Expression of CD27 and its Ligand, CD70, on Chronic Lymphocytic Leukemia B Cells

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Crosslinking the CD27 antigen on T cells provides a costimulatory signal that, in concert with T-cell receptor crosslinking, can induce T-cell proliferation and cellular immune activation. We find that chronic lymphocytic leukemia (CLL) B cells from most patients coexpress both membrane-bound and soluble CD27, along with its newly identified ligand, CD70. The expression of soluble CD27 may preclude leukemic B cells from stimulating T cells via CD70, thereby potentially impairing their ability to function as effective antigen-presenting cells. We find that leukemic B-cell expression of soluble and membrane-bound CD27 can be downmodulated through a CD40-dependent signal. This signal also induces increased expression of CD70 on both normal and leukemic B cells. We find that tumor necrosis factor (TNF)-α, or the Th1 cytokine interferon (IFN)-γ, also can induce downmodulation of CD27, whereas Th2-associated cytokines interleukin-4 (IL-4) or IL-10 can enhance leukemic B-cell expression of this accessory molecule. The modulation of CD27 induced by these conditions is accompanied by reciprocal changes in the expression levels of CD70, suggesting that these accessory molecules may be engaged in reciprocal receptor-ligand downmodulation. Consistent with this, we observe that co-culture of CLL B cells with transfected murine plasmacytoma cells that express human CD70 affects downmodulation of CD27 and enhanced expression of CD70 on leukemic B cells, but does not affect expression of CD27 mRNA. However, we find that CD40-crosslinking, in addition to reducing the level of CD27 protein, also reduces leukemic B-cell expression of CD70 mRNA. This argues that the changes in the expression levels of CD27 following CD40-signaling are not simply due to induced increases in the expression levels of CD70. Finally, we demonstrate that reciprocal changes in expression of CD27 and CD70 may contribute to the enhanced antigen-presenting capacity of CLL B cells after CD40-dependent leukemic B-cell activation. These findings expand the understanding of the regulation of costimulatory molecules important in antigen presentation and also have implications for the immunobiology of and therapy for CLL.

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CD54 (ICAM-1) MoAbs were purchased from AMAC, Inc (Westbrook, ME). FITC-conjugated CD27 MoAb and rat-antimouse IgG MoAbs were purchased from Pharmingen, Inc (San Diego, CA). MOPC 21 (mouse IgG,) and phycoerythrin (PE)-conjugated CD19 MoAb were purchased from Callig, Inc (South San Francisco, CA). Anti-B7 MoAb, recognizing the CD80 protein (clone L307.4), was purchased from the Advanced Cellular Biology Division, Becton Dickinson (San Jose, CA). The CD40 MoAb G28-5 was provided by Dr. Edward Chang (University of Washington, Seattle, WA). The CTLA-4 Ig fusion protein was provided by Dr. Peter Linsley (OncoGene Division, Bristol-Myers Squibb, Seattle, WA). The CD27-Ig fusion protein was provided by Dr Raymond Goodwin (Immunix Corp, Seattle, WA). The rat-antimouse CD8 MoAb (IgM) 3.168 was obtained from Zen-Enzyme Research (La Jolla, CA) and was purified from ascites fluid as described. Some MoAbs were conjugated to FITC for use in flow cytometric analysis, as described. Recombinant human (rh) TNF-α was purchased from R & D Systems (Minneapolis, MN); rhIL-2 and rhIL-10, from Biosource International (Cabrillo, CA); rhTNF-β, from Genzyme (Cambridge, MA); and rh interferon-γ (rhIFN-γ), from Sigma Chemicals (St Louis, MO).

CLL B cells and cell lines. After informed consent, blood was obtained from patients satisfying diagnostic criteria for B-CLL. Peripheral blood mononuclear cells (PBMC) were isolated from blood collected into heparinized tubes by centrifugation over Ficoll (Sigma Chemical Co). Such cells were typically greater than 97% CD19+CD5+ CLL B cells, as determined by flow cytometric analysis. To produce the human CD70-expressing cell line designated CT70-P3, the human CD70 cDNA was cloned by polymerase chain reaction (PCR) amplification of total cDNA from the human T-cell lymphotropic virus type I (HTLV-I)-transformed human T cell line, SLB-1, using a reverse transcription (RT)-PCR kit from Gibco BRL (Grand Island, NY), according to the manufacturer’s instructions. The primers used for PCR amplification of SLB-1 cDNA were as follows: 5′GCCAAGCTTATGCCGAAGAAGGTGCG3′ (containing an HindIII restriction site) and 5′GCCCTCATAGTCA-GGGCGGACCACTG3′ (containing an Xho I restriction site). The CD70 cDNA was subcloned into the pcDNA3 mammalian expression vector (Invitrogen Corp, La Jolla, CA). The murine nonsecreting myeloma cell line P3X63Ag8.653 (American Type Culture Collection, Rockville, MD) was transfected with linearized, empty pcDNA3 or CD70-pcDNA3 vector by electroporation, and selected in 1 mg/mL G418. Uniform, high-level expression of CD70 protein was confirmed by flow cytometric analysis (data not shown).

