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Demethylation of the Same Promoter Sequence Increases CD70 Expression in Lupus T Cells and T Cells Treated with Lupus-Inducing Drugs

Qianjin Lu, Ailing Wu, and Bruce C. Richardson

Exposing genetically predisposed individuals to certain environmental agents is believed to cause human lupus. How environmental agents interact with the host to cause lupus is poorly understood. Procainamide and hydralazine are drugs that cause lupus in genetically predisposed individuals. Understanding how these environmental agents cause lupus may indicate mechanisms relevant to the idiopathic disease. Abnormal T cell DNA methylation, a repressive epigenetic DNA modification, is implicated in procainamide and hydralazine induced lupus, as well as idiopathic lupus. Procainamide is a competitive DNA methyltransferase (Dnmt) inhibitor, hydralazine inhibits ERK pathway signaling thereby decreasing Dnmt expression, and in lupus T cells decreased ERK pathway signaling causing a similar Dnmt decrease. T cells treated with procainamide, hydralazine, and other Dnmt and ERK pathway inhibitors cause lupus in mice. Whether the same genetic regulatory elements demethylate in T cells treated with Dnmt inhibitors, ERK pathway inhibitors, and in human lupus is unknown. CD70 (TNFSF7) is a B cell costimulatory molecule overexpressed on CD4+ lupus T cells as well as procainamide and hydralazine treated T cells, and contributes to excessive B cell stimulation in vitro and in lupus. In this report we identify a genetic element that suppresses CD70 expression when methylated, and which demethylates in lupus and in T cells treated with Dnmt and ERK pathway inhibitors including procainamide and hydralazine. The results support a model in which demethylation of specific genetic elements in T cells, caused by decreasing Dnmt expression or inhibiting its function, contributes to drug-induced and idiopathic lupus through altered gene expression. The Journal of Immunology, 2005, 174: 6212–6219.

M ethylation of DNA, the methylation of dC bases in CG pairs, promotes a repressive chromatin structure inaccessible to transcription factors, suppressing gene expression. Most CG pairs in mammalian DNA are methylated, with exceptions in or near the promoters of active genes, in which expression requires a chromatin configuration permissive of transcription factor binding. Abnormalities in DNA methylation result in the aberrant increase or decrease in gene expression, and are implicated in the development of a variety of diseases (1). Recently, abnormal decreases in T cell DNA methylation have been implicated in the development of drug-induced and idiopathic lupus (1, 2). The lupus-inducing drugs procainamide (Pca) and hydralazine (Hyd) inhibit T cell DNA methylation, and T cells from patients with active lupus have global decreases DNA methylation and changes in gene expression and methylation resembling those caused by DNA methylation inhibitors (3–7). Further, demethylating CD4+ T cells with DNA methylation inhibitors like 5-azacytidine (5-azaC), Pca, and Hyd causes autoreactivity, and injecting the autoreactive cells into syngeneic recipients causes a lupus-like disease (8, 9). These observations suggest that T cell DNA hypomethylation may be fundamental to drug-induced and idiopathic lupus.

We recently reported (7) that CD70, a T cell costimulatory molecule encoded by the TNFSF7 gene, is overexpressed on CD4+, but not CD8+ T cells treated with a panel of DNA methylation inhibitors including 5-azaC, Pca, PD98059, U0126, and Hyd. 5-azaC and Pca are irreversible and competitive DNA methyltransferase (Dnmt) inhibitors, respectively (10, 11), and PD98059, U0126, and Hyd decrease Dnmt expression by inhibiting ERK pathway signaling (12–14). CD70 is similarly overexpressed on CD4+ T cells from lupus patients (7), where DNA hypomethylation is due to impaired ERK pathway signaling (14). Whether the same TNFSF7 sequences demethylate in CD4+ T cells treated with Dnmt and signaling inhibitors and in CD4+ T cells from lupus patients is unknown.

