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Regulatory Defects in Cbl and Mitogen-Activated Protein Kinase (Extracellular Signal-Related Kinase) Pathways Cause Persistent Hyperexpression of CD40 Ligand in Human Lupus T Cells[1]

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To identify intrinsic defects in lupus, we studied short-term, $CD4^+$ T cell lines that were established from 16 lupus patients (active or inactive) and 15 normal subjects by stimulating once with anti-CD3, anti-CD28, and IL-2. After resting, the pure $CD4^+$ T cells were exposed to anergy-inducing stimulation with plate-bound anti-CD3 mAb in the absence of APC. Lupus T cells showed prolonged high level expression of CD40 ligand (CD40L, CD154) even in the face of anergy protocol, which shut down CD40L expression in normal T cells. The sustained CD40L expression in lupus T cells did not correlate with memory status or Th deviation, and was relatively independent of IL-2 or other autocrine or paracrine signals via CD28 or CTLA-4. Cyclosporin A could block CD40L expression by lupus T cells when added early during the anti-CD3 stimulation period, but only partially when added later, indicating that another mechanism regulates the prolonged hyperexpression of CD40L besides the $Ca^{2+} \Rightarrow$ calcineurin-dependent NF-AT pathway. When exposed to the anergy protocol, lupus T cells, in marked contrast to normal T cells, did not phosphorylate Cbl/Cbl-b but continued to express strongly phosphorylated extracellular signal-regulated kinase (ERK); U0126, a specific inhibitor of mitogen-activated protein kinase kinase $\Rightarrow$ ERK, could block both the early and the prolonged hyperexpression of CD40L. Thus, pathways regulating the activities of Cbl and one particular mitogen-activated protein kinase, ERK, are involved in the prolonged hyperexpression of CD40L in lupus T cells. ***The Journal of Immunology*****, 2000, 165: 6627–6634.**

In systemic lupus erythematosus, a polyclonal hyperactivity of T and B cells appears to accompany a more focused autoimmune response directed against nuclear constituents from apoptotic cells (1–9). The two classes of defects are interconnected, probably because the potential repertoire of both T cells and B cells for nuclear autoantigens is vast due to promiscuity or cross-reactivity of their receptors (9–13). For this reason, almost any means of lowering the activation threshold, interfering with activation-induced cell death of peripheral T and/or B cells, or providing inappropriate help to B cells, leads to the production of anti-nuclear Abs and even lupus-like nephritis. But which of these mechanisms is specifically important for the pathogenesis of spontaneous systemic lupus erythematosus remains to be established.

The cognate interaction between autoimmune Th and B cells of lupus in the production of pathogenic anti-DNA autoantibodies (14, 15) is dependent on costimulatory signals (4, 16). Blocking the interaction between CD40 ligand or CD154 (CD40L)[3] on the autoimmune Th cells and CD40 on autoimmune B cells, even briefly with anti-CD40L Ab therapy in lupus-prone mice, produces unexpected long-term benefits (4, 5). But, even more interesting is the prolonged hyperexpression of CD40L in lupus T cells that occurs irrespective of disease status. In normal T cells, CD40L expression is tightly regulated (17, 18). Persistent and increased expression of CD40L could disrupt this regulatory checkpoint and allow autoimmune cells to expand. T cells in lupus-prone SNF_1 mice (4) and not only T cells (5, 6), but also B cells (5, 19), of lupus patients express abnormally high levels of CD40L without any deliberate stimulation (5). Moreover, upon stimulation with suboptimal doses of mitogens, the up-regulation of CD40L in T cells (5) and in B cells (5) from lupus patients in long-term remission, and in T cells from preautoimmune lupus-prone mice (4), is markedly greater than normal, suggesting an intrinsic regulatory defect associated with a lowered threshold of activation of T cells as well as B cells of lupus (5, 20–22). The possession of promiscuous Th cell receptors (10) and abnormally prolonged expression of CD40L by lupus T cells probably lower the threshold for recognition of apoptotic cell Ags, resulting in a pathogenic autoimmune response (9).

Herein, we have begun to study the mechanisms underlying the prolonged expression of CD40L in lupus T cells. T cells from patients with active lupus might be exhausted by continuous stimulation in vivo and thus become refractory to further stimulation in vitro. However, these T cells recover after resting in vitro (23). Therefore, to reveal intrinsic T cell defects we have studied short-term T cell lines that were expanded by one-time stimulation and then fully rested (5, 24, 25). Moreover, half of these T cell lines were obtained from patients in long-term remission of their disease (5, 25). We have previously shown that the one-time expansion to make these short-term lines by anti-CD3, anti-CD28, and IL-2 stimulation yields a polyclonal population that retains the original

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[3] Abbreviations used in this paper: CD40L, CD40 ligand (CD154); CsA, cyclosporin A; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MKK (MEK), MAPK kinase; MS, multiple sclerosis.

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fraction of autoimmune T cells with their autoantibody-inducing ability and specificity for nucleosomal histone peptides (5, 10, 25, 26). We found herein that in contrast to T cell lines from normal subjects, T cell lines from lupus patients, even those from patients with inactive disease, do not down-regulate CD40L expression in the face of anergy-inducing conditions.

Materials and Methods

Patients and healthy donors

T cells from eight patients with active lupus (all females, aged 18–50 years), eight patients in long-term remission, and 15 normal (healthy) subjects (10 females and 5 males, aged 22–40 years) were studied. Disease activity by System Lupus Activity Measure ranged between 7 and 20 for the active patients. None of the patients in remission had detectable proteinuria or serum anti-DNA autoantibodies at the time of testing, and their System Lupus Activity Measure ranged between 0 and 4. The patients in remission had never received any cytotoxic drugs, and they were not receiving any steroids at the time their blood samples were drawn for the assays. Steroid had been discontinued for several years in the remission patients, as reported previously (5, 25).

