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Research Article

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Increased Expression of CD40 Ligand on Systemic Lupus Erythematosus Lymphocytes

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Abstract

The specificity of T cell help for B cell activation and differentiation is maintained by the brief expression on the T cell surface, following T cell receptor-mediated triggering, of CD40 ligand (CD40L). Interaction of T helper (T_h) cell CD40L with B cell CD40 induces B cell activation, cell surface expression of activation antigens, proliferation, and initiation of immunoglobulin isotype switch. We predicted that in patients with systemic lupus erythematosus (SLE), in whom T_h cell-dependent production of autoantibodies results in immune complex-mediated tissue damage, CD40L expression might be augmented, prolonged, or abnormally regulated. Baseline expression of CD40L was increased in some SLE patients studied, when compared with control subjects. While T_h cells from normal subjects (n = 14) and rheumatic disease control patients (n = 9) showed maximal expression of CD40L, after in vitro activation with phorbol myristate acetate (PMA) and ionomycin, at 6 h of culture with diminished levels observed at 24 and 48 h, T_h cells from SLE patients (n = 19) maintained high level cell surface expression of CD40L through 24 and 48 h of culture. The prolonged expression of CD40L was functionally significant, as 24 h–activated SLE T cells, when cocultured with target B cells, induced greater B cell surface CD80 (B7-1) expression than did 24 h–activated normal T cells. These results document impaired regulation of CD40L expression in SLE T cells and identify an important potential target for therapy in this systemic autoimmune disease. (*J. Clin. Invest.* 1996. 98:826–837.) Key words: CD40 ligand • T lymphocytes • lupus erythematosus • systemic • lymphocyte activation • autoimmunity

Introduction

In situ formation or deposition of circulating immune complexes mediates the tissue and organ damage that results in disease in patients with systemic lupus erythematosus (SLE).[1] Recent investigations have supported the important roles of defined autoantigens, as well as autoantigen-specific T helper (T_h) cells, in the induction of the autoantibodies that comprise these pathogenic immune complexes (1–10). As in specific immune responses to most antigens, autoantibody production is likely to depend on interaction of self antigen with B cell surface immunoglobulin receptors and the receipt of B cell surface signals mediated by direct interaction with the T_h cell surface, as well as T_h cell-derived cytokines (11, 12).

The binding of the CD40 ligand (CD40L; TBAM; gp39) molecule on the T_h cell surface to B cell surface CD40 is the molecular event that mediates direct T cell help for B cell activation (13–21). Studies of B cell CD40 ligation, along with delineation of the immunologic consequences of the hyperIgM syndrome, attributable to a variety of mutations in the X chromosome-encoded gene for CD40L (22–28), have defined the important role of CD40L–CD40 binding in B cell function. The induction of B cell surface expression of CD23, CD80 (B7-1), and Apo-1 (Fas) (16, 29–32); B cell proliferation (30, 33); germinal center formation (34, 35); and the initiation of Ig class switch from IgM to more mature B cell isotypes (16–21, 34–36) are among the effects of CD40 ligation. Given the important consequences of CD40 signaling for regulation of humoral immunity, rigorous regulation of T cell CD40L expression is necessary to maintain the antigen specificity of the immune response. Consistent with this suggestion, T cell receptor (TCR)–mediated T cell activation results in rapid, but brief, expression of cell surface CD40L on CD4+ T cells (37). Studies using phorbol myristate acetate (PMA) and ionomycin, to mimic the biochemical consequences of TCR signaling, have shown maximal expression of CD40L at 6 hours, with diminution of expression by 24 h of culture (37–39).

We have investigated the regulation of CD40L expression in patients with SLE, a systemic autoimmune disease characterized by B cell activation, hypergammaglobulinemia, and unregulated production of IgG autoantibodies. We predicted that increased, prolonged, or abnormally regulated expression of CD40L after T cell activation might contribute to the humoral immune dysfunction that is the basis for the pathophysiology of SLE.

Methods

Study subjects. Patients with SLE (n = 19), meeting at least four of the American College of Rheumatology revised criteria for the diagnosis of SLE (40), were followed at The Hospital for Special Surgery Rheumatic Disease Clinic. Peripheral blood samples were obtained with permission of the patients. Disease control subjects (n = 9) included five patients with seropositive rheumatoid arthritis (RA), one patient with progressive systemic sclerosis, two patients with Reiter's syndrome, and one patient with autoimmune thyroid disease. Normal controls (n = 14) included healthy approximately age-matched volunteers. Table I shows the age, sex, race, and medical therapy being administered to the subjects in each study group. In addition, relative disease activity for SLE and disease control groups is noted. For SLE patients, activity rating of 3 represents nephritis, serositis, or central

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1. *Abbreviations used in this paper:* CD40L, CD40 ligand; CLL, chronic lymphocytic leukemia; MCF, mean channel fluorescence; PDB, phorbol dibutyrate; TCR, T cell receptor; T_h, T helper.

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Table I. Summary of Study Subjects

Subjects	Diagnosis	Age	Race	Sex	Disease activity	Medications	Change in percent CD40L+ from 6 to 24 h
A. SLE Patients							
1		30	C	F	3	MTX, Pred > 10	⇑12
2		46	H	F	2	Pred ≤ 10	⇑12
3		60	C	F	3	Pred ≤ 10	⇑11
4		39	AA	F	3	Hycl, Pred ≤ 10	⇑10
5		29	AA	F	2	Hycl, Pred > 10	⇑10
6		33	C	M	2	Hycl, Pred ≤ 10	⇑7
7		34	AA	F	2	NA	⇑3
8		50	C	M	2	Aza, Pred > 10	⇑2
9		47	AA	F	2	Pred > 10	⇑2
10		35	AA	F	3	MTX, Pred ≤ 10	0
11		51	AA	F	3	Pred ≤ 10	0
12		41	C	F	3	Cyt, Pred >10	0
13		40	AA	F	1	NA	⇓2
14		41	C	F	3	Pred ≤ 10	⇓2
15		31	AA	F	3	Hycl, Pred > 10	⇓2
16		40	H	F	2	NA	⇓5
17		42	H	F	3	Aza, Pred ≤ 10	⇓7
18		29	H	F	1	none	⇓10
19		49	C	F	3	Aza, Hycl, Pred ≤ 10	⇓24
B. Disease Controls							
1	RA	70	C	F	NA	NA	⇑14
2	RA	45	AA	F	3	Pred ≤ 10	0
3	Autoimm thyroiditis	33	C	F	1	none	⇓10
4	PSS	38	H	F	3	ASA	⇓10
5	RA	35	H	F	2	MTX	⇓15
6	Reiter's	50	C	F	2	NSAID	⇓22
7	RA	48	AA	F	NA	Pred ≤ 10	⇓25
8	RA	55	H	F	NA	Pred ≤ 10	⇓25
9	Reiter's	50	C	F	1	NSAID	⇓35
C. Healthy Controls							
1		22	C	M	0	none	⇑9
2		55	H	F	0	none	⇑3
3		23	AA	F	0	none	⇓8
4		47	C	F	0	none	⇓8
5		33	C	F	0	none	⇓9
6		58	C	F	0	none	⇓11
7		35	H	M	0	none	⇓19
8		22	C	M	0	none	⇓25
9		43	C	F	0	none	⇓25
10		20	C	M	0	none	⇓26
11		38	C	F	0	none	⇓27
12		28	A	M	0	none	⇓27
13		55	AA	F	0	none	⇓32
14		27	C	M	0	none	⇓34