In this report we tested the hypothesis that the same sequences in the TNFSF7 promoter demethylate in response to Dnmt inhibitors, ERK pathway inhibitors, and in T cells from lupus patients, and that methylation of the affected sequences suppresses TNFSF7 gene expression. We found that a region flanking the TNFSF7 promoter is demethylated by Dnmt and ERK pathway inhibitors and in T cells from lupus patients, and contributes to the CD70 overexpression observed in T cells treated with these drugs and in lupus. These results provide further support to the hypothesis that DNA hypomethylation is fundamental to the pathogenesis of drug-induced and idiopathic lupus.

Materials and Methods

Human subjects

Patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) were recruited from the outpatient rheumatology clinics and inpatient...
CD3 and soluble anti-CD28. Briefly, 24-well plates were coated with 300 μg/ml mycophenylate mofetil. Purified human CD4+ T cells were cultured as previously described relative to the predicted transcription start site: forward (567): GCTCTCGAGTGTTGAATTCATTTTTATTAAAAATATAAAAAGTTAGTAT-3′; reverse primer (920): 5′-TGAGAATTCATTTTTATTAAAAATATAAAAAGTTAGTAT-3′. The amplified fragment was cloned into pGL3-luciferase reporter gene (Stratagene), and transfection experiments were conducted using Lipofectamine 2000 (Invitrogen). Cell lysates were analyzed for luciferase activity using a luciferase assay kit (Promega). The constructs were then transfected into Jurkat cells by electroporation using previously described protocols and a previously described β-galactosidase expression construct (19) as control.

**Bisulfite sequencing**

Genomic DNA was isolated from T cells, treated with sodium bisulfite (19), and the 1-kb TNFSF7 promoter fragment amplified in three fragments by nested PCR. The fragments were cloned into PBS+ (Stratagene), and five independent clones were sequenced for each of the amplified fragments.

The following primers were used: fragment 1: round I: forward primer (192 bp to 318 bp) 5′-CACCTCGAGCTGTCTATCTCTACTAG; reverse primer (318 bp to 432 bp) 5′-CCGCTCGAGCTGCCTGGGGGCTCTTTTGTATTAT-3′; fragment 2: round I: forward primer (432 bp to 545 bp) 5′-CATCTCGAGCATTTATTAAAAATATAAAAAGTTAGTAT-3′; reverse primer (545 bp to 658 bp) 5′-GTTGAATTCCTTTAAGGTTAGGAGTTTAAGTTT-3′; fragment 3: round I: forward primer (658 bp to 771 bp) 5′-GGATCCCTTCTTCCTTTTTTTTTAGTT-3′; reverse primer (771 bp to 884 bp) 5′-GTTGAATTCCTTTAAGGTTAGGAGTTTAAGTTT-3′; fragment 4: round I: forward primer (884 bp to 997 bp) 5′-GTGGATCCCTTCTTCCTTTTTTTTTAGTT-3′; reverse primer (997 bp to 1110 bp) 5′-GTTGAATTCCTTTAAGGTTAGGAGTTTAAGTTT-3′.

**Promoter characterization**

A 1018-bp fragment containing the TNFSF7 promoter and predicted transcription start site, identified using Trinsect software, was amplified from primary human CD4+ T cells by PCR using the following primers, numbered relative to the predicted transcription start site: forward primer (966): GCTCTCGAGGTGAAAAACCCATCTCTAC; reverse (+52): TCCAGCTTTCATGTCGTTACGCT.

The forward primer contains an XhoI site at the 5′ end, and the reverse a HindIII site at the 3′ end. The amplified fragment was cloned into pGL3-Basic, and sequenced by the University of Michigan DNA Sequencing Core to exclude Taq error. TNFSF7 promoter constructs with 5′ deletions were generated by PCR amplification of genomic DNA using the following forward primers: F1(966): GCTCTCGAGGTGAAAAACCCATCTCTAC; F2(572): AGCAGCTTGAAAAATCTCTAC.

