

CD40 Ligation Induces Apo-1/Fas Expression on Human B Lymphocytes and Facilitates Apoptosis through the Apo-1/Fas Pathway

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Summary

The Apo-1/Fas antigen (CD95) mediates programmed cell death of lymphocytes when bound by Fas ligand or anti-Apo-1/Fas antibody. In contrast, the CD40 antigen provides a potent activation and survival signal to B lymphocytes when it is engaged by its T cell ligand (CD40L, gp39) or cross-linked by anti-CD40 antibody. In this study, we use human tonsillar B cells and the Ramos Burkitt's lymphoma B cell line, which serves as a model for human germinal center B lymphocytes, to study the effectors of Apo-1/Fas expression and apoptosis of human B cells. We found that Apo-1/Fas expression was upregulated on both malignant and normal human B lymphocytes after CD40 ligation induced by (a) cognate T helper-B cell interaction mediated by microbial superantigen (SAg); (b) contact-dependent interaction with CD40L⁺, but not CD40L⁻ Jurkat mutant T cell clones; and (c) monoclonal anti-CD40, but not any of a panel of control antibodies. Enhanced B cell Fas/Apo-1 expression is functionally significant. Coculture of Ramos Burkitt's lymphoma line cells with irradiated SAg-reactive CD4⁺ T cells with SAg or CD40L⁺ Jurkat T cells results in B cell apoptosis, evidenced by reduced cell viability and DNA laddering. This process is augmented by the addition of anti-Apo-1/Fas monoclonal antibody, consistent with an acquired susceptibility to Apo-1/Fas-mediated apoptosis. These data support an immunoregulatory pathway in which seemingly contradictory signals involving the B cell proliferation/survival antigen CD40, as well as the Apo-1/Fas molecule, which mediates programmed cell death of lymphocytes, are linked in the process of human B cell activation.

Apo-1/Fas (CD95) is a 48-kD cell surface antigen of the ATNF/nerve growth factor (NGF)¹ receptor superfamily that mediates apoptosis of T and B lymphocytes (1). The interaction of Apo-1/Fas and its ligand have been shown to play a central role in the regulation of programmed cell death of mature peripheral murine and human T lymphocytes. Defects in both Fas and its ligand have been described in murine models of lymphoproliferative disease characterized by features of SLE (2-4), suggesting that this pathway may be responsible for the deletion of au-

toxic clones. In peripheral T lymphocytes, TCR signaling leads to upregulation of Apo-1/Fas and, on Th1 cells, Fas ligand expression, resulting in fratricide or even suicide of antigen-activated T cells (5-8). Although Apo-1/Fas expression occurs early after antigen stimulation, susceptibility to Apo-1/Fas-mediated apoptosis develops slowly (9), allowing for the clonal expansion and effector function of antigen-activated T cells before their apoptotic elimination. B lymphocytes also express the Apo-1/Fas receptor (10, 11), and they are induced to undergo apoptosis when bound by Fas ligand, which is secreted selectively from Th1 cells (12, 13). The observations that the mAb to human Apo-1/Fas can induce apoptosis of B lymphoblastoid tumor cells in vitro (14) and of xenografted human B cell tumors in vivo (15) suggest that the Apo-1/Fas signal-

¹Abbreviations used in this paper: CD40L, CD40 ligand; MAM, *Mycoplasma arthritidis*-derived microbial superantigen; MHC II, MHC class II; NGF, nerve growth factor; SAg, microbial superantigen; TSST-1, toxic shock syndrome toxin 1; T_{MAMxT}, irradiated MAM-reactive CD4⁺ cells; T_{TSSTxT}, TSST-1-reactive CD4⁺ cells.

ing pathway may function in the immunosurveillance of B cell tumors.

The CD40 antigen is a 50-kD cell surface protein, also of the TNF/NGF receptor superfamily, which promotes B cell proliferation and survival. Germinal center B cells can be rescued from spontaneous apoptosis *in vitro* by soluble anti-CD40 mAb (16). When engaged by the T cell activation molecule CD40L (gp39) or when cross-linked by anti-CD40 antibody, CD40 induces a potent B cell activation signal (17, 18) that is characterized by expression of activation antigens (19, 20), increased DNA synthesis (21), tyrosine kinase activity (22), and Ig class switching (23–25).

The need to control the magnitude and duration of humoral immune responses after antigenic stimulation suggests that the pathways of B cell activation and apoptosis may be closely linked. In this study, we demonstrate that cognate Th–B cell interaction mediated by CD40 ligation leads to marked upregulation of Apo-1/Fas expression on normal and malignant human B lymphocytes. Using the Burkitt's lymphoma Ramos B cell line as a model of germinal center B cells (26, 27), we demonstrate that this interaction both upregulates Apo-1/Fas expression and facilitates susceptibility to Apo-1/Fas-mediated programmed cell death.

