

Overexpression of CD70 and Overstimulation of IgG Synthesis by Lupus T Cells and T Cells Treated With DNA Methylation Inhibitors

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Objective. Generalized DNA hypomethylation contributes to altered T cell function and gene expression in systemic lupus erythematosus (SLE). Some of the overexpressed genes participate in the disease process, but the full repertoire of genes affected is unknown. Methylation-sensitive T cell genes were identified by treating T cells with the DNA methyltransferase inhibitor 5-azacytidine and comparing gene expression with oligonucleotide arrays. CD70, a costimulatory ligand for B cell CD27, was one gene that reproducibly increased. We then determined whether CD70 is overexpressed on T cells treated with other DNA methylation inhibitors and on SLE T cells, and determined its functional significance.

Methods. Oligonucleotide arrays, real-time reverse transcription–polymerase chain reaction, and flow cytometry were used to compare CD70 expression in T cells treated with 2 DNA methyltransferase inhibitors (5-azacytidine and procainamide) and 3 ERK pathway inhibitors known to decrease DNA methyltransferase expression (U0126, PD98059, and hydralazine). The consequences of CD70 overexpression were tested by coculture of autologous T and B cells with and without anti-CD70 and measuring IgG production by enzyme-linked immunosorbent assay. The results were compared with those of T cells from lupus patients.

Results. SLE T cells and T cells treated with DNA methylation inhibitors overexpressed CD70 and overstimulated B cell IgG production. The increase in IgG synthesis was abrogated by anti-CD70.

Conclusion. SLE T cells and T cells treated with DNA methyltransferase inhibitors and ERK pathway inhibitors overexpress CD70. This increased B cell costimulation and subsequent immunoglobulin overproduction may contribute to drug-induced and idiopathic lupus.

CD4+ T cell DNA hypomethylation may contribute to the development of drug-induced and idiopathic systemic lupus erythematosus (SLE). DNA methylation refers to the methylation of deoxycytosine (dC) bases in CG pairs, and it is one of the mechanisms by which gene expression is suppressed (1). CD4+ T cells treated in vitro with the DNA methylation inhibitors 5-azacytidine (5-azaC), procainamide, or hydralazine became autoreactive, killing autologous or syngeneic macrophages and promoting antibody production (2–5). Adoptive transfer of the autoreactive cells caused a lupus-like disease (4,5). The autoreactivity was found to be due in part to an overexpression of the adhesion molecule lymphocyte function–associated antigen 1 (LFA-1; CD11a/CD18) (6,7), and abnormal perforin expression contributed to the macrophage killing (8,9). The mechanisms by which the demethylated T cells promote antibody synthesis are not completely understood.

The genomic deoxymethylcytosine (d^mC) content was also shown to be decreased in T cells from patients with active SLE, similar to that in T cells treated with 5-azaC, procainamide, and hydralazine (10). LFA-1 was also shown to be overexpressed on a CD4+, perforin-expressing, cytotoxic, autoreactive lupus T cell subset with major histocompatibility complex specificity identi-

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cal to that of T cells treated with DNA methylation inhibitors (8,11). Furthermore, the same LFA-1 and perforin regulatory sequences were shown to be demethylated in CD4+ T cells from patients with active SLE as in T cells treated with 5-azaC or procainamide (8,12). Together, these studies suggest that T cell DNA hypomethylation may be fundamental to the pathogenesis of autoimmunity in the adoptive transfer model (4,5) and in humans with drug-induced and idiopathic lupus. While overexpression of LFA-1 and, perhaps, perforin is important to the disease process in the DNA hypomethylation model (7,13) and possibly in human lupus (8,11,14), the repertoire of genes that are affected is unknown, and other genes may also contribute to disease pathogenesis through mechanisms that promote antibody synthesis.

We identified additional methylation-sensitive genes by treating phytohemagglutinin (PHA)-stimulated human T lymphocytes with 5-azaC, then analyzing gene expression using oligonucleotide arrays. One gene that reproducibly increased >2-fold was CD70, which is also known as CD27 ligand (CD27L). CD70 is a member of the tumor necrosis factor (TNF) family that is expressed on activated CD4+ and CD8+ T cells and B cells (15). Adding cells transfected with CD70 was shown to increase pokeweed mitogen (PWM)-stimulated IgG synthesis in T cell-dependent B cell assays (16), indicating that CD70 has B cell-costimulatory functions resembling those of CD40L (16). This suggests that T cells overexpressing CD70 as a result of either DNA methylation inhibitor treatment or the DNA hypomethylation associated with lupus may also provide additional B cell-costimulatory signals.

In these studies, we sought to determine whether CD70 expression is increased on T cells treated with a panel of DNA methylation inhibitors and, if so, whether the hypomethylated T cells overexpressing CD70 could overstimulate the production of IgG by B cells. The DNA methylation inhibitors we used included the direct DNA methyltransferase inhibitors 5-azaC and procainamide (17), as well as PD98059, U0126, and hydralazine, which decrease DNA methyltransferase expression by inhibiting ERK pathway signaling (18). It is likely that ERK pathway inhibition is more relevant to idiopathic SLE in humans than is direct DNA methyltransferase inhibition, because T cells from patients with active lupus have impaired ERK pathway signaling, associated with decreased DNA methyltransferase levels and hypomethylated DNA (19). Similar studies were then performed on T cells from SLE patients. The results suggest that CD70 overexpression may contribute to B

cell IgG overproduction induced by experimentally hypomethylated T cells and by T cells from patients with idiopathic SLE.

MATERIALS AND METHODS

Subjects. SLE patients (n = 14) were recruited from the outpatient and inpatient services at the University of Michigan. Age-, race-, and sex-matched control subjects (n = 17) were recruited by advertising. The study protocol was approved by the University of Michigan Institutional Review Board. Patients with SLE met 4 criteria for the classification of lupus (20), and disease activity was assessed using the SLE Disease Activity Index (SLEDAI) (21). Active disease was defined as a SLEDAI score ≥ 5 . Relevant clinical information regarding the study subjects is shown in Table 1.