**Real-time RT-PCR**

Total cellular RNA was isolated using TRIzol (Invitrogen Life Technologies), then treated with DNaseI (Ambion) using published protocols (18). Real-time semiquantitative RT-PCR was performed using a Rotor-Gene 3000 (Corbett Research) and previously published protocols (7). CD70 mRNA levels were quantitated relative to β-actin transcripts. The following primers were used: CD70 forward: 5′-TTGTTGAATTCTTTAAGGTTAGGAGTTTAAGTTT-3′; reverse: 5′-GAGCAGCTTGAAAAATCTCTAC-3′; β-actinforward: 5′-GAGCAGCTTGAAAAATCTCTAC-3′; reverse: 5′-AGCAGCTTGAAAAATCTCTAC-3′. Fragment 2: round I: forward primer (321): ATTCTCGAGTGTTCAGGAGGCTCT; reverse primer (572) 5′-AACTCTAGATTAATTAATGACTATCTATA-3′; fragment 3: round I: forward primer (321): ATTCTCGAGTGTTCAGGAGGCTCT; reverse primer (572) 5′-AACTCTAGATTAATTAATGACTATCTATA-3′; fragment 4: round I: forward primer (321): ATTCTCGAGTGTTCAGGAGGCTCT; reverse primer (572) 5′-AACTCTAGATTAATTAATGACTATCTATA-3′. The amplified fragment was cloned into pGL3-vector (Promega). The constructs were then transfected into Jurkat cells by electroporation using previously described protocols and a previously described β-galactosidase expression construct (19) as control.

**Patch methylation and transfections**

The 1018-bp (966 to +52) TNFSF7 gene promoter fragment, cloned into the luciferase-containing vector pGL3-Basic, was digested with the following restriction endonucleases: region 1 (966 to −490): XhoI and NrdI, region 2 (−490 to −229): NrdI and ApaI, region 3 (−229 to +52): ApaI and HindIII.

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**Table I. Patient demographics and treatment**

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<th>Medications</th>
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<td>3</td>
<td>31/W/F</td>
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<td>Quin/Plaq</td>
</tr>
<tr>
<td>4</td>
<td>56/W/F</td>
<td>6</td>
<td>Pred 10</td>
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<tr>
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<td>4</td>
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</tr>
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</table>

Quin, quinacrine; Plaq, plaquenil; MTX, methotrexate; Pred, prednisone; MM, mycophenolate mofetil.

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**FIGURE 1.** Dnmt and ERK pathway inhibitors increase CD70 mRNA in CD4+ T cells. CD4+ T cells were stimulated with anti-CD3 + anti-CD28 and 18–24 h later treated with the indicated Dnmt inhibitors (5-azaC and Pca) and ERK pathway inhibitors (PD98059, U0126, and Hyd). Three days later, CD70 transcripts were measured in untreated (□) and treated (●) cells relative to β-actin by real-time RT-PCR. Results represent the mean ± SEM of the indicated number of repeats, normalized to the untreated control.
The three fragments were gel purified, methylated with SssI and 5-adenosylmethionine as described (19), and then religated back into the reporter construct. Completeness of methylation was tested by digestion with NraI for regions 1 and 2, and EaqM for region 3. Controls included a mock-methylated construct, prepared by omitting the SssI. The methylated or mock-methylated constructs were transfected into Jurkat cells by electroporation using previously described protocols (19) and expression measured relative to β-galactosidase controls (19).

Statistical analysis

Differences between groups were tested using Student’s t test or ANOVA with post hoc testing using the Bonferroni correction.

Results

Effect of DNA methylation inhibitors on CD70 mRNA

Our previous studies demonstrated that 5-azaC, Pca, Hyd, U0126, and PD98059 increased CD70 expression on CD4⁺ T cells. Initial studies determined whether CD70 mRNA levels increased as well. Maintenance DNA methylation is a postsynthetic event (1), and Dnmt inhibitors must be present during S phase to inhibit methylation of the daughter cells. Purified CD4⁺ T cells were stimulated for 18 h, cultured with 5 μM 5-azaC, 50 μM Pca, 20 μM Hyd, 40 μM U0126, or 25 μM PD98059 for 3 days as before, then CD70 transcripts were quantitated by real-time RT-PCR. Fig. 1 confirms that all five drugs increase CD70 transcripts.

