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Demethylation of CD40LG on the Inactive X in T Cells from Women with Lupus

Qianjin Lu,* Ailing Wu,† Laura Tesmer,† Donna Ray,† Neda Yousif,‡ and Bruce Richardson2†§

Why systemic lupus erythematosus primarily affects women is unknown. Recent evidence indicates that human lupus is an epigenetic disease characterized by impaired T cell DNA methylation. Women have two X chromosomes; one is inactivated by mechanisms including DNA methylation. We hypothesized that demethylation of sequences on the inactive X may cause gene overexpression uniquely in women, predisposing them to lupus. We therefore compared expression and methylation of CD40LG, a B cell costimulatory molecule encoded on the X chromosome, in experimentally demethylated T cells from men and women and in men and women with lupus. Controls included TNFSF7, a methylation-sensitive autosomal B cell costimulatory molecule known to be demethylated and overexpressed in lupus. Bisulfite sequencing revealed that CD40LG is unmethylated in men, while women have one methylated and one unmethylated gene. 5-Azacytidine, a DNA methyltransferase inhibitor, demethylated and overexpressed in lupus. Bisulfite sequencing revealed that CD40LG doubled its expression on CD4+ T cells from women but not men, while increasing TNFSF7 expression equally between sexes. Similar studies demonstrated that CD40LG demethylates in CD4+ T cells from women with lupus, and that women but not men with lupus overexpress CD40LG on CD4+ T cells, while both overexpress TNFSF7. These studies demonstrate that regulatory sequences on the inactive X chromosome demethylate in T cells from women with lupus, contributing to CD40LG overexpression uniquely in women. Demethylation of CD40LG and perhaps other genes on the inactive X may contribute to the striking female predilection of this disease. The Journal of Immunology, 2007, 179: 6352–6358.

Systemic lupus erythematosus is a female predominant disease characterized by autoantibody formation and immune complex deposition. Genetic elements predispose to lupus, but incomplete concordance in identical twins, and reports that UV light and drugs like procainamide and hydralazine trigger a lupus-like disease, indicate an environmental contribution (1).

DNA methylation is an epigenetic modification that suppresses gene expression (2). CD4+ T cell DNA methylation is impaired in human lupus, due to decreases in DNA methyltransferase expression and activity (3, 4), causing genome-wide DNA hypomethylation and aberrant gene overexpression that contributes to autoantibody formation. The lupus-inducing drugs procainamide and hydralazine as well as UV light are DNA methylation inhibitors, and the same regulatory elements demethylate in T cells treated with these drugs as in CD4+ T cells from lupus patients, causing overexpression of autosomal genes that contribute to autoimmunity (5–7). Furthermore, CD4+ T cells treated with DNA methylation inhibitors including procainamide and hydralazine cause lupus-like autoantibodies and kidney disease in murine models (8). These observations suggest that DNA hypomethylation may be a mechanism contributing to the development of idiopathic and some forms of drug-induced human lupus.

Idiopathic lupus afflicts women nine times more often than men (9). The reason for the female predominance is unknown. DNA methylation also contributes to the silencing of one X chromosome in women (10). We hypothesized that autoantibody promoting genes on the inactive X may also demethylate in CD4+ T cells, causing greater expression in women than men with lupus. CD40LG is a B cell costimulatory molecule encoded on the X. CD40LG is overexpressed on lupus lymphocytes, contributing to overproduction of pathogenic autoantibodies (11–14). We therefore characterized the role of DNA methylation in silencing CD40LG on the inactive X, and determined whether CD40LG on the inactive X demethylates and is overexpressed uniquely in women with lupus.

Materials and Methods

Subjects

Healthy subjects were recruited by advertising. The average age of the men was 38 ± 13 years and 41 ± 14 for the women (mean ± SD). Lupus patients met criteria for lupus (15) and were recruited from the Michigan Lupus Cohort. Disease activity was quantitated using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (16). The average age of the lupus women was 39 ± 11 and the men were 44 ± 10 (mean ± SD). Patient demographics and medications are shown in Table I. The protocols were reviewed and approved by the University of Michigan Institutional Review Board.

