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Overexpression of the Growth Arrest and DNA Damage–Induced 45α Gene Contributes to Autoimmunity by Promoting DNA Demethylation in Lupus T Cells

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Objective. **Demethylation of CD11a and CD70 regulatory regions in CD4+ T cells contributes to the development of autoreactivity and overstimulation of autoantibodies. Because growth arrest and DNA damage–induced 45α (GADD45α) reduces epigenetic silencing of genes by removing methylation marks, this study examined whether the *gadd45A* gene could contribute to autoimmunity by promoting DNA demethylation in T cells from patients with systemic lupus erythematosus (SLE).**

Methods. **Levels of GADD45α, CD11a, and CD70 messenger RNA (mRNA) and protein were detected by real-time reverse transcription–polymerase chain reaction and Western blotting or flow cytometry. Global DNA methylation was evaluated using Methylamp global DNA methylation quantification kits. Detection of CD4+ T cell proliferation and autologous B cell IgG antibodies was performed using commercially available kits. CD11a and CD70 promoter methylation was determined with bisulfite sequencing.**

Results. **Elevated *gadd45A* mRNA expression and global DNA hypomethylation were observed in CD4+ T cells from SLE patients. The levels of *gadd45A* mRNA were inversely proportional to the levels of DNA methylation. Positive correlations were found between *gadd45A* and CD11a/CD70 mRNA levels. Expression of *gadd45A* mRNA was increased in CD4+ T cells following ultraviolet B irradiation, and this was accompanied by increased levels of CD11a and CD70 mRNA. Moreover, increased expression of *gadd45A*, CD11a, and CD70 mRNA was accompanied by increased autoreactivity and excessive B cell stimulation in *gadd45A*-transfected CD4+ T cells. CD11a promoter methylation was also significantly reduced in transfected cells. Transfection of *gadd45A* small interfering RNA inhibited the autoreactivity of SLE CD4+ T cells and led to significant increases in the methylation levels of the CD11a and CD70 promoter regions.**

Conclusion. **These findings indicate that *gadd45A* may contribute to lupus-like autoimmunity by promoting DNA demethylation in SLE CD4+ T cells.**

Systemic lupus erythematosus (SLE) is an autoimmune disease that involves multiple organ systems. The pathogenic mechanisms that cause lupus are unclear. However, recent studies have shown that T cell DNA demethylation plays an important role in the pathogenesis of SLE (1). In previous studies, we demonstrated that inhibiting T cell DNA methylation in vitro causes demethylation of regulatory sequences and overexpression of genes, including CD11a (ITGAL) (2), CD70 (TNFSF7) (3), perforin (PRF1) (4), and CD40 ligand (CD40LG) (5). Overexpression of these genes results in T cell autoreactivity, spontaneous monocyte/macrophage killing, and B cell immunoglobulin overproduction. We also reported that inhibitors of DNA methylation can induce lupus-like autoimmunity in vitro and

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in vivo (6,7). Furthermore, T cells from patients with active lupus have genome-wide decreases in deoxymethylcytosine content and gene-specific DNA hypomethylation. The process of DNA hypomethylation causes overexpression of autoimmune-related genes, resulting in autoreactive monocyte/macrophage killing and excessive B cell stimulation (3,8–10). DNA demethylation also contributes to the striking predilection for development of SLE in women, since the process induces expression of CD40LG on the inactive X chromosome (5).

The molecular basis of DNA demethylation in SLE T cells remains incompletely understood. A recent study showed that the growth arrest and DNA damage–induced 45α (GADD45α) gene can promote DNA repair and remove methylation marks, and thereby reduce epigenetic gene silencing (11). In addition, ultraviolet (UV) irradiation can up-regulate *gadd45A* transcript expression, and UV light can trigger lupus flares (12–14). Therefore, to determine whether the *gadd45A* gene contributes to the development of autoimmunity by promoting DNA demethylation in SLE CD4+ T cells, we compared the expression of GADD45α protein and messenger RNA (mRNA) in CD4+ T cells between patients with SLE and healthy controls, and studied the effects of UV irradiation on *gadd45A* mRNA expression and DNA methylation in CD4+ T cells. We also investigated the consequences of either increased or decreased *gadd45A* mRNA expression in CD4+ T cells from patients with SLE and healthy controls. The results of these studies provide new insights into the mechanisms causing DNA demethylation in lupus T cells, and suggest novel approaches for the treatment of SLE.

PATIENTS AND METHODS

Subjects. The demographic characteristics of the subjects are shown in Table 1. SLE patients (mean ± SD age 26.47 ± 5.61 years) were recruited from the outpatient clinics and inpatient services at the Second Xiangya Hospital of Central South University in China. All patients fulfilled at least 4 of the American College of Rheumatology classification criteria for SLE (15). Lupus disease activity was quantified using the SLE Disease Activity Index (SLEDAI) (scale of 0–26, with higher scores indicating worsening disease activity) (16). The mean ± SD overall SLEDAI score in the patients with SLE enrolled in this study was 7.53 ± 5.77. Healthy controls (mean ± SD age 27.75 ± 6.20 years) were recruited from medical staff at the Second Xiangya Hospital. This study was approved by the human ethics committee of the Central South University Xiangya Medical School, and written informed consent was obtained from all subjects. Patients and controls were age- and sex-matched in all experiments.

Cells. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density-gradient centrifugation (Shanghai Hengxin Chemical Reagent), and CD4+ T cells as well as B cells were isolated by positive selection using Miltenyi beads, according to protocols provided by the manufacturer. The purity of the enriched cell subsets was evaluated by flow cytometry, which generally showed a purity of >95% for both cell types.