Abs and reagents

Anti-CD3 (OKT3) mAb-secreting hybridomas were obtained from American Type Culture Collection (Manassas, VA). The hybridoma supernatants were concentrated by 47% saturated ammonium sulfate precipitation and dialysis before use. Anti-CD28 Ab (mAb 9.3) containing ascites was provided by Kathy Cabrian (Bristol-Myers Squibb, Seattle, WA). Anti-CD3 (clone UCTH1) and PE-conjugated anti-CTLA-4 (clone BNI3) mAbs were obtained from Immunotech (Marseille, France). Purified anti-human CD40L (CD154, clone 24-31) FITC, anti-human CD80 (B7-1, clone BB1), anti-CD152 (CTLA-4, clone ANC152.2/8H5), as well as isotype-matched control IgG1 Ab were obtained from Ancell (Bayport, MN). Anti-CD86 (B7.2, clone IT2.2, clone FUN-1), anti-CTLA4 (clone BNI3), anti-IL2 neutralization Ab (clone MQ1-17H12), and isotype control rat IgG2a (clone R35-95) were obtained from PharMingen (San Diego, CA). Purified anti-human CD28 (clone L293), PE-conjugated mAb to CD69 and anti-CD45RO-PE were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). Mitogen-activated protein kinase (MAPK) kinase (MKK (MEK)) Inhibitor U0126 and anti-active MAPK polyclonal Ab were obtained from Promega (Madison, WI). SB202190, an inhibitor of P38 MAPK, was purchased from Calbiochem (San Diego, CA). All mAbs were used at optimal saturating concentrations as recommended by the manufacturers. c-Jun (N-terminal amino acid positions: 1–79) recombinant proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell preparations and short-term T cell lines

PBMC were obtained from lupus patients and healthy donors by centrifugation of heparinized blood over Ficoll-Hypaque (Pharmacia LKB Biotechnology, Piscataway, NJ) gradient. CD4 cells were selected from PBMC using anti-CD4 mAb-coupled magnetic beads (Dynal, Oslo, Norway). CD4 T cell lines were made within 10–14 days, after one round of stimulation of the CD4 T cells with plate-bound anti-CD3 and anti-CD28 mAb and rIL-2 (20 U/ml), as described (5, 25).

Flow cytometry analysis

Viable T cells were harvested and stained with various FITC-conjugated or PE-conjugated mAbs as described (5, 25). The cells were analyzed by flow cytometry using FACScan and CellQuest software (Becton Dickinson). For intracellular CTLA-4 staining, the T cells were first treated with IntraPrep permeabilization reagent obtained from Immunotech as recommended by the manufacturer, and then were incubated with PE-conjugated anti-CTLA-4 Ab.

Induction of anergy

$CD4^+$ short-term T cell lines that were previously expanded by OKT3 and anti-CD28 were fully rested for 7–10 days (first rest). These rested T cells (1×10^6/ml) were then stimulated in complete RPMI 1640 medium with plate-bound anti-CD3 (OKT3; coated at 1 μg/ml) for 16–18 h (first stimulation). After this anergy induction, cells were transferred into fresh uncoated plates and incubated for 2 days (second rest), and then were rechallenged with the same concentrations of plate-bound anti-CD3 (second stimulation). At different time points, the T cells were harvested to determine expression of the CD40L and other markers by flow cytometry.

Cytokine assays

Concentrations of IL-2 were measured by ELISA using anti-human IL-2 (clone B33-2; PharMingen) as a detection Ab and anti-human IL-2 (clone 5344.11; PharMingen) as a capture Ab (25). Concentrations of IL-4 and IFN-γ in T cell culture supernatants were determined by Quantikine per manufacturer instructions (R&D Systems, Minneapolis, MN).

Western blot and immunoprecipitation analysis

T cells were stimulated for the indicated times by anti-CD3 Ab at 37°C. Stimulation was stopped with cold wash buffer containing PBS, 5 mM EDTA, and 0.5 mM vanadate, then cells were lysed in a 1% Nonidet P-40 (Sigma, St. Louis, MO) lysis buffer containing Tris-HCl, pH 7.6; 150 mM NaCl; 1 mM EGTA; 5% glycerol; 10 mM tetrasodium pyrophosphate; 0.5% sodium deoxycholate; 10 mM NaF; aprotinin (5 μg/ml); pepstatin (1 μg/ml); soybean trypsin inhibitor (2 μg/ml); and 1 mM PMSF for 20 min on ice. Protein content of the lysate was determined with the BCA protein assay kit (Pierce, Rockford, IL), and 30 μg was subjected to SDS-PAGE. Immunoblotting, stripping, and reprobing of the immunoblots were performed with various Abs, such as anti-phosphotyrosine mAb (PY99), mAb anti-Fyn, mAb anti-extracellular signal-regulated kinase 2 (ERK2), and rabbit polyclonal Ab against Cbl/Cbl-b, which were all obtained from Santa Cruz Biotechnology, and HRP-conjugated anti-phosphotyrosine mAb (clone 4G10), rabbit anti-human Fyn polyclonal Ab, rabbit anti-human Vav polyclonal Ab, mAb anti-Vav, and mAb anti-Cbl, which were obtained from Upstate Biotechnology (Lake Placid, NY). All blots were developed with enhanced chemiluminescence substrate (Amersham, Arlington Heights, IL). Immunoprecipitations of lysates from 5×10^6 cells were performed with anti-Cbl, anti-Fyn, or anti-Vav antiserum (5 μg/sample), respectively, followed by protein A/G-conjugated agarose (Santa Cruz Biotechnology), and analyzed by SDS-PAGE and immunoblotting, as above.

Jun kinase assay

Solid state c-Jun N-terminal kinase assay was performed by incubating cell lysates with GST-c-Jun (1–79) agarose beads as described (27).

