Demethylation of Promoter Regulatory Elements Contributes to Perforin Overexpression in CD4+ Lupus T Cells

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Inhibiting DNA methylation in CD4⁺ T cells causes aberrant gene expression and autoreactive monocyte/macrophage killing in vitro, and the hypomethylated cells cause a lupus-like disease in animal models. Similar decreases in T cell DNA methylation occur in idiopathic lupus, potentially contributing to disease pathogenesis. The genes affected by DNA hypomethylation are largely unknown. Using DNA methylation inhibitors and oligonucleotide arrays we have identified perforin as a methylation-sensitive gene. Our group has also reported that DNA methylation inhibitors increase CD4⁺ T cell perforin by demethylating a conserved methylation-sensitive region that is hypomethylated in primary CD8⁺ cells, which express perforin, but is largely methylated in primary CD4⁺ cells, which do not. As lupus T cells also have hypomethylated DNA and promiscuously kill autologous monocytes/macrophages, we hypothesized that perforin may be similarly overexpressed in lupus T cells and contribute to the monocyte killing. We report that CD4⁺ T cells from patients with active, but not inactive, lupus overexpress perforin, and that overexpression is related to demethylation of the same sequences suppressing perforin transcription in primary CD4⁺ T cells and demethylated by DNA methylation inhibitors. Further, the perforin inhibitor concanamycin A blocks autologous monocyte killing by CD4⁺ lupus T cells, suggesting that the perforin is functional. We conclude that demethylation of specific regulatory elements contributes to perforin overexpression in CD4⁺ lupus T cells. Our results also suggest that aberrant perforin expression in CD4⁺ lupus T cells may contribute to monocyte killing. The Journal of Immunology, 2004, 172: 3652–3661.

A normal hypomethylation of CD4⁺ T cell DNA can contribute to the pathogenesis of lupus-like diseases. Human and murine CD4⁺ T cells treated with the DNA methylation inhibitor 5-azacytidine (5-azaC) become autoreactive in vitro, and CD4⁺ murine T cells made autoreactive with 5-azaC induce a lupus-like disease in vivo (1–4). The autoreactivity is due in part to LFA-1 (CD11a/CD18) overexpression, because LFA-1-transfected CD4⁺ T cells have an identical autoreactivity in vitro and cause a similar lupus-like disease in vivo (5). Further, some lupus-inducing drugs, including procainamide and hydralazine, are DNA methylation inhibitors (2), and CD4⁺ T cells treated with these drugs overexpress LFA-1 and become autoreactive in vitro, and induce autoimmunity in vivo, similar to the effects of 5-azaC (6). In addition, CD4⁺ T cells from patients with active lupus have demethylated DNA (7) and overexpress LFA-1 on an autoreactive subset (8). Finally, the same LFA-1 regulatory sequences are demethylated in T cells treated with 5-azaC and in T cells from patients with active lupus (9). However, the DNA hypomethyl-

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4 Abbreviations used in this paper: 5-azaC, 5-azacytidine; CYC, CyChrome; DMF, dimethylformamide; MCF, mean channel fluorescence; 6-MP, 6-mercaptopurine; RA, rheumatoid arthritis; SB, standard buffer; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index.

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Materials and Methods

Abs and reagents

Purified PHA was obtained from Murex (Norcross, GA). The following reagents were obtained from BD Pharmingen (San Diego, CA) and unless specified otherwise, the Abs were all mouse anti-human mAbs: PE-anti-human perforin kit, unconjugated anti-human perforin, CyChrome (CYC) anti-CD3, allophycocyanin- and CYC-anti-CD4, allophycocyanin-CD11a, FITC-anti-CD25, CYC- and FITC-anti-CD8, allophycocyanin-mouse IgG2a, PE-mouse IgG1, FITC-mouse IgG1, and CYC mouse IgG1. A pan-T cell isolation kit, CD4 T cell isolation kit, monocyte isolation kit, and CD8 microbeads were obtained from Miltenyi Biotech (Auburn, CA). Chloroquine, hydrocortisone, indomethacin, 6-mercaptopurine, and concanamycin A were obtained from Sigma-Aldrich (St. Louis, MO). 51Cr was obtained from PerkinElmer Life Sciences (Boston, MA).