Multiparameter flow cytometric analysis. Cells were washed and then suspended in staining media (SM), consisting of 1% Hanks’ balanced salt solution (HBSS); 3% fetal calf serum (FCS), 0.1% Na2EDTA, and 1 μg/mL propidium iodide (Calbiochem, La Jolla, CA), plus saturating amounts of FITC- or PE-conjugated MoAbs specific for the cell surface antigens of interest. After 30 minutes at 4°C, cells were washed in SM and analyzed on a FACScan (Becton Dickinson). Dead cells and debris were excluded from analysis by characteristic forward and side scatter properties and propidium iodide staining as described. The relative level of antigen detected on the B cell surface is quantified as the mean fluorescence intensity ratio (MFI). MFI equals the mean fluorescence intensity (MFI) of cells stained with a specific FITC-conjugated MoAb, divided by the MFI of cells stained with a control IgG-FITC. This method controls for the nonspecific increase in autofluorescence seen in larger, more activated cells. We designated a CLL B-cell population as being positive for expression of an antigen if its MFI was at least 1.25 times that of leukemia cells stained with an isotype control MoAb, and when over 10% of the specific MoAb-stained cells exhibited higher fluorescence than 99% of control MoAb-stained cells. The statistical significance of data that appeared to fall in a normal distribution was assessed by Student’s t-test. For those data that did not fit a normal distribution, results of both the nonparametric sign test and the more commonly used Student’s t-test are given.

B-cell stimulation with soluble, crosslinked CD40L. Hybridoma cells expressing a murine CD40L-CD8 fusion protein were obtained from F. Lane (Basel Institute for Immunology, Basel, Switzerland). Supernatants of these cells were collected and pooled. The rat-antimouse CD8 IgM MoAb 3.168, was added to CD40L-CD8 supernatant at 20 μg/mL to enhance CD40 crosslinking. B cells were suspended in RPMI-10 (RPMI-1640 + 10% FCS + 4 mmol/L L-glutamine) at a final density of 2 x 10^6/mL, and the supernatant–anti-CD8 solution was added at a final concentration of 25%. Cells were cultured, as above, for 48 to 72 hours before harvesting for analysis.

CD32-L cell assay. To crosslink the cell surface antigens CD40 or CD120b, we used a modified version of the B-cell in vitro culture system of Banchereau et al., as previously described.

Soluble CD27 enzyme-linked immunosorbent assay (ELISA). Determination of soluble CD27 levels in patient serum or cell supernatants was performed using a previously described ELISA kit (CLB, Amsterdam, The Netherlands) according to the manufacturer’s instructions.