Bead preparation to test CTLA-4 function

Anti-CD3, anti-CD28, and one of the anti-human CTLA-4 mAbs were covalently attached to polyurethan-coated tosyl-activated Dynabeads (Dynal, Lake Success, NY) according to the manufacturer's instruction (bead/cell ratio, 1:1). Beads were prepared with a constant amount of anti-CD3 mAb that represented 5% of the total protein bound and a titration of anti-CD28 and anti-CTLA-4 or control mAb to make up the remaining 95%. Ab-coated beads were tested at anti-CD28 and anti-CTLA-4 ratios of 10:0, 9:1, 5:5, 3:7, 1:9, and 0:10, as described (28, 29). $CD4^+$ T cells from rested cell lines were cultured in 96-well plates at a density of 5×10^5 cells with the beads coated with a constant amount anti-CD3 and various concentrations of anti-CD28 and anti-CTLA4 mAbs. T cell proliferation was determined in 72-h culture assays that were pulsed with 1 μCi of [^{3}H]TdR during the last 18 h. Staining of T cells for CD40L in duplicate cultures with the beads were performed after 5 h.

Blocking with anti-B7 mAbs

$CD4^+$ T cells were cultured in the presence of anti-B7 (B7.1 and B7.2) mAbs or control Ab (20 μg/ml) during the entire anti-CD3 stimulation and anergy induction period. CD40L expression levels were measured by flow cytometry.

Blocking with anti-IL2 mAb

Rested $CD4^+$ T cells were stimulated by plate-coated anti-CD3 and anti-CD28 in the presence of soluble anti-IL2 neutralizing mAb or rat IgG2a control Ab (20 μg/ml) for 48 h. CD40L expression levels were measured by flow cytometry.

Results

Prolonged hyperexpression of CD40L (CD154) and resistance to anergy induction

Short-term CD4 T cell lines from 16 lupus patients and 15 normal subjects were analyzed. Kinetics from a representative experiment are shown in Fig. 1. The CD4 T cell lines were fully rested for 7–10 days in uncoated wells and then stimulated in vitro by plate-bound anti-CD3 Ab for 5, 18, or 48 h (first stimulation). The T cells from either normal or lupus subjects had similar resting levels

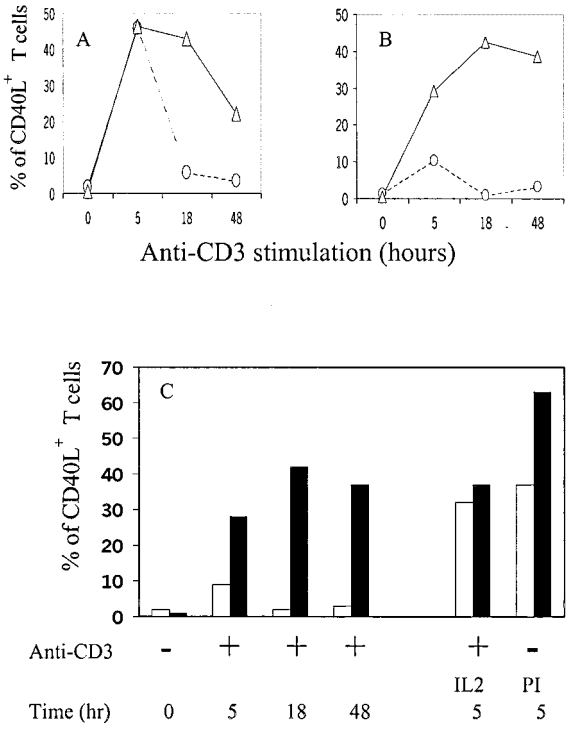

FIGURE 1. Kinetics of CD40L expression in short-term CD4 T cell lines from normal (○) and lupus (△) subjects. Representatives from 15 normal and 16 lupus T cell lines are shown. *A*, First stimulation of fully rested T cell lines with plate-bound anti-CD3 for 5, 18, or 48 h. *B*, After anergy induction and second rest, the T cells were rechallenged with plate-bound anti-CD3 (second stimulation) for 5, 18, or 48 h. *C*, The normal (open columns) and lupus T cells (filled columns) after their second rest were restimulated by plate-coated anti-CD3 (second stimulation) alone for different durations (*left*), and also for 5 h with anti-CD3 in the presence of IL2 (50 U/ml) or PI (10 ng/ml PMA + 100 ng/ml ionomycin) (*right*). This is a representative of six experiments.

of CD40L-positive T cells (2 and 1%, respectively). After stimulation, both types of cell lines showed almost identical early response at 5 h with a 23- to 46-fold increase in the percentage of CD40L-positive T cells. However, in the case of normal T cells, the percentage of CD40L-positive cells dropped quickly from 46 to 6% by 18 h and went down further to background level (3%) at 48 h. By contrast, in the case of lupus T cells, the expression of CD40L was prolonged, as 22% of the T cells were CD40L positive even at 48 h. Next, the same T cells that were stimulated by plate-bound anti-CD3 for 48 h were rested for 2 days in fresh uncoated wells (second rest) when surface CD40L came down to background levels also in the lupus T cells in the absence of continued anti-CD3 stimulation. At this stage, viability was comparable between the normal and lupus T cell cultures with ~20–30% dead cells. Only viable T cells were harvested by Ficoll-Hypaque centrifugation and then restimulated by plated-bound anti-CD3 (second stimulation) (Fig. 1*B*). The normal T cell lines showed weak response to the second anti-CD3 stimulation with a peak level of CD40L-positive T cells reaching 10% at 5 h, which declined to background levels thereafter (Fig. 1*B*). However, when these anergic normal T cells were treated with either IL-2 (50 U/ml) or PMA (10 ng/ml) plus ionomycin (100 ng/ml), the hyporesponsiveness to anti-CD3 could be reversed completely, demonstrating that the cells were still viable (Fig. 1*C*). In contrast to normal T cells, lupus T cells behaved differently after exposure to the anergy induction protocol; they responded to the second anti-CD3 stimulation with prolonged hyperexpression of CD40L with kinetics similar to the first anti-CD3 stimulation.

CD40L expression from all the T cell lines are shown in Fig. 2, *A* and *B*, at two time points; one is at 5 h of the first anti-CD3 stimulation when optimal expression occurs before anergy sets in, and the other is at 18 h of second stimulation when anergy is well established. Consistent with the kinetics in Fig. 1, the normal T cells failed to up-regulate CD40L after exposure to the anergy induction protocol, in marked contrast to the lupus T cells ($p < 0.01$). We also measured another T cell activation marker, CD69, in these experiments (Fig. 2, *C* and *D*). In marked contrast to the pattern of CD40L expression, both types of cell lines had similar increases in CD69 expression ($p > 0.1$), either on first stimulation with anti-CD3 after being fully rested or after second stimulation following the anergy induction protocol.