Characterization of the TNFSF7 promoter

We then determined whether the five DNA methylation inhibitors affect the same regulatory sequences. The TNFSF7 promoter has not been characterized, but the TNFSF7 genomic sequence is available from the human genome database (NT 011255). Fig. 2A shows the sequence analyzed, with the location of the predicted start site, CAAT boxes, and potential transcription factor binding sites. Fig. 2B is a graphic representation of the same sequence, identifying the locations of the potentially methylatable CG pairs, start site, CAAT boxes, and putative transcription factor binding motifs.

Promoter activity was tested by amplifying a 1018-bp fragment containing the predicted TNFSF7 promoter and transcription start from human T cells, and verified by sequencing. The fragment was then cloned into pGL3 and transfected into Jurkat cells using the empty vector and a β-galactosidase construct as controls. Fig. 3A demonstrates that the TNFSF7 fragment has promoter activity (p = 0.02 by t test). Two 5’ truncated fragments were similarly generated by PCR, and the entire fragment (−966 to +52) or the truncated mutants (−572 to +52 and −360 to +52) were transfected into Jurkat cells (Fig. 3B). The first 321 bp 5’ fragment to the predicted start site has promoter activity essentially identical to the longer fragments, suggesting that the majority of the promoter activity is located within this region.

Methylation patterns of the TNFSF7 promoter and 5’ flanking region

Fig. 4 shows the methylation pattern of the 1-kb fragment in CD4⁺ and CD8⁺ T cells. CD4⁺ and CD8⁺ T cells were isolated from the peripheral blood of healthy subjects, DNA isolated, treated with bisulfite, then the region shown in Fig. 2 was amplified in three sequential fragments as described in Materials and Methods. The amplified fragments were cloned and five clones sequenced from each amplified fragment from each subject. Fig. 4A shows the average methylation of each of the 32 CG pairs in CD4⁺ T cells from four donors (bp −211 to +29) or eight donors (bp −956 to −288), thus representing a total of 20 to 40 determinations per CG pair. Fig. 4B shows a similar analysis of the same region in CD8⁺ T cells from four healthy donors, representing 20 determinations for each CG pair. In both subsets, the region from the transcription

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**FIGURE 2.** **TNFSF7** promoter and 5’-flanking region: sequence and relevant features. A, Sequence, potentially relevant elements, and predicted transcription start site of the TNFSF7 promoter and 5’-flanking region. B, Schematic diagram of the region shown in A. ● represent the potentially methylatable CG pairs, and the broken arrow the putative transcription start site. The locations of potential transcription factor binding sites and CAAT boxes are also shown.
Electroporation using or the pGL3 vector without insert was then transfected into Jurkat cells by PCR, verified by sequencing, then cloned into pGL3-Basic. The construct to

\[ /H11001 \]

experiments. Control (f) and represent the mean methylation of each CG pair in the methylated region (−956 to −288) in CD4⁺ T cells from seven healthy controls stimulated with anti-CD3 and anti-CD28. Again, we sequenced five cloned fragments from each control, for a total of 35 determinations per CG pair. Compared with Fig. 4, stimulation has no significant effect on the methylation status of this region, consistent with the effects of stimulation on other T cell genes like ITGAL and PRF1 (5, 20). Fig. 5B shows the effect of 5-azaC on the methylation pattern of the same region in stimulated CD4⁺ cells from five healthy donors. The 10 CG pairs in the region between −515 and −423 are hypomethylated compared with controls (Fig. 5A). The same region appears to demethylate in T cells treated with Pca (Fig. 5C), U0126 (Fig. 5D), and Hyd (Fig. 5F). Fig. 6 compares the average methylation of the 10 CG pairs in this region relative to stimulated, untreated controls. All five methylation inhibitors, whether signaling inhibitors or Dnmt inhibitors, significantly decrease the overall methylation of this region.