Cells and cell culture. Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation. T cells were then isolated by E-rosetting, as previously described (22). Purity, assessed by staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and flow cytometry, was typically 87–94%. Where indicated, the cells were cultured in RPMI 1640/10% fetal calf serum (FCS) supplemented with interleukin-2 (IL-2), as described previously (3), in round-bottomed 5-ml culture tubes (Falcon, Franklin Lakes, NJ). Cells were stimulated with 1 $\mu\text{g}/\text{ml}$ of PHA (Remel, Lenexa, KS) for 16 hours, then cultured in 24-well plates at a density of 1×10^6 for an additional 72 hours in the presence of 2-deoxy-5-azaC or 5-azaC (Aldrich, St. Louis, MO), procainamide (Aldrich), hydralazine (Aldrich), or the MEK inhibitors U0126 (Promega, Madison, WI) or PD98059 (Promega).

In other studies, PHA-stimulated PBMCs were cultured in RPMI 1640/10% FCS and treated with indomethacin, chloroquine, hydrocortisone, and 6-mercaptopurine (6-MP), all from Sigma (St. Louis, MO). TT48E, a cloned, CD4+, tetanus toxoid-reactive human T cell line, was cultured as previously described (2,11).

Oligonucleotide array analysis. Messenger RNA (mRNA) was isolated from untreated or 2-deoxy-5-azaC-treated T cells, and analyzed using Affymetrix U95A oligonucleotide arrays, as previously described (9).

Real-time reverse transcription-polymerase chain reaction (RT-PCR). CD70 transcripts were quantitated by real-time RT-PCR using a LightCycler (Roche, Indianapolis, IN) and previously published protocols (9). The following primers were used: forward, 5'-TGCTTTGGTCCCATTTGGTCG-3' and reverse, 5'-TCCTGCTGAGGTCCTGTGTGATTTC-3'. The transcripts were measured relative to β -actin as described previously (9).

Flow cytometric analysis. The following fluorochrome-conjugated monoclonal antibodies were obtained from BD PharMingen (San Diego, CA): FITC-conjugated anti-human CD70, CD2, or isotype-matched controls; phycoerythrin (PE)-conjugated anti-CD2, CD4, and CD8; and CyChrome-conjugated anti-HLA-DR, CD2, and isotype controls. Staining and multicolor flow cytometric analysis were performed as previously described (23), using saturating concentrations of antibody.

T cell and B cell costimulation assays. E-rosette-purified T cells were stimulated for 16 hours with PHA and

Table 1. Demographics, disease activity, and treatment in the SLE patients and control subjects*

Patient	Age/race/sex	SLEDAI score or diagnosis	Medications
SLE patients			
1	49/W/F	6	HCO
2	28/B/F	10	MMF 2 gm/day, HCO, Pred. 15 mg/day
3	38/B/F	10	MMF 2.5 gm/day, HCO, Pred. 12 mg/day
4	25/W/F	7	Pred. 5 mg/day
5	23/W/M	5	HCO, Pred. 5 mg/day
6	53/W/F	12	Pred. 60 mg/day
7	23/W/F	12	HCO, Pred. 20 mg/day
8	30/W/F	8	MMF 2.5 gm/day, HCO, Pred. 20 mg/day
9	31/W/F	6	MMF 2 gm/day, HCO, quinacrine, Pred. 10 mg/day
10	24/W/F	8	MMF 2 gm/day, HCO, Pred. 36 mg/day
11	41/W/F	10	Pred. 15 mg/day
12	54/W/F	2	HCO
13	38/W/F	0	Pred. 1.5 mg/day
14	43/W/F	0	Pred. 5 mg/day, MTX, MMF
Control subjects			
15	65/W/F	Dermatomyositis	Pred. 10 mg/day, MTX, MMF
16	39/W/F	CNS vasculitis	Pred. 15 mg/day, CYC
17	34/W/F	WG	Pred. 40 mg/day, CYC, etanercept

* SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; HCO = hydroxychloroquine; MMF = mycophenolate mofetil; Pred. = prednisone; MTX = methotrexate; CNS = central nervous system; CYC = cyclophosphamide; WG = Wegener's granulomatosis.

then treated with the indicated chemicals for an additional 72 hours as described above. Where indicated, T cell subsets were isolated by negative selection using magnetic beads (Miltenyi Biotec, Sunnyvale, CA). B cells ($1-4 \times 10^5$) enriched by negative selection using magnetic beads (Miltenyi Biotec) and assessed to be 70–85% pure using PE-conjugated anti-human CD21 (PharMingen), were added to washed, drug-treated autologous T cells, at T cell to B cell ratios of 4:1, 2:1, 1:1, 1:2, and 1:4. Where indicated, 0.625 $\mu\text{g/ml}$ of PWM (Aldrich) was added. The cells were cultured in RPMI 1640/10% FBS/penicillin/streptomycin for 8 days in 96-well round-bottomed plates (Costar, Corning NY) containing a 200- μl total volume (performed in duplicate). Cells were supplemented with 50 μl of medium on day 4. Where indicated, 1 $\mu\text{g/ml}$ of anti-CD70 monoclonal antibody (HNE51; Dako, Glostrup, Denmark) was added to the cultures.

TT48E cells were similarly stimulated with PHA (1 $\mu\text{g/ml}$) for 18 hours, treated with the indicated drugs for 3 days, then similarly cultured with autologous B cells for 8 days. Where indicated, the TT48E cells were pretreated with 1 $\mu\text{g/ml}$ of anti-CD70 for 30 minutes at 4°C, then washed and added to the B cells, according to protocols described by others (16).

CD4+ T cells were similarly isolated from lupus patients by first purifying the T cells by E-rosetting, then depleting the CD8+ T cells using magnetic beads (Miltenyi Biotec). These cells were then similarly cultured with purified autologous B cells. Where indicated, the T cells were pretreated with anti-CD70.