Prolonged CD40L expression by lupus T cells does not correlate with IL-2 dependence, Th deviation, or memory status

Because anergy is associated with a block in IL-2 production, we measured IL-2 production by the lupus and normal T cells at different time points (Fig. 3). In general, normal and lupus T cell lines produced comparable level IL-2 on the first anti-CD3 stimulation, but after exposure to the anergy protocol, normal T cell lines produced IL-2 at background levels after second stimulation, in marked contrast to lupus T cell lines ($p < 0.01$). Indeed, addition of IL-2 could restore CD40L expression by the anergized, normal T cells (Fig. 1*C*). This data suggested that resistance to anergy induction was associated with prolonged CD40L expression in lupus T cells. When T cells are optimally stimulated with both anti-

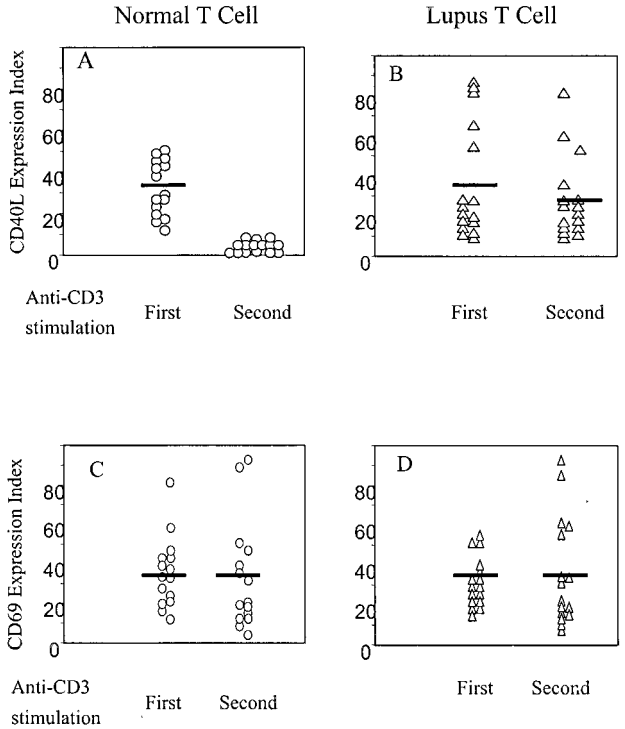

FIGURE 2. Collective data from 15 normal (○) and 16 lupus (△) T cell lines showing expression of CD40L (*A* and *B*) and CD69 (*C* and *D*) at two time points: at 5 h after the first stimulation and 18 h after the second stimulation with plate-bound anti-CD3. To account for any background variation at resting state, the expression is shown as an index, which is the ratio of % CD40L$^+$ T cells after stimulation over % CD40L$^+$ T cells at rest, i.e., first stimulation/first rest, and second stimulation/second rest.

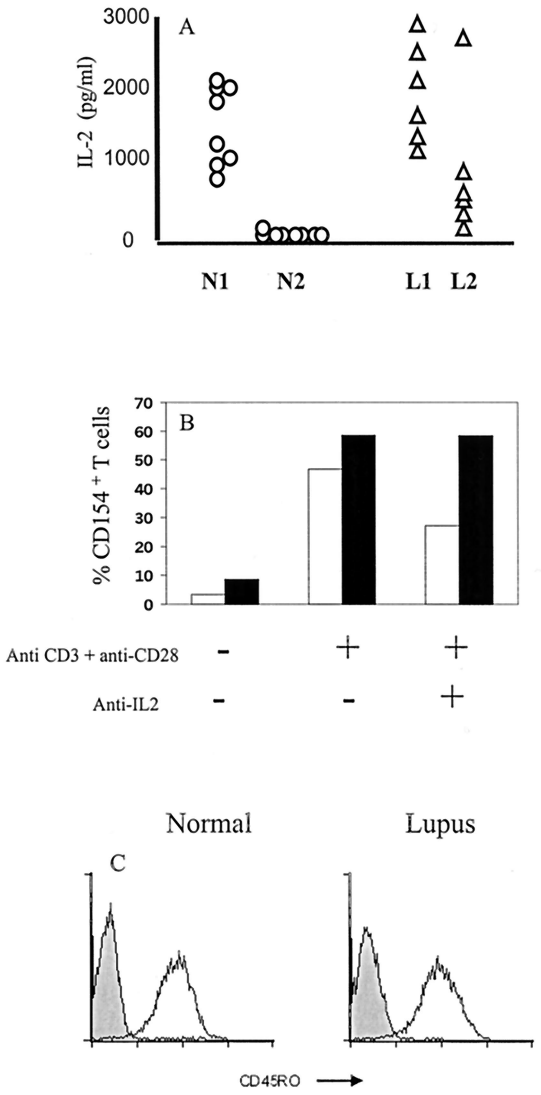

FIGURE 3. Prolonged expression of CD40L in lupus T cells is independent of IL-2 production and memory markers. *A*, Normal T cell (○) produced IL-2 on first anti-CD3 stimulation (N1) comparably to lupus T cells (L1), but on second stimulation, IL-2 production was diminished by 98 ± 0.71% (mean ± SEM) in the case normal (N2) in contrast to lupus T cells where the reduction was 51 ± 11.43%. *B*, Fully rested T cells (normal T cells = open columns, lupus T cells = filled columns) were either untreated or stimulated optimally with both anti-CD3 and anti-CD28 in the presence or absence of anti-human IL-2-neutralizing Ab for 48 h. This is a representative of four experiments. *C*, CD45RO surface expression on normal and lupus $CD4^+$ T cells. The cells were rested and stained with either anti-CD45RO mAb (open histogram) or isotype control (shaded histogram). In this example, 97.44% of the normal and 96.65% of the lupus T cells were $CD45RO^+$. The experiment was repeated with five normal and five lupus T cell lines with similar results.