Patient selection

Patients with active systemic lupus erythematosus (SLE), inactive SLE, and rheumatoid arthritis (RA) were recruited from the out-patient rheumatology clinic and in-patient services at the University of Michigan. Healthy controls were obtained by advertising. This protocol was approved by the University of Michigan internal review board, and written informed consent was obtained from all subjects. All SLE and RA patients fulfilled the American College of Rheumatology criteria for these diseases (13, 14).

PBMC and T cell isolation

PBMC were isolated by Ficol-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation as previously described (1). T cells were isolated by negative selection using magnetic beads and instructions provided by the manufacturer (pan T cell isolation kit; Miltenyi Biotech) or by rosetting with sheep erythrocytes (Colorado Serum, Denver, CO) as previously described (7). When indicated, CD4+ or CD8+ T cells were isolated using negative selection with magnetic beads after T cell purification.

Drug treatment

Unstimulated and PHA-stimulated human T cells were cultured in 24-well plates in RPMI 1640/5% FBS (100,000 cells/ml) in the presence or the absence of graded concentrations (0.1–100 μM) of indomethacin, chloroquine, hydrocortisone, or 6-mercaptopurine (6-MP). A stock solution of 6-MP was prepared in dimethylformamide (DMF) at a concentration of 10 mg/ml. The stock solution was diluted in assay diluent (80% culture medium/20% ethanol) to yield a 6-MP working solution of 80 mg/ml or less.

Immunofluorescent staining

PBMCs were isolated from SLE patients, healthy controls, and RA patients. PBMCs or purified T cells (isolated with magnetic beads) were washed with SB and incubated for 30 min at 4°C with 0.06–0.15 μg/ml fluorochrome-conjugated mAb following the manufacturer’s directions. For perforin expression, cells were then permeabilized with 0.1% saponin, stained with mouse anti-human perforin-PE or control, washed and re suspended in 10% formaldehyde. For perforin expression, cells were then permeabilized with 0.1% saponin, stained with mouse anti-human perforin-PE or control, washed and re suspended in 10% formaldehyde, and analyzed in a FACScan flow cytometer (BD Biosciences, Mountain View, CA).

Cytotoxicity assays

PBMCs or purified T cells were adhered to round-bottom microtiter wells in 100 μl of RPMI 1640/10% FBS for 1–2 h, after which the non-adherent cells were removed. The adherent cells were labeled with 2 μCi 51Cr/well for 3 h and then washed. T cells were obtained by rosetting or negative selection using magnetic beads. Where indicated, fractionated CD4+ or CD8+ T cells were used after isolation with magnetic beads. T cells were preincubated with 100 nM concanamycin A or DMSO for 1 h and added to the labeled monocytes at a ratio of 25:1. Cells were then incubated in the presence or the absence of 100 nM concanamycin A or DMSO. 51Cr release was determined 18 h later as previously described (12).

Statistical analysis

The difference between means was analyzed using Student’s t test or ANOVA with post-hoc analysis and Bonferroni correction, using Systat 10 software (Systat, Evanston IL) and Stata 6.0 (Stata Corp., College Station, TX). Differences in the degree of methylation of various DNA segments between groups was tested using the method of generalized estimating equations. This method fits a logistic regression model for correlated data (17). Robust SEs were used to assess the statistical significance. To determine whether current treatment with immunosuppressive agents was associated with perforin expression, univariate linear regression was performed. Control subjects were excluded from this portion of the analysis because a high degree of colinearity existed between case/control status and treatments due to the lack of immunosuppressive use in controls.