Materials and Methods

Cell Culture. B cells were isolated from fresh human tonsils as described previously (28). Briefly, T cells were eliminated by rosetting overnight with SRBCs, and the isolated B cell population was frozen in liquid nitrogen and cryopreserved at -70°C . B cells were thawed, washed, and cultured in C50 medium (RPMI 1640 medium with penicillin, streptomycin, glutamine, and 10% fetal bovine serum). The Burkitt's lymphoma Ramos B cell line was obtained from American Type Culture Collection (Rockville, MD) and grown in C50 as described above. Mutant clones of the Jurkat lymphoblastoid T cell lines B2.7 and D1.1 were kindly provided by Dr. Seth Lederman (Columbia University College of Physicians and Surgeons, New York, NY). B2.7 is deficient in CD40L expression, while clone D1.1 constitutively expresses CD40L (17, 29), which were confirmed by staining with antibody to CD40L (Genzyme Corp., Cambridge, MA). As indicated, cells were cultured in the presence of highly purified *Mycoplasma arthritidis*-derived microbial superantigen (SAG) (MAM) at a final concentration of 1:80,000 of stock solution, which was generously provided by Dr. B. Cole (University of Utah College of Medicine, Salt Lake City, UT) (30), or toxic shock syndrome toxin-1 (TSST-1) at a final concentration of 10 ng/ml (Toxin Technology, Sarasota, FL).

Coculture experiments were performed using 10^6 B cells and 2×10^5 irradiated MAM- or TSST-1-reactive CD4⁺ cells (T_{MAMxr} or T_{TSSTxr} , respectively), or with Jurkat T cells (B2.7_{xr} or D1.1_{xr}) in a final volume of 1 ml C50 media. After irradiation with 500 (SAG-reactive T cells) or 1,000 rads (Jurkat mutants), the T cells were still functional in cognate interaction, as assessed by CD23 and Apo-1/Fas induction on the B cells, but they died in culture within 24–48 h, as determined by FACS[®] analysis (Becton Dickinson & Co., Mountain View, CA) of viable cells for B and T cell markers.

mAbs. Sterile antibodies used in cell culture experiments include anti-human Fas (murine IgM; Upstate Biotechnology Inc.,

Lake Placid, NY) used at 100 ng/ml, and anti-CD40 (mouse IgG_{1k}, clone B-B20; Biosource International, Camarillo, CA), anti-CD40L (Genzyme), anti-CD71 (anti-transferrin receptor, mouse IgG_{1k}; Biosource), anti-CD54 (anti-ICAM; AMAC Inc., Westbrook, ME), and anti-CD37 (mouse IgG_{2a}; Biosource), W6/32 (anti-MHC I), NAMBI (anti- β 2-microglobulin), and L-243 (anti-MHC class II [MHC II]; gift of Dr. Carlo Russo, Cornell University Medical College), OKT3 (mouse IgG_{2a}; ATCC), anti-CD20 (PharMingen, San Diego, CA), and anti-CD23 IgG (EBVCS2; ATCC) or anti-CD23 IgM (EBVCS3; ATCC). For culture, these antibodies were used at 1 $\mu\text{g}/\text{ml}$ unless otherwise specified. Directly conjugated antibodies used in cell staining experiments include anti-Apo-1-biotin (IgG_{3k}) or anti-Apo-1/Fas-FITC (IgG₁; Immunotech, Inc., Westbrook, ME), B1 (Coulter Immunology, Hialeah, FL), anti-CD19-FITC (PharMingen), and anti-CD4-biotin (PharMingen). For blocking experiments, an F(ab)[']₂ preparation of the anti-Apo-1 IgG_{3k} antibody was used at 200 ng/ml (5).

Indirect Immunofluorescence Analysis. Cells were washed in cold HBSS and stained in primary antibodies (or control, 1% BSA in PBS with azide) for 45 min at 4°C . Cells were washed 3 \times in cold HBSS, incubated with secondary antibodies or FITC-avidin, as indicated, for 25 min at 4°C , and then washed 3 \times again in cold HBSS. Alternatively, cells were washed in cold HBSS and stained with a single, directly conjugated primary antibody before washing 3 \times before analysis. Cell fluorescence was analyzed on a FACScan[®] (Becton Dickinson) and analyzed with CellQuest (Becton Dickinson).

Superantigen-reactive CD4⁺ T Cell Lines. As described previously (29), PBL from normal donors were cultured at $2 \times 10^6/\text{ml}$ in the presence of highly purified MAM at a final dilution of 1:80,000 or TSST-1 at a final concentration of 10 ng/ml. After 3 d, the cultures were supplemented with human IL-2 (Schiapparelli, Columbia, MD), and 1 d later, blast and resting cells were separated by Ficoll gradient centrifugation. Purified CD4⁺ T cells were obtained by immune rosette depletion of CD8⁺ cells. These CD4⁺, SAG-reactive, T cell lines (T_{MAM} or T_{TSST}) were frozen -70°C . After thawing and washing, they were grown in the presence of IL-2, and periodically expanded by retriggering with the relevant SAG in the presence of irradiated, autologous non-T cells.

Viability Assay. Aliquots of cultured cells were diluted 1:4 in 0.4% trypan blue and were analyzed for the exclusion of dye by light microscopy.