CD3 and anti-CD28 mAbs, prolonged expression of CD40L occurs even in normal T cells (30). However, although the prolonged CD40L expression by the optimally stimulated, normal T cells could be significantly inhibited by anti-IL2 Ab (Ref. 30; Fig. 3*B*), that was not the case in lupus T cells (Fig. 3*B*). There were no particular deviations to either Th1 or Th2 type among the lupus or normal T cell lines. All of the short-term T cell lines produced both IFNγ and IL-4 on optimal stimulation with anti-CD3 (data not shown), which was consistent with previous observations (25). Moreover, there were no differences in memory markers between the lupus and normal T cell lines (Fig. 3*C*).

Calcineurin-NF-AT pathway and prolonged CD40L expression in lupus T cells

Because it is well known that $Ca^{2+} \Rightarrow$ calcineurin-dependent NF-AT activation is responsible for inducing CD40L expression (17, 31, 32), we tested lupus T cells for abnormalities in this pathway. Cyclosporin A (CsA) is a specific inhibitor that blocks calcineurin-dependent NF-AT activation. Therefore, CsA was added along with plate-bound anti-CD3 stimulation to fully rested T cell lines, and CD40L expression was measured. At earlier time points, up to18 h, both lupus and normal T cells were very sensitive to inhibition by CsA, and titration of CsA failed to show differences between normal and lupus T cell lines in dose response (data not shown). Representative results from a lupus T cell line is shown in Fig. 4. When lupus T cell lines were treated with CsA (200 ng/ml) at early stages of activation (added at time 0 or 6 h of stimulation by anti-CD3) the degree of inhibition was very high (between 81 and 97%). However, when CsA was added at later stages (18 h or later during continued anti-CD3 stimulation), it was much less effective; inhibition was only 11% when CsA was present in the cultures during the last 18- to 26-h period of anti-CD3 stimulation, or 35% when CsA was present during the last 18–42 h of anti-CD3 stimulation. Normal T cells were not tested with CsA at these later stages of anti-CD3 stimulation because they had spontaneously down-regulated CD40L expression (Fig. 1*A*). The data so far suggested that other factors might determine the prolonged expression of CD40L in lupus T cells besides $Ca^{2+} \Rightarrow$ calcineurin-dependent NF-AT pathway.

Tyrosine phosphorylation after TCR signaling, pre- and postanergy

We searched for possible mechanisms in TCR signal transduction pathways that could be responsible for persistent CD40L expression in lupus T cells following exposure to the anergy induction protocol. Representative results are shown in Fig. 5. In Fig. 5*A*, preanergy samples from fully rested T cells before first stimulation and postanergy samples from T cells that were rested for 2 days after 18-h exposure to anergy induction protocol are shown side by side. The phosphotyrosine immunoblots were obtained from the respective T cells either before stimulation (−) or 10 min after stimulation (+) with anti-CD3. There were no obvious differences in the preanergy samples from normal (*lanes 1* and *2*) and lupus T cells (*lanes 5* and *6*) up to 10 min of anti-CD3 stimulation (first

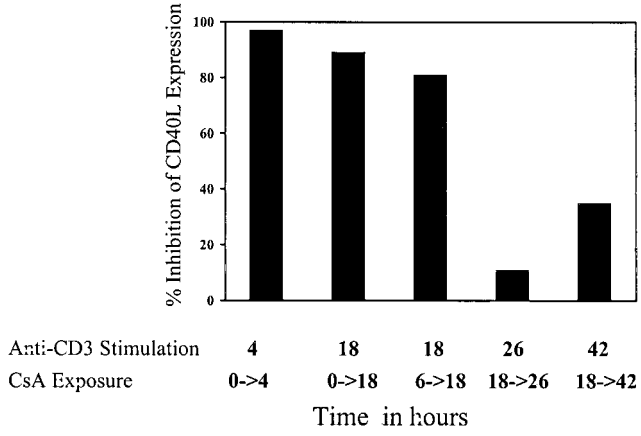

FIGURE 4. Effect of CsA on CD40L expression by lupus T cells. Rested lupus T cell lines were stimulated by plate-bound anti-CD3 (first stimulation) either in the absence or presence of CsA (200 ng/ml) for 4 or 18 h, or during the last 12, 8, or 24 h of an 18-, 26-, or 42-h stimulation period. This is a representative of three experiments.

