

Concurrent Engagement of CD40 and the Antigen Receptor Protects Naive and Memory Human B Cells from APO-1/Fas-mediated Apoptosis

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Summary

Naive and memory B cells were isolated from human tonsils and examined for expression of APO-1/Fas and for their sensitivity to the APO-1-dependent apoptosis. APO-1 was found to be constitutively expressed on memory but not on naive B cells. The susceptibility of both cell types to the APO-1 apoptotic pathway was acquired upon CD40 triggering and was correlated with increased expression of the APO-1 receptor. Both naive and memory B cells were protected from the APO-1-mediated death signal after dual ligation of the Ag receptor and CD40. Our findings suggest that the APO-1 pathway controls the specificity of B cell responses to T-dependent Ags and that occupancy of the Ag receptor dictates the outcome of APO-1 ligation on B cell survival.

Immunization with a T cell-dependent Ag induces the B cell clones bearing the appropriate antigenic specificity to initiate the formation of germinal centers (GC)¹ where they differentiate into either precursors of plasma cells or memory B cells (1). There is no dispute that the CD40 molecule expressed on B cells and its T cell-bound ligand gp39 play a critical role in this maturation process. In particular, ligation of CD40 has been shown to be instrumental in B cell proliferation (2), Ig isotype switching (3), rescue of high affinity B cell mutants in GC (4), and generation of B cell memory (5, 6). A distinctive feature of the CD40 signaling pathway is that it is Ag nonspecific and genetically unrestricted (7). This implies that interactions between T and B cells have to be tightly regulated in order to prevent the stimulation of bystander or self-reactive B cells. It is generally accepted that the rapid turnover and CD40-dependent downregulation of the CD40 ligand (CD40-L) on activated T cells (8–10) prevent the activation of B cells with unwanted specificity. However, several lines of evidence suggest that the APO-1 (Fas/CD95)/APO-1 ligand system may be an additional backup mechanism of immunosuppression operating to preserve the specificity of T-dependent Ab responses and to censor autoreactive B cells in the periphery. First, *lpr/lpr* mice, genetically deficient for the

expression of APO-1 (11), suffer from an autoimmune disease analogous to systemic lupus erythematosus (12) which has been described to be partly attributable to an intrinsic B cell defect (13, 14). This has given rise to the idea that the defective expression of APO-1 on B cells is directly associated with the emergence of autoreactive B cells in these animals. Second, the ligands for both CD40 and APO-1 are inducible on CD4⁺ T cells after engagement of the TCR (2, 15, 16), suggesting that helper T cells are equally capable of providing activation and death signals to B cells after cognate interaction. Finally, we have recently reported that GC B cells express high levels of the APO-1 molecule and acquire sensitivity to the APO-1-mediated death signal upon engagement of CD40 (17).

The present study was undertaken in order to gain insight into the expression and function of APO-1 at different steps of the Ag-driven B cell maturation pathway in humans. Experimental procedures allowing the isolation of distinct developmental stages of the mature human B cell compartment, based on the differential expression of a set of surface markers, are now available. In particular, we and others (18–20) have reported that human memory B cells can be identified according to their unique pattern of expression of IgD, CD38, and CD44. In this report, we have compared naive and memory B cells for their expression of APO-1 and have examined the activation requirements of both cell types for the acquisition of susceptibility to the APO-1-mediated death signal. Our results demonstrate

¹Abbreviations used in this paper: GC, germinal center; HEL, hen egg lysozyme; ISNT, in situ nick translation assay; MV, measles virus.

that: (a) APO-1 is constitutively expressed on memory B cells but not on naive B cells; and (b) triggering of CD40 but not surface immunoglobulin ligation upregulates APO-1 on both B cell subsets and induces their responsiveness to APO-1-dependent apoptosis. Furthermore, dual ligation of sIgs and CD40 was found to protect naive and memory B cells from APO-1-mediated cell death. In contrast, CD40-activated GC B cells were resistant to the protective effect afforded by engagement of the Ag receptor, thus suggesting that additional molecular mechanisms may operate to block the APO-1 signaling pathway in GC. We propose that the protection against APO-1-dependent apoptosis induced by concurrent ligation of CD40 and the Ag receptor allows for the targeting of the APO-1-mediated death signal to B cells of unrelated antigenic specificity activated through bystander interaction with CD40-L-bearing T cells in the course of a normal immune response.

Materials and Methods

Abs. The sources of the Abs used for phenotyping, isolation of B cell subsets and functional assays are listed in Table 1.

Reagents. Purified recombinant IL-2 and IL-10 were generous gifts from Drs. A. Minty (Sanofi, Labège, France) and J. Banchereau (Schering-Plough, Dardilly, France), respectively. IL-2 and IL-10 were used at 10 U/ml and 100 ng/ml, respectively, throughout the study. A pool of human sera (a gift from Dr. F. Wild, INSERM U 404, chosen for their high measles Ab titer, was used for calibration of the measles-specific ELISA. The Hallé strain of measles virus (MV) was grown on Vero cells, then semipurified on sucrose gradients as described elsewhere (21), and partially inactivated by UV irradiation before addition to B cell cultures. A 1/10⁵ dilution (equivalent to 0.02 PFU/ml) of the MV preparation was used for activation of the MV-specific memory B cells.

Cells. Purified tonsillar B cells were obtained after two rosetting steps with sheep red blood cells followed by depletion of residual T cells using pan-T cell Abs (CD2, CD3) and magnetic beads coated with anti-mouse IgG Abs (Dynabeads, Dynal, Oslo, Norway). A preparative magnetic cell separation system (MACS; Miltenyi Biotec, Bergish Gladbach, Germany) was used to purify IgD⁻ B cells according to the protocol described previously (18). GC (CD38⁺/CD44⁻) and memory B cells (CD38⁻/CD44⁺) were isolated from the IgD⁻ population as previously described (18). For purification of GC B cells, IgD⁻ B cells were submitted to two successive rounds of depletion, each one involving labeling with an anti-CD44 mAb (clones NKI-P2 and J 173) and incubation with magnetic beads coated with anti-mouse IgG. A similar procedure was applied for the isolation of memory B cells; this time, labeling of the cells was performed with two different anti-CD38 mAbs (clones IB4 and HB-7). Cross-contamination of GC and memory B cells was routinely <2% as estimated by the expression of CD38 and CD44 revealed by stainings performed with FITC or PE-conjugated anti-CD38 and CD44 mAbs.

Naive (IgD⁺) B cells were purified from the unfractionated B cell population by a negative selection procedure based on the selective depletion of GC and memory B cells using the anti-CD38 mAb IB4 (GC B cells) in combination with the anti-CD80 (B7-1) mAb 104 (GC and memory B cells) and magnetic beads coated with anti-mouse IgG. In some experiments, IgD⁺ B cells

were negatively selected by sorting after labeling of the GC and memory B cells with a combination of PE-conjugated anti-CD80 and anti-CD38 mAbs. The mouse Ltk⁻ cell line HR4C6, stably expressing the human FcγRII (CD32), was kindly provided by Dr. C. Sautès (INSERM U 255, Institut Curie, Paris, France). The CD32 transfectants were treated with 75 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, MO) 1 h at 37°C before their addition to B cell cultures.