Results

Perforin is abnormally expressed in CD4+ lupus T cells

Initial experiments used flow cytometry to compare perforin expression in CD4+ and CD8+ cells from lupus patients and controls. Fig. 1A shows perforin staining in purified CD4+ T cells from a healthy control, a patient with lupus, and a patient with RA. Fig. 1b similarly shows perforin staining in purified CD8+ T cells from the same three subjects. More CD4+ T cells from the lupus patient express perforin than those from controls, whereas perforin expression was similar in the CD8+ populations. Fig. 1C compares the percentage of perforin-expressing T cells in the CD4+ and CD8+ subsets from 16 healthy controls, 25–26 lupus patients, and nine RA patients. More CD4+ T cells from lupus patients express perforin than those from normal controls (p = 0.03), whereas perforin expression in the CD8+ subset was not significantly

Table I. Patient characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lupus</th>
<th>Control</th>
<th>RA</th>
</tr>
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<tbody>
<tr>
<td>Number studied</td>
<td>42</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>Age (mean ± range)</td>
<td>37 ± 15</td>
<td>35 ± 9.5</td>
<td>48 ± 21</td>
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<tr>
<td>Females n (%)</td>
<td>34 (81)</td>
<td>17 (74)</td>
<td>9 (82)</td>
</tr>
<tr>
<td>Males n (%)</td>
<td>8 (19)</td>
<td>6 (26)</td>
<td>2 (18)</td>
</tr>
<tr>
<td>Disease activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLEDAI ≤ 5 n (%)</td>
<td>20 (48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLEDAI &gt; 5 n (%)</td>
<td>22 (52)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medications</td>
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<tr>
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</tr>
<tr>
<td>Azathioprine (%)</td>
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<td></td>
</tr>
<tr>
<td>Mycophenolate mofetil (%)</td>
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<tr>
<td>Cyclophosphamide (%)</td>
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<td>Methotrexate (%)</td>
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<td>Prednisone (&lt;0.5 mg/kg/day) (%)</td>
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<td>45</td>
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<td>Prednisone (0.5–1 mg/kg/day) (%)</td>
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<tr>
<td>Prednisone (&gt;1 mg/kg/day) (%)</td>
<td>2</td>
<td>0</td>
<td>10</td>
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</table>
FIGURE 1. Perforin expression in control and lupus T cells. CD4+ and CD8+ T cells were purified using magnetic beads and negative selection, then fixed, permeabilized, and stained with anti-perforin-PE and analyzed by flow cytometry. A, Representative histograms of CD4+ T cells from one control (light solid line), one lupus patient (heavy solid line), and one RA patient (broken line) comparing perforin staining (x-axis). B, Representative histograms of CD8+ T cells from one control (light solid line), one lupus patient (heavy solid line), and one RA patient (broken line) comparing perforin staining (x-axis). C, Perforin expression was measured by three-color flow cytometry in CD4+ and CD8+ T cells relative to an isotype control, using PBMC from 16 controls (■), 25 (CD4+) or 26 (CD8+) lupus patients (□), and 9 RA patients (□). Results are presented as the mean ± SEM.
different between lupus patients and controls. As the extent of T cell DNA hypomethylation increases with lupus disease activity, the patients shown in Fig. 1C were classified as inactive (SLEDAI = ≤5; n = 12) or active (SLEDAI = >5; n = 13). Fig. 2A shows that perforin is preferentially expressed in CD4⁺ T cells from patients with active lupus, whereas expression in T cells from patients with inactive lupus is not significantly different from that in controls (p < 0.001 overall, by ANOVA; p = 0.001, active vs control and active vs inactive, by post-hoc analysis). In Fig. 2B the percentage of CD4⁺ T cells expressing perforin is plotted against the SLEDAI. In the CD4⁺ subset, perforin expression increased with disease activity (p < 0.001, by regression analysis).