DNA Analysis. After 2 d of culture with irradiated CD4⁺ T cells, viable B cell populations were purified by Ficoll Hypaque centrifugation. These cells were cultured for an additional 16 h in the presence of media alone, anti-Apo-1/Fas IgM or anti-CD23 IgM at a concentration of 200 ng/ml. Low molecular weight DNAs prepared from 10^6 cells by lysis in 20 μl buffer (10 mM EDTA, 50 mM Tris-Cl, pH 8.0, 0.5% SDS, 0.5 mg/ml proteinase K) at 50°C for 1 h, followed by the addition of RNase A (0.5 mg/ml) at 50°C for 1 h. After heating (70°C , 5 min), DNAs were analyzed by 1.6% agarose gel electrophoresis in the presence of ethidium bromide (0.16 $\mu\text{g}/\text{ml}$), and they were photographed under UV light.

Results

Cognate Th–B Cell Interactions Upregulate Apo-1/Fas Expression on Human Tonsillar and Ramos B Cells. To investigate the effect of Th–B cell interaction on B cell Apo-1/Fas

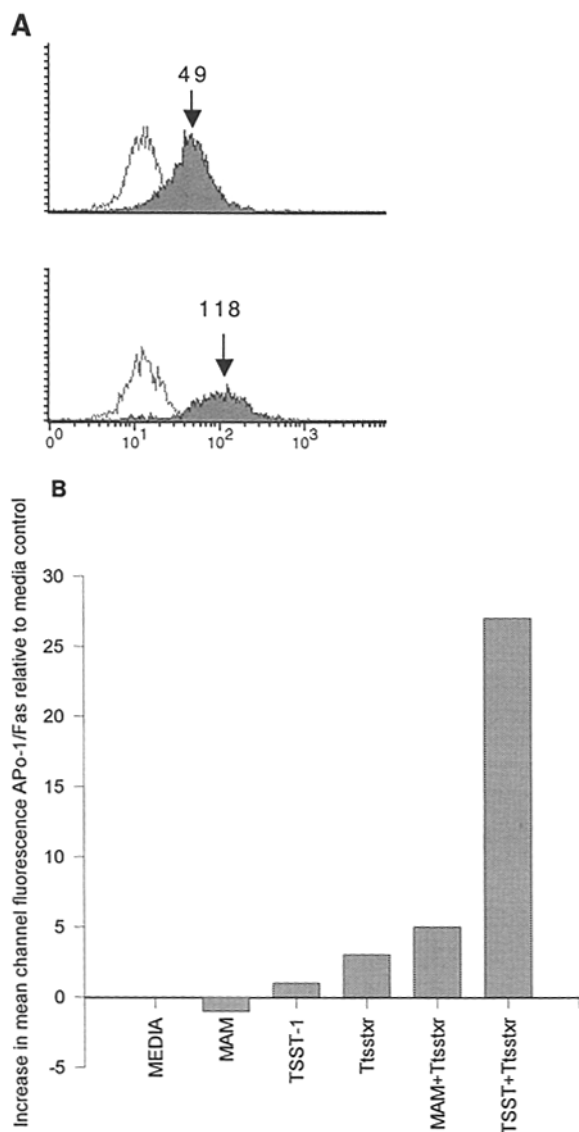


Figure 1. Superantigen-induced cognate T-B cell signaling upregulates Apo-1 expression on tonsillar B lymphocytes. (A) Flow cytometric analysis (FACS[®]) of human tonsillar B cells cultured for 48 h with (a) media or (b) CD4⁺ T_{MAMxr} in the presence of MAM. Cells were stained with a biotin-conjugated antibody to Apo-1, followed by FITC-avidin (shaded areas), or with a biotin-conjugated control antibody, followed by FITC-avidin (unshaded areas). The mean channel fluorescence for each Apo-1 stain is indicated. At the time of analysis, >99% of the gated cells were B1 positive, and <1% were CD4 positive. (B) Tonsillar B cells were cocultured with media, MAM, TSST-1, T_{TSSTxr}, MAM + T_{TSSTxr}, or TSST-1 + T_{TSSTxr}, and were analyzed for Apo-1/Fas expression by flow cytometry. The increase in mean channel fluorescence of Apo-1/Fas staining is shown relative to a mean channel fluorescence of 58, which was observed for the tonsillar B cells cultured with media alone.

expression, we took advantage of the dual affinity of SAg for MHC II molecules and particular TCR- β chain variable gene products (31, 32). These properties allow SAg to facilitate a nonphysiologic form of cognate Th-B cell interaction between SAg-bearing B cell populations and SAg-reactive CD4⁺ T cells (33). To determine if SAg-mediated Th-B interaction would influence B cell Apo-1/Fas ex-

pression, tonsillar B lymphocytes were cocultured with CD4⁺ T_{MAMxr} in the presence of MAM. Flow cytometric analysis of the B cell populations was performed after 48 h of culture. The tonsillar B cells constitutively express a relatively low level of Apo-1/Fas, which is augmented by the Sag bridge (Fig. 1 A). As shown in Fig. 1 B, this effect is specific, since TSST-reactive CD4⁺ T cells are effective in upregulating Apo-1/Fas expression only in the presence of TSST-1, and not when non-cross-reactive SAg (MAM) is present in the culture system.

To study the effect of cognate T-B cell interaction on Apo-1/Fas expression in a more homogeneous target B cell population, we focused on the Burkitt's lymphoma Ramos B cell line. Burkitt's lymphoma cell lines such as Ramos bear phenotypic and functional similarities to human germinal center B cells (26, 27). As shown in Fig. 2, although there was modest upregulation of Apo-1/Fas when the B cells were cultured with SAg-reactive CD4⁺ T cells alone, Apo-1/Fas expression was dramatically increased on Ramos B cells cultured with both MAM and T_{MAMxr}.