Cultures. All cultures were performed in RPMI 1640 medium enriched with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2% Hepes (all from GIBCO BRL, Gaithersburg, MD). For functional assays, B cells were seeded at 10⁵ cells per well in round-bottomed 96-well microtiter trays at a final culture volume of 0.1 (evaluation of DNA synthesis) or 0.25 ml (quantitation of secreted Igs), except for the MV-specific culture system, in which B cells were seeded at 5 × 10⁵ cells per well in 0.25 ml. For the CD40-mediated activation of B cells, the cultures were performed with the anti-CD40 mAb G28-5 (22) (100 ng/ml) in the presence of 1 × 10⁴ mitomycin C-treated HR4C6 cells. The anti-CD95/Fas mAb APO-1 (23) and its unrelated IgG3-κ control were used at a final concentration of 200 ng/ml, which was determined, in pilot experiments, to be optimal for the induction of apoptosis. For two-step cultures, B cells were first stimulated for 48 h at a density of 10⁷ cells per well in 6-well plates with either the anti-CD40 mAb G28-5 or a combination of anti-κ and anti-λ IgG1 mAbs (both used at a final concentration of 2 μg/ml) in the presence of mitomycin C-treated HR4C6 cells. B cell blasts recovered at the end of the primary culture were washed and recultured in 96-well plates, at a density of 10⁵ cells per well with the indicated stimuli. DNA synthesis was determined by pulsing the cells with [³H]thymidine ([³H]TdR) for the last 16 h of the culture period. Ig secretion (IgG, IgM, and IgA) was determined in 12-d culture supernatants by standard ELISA techniques, as described elsewhere (24).

In some experiments, the results of the proliferation assays were expressed as percent response in control cultures, calculated as follows: % of the response in control cultures = [(cpm in stimulated cultures supplemented with the anti-APO-1 mAb) - (cpm in unstimulated cultures)] / [(cpm in stimulated cultures supplemented with the IgG3-κ control) - (cpm in unstimulated cultures)] × 100.

Detection of Anti-MV IgG Abs. For detection of anti-MV IgG Ab, flat-bottomed microtiter plates (Immunoplate Maxisorp F96; Nunc, Roskilde, Denmark) were first coated with 0.1 ml of a 1/800 dilution from a lysate of MV-infected Vero cells prepared in carbonate buffer, pH 9.6, and allowed to incubate overnight at 4°C. Plates were then washed three times with PBS containing 0.05% Tween 20 (Sigma Chemical Co.). Samples were appropriately diluted, added to the wells in a final volume of 0.1 ml, and incubated at room temperature for 90 min. The plates were then washed as before and 0.2 ml of a 1/2, 500 dilution of a rabbit anti-human IgG Ab conjugated to alkaline phosphatase (Dako, Glostrup, Denmark) was added to each well. After a 90-min incubation, plates were washed and developed with a solution of p-nitrophenylphosphate (Sigma Chemical Co.) prepared in diethanolamine buffer. The subsequent development of color at 490 nm was detected on a multichannel spectrophotometer. The values were related to a standard serum containing high amounts of anti-MV IgG Ab. One ELISA unit (EU) was defined as the amount of anti-MV IgG Ab present in a 1/160,000 dilution of the standard serum.

Assay for Programmed Cell Death (In Situ Nick Translation Assay). For measurement of apoptosis, B cells were seeded in 12-

well plates (3×10^6 cells per well) in 1 ml of complete RPMI medium. At the times specified in the text, the cultures were harvested and apoptotic cells were quantified using the in situ nick translation assay (ISNT), allowing the detection of cells carrying DNA single strand breaks, as described elsewhere (25). Briefly, the cells were fixed successively with 1% formaldehyde and 70% ethanol. The fixed cells were washed with nick buffer (50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 0.1 M β -mercaptoethanol, and 10 μ g/ml BSA) and incubated with a mixture of 1.3 μ l dATP, dGTP, dCTP, each 0.2 mM (Boehringer Mannheim, Mannheim, Germany), 1.6 μ l nick buffer, 0.3 μ l 1 mM biotinylated-11-dUTP (Boehringer Mannheim), and 1 U *Escherichia coli* polymerase I (New England Biolabs, Beverly, MA) for 90 min at 15°C to complete broken DNA strands. The cells were stained with avidin-FITC (Immunotech, Marseille, France) and DNA was counterstained with 10 μ g/ml propidium iodide before analysis on a FACScan® cytofluorimeter (Becton Dickinson & Co., Mountain View, CA). Percent DNA fragmentation corresponds to the percent green fluorescence in the propidium iodide-positive population.

Immunofluorescence Stainings. Surface expression of APO-1 was measured after labeling performed with the IgG3 APO-1 mAb revealed by PE-conjugated goat anti-mouse IgG3 Abs. The staining control was performed with a nonbinding IgG3 mAb. Immunofluorescence stainings were analyzed on a FACScan® flow cytometer using Lysis software (Becton Dickinson & Co.). To estimate the modulation of the mean fluorescence intensity (MFI) of the APO-1 fluorescence peak in cultures of activated B cells, the cytofluorimeter settings were adjusted so that the fluorescence histograms of the negative controls, for the different time points considered, overlapped completely.

Results

CD40 Cross-linking Upregulates APO-1 Expression on B Cells, Irrespective of their Maturational Stage. We first compared the surface expression of APO-1 on different B cell subsets, before and after stimulation by either immobilized

Table 1. Clone Numbers and Sources of the Abs Used for Phenotypical and Functional Studies

Abs	Clone	Source
CD2	OKT11	American Type Culture Collection, Rockville, MD
CD3	OKT3	American Type Culture Collection
CD3-FITC	UCHT1	Dako
CD10	44C10	Dr. M. Letarte, The Hospital for Sick Children, Toronto, Canada
CD10-PE	SS2/36	Dako
CD19-PE	HD37	Dako
CD20-FITC	B-Ly-1	Dako
CD38	IB4	Dr. F. Malavasi, Laboratorio di Biologia Cellulare, Torino, Italy
CD38	HB-7	Becton Dickinson
CD38-PE	HB-7	Becton Dickinson
CD40	G28-5	Dr. E.A. Clark, University of Washington, Seattle, WA
CD44	J173	Immunotech
CD44-FITC	J173	Immunotech
CD44	NKI-P2	Dr. C. Figdor, The Netherland Cancer Institute, Amsterdam, The Netherlands
CD80	104	Immunotech
CD80-PE	L307.4	Becton Dickinson
CD95/Fas	APO-1	Dr. P. Krammer, German Cancer Research Center
IgD biotin	Goat Abs	Sigma Chemical Co.
IgD-PE	Goat Abs	Southern Biotechnology Associates, Birmingham, AL
Igs (H + L) coupled to beads	Rabbit Abs	Bio-Rad Laboratories, Richmond, CA
κ light chain	6E1	Immunotech
λ light chain	C4	Immunotech
Isotype controls		
Mouse IgG1		Sigma Chemical Co.
Mouse IgG3- κ		Sigma Chemical Co.
Conjugates		
Goat anti-mouse Igs-FITC		Dako
Streptavidin-FITC		Immunotech

anti-Ig Abs, or the anti-CD40 mAb G28-5 “presented” on CD32 transfectants. Naive B cells were negatively selected from the purified tonsillar B cell population by sorting, after gating out cells labeled with the anti-CD80 and anti-CD38 Abs. GC and memory B cells were recovered from the IgD⁻ population after immunomagnetic depletion of CD44 and CD38-bearing cells, respectively. The results of a representative experiment, shown in Fig. 1, indicate that both GC and memory B cells, but not naive B cells, constitutively express APO-1. However, in contrast with the homogeneous staining profile obtained with GC B cells, APO-1 is expressed with variable levels of intensity on memory B cells. Triggering of CD40 induced most naive B cells to express APO-1 and strongly enhanced the levels of expression of this molecule on both GC (sevenfold increase) and memory B cells (fourfold increase). Among the three B cell subsets examined, GC B cells were reproducibly found to express the highest density of APO-1 after CD40 stimulation. Conversely, engagement of the B cell receptor by immobilized polyclonal anti-Ig Abs did not induce APO-1 on naive B cells and reduced the levels of