Study subjects are characterized by age, sex and race (C, Caucasian; H, Hispanic; AA, African American; A, Asian). Relative disease activity is described as 0 (no disease), 1 (inactive disease), 2 or 3 (active disease, as defined in Methods). Immunosuppressive medications used at the time of lymphocyte assay are noted as MTX (methotrexate), Hycl (hydroxychloroquine), Aza (azathioprine), Cyt (cytoxan), Pred (prednisone, either ≤ 10 mg/day or > 10 mg/day). The change in percent of mononuclear cells positive for CD40L from 6 to 24 h after in vitro activation with PMA and ionomycin is noted (⇑ designates an increase in percent CD40L+; 0 designates no change; and ⇓ designates a decrease in percent CD40L+ over this time period). *NA*, not available.

nervous system disease; activity rating of 2 represents active skin disease, arthralgias or arthritis, or mild proteinuria; activity rating of 1 represents inactive SLE. For RA patients, activity rating of 3 signifies severe polyarticular synovitis with nodules or organ involvement; activity rating of 2 represents polyarticular arthritis. The PSS patient, with an activity rating of 3, had facial and upper extremity skin tightening, digital ulcerations, mild restrictive pulmonary disease, and serum anti-Scl70 autoantibody. One of the Reiter's syndrome patients (rating of 2) had lower extremity arthritis and iritis and the other (rating of 1) had mild arthralgias. Immunosuppressive medications administered at the time of assay are noted.

Cell cultures. Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Hypaque and incubated at 37°C, 5% CO_2 for 6, 24, or 48 h in culture medium containing RPMI 1640 (GIBCO BRL, Gaithersburg, MD), 10% fetal calf serum (Whittaker Bioproducts, Inc., Walkersville, MD), 2mM glutamine, and 50 U/ml penicillin and streptomycin (GIBCO BRL). 1×10^6 PBMC were cultured in 1 ml culture medium alone or with 5 ng/ml PMA or phorbol dibutyrate (PDB; Sigma Chemical Co., St. Louis, MO) and 500 ng/ml ionomycin (Sigma Chemical Co.).

Monoclonal antibodies (mAb). mAb used included OKT3 (anti-CD3, pan-T); OKT4 (anti-CD4, helper/inducer subset); and OKT8 (anti-CD8, suppressor/cytotoxic subset) (American Type Culture Collection, Rockville, MD). mAb anti-Tac, reactive with the p55 chain of the IL-2 receptor (CD25), was obtained from Dr. Thomas Waldmann (National Institutes of Health). mAb reactive with human CD40L included mAb 5c8 (13; kindly provided by Drs. Seth Lederman and Michael Yellin, Columbia University College of Physicians and Surgeons, NY) and anti-CD40L (PharMingen, San Diego, CA). mAb EBVCS (anti-CD23) was obtained from Drs. Stan Metzenberg and Bill Sugden (McCardle Institute for Cancer Research, Madison, Wisconsin), anti-B7-1 (CD80) was obtained from Becton Dickinson, Cockeysville, MD, and anti-CD19 (pan-B cell) was purchased from PharMingen.

Indirect immunofluorescence. 1×10^5 PBMC or B cells were incubated with buffer alone or with a saturating concentration of mAb at 4°C for 30 min. After three washes in buffer, cells were incubated with a saturating concentration of fluorescein-labeled $F(ab')_2$ fragments of goat anti–mouse IgG (Tago, Inc., Burlingame, CA) at 4°C for 30 min. After three washes in buffer, cell fluorescence was analyzed on a FACScan® cytofluorograph or an Ortho cytofluorograph, gating on the lymphocyte population. Percent of cells positive and mean channel fluorescence, above the level of fluorescence of cells stained with the fluorescein-labeled goat anti-mouse IgG alone, were recorded for each sample. Most FACS analyses were performed by a single investigator (MK) using standard cytofluorograph parameters. Peak and mean background fluorescence and setting of regions for positive fluorescence were comparable in different experimental settings. When possible (in nine experiments), SLE and control subjects were studied in parallel in a single assay.

Multiparameter analysis was performed using 2-fluorochromes to determine CD40L expression on lymphocyte populations. Propidium iodide binding cells, representing poorly viable cells, were excluded from the analysis.

Induction of cell surface activation antigen expression on target B cells. In some experiments, 1×10^6 normal or SLE PBMC were incubated for 6, 24, or 48 h with culture medium alone or with PDB and ionomycin, as described above. The cells were then washed three times with Hank's Balanced Salt Solution, resuspended in 1 ml fresh culture medium, and irradiated with 1000R from a Cesium source. 1×10^6 precultured cells were incubated with 0.5×10^6 Ramos B cells (a Burkitt's lymphoma-derived cell line; ATCC), human tonsillar B cells, or B cells from a patient with chronic lymphocytic leukemia (CLL) in a total volume of 2 ml culture medium at 37°C, 5% CO_2. After an additional 24 h of culture, the cells were harvested, prepared for indirect immunofluorescence analysis as above, and assessed for expression of CD23, CD80, and CD19.

Statistical analysis. The mean±standard deviation (SD) for %CD40L+ mononuclear cells and for MCF was determined for each subject group, at each time point, and for each culture condition. Statistically significant differences between SLE and control groups were defined using the Student's *t* test.

Results

Expression of CD40L on SLE PBMC. Previous experiments by others have documented the rapid kinetics of expression of CD4+ T cell surface CD40L after T cell activation through the TCR (37–39). In most studies, TCR signaling has been mim-