Apo-1/Fas Expression on B Cells Is Facilitated by the Interaction of CD40 with Its T Cell Ligand, CD40L. Cognate Th-B cell interaction is mediated, in large measure, by signals delivered to the B cell by the CD40 antigen (17). To investigate the role of CD40 ligation in T cell-induced B cell Apo-1/Fas expression, we used two well-characterized mutant clones of the Jurkat T cell line (17, 29). Clone D1.1 cells constitutively express high levels of gp39, the physiologic ligand of CD40 (CD40L), while clone B2.7 is deficient in CD40L expression. As shown (Fig. 3), coculture of Ramos B cells with the CD40L⁺ Jurkat clone, but not the CD40L⁻ Jurkat clone, induced marked upregulation of Apo-1/Fas expression on B cells. Similar results were obtained using tonsillar B lymphocytes (data not shown). In

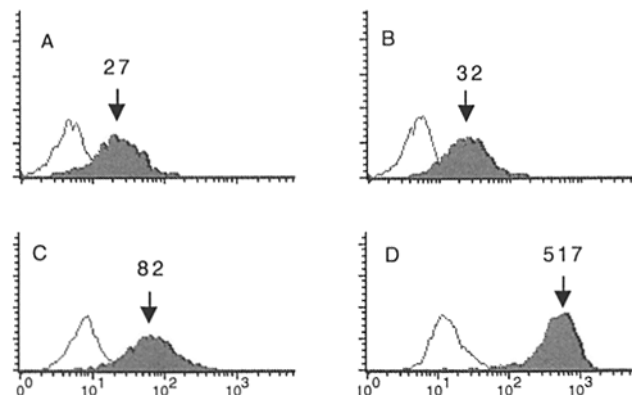


Figure 2. Superantigen-induced cognate T-B cell signaling upregulates Apo-1 expression on Ramos B lymphocytes. Flow cytometric analysis (FACS[®]) of Ramos B cells cultured for 72 h with (A) media, (B) MAM, (C) CD4⁺ T_{MAMxr}, or (D) MAM + CD4⁺ T_{MAMxr}. Cells were stained with a biotin-conjugated antibody to Apo-1, followed by FITC-avidin (shaded areas) or with a biotin-conjugated control antibody followed by FITC-avidin (unshaded areas). The mean channel fluorescence for each Apo-1 stain is indicated. At the time of analysis, >99% of the gated cells were B1 positive, and <1% were CD4 positive.

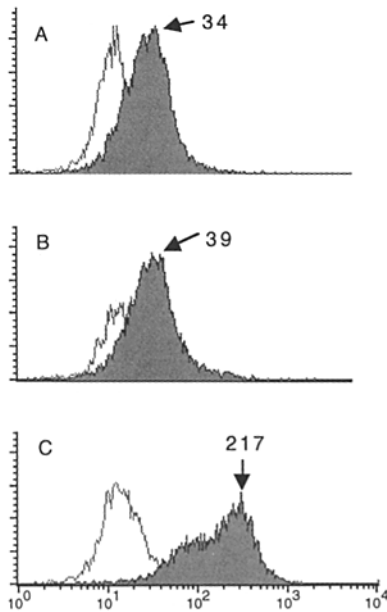


Figure 3. Apo-1 expression is induced on Ramos B cells by CD40L⁺ Jurkat T cells, but not CD40L⁻ Jurkat T cells. Ramos B cells were cultured for 48 h with (A) media alone, (B) with CD40L⁻ Jurkat T cells (B2.7_{sr}), or (C) with CD40L⁺ Jurkat T cells (D1.1_{sr}), and stained with a biotin-conjugated antibody to Apo-1 followed by FITC-avidin (shaded areas), or with a biotin-conjugated control antibody followed by FITC-avidin (unshaded areas). The mean channel fluorescence for each Apo-1 stain is indicated. At the time of analysis, >99% of the gated cells were B1 positive, and <1% were CD4 positive.

addition, phenotypic analysis of Ramos B cells cultured for 48 h with the Jurkat mutants (Fig. 4) demonstrated that increased Apo-1/Fas expression occurs in concert with enhanced expression of other CD40-inducible cell surface antigens including B7, CD23, MHC II, and CD54. CD19 and CD71 expressions were not affected. Experiments performed using mAb to CD40L demonstrate that the induction of Apo-1/Fas by CD40L⁺ Jurkat T cells is impeded by antibody to CD40L (Table 1).