IgG enzyme-linked immunosorbent assays (ELISAs). IgG was measured in the supernatants of the T cell-B cell cultures as previously described (3). Briefly, 96-well flat-bottomed polystyrene plates (Costar) were coated with 1 $\mu\text{g/ml}$ of goat anti-human IgG (Southern Biotech, Birmingham AL) and washed. Unreacted combining sites were sealed with 3%

bovine serum albumin (BSA) in phosphate buffered saline (PBS) by incubation at 4°C for 16 hours. Pooled supernatants from duplicate wells were diluted 1:5 in PBS/1% BSA, and 50 μl was added to the wells. Serial dilutions of purified human IgG (Sigma) were used for quantitation. Following incubation and washing, goat anti-human IgG conjugated with horseradish peroxidase (Southern Biotech) was added, and cells were incubated for 2 hours at room temperature. The wells were washed 3 times with PBS/0.1% Tween 20, and color was developed using Sigma Fast tablets. The plates were read at 405 nm using a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA). All determinations were performed in quadruplicate.

Statistical analysis. The difference between means was tested by Student's unpaired *t*-test. Power, regression analyses, and analysis of variance were performed using Systat 10 software (Richmond, CA).

RESULTS

Identification of methylation-sensitive T cell genes. In preliminary studies, we used oligonucleotide arrays to identify T cell genes affected by DNA methylation inhibition. Purified T cells were stimulated with PHA and treated with 2-deoxy-5-azaC as described in Materials and Methods. Three days later, gene expression was compared in treated and untreated cells using oligonucleotide arrays. Overall, 118 genes reproducibly increased ≥ 2 -fold, and 12 genes decreased ≥ 2 -fold. In 2 independent experiments, CD70 expression increased 2.6 ± 0.6 -fold (mean \pm SEM) in treated cells relative to untreated controls (Figure 1A). These results were

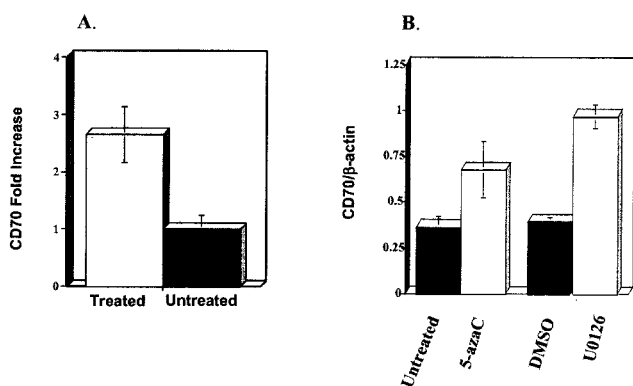


Figure 1. A, Effect of DNA methylation inhibition on CD70 expression. Phytohemagglutinin (PHA)-stimulated T cells were treated with 1 μ M 2-deoxy-5-azacytidine (5-azaC) for 3 days, and CD70 expression was analyzed using oligonucleotide arrays. Results are expressed relative to untreated cells. B, PHA-stimulated T cells were untreated (medium alone) or were treated with 1 μ M 5-azaC, DMSO, or 40 μ M U0126 dissolved in DMSO. Three days later, the cells were harvested, and CD70 and β -actin mRNA were measured by real-time reverse transcription-polymerase chain reaction. Results are presented as the ratio of CD70 to β -actin. All values are the mean \pm SEM of 2 experiments.

confirmed using real-time RT-PCR to compare CD70 mRNA levels in untreated cells and cells treated with 5-azaC and the ERK pathway inhibitor U0126. U0126 inhibits DNA methylation by decreasing levels of DNA methyltransferase 1 (Dnmt1) and Dnmt3a (18). Figure 1B shows that both drugs increased the expression of CD70 mRNA relative to that of β -actin.

Comparison of DNA methylation inhibitors on CD70 expression. The effects of DNA methylation inhibitors on T cell CD70 expression were further confirmed by treating T cells with a panel of DNA methylation inhibitors and measuring CD70 by flow cytometry. The panel of inhibitors we used included 5-azaC, an irreversible DNA methyltransferase inhibitor (24), procainamide, a competitive DNA methyltransferase inhibitor (17), and the ERK pathway inhibitors PD98059, U0126, and hydralazine.

Kinetic analyses performed by flow cytometry on days 1, 3, 5, and 7 after treatment with all 5 drugs demonstrated that the increase in CD70 expression was maximal at 3 days after treatment (data not shown). Figure 2A shows representative histograms of the CD70