RNA analysis. CLL B cells at greater than 99% purity were stimulated using the CD32-L cell system, as above, and total RNA was isolated using the RNA STAT-60 kit (Tel-Test “B” Inc, Friendswood, TX). Total RNA was slot-blotted onto nylon membranes at increasing dilutions as described. Membranes were incubated in prehybridization buffer consisting of 5 x SSPE (0.9 mol/L NaCl, 50 mmol/L Na2HPO4, and 5 mmol/L EDTA, pH 7.7), 5 x Denhardt’s solution (0.1% bovine serum albumin (BSA), 0.1% Ficoll, and 0.1% polyvinylpyrrolidone), and 0.5% sodium dodecyl sulfate (SDS) for 6 hours at 65°C, followed by addition of 50 ng of 3P-labeled CD27 cDNA and overnight incubation at 65°C. Membranes were then washed twice in 2 x SSPE + 0.1% SDS at room temperature for 10 minutes, once with 1 x SSPE + 0.1% SDS at 65°C for 15 minutes, and once with 0.1 x SSPE + 0.1% SDS at 65°C for 10 minutes. Membranes were exposed to film and/or a phosphor-imaging cassette for 24 hours and read on a 425E Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Mixed lymphocyte reaction. CLL B cells were stimulated for 48 hours with CD40 MoAb or control MoAb as above, but in six-well culture plates with 1.5 x 10^6 CD32-L cells per well and 6 x 10^5 B cells per well in a total volume of 4 mL RPMI-10. B cells were stimulated from adherent CD32-L cells by gentle rinsing wells with RPMI-10, resulting in greater than 99% pure leukemia B cells as assessed by FACS (data not shown). B cells were treated with mitomycin-C as above, washed four times in RPMI-10, and then suspended in RPMI + 10% pooled human AB serum (RPMI-10 HAB; Gibco BRL Life Technologies). B cells were plated with various blocking or control MoAbs in 150 μL RPMI-10 HAB in triplicate in 96-well round-bottom plates (Corning Glass Works, Corning, NY) at 10^4 to 10^5 cells per well to give final responder T cell-to-stimulator B cell (R:S) ratios of between 1:1 and 25:1. After allowing MoAbs or fusion proteins to bind for 1 hour at 37°C, responder alloreactive formal PBL cells, isolated as previously described, were added at 10^5 per well in a final total volume of 200 μL, and plates were incubated at 37°C for 5 days. Proliferative response was measured by pulsing the cultures with 1 μCi [3H]-thymidine (ICN Biomedicals, Inc, Costa Mesa, CA) per well, 18 hours before harvesting onto glass fiber filters (PHD Cell Harvester, Cambridge Technology, Watertown, MA). Filter-bound [3H]-thymidine that had been incorporated into newly synthesized DNA was measured using a Beckman LS1801 scintillation counter (Beckman Instruments, Inc, Fullerton, CA).

RESULTS

Regulation of CD27 expression on CLL B cells by CD40L and TNF-α. Membrane CD27 antigen (mCD27) was con-
Fluorescence Intensity

constitutively expressed by leukemic cells from 18 of 18 B-CLL patients tested. However, the MFI of CLL B cells stained with CD27 MoAb varied between the leukemic cell populations of different patients (Fig 1), as noted previously. We find that crosslinking CD40 with CD40 MoAbs presented on CD32-L cells, or with soluble, crosslinked CD40L, decreased the level of mCD27 expressed on CLL B cells by an average of 47% ± 23% (mean percent decrease in CD27 MFI of control treated cells ± SD; n = 15 experiments on nine different patients, P < .0001 by Student’s t-test; Fig 2). Constitutive mCD27 expression also is reduced on CLL B cells cultured with rhTNF-α (Figs 2 and 3) or rhIFN-γ (not shown) by an average of 39% ± 15% (n = 12, P < .0001) or 33% ± 7% (n = 7, P < .0001), respectively.

Fig 1. CLL B cells constitutively express CD27 and CD70. Freshly isolated CLL B cells from three different, representative patients were analyzed for expression of CD27 (top row) or CD70 (bottom row) by flow cytometry. Shaded histograms represent staining of live, CD19+ cells with FITC-conjugated isotype control MoAb. Open histograms represent staining with FITC-conjugated MoAbs specific for CD27 or CD70.

Fig 2. Summary of reciprocal changes induced in expression of CD27 and CD70 by various culture conditions. Depicted is the average percent change in MFI of CD27 or CD70 after 72-hour culture in each of the conditions listed (see Materials and Methods for details). Culture in media without added exogenous cytokine or with control Ig did not affect any changes in the MFI of cells stained with MoAbs specific for either of the two surface antigens.