**Effect of methylation on TNFSF7 promoter function**

The transcriptional relevance of the methylation changes was determined using regional or “patch” methylation. The 1018-bp promoter fragment was cloned into pGL3-Basic, then the regions from −996 to −490, −490 to −229, or −229 to +52 were individually excised, methylated in vitro with SsII and S-adenosylmethionine, ligated back into the expression construct, and transfected into Jurkat cells. Controls included β-galactosidase transfection controls as well as mock-methylated constructs, similarly generated but omitting the SsII. The results are illustrated in Fig. 7. Methylation of each fragment suppressed promoter function relative to mock-methylated controls (p = 0.019, by paired t test for −996 to −490, p = 0.009 for −490 to −229, and p = 0.025 for −229 to

start site to −300, corresponding to the region with promoter activity (Fig. 3B), is nearly completely demethylated, consistent with an active gene. The region from −400 to −700 is partially methy-

lated, whereas the more distal region (−750 to −1000) is nearly completely methylated. Although there appears to be a small decrease in methylation in the region from −515 to −300 in CD4⁺ T cells, the average methylation in this region was not significantly different from CD8⁻ T cells (p = 0.175), and overall, the pattern of methylation in CD4⁺ and CD8⁻ T cells is essentially the same.

**Effect of DNA methylation inhibitors on TNFSF7 promoter methylation**

The effects of the DNA methylation inhibitors on the methylation status of this region were then compared. Fig. 5A shows the average methylation of each CG pair in the methylated region (−956 to −288) in CD4⁺ T cells from seven healthy controls stimulated with anti-CD3 and anti-CD28. Again, we sequenced five cloned fragments from each control, for a total of 35 determinations per CG pair. Compared with Fig. 4, stimulation has no significant effect on the methylation status of this region, consistent with the effects of stimulation on other T cell genes like ITGAL and PRF1 (5, 20). Fig. 5B shows the effect of 5-azaC on the methylation pattern of the same region in stimulated CD4⁺ cells from five healthy donors. The 10 CG pairs in the region between −515 and −423 are hypomethylated compared with controls (Fig. 5A). The same region appears to demethylate in T cells treated with Pca (Fig. 5C), U0126 (Fig. 5D), PD98059 (Fig. 5E), and Hyd (Fig. 5F). Fig. 6 compares the average methylation of the 10 CG pairs in this region relative to stimulated, untreated controls. All five methylation inhibitors, whether signaling inhibitors or Dnmt inhibitors, significantly decrease the overall methylation of this region.

### FIGURE 3. TNFSF7 promoter activity. A. A 1018-bp fragment (−996 to +52) containing the putative transcription start site was amplified by PCR, verified by sequencing, then cloned into pGL3-Basic. The construct or the pGL3 vector without insert was then transfected into Jurkat cells by electroporation using β-galactosidase as a control. The results are presented relative to β-galactosidase, and represent the mean ± SEM of four independent experiments. B. Fragments spanning the indicated regions were amplified by PCR, verified by sequencing, cloned into pGL3, then the constructs or pGL3 without insert were transfected into Jurkat cells with β-galactosidase controls as in A. The results of the constructs containing the promoter fragments (■) are normalized to the paired empty vector control (▲) and represent the mean ± SEM of two independent experiments.
However, methylation of the region from −490 to −229, which was affected by the methylation inhibitors, inhibits promoter function to a greater extent than does methylation of the distal sequences (−996 to −490; \( p = 0.013 \) by ANOVA with post hoc testing and Bonferroni correction). Methylation of the core promoter also suppresses promoter function to a greater extent than the distal sequence, but this was of marginal significance (\( p = 0.070 \)). These studies indicate that methylation of the CG pairs between −490 and −229 is transcriptionally relevant, and suppresses promoter function to a greater degree than methylation of the more distal sequences.

**Demethylation of the CD70 promoter and the 5′-flanking region in lupus T cells**

The previous study demonstrated that CD70 is overexpressed on the surface of CD4\(^+\) T cells from patients with active lupus (7). Initial studies therefore confirmed that the increase was associated with an increase in CD70 mRNA levels. Fig. 8 compares the level of CD70 transcripts in CD4\(^+\) T cells from 10 patients with lupus (5 inactive, 5 active), 3 patients with RA, and 9 healthy controls (Table I). CD70 is also significantly (\( p = 0.03 \) lupus vs controls) increased at the mRNA level in T cells from lupus patients. The difference in CD70 mRNA levels between patients with active and inactive lupus was not significant (1.52 ± 0.74 vs 0.49 ± 0.09, mean ± SEM, active vs inactive). We previously excluded an effect of medications on CD70 expression (7), and again no correlation between medications and CD70 expression was observed (Table I).