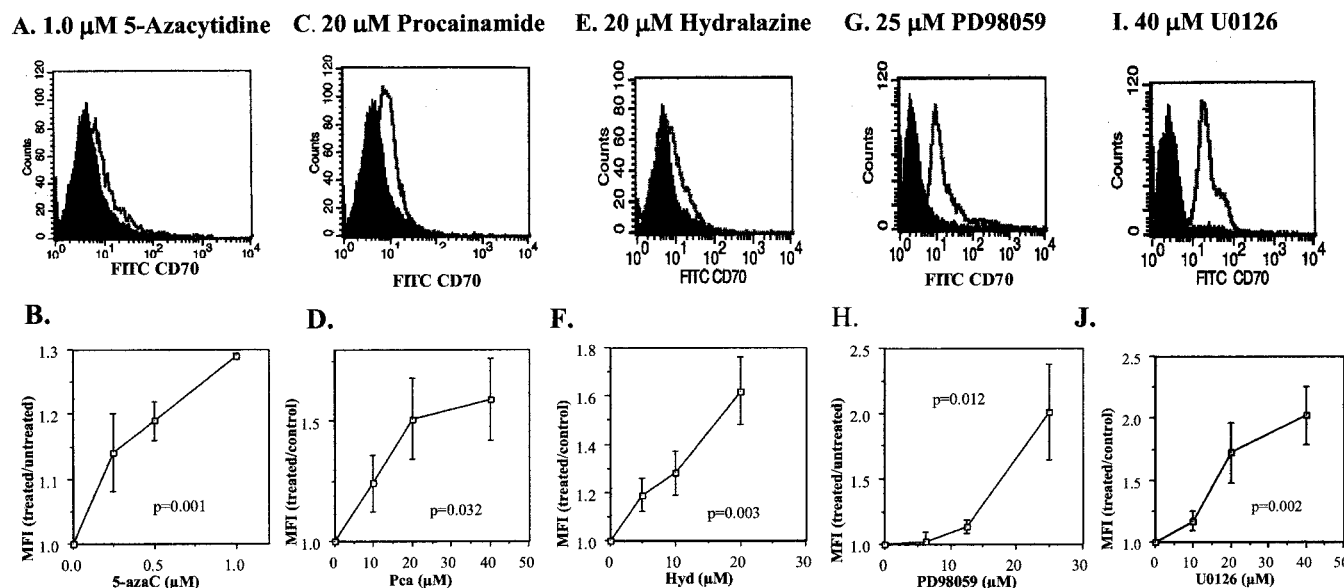


Figure 2. Increased CD70 expression induced by DNA methylation inhibitors. Phytohemagglutinin-stimulated T cells from normal subjects were treated with the indicated drugs, and 3 days later, treated and untreated cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD70 and analyzed by flow cytometry. Solid histograms show CD70 expression on untreated T cells; open histograms show expression on T cells treated with A, 5-azacytidine, C, procainamide, E, hydralazine, G, PD98059, and I, U0126. Dose-response curves show the ratio of the mean fluorescence intensity (MFI) of T cells treated with B, 5-azacytidine (5-azaC), D, procainamide (Pca), F, hydralazine (Hyd), H, PD98059, and J, U0126 to the MFI in the respective control groups. Values are the mean \pm SEM (n = 5 experiments in B, H, and J; n = 6 experiments in D and F). P values were determined by analysis of variance.

expression in untreated, PHA-stimulated T cells and in T cells treated with 1 μM 5-azaC for 3 days. A small increase was seen. Figure 2B shows the effect of a range of 5-azaC concentrations on CD70 expression, with 1 μM producing the greatest effect ($P = 0.001$ overall by analysis of variance; $n = 5$ experiments). The relatively small magnitude of the change probably reflects the fact that 5-azaC has significant toxicities (24). Figure 2C shows histograms of CD70 expression on untreated T cells and T cells treated with 20 μM procainamide, and Figure 2D shows an increase in the ratio of the mean fluorescence intensity (MFI) of CD70 expression in treated cells to that in untreated cells ($P = 0.032$; $n = 6$ experiments).

Similarly, Figure 2E shows the effect of 20 μM hydralazine on CD70 expression, and Figure 2F shows the dose-response curve of 6 experiments. A significant increase was seen ($P = 0.003$). Figure 2G shows the effect of 25 μM PD98059 on CD70 expression, and 2H shows the dose-response curve, demonstrating an increase in CD70 MFI ($P = 0.012$; $n = 5$). Figure 2I shows the effect of 40 μM U0126 on CD70 expression, and Figure 2J shows the dose-response curve, demonstrating an increase ($P = 0.002$; $n = 5$). In this series of experiments, there was no significant difference in the maximum increase caused by the DNA methyltransferase inhibitor procainamide and the ERK pathway inhibitors PD98059 and U0126.

Similar studies were performed examining the effects of the DNA methylation inhibitors on CD70 expression in CD4⁺ and CD8⁺ T cell subsets. We found that 1 μM 5-azaC increased CD70 MFI on CD4⁺ T cells by 1.53 ± 0.45 -fold ($P = 0.025$; $n = 5$ experiments), 25 μM PD98059 increased the MFI by 1.63 ± 0.43 -fold ($P = 0.032$; $n = 3$), and 40 μM U0126 increased the MFI by 3.20 ± 0.44 -fold ($P = 0.039$; $n = 4$). In contrast to the CD4⁺ population, the increase in CD70 MFI was smaller on CD8⁺ T cells and did not reach statistical significance for any of the drugs tested. However, this smaller increase may account for the suggestion of 2 populations seen in T cells treated with U0126 (Figure 2I, where CD70 MFI increased 2.83 ± 0.95 -fold [$P = 0.085$]). This also most likely accounts for the greater increase in expression observed on the CD4⁺ population relative to the polyclonal cells, particularly for the cells treated with U0126.

It was possible that the drug treatments selected for overgrowth or survival a T cell subset that overexpressed CD70. To exclude this possibility, the cloned human tetanus toxoid-reactive T cell clone TT48E was treated with 1 μM 5-azaC and 40 μM U0126 for 3 days

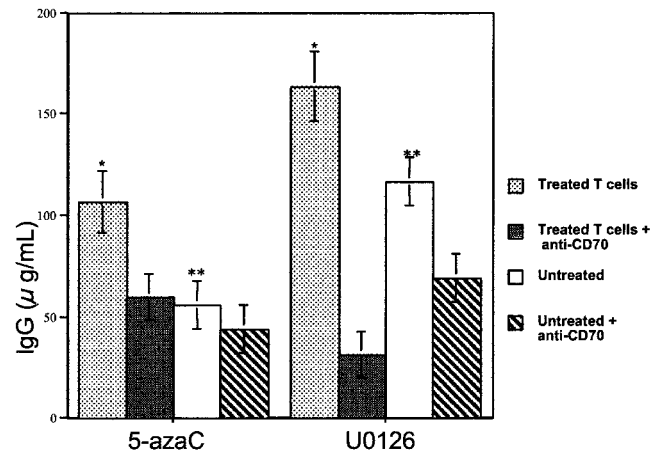


Figure 3. Increased B cell costimulation by polyclonal T cells treated with DNA methylation inhibitors, and reversal with anti-CD70. Phytohemagglutinin-stimulated T cells were treated with 1 μM 5-azacytidine (5-azaC) or 40 μM U0126 for 3 days and then cocultured with pokeweed mitogen and autologous B cells at a ratio of 1:4. Values are the mean \pm SEM of 3 independent experiments. * = $P < 0.05$ versus cultures with and without anti-CD70. ** = $P < 0.05$ versus treated T cells. Controls included B cells cultured alone (IgG 10 ± 5 $\mu\text{g/ml}$), B cells plus lipopolysaccharide (LPS; IgG 136 ± 9 $\mu\text{g/ml}$), and B cells plus LPS and anti-CD70 (IgG 125 ± 8 $\mu\text{g/ml}$).

as above. In 6 serial experiments, CD70 expression increased 1.69 ± 0.33 -fold ($P = 0.048$) on the 5-azaC-treated cells and 1.87 ± 0.37 -fold ($P = 0.004$) on the U0126-treated cells. This is evidence against subset selection by the drug treatment. The smaller increase observed in the U0126-treated cloned cells relative to the uncloned cells may reflect differences between the cloned line and primary polyclonal cells.