APO-1 expression on both GC and memory B cells. Similar results were obtained when a combination of anti- κ and anti- λ mAbs presented by the CD32 transfectants was substituted to the immobilized polyclonal anti-Ig Abs (data not shown).

APO-1 Ligation Blocks the CD40-dependent Proliferation of Naive and Memory B Cells. We have previously reported that APO-1 ligation inhibits the DNA synthesis promoted by anti-CD40 Abs in purified GC B cells (17). Thus, we next examined the influence of the agonistic anti-APO-1 mAb on the growth-response of naive and memory B cells promoted either by engagement of CD40 (G28-5 mAb plus CD32 transfectants) or triggering of the Ag receptor (immobilized polyclonal anti-Ig Abs). For this purpose, the proliferative responses of B cells in both activation systems was estimated in cultures supplemented or not with either the anti-APO-1 mAb or the unrelated IgG3- κ control. The results of a representative experiment depicted in Fig. 2 show that the CD40-mediated proliferation of naive and memory B cells was profoundly inhibited by addition of the anti-APO-1 mAb, whereas it was unaffected by the isotype-matched control Ab. In the majority of experiments, the maximal levels of inhibition of the proliferative response of CD40-stimulated B cells brought about by the anti-APO-1 mAb were observed between 48 and 72 h of culture. Although the anti-APO-1 mAb induced some inhibition of the DNA synthesis promoted by immobilized anti-Ig Abs, the degree of suppression evoked by the anti-APO-1 mAb was consistently higher in CD40-stimulated cultures.

APO-1 Ligation Inhibits CD40- but Not Ag-driven Ig Synthesis. We next addressed the question of the ability of the anti-APO-1 mAb to suppress Ig synthesis. The choice of the combination of IL-2 and IL-10 for these experiments was motivated by the fact that these cytokines efficiently support the differentiation of B cells into Ig-secreting cells, both in CD40-dependent culture systems (polyclonal stimulation; 26) and in CD40-independent models in which memory B cells are selectively induced for specific Ab production by the Ag. Thus, polyclonal Ig synthesis was first measured in cultures of naive and memory B cells costimulated with anti-CD40 Abs, IL-2 and IL-10, in the presence or absence of either the anti-APO-1 mAb or the isotype-matched unrelated control Ab. As shown in Table 2, APO-1 ligation on CD40-activated naive and memory B cells dramatically impaired their ability to differentiate into Ig-secreting cells in response to IL-2 and IL-10, thus excluding the possibility that APO-1 triggering may favor the maturation of B cells into terminally differentiated plasma cells.

We have recently reported that a typical recall Ag such as MV can be used to generate secondary Ab responses in vitro (26a). This approach has permitted us to conclude that the differentiation of memory B cells along the plasmacytoid pathway does not rely upon engagement of CD40 and can be achieved by costimulation with the Ag and the combination of IL-2 and IL-10. This experimental system was thus used to examine the influence of APO-1 trigger-

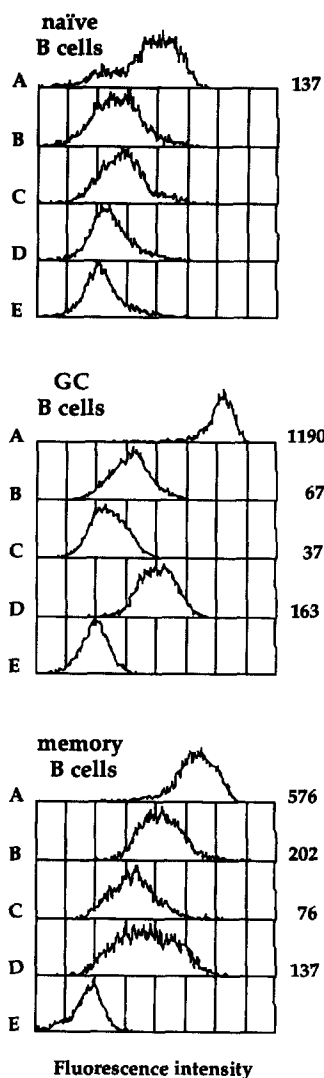


Figure 1. Constitutive and activation-induced expression of APO-1 on naive, GC, and memory B cells. 2×10^6 B cells from each subset were stimulated for 48 h with: the anti-CD40 mAb G28-5 (A), an unrelated IgG1 control mAb (B) in the presence of 2×10^5 mitomycin C-treated HR4C6 cells, immobilized polyclonal anti-Ig Abs (C). Viable cells were gated according to forward versus side scatter profiles for the flow cytometry analysis. The bindings of the APO-1 mAb and of an isotype-matched control mAb, on freshly isolated cells, are illustrated by the fluorescence profiles shown in D and E, respectively. (Horizontal axes) log fluorescence; (vertical axes) relative cell numbers. MFI values of the fluorescence peaks of APO-1⁺ cells are indicated next to the staining profiles.