Table 1. Demographic characteristics and medication regimens of the patients with systemic lupus erythematosus*

Patient/age/sex	SLEDAI score†	Medication and dose
1/30/F	4	Pred. 15 mg/day
2/32/F	18	Pred. 20 mg/day + TG
3/30/F	4	Pred. 15 mg/day
4/31/F	4	Pred. 10 mg/day
5/28/F	6	None
6/28/F	12	None
7/24/M	0	Pred. 15 mg/day
8/23/F	20	None
9/18/F	2	CYC
10/26/F	4	TG
11/25/F	2	TG
12/28/F	4	Pred. 15 mg/day
13/25/F	14	Pred. 20 mg/day
14/21/F	6	Pred. 10 mg/day
15/41/F	8	Pred. 5 mg/day
16/19/F	8	Pred. 5 mg/day
17/21/F	12	Pred. 15 mg/day

* SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; Pred. = prednisone; TG = tripterygium glycoside; CYC = cyclophosphamide.
† Scale 0–26.

UVB irradiation. CD4+ T cells were irradiated with 1.5 joules/cm^2 UVB (emission peak 312 nm) using the KN4003 UVB irradiation treatment system (Xuzhou Kernel Medical Equipment). After irradiation, the cells were cultured in fresh medium and incubated for 6, 24, or 48 hours. Controls were treated the same way, but without UVB exposure.

Real-time reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was isolated from CD4+ T cells using a MiniRNA kit (Qiagen), and then *gadd45A*, CD11a, CD70, and β-actin transcript expression was measured using a Rotor-Gene 3000 thermocycler (Corbett Research). Messenger RNA levels were quantified using a SYBR PrimeScript RT-PCR kit (Takara). The primers used were as follows: for *gadd45A*, forward 5′-AAGGGGCTGAGTGAGTTCAA-3′ and reverse 5′-TTTTCCTTCCTGCATGGTTC-3′; for CD11a, forward 5′-TGAGAGCAGGCTATTTGGGTTAC-3′ and reverse 5′-CGGCCCATGTGCTGGTAT-3′; for CD70, forward 5′-CACACTCTGCACCTCACT-3′ and reverse 5′-CACCCACTGCACTCCAAAGA-3′; and for β-actin, forward 5′-CGCGAGAAGATGACCCAGAT-3′ and reverse 5′-GCACTGTGTTGGCGTACAGG-3′.

Immunoblotting. CD4+ T cell proteins were denatured, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membranes (Amersham Biosciences or GE Healthcare). The membranes were then incubated overnight at 4°C with a 1/300 dilution of rabbit anti-human GADD45α polyclonal antibody (Santa Cruz Biotechnology), followed by incubation

with a 1/2,000 dilution of peroxide-conjugated secondary mouse anti-rabbit antibody (Santa Cruz Biotechnology). The binding of antibodies was detected using an enhanced chemiluminescence detection system (SuperSignal). Anti–β-actin was used as a control for protein loading and transferring.

Transfections. A pcDNA3.1(+) blank plasmid and pcDNA3.1-*gadd45A*–expressing plasmid were constructed as described previously (17). Control small interfering RNA (siRNA) and siRNA targeting *gadd45A* were designed and synthesized at Guangzhou RiboBio in China. The sequences of the siRNA for *gadd45A* were as follows: forward (5′–3′) 5′-GGAGGAAGUGCUCAGCAAA–DNA terminal deoxynucleotidyl transferase (dTdT)–3′ and backward (3′–5′) 3′-dTdT-CCUCCUUCACGAGUCGUUU-5′. Plasmid and siRNA transfections were performed using the Human T Cell Nucleofector kit (Amaxa) according to the manufacturer's instructions.

Flow cytometric analysis. CD4+ T lymphocytes were analyzed for expression of CD11a and CD70 using flow cytometry and fluorescein isothiocyanate–conjugated monoclonal antibodies (Becton Dickinson). Data were acquired using a FACScalibur, with results analyzed using CellQuest software (Becton Dickinson).

Cell proliferation assays. CD4+ T cells (4×10^5) were cultured in 96-well plates, and cell proliferation was measured using a cell proliferation enzyme-linked immunosorbent assay (ELISA) bromodeoxyuridine (colorimetric) kit (Roche Diagnostics) according to the manufacturer's instructions. Absorbance was measured using an ELISA reader (Bio-Tek Elx800 automatic microplate reader) at a wavelength of 370 nm (reference wavelength 492 nm).

ELISAs for IgG. IgG levels in the supernatants of the T–B cell cultures were measured using an ELISA kit (Columbia Bio) following the manufacturer's protocol. All determinations were performed in quadruplicate.

Global DNA methylation detection. Genomic DNA was isolated from CD4+ T cells using the TIANamp Genomic DNA kit (Tiangen Biotech). Global methylation levels were measured using the Methylamp Global DNA Methylation Quantification Ultra kit (Epigentek Group) according to the manufacturer's instruction. In this assay, DNA is immobilized to wells specifically coated with a specific DNA affinity substratum. The methylated fraction of DNA can be recognized with a 5-methylcytosine antibody and quantified through an ELISA-like reaction. Absorbance was measured at 450 nm.