We then compared CD70 promoter methylation patterns in CD4\(^+\) T cells from patients with active and inactive lupus with controls. Fig. 9A shows the methylation pattern in T cells from four healthy age and gender-matched controls, whereas Fig. 9B shows the methylation pattern in T cells from five women with inactive lupus, and Fig. 9C shows the pattern in six women with active lupus. The region from −515 to −423, demethylated by the panel of methylation inhibitors, is also demethylated in CD4\(^+\) T cells from patients with active and inactive lupus.
cells from lupus patients with both active and inactive disease relative to controls. Fig. 9D compares the average methylation of this region across the three groups. The overall methylcytosine content is significantly less in lupus than in controls ($p = 0.004$ and 0.002 for inactive and active patients vs controls, respectively, by ANOVA and post hoc testing with Bonferroni correction), similar to T cells demethylated with methylation inhibitors. Again, no correlation with medications was observed (Table I).

Discussion

The regulation of TNFSF7 gene expression is poorly understood. CD70 is not expressed on unstimulated T cells, but expression is rapidly up-regulated following mitogenic stimulation (21) or stimulation with TNF-$
\alpha$ (22). The signaling pathways and transcription factors involved are unknown.

Our previous studies demonstrated that a panel of DNA methylation inhibitors, including direct Dnmt inhibitors as well as ERK pathway inhibitors, all increased CD70 expression on the surface of CD4$^+$ T cells (7). The same study reported that CD4$^+$ T cells from patients with active lupus, characterized by impaired ERK pathway signaling, decreased Dnmt expression and DNA hypomethylation (4), similarly overexpress CD70 (7). The present studies confirmed that the methylation inhibitor panel also increases CD70 mRNA. This suggests that the drugs may affect transcriptional regulatory mechanisms. Similarly, CD70 transcripts were also increased in T cells from lupus patients.

An $\sim$300-bp fragment of the TNFSF7 gene possessing promoter activity was identified using deletion analysis and transient transfection of reporter constructs. The promoter region contains binding sites for several transcription factors including AP-1, Sp1, NF-$\kappa$B, and AP-2. Further studies will be required to determine whether these transcription factors contribute to CD70 expression in T lymphocytes. Bisulfite sequencing of primary CD4$^+$ and CD8$^+$ T cells revealed complete demethylation of the promoter sequence, with progressively greater methylation in the more distal 5' regions. Hypomethylation of regulatory regions is characteristic of a transcriptionally permissive chromatin configuration, and active promoters are typically hypomethylated (1). The colocalization of promoter activity and regional DNA hypomethylation indicate that the region is likely to be the TNFSF7 promoter.
Treating CD4⁺, but not CD8⁺, T cells with two direct Dnmt inhibitors (5-azaC and Pca) (10, 11) or three ERK pathway inhibitors (PD98059, U0126, and Hyd) known to decrease Dnmt expression (12), all increased steady-state levels of CD70 mRNA. Because the only property common to all five agents is DNA methylation inhibition, it is reasonable to propose that the effect is mediated at least in part by demethylation of sequences affecting gene expression. This was confirmed by demonstrating that all five agents demethylate a sequence located within ~200 bp upstream of the promoter. Patch methylation of reporter constructs indicated that methylation of the affected region can suppress promoter function, as reflected by transient transfection assays. Thus, it is likely that the decreased methylation contributes to the increased CD70 expression in the treated cells. Whereas the sequences affected are upstream of the promoter, DNA methylation can suppress gene expression from a distance (23). This appears to be due to the effects of methylcytosine-binding proteins such as MeCP2 and MBD1. These proteins recruit chromatin inactivation complexes containing histone deacetylases to methylated sequences, promoting condensation of the chromatin into a transcriptionally repressive state.