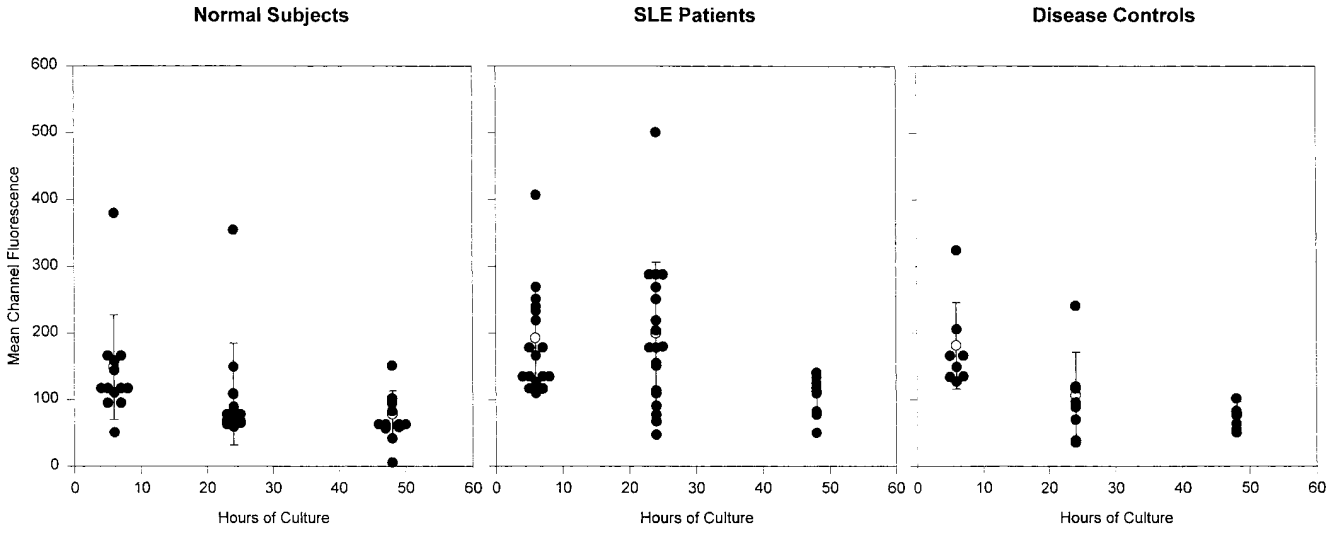

Figure 1. Kinetics of mean fluorescence intensity CD40L expression on in vitro activated PBMC. PBMC from normal subjects (*left panel*), SLE patients (*center panel*), or disease control subjects (*right panel*) were cultured for 6, 24, or 48 h with PMA and ionomycin and then assessed by indirect immunofluorescence for expression of CD40L. Results are expressed as MCF of the population of gated cells that were in the region to the right of the upper limit of the background fluorescence histogram. The mean (*open circles*) and standard deviation for each study population at each time point are shown.

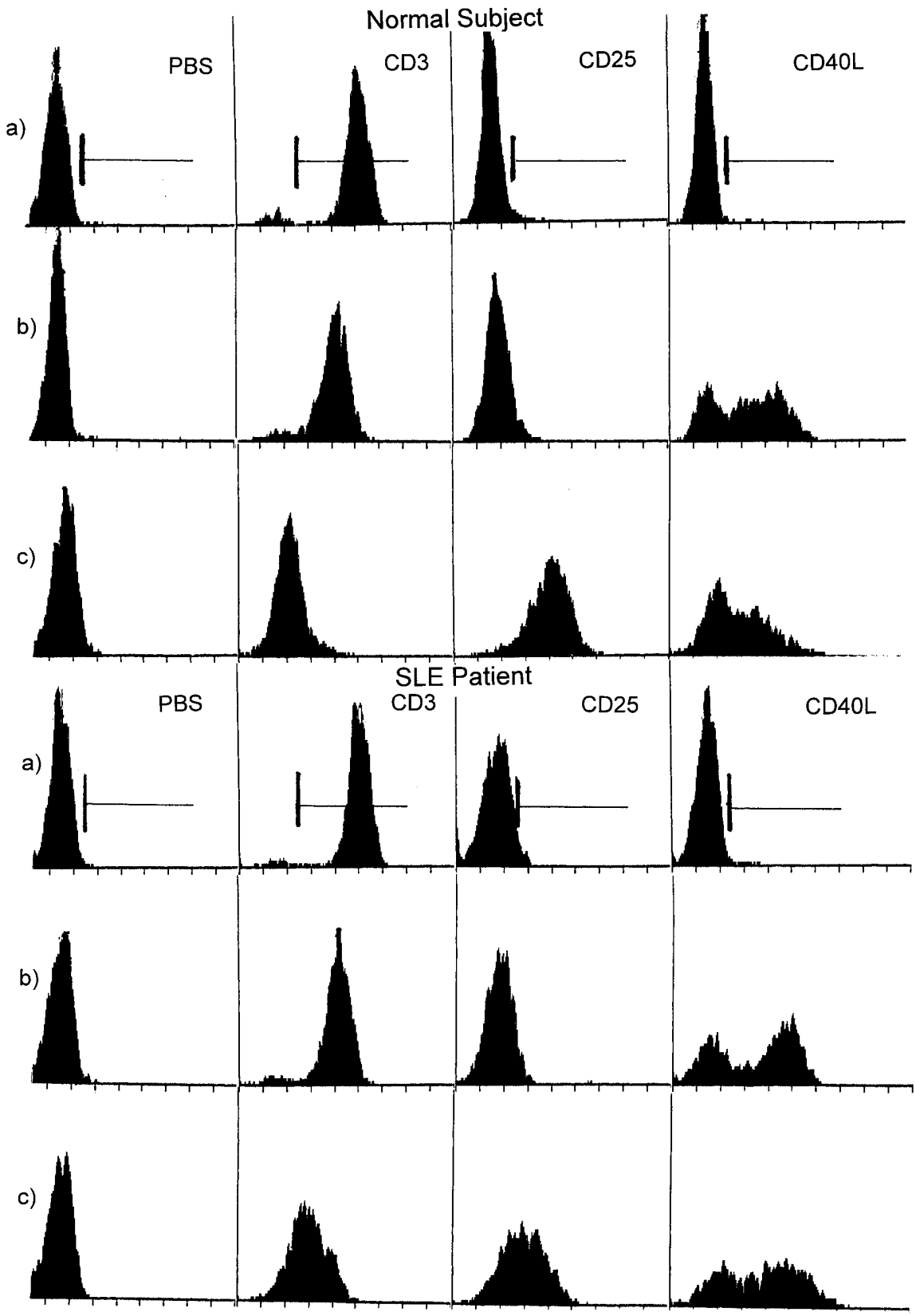

Figure 2. Kinetics of CD40L expression on PMA and ionomycin-activated PBMC from a normal subject (*top*) and a patient with SLE (*bottom*). PBMC from a healthy subject and a patient with SLE were cultured in parallel for 6 hours with medium alone (*a*) or with PMA and ionomycin for 6 h (*b*) or 24 h (*c*) and then analyzed by indirect immunofluorescence and FACS for background staining (PBS), or CD3, CD25, or CD40L expression. Cell number is shown on the Y axis and fluorescence intensity (log scale) on the *x*-axis of each histogram.

Table II. Kinetics of CD40 Ligand and CD25 Expression on PBMC Activated with PMA and Ionomycin

	Average mean channel fluorescence for study population							
	CD40 ligand				CD25			
Hours of activation	0	6	24	48	0	6	24	48
Normal subjects	49.6±16.7 (10)	141.0±75.6 (13)	100.9±73.9 (14)	76.5±28.9 (11)	50.4±11.5 (12)	48.8±14.4 (13)	162.6±69.6 (14)	410±275.8 (12)
SLE	65.8±28.7 (16)	185.4±75.1 (17)	192.6±104.9* (19)	105.3±29.5* (8)	55.5±21.5 (18)	56.1±34.5 (17)	145.6±84.7 (19)	564.9±247.1 (8)
Disease controls	51.1±24.6 (8)	175.9±60.8 (8)	101.0±60.6 (8)	72.0±17.5 (6)	63.0±32.1 (8)	75.8±39.3‡ (8)	167.9±101.2 (8)	546.5±191.7 (6)

1×10^6 PBMC were cultured with medium or with PMA and ionomycin for 6, 24, or 48 h, then assessed by indirect immunofluorescence for expression of CD40L and CD25. Mean channel fluorescence (MCF), above that of PBS-BSA/FITC goat anti-mouse Ig-stained cells, was recorded for the gated lymphocyte population. The mean±standard deviation of the MCF and the number of individuals studied ( ) is shown for each study population and time point. *MCF CD40L expression was statistically significantly higher for SLE patients after 24- and 48-h culture with PMA and ionomycin when compared with normal subjects ($P = 0.011$ and 0.038 for 24 and 48 h, respectively) and with disease controls ($P = 0.035$ and 0.042). ‡MCF CD25 expression was statistically significantly higher for disease control patients after 6 h of culture with PMA and ionomycin when compared with normal subjects ($P = 0.046$).

icked using either anti-CD3 mAb, reactive with the signal transducing component of the TCR complex, or PMA (or PDB) plus ionomycin, which activate protein kinase C and increase intracellular Ca^{2+}. These stimuli obviate the need for antigen-specific TCR triggering and bypass the earliest molecular events in TCR-mediated T_h cell activation.