Bisulfite genomic sequencing. Bisulfite conversion was performed using the EpiTect Bisulfite kit (Qiagen). The 310-bp (−1,289 to −979 bp) CD11a promoter and 294-bp (−581 to −288 bp) CD70 promoter fragments were amplified using nested PCR, and then cloned into a pGEM-T vector (Promega). The 5 independent clones were then sequenced for each of the amplified fragments. The primers for CD11a were as follows: first round, forward (−1,465 to −1,424 bp) 5′-GGTGAATTCTTTAAGGTTAGGAGTTTAAGTTTAGTT-3′ and reverse (−858 to −899 bp) 5′-CAATCTAGAACTACACATTTATTAAAAATTAAATTA-3′; and second round, forward (−1,330 to −1,289 bp) 5′-GTTGAATTCGGTTAATATGGTGAAATTTTATTTTTAT-3′ and reverse (−938 to −979 bp) 5′-CACTCTAGATACAACAAACATCCAAAAATTAAAAATA-3′. The primers for CD70 were as follows: first round, forward (−609 to −580 bp) 5′-GGTGAATTCTTTAAGGTTAGGAGTTTAAGTTTAGTT-3′ and reverse (−278

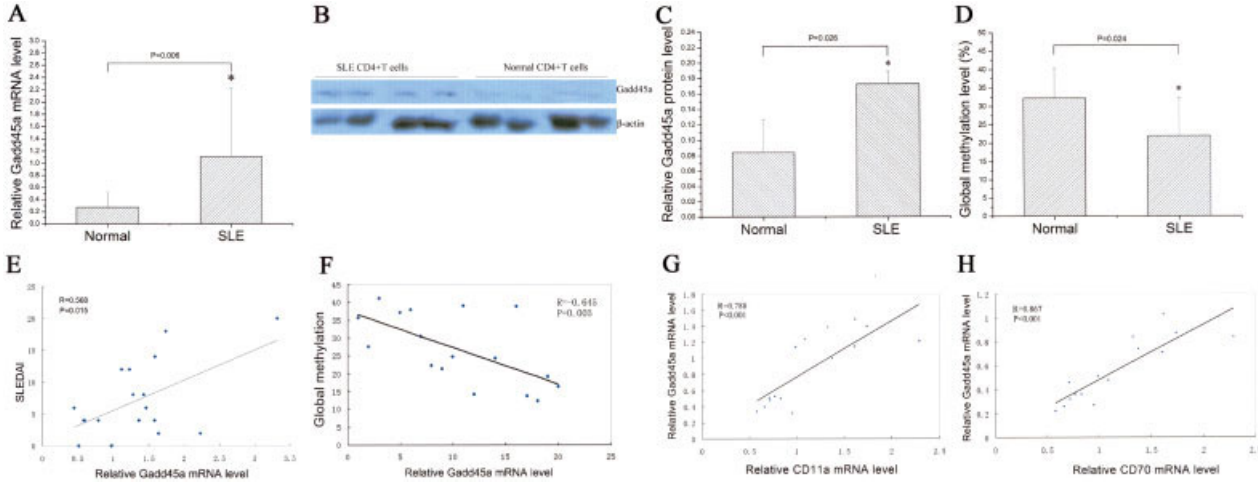

Figure 1. Growth arrest and DNA damage–induced 45α (GADD45α) gene and protein expression and global DNA methylation in patients with systemic lupus erythematosus (SLE) compared with healthy controls. **A,** Relative expression of *gadd45A* mRNA in CD4+ T cells from SLE patients and healthy controls was measured by quantitative real-time polymerase chain reaction, with results normalized to the levels of β-actin. **B** and **C,** Protein levels of GADD45α, relative to the levels of β-actin, in CD4+ T cells from SLE patients and healthy controls were assessed by Western blotting. Representative Western blot results are shown. **D,** Global DNA demethylation was observed in lupus CD4+ T cells, with significant differences compared with controls. **E,** Levels of *gadd45A* mRNA are plotted against the SLE Disease Activity Index (SLEDAI). **F,** Levels of *gadd45A* mRNA are plotted against the global DNA methylation levels. **G** and **H,** A significant positive correlation was observed between *gadd45A* mRNA levels and CD11a (**G**) or CD70 (**H**) mRNA levels. Bars show the mean and SD. Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

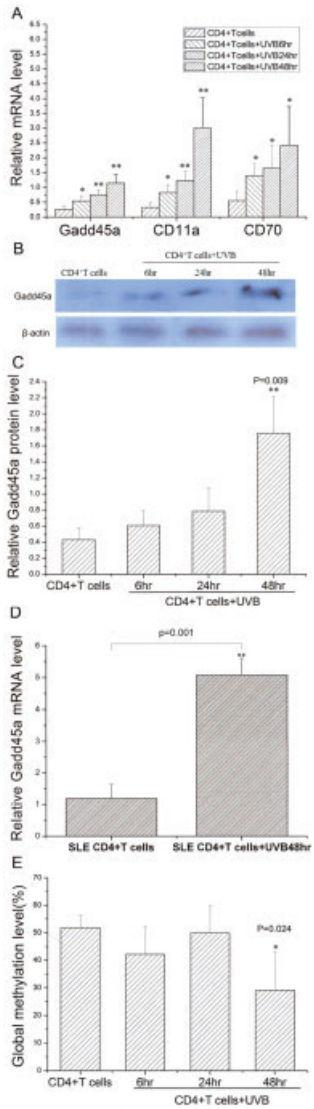

Figure 2. The effect of ultraviolet B (UVB) irradiation (1.5 joules/cm^2) on the expression of *gadd45A*, CD11a, and CD70 mRNA and global DNA methylation in CD4+ T cells. **A,** Relative expression of *gadd45A*, CD11a, and CD70 mRNA was measured in normal CD4+ T cells by quantitative real-time polymerase chain reaction at baseline and after 6, 24, and 48 hours of irradiation with UVB, with results normalized to the levels of β-actin. $* = P < 0.05$; $** = P < 0.01$, versus baseline. **B** and **C,** Protein levels of GADD45α, relative to the levels of β-actin, in normal CD4+ T cells were assessed by Western blotting at baseline and after 6, 24, and 48 hours of irradiation with UVB. Significant differences were seen at 48 hours in comparison with baseline. Representative Western blot results are shown. **D,** Relative levels of *gadd45A* mRNA were also determined in CD4+ T cells from patients with systemic lupus erythematosus (SLE) at 48 hours after irradiation with UVB. **E,** Global DNA methylation levels were determined in normal CD4+ T cells at baseline and after 6, 24, or 48 hours of irradiation with UVB. Significant differences were seen at 48 hours in comparison with baseline. Bars show the mean and SD. Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

to −242) 5′-CAATCTAGAACTACACATTTATTAAAAATTAAATTA-3′; and second round, forward (−581 to −545) 5′-GTTGAATTCGGTTAATATGGTGAAATTTTATTTTTAT-3′ and reverse (−330 to −288) 5′-CACTCTAGATACAACAAACATCCAAAAATTAAAAATA-3′.