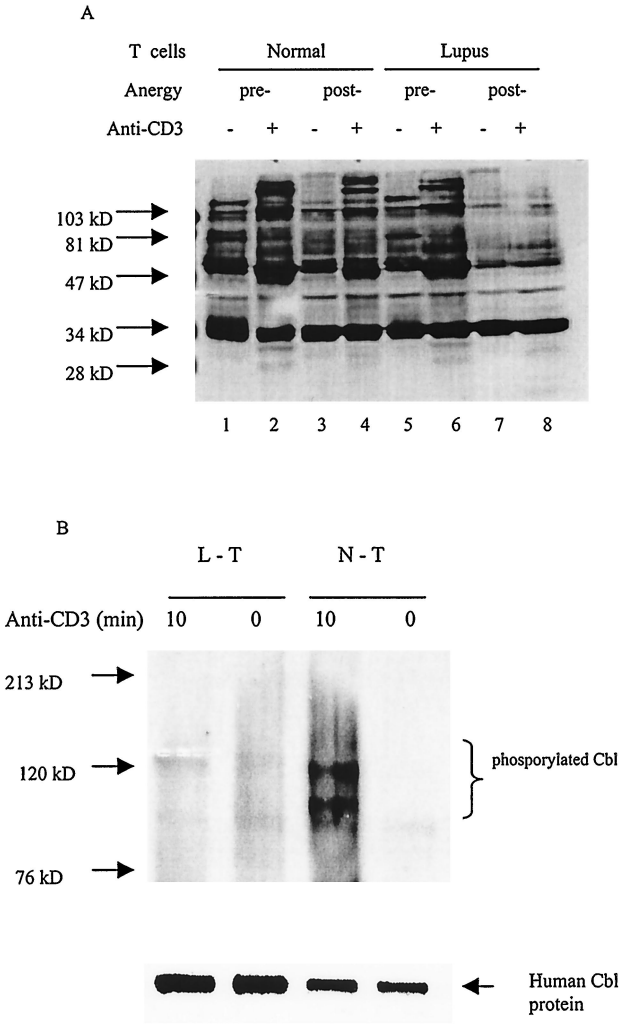

FIGURE 5. Tyrosine phosphorylation of proteins in lysates from normal and lupus T cells. *A*, Phosphotyrosine immunoblots before (pre-) or after (post-) anergy induction of T cells that were either left untreated (−) or stimulated by anti-CD3 (+) for 10 min. *B*, Deficient phosphorylation of Cbl in lupus T cells after exposure to anergy induction protocol. Postanergy normal T cells (N-T) and lupus T cells (L-T) were restimulated with anti-CD3 for 10 min. Cell lysates were immunoprecipitated with rabbit anti-Cbl Ab and immunoblotted sequentially with anti-pTyr (*upper panel*) and anti-human Cbl mAb (*lower panel*). This is a representative of four experiments.

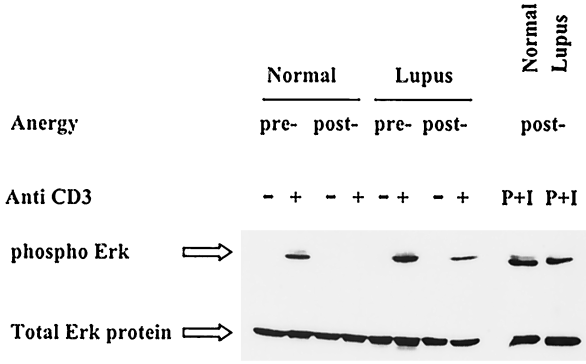

FIGURE 6. Ability to phosphorylate ERK is preserved in lupus T cells upon second anti-CD3 stimulation after exposure to anergy protocol. Before (pre-) or after (post-) anergy induction, normal and lupus T cell lines were left untreated (−) or stimulated by anti-CD3 (+) for 10 min, or by PMA and ionomycin (P+I) for 2 min. Western immunoblot with anti-phospho-ERK and reprobing with anti-ERK mAb for total ERK protein were performed. This is a representative of four experiments.

stimulation). However, among postanergy samples, when the normal T cells were stimulated for the second time with anti-CD3 (*lanes 4* and *8*), they showed increased tyrosine phosphorylation of bands, including those at higher molecular mass regions (110–140 kDa), in marked contrast to lupus T cells under this condition. Total protein loadings in the last two lanes of Fig. 5*A* were comparable to the other lanes (as shown in Fig. 6). Similar results were obtained with a 5- or 30-min stimulation of the T cells by anti-CD3; the differences in phosphorylation of proteins on second stimulation, particularly at the higher molecular mass regions were evident also at these time points (data not shown). Because the high molecular mass bands could include phosphorylated Cbl, which is important for anergy maintenance (33), we performed Cbl-specific immunoprecipitation and immunoblotting, which showed that in marked contrast to normal T cells, Cbl/Cbl-b was not phosphorylated in lupus T cells under anergy-inducing conditions (Fig. 5*B*). Moreover, normal T cells showed an increase in phosphorylated Fyn as compared with lupus T cells after exposure to anergy protocol, but no differences in phosphorylation of Vav could be detected under those conditions (data not shown).

Differential activation of MAPK and prolonged CD40L expression in lupus T cells

Inactivation of the ERK pathway is clearly involved in T cell anergy (34, 35). We measured ERK activity in normal and lupus T cells under preanergy or postanergy conditions using anti-active or phospho-ERK Ab. The same membrane from Fig. 5*A* was reprobed with anti-phospho ERK Ab, as shown in Fig. 6 (*upper panel*, labeled as phospho ERK), stripped, and further reprobed with anti-total ERK protein Ab, as shown in Fig. 6 (*lower panel*), demonstrating equal loading of total Erk protein among the different samples. The phosphorylated ERK band was not detectable in any of the unstimulated T cell lysates. Strongly phosphorylated ERK bands were found in preanergy samples of both normal and lupus T cells upon stimulation by anti-CD3 for 10 min. After anergy induction protocol (postanergy), the active ERK bands in lupus T cell lysates persisted and were markedly greater in intensity than in normal T cells after stimulation by anti-CD3 (second stimulation). Indeed, the same difference between lupus and normal T cells in the level of active ERK was seen as early as 60 min after the first stimulation with plate-bound ant-CD3 (data not shown). The down-regulation of ERK activity in normal T cells could be bypassed by treating the cells with PMA and ionomycin (P+I) for 1 min, which also indicated that the T cells were not apoptosed after anergy induction. These data suggested that there is a possible defect in down-regulation of the Ras-MAPK pathway in lupus T cells that causes resistance to induction of anergy, which may also be responsible for abnormally prolonged expression of CD40L by lupus T cells.

There are at least three known MAPK family members, ERKs, c-Jun N-terminal kinases, and p38 MAPK. We failed to detect any difference in p38 MAPK activity between normal and lupus T cell lysates under the above conditions using anti-phospho p38 Ab (data not shown). Moreover, addition of up to 5 μM SB202190, which specifically inhibits p38 MAPK at a 4 μM range (36), did not block the prolonged hyperexpression of CD40L in lupus T cells (data not shown). Furthermore, using the Jun kinase assay we did not detect any differences in phosphorylation of c-Jun between normal and lupus T cells during the first stimulation (preanergy) or second stimulation (postanergy) (data not shown).