To assess the requirement for cell-cell contact in this response, two-chamber experiments were performed in

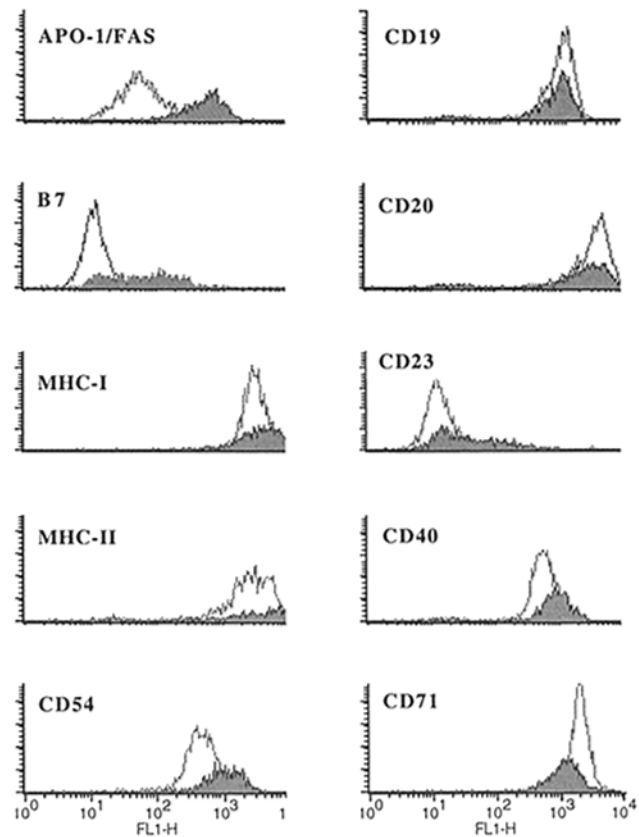


Figure 4. Activation antigen expression on Ramos B cells after coculture with CD40L⁻ and CD40L⁺ Jurkat T cells. Ramos cells were cultured with CD40L⁻ Jurkat T cells (B2.7_{sr}) or CD40L⁺ Jurkat T cells (D1.1_{sr}) for 48 h and then analyzed by indirect immunofluorescence with a panel of mAbs, as indicated.

which B cells were separated from Th cells by a semipermeable membrane. Ramos B cells were cultured in the lower chambers of wells with cytokine-permeable inserts (upper chambers) containing irradiated D1.1 or B2.7 Jurkat T cells with and without Ramos B cells. As shown (Table 2), Apo-1/Fas was induced on Ramos cells only when

Table 1. Apo-1 Induction on Ramos B cells by CD40L⁺ Jurkat Mutants Is Inhibited by Antibody to CD40L

Cell culture	B cell Apo-1/Fas expression	
	% +	Mean channel fluorescence
Ramos + media	16	27
Ramos + media + anti-CD40L	18	28
Ramos + media + anti-MHCI	17	27
Ramos + D1.1 _{sr}	83	100
Ramos + D1.1 _{sr} + anti-CD40L	19	28
Ramos + D1.1 _{sr} + anti-MHC I	83	96

5×10^5 Ramos B cells were cultured with media alone or with 10^5 irradiated CD40L⁺ Jurkat T cells (D1.1_{sr}) for 48 h in the presence or absence of blocking antibody (anti-CD40L), or a control antibody (anti-MHC I, W6/32), each at a concentration of 1 μ g/ml.

Table 2. *Apo-1 Induction on Ramos B Cells Requires Direct Contact with CD40L⁺ Jurkat Mutants*

Upper chamber	Lower chamber	B cell Apo-1/Fas expression	
		% +	Mean channel fluorescence
Media	Ramos	10	28
CD40L ⁻ T _{xr}	Ramos	14	28
CD40L ⁻ T _{xr} +Ramos _{xr}	Ramos	17	28
Media	Ramos+D1.1 _{xr}	52	59
CD40L ⁺ T _{xr}	Ramos	12	26
CD40L ⁺ T _{xr} +Ramos _{xr}	Ramos	10	26

5×10^5 Ramos B cells were cultured with media alone or with 10^5 irradiated, CD40L⁺ Jurkat T cells for 48 h. Each well (lower chamber) contained a cytokine-permeable insert (upper chamber) with 10^5 irradiated CD40L⁻ (B2.7_{xr}) or CD40L⁺ (D1.1_{xr}) Jurkat mutants with or without 10^5 irradiated Ramos cells (Ramos_{xr}), as indicated. Flow cytometry for Apo-1 expression on cells in the lower chamber was performed at 48 h. In the well with both Ramos and D1.1_{xr} Jurkat cells in the lower chamber, 94% of the cells were B1⁺, and 0.9% were OKT3⁺ at the time of analysis.

CD40L⁺ D1.1 Jurkat T cells were directly present in the lower chamber. There was no induction of Apo-1/Fas expression on Ramos cells in the lower chamber when B2.7 or D1.1 cells were present in the inserts, even when irradiated Ramos cells were added to the insert. The specific induction of Apo-1/Fas by the CD40L⁺ mutant Jurkat T cell line, in a process that requires direct cell-cell contact, supports the conclusion that CD40L-CD40 binding is sufficient for the upregulation of Apo-1/Fas on human B cells.

Soluble mAb to CD40 Induces Apo-1/Fas Expression on Normal and Malignant Human B Lymphocytes. To examine directly whether ligation of CD40 enhances B cell Apo-1/Fas expression, tonsillar or Ramos B cells were cultured in the presence of anti-CD40 or a control antibody and stained for Apo-1/Fas expression. As shown in Fig. 5 A, soluble anti-CD40 antibody upregulates Apo-1/Fas expres-

sion on tonsillar B cells during a 48-h period. Experiments using the Ramos system demonstrate that this effect is dose-dependent and occurs over a wide range of antibody concentrations (Fig. 5 B). Specificity of CD40 signaling was supported by the lack of Apo-1/Fas induction after treatment of Ramos B cells with a panel of mAbs to other B cell surface molecules, including MHC I, MHC II, and B7.