Effect of DNA methylation inhibitors on CD70-dependent B cell help. Since CD70 participates in T cell-dependent B cell stimulation (16), the effects of DNA methylation inhibitors on CD70-dependent B cell help were examined. Unfractionated T cells were stimulated with PHA, treated with 5-azaC or U0126 as above, and 3 days later, the treated cells were cultured with PWM and varying numbers of autologous B cells, with and without anti-CD70. Eight days later, total IgG in the supernatants was measured by ELISA. Optimal results were routinely observed at T cell to B cell ratios of 1:4 (see below). B cells cultured with 5-azaC-treated T cells and with U0126-treated T cells secreted greater amounts of IgG than did B cells cultured with the same numbers of untreated T cells ($P < 0.05$) (Figure 3). This finding is consistent with earlier reports that increasing the CD70 expression by transfection increases B cell IgG production in similar systems (16). Furthermore, the

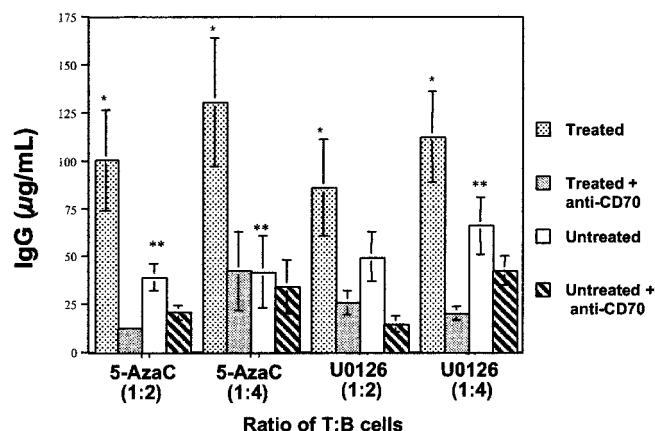


Figure 4. Increased B cell costimulation by cloned T cells treated with DNA methylation inhibitors, and reversal with anti-CD70. TT48E cells were treated with 1 μ M 5-azacytidine (5-azaC) or 40 μ M U0126 for 3 days and then cocultured with autologous B cells at the indicated ratios. T cells were pretreated with anti-CD70 where indicated. Values are the mean \pm SEM of 4 independent experiments. * = $P < 0.05$ versus cultures with and without pretreatment of T cells with anti-CD70. ** = $P < 0.05$ versus untreated T cells. Controls consisted of B cells cultured alone (IgG 7.5 ± 5 μ g/ml), B cells plus lipopolysaccharide (LPS; IgG 114 ± 8 μ g/ml), and B cells plus LPS and anti-CD70 (IgG 112 ± 8 μ g/ml).

addition of anti-CD70 decreased IgG production by the treated cells ($P < 0.05$). A suppressive effect of anti-CD70 on B cells was unlikely, because stimulating purified B cells with lipopolysaccharide (LPS) then adding the same amount of anti-CD70 yielded no significant inhibition of IgG synthesis (B cells plus LPS 136 ± 9 μ g/ml and B cells plus LPS and anti-CD70 125 ± 8 μ g/ml).

These results were confirmed using the cloned, CD4+, tetanus toxoid-reactive human T cell line TT48E. The T cells were again treated for 3 days with 5-azaC or U0126. To further exclude the possibility that anti-CD70 interacted with CD70 on B cells, the T cells were pretreated with anti-CD70 for 30 minutes at 4°C, washed, and then cultured with autologous B cells. Since our group has reported that T cells treated with DNA methylation inhibitors also induce T cell autoreactivity and that the autoreactive cells can directly stimulate B cell IgG secretion (3), these studies were performed without the addition of PWM. Figure 4 shows that the cloned T cells treated with either 5-azaC or U0126 induced B cells to produce greater amounts of IgG than did untreated T cells ($P < 0.05$), similar to our previous report. Furthermore, pretreatment of the T cells with anti-CD70 decreased IgG synthesis, indicating a direct effect on T cells.

Overexpression of CD70 on T cells from patients with active lupus. T cells from patients with active lupus have decreased levels of total genomic d^mC (10), and the same CD11a and perforin sequences demethylate in lupus T cells as in T cells treated with 5-azaC (8,12). We therefore sought to determine whether CD70 is also overexpressed on lupus T cells. Figure 5A compares representative histograms showing CD70 expression on T cells from a patient with active lupus (SLEDAI score 12) and a matched control subject. Figure 5B shows CD70 expression on PHA-stimulated normal T cells with and without U0126 treatment. A similar pattern of overexpression was seen in lupus T cells as in the drug-treated T cells.

Figure 5C compares the percentage of peripheral blood T lymphocytes expressing CD70 in 11 patients with active lupus and 11 healthy controls. Significantly more T cells from lupus patients expressed CD70 ($P = 0.047$). Figure 5D compares CD70 expression on CD4+ and CD8+ T cells from normal controls and lupus patients. Significantly more CD4+ T cells from the lupus patients expressed CD70 than did those from the controls ($P < 0.05$), and relatively few CD8+ T cells expressed CD70.