To determine whether persistent ERK activity in lupus T cells was involved in prolonged expression of CD40L, one of MKK (MEK) ⇒ ERK-specific inhibitors, U0126, was used. This inhibitor specifically inhibits MKK1/2 activation at a range of 10–100 μM (Promega). After exposure to anergy induction protocol, lupus T cell lines were treated with either CsA (200 ng/ml) or U0126 (25 μM) either from the beginning of plate-bound anti-CD3 stimulation or 18 h later (Fig. 7). When cells were treated with inhibitors from the beginning of anti-CD3 stimulation, inhibition of CD40L expression was comparable between CsA (down from 32% CD40L$^+$ T cells to 6.7%) and U0126 (from 32 to 7.3%), but when the inhibitors were added into cell cultures after 18 h of activation with anti-CD3, U0126 was still a potent inhibitor (from 32% CD40L$^+$ T cells to 6.5%), but CsA was less effective at this time (from 32 to 18.7%). Thus, prolonged CD40L expression in preactivated lupus T cells was only partially inhibited by CsA but almost completely inhibited by U0126.

Prolonged expression CD40L by lupus T cells is independent of CD28 and CTLA-4 signals

Because activated T cells can express B7 molecules as well as CD28, autocrine or paracrine signaling could make them relatively resistant to anergy, i.e., independent of exogenous CD28 signals. Moreover, CTLA-4-mediated negative signaling is important for anergy in mouse T cells (37). Therefore, we addressed the correlation between resistance to anergy and expression of costimulatory molecules (CD28, B7) or CTLA-4 in human lupus T cell lines before and after anergy induction, as compared with the normal T cell lines (Fig. 8). The expression of CD28 in T cells was constitutive and high at all time points we measured without any major difference between normal and lupus T cells (consistently around 70–90%). However, B7.1 and B7.2 expression on normal T cells was markedly lower than that on lupus T cells during the postanergy period ($p < 0.01$).

Before anergy induction, the CTLA-4 expression pattern was very similar between normal and lupus T cells (<10% CTLA4$^+$ T cells at resting state and 20–60% after stimulation by anti-CD3). However, after anergy induction there were marked differences in expression of CTLA-4 between lupus and normal T cells. Lupus T cell population retained on the average 44% CTLA-4$^+$ T cells, as compared with ~10% CTLA-4$^+$ in the case of normal T cells ($p < 0.01$). The above stainings included both surface and intracellular pool of CTLA-4. Because maximal CTLA-4 surface expression can be achieved by treating cells with the phosphatase inhibitor pervanadate, we treated both normal and lupus T cells with pervanadate as described (38). We found that, after anergy induction, the percentage of CTLA-4$^+$ T cells in lupus cell lines was 3-fold higher than that among normal T cell lines upon pervanadate treatment (data not shown).

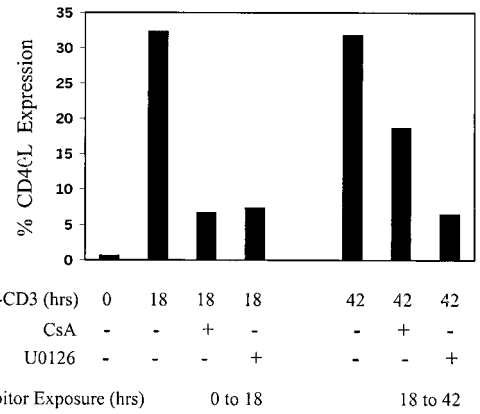

FIGURE 7. Efficient inhibition of prolonged CD40L expression by MKK-ERK inhibitor U0126. Rested lupus T cells after anergy induction were restimulated by plate-bound anti-CD3 (second stimulation), either in the absence or presence of CsA (200 ng/ml) or U0126 (25 μM) for 18 h or during the last 24 h of a 42-h stimulation period. This is a representative of three experiments.

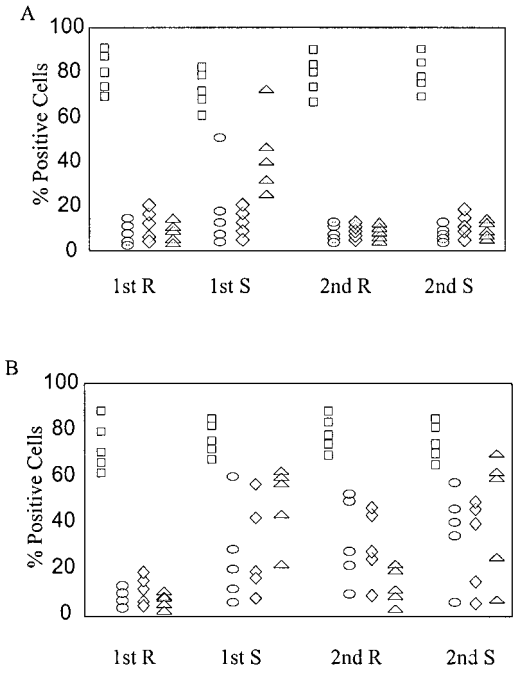

FIGURE 8. Expression of various costimulatory markers (□, CD28; ○, B7.1; ◇, B7.2), and total CTLA-4 (△) were measured at different time points (1st R, first rest; 1st S, first stimulation; 2nd R, second rest; 2nd S, second stimulation) in five normal T cell lines (*A*) and five lupus T cell lines (*B*) by flow cytometry.

Although there were differences in expression of B7 and CTLA-4 between lupus T cells and normal T cells, we failed to block the anti-CD3-induced prolonged expression of CD40L on lupus T cells using any of the mAbs available against B7.1 and B7.2 that are known to block costimulatory function (39, 40) (data not shown). We also tested various Abs against CTLA-4 (clone ANC152.2/8H5, Ancell; clone BNI3, PharMingen) using published protocols (28, 29), and none of them showed inhibition of either proliferation or CD40L expression in normal or lupus T cells upon optimal anti-CD3 stimulation (data not shown).