Cells and cell culture

T cells were isolated, PHA stimulated and treated with 5-azacytidine (5-azaC) as previously described (7). For CD40L expression, the cells were then restimulated with 5 ng/ml PMA and 500 ng/ml ionomycin for an

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3 Abbreviations used in this paper: SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; 5-azaC, 5-azacytidine; MFI, mean fluorescence intensity; MS-PCR, methylation-sensitive PCR.
### Table I. Patient demographics and medications

<table>
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<th>Age</th>
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<th>Medications</th>
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</table>

*F*: Female; *M*: male; *Plaq*: plaquinyl; *Pred*: prednisone; *MTX*: methotrexate.

additional 6 h (17). Where indicated, the cells were fractionated by negative selection into CD4+ and CD8+ subsets using magnetic beads and protocols provided by the manufacturer (Miltenyi Biotec).

### Flow cytometric analysis

Cells were stained with anti-CD40LG-FITC, anti-CD8-allophycocyanin, and anti-CD3-PE then analyzed by flow cytometry using published protocols (7). CD3+CD8+ cells were referred to as CD8+. Because CD4 expression diminishes following PMA and ionomycin stimulation (17), the CD3+CD8+ population is referred to as the CD4+ population.

### Real-time RT-PCR

CD40L, CD70, and β-actin transcripts were measured in magnetic bead purified CD4+ and CD8+ T cells using a Rotor-Gene (Corbett) thermocycler. CD70 and β-actin primers and amplification were as published (5, 7). CD40L primers were: forward: CACCCCCGTTAATCTGCTA, reverse: CTGGATGTGTCGATCATGTTG, and FoxP3 primers were forward: CAAATGGTGT TTTTCATACTAATAAACTATCCAATAA. The enhancer was amplified between bp 11,311–11,355: AAATCTAGACCCAATCATCTAAATAATAAAAAAAGAAAAAA AA. Round I: forward: (bp 11,311 to 11,355): TAATCTAGAAAATTAAAAAAAAAACTAAAAAAAAA reverse: (bp 11,356 to 11,400): TTTGAATTCAAAAACAAAATTTATAAAAACATCACAA. Round II: forward: (bp 11,401 to 11,498): TTTGAATTCGATAAAATTGGTTAAGGTTT reverse: (bp 11,499 to 11,536): TTTGAATTCGATAAAATTGGTTAAGGTTT. The downstream enhancer, numbered relative to the transcription start site (bent arrow), is shown. The promoter is identified by the broad arrow. The balloons mark the CG pairs. B. The downstream enhancer, numbered relative to the transcription start site, is similarly shown, with balloons identifying CG pairs and the bent line the transcriptionally relevant NF-κB-binding site.

### Statistical analysis

Statistical analysis was performed using the t test or ANOVA and post-hoc testing with Fisher’s least significant difference test. Unless otherwise noted, results are presented as the mean ± SEM.

### Results

#### DNA methylation and CD40LG expression

CD40LG is regulated by a core promoter and upstream and downstream enhancers (19). Fig. IA shows the transcription start site, promoter, three NF-AT sites, and all CG pairs. There are 10 CG pairs in the promoter and surrounding the start site and 3 in the upstream enhancer. Fig. IB similarly shows the downstream enhancer with 11 CG pairs and an NF-κB site.
there was a small increase in CD4+ T cells when comparing women with or without 5-azaC treatment, although only one inactive gene. In contrast, all cloned fragments from a representative male subject are largely methylated, consistent with one active and one inactive gene. The average methylation of each CG pair is shown in the y-axis. Each point thus represents the mean of 50 determinations for the women and 30 for the men (*, overall methylation 0.42 ± 0.02 vs 0.06 ± 0.01, mean ± SEM, women vs men, p < 0.001). The downstream enhancer was amplified in three segments, bisulfite treated, cloned, and sequenced using the same subjects and approach as in C and D. Each point represents the average of 40–50 determinations for the (E) women and 30 for the (F) men (∆, overall methylation 0.52 ± 0.06 vs 0.26 ± 0.08%, mean ± SEM, women vs men, p = 0.008).