Fig 3. Differential regulation of CD27 expression by CD40 crosslinking, rhTNF-α, rhIL-4, or rhIL-10. CLL B cells from two representative patients were cultured for 72 hours on a monolayer of CD32-L cells before harvesting for flow cytometric analysis of CD27 expression. Staining with FITC-conjugated isotype control MoAb is represented by the solid histograms, and staining with CD27-MoAb FITC is represented by shaded and open histograms. Shaded histograms represent CD27 expression on control-treated CLL B cells. Open histograms represent CD27 staining after culture with 20 ng/mL of rhIL-4, rhIL-10, or rhTNF-α or 250 ng/mL CD40 MoAb, as labeled in each histogram. The four histograms are representative data obtained from analysis of the leukemia cells from one patient with B-CLL.
Leukemia cell expression of mCD27 was enhanced, however, after culture in media with exogenous rhIL-4 or rhIL-10 (Figs 2 and 3). Each cytokine induced a dose-dependent increase in three of three CLL B-cell populations tested. Culture in the presence of rhIL-4 for 48 to 72 hours increased the MFIR of cells stained with CD27-specific MoAbs in 11 of 14 experiments by an average of 38% (range, 15% to 130%; median, 23%; Fig 2). This increase in specific staining was significant (P = .017 by Student’s t test or P = .001 by nonparametric sign test). Culture with rhIL-10 also induced higher expression levels of mCD27 in 9 of 13 experiments by an average of 24% (Fig 2). However, the median increase in staining intensity was only 10%, ranging from 1% to 79%, and was of borderline significance (P = .056 by Student’s t test and P = .004 by sign test). The increase in mCD27 expression induced by these cytokines is nearly abrogated by simultaneous CD40 crosslinking by MoAb on CD32-L cells or by soluble, crosslinked CD40L (Fig 4 and data not shown). We find that mCD27 was regulated similarly in all CLL samples tested, regardless of their initial expression level of mCD27.

One mechanism whereby cytokines or CD40 crosslinking could affect an increase or decrease in the levels of mCD27 is by respectively enhancing the retention or release of mCD27 as a soluble molecule. However, we find that changes in the levels of soluble CD27 (sCD27) released by CLL B cells into the culture supernatants after stimulation with IL-4 or CD40 crosslinking paralleled those noted for cell-surface expression of mCD27 (Fig 4).

Conditions that affect changes in CD27 affect reciprocal changes in expression of its ligand, CD70. Freshly isolated CLL B cells from different patients are heterogeneous in their expression levels of CD70. However, 17 of 19 CLL patients tested had leukemic cells expressing detectable levels of this surface antigen. The leukemic cells of three CLL patients representative of high, moderate, and negative CD70 expression are shown in Fig 1. In contrast to CLL B cells, only a small fraction of normal, freshly isolated blood B cells express CD70 (Fig 5). Crosslinking surface CD40 with CD40 MoAbs presented on CD32-L cells (Fig 5), or soluble, crosslinked CD40L-CD8 fusion protein (data not shown) increased the expression of CD70 on 10 of 10 different CLL and five of five normal B cell samples tested (Figs 2 and 5). CLL B cells from different patients responded similarly to the stimuli used, regardless of their initial level of CD70 expression (Fig 6). The kinetics of CD70 induction after CD40 crosslinking are similar to those of CD80 induction: apparent at 24 hours and reaching a maximum at 48 to 72 hours (data not shown).

Culture in the presence of rhTNF-α also significantly increased the MFIR of CD70 expression on eight of eight different CLL B-cell populations by an average of 43% over control treated cells (n = 19 independent experiments; Fig 2), with increases ranging from 0% to 130% (Fig 6). The median increase in the CD70 MFIR for all rhTNF-α-treated CLL cell samples was 46%. This increase was highly significant (P = .0001 via the nonparametric sign test or the Student’s t test). In a limited number of experiments, rhTNF-
CD70 also increased CD70 expression in two of two CLL samples tested (data not shown). The combination of CD40 crosslinking and rhTNF-α induced greater levels of CD70 than either one alone (data not shown). The presence of rhIFN-γ during CD40 crosslinking also significantly increased CD70 expression by an average of 23% ± 17% (n = 4; P = .037). Acting alone, rhIFN-γ also induced an increase in the expression of CD70 (Figs 2 and 6).

On the other hand, upon culture with exogenous rhIL-4, CLL B cells decreased their baseline expression of CD70 in 12 of 15 experiments (Figs 2 and 6). The average decrease in CD70 expression in IL-4–treated cultures compared with control cultures was 25% ± 15% (SD). This decrease was highly significant (P = .0001). Culture in the presence of rhIL-10 also resulted in reduced expression of CD70 in seven of nine experiments. The average decrease in CD70 expression by IL-10–treated cells versus control cultured cells was 28% ± 17% (P = .0005; Fig 2). In addition, rhIL-10 significantly inhibited CD70 upregulation mediated by CD40 crosslinking by 69% ± 33% (n = 8, P = .0006). The effect of rhIL-4 on CD40-mediated CD70 upregulation, however, was inconsistent and varied between different CLL patient samples.