The Induction of Apo-1/Fas by Cognate T-B Cell Signaling Correlates with B Cell Apoptosis In Vitro. One of the most striking features of these experiments was the consistent observation that after cognate interaction with SA_g-reactive CD4⁺ T cells, a significant fraction of both tonsillar and Ramos B cells died in culture (Table 3). To examine the mechanism of B cell death after CD40 ligation and increased Apo-1 expression, Ramos B cells were cocultured with irradiated CD40L⁻ (B2.7_{xr}) or CD40L⁺ (D1.1_{xr}) Jur-

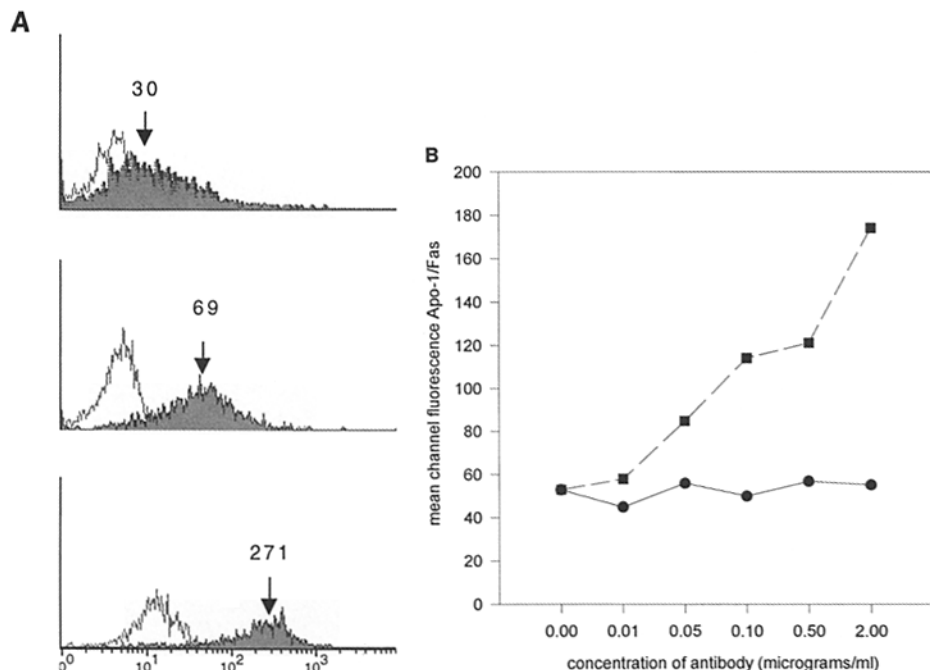


Figure 5. Apo-1 induction on human B cells by soluble antibody to CD40. (A) Human tonsillar B cells were cultured with media alone (a) or with mAb to CD40. After 24 (b) and 48 h (c), cells were stained using a biotin-conjugated antibody to Apo-1 (shaded areas) or with a biotin-conjugated control antibody (unshaded areas) followed by FITC-avidin. (B) Apo-1 expression is specifically induced on Ramos B cells by anti-CD40 antibody in a dose-dependent manner. Ramos B cells were cultured in the presence of media alone or with serial dilutions of anti-CD40 or anti-CD71 ranging from 2 μ g/ml to 10 ng/ml, and they were stained for Apo-1 expression using a direct FITC-conjugated anti-Apo-1/Fas mAb. ■, anti-CD40; ●, anti-CD71.

Table 3. Cognate Signaling Induced by Superantigen or by DC40L⁺ Jurkat T Cells Is Associated with B Cell Death

Culture conditions	Viability at 48 h
Tonsillar B cells + media	50%
Tonsillar B cells + CD40L ⁻ T _{xr}	20%
Tonsillar B cells + CD40L ⁺ T _{xr}	<5%
Tonsillar B cells + MAM + CD4 ⁺ T _{MAMxr}	<1%
Ramos + media	76%
Ramos + Jurkat CD40L ⁻ T _{xr}	69%
Ramos + Jurkat CD40L ⁺ T _{xr}	41%
Ramos + MAM	60%
Ramos + CD4 ⁺ T _{MAMxr}	84%
Ramos + MAM + CD4 ⁺ T _{MAMxr}	16%

Tonsillar or Ramos B cells were cultured under conditions as indicated, and assessed for viability by trypan blue exclusion after 48 h. These results are representative of three separate experiments.

kat mutants, or CD4⁺ T_{MAMxr} in the presence or absence of MAM. After 48 h, viable B cells were isolated from each culture by Ficoll-Hypaque gradient centrifugation. After overnight incubation with medium, IgM anti-Apo-1/Fas, or IgM anti-CD23 as control, low molecular weight DNAs were isolated and assessed for fragmentation (Fig. 6). As shown, Ramos B cells cultured with CD40L⁻ Jurkat T cells do not exhibit DNA fragmentation. In the case of Ramos B cells cultured with CD4⁺ T_{MAMxr} in the absence of MAM, modest upregulation of Apo-1/Fas antigen is induced (Fig. 2 C) and DNA fragmentation occurs after the

addition of anti-Apo-1/Fas, but there is no spontaneous apoptosis. In contrast, Ramos B cells cultured with D1.1_{xr} or CD4⁺ T_{MAMxr} in the presence of MAM, are induced to undergo apoptosis. This process is augmented by the addition of IgM anti-Apo-1/Fas antibody, but not of the isotype-matched control antibody. Pretreatment of the Apo-1/Fas-expressing Ramos B cells with a blocking F(ab)₂' preparation of the anti-Apo-1 IgG_{3k} antibody abrogated this effect (Table 4 B). Taken together, these results suggest that cognate signaling through the CD40 pathway both upregulates Apo-1/Fas expression on B cells and renders these B cells susceptible to Apo-1/Fas-mediated apoptosis.