CD40LG promoter and downstream enhancer methylation patterns were compared in CD4+ T cells from healthy men and women. Fig. 2A shows representative methylation patterns of the eight CG pairs surrounding the start site in 10 cloned fragments from a woman. Half the fragments are unmethylated, while the other half are largely methylated, consistent with one active and one inactive gene. In contrast, all cloned fragments from a representative male subject are largely unmethylated (Fig. 2B), consistent with their one active gene. The average methylation of each CG pair in the CD40LG promoter of CD4+ T cells from five women is shown in Fig. 2C, and of three men in Fig. 2D. Approximately half the pairs are methylated in women, while almost none are in men. Fig. 2E shows a similar analysis of the CD40LG enhancer in T cells from the same women; Fig. 2F shows the men. Again, ~half the CGs in the female enhancer are methylated and half are not. In men, the proximal enhancer is somewhat more methylated than the promoter although overall methylation is still less than in women.

We then determined whether 5-azaC, an irreversible DNA methyltransferase inhibitor, increases CD40LG expression more in women than men. PBMC from five to six men or women were stimulated with PHA, treated with graded amounts of 5-azaC, and T cell CD40L expression was compared between the men and women using flow cytometry. Fig. 3A shows the effect on percent CD4+ CD40L+ cells. No significant differences were found between men and women with or without 5-azaC treatment, although there was a small increase in CD4+ CD40L+ cells in women. Fig. 3B shows the effect of 5-azaC on CD40L mean fluorescence intensity (MFI). There was a 27% increase in CD40L levels on male CD4+ cells. In contrast, CD40L levels approximately doubled (216%) on 5 μM 5-azaC-treated female CD4+ cells. Higher concentrations gave no further increase (data not shown). No difference was seen in CD40L MFI on untreated CD4+ T cells from men and women (female/male 0.86 ± 0.09, n = 7 overall). Fig. 3C shows the effect on percent CD8+ CD40L+ cells. Relatively few CD8+ T cells express CD40L, and 5-azaC increased CD8+ CD40L+ cell numbers equally in men and women. No significant difference in CD40L expression levels was seen in untreated CD8+ cells from men and women (female/male 0.90 ± 0.08, n = 7), or on 5-azaC-treated CD8+ cells from men and women (Fig. 3D).

These results were confirmed at the mRNA level in four men and four women. 5-azaC approximately doubled CD40L mRNA in CD4+ cells from the women but not the men (fold increase 2.32 ± 0.19 vs 1.43 ± 0.23, women vs men, p = 0.02). Others reported that ~1.8-fold increases in CD40L cause a significant increase in high-affinity IgG autoantibodies (14), suggesting that the increase in CD40L expression on CD4+ T cells from women is functionally significant. In contrast, and similar to results seen at the protein level, CD40L mRNA increased equally in male and female CD8+ cells (fold increase 2.52 ± 0.85 vs 2.38 ± 0.85, women vs men). As a control, we compared 5-azaC effects on an autosomal gene, TNFSF7 (CD70) in CD4+ T cells from the same men and women. We previously reported that 5-azaC demethylates the TNFSF7 promoter and increases CD70 expression in women and men (7). In contrast to CD40LG, no significant difference was seen in the TNFSF7 increase caused by 5-azaC in CD4+ cells from women and men (CD4 fold increase 2.9 ± 0.9 vs 3.3 ± 0.6, women vs men).

We compared 5-azaC effects on CD40LG promoter and enhancer methylation in T cells from men and women. Fig. 4A shows the promoter methylation pattern in untreated CD4+ T cells from four women, and Fig. 4B shows the pattern in their 5-azaC-treated cells. 5-azaC causes a decrease in methylation across the region. Fig. 4, C and D, similarly compare enhancer methylation in the
similarly analyzed for the percentage of CD3\textsuperscript{+}/H11569, expression in female than male CD4\textsuperscript{+}/H11005 (p).