Statistical analysis. Student's *t*-test for equality of means was used to compare values. Pearson's correlation coefficient was used to determine the relationship between lupus disease activity and other parameters. *P* values less than 0.05 were considered significant. All analyses were performed with SPSS version 12.0 software.

RESULTS

Overexpression of *gadd45A* in CD4+ lupus T cells. Initially, we compared the levels of *gadd45A* mRNA in CD4+ T cells from SLE patients and healthy controls. SLE T cells had significantly higher *gadd45A* mRNA levels as compared with healthy donor T cells ($P = 0.006$) (Figure 1A). The increase in *gadd45A* mRNA in lupus T cells was confirmed at the protein level using immunoblotting (Figures 1B and C). Global DNA hypomethylation was also observed in CD4+ lupus T cells ($P = 0.024$ versus controls) (Figure 1D), as has been reported by others (18).

There was a significant positive correlation between *gadd45A* mRNA levels and lupus disease activity as measured by the SLEDAI ($R = 0.568$, $P = 0.015$) (Figure 1E). Levels of *gadd45A* mRNA also correlated significantly with the extent of overall CD4+ T cell DNA hypomethylation ($R = -0.645$, $P = 0.005$) (Figure 1F). Significant positive correlations were also found between *gadd45A* and CD11a mRNA expression ($R = 0.788$, $P < 0.001$) (Figure 1G), as well as between *gadd45A* and CD70 mRNA expression ($R = 0.867$, $P < 0.001$) (Figure 1H). No significant differences in *gadd45A* mRNA levels between SLE patients and healthy controls were observed in CD8+ T cells (results not shown).

Increase in *gadd45A*, CD11a, and CD70 mRNA expression and increased DNA methylation after UV irradiation of CD4+ T cells. We then examined the effects of UVB irradiation (1.5 joules/cm^2) both on *gadd45A*, CD11a, and CD70 mRNA expression and on DNA methylation in normal CD4+ T cells. Levels of *gadd45A*, CD11a, and CD70 mRNA increased significantly at 6, 24, and 48 hours following irradiation (for *gadd45A*, $P = 0.041$, $P = 0.004$, and $P = 0.002$, respectively; for CD11a, $P = 0.027$, $P = 0.002$, and $P = 0.004$, respectively; for CD70, $P = 0.034$, $P = 0.032$, and $P = 0.031$, respectively) (Figure 2A). In addition, the overexpression of *gadd45A* mRNA after UVB irradiation