Discussion

The present report provides evidence for a new outcome of CD40 signaling. Upregulation of Fas/Apo-1 antigen on normal and malignant human B cells occurs after (a) cognate Th-B cell interaction mediated by SAg; (b) coculture with CD40L⁺ but not CD40L⁻ Jurkat T cell line mutant clones; or (c) soluble anti-CD40 mAb. Enhanced B cell Fas/Apo-1 expression after CD40 ligation is functionally significant. Coculture of Ramos Burkitt's lymphoma line cells with irradiated SAg-reactive CD4⁺ T cells with SAg or CD40L⁺ Jurkat T cells resulted in B cell apoptosis, demonstrated by reduced cell viability and DNA laddering. This process was augmented by the addition of anti-Apo-1/Fas mAb, consistent with an acquired susceptibility to Apo-1/Fas-mediated apoptosis. These data support an immunoregulatory pathway in which seemingly contradictory signals involving the B cell proliferation/survival antigen CD40 and the Apo-1/Fas molecule that mediates pro-

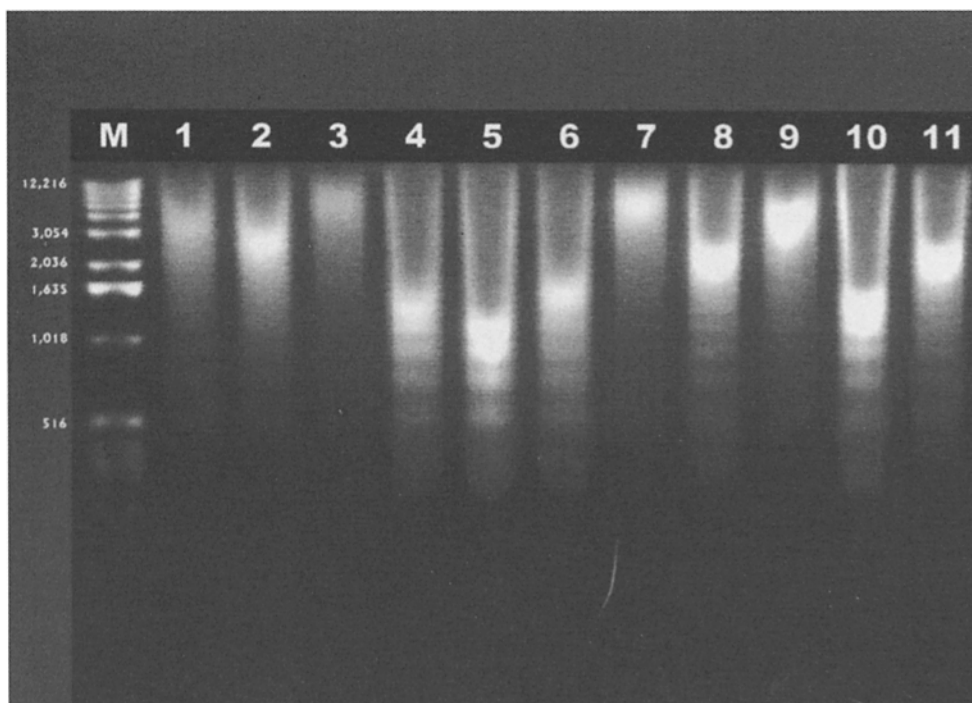


Figure 6. Cognate T-B cell signaling induces apoptosis of Ramos B cells and renders them susceptible to Apo-1/Fas-mediated apoptosis. Ramos B cells were cultured for 48 h with irradiated CD40L⁻ (B2.7_{xr}, lanes 1-3) or CD40L⁺ (D1.1_{xr}, lanes 4-6) Jurkat mutants, with CD4⁺ T_{MAMxr} without (lanes 7-9), or with (lanes 10 and 11) MAM. After Ficoll Hypaque centrifugation, viable Ramos B cells were cultured overnight after the addition of media (1, 4, 7), anti-Apo-1 mAb (2, 5, 8, 10), or anti-CD23 (3, 6, 9, 11), each at a concentration of 200 ng/ml, and low molecular weight DNAs were prepared and analyzed by ethidium bromide agarose gel electrophoresis. Molecular weight markers are indicated.