T cell DNA was isolated and promoter methylation was analyzed as in Fig. 2. Effect of 5-azaC on

Inhibiting DNA methylation causes greater CD40L overexpression in women than men. CD40L levels increased in men (p < 0.074), but the difference between men and women was not significant.

FIGURE 3. Inhibiting DNA methylation causes greater CD40L overexpression in female than male CD4\textsuperscript{+} T cells. A, PBMC from five men (□, M) and five women (●, F) were stimulated with PHA, treated with the 5-azaC concentrations indicated on the x-axis, restimulated with PMA plus ionomycin then stained with anti-CD40L-FITC, anti-CD8-allophycocyanin, and anti-CD3-PE and analyzed by flow cytometry. The y-axis shows the percentage of CD3\textsuperscript{+}CD8\textsuperscript{+} CD40L\textsuperscript{+} (CD4\textsuperscript{+}CD40L\textsuperscript{+}) relative to total CD4\textsuperscript{+} cells, and represent the mean ± SEM of the five determinations. The increase in percentage of positive female cells was significant (p = 0.002, 0 vs 5 μM), while the increase in men was not. There was no significant difference in the percentage of positive cells between men and women. B, CD40L MFI was measured on 5-azaC-treated CD4\textsuperscript{+} T cells, using T cells from six men and six women as in A. The y-axis represents the CD40L MFI relative to untreated cells, and error bars the SEM. CD40L levels increased on both male (p = 0.043) and female (p = 0.003) cells, and the increase on female cells was greater than on male (*, p = 0.034). C, The cells shown in A were similarly analyzed for the percentage of CD3\textsuperscript{+}CD8\textsuperscript{+} CD40L\textsuperscript{+} relative to total CD3\textsuperscript{+}CD8\textsuperscript{+} cells. The number of CD8\textsuperscript{+} cells from men and women expressing CD40L increased significantly (†, p = 0.039 and p = 0.023, respectively). D, The cells shown in B were analyzed for CD40L MFI on CD8\textsuperscript{+} T cells. CD40L levels increased in men (p = 0.015) and women (p = 0.074), but the difference between men and women was not significant.

CD40LG methylation and expression in lupus

We then determined whether CD40LG demethylates in T cells from women with lupus. Fig. 6, A–E, compare CD40LG promoter and enhancer methylation in CD4\textsuperscript{+} cells from women with lupus and controls. Fig. 6A shows the promoter methylation of five female controls, and Fig. 6B the methylation of the same region in CD4\textsuperscript{+} cells from five women with mildly (SLEDAI = 4) active lupus. There is modest demethylation of the five CG pairs just 5' to the start site. Fig. 6C similarly shows the methylation pattern in CD4\textsuperscript{+} cells from three women with more active lupus (SLEDAI = 8.7). Methylation of the eight CG pairs surrounding the start site is less than controls and patients with less active lupus. Fig. 6, D and E,
compare enhancer methylation patterns in CD4+ cells from the patients and controls. Overall methylation is diminished in the lupus patients. There was no significant effect of disease activity on enhancer methylation (data not shown).

Together, these results suggest that promoter and enhancer de-methylation may cause CD40LG overexpression on CD4+ T cells from women but not men with lupus. CD40LG expression was therefore compared on CD4+ cells from men and women with lupus. Promoter demethylation is proportional to disease activity, so the men and women were matched for SLEDAI scores (4.3 ± 0.61 vs 3.86 ± 0.77, mean ± SEM, male vs female). Each subject was paired with a healthy age- and sex-matched control, and CD40L levels on CD4+ cells measured as in Fig. 3. Fig. 6F compares CD40L levels in the men and women with lupus, standardized to the healthy controls. CD4+ cells from women but not men with lupus overexpress CD40L.