To assess the level and kinetics of CD40L expression after T cell activation in patients with SLE, PBMC from SLE patients, disease control patients, and healthy subjects were cultured for 6, 24, or 48 h with culture medium alone or with PMA and ionomycin, and MCF CD40L and CD25 determined by indirect immunofluorescence and FACS analysis (Figs. 1 and 2 and Table II). While activated cell populations from healthy subjects and disease controls showed high intensity expression (MCF) of CD40L after 6 h of culture, with a gradual decrease to near baseline at 48 h of culture, activated lymphocytes from SLE patients continued to demonstrate high level CD40L at 24 and 48 hours ($P = 0.01$ and 0.038 at 24 and 48 h, respectively, when compared with normal subjects, and $P = 0.035$ and 0.042 at 24 and 48 h, when compared with disease controls) (Fig. 1 and Table II). Representative histograms showing CD3, CD40L, and CD25 expression on untreated and PMA and ionomycin-activated PBMC from a healthy subject and a SLE patient assessed in parallel after 6 and 24 h of culture are demonstrated in Fig. 2 and illustrate the persistent high level cell surface CD40L at 24 hours in the SLE, but not in the healthy, subject. Of particular interest, MCF above background of CD40L on untreated PBMC was increased in the SLE group, but not statistically different than the normal or disease controls (Table II). Multiparameter fluorescence analysis of paired SLE and control subjects (Fig. 3) showed CD40L on untreated CD4+ SLE T cells, as well as on some CD8+ T cells (not shown), and fluorescence intensity was further increased after in vitro activation, while the normal subject showed high level CD40L expression on CD4+ T cells only after in vitro activation. In contrast to the results with CD40L, the intensity and kinetics of CD25 (Tac) expression were comparable in normal control and SLE subject groups, with MCF gradually increasing over 48 h of culture (Table II).

Assessment of CD40L expression in terms of the percentage of the mononuclear cells positive, above background fluorescence, was also determined and gave comparable results (Figs. 4 and 5). The % of CD40L+ cells in the absence of in vitro activation was increased in some of the SLE patients compared to the normal subjects ($P = 0.06$) (Fig. 4). While these results did not achieve statistical significance, it was of interest that the three SLE patients with the highest baseline CD40L expression had active nephritis or end stage renal disease. CD40L expression after in vitro activation with PMA and ionomycin was significantly greater at 24 ($P = 0.038$) and 48 h ($P = 0.004$) in the SLE patients as compared with the normal subjects (Fig. 5). Normal and disease control groups had nearly overlapping patterns of CD40L expression over time when the % CD40L+ cells at 24 and 48 h of culture was compared with expression at the optimal (6 h) time point after initiation of activation, while the SLE group showed a slight increase in CD40L expression from the 6 to the 24 h time point Fig. 5 *B*). As CD40L is expressed predominantly on CD4+ T cells, it was possible that differences in the relative composition of the mononuclear cell populations between the SLE and control subject groups accounted for the prolonged kinetics of CD40L expression observed on activated SLE cells. However, the percent of CD4+ mononuclear cells in the SLE patients was not statistically different than that in the normal or disease control groups (data not shown). These data clearly demonstrate that while early after T cell activation by PMA and ionomycin, both SLE and normal T_h cells rapidly express high levels of CD40L, in normal subjects, CD40L is quickly downregulated and nearly gone from the cell surface by 48 h. In contrast, SLE T cells show prolonged high level expression of CD40L for more than 24 h. Moreover, lymphocytes from some SLE patients express some CD40L even in the absence of in vitro activation.

Induction of activation antigen expression on target B cells cultured with CD40L-positive T cells. To evaluate the functional potential of prolonged CD40L expression, the capacity of activated T cells to induce cell surface activation antigen expression on allogeneic target tonsil, Ramos, or CLL B cells was determined. PBMC were cultured with water soluble PDB and ionomycin for 6, 24, or 48 h, the cells were washed, irradiated, and then recultured with Ramos B cell line cells. CD40L and CD25 expression were determined on the activated T cells at each time point (Fig. 6, *left panel*), and CD23 and B7-1 expres-