Table 4. Cognate Signaling Induced by Superantigen or by CD40L⁺ Jurkat T Cells Confers Susceptibility to Apo-1/Fas-mediated B Cell Death

Culture conditions	Viability at 72 h (media or antibody added after 48 h)		
	Media	Anti-Apo-1	Anti-CD71
A. Ramos + media	83%	76%	72%
Ramos + CD40L ⁻ T _{xr}	77%	65%	77%
Ramos + CD40L ⁺ T _{xr}	60%	18%	77%
Ramos + MAM + T _{MAMxr}	52%	22%	70%
		Anti-Apo-1	
	Media	Media	F(ab) ₂ ' anti-Apo-1
B. Ramos + CD40L ⁻ T _{xr}	79%	81%	78%
Ramos + CD40L ⁺ T _{xr}	70%	42%	64%
Ramos + T _{MAMxr}	67%	60%	77%
Ramos + MAM + T _{MAMxr}	10%	0%	12%

(A) Ramos B cells were cultured as indicated for 48 h, after which cultures were supplemented with media, mAb to Apo-1/Fas (200 ng/ml), or with antibody to CD71 as a control. After an additional 24 h, each culture was assessed for viability by trypan blue exclusion. (B) Ramos B cells were cultured as indicated for 48 h, after which cultures were supplemented with media alone or with an mAb to Apo-1/Fas (200 ng/ml), in the presence or absence of an F(ab)₂'₂, nonsignaling preparation of antibody to Apo-1/Fas, also at a concentration of 200 ng/ml. After an additional 24 h, each culture was assessed for viability by trypan blue exclusion.

grammed cell death of lymphocytes are linked in the process of human B cell activation.

Productive B cell activation requires the delivery of at least two signals, the binding of antigen to surface Ig receptors (signal 1), followed by cognate interaction with an antigen-specific T cell (signal 2) (34, 35). The studies reported here suggest that in the absence of slg signaling, B cells stimulated via CD40 upregulate Apo-1/Fas and are prepared for the delivery of death signals. This pathway could serve to eliminate bystander B cells that are inappropriately activated. Presumably, a B cell that has received both signals 1 and 2 would be resistant to this form of elimination and respond by proliferation and differentiation into an antibody-forming cell. Consistent with this hypothesis, we have determined that cross-linking of surface Ig on Ramos B cells by anti-IgM antibody does not affect Apo-1/Fas expression induced by CD40 ligation, but does render these cells resistant to apoptosis (Schattnner, E., unpublished data).

The results described in this study must be considered in the context of previous reports from our laboratories and others that direct T cell help for B cell activation mediated by allospecific T cell lines or SAg-induced Th-B cell bridge can result in polyclonal activation of both human and murine B cells (29, 36-38). Heterogeneity in the response of B cell subpopulations to CD40 ligation may be a factor in determining the outcome of the Th-B cell interaction. For example, it is the high density, resting B cell subset that expresses the activation antigen CD23 in re-

sponse to cognate Th-B cell interaction (39). An additional determinant of the B cell's fate may be the relative abundance of Th1 and Th2 cells in culture. Th1 cells secrete cytokines central to the cellular immune response, such as IL-2 and IFN- γ (40), and they selectively express Fas ligand after activation (12), while CD4⁺ Th2 cells secrete cytokines that support the humoral immune response. A role for CD4⁺ Th1 cells in Apo-1/Fas-mediated B cell death is consistent with this functional dichotomy, and it supports a mechanism other than antagonistic cytokine effects by which Th1 cells can downregulate B cell responses. The association of defects in the Fas/Apo-1/Fas ligand pair in the development of SLE-like disease in several strains of mice (2-4) suggests that this pathway may be involved in the control of autoreactive B cells. SAg-mediated, CD4⁺ T cell-induced B cell apoptosis should provide a model system for assessing the integrity of this signaling pathway in human SLE.

Our data suggest a possible mechanism for the development of B cell lymphomas in patients with X-linked hyper-IgM, an immunodeficiency syndrome defined by molecular defects in the T cell ligand for CD40 (41, 42). In these patients, impaired signaling through CD40 might allow for uncontrolled B cell expansion due to a lack of CD40L-mediated upregulation of Apo-1/Fas on B cells. This signaling pathway, which primes B cells for CD4⁺ cell-mediated B cell apoptosis, may also relate to the high incidence of B cell lymphomas among patients with HIV disease, where a high proportion of tumors are of Burkitt's

or Burkitt's-like (small, noncleaved cell) subtypes (43), similar to those of the Ramos B cell line used in our experiments. In HIV disease, the progressive decline of CD4⁺ T cells would diminish the capacity for Apo-1/Fas induction on B cells via the CD40L T cell-dependent pathway we have described. Moreover, the reduction of CD4⁺ T cells and shift

from a Th1- to Th2-dominant population would specifically deplete the host of those T cells that express and secrete human Fas ligand and are responsible for Fas-mediated B cell cytotoxicity. In that setting, B cell malignancies would arise due to a lack of CD4⁺ T cell-mediated induction of Apo-1/Fas, with subsequent failure of B cell apoptosis.

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Note Added in Proof: While this manuscript was under review, another group reported similar findings that CD40 ligation results in increased B cell Fas expression in a murine system (Rothstein, T. L., J. K. Wang, D.J. Panka, L. C. Foote, Z. Wang, B. Stanger, M. Cui, S.-T. Ju, and A. Marshak-Rothstein. 1995. *Nature (Lond.)*. 374:163-165). Subsequently, as reported in this issue, Garrone et al. have observed findings similar to ours in human B lymphocytes (see pp. 1265-1273).

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