CD40 crosslinking reduces expression of CD27 protein and CD27 mRNA. Because CD40 crosslinking enhances the expression levels of CD70, we examined whether the concomitant reduction in expression of CD27 was simply a consequence of ligand-receptor crosslinking. We observed that 48-hour coculture of CLL B cells from any one of five randomly selected CLL patients with a transfected murine myeloma cell line expressing CD70 (CD70-P3) resulted in a 75% ± 11% decrease in mCD27 expression on the CLL B cells (mean percent decrease in CD27 MFIR ± SD, n = 5; P < .0001; Fig 7). This treatment also resulted in an 11 ± 6.5-fold increase, on average, in the expression of CD70 on the CLL B cells (n = 5, P = .021; Fig 7). As shown in Fig 7, culture with P3 cells transfected with a control plasmid had no effect on CD27 or CD70 expression. These results indicate that CD70 can directly downmodulate mCD27 expression. As such, CD40 crosslinking conceivably could reduce expression of CD27 indirectly by enhancing expression of CD70.

To assess the effects of crosslinking CD40 on CD27 gene expression, we stimulated CLL B cells with control MoAb or CD40 MoAb on CD32-L cells for 12 hours and isolated total RNA for analysis of CD27 mRNA expression by slot blot. As shown in Fig 8A, CD40 crosslinking decreased leukemic B-cell expression of CD27 mRNA approximately 10-fold compared with control treated cultures in two separate experiments with two different CLL B-cell clones. In contrast, coculture with CD70-P3 cells or control transfected P3 cells had no effect on the expression of CD27 mRNA (Fig 8A). On the other hand, CD40 crosslinking increased leukemic B-cell expression of CD70 mRNA approximately 10-fold compared with control treated cultures (Fig 8B). These results indicate that the downregulation of CD27 antigen expression that is effected by CD40-crosslinking does not occur simply through CD70-mediated CD27 ligand-receptor downmodulation.

CD70 acts as a costimulatory molecule for T-cell proliferation. We previously reported that CLL B cells act as more stimulatory presenters of alloantigen to normal T cells in the mixed lymphocyte reaction (MLR) when they are previously stimulated through CD40.11 Upregulation of CD80 (B7-1) and B7-2 expression in part accounts for this increased antigen-presenting cell (APC) activity, as it can be partially inhibited by CTLA4-Ig, a fusion protein that blocks the interaction of CD28 or CTLA-4 with CD80 or B7-2.11,35

To determine whether CD70 expression affects the APC function of CLL B cells, we performed MLRs using CD40 MoAb-activated CLL B cells as stimulators and blocked CD70-cD27 interactions with CD70 MoAb or a CD27-Ig fusion protein.17 We find that the addition of either CD70 MoAb or CD27-Ig fusion protein significantly inhibits the ability of MoAb CD40-stimulated CLL B cells to induce T-cell proliferation in the MLR (Fig 9). Optimal amounts of CD70 MoAb inhibited, on average, 51% ± 27% of T-cell proliferation induced by activated CLL B cells (n = 7, mean ± SD; P = .003 by Student’s t test). However, CD70 MoAbs do not inhibit IL-2–induced T-cell proliferation in the absence of costimulatory cells, indicating that CD70 MoAbs are not nonspecifically inhibitory (Fig 9A and data not shown). The combination of CTLA4-Ig and either CD70 MoAb or CD27-Ig is significantly more inhibitory than either of these agents alone (Fig 9B; P = .037). In four independent experiments, addition of CD70 MoAb with CTLA4-Ig resulted in an average 55% greater inhibition in the MLR than
that observed with CTLA4-Ig alone. Similar results were obtained when Epstein-Barr virus (EBV)-transformed B cell lines that co-express CD80 and CD70 were used as stimulators in the MLR (data not shown).

Because CD70 MoAbs can react specifically with a subset of activated T cells and can directly affect their ability to proliferate in vitro, we evaluated whether the CD70 MoAb used in our studies could react with the T cells in the MLR. After 3 days' coculture with allogeneic CD40 MoAb-activated CLL B cells, the CD2-reactive T cells did not have detectable binding activity for the CD70 MoAb (Fig 9C). On the other hand, the CD70 MoAb still had binding activity

![CD27 and CD70 Expression](image)

Fig 7. Coculture with CD70-expressing cells downmodulates CD27 and increases CD70 expression on CLL B cells. CLL B cells (2 x 10^6) were cultured for 48 hours in media alone (left), with 10^6 control-transfected P3 cells (center), or 10^6 CD70-P3 cells (right). P3 and CD70-P3 cells were pretreated with mitomycin-C to prevent proliferation. Expression of CD27 (top row) and CD70 (bottom row) on CLL B cells was assessed by flow cytometry. B-CLL cells were specifically analyzed by electronic gating on cells expressing CD19, that had forward and side scatter profiles appropriate for the size CLL B cells, and excluded propidium iodide (viable cells). Shaded histograms represent staining of gated cells with FITC-conjugated control MoAb. Open histograms represent staining with FITC-conjugated MoAbs specific for CD27 (top row) or CD70 (bottom row).