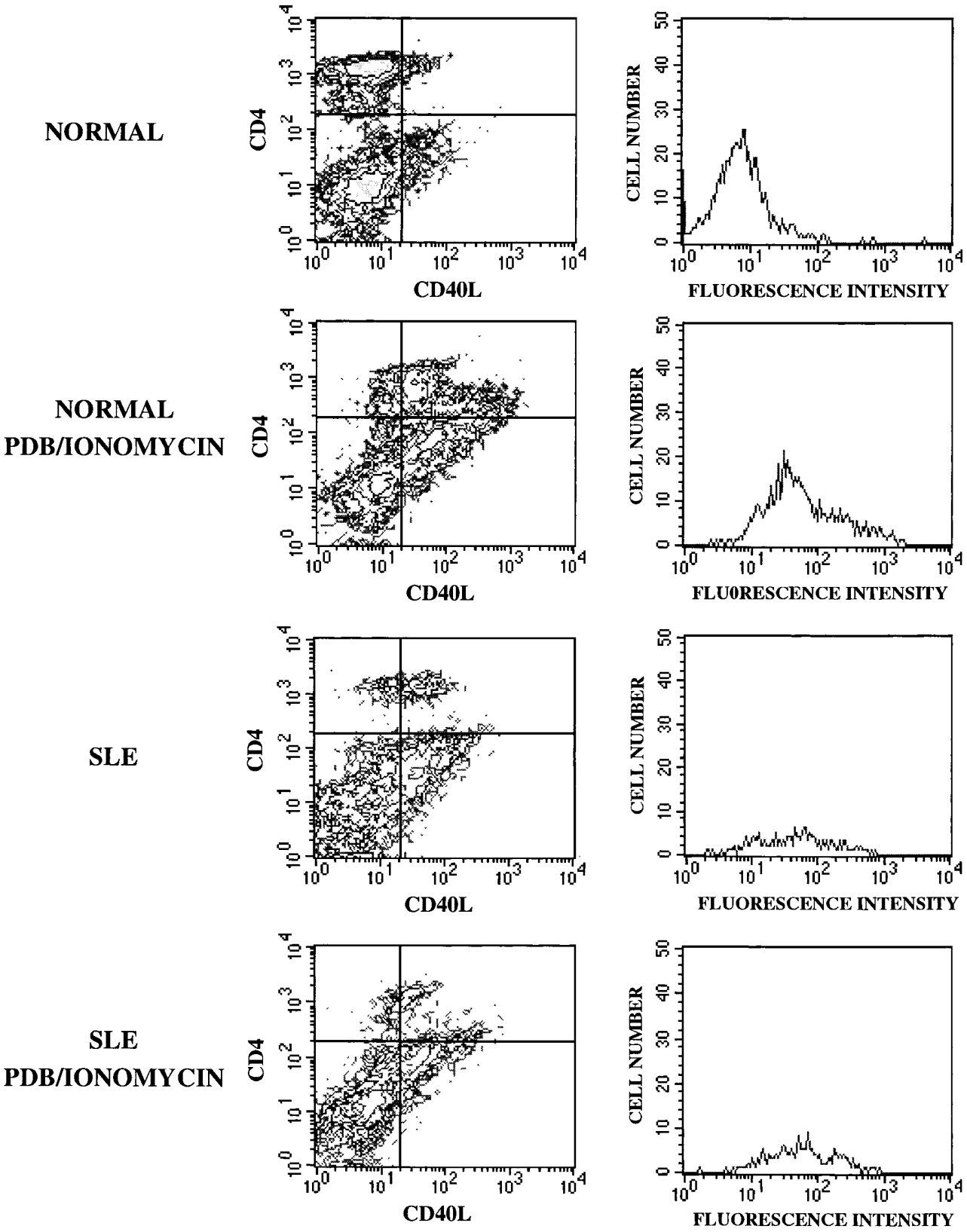

Figure 3. Expression of CD40L on untreated SLE CD4+ T cells. Normal or SLE PBMC were analyzed by multiparameter flow cytometry after 6 h of culture with medium alone or with PDB and ionomycin. Propidium iodide positive cells and cells with the scatter properties of monocytes were excluded from the analysis. Left panels show CD4 expression on the Y axis and CD40L expression on the *x*-axis. The horizontal line in the left panels defines the CD4 positive and negative cells, and the vertical line designates the CD40L positive and negative cells. Right panels show histograms indicating the fluorescence intensity of CD40L staining on the CD4+ T cells.

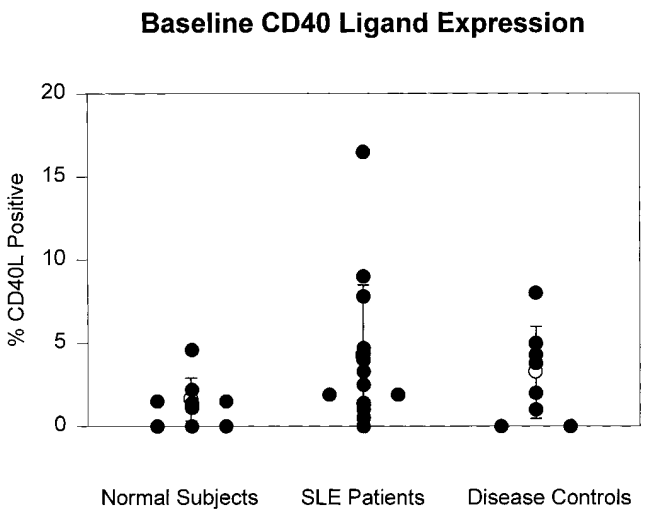

Figure 4. Baseline expression of CD40L on PBMC. PBMC from normal subjects, SLE patients, and disease control subjects were analyzed by indirect immunofluorescence for CD40L expression. Results are expressed as the percent of PBMC CD40L+ after subtraction of the percent of PBMC positive with goat anti–mouse IgG. The mean (*open circle*) and standard deviation for each study population are shown.

sion determined on the Ramos B cells cultured for an additional 24 h with the PBMC preparations (Fig. 6, *right panel*). Normal PBMC cultures activated with PDB and ionomycin for 6 h, the time point at which CD40L is optimally expressed, induced maximal levels of CD23 and B7-1 when cocultured with Ramos B cells, while the 24 and 48 h activated cultures induced less CD23 and B7-1 on the cocultured Ramos B cells (Fig. 6, *right panel*). Comparable data were obtained after culture of activated PBMC with tonsillar B cells (not shown). Thus, capacity of the activated PBMC populations to induce expression of CD23 and B7-1 on target B cells correlated with the kinetics of T_h cell expression of CD40L, rather than expression of CD25.

To further implicate CD40L-CD40 interactions in the induction of B cell activation antigens in the PDB and ionomycin-treated PBMC culture system, the capacity of a mAb specific for human CD40L to inhibit the induction of B cell CD23 was studied (Fig. 7). While anti-CD40L mAb did not alter expression of the pan-B cell marker CD19 on a CLL target B cell population cultured with 24 h PDB and ionomycin-activated PBMC, CD23 expression was inhibited nearly to baseline levels by inclusion of the anti-CD40L mAb in the activated PBMC/CLL B cell culture. These experiments suggest that the functional consequences of CD40L expression on activated T_h cells for B cell activation can be measured by the induction of B cell activation antigens on cocultured target B cells. They do not, however, rule out a contribution of T cell-derived cytokines, such as interleukin 4 or tumor necrosis factor α, to the level of B cell activation antigens expressed.

Augmented expression of CD80 (B7-1) on target B cells cultured with PDB and ionomycin-activated SLE PBMC. To assess whether the prolonged expression of CD40L on activated SLE T_h cells might result in augmented help for B cell activa-