We confirmed and extended these results by comparing CD40LG and TNFSF7 transcripts in three healthy men and women and three men and women with lupus (SLEDAI 4.7 ± 1.8 and 4.3 ± 1.2, respectively). Fig. 7A shows that CD4+ cells from lupus women have higher CD40LG levels than the other three groups (p = 0.023 by ANOVA). The relationship between CD40LG enhancer methylation status, measured by MS-PCR, and transcript levels was assessed in the control and lupus women. Similar to the 5-azaC-treated cells, there was an inverse relationship between the enhancer methylation index and CD40LG mRNA (R² = 0.866, p = 0.007).

**FIGURE 5.** Effect of methylation on CD40LG promoter and enhancer function. PCR-amplified fragments containing the upstream enhancer and promoter or downstream enhancer were cloned into reporter constructs, the indicated regions excised, methylated with SssI and S-adenosylmethionine, religated back into the reporter construct, and transfected into Jurkat cells. The effect of fragment methylation (dark bars) is expressed relative to the mock-methylated controls (light bars), similarly constructed but omitting the SssI. The results represent the mean ± SEM of three independent experiments.

**FIGURE 6.** CD40LG is demethylated and overexpressed in women with lupus. A. The average methylation for each CG pair in the CD4+ T cell CD40LG promoter from five healthy women is shown as in Fig. 2A (overall methylation 42 ± 2%, mean ± SEM). B. Average methylation of each CG pair in the CD4+ T cell CD40LG promoter in CD4+ T cell DNA from five women with lupus and average SLEDAI 4 (overall methylation 33 ± 5%). C. The same analysis as in B using CD4+ T cell DNA from three women with active lupus and average SLEDAI 8.7 (overall methylation 18 ± 5%). The overall average methylation of the CD40LG promoter decreased with increasing disease activity in the lupus patients (*, p = 0.018 overall by ANOVA, and p = 0.006 SLEDAI 8.7 vs control and p = 0.05 SLEDAI 8.7 vs 4.0 by post-hoc analysis). D. The average methylation for each CG pair in the CD40LG enhancer in CD4+ T cells from four to five healthy women is shown as in A (overall methylation 52 ± 6%). E. The average methylation of each CG pair in the CD40LG enhancer in CD4+ T cell DNA from all eight lupus patients is shown (overall methylation 34 ± 3%, †, p = 0.01 vs controls). F. Six men and seven women with lupus were paired with age- and sex-matched controls, and CD40L MFI measured on CD4+ T cells as in Fig. 3. Results are presented as the mean ± SEM of the CD40L MFI of each lupus patient relative to his or her matched control (p = 0.009, men vs women).
CD40LG and TNFSF7 mRNA in lupus women and men and controls. A, CD4+ T cells from three healthy women, three lupus women, three healthy men, or three lupus men were stimulated and CD40LG transcripts measured relative to β-actin. The light bars represent the controls and dark bars the lupus patients. Results are presented as the mean ± SEM of the three determinations per group (p = 0.023). B, TNFSF7 transcripts were measured relative to β-actin in mRNA from the same subjects, and results presented as in A (p = 0.01 overall lupus vs control).

Fig. 7B compares TNFSF7 transcripts in CD4+ cells from the same subjects. Similar to experimentally demethylated cells, TNFSF7 mRNA was not significantly different in T cells from healthy men and women, and increased equally in CD4+ from both men and women with lupus (p = 0.01 lupus vs controls).

Additional studies compared expression of FOXP3, also encoded on the X chromosome, in CD4+ and CD8+ T cells from the same lupus patients and controls. No significant differences in FOXP3 transcripts were seen between male and female CD4+ lupus T cells (data not shown). However, CD8+ T cells from lupus women had higher FoxP3 levels than lupus men (0.42 ± 0.07 vs 0.18 ± 0.003, mean ± SEM, p = 0.03).