Discussion

We have focused here on those molecules in the signaling pathway of lupus T cells that could be involved in resistance to anergy and prolonged expression of CD40L. Lupus susceptibility is not linked to an X chromosome (41–43), ruling out a role of CD40L gene mutation or polymorphism. However, the hyperexpression of CD40L could be due to abnormal regulation. Optimal expression of CD40L in T cells requires a rise in intracellular calcium. CsA blocks CD40L gene expression in T cells, indicating a key role of the phosphatase, calcineurin, which is specifically inhibited by CsA (17). When T cells are activated, calcineurin becomes essential for mediating the translocation of the NF-AT family of transcription factors from the cytosol to the nucleus, where the latter bind to specific DNA recognition sites and, in cooperation with activated AP-1 (Fos/Jun), activate transcription of cytokine genes (44, 45). CD40L is related to the TNF family of cytokine genes

and the 5′ regulatory region of CD40L gene contains several NF-AT binding motifs (31, 32). We found that CsA can shut down prolonged expression of CD40L in lupus T cells when added during early stages of anti-CD3 stimulation, but it was not so effective later. Thus noncalcineurin-dependent pathways may also be operating for increased transcription and prolonged expression of CD40L in lupus. Moreover, several studies have shown that activated NF-AT can be normally induced and translocated to the nucleus in anergic T cells (34), yet we found that induction of CD40L expression is blocked in such T cells from normal subjects, as reported (46). Therefore, NF-AT-independent mechanisms of CD40L transcription and expression have to be considered.

Indeed, we have found that selective inhibition of one of the MAPKs, ERK, blocks the prolonged expression of CD40L in lupus T cells. Our results suggest that the early expression of CD40L requires the CsA-sensitive NF-AT pathway, but prolonged expression of CD40L is more dependent on ERK. Increased and persistent ERK activity in lupus T cells even after exposure to the anergy induction protocol, could contribute to CD40L hyperexpression via activation of AP-1 transcription factors and possibly also by stabilizing CD40L mRNA (27, 47). Indeed, PMA + ionomycin greatly increases the stability and expression of CD40L mRNA, even in normal T cells (6, 47, 48), which could have been due to PKC-mediated Ras-MAPK activation.

Thus, prolonged expression of CD40L by lupus T cells is associated with defects in anergy induction and maintenance. Ligation of TCR without costimulation leads to functional unresponsiveness or anergy. Anergic T cells have a block in Ras activation by TCR signaling, but the pathway downstream of Ras is intact because PMA restores normal function in such cells by activating Ras directly (34, 35, 49). In anergic T cells from normal subjects MAPKs are down-regulated, but the mechanism is upstream because direct inhibition of the MAPKs does not lead to anergy (34, 35, 50). One proposed mechanism of anergy is that mediated by Fyn kinase-phosphorylating Cbl, which then activates Rap1. Rap-1 antagonizes Ras function by sequestering Raf-1 kinase and thus preventing activation of MAPK cascade and downstream transcription factors (33, 51, 52). We found that the Fyn-Cbl-Rap1 pathway, as evidenced by phosphorylation status of Cbl, is operative in normal T cells after exposure to anergy induction protocol, but not in the lupus T cells. In anergic T cells, phosphorylated Cbl may interact with a negative regulatory tyrosine in ZAP-70 to down-regulate its activity and, consequently, lead to inactivation of Ras-MAPK pathway (53–55). Consistent with this scenario, we found persistently active or phosphorylated ERK in lupus T cell lines exposed to anergy induction protocol, in marked contrast to normal T cell lines. However, phosphorylated Cbl might also mediate ubiquitination and degradation of activating molecules in the T cell signaling pathway (56, 57). Although decreased tyrosine phosphorylation of other bands was apparent in the lupus T cells after exposure to the anergy protocol, we did not examine phosphorylation status of TCRζ chain, as its expression is variable in lupus T cells and its functional significance is uncertain because other TCR subunits may take over the job of the ζ chain (58–60).

Furthermore, we did not detect any gross differences in cytokine profile (Th1 or Th2 deviation), memory marker (CD45RO), or rates of apoptosis between the T cell lines from lupus and normal subjects. Resistance to anergy could be due to high level expression of B7 (B7.1 and B7.2) molecules by the lupus T cells, which could cause autocrine and paracrine stimulation via constitutively expressed CD28 as has been thought to be the case in another autoimmune disease, multiple sclerosis (MS; Refs. 61–63). However, the situation in lupus is different. In the case of MS, anergy resistance was seen only in T cells from patients with actively progressive disease, whereas in lupus, we find resistance to anergy in T cells from patients in long-term remission, suggesting an intrinsic defect rather than some adjuvant effect during relapse of disease (63, 64). Interestingly, the resistance to anergy coincides with hyperinducibility of CD40L expression in lupus T cells from patients with inactive disease (5), and these phenomena are probably connected. Moreover, anergy resistance is not just a feature of any T cell that has been activated before (61, 62), because we and others (65) do not observe it in established T cell lines and clones from normal subjects. In conflict with the possibility proposed for MS T cells (61–63), constitutive overexpression of transgenic B7 molecules in T cells of mice did not precipitate any autoimmune disease or alter susceptibility to anergy induction (66). Indeed, we found that blocking of B7-CD28 interaction among the lupus T cells fails to diminish the prolonged CD40L expression in lupus T cells, indicating that other mechanisms are at work. Moreover, B7 $\Rightarrow$ CTLA-4 mediated negative signaling, which is thought to be important for anergy induction and maintenance, and inactivation of MAPK in mouse T cells (29, 37, 67, 68), was not important in down-regulating the prolonged hyperexpression of CD40L in lupus T cells.

Common underlying mechanisms could determine increased Ca^{2+} mobilization on TCR signaling (3), deficiency of PKA I activity (69), various effects caused by lupus-inducing drugs (70, 71), and the lowered activation threshold with prolonged hyperexpression of CD40L we find in lupus T cells (4–6). However, Ca^{2+} $\Rightarrow$ calcineurin-dependent NF-AT pathway is not solely involved for CD40L hyperexpression as expected. We found that prolonged expression of CD40L in lupus T cells is associated with a lack of Cbl/Cbl-b phosphorylation and persistent activation of one particular MAPK, ERK, under conditions that would induce anergy in normal T cells. These observations open up the possibility of using ERK-specific inhibitors for therapy of lupus.

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