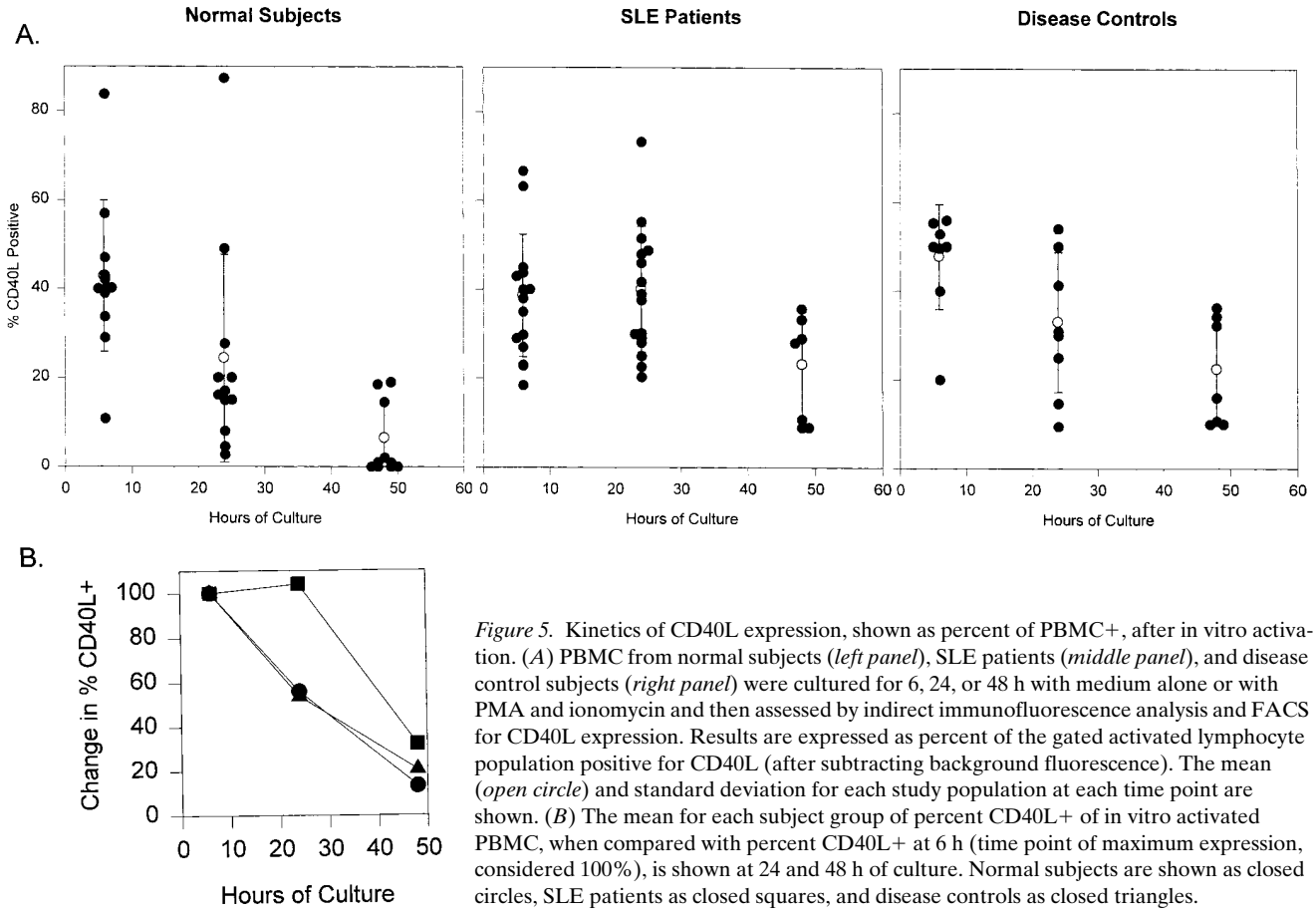

Figure 5. Kinetics of CD40L expression, shown as percent of PBMC+, after in vitro activation. (*A*) PBMC from normal subjects (*left panel*), SLE patients (*middle panel*), and disease control subjects (*right panel*) were cultured for 6, 24, or 48 h with medium alone or with PMA and ionomycin and then assessed by indirect immunofluorescence analysis and FACS for CD40L expression. Results are expressed as percent of the gated activated lymphocyte population positive for CD40L (after subtracting background fluorescence). The mean (*open circle*) and standard deviation for each study population at each time point are shown. (*B*) The mean for each subject group of percent CD40L+ of in vitro activated PBMC, when compared with percent CD40L+ at 6 h (time point of maximum expression, considered 100%), is shown at 24 and 48 h of culture. Normal subjects are shown as closed circles, SLE patients as closed squares, and disease controls as closed triangles.

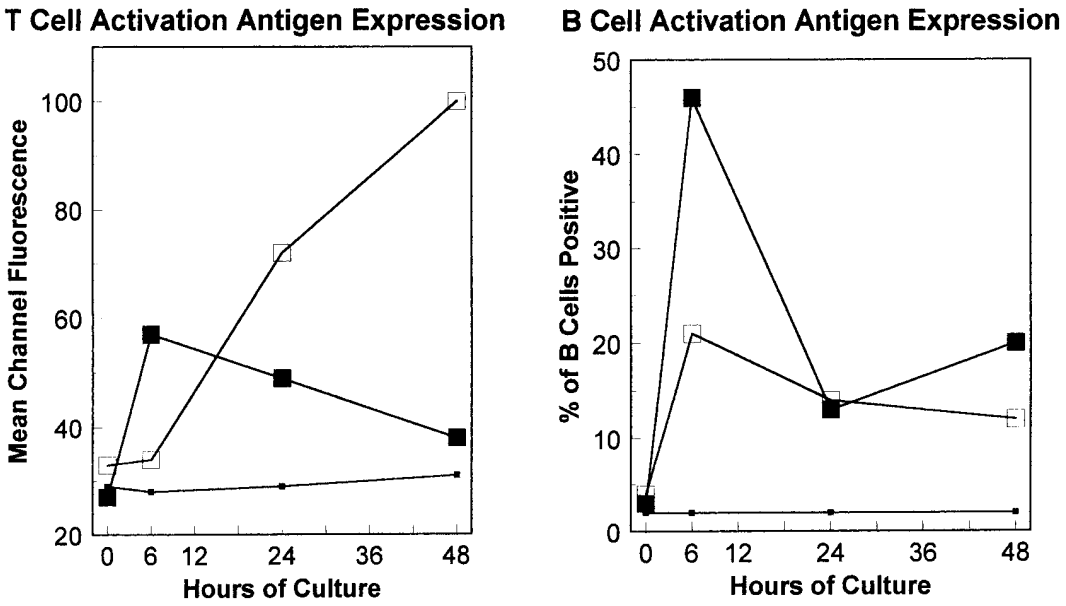

Figure 6. CD23 and B7-1 are maximally expressed on target B cells cultured with 6 hour PDB and ionomycin-activated PBMC. PBMC from a normal subject were cultured for 0, 6, 24 or 48 h with PDB and ionomycin. At each time point, an aliquot of the PBMC was stained for background fluorescence (*left panel*, *closed small squares*), CD40L (*left panel*, *closed large squares*), or CD25 (*left panel*, *open large squares*). The activated PBMC were washed, irradiated with 1000R from a Cesium source, and 1×10^6 cells added to 0.5×10^6 Ramos B cell line cells. After 24 additional hours of culture, the Ramos B cells were assessed for background fluorescence (*right panel*, *closed small squares*), CD23 (*right panel*, *closed large squares*), or B7-1 (*right panel*, *open large squares*).

tion, PBMC from healthy subjects and SLE patients were cultured for 24 h with medium or with PDB and ionomycin, the cells washed, irradiated, and then cocultured for an additional 24 h with Ramos B cell line cells or with, in one experiment, tonsil B cells (Figs. 8 and 9). The mean data derived from 5 experiments show that normal PBMC activated in vitro for 24 h increased the MCF of B cell B7-1 by 4 channels, when compared with untreated normal PBMC, while SLE PBMC, activated in vitro for 24 h, induced an increase of 15 channels B7-1 expression on target B cells when compared with untreated SLE PBMC. The MCF of B7-1 induced by the activated SLE PBMC was significantly greater than the low level B7-1 induced by activated normal PBMC ($P = 0.013$). Cytofluorograph histograms of Ramos B cells cultured with PBMC from a normal subject and a SLE patient analyzed in this system are shown in Fig. 9, with the activated SLE cells inducing high level B7-1 expression on the target Ramos B cells. These data suggest that the level of CD40L that persists on the SLE T_h cell surface after 24 h of in vitro activation, a time point at which normal T_h cells are rapidly modulating their CD40L, may be functionally significant and contribute to excessive B cell activation, as measured by B7-1 expression.