These results were confirmed at the protein level by immunoblotting and at the mRNA level by real-time RT-PCR. Fig. 3A shows a representative immunoblot of perforin and β-actin using CD4⁺ T cells, isolated by negative selection, from a healthy control and a patient with active (SLEDAI = 6) lupus. There was a relative increase in perforin protein in the lupus T cells. Fig. 3B shows perforin protein expressed relative to β-actin in CD4⁺ T cells from eight controls, five patients with inactive lupus, and six patients with active lupus. Overall, CD4⁺ T cells from patients with active lupus expressed greater amounts of perforin than those from healthy controls (p = 0.03, by t test, control vs active lupus), and the relationship between disease activity and perforin expression detected by flow cytometry was confirmed (p < 0.001, by ANOVA). The difference between active and inactive lupus patients was not significant (p > 0.05). Fig. 3C shows immunoblot analyses of perforin expression in CD8⁺ T cells from four of the same controls and seven of the lupus patients. Although there was an apparent increase in perforin in T cells from patients with active lupus, this was due to a large increase in one patient, whereas the rest were lower (60%) relative to the controls, and the average increase was not statistically significant. Fig. 3D shows a representative real-time RT-PCR amplification curve of perforin transcripts from CD4⁺ T cells of a lupus patient and a healthy control. Fewer cycles were required to amplify the transcripts from the lupus patient relative to the control. Fig. 3E shows perforin mRNA levels in CD4⁺ T cells from 14 of the same subjects shown in Fig.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Effect of disease activity on perforin expression in CD4⁺ lupus T cells. A, PBMCs from 16 controls, 12 patients with inactive lupus (SLE-i; SLEDAI = ≤5); or 13 patients with active lupus (SLE-a; SLEDAI = >5) were stained for CD4 and perforin expression and analyzed by flow cytometry as described in Fig. 1C. The results represent the percentage of cells expressing both CD4 and perforin, and are presented as the mean ± SEM (p = 0.001, active lupus vs control and inactive lupus). B, The percentage of CD4⁺ perforin⁺ cells for each lupus patient shown in A is plotted against the patient’s SLEDAI score, then analyzed by linear regression.
Results are presented as the ratio of perforin/β-actin transcripts in were measured in CD4+ T cells from the indicated groups as described in B. D, RNA was isolated from CD4+ T cells of a healthy control (C) and a patient with lupus (P), and perforin transcripts were measured by real-time RT-PCR. Fluorescence is shown on the y-axis, and cycle number is shown on the x-axis. E, Perforin and β-actin transcripts in were measured in CD4+ T cells isolated from the indicated numbers of controls, lupus patients with inactive disease, and lupus patients with active disease as described in D. Results are presented as the ratio of perforin/β-actin transcript levels and represent the mean ± SEM (%). F, Perforin and β-actin transcripts were measured in CD8+ T cells from the indicated groups as described in B. Results again represent the mean ± SEM of the indicated numbers of subjects.

3B, again expressed relative to β-actin. CD4+ T cells from patients with active lupus also had greater amounts of perforin mRNA relative to CD4+ T cells from normal controls (p = 0.006) or patients with inactive lupus (p = 0.019). Fig. 3F compares perforin mRNA in CD8+ T cells from nine controls and 10 lupus patients. In contrast to CD4+ T cells, there was no difference in perforin mRNA levels in T cells from patients with active or inactive disease, and perforin mRNA levels were significantly higher in CD8+ T cells from the controls relative to the combined lupus patients (p = 0.013). The greater decrease in perforin mRNA expression relative to protein suggests post-transcriptional control in the CD8+ subset.