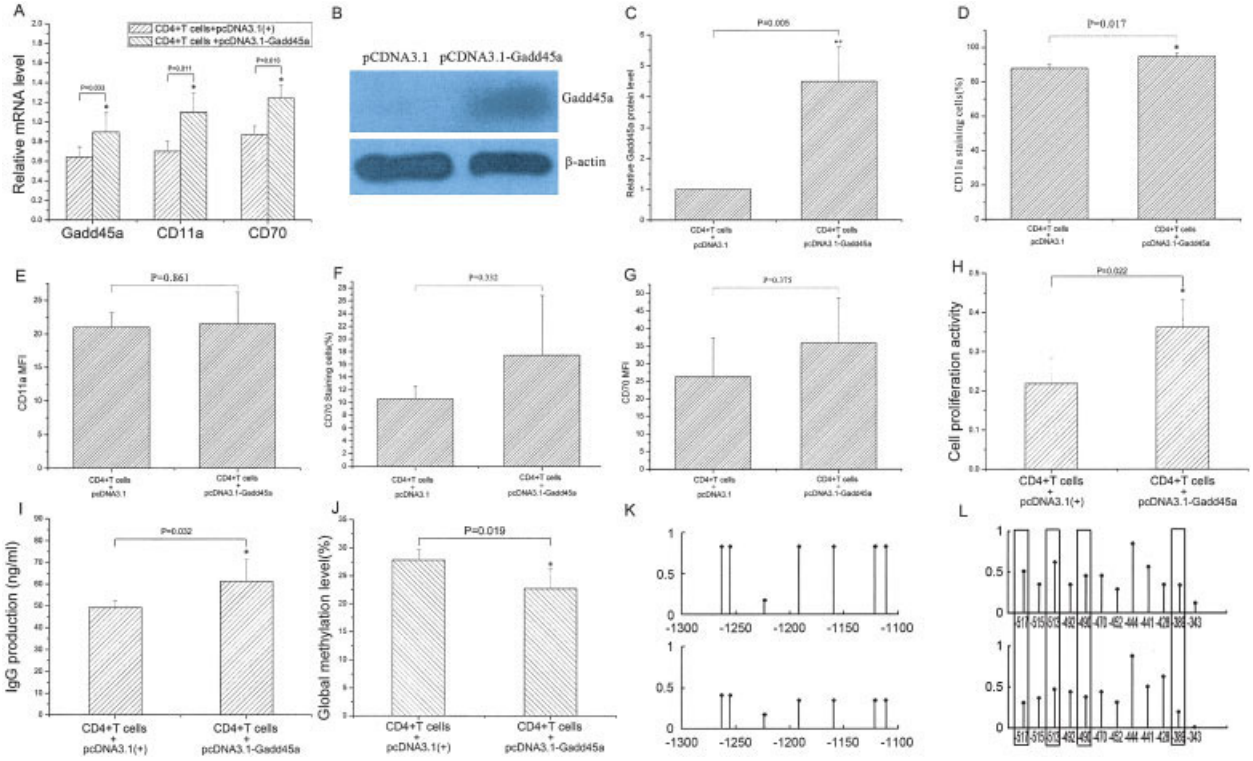

Figure 3. Effects of *gadd45A* overexpression on CD4+ T cell DNA methylation, gene expression, and autoreactivity. **A,** Overexpression of *gadd45A*, CD11a, and CD70 mRNA was induced in CD4+ T cells by transfection with pcDNA3.1-*gadd45A*; a blank plasmid (pcDNA3.1[+]) was used as control. **B** and **C,** GADD45α protein overexpression in CD4+ T cells transfected with pcDNA3.1-*gadd45A* was confirmed by Western blot analysis, with results expressed relative to the levels of β-actin. **D–G,** CD4+ T cells transfected with pcDNA3.1-*gadd45A* or control plasmid were stained with anti-CD11a–fluorescein isothiocyanate (FITC) or anti-CD70–FITC, and then analyzed for expression of CD11a (**D**) and CD70 (**F**) by flow cytometry. The CD11a mean fluorescence intensity (MFI) (**E**) or CD70 MFI (**G**) were also compared between transfection groups. **H–J,** Increased cell proliferation (**H**), enhanced B cell costimulation (**I**), and global DNA demethylation (**J**) were observed in CD4+ T cells transfected with pcDNA3.1-*gadd45A*. **K,** The average methylation levels of CpG pairs in the −1,289 to −979–bp CD11a promoter fragment were compared between CD4+ T cells transfected with pcDNA3.1-*gadd45A* (bottom) and those transfected with control plasmid (top). **L,** Compared with CD4+ T cells transfected with control plasmid (top), the 4 CpG sequence motifs (−517, −513, −490, and −389 bp; indicated by boxes) of CD70 were hypomethylated in CD4+ T cells transfected with pcDNA3.1-*gadd45A* (bottom). This decrease in methylation was not statistically significant in comparison with controls. Vertical lines with diamonds represent the average methylation of each CpG pair. Bars in **A** and **C–J** show the mean and SD. Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

was confirmed at the protein level by Western blotting. As shown in Figures 2B and C, GADD45α protein levels were significantly higher in irradiated CD4+ T cells 48 hours after UVB irradiation as compared with cells before UV treatment ($P = 0.009$). In addition, the global DNA methylation levels were significantly reduced 48 hours after irradiation ($P = 0.024$ versus before UV treatment) (Figure 2E). The effects of UVB exposure on *gadd45A* mRNA levels were also examined in CD4+ T cells from SLE patients, and the results showed that *gadd45A* mRNA levels were significantly increased following 48 hours of irradiation ($P = 0.001$ versus before UV treatment) (Figure 2D).

Association of *gadd45A* overexpression with DNA demethylation, CD11a and CD70 overexpression, and T cell autoreactivity. We also determined the effects of *gadd45A* overexpression on DNA methylation, the expression of CD11a and CD70, and T cell autoreactivity. A *gadd45A* expression plasmid (pcDNA3.1-*gadd45A*) or control plasmid (pcDNA3.1[+]) was transfected into unstimulated normal CD4+ T cells. The CD4+ T cells transfected with pcDNA3.1-*gadd45A* expressed significantly higher amounts of *gadd45A*, CD11a, and CD70 mRNA compared with cells transfected with blank control plasmid ($P = 0.033$, $P = 0.011$, and $P = 0.016$, respectively) (Figure 3A). Western blot analysis showed