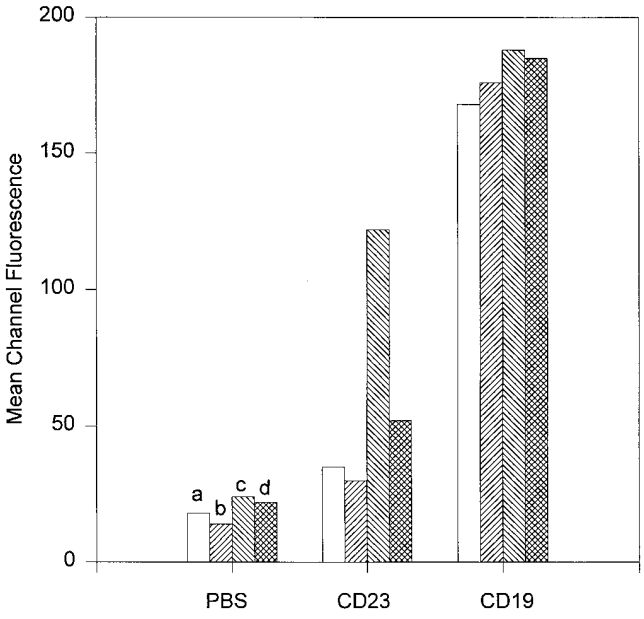

Figure 7. mAb reactive with CD40L inhibits the induction of B cell CD23 expression by PDB and ionomycin-activated PBMC. Normal PBMC were cultured for 24 h with medium or with PDB and ionomycin, the cells washed, irradiated, and then recultured with target B cells from a CLL patient. After an additional 48 h of culture, CLL B cells were assessed by indirect immunofluorescence for background fluorescence (PBS), CD23, or CD19, as indicated. For each staining assay, culture conditions included (*a*) B cells plus PBMC; (*b*) B cells plus PBMC, in the presence of 10 ug/ml mouse IgG anti–human CD40L mAb; (*c*) B cells plus PBMC activated with PDB and ionomycin; and (*d*) B cells plus PBMC activated with PDB and ionomycin, in the presence of anti-CD40L mAb.

Discussion

A consistently observed manifestation of immune dysfunction in SLE is the hyperactivity of the humoral immune system, both in vitro and in vivo. In vitro, SLE B cells are noted to be enlarged and of lower density than normal B cells; they express some B cell activation antigens in the absence of experimental stimulation; and they are hyperresponsive to T cell-derived cytokines (41, 42). In vivo, the SLE humoral immune system is characterized by hypergammaglobulinemia, immune complex formation, and complement system activation (43). While highly specific autoantigen-driven responses, particularly those directed at protein and nucleic acid components of intracytoplasmic and intranuclear particles, are characteristic of the antibodies produced in patients with SLE (1, 3, 4), recent evidence suggests that determinant spreading of the antibody response from an early narrow range of specificities to a later broad range of specificities may be operative (44–47).

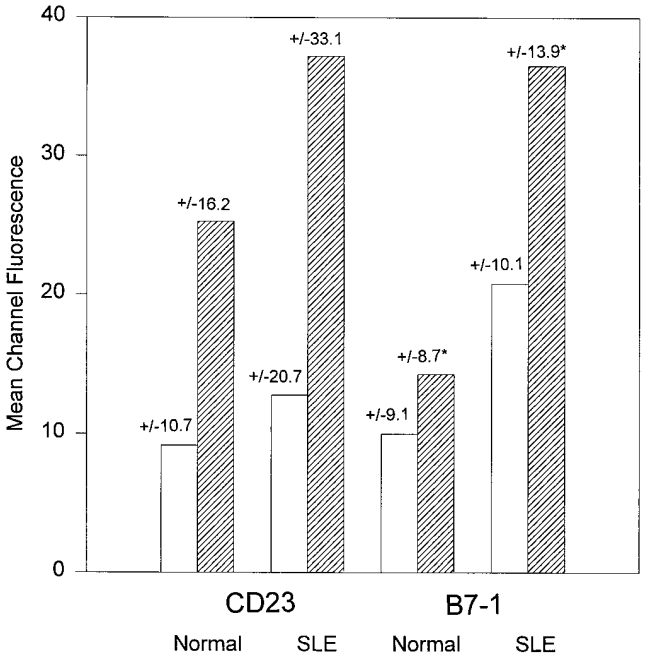

Figure 8. Induction of B7-1 expression on target B cells cultured with 24 h–activated SLE PBMC. Five normal and five SLE PBMC preparations were incubated for 24 h with medium (*open bars*) or with PDB and ionomycin (*hatched bars*), washed, irradiated, and then recultured with target B cells (Ramos B cell line cells in four experiments and tonsillar B cells in one experiment) for an additional 24 h. CD23 and B7-1 expression on the target B cells were assessed by indirect immunofluorescence and expressed as MCF, after subtraction of background fluorescence. Results shown are the mean MCF values (±standard deviation) for the B cells activated with normal control or SLE PBMC. A statistically significant difference (*) was observed between B7-1 expression induced by activated normal PBMC and activated SLE PBMC ($P = 0.01$).

To investigate T_h cell-dependent B cell activation in SLE, and to understand the mechanisms by which a presumably well-focused early autoreactive immune response might generate a more generalized humoral response of persistent high magnitude, we studied the regulation of CD40L expression on untreated and in vitro-activated SLE PBMC. In an effort to observe the phenotypic change and functional response of SLE T_h cells to a successful activating stimulus, we chose to stimulate the PBMC with PMA or PDB plus ionomycin, thus bypassing the requirement for successful TCR ligation. While this stimulus is unquestionable artificial, it allowed us to effectively compare normal and SLE T_h cell responses in the system best studied by others who have investigated the regulation of CD40L expression.

Our data demonstrate that after in vitro activation of PBMC with PMA and ionomycin, T cells from normal subjects, SLE patients, and patients with systemic inflammatory disease show comparable high level expression of CD40L at 6 h of culture and similar kinetics of CD25 expression at all time points studied. Importantly, SLE T cells, but not T cells from the healthy or disease control groups, showed persistent near maximal expression of CD40L at 24 h of culture, and continued expression of CD40L after 48 hours of culture. CD40L expression was significantly prolonged in SLE whether assessed in terms of MCF or as percent CD40L+ PBMC, and