As DNA hypomethylation contributes to LFA-1 overexpression in lupus T cells (18), we tested whether those cells overexpressing LFA-1 also overexpress perforin. Fig. 4, A and B, shows representative histograms of purified T cells from a control and a lupus patient, stained for LFA-1 and perforin expression. Relatively few control T cells expressed perforin. In contrast, greater numbers of T cells from a patient with active lupus (SLEDAI = 12) expressed perforin, and perforin was preferentially expressed in cells with high LFA-1 levels. Fig. 4C compares the mean channel fluorescence (MCF) of CD11a expression on CD4+ perforin+ and CD4+ perforin− cells from 10 lupus patients and eight controls. In lupus patients, the CD11a MCF was significantly greater in perforin-expressing CD4+ T cells compared with perforin− cells (p = 0.024). In contrast, there was no significant difference in CD11a MCF in perforin+ and perforin− CD4+ T cells from the controls. This suggests that the perforin-expressing lupus T cells also overexpress LFA-1, which was previously shown to be due to DNA hypomethylation (9). To exclude the possibility that T cell activation contributed to LFA-1 and perforin overexpression, perforin was correlated with IL-2R (CD25) expression on T cells from 17 lupus patients. No correlation was observed (p = 0.89). Similarly, there was no relationship with CD4+ T cell class II MHC expression (p = 0.55). However, perforin was preferentially expressed in CD4+ CD69+ cells (8.4 ± 3.2% perforin+CD69+ vs 1.4 ± 0.4% perforin−CD69+; n = 12; p = 0.015, by paired t test) and in CD4+ CD40L− cells (5.4 ± 2.2% perforin+CD40L− vs 1.9 ± 1.3% perforin−CD40L−; n = 12; p = 0.07), arguing against this interpretation.
Alternatively, the increase in perforin expression may be related to the medications used to treat the lupus patients. However, patients with rheumatoid arthritis were receiving similar medications, and no effect on perforin was noted (Table I). To further exclude this possibility, unstimulated and stimulated human T cells were cultured in the presence or the absence of graded concentrations (0.1–100 μM) of medications that are commonly used in lupus treatment: indomethacin (for nonsteroidal anti-inflammatory drugs), chloroquine (for antimalarial agents), hydrocortisone (for steroids), 6-MP (for azathioprine), and their combinations, as previously reported (12). After culture for 6 and 24 h, perforin expression was determined by flow cytometry and compared with that in untreated cells. None of these medications caused significant changes in perforin expression, suggesting that medications do not account for the differences in expression (data not shown).

To determine whether treatment with immunosuppressive agents was associated with abnormal perforin expression, univariate linear regression was performed. Perforin was modeled as a dependent variable, with medications modeled as dichotomous independent predictors. There was no correlation of specific medications with perforin expression.

The perforin promoter is demethylated in CD4+ T cells from lupus patients

To test whether changes in DNA methylation might contribute to perforin overexpression in CD4+ lupus T cells, the methylation status of a 1.3-kb region encoding the perforin promoter, enhancer, and 5′-flanking region was analyzed using bisulfite sequencing. Fig. 5A shows the key elements of the PRF1 promoter and 5′-flanking region. The first 1300 bp 5′ to the transcription start site contains 29 potentially methylatable CG pairs. The first 55 bp 5′ to the start site contains a core promoter with a GC box. A series of repetitive elements is located between −396 and −83, and a region containing enhancer elements is located between −1089 and −835 (19). Earlier studies demonstrated that hypomethylation of the seven CG pairs located between −929 and −577, indicated by the double arrow in Fig. 5A, is associated with perforin expression, and that methylation of the region suppresses promoter function in reporter constructs (10). Therefore, particular attention was focused on the methylation status of this methylation sensitive region.