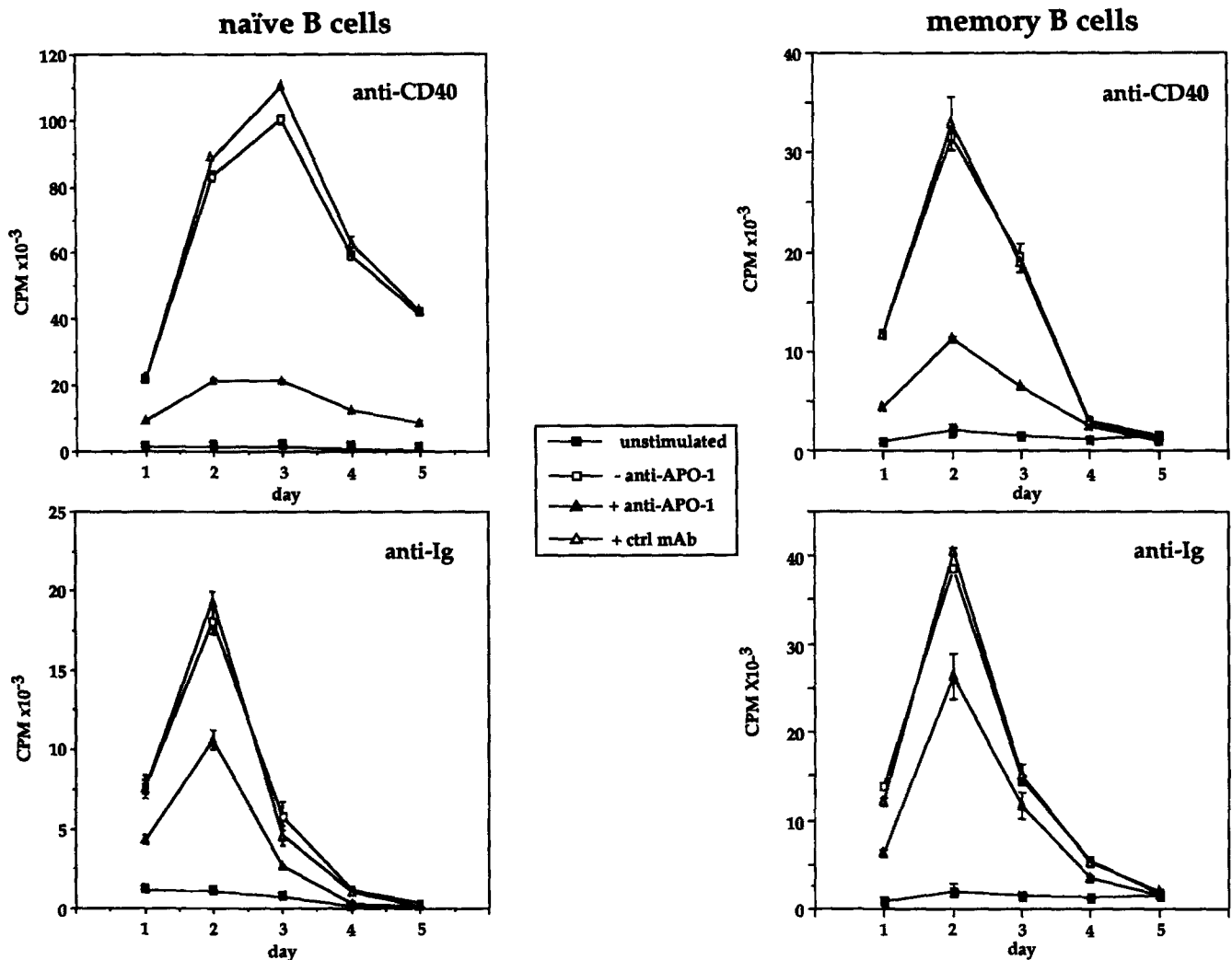


Figure 2. APO-1 ligation inhibits the proliferative response of anti-CD40-activated naive and memory B cells. Isolated naive and memory B cells (10^5 /well) were stimulated with the anti-CD40 mAb G28-5 together with mitomycin C-treated HR4C6 (3×10^4 /well) cells, or with immobilized polyclonal anti-Ig Abs, in the presence or absence of either the anti-APO-1 mAb or the IgG3- κ control mAb (both used at 200 ng/ml). Cells were pulsed with [3 H]thymidine at the indicated times; cultures were terminated 16 h later. Results correspond to the mean \pm SD values of triplicate determinations. The figure is representative of three separate experiments. The [3 H]thymidine incorporation levels in unstimulated control cultures are also indicated.

ing on the MV-driven secondary Ab response. The results of a representative experiment depicted in Fig. 3 indicate that addition of the anti-APO-1 mAb does not affect the development of the in vitro secondary anti-MV Ab response. This observation suggests that the APO-1 signaling pathway has no impact on the Ag-driven differentiation of memory B cells into plasma cells, and further supports the hypothesis that CD40 ligation is instrumental in inducing the susceptibility of B cells to APO-1-mediated apoptosis.

APO-1 Ligation on CD40-activated Naive and Memory B Cells Induces Their Apoptosis. We next determined whether the inhibitory effect of the anti-APO-1 mAb on B cell growth and differentiation was mediated through the induction of apoptosis. For this purpose, the proportion of apoptotic cells was estimated by ISNT in cultures of naive and memory B cells activated by anti-Ig or anti-CD40 Abs,

supplemented or not with either the agonistic anti-APO-1 mAb or the unrelated IgG3- κ control mAb. As shown in Table 3, APO-1 ligation on either unstimulated or anti-Ig-activated B cells did not affect the levels of apoptosis occurring in these cultures. Experiments performed at earlier time points (6, 12, and 24 h) demonstrated that addition of the anti-APO-1 mAb did not modify the kinetics of entry of the cells into apoptosis in these cultures (data not shown), thus indicating that unstimulated and anti-Ig-activated B cells are resistant to anti-APO-1-induced apoptosis. On the contrary, engagement of APO-1 promoted a significant rise in the proportion of apoptotic cells in anti-CD40-stimulated cultures for both naive and memory B cells. Taken together, these results suggest that: (a) the APO-1 molecule, although constitutively expressed on memory B cells, is not readily connected to the cell death

Table 2. APO-1/Fas Ligation Inhibits the Ig Synthesis Elicited by IL-2 and IL-10 from Anti-CD40-activated Naive and Memory B Cells

	Ig synthesis					
	Naive B cells			Memory B cells		
	-Anti-APO-1	+Anti-APO-1	+Ctrl mAb	-Anti-APO-1	+Anti-APO-1	+Ctrl mAb
	$\mu\text{g/ml}$					
Unstimulated	0.5	0.3	0.5	<0.1	<0.1	<0.1
Anti-CD40	1.4	0.4	0.8	<0.1	<0.1	<0.1
IL-2+IL-10	5.8	3.1	4.7	1.1	0.9	1.2
Anti-CD40+IL-2+IL-10	476.0	181.0	483.0	94.1	17.0	101.0

Isolated naive and memory B cells were either stimulated with or without: (a) the anti-CD40 mAb G28-5 “presented” by the CD32 transfectants; (b) IL-2+IL-10; (c) the combination of both stimuli, in the presence or absence of either the anti-APO-1 mAb or the IgG3- κ control mAb. The cumulative IgM, IgG, and IgA levels (expressed in $\mu\text{g/ml}$) were evaluated in 12-d culture supernatants. Data are shown as means of quadruplicate determinations. Standard deviations did not exceed 10% of the mean values. The table is representative of three separate experiments. *ctrl*, control.

pathway; and (b) both naive and memory B cells become sensitive to anti-APO-1-induced apoptosis upon activation of the CD40 signaling pathway.