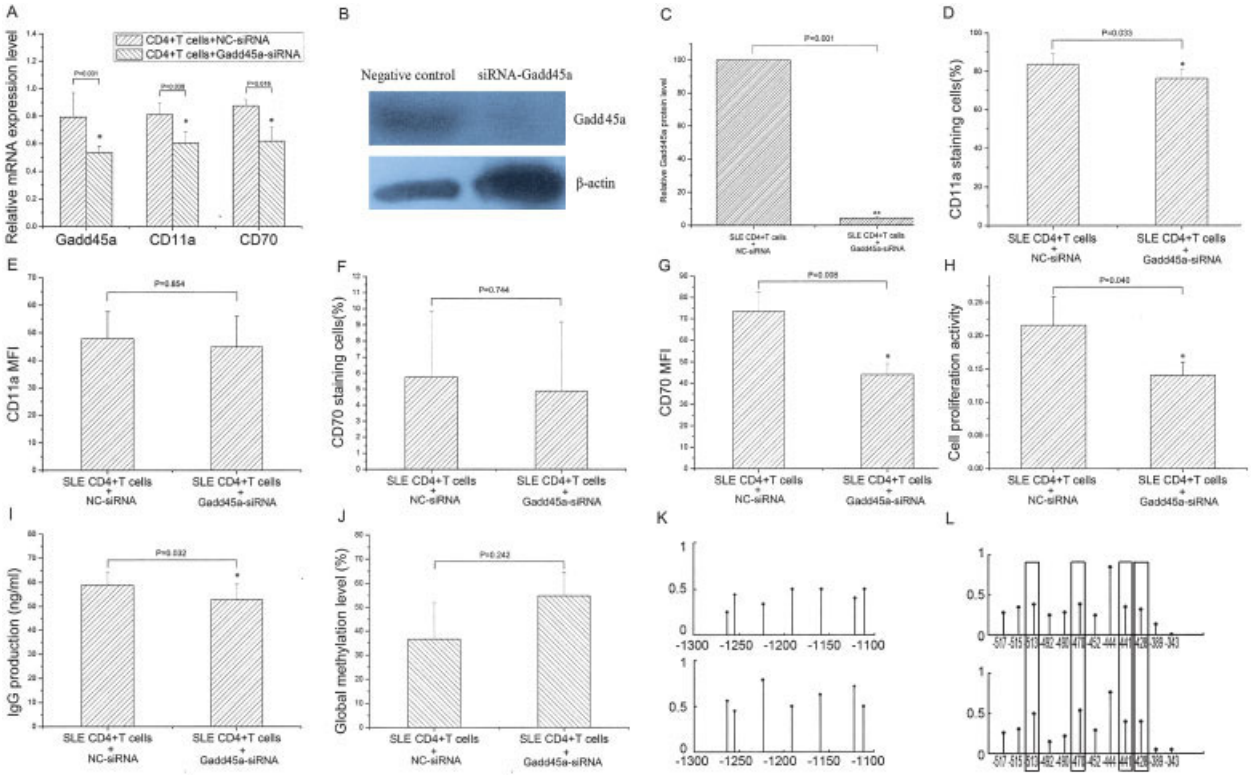

Figure 4. Effects of *gadd45A* inhibition on lupus CD4+ T cell DNA methylation, gene expression, and autoreactivity. **A,** Down-regulation of *gadd45A*, CD11a, and CD70 mRNA was observed in lupus CD4+ T cells transfected with *gadd45A* small interfering RNA (siRNA) as compared with that in lupus CD4+ T cells transfected with negative control (NC) siRNA. **B** and **C,** Down-regulation of GADD45α protein by *gadd45A* siRNA was confirmed in CD4+ lupus T cells by Western blot analysis, with results expressed relative to the levels of β-actin. **D–G,** CD4+ lupus T cells transfected with *gadd45A* siRNA or control siRNA were stained with anti-CD11a–fluorescein isothiocyanate (FITC) or anti-CD70–FITC, and then analyzed for expression of CD11a (**D**) or CD70 (**F**) by flow cytometry. The CD11a mean fluorescence intensity (MFI) (**E**) or CD70 MFI (**G**) were also compared between siRNA transfection groups. **H–J,** Decreased cell proliferation (**H**), reduced B cell costimulation (**I**), and increased global DNA methylation (**J**) were observed in lupus CD4+ T cells transfected with *gadd45A* siRNA. **K,** The average methylation levels of CpG pairs in the CD11a promoter (−1,289 to −979 bp) were compared between CD4+ lupus T cells transfected with *gadd45A* siRNA (bottom) and those transfected with control siRNA (top). **L,** Compared with the control siRNA–transfected group (top), the 4 CpG sequence motifs (−513, −470, −441, and −428 bp; indicated by the boxes) of CD70 were significantly hypermethylated in CD4+ lupus T cells transfected with *gadd45A* siRNA (bottom). Vertical lines with diamonds represent the average methylation of each CpG pair. Bars in **A** and **C–J** are the mean and SD.

that GADD45α protein levels were also significantly up-regulated in the pcDNA3.1-*gadd45A*–transfected group ($P = 0.005$ versus controls) (Figures 3B and C).

Flow cytometry revealed that, compared with CD4+ T cells transfected with control plasmid, a higher percentage of pcDNA3.1-*gadd45A*–transfected cells expressed CD11a ($P = 0.017$) (Figure 3D), without a significant change in the mean fluorescence intensity (MFI) of CD11a (Figure 3E). Both the percentage of cells expressing CD70 and the MFI of CD70 were increased in CD4+ T cells transfected with pcDNA3.1-*gadd45A* compared with control cells transfected with blank plasmid, but the differences were not significant (Figures 3F and G). We also observed significantly greater T cell proliferation (Figure 3H) and enhanced stimulation of autologous B cell IgG production (Figure 3I) in CD4+ T cells transfected with pcDNA3.1-*gadd45A* compared with that in controls ($P = 0.022$ and $P = 0.032$, respectively).

We next determined the effect of *gadd45A* overexpression on genome-wide DNA methylation levels and gene-specific DNA methylation in CD4+ T cells. First, we found that global DNA methylation levels were significantly decreased in CD4+ T cells transfected with pcDNA3.1-*gadd45A* compared with control cells transfected with blank plasmid ($P = 0.019$) (Figure 3J). We then analyzed the methylation status of the CD11a (−1,263 to −1,111 bp) promoter and CD70 (−581 to

−288 bp) promoter, using bisulfite sequencing. Specific hypomethylation within these promoter areas is characteristic of lupus T cells (8). As shown in Figure 3K, the average level of methylation of the CD11a promoter region was significantly reduced in CD4+ T cells transfected with pcDNA3.1-*gadd45A* as compared with blank plasmid–transfected controls (mean ± SD ratio of methylated CG pairs 0.3333 ± 0.0833 versus 0.7381 ± 0.2520 in controls; $P = 0.005$). Six of the 7 CpG sequence motifs (−1,263, −1,255, −1,191, −1,159, −1,121, and −1,111 bp) were hypomethylated in CD4+ T cells transfected with pcDNA3.1-*gadd45A* as compared with blank plasmid–transfected controls. In the CD70 promoter region, 4 CpG sequence motifs (−517, −513, −490, and −389 bp) were hypomethylated in CD4+ T cells transfected with pcDNA3.1-*gadd45A* as compared with that in controls. However, this decrease in the average methylation levels for the 4 CpG motifs of CD70 was not statistically significant in comparison with controls (mean ± SD 0.3354 ± 0.1172 versus 0.4722 ± 0.1157 in controls; $P = 0.148$) (Figure 3L).