FIGURE 4. Lupus T cells expressing perforin also overexpress LFA-1. Purified T cells from healthy controls and lupus patients were stained for CD11a and perforin expression, then analyzed by flow cytometry. A. Representative histogram from a normal subject. Perforin staining is shown on the x-axis, and CD11a is shown on the y-axis. B, T cells from a patient with active lupus (SLEDAI = 12), analyzed as described in A. C. The mean ± SEM of CD11a MCF in CD4+perforin− (□) and CD4+perforin+ (■) T cells from eight controls and 10 lupus patients, analyzed as described in A and B, are shown.
FIGURE 5. Perforin promoter methylation patterns in CD4\(^+\) and CD8\(^+\) T cells from lupus patients and controls. A, Map of the perforin promoter. The positions of the transcription initiation site, promoter, repetitive elements, and enhancer elements are identified. The “balloons” represent CG pairs. The double arrow identifies the region that suppresses promoter function when methylated. B, DNA was isolated from CD4\(^+\) T cells of normal controls and treated with bisulfite, then the region shown in A was amplified in two fragments (−1326 to −599 and −457 to −4). Five clones of each amplified fragment from each individual were then cloned and sequenced. C, The methylation status of the perforin promoter was determined in CD4\(^+\) T cells from four patients with lupus (SLEDAI = 0–2) using the methods and approach described in B. The results thus represent the mean of 20 determinations for each dC base. D, Methylation profile of the perforin promoter in CD4\(^+\) T cells from four patients with lupus (SLEDAI = 4–6), determined as described in B. E, Methylation profile of the perforin promoter in CD4\(^+\) T cells from four patients with lupus (SLEDAI = 8–12). F, DNA from control CD8\(^+\) T cells was analyzed as described in B. G, Methylation profile of the perforin promoter in CD8\(^+\) T cells from three patients with lupus (SLEDAI = 0–2). H, Methylation profile of the perforin promoter in CD8\(^+\) T cells from three (−1326 to −599 bp) or two (−457 to −4 bp) patients with lupus (SLEDAI = 4–6). I, Methylation profile of the perforin promoter in CD8\(^+\) T cells from four patients with lupus (SLEDAI = 8–12).
CD4+ cells

Fig. 5B shows the promoter/enhancer methylation pattern in normal CD4+ T cells determined using five cloned and sequenced fragments from seven (−1326 to −599 bp) or six (−457 to −4 bp) healthy individuals for a total of 35 or 30 fragments, respectively. The region from −1326 through the CG pair at −650 is relatively heavily methylated, whereas the more proximal CG pairs become progressively less methylated the closer they are to the transcription start site, with most of the methylated residues in the proximal sequences located in the region containing repetitive elements. Overall, the average methylation is 47%.

We then examined the methylation pattern of the perforin promoter on T cells from lupus patients with a range of SLEDAI scores. Fig. 5C shows the methylation status of the same region in CD4+ T cells from four lupus patients with SLEDAI scores of 0–2. Again, five fragments from each donor were cloned and sequenced, so each point represents the mean of 20 determinations. The overall methylation was 51%, slightly higher than that in controls, and the difference was not statistically significant (p = 0.72). The region from approximately −900 to −600, responsible for methylation-induced suppression (10), was also not significantly different from that in controls (overall methylation, 0.80 vs 0.79; control vs lupus, p = 0.92).

Fig. 5D shows the methylation patterns of the same region in CD4+ T cells from three or four lupus patients with SLEDAI scores of 4–6, determined from five sequenced fragments from each region. The average methylation of the region is less than that in controls (41%), but this was not statistically significant (p = 0.63). The enhancer 3’-flanking region was somewhat, but not significantly, hypomethylated (average methylation, 0.80 vs 0.63; control vs lupus, p = 0.31). The region closest to the promoter (−500 to the start site) was slightly less methylated than controls (total methylation, 0.21 vs 0.19; control vs lupus), but the overall difference was not statistically significant (p = 0.69) and methylation of this region had a lesser effect on gene expression relative to the enhancer (10).

Fig. 5E shows the methylation pattern of the same region in CD4+ T cells from four lupus patients with SLEDAI scores of 8–12. The average methylation was 0.28, less than that in controls or patients with less active lupus (p = 0.006, control vs active lupus). The methylation-sensitive enhancer region was also significantly hypomethylated relative to controls (0.80 vs 0.50; control vs lupus, p = 0.05).