Dual Ligation of CD40 and sIgs Protects Naive and Memory B Cells from APO-1-mediated Cell Death. As outlined in the introduction, it is conceivable that B cells could potentially be signaled both through CD40 and APO-1, in the context of a cognate interaction with T cells. If this proposal is correct, it implies the existence of a mechanism that would protect Ag-specific B cell clones from being deleted through activation of the APO-1-signaling pathway, in the course of an immune response. As the occupancy of the Ag receptor itself allows discrimination between the appropriate B cell clones and those bearing an unrelated antigenic specificity, we speculated that the sIg signaling pathway

might influence the outcome of APO-1 ligation on B cell survival. To test this hypothesis, we first examined the influence of the agonistic anti-APO-1 mAb on the proliferation and differentiation of unfractionated B cells activated by: (a) engagement of the Ag receptor; (b) ligation of CD40; and (c) coligation of the Ag receptor and CD40. The results of a representative experiment, illustrated in Fig. 4, demonstrate that the inhibitory effect of the anti-APO-1-mAb on the proliferation and Ig response of CD40-activated B cells is reversed when sIgs and CD40 are simultaneously cross-linked. To confirm that activation of the sIg signaling pathway confers protection against the cell death signal mediated by APO-1, we next compared anti-CD40-activated B cells with B cells activated by coligation of CD40 and sIgs for their sensitivity to the APO-1 apop-

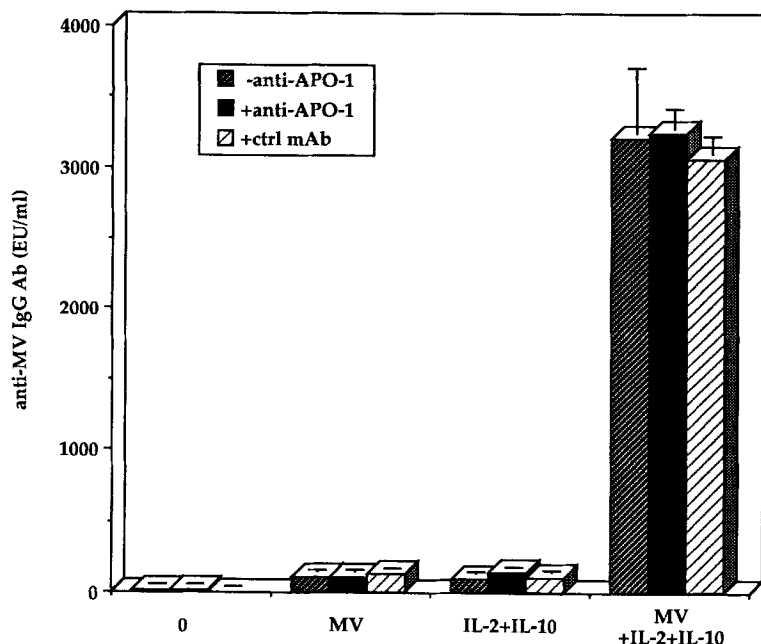


Figure 3. APO-1 ligation on Ag-activated memory B cells does not affect the induction of a secondary Ab response. Isolated memory B cells (4×10^5 cells/well in 96-well round-bottomed microtiter trays) were stimulated for 12 d with: (a) measles virus (MV); (b) the combination of IL-2 (10 U/ml) and IL-10 (100 ng/ml); and (c) MV+IL-2+IL-10. Cultures were further supplemented or not with either the anti-APO-1 mAb or the IgG3- κ control mAb (used at 200 ng/ml). The levels of specific anti-MV IgG Ab production were evaluated in 12-d culture supernatants by ELISA. The results correspond to the mean \pm SD values of triplicate determinations and are expressed in ELISA units per ml (EU/ml).

Table 3. Sensitivity of Naive and Memory B Cells to the APO-1/Fas Apoptotic Pathway Is Acquired upon CD40 Ligation

Stimuli	Percent apoptotic cells					
	Naive B cells			Memory B cells		
	-Anti-APO-1	+Anti-APO-1	+Ctrl mAb	-Anti-APO-1	+Anti-APO-1	+Ctrl mAb
	%					
None	77	77	77	80	80	80
Anti-Ig	64	67	69	69	70	70
Anti-CD40	35	59	34	47	69	48

Isolated naive and memory B cells were cultured either with or without: (a) the anti-APO-1 mAb; (b) the IgG3- κ control, in the presence or absence of either the anti-CD40 mAb G28-5 presented by the CD32 transfectants or immobilized polyclonal anti-Ig Abs. The proportion of apoptotic cells was estimated by ISNT after a 48-h culture. Results are expressed as percent apoptotic cells and are representative of three separate experiments.

otic pathway. For this purpose, the proportion of apoptotic cells was estimated in cultures of unfractionated tonsillar B cells stimulated for 48 h either with anti-CD40 Abs or with the combination of anti-CD40 and immobilized anti-Ig Abs and supplemented or not with the anti-APO-1 mAb or the IgG3- κ control mAb. As shown in Fig. 5, CD40-activated B cells were induced for apoptosis after engagement of APO-1, whereas the simultaneous triggering of CD40 and sIgs protected B cells from the APO-1-mediated programmed cell death. To dissect the sequence of events leading to the acquisition of an APO-1-resistant phenotype, the sensitivity of B cells to APO-1-mediated apoptosis was estimated in a two-step culture system in which the Ag receptor was engaged either before or after ligation of CD40. For this purpose, both anti-Ig and anti-CD40 B cell blasts were generated by stimulating B cells for 48 h, in the presence of the CD32 transfectants, either with a combination of anti- κ and anti- λ mAbs, or with the anti-CD40 mAb. We deliberately avoided the use of Sepharose-bound polyclonal anti-Ig Abs for these experiments because cells cannot be washed free of this particular anti-Ig reagent. At the end of the primary culture, anti-Ig and anti-CD40 B cell blasts were washed, seeded on mitomycin C-treated CD32 transfectants, and recultured with anti-CD40 and anti- κ/λ mAbs, respectively. The secondary cultures were further supplemented or not with either the anti-APO-1 mAb or the unrelated IgG3- κ control mAb. The susceptibility of the two types of blasts to APO-1-dependent apoptosis was estimated in a proliferation assay. The same B cell preparation was tested in parallel for its sensitivity to APO-1-dependent apoptosis when anti-CD40 and anti- κ/λ Abs were simultaneously provided at the onset of the culture. The results of a representative experiment, shown in Fig. 6, indicate that neither anti-Ig nor anti-CD40 B cell blasts are efficiently protected from APO-1-mediated apoptosis, whereas the simultaneous addition of anti-CD40 and anti- κ/λ mAbs at the initiation of the culture completely abrogates the growth-inhibitory effect of the anti-APO-1 mAb.

To determine whether the protective signal evoked by

concurrent engagement of the B cell receptor and CD40 is operative at all B cell maturational stages, naive, GC, and memory B cells were tested for their sensitivity to the APO-1 apoptotic pathway after cross-linking of CD40 or dual ligation of CD40 and sIgs. B cell proliferation was chosen as the readout system for these experiments. As shown in Fig. 7, both naive and memory B cells acquired resistance against APO-1-mediated cell death when they were costimulated by anti-Ig and anti-CD40 Abs, whereas GC B cells remained sensitive to the anti-APO-1 mAb in the latter culture condition. Taken together, these findings suggest that: (a) the protection of B cells from APO-1-mediated apoptosis requires concurrent ligation of CD40 and sIgs; and (b) the protective signal generated by engagement of the Ag receptor is defective in GC B cells.

Discussion

The present study highlights three important features of the APO-1 apoptotic pathway in mature human B cells: (a) APO-1 is not constitutively connected to the intracellular death-inducing pathway; (b) the transduction of a death signal through APO-1 requires prior activation through CD40; and (c) the dual engagement of the Ag receptor and CD40 protects naive and memory B cells from APO-1-induced apoptosis.