![CD27 and CD70 mRNA Expression](image)

Fig 8. Effect of CD40 crosslinking on levels of CD27 and CD70 mRNA in CLL B cells. CLL B cells at greater than 98% purity were cultured on a monolayer of CD32-L cells with 250 ng/mL isotype control MOPC 21 MoAb (MOPC) or CD40 MoAb (α-CD40) for 12 hours, followed by isolation of total cellular RNA. In a separate experiment, 10^7 CLL B cells were cocultured for 48 hours with 5 x 10^5 P3 cells transfected with control vector (P3) or a vector directing expression of human CD70 (CD70-P3). When surface CD27 expression was fully downmodulated, total cellular RNA was isolated. (A) RNA (5, 1, or 0.2 μg) from each sample was slot-blotted onto a nylon membrane and probed using the human CD27 cDNA (CD27). A Phosphorimager was used to expose the blots. To control for RNA loading, the membrane was stripped and reprobed for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH). Bands in the P3 rows appear lighter due to the dilutional effect of the P3 RNA. Data are from one representative experiment of three performed on cells from two different CLL patients. (B) RNA (5, 1, or 0.2 μg) from each sample was slot-blotted onto a nylon membrane and probed using the human CD70 cDNA (CD70). Again, to control for RNA loading, the membrane was stripped and reprobed for G3PDH.
above that noted for an isotype control MoAb for the CD2-negative, mitomycin-treated CLL B cells used as stimulators in the MLR. This makes it unlikely that the inhibitory effects of the CD70 MoAb on T-cell proliferation in the MLR are secondary to direct inhibition of T-cell/T-cell interactions.

**DISCUSSION**

We note that CLL B cells express the CD27 antigen, confirming a recent study by Van Oers et al.\(^3\) and earlier reports.\(^3,32\) CD27 is expressed on a subset of normal B cells that are present in blood and may be localized to the germinal centers of lymphoid organs.\(^39\) Maurer et al.\(^43-44\) have suggested that mCD27 expression on normal B cells is associated with the phenotype of a previously activated B cell, being surface IgD\(^-\) and primed to secrete Ig after in vitro stimulation.

In this study, we also find that the leukemic cells from most patients with CLL coexpress the ligand for CD27, namely, CD70. Our results contrast with those reported previously by other investigators that indicated that only a minority (range, 10% to 32%) of CLL patients have CD70-expressing leukemic cells.\(^33,34\) Perhaps this apparent contrast results from differences in criteria used to define a leukemia population as being positive for CD70 expression. However, we consider this unlikely. Even if we increase the threshold for defining a leukemia cell population as being positive for CD70 to when it displays an MFIR of 2, or when \(\geq 20\%\) of the CD70-stained cells fluoresce more brightly than 99% of control-stained cells, 12 of 19 (63%) patients still would be classified as being positive for CD70 in our survey. It is possible that our use of directly FITC-conjugated CD70 MoAb resulted in greater sensitivity in our flow cytometric analyses compared with that of previous studies.

We find that the expression levels of these two colligands on the leukemic cells from a single CLL patient do not appear to be correlated, either positively or negatively. Some patients' cells express high levels of both mCD27 and CD70, while others express only CD27 at high levels or express low levels of both CD27 and CD70. However, in no case did we observe resting leukemia cells to express high levels of CD70 and low or negligible levels of CD27.