Inhibition of CD11a and CD70 expression and T cell autoreactivity and increase in DNA methylation following down-regulation of *gadd45A* expression. The effects of *gadd45A* on T cell autoreactivity were also confirmed using *gadd45A* siRNA, which induced a decrease in *gadd45A* expression in CD4+ T cells from patients with SLE. Compared with that in control siRNA–transfected lupus T cells, *gadd45A*, CD11a, and CD70 mRNA levels were significantly reduced in *gadd45A* siRNA–transfected lupus T cells ($P = 0.031$, $P = 0.038$, and $P = 0.016$, respectively) (Figure 4A). GADD45α protein levels were also markedly down-regulated after transfection of lupus T cells with *gadd45A* siRNA ($P < 0.001$ versus control siRNA–transfected lupus T cells) (Figures 4B and C).

CD11a and CD70 protein levels in siRNA transfection experiments were measured by flow cytometry. Compared with CD4+ lupus T cells transfected with control siRNA, the percentage of CD4+ lupus T cells expressing CD11a was reduced following transfection with *gadd45A* siRNA ($P = 0.033$) (Figure 4D), but there was no significant difference in the MFI of CD11a between *gadd45A* siRNA–transfected and control siRNA–transfected CD4+ lupus T cells (Figure 4E). The MFI of CD70 was significantly decreased in CD4+ T cells transfected with *gadd45A* siRNA as compared with controls ($P = 0.008$) (Figure 4G). However, the percentage of cells expressing CD70 was not significantly different between CD4+ lupus T cells transfected with *gadd45A* siRNA and those transfected with control siRNA (Figure 4F).

In addition, reductions in T cell proliferation and IgG stimulation were found in SLE CD4+ T cells transfected with *gadd45A* siRNA as compared with that in control siRNA–transfected SLE CD4+ T cells ($P = 0.040$ and $P = 0.032$, respectively) (Figures 4H and I). Moreover, global DNA hypermethylation was observed in *gadd45A* siRNA–transfected SLE CD4+ T cells, but there was no significant difference in the global DNA methylation levels between CD4+ T cells transfected with *gadd45A* siRNA and those transfected with control siRNA (Figure 4J).

To explore whether the down-regulation of *gadd45A* causes gene-specific methylation, we also analyzed the methylation status of the CD11a promoter (−1,263 to −1,111 bp), using bisulfite sequencing. The average level of methylation of the CpG pairs in this region in SLE CD4+ T cells transfected with *gadd45A* siRNA was significantly increased (mean ± SD 0.6296 ± 0.1400 versus 0.4471 ± 0.1469 in controls; $P = 0.035$), and 4 of 7 CpG motifs (−1,263, −1,223, −1,159, and −1,121 bp) were hypermethylated in the cells transfected with *gadd45A* siRNA (Figure 4K). In the CD70 promoter region (−581 to −288 bp) of SLE CD4+ T cells transfected with *gadd45A* siRNA, 4 CpG motifs (−513, −470, −441, and −428 bp) were hypermethylated, and the average methylation level of the 4 CpG motifs was significantly reduced as compared with that in the control siRNA group (mean ± SD 0.3534 ± 0.0330 versus 0.4554 ± 0.0736; $P = 0.029$) (Figure 4L).

DISCUSSION

Evidence linking T cell DNA demethylation to idiopathic and drug-induced lupus has been growing over the past 2 decades. Global DNA hypomethylation and gene-specific DNA demethylation are common characteristics of T cells in patients with active lupus, and the degree of demethylation is proportional to the extent of disease activity (19). However, the molecular basis of the DNA demethylation in lupus is unclear. GADD45α is a nuclear protein involved in the maintenance of genomic stability, DNA repair, and suppression of cell growth (20). Barreto et al found a key role for GADD45α in the process of DNA demethylation (11). Although another study, by Jin et al, was unable to confirm this observation with the use of methylated reporter plasmids and analysis of several candidate genes (21), Rai et al found that GADD45α participates in active DNA demethylation through a mechanism involving a 5-methylcytosine deaminase and a G:T

mismatch–specific thymine glycosylase in single-cell zebra fish embryos (22). Similarly, in a study by Schmitz et al, demethylation of the ribosomal DNA promoter in mammalian cells was initiated by TAF12-mediated recruitment of GADD45α, which then recruited the nucleotide excision-repair machinery (23). It thus appears that GADD45α does participate in active DNA demethylation in mammalian cells.

The current study demonstrates several novel observations indicating that *gadd45A* may also participate in lupus T cell DNA demethylation. First, CD4+ T cells from patients with SLE abnormally overexpressed GADD45α at both the gene and protein levels, and *gadd45A* mRNA expression correlated with disease activity in lupus patients. The levels of *gadd45A* mRNA were proportional to the mRNA level of the methylation-sensitive genes CD11a and CD70. Second, UV irradiation–induced expression of *gadd45A* caused overexpression of the methylation-sensitive genes CD11a and CD70, and caused a reduction in DNA methylation in normal CD4+ T cells. In SLE CD4+ T cells, *gadd45A* mRNA was also up-regulated by UV irradiation. Third, T cell autoreactivity was induced through DNA demethylation caused by overexpression of *gadd45A*. Finally, down-regulation of *gadd45A* mRNA efficiently inhibited lupus CD4+ T cell autoreactivity by increasing DNA methylation. These results suggest that *gadd45A* may be involved in aberrant T cell gene demethylation in the pathogenesis of SLE.