The observation that APO-1 is absent on naive B cells, whereas it is expressed on both GC and memory B cells, strongly suggests that APO-1 expression in the mature B cell compartment is acquired as the consequence of antigenic stimulation *in vivo*. However, the finding that cross-linking of CD40 can induce expression of APO-1 on naive B cells and their subsequent apoptosis after APO-1 ligation indicates that APO-1 can exert its regulatory function both at early and late stages of the Ag-driven B cell maturation process.

The notion that activation of human B cells is a prerequisite for the induction of an APO-1-sensitive phenotype has been suggested in a previous report (27). Here, we provide evidence that triggering of CD40 but not cross-link-

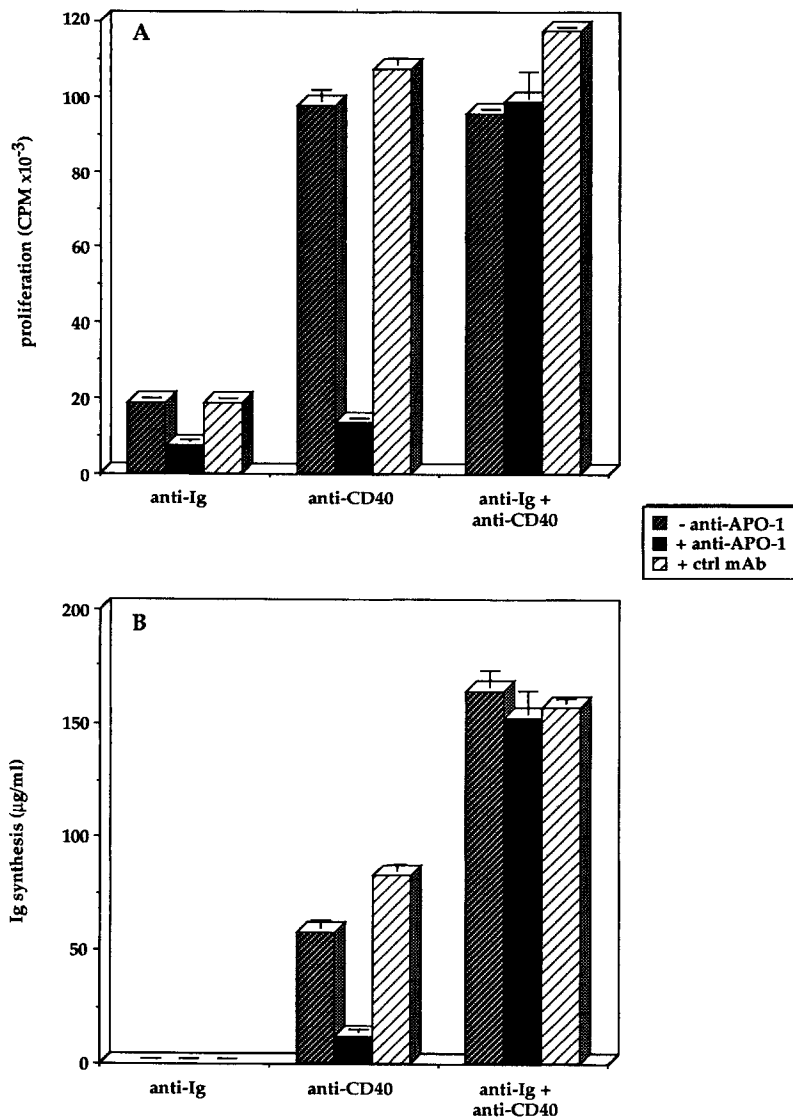


Figure 4. Engagement of the Ag receptor counteracts the inhibitory effect of the anti-APO-1 mAb on the proliferation and differentiation of CD40-activated B cells. Unfractionated tonsillar B cells were activated by: (a) polyclonal anti-Ig Abs; (b) the anti-CD40 mAb; or (c) the combination of anti-CD40 and anti-Ig Abs, in the presence or absence of either the anti-APO-1 mAb or IgG3- κ control mAb (used at 200 ng/ml). DNA synthesis (A) was assessed at day 2, after a 16-h pulse with [3 H]thymidine. Cultures made for evaluation of Ig secretion (B) were further supplemented with the combination of IL-2 and IL-10. All cultures were performed in the presence of mitomycin C-treated HR4C6 cells. The cumulated IgM, IgG, and IgA levels were measured in 10-d culture supernatants and are expressed in μ g/ml. The results shown correspond to the mean \pm SD values of triplicate (proliferation) and quadruplicate (Ig synthesis) determinations and are representative of three such experiments.

ing of the Ag receptor confers on B cells the susceptibility to APO-1-mediated apoptosis. First, the anti-APO-1 mAb suppressed the growth and differentiation of anti-CD40-activated B cells through the induction of apoptosis but only marginally affected the responses of B cells stimulated by anti-Ig Abs. Second, APO-1 ligation did not interfere with the CD40-independent induction of a secondary anti-MV Ab response in vitro. Thus activation of the CD40 signaling pathway appears to be essential for the development of both APO-1-induced apoptosis and T-dependent Ab responses. This finding implies that the fate of B cells after physical contact with T helper cells could equally be differentiation or death. It can thus be speculated that APO-1-mediated apoptosis has to be tightly regulated in vivo in order to avoid deletion of the responder B cell clones in the course of an ongoing immune response. Our current data indicate that occupancy of the Ag receptor itself dictates the outcome of APO-1 ligation on the survival of activated human B cells. This conclusion is coherent with a recent

study (28) in which murine B cells activated through the Ag receptor were found to be resistant to APO-1/Fas-dependent Th1-mediated apoptosis. Our hypothetical model (depicted in Fig. 8) predicts that the APO-1 signaling pathway on B cells is silenced when triggered in the context of an Ag-specific, cognate T-B cell interaction, due to the engagement of the Ag receptor by the Ag. On the contrary, the APO-1 signaling pathway should be fully operational if B cells bearing an irrelevant antigenic specificity are stimulated through CD40 upon interaction with activated T cells.

Several lines of evidence indicate that APO-1 may also play a critical role in the censoring of autoreactive B cells in the periphery. In particular, Rathmell et al. (29) have recently demonstrated that, in double transgenic mice coexpressing the hen-egg lysozyme (HEL) and a B cell receptor specific for HEL, self-reactive B cells are eliminated through an APO-1/Fas-mediated mechanism upon interaction with HEL-specific CD4 $^+$ T cells. At face value, our obser-