Despite the finding that there is no apparent relationship between the relative levels of CD27 and CD70 on freshly isolated leukemia cells, we found that the induced expression of the molecules is reciprocally regulated. IL-4 and IL-10, generally associated with a Th\(2\) or antibody-dominated immune response,\(^45-48\) enhanced mCD27 expression but down-modulated the constitutive expression of CD70 on CLL B cells. In contrast, the Th\(1\)-associated cytokine IFN-\(\gamma\)\(^49-50\) decreased constitutive mCD27 expression, as did TNF-\(\alpha\) and crosslinking of surface CD40. CD40 crosslinking and TNF-\(\alpha\) upregulated CD70 expression when acting alone, and IFN-\(\gamma\) could augment this activity. These findings are consistent with the fact that activated T cells, which may express

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**Fig 9.** CD70-CD27 interactions contribute to the T-cell proliferation induced by MoAb CD40-activated CLL B cells. CLL B cells were cultured for 72 hours on a monolayer of CD32-L cells in the presence of 250 ng/ml CD40 MoAb before use as stimulator cells in the MLR. Normal, allogeneic T cells (10\(^5\)) and 3 \(\times\) \(10^4\) CLL B cells were cocultured for 5 days, and T-cell proliferation was measured by uptake of \(3^H\)-thymidine. (A) Increasing amounts of ascites fluid containing CD70 MoAb were added at the beginning of culture in the MLR (open circles, left ordinate) or to cultures of T cells plus 50 U/ml of rhuIL-2 (solid circles, right ordinate). Symbols represent the mean \(\pm\) SD of triplicate measurements. The addition of MOPC 21 isotype control MoAb had no inhibitory effect in this experiment (data not shown). Data are from one representative experiment of four performed. (B) Control MoAb, CD27-Ig, and/or CTLA4-Ig fusion proteins were added at the beginning of the MLR. Antibodies were used at a concentration of 2 \(\mu\)g/ml, unless otherwise noted. Bars represent the mean \(\pm\) SD of triplicate measurements. Data are from one representative experiment. (C) At day 3 of the MLR, cells were harvested and stained with PE-conjugated CD2 MoAbs and FITC-conjugated CD70 MoAb or an isotype control myeloma protein, MOPC21. Cells were analyzed on the FACS. The left and right histograms, respectively, display the green fluorescence activity of the electronically gated CD2-reactive T cells (CD2\(^+\)) and the CD2-reactive CLL B cells (CD2\(^-\)).
CD40L\textsuperscript{10} and membrane or soluble TNF-\textalpha\textsubscript{1,2,23,51} can induce B cells to express CD70.\textsuperscript{16} We find that such stimulation also simultaneously reduces the expression of mCD27 on CLL B cells. As found in T cells after CD3 crosslinking,\textsuperscript{22} the expressions of soluble and membrane CD27 in CLL B cells vary coordinately after stimulation with CD40 or IL-4. Thus, an increased release of CD27 cannot account for the CD40L-mediated downmodulation of mCD27.

It is possible that some mCD27 downmodulation occurs indirectly in response to interaction with CD70, the latter being upregulated by the same mediators that decrease mCD27 expression. We observed that coculture of CLL B cells with cells expressing high amounts of CD70 downmodulated mCD27, as has been observed in T cells.\textsuperscript{16} This may be due to shedding of CD27 or to ligand-receptor downmodulation. However, this mechanism appears not to fully account for the regulation of these molecules on CLL B cells. First, CD27 and CD70 are coexpressed at high levels on many CLL B-cell populations. Second, CD40 crosslinking decreases levels of CD27 mRNA approximately 10-fold in CLL B cells. In contrast, downmodulation of mCD27 from the cell surface in response to ligation by CD70-expressing P3 cells is not accompanied by decreased CD27 mRNA levels. To our knowledge, this is the first demonstration that CD40-mediated signaling can affect negative regulation of gene expression.

Although CLL and normal B cells respond similarly to CD40 crosslinking in terms of CD70 and CD80\textsuperscript{11} upregulation, it is possible that the some of the findings on the regulation of CD27 antigen expression are unique to CLL B cells. In preliminary studies, neither IL-4, IL-10, nor CD40 crosslinking affected a change in the heterogeneous expression of mCD27 on normal B cells. On T cells, CD27 expression is increased after TCR crosslinking\textsuperscript{22} but was reported not to be affected by some of the cytokines used in this study.\textsuperscript{74}

Previously, both positive\textsuperscript{23,26} and negative\textsuperscript{72} autocrine cytokine cytokine loops have been described for CLL B cells. However, although CD27 has been shown to provide costimulatory signals for T-cell proliferation and differentiation,\textsuperscript{16,17,58} the functional effects of CD27 signaling in normal or leukemic B cells are unknown. The data presented here do suggest a potentially important but indirect effect of membrane and/or soluble CD27 protein expression on B cells; ie, inhibition of the costimulatory effect of CD70 on T-cell proliferation. Another possibility not excluded in this study is that CD27 signaling may act directly to enhance expression of its own ligand.

