Effect of CD8+ T cells on activating autologous naïve CD4+ T cells. (A) Lupus and control CD8+ T cells, with and without IFN α , were cocultured with autologous naïve CD4+ T cells and the percentage of CD3+ CD4+ CD69+ T cells were evaluated. A representative dot plot is shown in panel (B). (C) Cytokine production from the CD8+/CD4+ T cell culture was analysed using Bio-Plex assay kit. (D) Blocking HLA-DR on IFN α -treated lupus CD8+ T cells abrogates costimulation of naïve CD4+ T cells compared with isotype IgG control. A representative dot plot of six independent experiments is shown in (E). Results are presented as mean $\pm$ SEM, and $p < 0.05$ was considered significant. N=number of subjects.

Table 1

Top 25 hypomethylated and hypermethylated loci in lupus CD8+ T cells

Probe ID	β	Patient average methylation (β)	Control average methylation (β)	Genomic locus (chr:position) (hg19)	DiffScore	Gene
cg11404906	-0.333	0.302	0.635	6:32551749	-337.887	<i>HLA-DRB1</i>
cg02453376	-0.330	0.402	0.732	1:152586240	-337.887	<i>LCE3B</i>
cg14392283	-0.319	0.445	0.765	8:144103587	-337.887	<i>LY6E</i>
cg10978274	-0.317	0.412	0.729	1:152572665	-337.887	<i>LCE3C</i>
cg10829391	-0.308	0.418	0.725	14:101069717	-337.887	
cg09949906	-0.270	0.361	0.630	6:32552350	-250.225	<i>HLA-DRB1</i>
cg00293330	-0.266	0.340	0.606	1:152572678	-242.598	<i>LCE3C</i>
cg17748470	-0.252	0.421	0.674	11:4969161	-219.081	<i>OR51A4</i>
cg12393503	-0.252	0.473	0.725	3:172468831	-232.588	
cg09139047	-0.247	0.462	0.709	6:32552042	-217.006	<i>HLA-DRB1</i>
cg16180556	-0.245	0.286	0.531	1:110230269	-204.564	<i>GSTM1</i>
cg21549285	-0.239	0.469	0.707	21:42799141	-201.317	<i>MX1</i>
cg26184673	-0.221	0.568	0.789	12:131520095	-201.400	<i>ADGRD1</i>
cg08822897	-0.215	0.348	0.564	1:1:64258103	-144.881	
cg27244972	-0.212	0.418	0.630	21:47716529	-140.917	<i>C21orf57</i>
cg02484732	-0.207	0.361	0.569	6:56096198	-132.102	<i>COL21A1</i>
cg20684491	-0.204	0.555	0.758	1:25596433	-156.792	
cg09234453	-0.199	0.375	0.575	6:18402672	-120.179	<i>RNF144B</i>
cg21158163	-0.198	0.681	0.879	20:59542578	-229.793	
cg16361921	-0.198	0.384	0.582	5:106599492	-118.102	
cg06650861	-0.197	0.379	0.576	4:169238926	-116.972	<i>DDX60</i>
cg01609658	-0.196	0.200	0.396	1:151694478	-143.422	<i>RIIAD1</i>
cg07815522	-0.194	0.480	0.675	3:122282157	-120.319	<i>PARP9-DTX3L</i>
cg24311634	-0.192	0.557	0.749	12:131519883	-134.197	<i>ADGRD1</i>
cg18220841	-0.188	0.344	0.531	16:88160246	-104.890	
cg11102724	0.182	0.293	0.112	5:150619039	340.551	
cg00256329	0.186	0.702	0.515	17:7 24 374	114.220	<i>NXN</i>

Probe ID	β	Patient average methylation (β)	Control average methylation (β)	Genomic locus (chr:position) (hg19)	DiffScore	Gene
cg24432675	0.186	0.603	0.417	10:1505472	102.562	<i>ADARB2</i>
cg19214707	0.187	0.406	0.219	7:3157722	125.122	
cg27246321	0.189	0.462	0.273	2:165924708	114.220	
cg02419513	0.189	0.299	0.110	8:90255978	340.551	
cg20083297	0.189	0.685	0.496	6:32528390	114.865	<i>HLA-DRB6</i>
cg09398595	0.190	0.751	0.561	14:98255618	340.551	
cg24919907	0.193	0.799	0.606	14:98255601	340.551	
cg14507403	0.196	0.550	0.355	7:97798005	115.218	<i>LMTK2</i>
cg11244641	0.207	0.591	0.384	2:241343025	340.551	
cg06559318	0.207	0.728	0.521	6:32526260	340.551	<i>HLA-DRB6</i>
cg10123377	0.212	0.584	0.373	3:42387524	340.551	
cg12022450	0.213	0.687	0.474	22:24353218	340.551	
cg21787089	0.213	0.806	0.593	3:46484250	340.551	<i>LTF</i>
cg01427108	0.225	0.668	0.443	3:46484379	340.551	<i>LTF</i>
cg25755428	0.227	0.570	0.343	19:13875111	340.551	<i>MR11</i>
cg14188831	0.233	0.299	0.066	1:25594523	340.551	
cg09232555	0.236	0.508	0.272	8:11619866	340.551	
cg22635523	0.239	0.682	0.443	6:32492390	340.551	<i>HLA-DRB5</i>
cg05176970	0.252	0.707	0.455	17:7 24 273	340.551	
cg12108278	0.291	0.892	0.601	17:15789774	340.551	<i>NXN</i>
cg12340462	0.297	0.650	0.353	9:46141878	340.551	
cg18213661	0.321	0.722	0.401	11:93681423	340.551	
cg01005486	0.325	0.802	0.477	3:13246006	340.551	