Since T cell DNA methylation decreases in proportion to lupus disease activity, we determined whether disease activity affects T cell CD70 expression. To minimize interexperimental variability, each lupus patient was paired with an age-, sex-, and race-matched control subject for this analysis. The ratio of the CD70 MFI on T cells from lupus patients and controls was determined and plotted against disease activity, as determined by the SLEDAI (Figure 5E). The increase in CD70 expression was directly related to disease activity ($P = 0.036$ by regression analysis). We similarly studied 3 patients with inactive lupus (SLEDAI score 2, 0, and 0, respectively). The CD70 MFI ratio in patients and controls was 0.94 ± 0.05 , indicating no overexpression in patients with inactive disease.

Since CD70 is preferentially expressed on activated T cells (15) and since T cells from patients with active lupus are frequently activated (25), we determined whether CD70 expression on T cells from patients with active lupus reflected T cell activation. Purified T cells from 4 patients with active lupus (patients 7, 8, 10, and 11 in Table 1) and 4 control subjects were stained with anti-HLA-DR and anti-CD70 and analyzed by flow cytometry (Figure 5F). CD70 was preferentially expressed on HLA-DR-negative lupus patients' T cells ($P < 0.05$). Using the data shown in Figure 5F, an unpaired *t*-test, and alpha level of 0.05, as few as 2

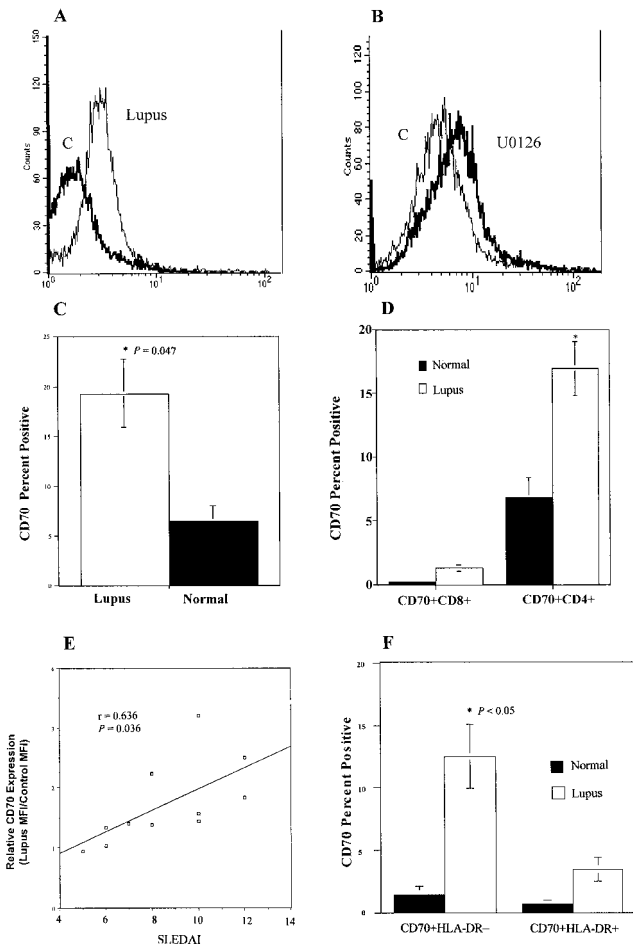


Figure 5. Overexpression of CD70 on T cells from patients with systemic lupus erythematosus (SLE). **A**, T cells were isolated from a patient with active SLE (SLE Disease Activity Index [SLEDAI] score 12) or a matched control subject (C), and CD70 expression was compared by flow cytometry. **B**, T cells from a healthy control subject were stimulated with phytohemagglutinin, treated with 40 μ M U0126 as described in Figure 2, and CD70 expression was compared with that in untreated (control [C]) cells as in **A**. **C**, Percentage of CD70+ T cells isolated from the peripheral blood of 11 patients with active SLE (SLEDAI score >5) and 11 healthy controls. Values are the mean \pm SEM of 11 experiments. **D**, Purified T cells from 4 patients with active SLE and 4 age- and sex-matched controls were stained for CD70, CD4, and CD8 expression, as indicated. Values are the mean \pm SEM of 4 experiments. * = $P < 0.05$ versus control and versus CD70+,CD8+ cells. **E**, Ratio of the mean fluorescence intensity (MFI) of CD70 expression by T cells from 11 patients with active SLE to the MFI of CD70 expression by T cells from age- and sex-matched controls are plotted against the SLEDAI score of the SLE patients. **F**, Purified T cells from the peripheral blood of 4 controls and 4 SLE patients were stained with anti-CD70 and anti-HLA-DR and then analyzed by flow cytometry. Values are the mean \pm SEM of the 4 experiments.

subjects per group would give 90% power to detect a difference in CD70 expression on HLA-DR-negative T cells. The CD70 overexpression on T cells lacking acti-

vation markers is similar to the overexpression of LFA-1 and perforin on T cells (8) and suggests that mechanisms other than T cell activation likely contribute to CD70 overexpression.

We considered the possibility that higher immunosuppression might contribute to this finding. However, the patients were taking different combinations of immunosuppressive agents, which does not support this possibility. Still, many of the patients were receiving prednisone. We therefore studied CD70 expression on CD4+ T cells from 3 patients receiving prednisone and various cytotoxic agents but with autoimmune diseases other than lupus (Table 1) and 3 matched healthy controls. No increase in CD70 was seen ($0.59 \pm 0.29\%$ CD4+,CD70+ cells in patients versus $0.65 \pm 0.51\%$ in controls). To further exclude this possibility, PBMCs were stimulated with PHA, then stimulated and unstimulated cells were cultured for 24 hours in the presence or absence of graded concentrations (1–100 μ M) of medications representative of the classes commonly used to treat lupus and not requiring metabolism for activation. These included indomethacin (for non-steroidal antiinflammatory drugs), chloroquine (for antimalarials), hydrocortisone (for steroids), and 6-MP (for azathioprine). CD70 and CD4 expression were then measured by flow cytometry. No increase in CD70 expression was seen on stimulated or unstimulated