We previously found that CD40 crosslinking induced CLL B cells to become more potent presenters of alloantigen in the MLR.\textsuperscript{11} This enhanced APC function is mediated in part by the upregulation of CD80 and CD54 expression on the B cells. Although the combination of CTLA4-Ig and CD11a MoAb could inhibit 95% of the enhanced T-cell stimulation afforded by using stimulator CLL B cells that had been preactivated via CD40 crosslinking,\textsuperscript{11} these studies cannot exclude the possibility that other accessory molecules also play a role in T-cell/B-cell alloantigen presentation. Indeed, in the present study, we find that the increased expression of CD70 after CD40 crosslinking also can contribute to the costimulatory activity of CD40-activated CLL B cells. The addition of either CD70 MoAb or CD27-Ig fusion protein to the MLR inhibits the T-cell proliferation induced by activated CLL B cells or CD70\textsuperscript{9} cell lines. Direct negative signaling through CD70 to T cells is unlikely, as treatment of T cells, in the absence of APCs, with CD70 MoAb or CD27-Ig did not inhibit their proliferative response to IL-2. Moreover, despite the fact that some activated T cells express CD70,\textsuperscript{16,42} we did not detect CD70 expression on T cells stimulated by alloantigen in the MLR (Fig 9C). Perhaps this could be secondary to allosteric changes or receptor-mediated downmodulation of the CD70 antigen on the activated T cells during coculture with the mitomycin-treated CLL B cells, thereby precluding the CD70 MoAb from binding the cultured T cells. In any case, the CD70 MoAb still had specific binding activity for the mitomycin-C--treated, activated CLL B cells in the MLR (Fig 9C), arguing that the inhibitory effects of the CD70 MoAb in the MLR were secondary to inhibition of T-cell/B-cell, rather than T-cell/T-cell, interactions.

In this regard, our results confirm and extend the findings of Hintzen et al,\textsuperscript{16} who observed an inhibitory effect of CD70 MoAb on the costimulatory function of transformed B cell lines. It is possible that CD70-CD27 interactions may enhance APC function primarily by increasing the avidity of T-cell/APC interactions. However, the noted ability of CD27L (CD70) to costimulate T-cell proliferation induced by CD3 antibodies\textsuperscript{16,17,59} makes it appear likely that CD70 expressed by APCs may provide a direct, positive, costimulatory signal through CD27. The combination of CD27-Ig or CD70 MoAb with CTLA4-Ig to block both CD27-CD27 interactions and the function of B7-like molecules results in greater inhibition of APC function than that observed with maximal amounts of CTLA4-Ig alone. This suggests that CD70-CD27 interactions may provide a proliferative signal to T cells that is distinct from that mediated through CD28. Since submission of this article for publication, Kobata et al\textsuperscript{6} have reported findings that support this hypothesis.

Collectively, the present study and our previous results\textsuperscript{11} indicate that at least two potentially important costimulatory molecules, CD80 and CD70, are expressed on B cells after interaction with activated T cells expressing CD40L and/or TNF-\textalpha. However, the cytokine profile of the response may add an additional layer of complexity to this schema. IL-4 and IL-10 both augment CD40-mediated expression of CD80.\textsuperscript{11} In contrast, both IL-4 and IL-10 downmodulate constitutive CD70 expression on CLL B cells and inhibit its induction by CD40 crosslinking. Meanwhile, the Th1 cytokines IFN-\gamma and TNF-\beta augment the expression of CD70 alone, or with CD40 crosslinking. In a murine system, other investigators have shown that the presence of IL-4 during T-cell activation results in Th2-type cells, while the inhibition of IL-4 or the presence of IFN-\gamma results in Th1 cells secreting a Th1 cytokine profile.\textsuperscript{51,62} In addition to the direct effects of these cytokines on T cells, indirect effects mediated through APCs also are possible. By regulating the expression of costimulatory molecules on monocytes and B cells, different cytokines may determine which cell type will be the major stimulatory APC during a response, thereby indirectly inducing a Th1 or Th2 T-cell phenotype.\textsuperscript{50} Alternatively, the nature and source of the costimulatory signal itself may direct T...
cells to secrete Th1 or Th2 cytokines. Direct evidence in support of these possibilities is not yet available, but further investigation of the regulation and function of the TNF/TNF-R family of proteins may yield novel methods for inducing, preventing, or redirecting the immune response in vivo.

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Expression of CD27 and its ligand, CD70, on chronic lymphocytic leukemia B cells

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