These studies also demonstrated the occurrence of *gadd45A* overexpression and genome-wide demethylation in lupus CD4+ T cells, and showed that both *gadd45A* expression and global DNA methylation levels were directly proportional to the extent of lupus disease activity. Patients with SLE are sensitive to UV irradiation, which triggers disease flares (14,24). However, the mechanism by which UV light aggravates lupus is unknown.

Our observations indicating that UV irradiation induces *gadd45A* overexpression and increases overexpression of autoimmune-related genes, including CD11a and CD70, in CD4+ T cells suggest that UV light may trigger lupus flares by increasing *gadd45A* expression. Interestingly, UV irradiation also reduced DNA methylation levels in CD4+ T cells. In addition, we found that *gadd45A* mRNA expression in irradiated CD4+ T cells increased to levels similar to those in SLE CD4+ T cells, and that irradiated SLE CD4+ T cells showed a similar fold increase in *gadd45A* mRNA levels when exposed to the same dose of UV irradiation. This could be attributed to the fact that lupus T cells already overexpress *gadd45A*. These results suggest that UV light induces autoimmune-related gene overexpression through T cell DNA demethylation, which then triggers antibody overproduction and provokes a lupus flare. It is important to note that ~10% of UVB light penetrates to the dermis and reaches the cutaneous vasculature (25). UVA and UVB phototherapy have been reported to decrease levels of HLA–DR, the interleukin-2 receptor, and CD30 in cutaneous common leukocyte antigen (CLA)–positive circulating T cell subsets in patients with atopic dermatitis (26), and to reduce the expression of CLA and very late activation antigen 4a on circulating T cells in psoriasis (27).

We were unable to demonstrate aberrant *gadd45A* expression in CD8+ lupus T cells. Our group has previously reported that gene-specific DNA demethylation occurs in CD4+, but not CD8+, lupus T cells (3,5,8,9), and that inhibiting DNA methylation in CD4+ T cells is sufficient to cause a lupus-like disease in animal models (6,7). These results suggest that the CD8+ T cell subset is not necessary for disease induction, and imply that there are differences in the regulation of some genes by DNA methylation between CD4+ and CD8+ T cells.

The present studies also revealed that *gadd45A* overexpression in normal CD4+ T cells, induced by transfection, led to CD11a and CD70 overexpression, genome-wide decreases in deoxymethylcytosine, and gene-specific demethylation accompanied by increased T cell proliferative activity and excessive B cell stimulation. Our previous studies demonstrated that CD11a overexpression contributes to autoreactive lupus T cell responses (8), while CD70 overexpression leads to overstimulation of IgG synthesis by lupus B cells (10), and both CD11a and CD70 are overexpressed and demethylated in lupus CD4+ T cells and in CD4+ T cells treated with DNA methylation inhibitors (8,10). Those previous results thus support the present findings, indicating that *gadd45A* may contribute to autoimmunity by promoting DNA demethylation.

Similarly, *gadd45A* knockdown in CD4+ SLE T cells decreased expression of CD11a and CD70, increased overall DNA methylation, increased methylation of the CD11a promoter (−1,263 to −1,111 bp) and CD70 promoter (CpG −513, −470, −441, and −428 bp), and decreased T cell proliferation and IgG stimulation. Thus, down-regulation of *gadd45A* may inhibit T cell autoreactivity and reduce IgG synthesis by increasing methylation of autoimmune-related genes. However, Salvador et al found that *gadd45A*$^{-/-}$ T cells were more susceptible to activation and proliferation than were

controls, when stimulated through the primary T cell receptor. Importantly, *gadd45A*$^{-/-}$ mice can develop autoimmunity that resembles human SLE (20). It is possible that congenital *gadd45A* deficiency modifies T cell maturation and differentiation, contributing to the effects observed, in contrast to the apparently acquired defects seen in lupus T cells. Alternatively, congenital *gadd45A* deficiency may cause autoimmunity through the effects on cells other than T lymphocytes.

Apart from *gadd45A*, other proteins, such as the methylcytosine binding domain proteins MBD-2 and MBD-4, may participate in the demethylation process. Expression of MBD-2 and MBD-4 mRNA was found to be significantly higher in CD4+ T cells from SLE patients compared with controls, and correlated negatively with overall DNA methylation (28). Rai et al proposed that DNA demethylation involves at least 3 molecules, namely *gadd45A*, activation-induced deaminase, and MBD-4–related GT glycosylase. The *gadd45A* gene may promote demethylation by interacting with deaminase and MBD-4 (22). Further studies will be needed to determine how *gadd45A* participates in DNA demethylation in lupus.

Other mechanisms may also contribute to T cell DNA demethylation in SLE. DNA methyltransferase levels are regulated, in part, by the ERK signaling pathway (29). Signaling via the ERK pathway is decreased in CD4+ T cells from patients with active lupus, causing decreased DNA methyltransferase 1 expression and overexpression of CD70 (30). Moreover, induced expression of a dominant-negative MEK in T cells causes DNA demethylation and lupus-like autoimmunity in animal models (30,31). Multiple mechanisms may thus contribute to DNA demethylation in lupus. The present study adds *gadd45A* overexpression as a possible mechanism.

Taken together, the results from the present study demonstrate that *gadd45A* overexpression can induce T cell autoreactivity and excessive B cell stimulation by inducing DNA hypomethylation, which then increases the expression of autoimmune-related genes. In contrast, *gadd45A* down-regulation can reverse T cell autoreactivity and overstimulation of IgG production by increasing methylation of autoimmune-related genes. Our results suggest that *gadd45A* may contribute to the development of SLE through DNA demethylation, and may be a target for more effective SLE therapy.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Lu had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Li, M. Zhao, Lu.

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