Discussion

These studies demonstrate differential CD40LG methylation in CD4+ T cells from healthy men and women. CD40LG is encoded on the X chromosome, so women have two copies and men have one. Bisulfite sequencing of DNA from CD4+ T cells revealed that women have one methylated and one unmethylated CD40LG gene, while the one gene in men is largely unmethylated. Because one X chromosome is inactivated in women by processes including DNA methylation (10), the methylated gene is from the inactive X. The unmethylated gene in men is consistent with reports that CD40LG is demethylated in unstimulated and PHA-stimulated T cells (5, 6, 22). Thus, 5-azaC induced demethylation in CD40LG increases only slightly in men because their single gene is largely unmethylated. Additional mechanisms contribute to X chromosome inactivation including coating with XIST RNA, histone modifications, and histone variant macro-H2A recruitment (10).

5-azaC is sufficient to reactivate CD40LG suggests that DNA methylation is a primary mechanism suppressing its expression. 5-azaC also demethylated the CD40LG promoter and induced expression equally in CD8+ cells from men and women. This suggests that methylation is a mechanism suppressing CD40LG expression in the CD8+ subset.

The CD40LG regulatory sequences demethylated by 5-azaC also demethylate in CD4+ T cells from women with lupus, correlating with overexpression in women but not men with lupus. Others have excluded T cell activation as a mechanism for the overexpression in lupus (19), and subset-specific effects are unlikely given the nearly complete demethylation observed in the women with more active lupus and lack of subset-specific differences in expression reported by others (21). The overexpression is unlikely to be due to medications, because the drugs commonly used to treat lupus do not inhibit DNA methylation (4, 23). T cell DNA also demethylates with age (24), but in this study the subjects were all within the same age range (Table I and Materials and Methods), and no effects of age were noted in individual subjects. We reported similar demethylation and overexpression of the ITGAL, PRF1, and TNFSF7 genes in 5-azaC-treated and lupus CD4+ cells (5–7), suggesting that demethylation occurs throughout the genome. Our present observation that TNFSF7 expression increases in 5-azaC-treated and lupus T cells from men and women confirms this. The mechanism causing DNA demethylation in idiopathic and hydralazine-induced lupus is decreased ERK pathway signaling, resulting in failure to up-regulate the maintenance DNA methyltransferase Dnmt1 during mitosis and consequently failure to replicate methylation patterns in the newly synthesized DNA (3, 25). Our present observation that the same CD40LG sequences demethylate in both 5-azaC-treated and lupus T cells supports our earlier report that proliferating T cells with treated DNA methyltransferase inhibitors, or ERK pathway inhibitors, demethylates the same DNA sequences (6).

T cells overexpressing ITGAL, PRF1, and TNFSF7 become autoreactive, killing autologous macrophages and overstimulating B cells (8). Macrophage killing releases apoptotic material and impairs its clearance, leading to generation of anti-chromatin Abs and accelerating lupus (26). CD40LG overexpression could amplify the anti-chromatin response more in women than men, increasing lupus susceptibility. The present observations also support the hypothesis that abnormal T cell DNA methylation contributes to lupus pathogenesis. There are >1000 genes on the X chromosome, some with immune function (27). CD40LG demethylation and overexpression raises the possibility that other X chromosome genes may also demethylate in lupus, amplifying autoimmune processes in women. This is supported by our observation that women with lupus also overexpress FOXP3 transcripts in CD8+ T cells relative to men. Others have reported a decrease in FoxP3 transcripts in CD4+ lupus T cells (28), but the functional significance of the gender-specific difference in the CD8+ subset is unknown.

Multiple mechanisms may contribute to female-biased diseases. DNA generally demethylates with age (24), so some female predominant diseases of aging could involve X chromosome reactivation. A recent report indicates that ~15% of genes on the inactive X escape inactivation to some degree in fibroblasts (29), suggesting another mechanism predisposing women to diseases. Both X chromosomes are active during early development, then one is randomly inactivated, and skewed X chromosome inactivation could also be a mechanism predisposing women to disease (30). Finally, estrogen likely contributes to the propensity of women to develop lupus, but does not completely explain the female predominance (9). The present work is the first to add X chromosome demethylation to these mechanisms.
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