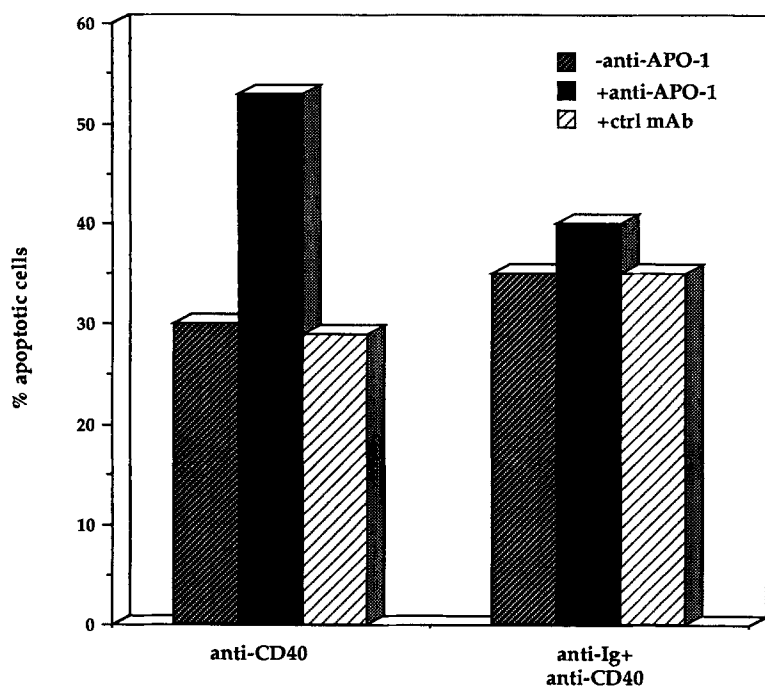


Figure 5. Engagement of the Ag receptor protects anti-CD40-activated B cells from APO-1-mediated apoptosis. Unfractionated tonsillar B cells were stimulated by the anti-CD40 mAb in the presence or absence of immobilized polyclonal anti-Ig Abs. Cultures were further supplemented or not with either the anti-APO-1 mAb or the IgG3- κ control mAb (used at 200 ng/ml). All culture conditions were performed in the presence of mitomycin C-treated HR4C6 cells. The proportions of apoptotic cells were evaluated after a 72-h culture by ISNT. The results are expressed as percent apoptotic cells and are representative of three such experiments. The proportion of apoptotic cells in unstimulated culture is 63%. Figure is representative of three such experiments.

vation that concurrent engagement of the Ag receptor and CD40 protects B cells from APO-1-mediated cell death seems difficult to reconcile with the fact that the binding of the self Ag does not prevent the APO-1-induced apoptosis of autoreactive B cells, in the experimental model described above. However, there is reason to believe that the protection mechanism afforded by sIgs does not operate in

peripheral self-reactive B cells. Indeed, it has been reported that B cells that express a transgenic HEL-specific receptor and that have been exposed to moderate concentrations of a soluble form of HEL, are tolerant to Ag stimulation but are still fully responsive to T cell-derived signals, including those delivered through CD40 (30-32). The molecular mechanism underlying their anergy has been shown to be

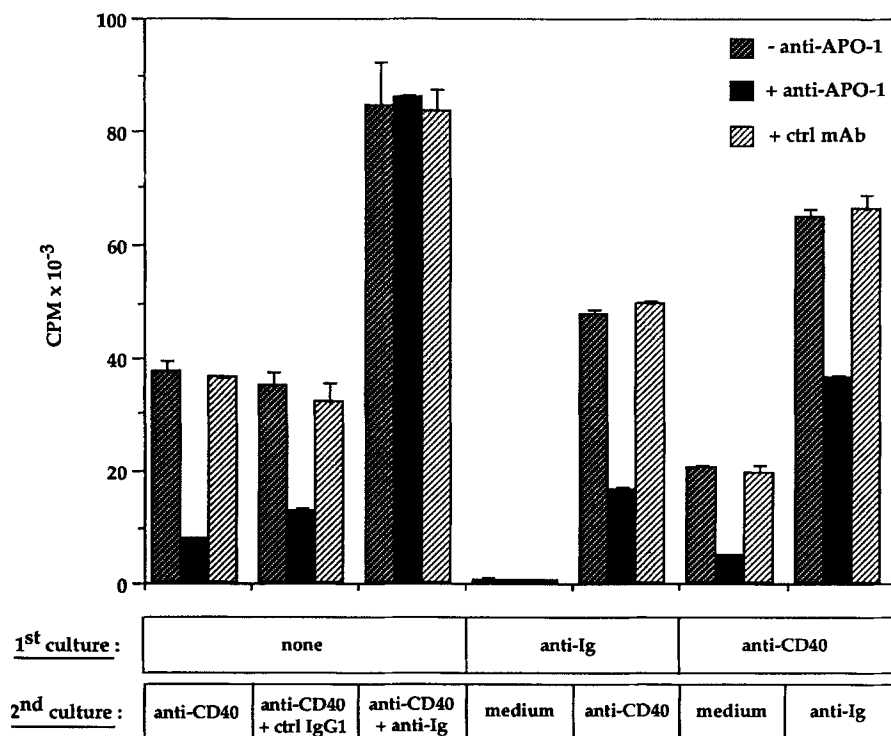


Figure 6. Protection of B cells from APO-1-dependent apoptosis is afforded by concurrent ligation of sIgs and CD40. Two culture systems were applied. In the first, the stimulating agents were simultaneously provided at the onset of the culture. In the second, B cells were precultured with either anti- κ/λ or anti-CD40 mAbs for 48 h (1st culture) and then recultured with anti-CD40 and anti- κ/λ mAbs, respectively (2nd culture). The anti-APO-1 and IgG3 control mAbs were added at the onset of the secondary cultures. All cultures were made in the presence of mitomycin C-treated HR4C6 cells. [³H]Thymidine incorporation levels were assessed 3 d after initiation of the secondary cultures, after a 16-h pulse. Note that the addition of an unrelated IgG1 mAb (*ctrl IgG1*) instead of the anti- κ/λ mAbs to CD40-stimulated cultures neither affected the levels of response to anti-CD40 nor the inhibitory effect of the anti-APO-1 mAb.

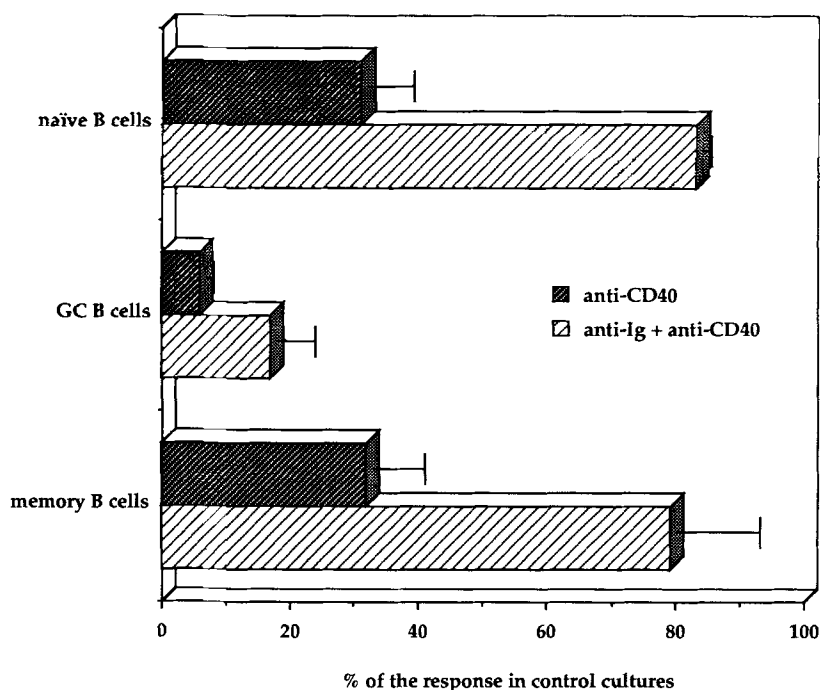


Figure 7. Engagement of the Ag receptor does not protect CD40-activated GC B cells from APO-1-mediated apoptosis. Isolated naive, GC, and memory B cells were stimulated with the anti-CD40 mAb or with the combination of anti-Ig and anti-CD40 Abs, in the presence of mitomycin C-treated HR4C6 cells. Cultures were further supplemented with either the anti-APO-1 mAb or the IgG3- κ control mAb (used at 200 ng/ml). DNA synthesis was assessed at day 2, after a 16-h pulse with [3 H]thymidine. Results are expressed as percent response in control cultures receiving the IgG3- κ control mAb, calculated as described in Materials and Methods. The results correspond to the mean \pm SD of the values calculated from three different experiments.