CD8+ cells

Fig. 5F shows the average methylation status of the same region in CD8+ T cells isolated from five (−1326 to −599 bp) or four (−457 to −4 bp) healthy individuals, for a total of 25 or 20 fragments, respectively. Overall, the entire region tended to be less methylated in normal CD8+ T cells compared with CD4+ cells (total methylation, 0.23 vs 0.47; p = 0.002). The region from −1326 to −1000 was less methylated than the corresponding region in the CD4+ T cells (mean fraction methylated, 0.89 vs 0.52; CD4 vs CD8; p = 0.002). The region from −900 to −599, containing the methylation-sensitive region, was also significantly (p = 0.004) less methylated in CD8+ T cells relative to CD4+ T cells (mean fraction methylated, 0.41 vs 0.71), although there was one exception at −720. The region from −457 to the transcription start site was also less methylated in the CD8+ T cells (CD4 vs CD8, 0.21 vs 0.07; p = 0.002). Interestingly, the overall pattern resembled the CD4+ T cells from the patients with the most active lupus (Fig. 5E).

**FIGURE 6.** Perforin promoter methylation in CD4+ T cells from the same patient with active and inactive disease. A, CD4+ T cells were isolated from a patient with inactive lupus (SLEDAI = 2), and bisulfite sequencing was performed on five cloned fragments as described in Fig. 5. At this time 3.4% of the subject’s CD4+ T cells expressed perforin by flow cytometry. B, CD4+ T cells were isolated from the same patient at a time of active disease (SLEDAI = 6), and methylation of the PRF1 promoter was analyzed as described in A. At this time, 21.1% of the CD4+ T cells expressed perforin. The region from −900 to −600 was significantly hypomethylated in the CD4+ T cells isolated during active disease (p < 0.001, by paired t test).
Overall, these results indicate progressive demethylation of the methylation-sensitive perforin enhancer flanking region in CD4\(^+\), but not CD8\(^-\), T cells from patients with active lupus. The progressive hypomethylation correlates well with perforin mRNA and protein levels in CD4\(^+\) lupus T cells.

**Role of perforin in monocyte killing**

As lupus T cells spontaneously kill autologous monocytes/macrophages (8, 12), we determined whether perforin plays a role in the monocyte killing. T cells from patients with active lupus were cultured with autologous monocytes with and without the addition of concanamycin A, a selective perforin inhibitor (20). Fig. 7A shows that concanamycin A inhibits monocyte killing in a dose-dependent fashion. Fig. 7B shows the effect of 100 nM concanamycin A on monocyte killing using T cells from 11 patients with active lupus. Concanamycin A causes significant \((p = 0.001, \text{by paired } t \text{ test})\) inhibition of killing, suggesting a role for perforin in this response. These studies were repeated using purified CD4\(^+\) and CD8\(^+\) T cells from five patients with active lupus (Fig. 7C). Concanamycin A (100 nM) caused nearly complete inhibition of killing by CD4\(^+\) T cells, but only partial inhibition by CD8\(^+\) T cells \((p = 0.036, \% \text{ killing in the presence of concanamycin A, CD4 vs CD8}). This suggests that nonperforin-mediated mechanisms may contribute to macrophage killing to a greater extent in CD8\(^+\) T cells than in CD4\(^+\) T cells.

**Discussion**

Several novel observations are described in this report. First, CD4\(^+\) T cells from patients with active lupus abnormally overexpress perforin. Second, methylation-sensitive perforin regulatory elements are hypomethylated in CD4\(^+\) T cells from patients with active lupus. Third, perforin may participate in the spontaneous monocyte/macrophage killing that characterizes lupus T cells. Finally, as DNA-hypomethylating agents cause LFA-1 and perforin overexpression in CD4\(^+\) T cells, and CD4\(^+\) lupus T cells have hypomethylated DNA and overexpress LFA-1 and perforin, the results suggest that genes overexpressed in experimentally hypomethylated CD4\(^+\) T cells may predict genes overexpressed in lupus T cells.