**FIGURE 9.** TNFSF7 promoter methylation in CD4⁺ T cells from lupus patients and controls. A, The methylation status of the region from −1000 to −200 was determined in CD4⁺ T cells from four healthy controls as described in Fig. 4. The results represent the average methylation of the 20 determinations for each CG pair analyzed. B, The methylation status of the same region was determined in CD4⁺ T cells from five patients with inactive (SLEDAI ≤ 5) lupus as in A. The results represent the mean of the 25 determinations for each CG pair. C, The methylation status of the same region was determined in CD4⁺ T cells from six patients with active (SLEDAI > 5) lupus as in A. The results represent the mean of the 30 determinations for each CG pair. D, The average methylation of the region between −515 and −423 was determined as in Fig. 6 for the individuals shown in A, B, and C. The results represent the mean ± SEM of the four controls, five patients with inactive lupus (SLE-i), and six patients with active lupus (SLE-a). Statistical analysis was by ANOVA with post hoc testing and Bonferroni correction.
configuration (23). Interestingly, one CG pair located at −439 was reproducibly resistant to demethylation. The reason for this localized effect is unknown. Further, the more distal CG pairs (−956 to −567) were also less affected by the methylation inhibitors. This indicates that DNA methylation is more stringently regulated in some regions than in others. The mechanism for this is incompletely understood, but may be affected by histone modifications (24).

Although all five DNA methylation inhibitors successfully demethylated the same region in the TNFSF7 gene, it is unlikely that all five demethylate the same sequences throughout the genome. Earlier studies demonstrated that 5-azaC caused a greater decrease in total T cell genomic deoxymethylcytosine content than did Pca or Hyd (3). This suggests differential susceptibility of some sequences to demethylation by different agents and also supports the concept that DNA methylation is more stringently regulated in some regions than in others.

CD4+ T cells from lupus patients were also found to have increased steady-state levels of CD70 mRNA and hypomethylation of the same sequences demethylated by the methylation inhibitors. Because T cells from patients with active lupus have impaired ERK pathway signaling and decreased Dnmt levels (14), it is possible that the same mechanism contribute to the DNA demethylation in the in vitro model and in patients with lupus. It should be noted that the sequence was demethylated in patients with both active and inactive disease. This is consistent with our observation that CD70 expression was increased on HLA-DR T cells (7), suggesting that T cell activation does not play a major role in the overexpression. However, we previously found a modest correlation with disease activity (r = 0.636) (7) suggesting the possibility of additional factors in the increased expression on lupus T cells. Further, the lack of HLA-DR expression does not necessarily exclude the possibility that the T cells might be in a state of limited or partial activation, as proposed by others (25).

The lack of correlation between disease activity and promoter demethylation is distinct from what we found for the ITGAL and PRF1 genes, where the degree of demethylation was proportional to disease activity (5, 6). However, earlier work by our group and others, examining remethylation kinetics following withdrawal of DNA methylation inhibitors, has shown that some sequences are rapidly remethylated whereas other sequences remain demethylated (3, 26). It is likely that the sequences flanking the TNFSF7 gene are in the latter category.

Overall, the observation that Pca and Hyd demethylate the same sequences demethylated in idiopathic lupus provides further support to the hypothesis that DNA hypomethylation contributes to the pathogenesis of drug-induced and idiopathic lupus. Further, identifying genes overexpressed in T cells treated with DNA methylation inhibitors has allowed us to predict both the genes and sequences affected for ITGAL and PRF1 genes, and now TNFSF7. These studies led to the functional consequences of overexpressing these molecules, with ITGAL overexpression causing auto-reactivity (27), PRF1 overexpression contributing to monocyte/macrophage killing by the T cells (6), and TNFSF7 contributing to B cell overstimulation (7). It is likely that this strategy may identify additional genes aberrantly expressed on lupus T cells and provide new insights into disease mechanisms and approaches to identify biomarkers and therapeutic targets for human lupus.

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Disclosures
The authors have no financial conflict of interest.

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