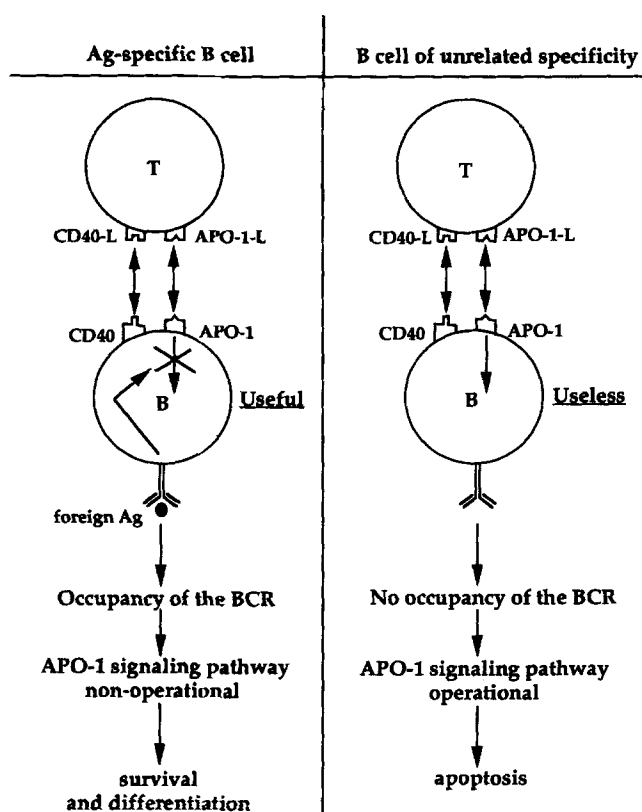


Figure 8. Hypothetical model for the immunoregulatory role of APO-1 on B cell responses. The assumption that the APO-1-L and CD40-L can both be expressed on Ag-activated CD4 $^+$ T cells predicts that B cells can be signaled both via APO-1 and CD40 upon interaction with T cells. Occupancy of the Ag receptor on B cells prevents the delivery of the death signal via APO-1 and thus ensures that the Ag-specific B cell clones

correlated with a defective sIg signaling consecutive to the loss of sIgM expression (33). Therefore, in a normal situation, the blocked sIg signaling in tolerant B cells would ensure that they remain vulnerable to the APO-1-mediated death signal, even after binding of the self-Ag. Consequently, specific or bystander interaction with primed T cells would result in their demise. On the contrary, the reduced expression of APO-1 in *lpr/lpr* mice precludes elimination of self-reactive B cells and allows for their stimulation consecutive to misdirected T cell help.

The observation that engagement of the Ag receptor failed to protect anti-CD40-activated GC B cells from APO-1-mediated apoptosis is intriguing and deserves further comment. The recent description that CD44 can be expressed at low levels on centrocytes (34) raised the possibility that our GC B cell isolation procedure, based on the depletion of CD44-bearing cells, could introduce a bias favoring the recovery of centroblasts to the detriment of centrocytes. Centroblasts are currently described as expressing low levels of sIgs (1) and are not expected to connect with T cells, which are mostly located in the light zone of GC (35). Thus, the use of centroblasts to study events that take place in the light zone of GC would have no physiological relevance. However, this hypothesis can be ruled out by two distinct observations. First, our GC B cell populations contained on average $46 \pm 5\%$ of centrocytes, identified on

are protected from APO-1-mediated apoptosis. The bystander activation of B cells whose specificity is unrelated to the ongoing response may be promoted by engagement of CD40. In this situation, because of the lack of sIg cross-linking, the dual triggering of CD40 and APO-1 will cause the demise of the unwanted B cell clones.

the basis of their lack of CD77 expression (19, 34). Second, engagement of the Ag receptor failed to protect CD40-activated centrocytes, (recovered after immunomagnetic depletion of CD77⁺ cells) from APO-1-induced apoptosis (data not shown). It is possible that additional costimulatory signals, provided by the GC microenvironment are required together with those delivered through sIgs to protect GC B cells from the APO-1-mediated death signal. This notion is supported by recent data showing that engagement of the Ag receptor on GC B cells by soluble Ag induces their apoptosis (36, 37), thus, suggesting that the Ag has to be taken up from follicular dendritic cells to allow delivery of a rescue signal through sIgs. A plausible scenario could be that some of the membrane receptors that are triggered when centrocytes bind to follicular dendritic cells are necessary to complement the signal transduced by the Ag receptor in order to afford protection against APO-1-mediated apoptosis. If this assumption is correct, GC B cells that fail to establish close interactions with follicular dendritic cells (such as the low affinity B cell mutants) would remain fully susceptible to APO-1-mediated cell death, even if the Ag receptor has been engaged.

The finding that B cells are no longer protected from APO-1-dependent apoptosis when the Ag receptor and CD40 are triggered separately, supports a model in which these two signaling pathways need to be contemporaneously activated to block the generation of the death signal transduced via APO-1. In vivo, coligation of the Ag receptor and CD40 is likely to occur at least during the primary

response, when naive B, T, and interdigitating cells (IDC) are brought into close proximity. In this particular cellular context, B cells can concomitantly be signaled by the native Ag presented by the IDC and by the CD40-L expressed on the adjacent activated T cell. We can only speculate on the molecular mechanism whereby triggering of sIgs protects anti-CD40-activated naive and memory B cells from APO-1-mediated apoptosis. Although we found that the coligation of sIgs and CD40 induced a moderate decrease in the density of APO-1 expression on B cells (data not shown), it remains difficult to conclude that downregulation of the APO-1 receptor is the sole determining factor accounting for the acquisition of resistance against APO-1-induced apoptosis. We favor the idea that the Ag receptor delivers short-lived intracellular signals that interfere with the CD40-mediated activation of the intracellular death pathway used by the APO-1 receptor. The signaling cascade that transmits the APO-1-mediated death signal has recently been shown to involve activation of tyrosine kinases (38) and Ras through the release of ceramide (39) and to converge on increasing the activity of an ICE-like protease (40, 41). It has also been reported that the expression of protein tyrosine phosphatases interacting with the cytosolic domain of APO-1 is required for transducing the APO-1-mediated apoptosis signal (42, 43). These findings should provide interesting clues for future studies aimed at unravelling the molecular mechanism responsible for the blockade of the APO-1 signaling pathway brought about by cross-linking of the Ag receptor.

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