Perforin expression was identified in CD4\(^+\) lupus T cells using flow cytometry, immunoblotting, and real-time RT-PCR and was directly proportional to disease activity. Perforin expression has been reported previously in CD4\(^+\) T cells, but in most instances has been in cultured T cell lines (19, 21). As lupus T cells are frequently activated (22), it is possible that T cell activation contributes to the increase in perforin expression. However, no correlation was seen between IL-2R and perforin expression, and perforin was preferentially expressed in CD69\(^+\) T cells, arguing against this interpretation. Further, we have not observed perforin expression in stimulated primary CD4\(^+\) T cells unless they were first treated with DNA methylation inhibitors (10). It thus seems reasonable to propose that the abnormal expression is due to mechanisms other than T cell stimulation alone. As the DNA methylation inhibitor 5-azaC induces perforin expression in CD4\(^+\) T cells by demethylating the enhancer 3'-flanking sequence (10), and the same sequences are demethylated in CD4\(^+\) lupus T cells, DNA hypomethylation may be contributing to the abnormal expression in CD4\(^+\) lupus T cells.

Our observation that the same sequences are not significantly hypomethylated in the CD8\(^-\) population suggests that perforin gene methylation is regulated differently in CD4\(^+\) and CD8\(^-\) T cells. Elucidating the mechanisms that establish and maintain methylation patterns in normal CD4\(^+\) and CD8\(^-\) T cells may help clarify the differential regulation in lupus. However, as we have reported that inhibiting DNA methylation in CD4\(^+\) T cells is sufficient to cause a lupus-like disease (3, 4), the contribution of the CD8 subset is not necessary for disease induction. Interestingly, we have been unable to induce autoreactivity in the CD8 subset using 5-azaC (23), suggesting differences in the regulation of some genes by DNA methylation in CD4\(^+\) and CD8\(^-\) T cells.

To the best of our knowledge there is only one other report of perforin expression in human lupus T cells. In this report, low expression was detected in CD3-stimulated lupus PBMC cultured for 13 days (24). However, in this report freshly isolated T cells did express perforin, although comparisons with controls were not made, and CD4\(^+\) and CD8\(^-\) subsets were not studied. Further, the decrease after 13 days of culture was also seen in the controls.
Thus, perforin expression in primary human lupus T cells has not previously been studied.

Perforin expression in CD4+ T cells may contribute to the pathogenesis of lupus. Others have reported that injecting apoptotic thymocytes into mice causes anti-DNA Abs (25), suggesting that increasing apoptosis could contribute to an autoimmune response. Other groups have also reported that mice genetically deficient in mechanisms involved in clearing apoptotic material will develop a lupus-like autoimmune disease (26), suggesting that decreased clearance of apoptotic material can also induce autoimmunity. As macrophages are responsible for the clearance of apoptotic material, their death would both increase the amount of apoptotic debris as well as decrease its clearance. We have reported that lupus T cells use FasL, TNF-like weak inducer of apoptosis, and TRAIL to induce monocyte apoptosis (12), and perforin now appears to contribute as well. Interestingly, perforin appears to play a greater role in macrophage killing mediated by CD4+ lupus T cells than CD8+, at least as assessed by concanamycin A inhibition. It should be noted that MRL/lpr mice deficient in perforin have a more severe disease, suggesting that in some instances perforin can be protective (27). However, perforin expression is usually confined to CD8+ T cells and NK cells, so the decreased expression in this animal model may influence a regulatory CD8+ population. An abnormal overexpression in CD4+ T cells, on the other hand, could participate in killing of the APC with which CD4+ T cells normally interact, with disease inducing results.

Finally, these studies are the second example in which a gene found to increase expression in experimentally hypomethylated T cells is also abnormally hypomethylated and expressed in lupus T cells, the first being CD11a (9). We have identified >100 additional methylation-sensitive genes using oligonucleotide arrays. It is possible that other genes contributing to the pathogenesis of human lupus may be identified using a similar strategy.

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References