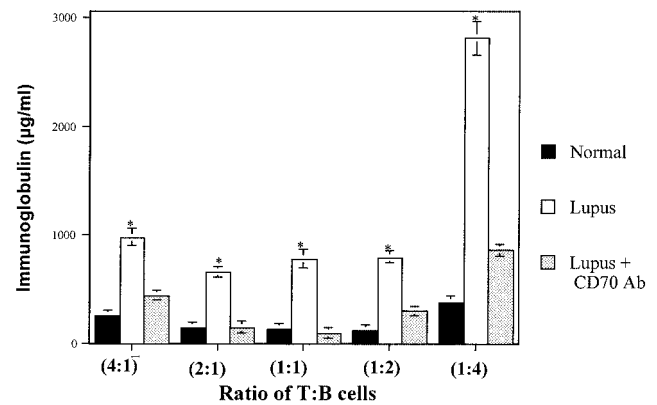


Figure 6. Anti-CD70 inhibition of IgG synthesis induced by lupus T cells. T cells from controls and patients with active lupus were cultured with autologous B cells at the indicated ratios for 8 days. Where indicated, the T cells were pretreated with anti-CD70 antibody (Ab). IgG was measured by enzyme-linked immunosorbent assay as described in Figure 3. Values are the mean \pm SEM of 3 independent experiments. * = $P < 0.05$ versus controls and versus anti-CD70 pretreatment. Controls included B cells cultured alone (IgG 37 ± 5 μ g/ml), B cells plus lipopolysaccharide (LPS; IgG $2,410 \pm 80$ μ g/ml), and B cells plus LPS and anti-CD70 (IgG $2,330 \pm 90$ μ g/ml).

CD4+ cells. Thus, other mechanisms, such as DNA hypomethylation, could play a role.

Contribution of CD70 to B cell activation by lupus T cells. To determine if CD70 overexpression on lupus T cells could contribute to B cell activation similar to T cells demethylated with 5-azaC or U0126, T cells from 3 patients with active lupus and 3 healthy controls were treated with anti-CD70 for 30 minutes at 4°C as above, then cultured for 8 days with purified autologous B cells at varying T cell to B cell ratios without PWM. Figure 6 shows that at all ratios tested, lupus T cells stimulated IgG synthesis significantly better ($P < 0.05$) than controls and that a T cell:B cell ratio of 1:4 resulted in optimal B cell activation. Using the results shown for a T cell:B cell ratio of 1:4, an unpaired *t*-test, and alpha level of 0.05, there was 94% power to detect a difference between the lupus patients and controls with 3 subjects per group. Furthermore, anti-CD70 significantly decreased ($P < 0.05$) IgG production to levels that were not significantly different from those in controls at all cell ratios tested, similar to the results in experimentally hypomethylated T cells (Figures 3 and 4).

DISCUSSION

The novel findings reported in this paper include the following. CD70 is overexpressed on polyclonal, as well as cloned, CD4+ T cells treated with a panel of DNA methylation inhibitors. CD70-overexpressing polyclonal and cloned CD4+ T cells overstimulate B cell IgG production, and the stimulation can be inhibited with anti-CD70. CD70 is overexpressed on CD4+ T cells from patients with SLE. Anti-CD70 inhibits the abnormal T cell-dependent IgG secretion that characterizes lupus B cells (26).

The initial oligonucleotide array studies as well as earlier work by our group and others have identified >100 T cell genes which reproducibly increase expression following treatment with 5-azaC. These include CD11a (11), interferon- γ (27), IL-6 (4), IL-4 (27), perforin (9), and now CD70. We selected CD70 for further study because of its potential for contributing to B cell activation. CD70 is expressed on activated T cells and B cells (15) and is expressed on 5–15% of the T cells in the peripheral blood of healthy individuals; these bear the activation marker HLA-DR (28). CD70 binds CD27 and provides costimulatory signals for B cell IgG production (16). B cells in the peripheral blood of patients with active lupus are abnormally activated and secrete polyclonal IgG, and this is a T cell-dependent process (26). We therefore hypothesized that CD70 might play a role in this abnormal B cell activation.

The array studies were confirmed by demonstrating that PHA-stimulated T cells treated with the irreversible DNA methyltransferase inhibitor 5-azaC or the MEK inhibitor U0126 also increased CD70 expression at both the mRNA and protein levels, consistent with an effect on DNA methylation. Increases at the protein level were further confirmed using procainamide and 2 additional ERK pathway inhibitors, PD98059 and hydralazine. Inhibiting ERK pathway signaling decreases the DNA methyltransferases Dnmt1 and Dnmt3a in stimulated T cells, which leads to DNA hypomethylation (18,19,29). Importantly, ERK pathway signaling and DNA methyltransferase expression are decreased in lupus T cells, suggesting that inhibiting DNA methylation with ERK pathway inhibitors may be relevant to idiopathic lupus (19). Similarly, since the lupus-inducing drugs procainamide and hydralazine both increased CD70 expression, the observations reported here may be relevant to drug-induced lupus syndromes as well. The effects of these drugs were greater on CD4+ T cells than on CD8+ T cells, although a small increase was detected in the CD8+ subset, which approached, but did not achieve, statistical significance, particularly with U0126. The reason for the differential effect is unknown. However, differential effects of 5-azaC on perforin expression have been observed in CD4+ and CD8+ T cells, reflecting differences in the methylation status of an upstream enhancer between the subsets (9). A similar mechanism could contribute to the differential effect in the CD70 response to 5-azaC in CD4+ and CD8+ cells.