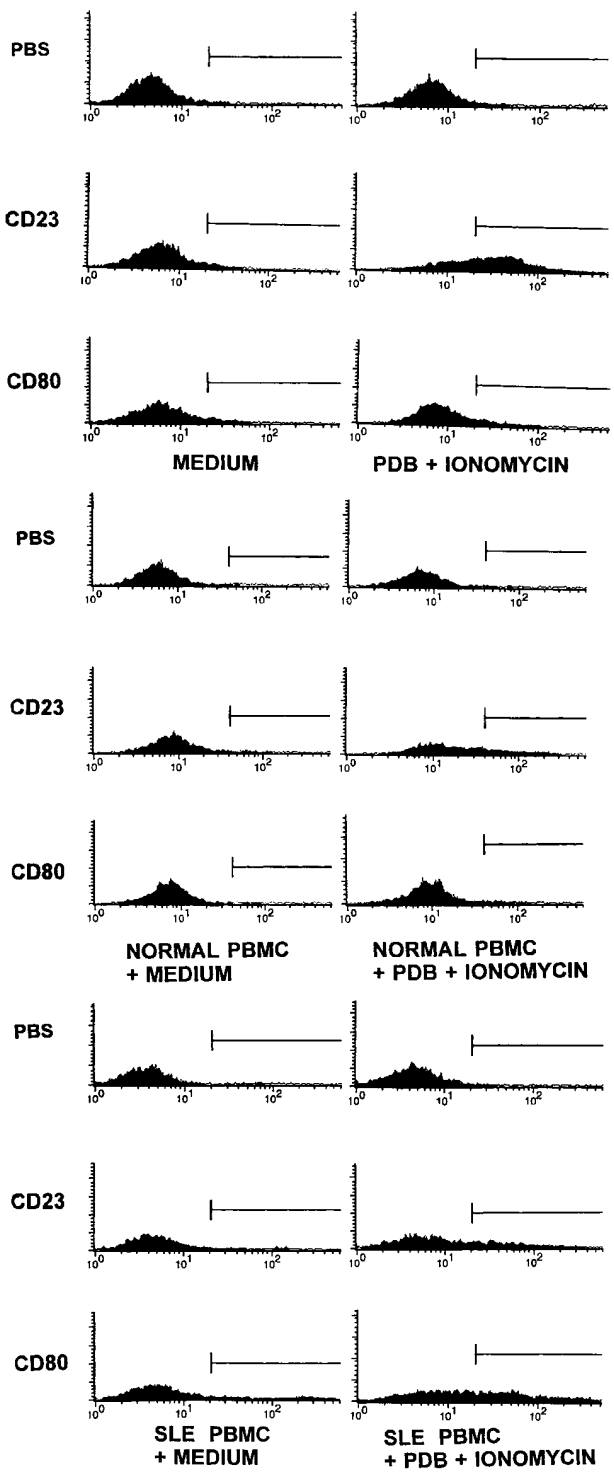

Figure 9. Induction of activation antigen expression on Ramos B cells. Ramos B cells were analyzed by indirect immunofluorescence for expression of CD23 and CD80 (B7-1) after 24-h culture with medium alone (*top left*) or PDB and ionomycin (*top right*), with normal PBMC precultured for 24 h with medium (*middle left*) or PDB and ionomycin (*middle right*), or with SLE PBMC precultured with medium (*lower left*) or with PDB and ionomycin (*lower right*).

was not attributable to differences in the proportion of CD4+ cells present. Also striking was the baseline expression of CD40L on untreated PBMC from some SLE patients. Preliminary analysis suggests that in addition to CD4+ T cell expression of CD40L, a population of CD8+ SLE T cells may also bear cell surface CD40L in the absence of in vitro activation in some patients (data not shown). While there was no apparent correlation between the kinetics of CD40L expression with disease activity in the SLE patients (see Table I), many of the patients did have active lupus at the time of study. Prolonged CD40L expression after in vitro activation may reflect chronic in vivo polyclonal T cell activation, likely attributable to T cell triggering by a spectrum of autoantigens. The mechanism responsible for the observed phenotype may be similar to that of the persistent cell surface CD40L on short term T cell lines from normal subjects that have been repeatedly retriggered through the TCR in vitro (data not shown). Disease activity may be more closely reflected in the baseline CD40L than in the kinetics of expression, as the three SLE patients with the highest CD40L expression in the absence of in vitro activation had active or end stage renal disease. Careful study and clinical characterization of additional patients will be necessary to support this possibility. A potential effect of corticosteroid treatment on CD40L expression is difficult to rule out. Most of the SLE patients, as well as some of the disease control patients, were receiving corticosteroid or other potentially immunosuppressive treatment at the time of study, although none was receiving cyclosporin A, an agent known to inhibit the expression of CD40L on T_h cells activated with PMA and ionomycin (38).

Definition of the cellular mechanisms that account for increased and prolonged CD40L expression in SLE will be a focus of our future studies. We favor the prediction that prolonged CD40L expression reflects persistent and polyclonal activation of T_h cells in the SLE patients with active disease, or impaired downregulation of CD40L, rather than an intrinsic abnormality in the CD40L molecule. SLE T_h cells may be enriched in memory T cells, which may have altered kinetics of CD40L expression when compared with naive T_h cells. Memory cells have recently been reported to rapidly express CD40L within minutes of in vitro activation, although in contrast to our results, in that study the memory cells lost their cell surface CD40L within hours (48). Altered levels of cytokines in patients with SLE may affect CD40L expression, as has been reported in the normal murine system (37). Yellin et al. have found a role for B cell CD40 binding to T cell CD40L in the downregulation of CD40L and its clearance from the T_h cell surface (39). Analysis of SLE B cell CD40 expression and function may elucidate the basis of altered SLE T cell CD40L kinetics. As B cells have recently been reported to express low level CD40L after activation (49), B cells may also contribute to either baseline or prolonged expression of CD40L in SLE.

The most important issue to be addressed is the functional significance of prolonged CD40L expression for SLE B cell activity and the generation of the autoantibody repertoire. To begin to investigate this question, we established an in vitro assay of T_h cell-mediated induction of target B cell activation antigen expression, similar to our assays that have measured direct T cell help mediated by T cell clones or anti-CD3 mAb-activated PBMC (50–53). Tonsillar B cells, CLL B cells, or the Ramos Burkitt's lymphoma B cell line served as responder cells to assess the functional effect of T_h cell CD40L expression on B cell CD40 ligation. CD23 and CD80 (B7-1) are induced after B cell signaling through CD40 (30–31, 54). While some T cell-derived cytokines can also stimulate the expression of these B cell activation molecules, ligation of CD40 may be the most significant physiologic stimulus (54). The capacities of 24 h–activated SLE and normal PBMC to induce CD23 and CD80 on target B cells were compared. At that time point, the level of CD40L on normal T cells was diminished from its peak, while SLE T cells still expressed high levels of CD40L. While the activated cell preparations from healthy subjects showed modest induction of CD80 on target B cells, activated SLE cell preparations stimulated statistically significantly higher levels of B cell CD80. These results suggest that the prolonged high level CD40L on activated SLE T_h cells may have functional consequences for autologous B cell activity, as was observed for the allogeneic target B cells used in our assay.

Among models of lymphocyte immunoregulation that have been applied to the analysis of autoantibody production are those that implicate intramolecular-intrastructural determinant spreading of antibody responses (44–47). The capacity of a B cell to specifically bind and process a complex particle can sometimes result in the activation of T_h cells specific for cryptic epitopes within that particle and the sequential generation of a range of antibody specificities reactive with components of the particle. While our experiments do not address the mechanisms through which autoantigen-specific T cells become activated in SLE, the prolonged expression of CD40L that we have observed may contribute to the maturation and generalization of T_h cell-dependent autoantibody secretion. Through the augmentation of B cell CD80 expression, CD40L may also contribute to inappropriate CD28 signaling and the activation of autoreactive T cells in the presence of adequate autoantigen (55).

In view of the striking and long lasting therapeutic effect of anti-CD40L mAb in a murine model of chronic graft versus host disease, clinically similar to SLE, and in the (SWR × NZB)F1 murine lupus model (56, 57), our results in patients with SLE support the CD40L-CD40 molecular pair as an important potential target for therapy in human disease.

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