While we have reported that the panel of drugs used in the present studies can inhibit T cell DNA methylation (2,18), the present studies did not identify the demethylated sequences that affect CD70 expression. In other studies, our group has used bisulfite sequencing and regional methylation of reporter constructs to characterize how DNA methylation inhibitors and lupus increase CD11a and perforin expression. We found that demethylation of sequences flanking the CD11a promoter increase CD11a expression in vitro and in lupus (12). Similarly, demethylation of a region linking an enhancer and minimal promoter, located 600–800 bp 5' of the perforin transcription start site, is responsible for the increase in perforin in vitro and in lupus, although methylation changes also occur elsewhere but are transcriptionally irrelevant (8,9). In more preliminary work, we have found that the 5 DNA-hypomethylating drugs used in these studies demethylate a region ~500 bp 5' of the CD70 transcription start site, and that the same region is hypomethylated in CD4+ T cells from patients with active lupus (Oelke K, et al: unpublished results). Whether these methylation

changes modify CD70 transcription, and whether the methylation is the same in CD4+ and CD8+ T cells, is currently unknown. Studies characterizing the CD70 promoter and the effects of methylation on its function are currently in progress.

Since other investigators have reported that cells transfected with CD70 will enhance PWM-induced IgG secretion, a T cell-dependent response (16), we wanted to determine whether T cells overexpressing CD70 may have similar effects on B cells. The initial studies compared untreated polyclonal T cells with the same cells treated with a DNA methyltransferase inhibitor and a MEK inhibitor. The drug-treated cells enhanced PWM-induced IgG secretion, and the effect was reversed with anti-CD70, which supports the hypothesis that T cell CD70 overexpression may contribute to the increase in IgG synthesis. The possibility that the effects might have been indirect due to effects of the drugs on a T cell subset lacking CD70, but requiring CD70+ cells, is unlikely because cloned T cells gave similar results. The possibility that anti-CD70 delivered a suppressive signal through B cell CD70 was tested by pretreating the T cell clones with anti-CD70 before adding them to the B cells, and we demonstrated suppression of the IgG response. Controls using LPS and purified B cells also indicated that anti-CD70 does not have a direct suppressive effect on B cells. Thus, these results, together with the earlier reports that cells transfected with CD70 overstimulate B cells, support the contention that CD70 on T cells contributes to the increased B cell IgG production.

Flow cytometry studies examining CD70 expression on T cells from patients with active lupus and age-, race-, and sex-matched healthy controls demonstrated that CD70 was overexpressed on CD4+ T cells from the lupus patients and that the degree of overexpression was directly proportional to disease activity. This is similar to the expression of CD11a and perforin, two other methylation-sensitive genes, and may reflect the DNA hypomethylation that characterizes T cells from patients with active disease. Again, the observation that T cells treated with DNA methylation inhibitors caused a lupus-like disease suggests that the DNA hypomethylation may induce the autoimmune disease, rather than reflect an effect secondary to the disease process. Since CD70 is expressed on stimulated T cells (15), it was possible that the increased expression was due to *in vivo* T cell activation. However, while CD70 is expressed on activated T cells in healthy individuals (30), CD4+ T cells overexpressing CD70 were largely HLA-DR-negative. There also appeared to be no correlation with medica-

tions. This suggests that the overexpression is due to other processes, such as DNA hypomethylation.

The role of CD70 in the abnormal B cell activation that characterizes lupus (26) was then examined. B cells in the peripheral blood of patients with active lupus are abnormally activated and secrete polyclonal IgG. While some of the antibodies secreted are the autoantibodies usually associated with SLE (26), other B cells secrete antibodies to antigens present on sheep erythrocytes and even keyhole limpet hemocyanin (31), suggesting that there is nonspecific polyclonal activation. Other investigators have reported that anti-IL-6 or anti-IL-6 receptor will inhibit this abnormal activation (32,33), but the mechanisms causing the IL-6 secretion have been unexplored. T cells from patients with active lupus stimulated IgG synthesis by autologous B cells in the absence of added antigen or mitogen, as reported by others (34). Pretreatment of the T cells with anti-CD70 abrogated this response. These studies thus suggest that T cell CD70 is required for the abnormal B cell stimulation in lupus. The present studies also suggest that CD70 overexpression on lupus T cells might contribute to B cell stimulation, together with other molecules, such as CD40L (35), and that inhibiting any of the costimulatory molecules is sufficient to decrease the antibody response to normal levels. Furthermore, IL-10, which is elevated in the serum of patients with SLE (36), is synergistic with the effects of CD27-CD70 interactions, which may lead to further increases in immunoglobulin synthesis (37).

Finally, the studies described above extend previous studies by our group examining mechanisms by which experimentally hypomethylated CD4+ T cells cause autoimmunity in animal models and determining whether the same mechanisms could contribute to idiopathic human lupus. In earlier work, we demonstrated that LFA-1 overexpression contributes to the autoimmune disease induced by T cells that had been experimentally demethylated with 5-azaC, procainamide, hydralazine, and U0126 (5,7,18) and that T cells from patients with active lupus overexpress LFA-1 (11,12). Comparing the magnitude of the effect of these drugs on CD70 and LFA-1 is difficult, because LFA-1 is a 2-chain molecule and 5-azaC only affects CD11a (11), so the increase is limited by the amount of CD18 available. More recently, we reported that perforin expression is also abnormally increased in CD4+ T cells treated with DNA methylation inhibitors (9) and in CD4+ T cells from patients with active lupus, and it may contribute to disease pathogenesis by participating in autologous macrophage killing (8,9,14). The magnitude

of the effect of 5-azaC on CD70 and perforin expression in CD4+ T cells is comparable (9). We report here that CD70 is also overexpressed in both experimentally hypomethylated CD4+ T cells and in CD4+ lupus T cells and may contribute to disease pathogenesis by augmenting B cell stimulation. Thus, DNA hypomethylation may contribute to lupus pathogenesis through effects on multiple genes that participate in multiple disease-augmenting mechanisms.

These observations also raise the possibility that administration of procainamide or hydralazine to patients with active lupus might worsen their disease. However, since these drugs only affect DNA methylation in the S phase (38) and since T cells from patients with active lupus are typically anergic (39), this question is difficult to address in vitro. Nonetheless, these results raise the possibility that the strategy of identifying methylation-sensitive T cell genes in experimentally hypomethylated T cells may predict genes that are abnormally expressed in lupus T cells and contribute to the disease process.

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