Fas Ligation Induces Apoptosis of CD40-activated Human B Lymphocytes

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Summary

Since CD40/CD40 ligand (CD40Lig) interactions are essential in vivo for the generation of germinal center B cells that express Fas (Apo-1/CD95), we explored whether CD40 engagement may modulate Fas expression and function on human B lymphocytes. Resting tonsil B cells, isolated by density gradient centrifugation, express either absent or low levels of Fas. They could be induced to promptly express Fas after ligation of their CD40, however, using either a recombinant human CD40Lig or a cross-linked anti-CD40 mAb. In contrast, engagement of the B cell antigen receptor by immobilized anti-κ and -λ antibodies did not turn on Fas expression. Addition of anti-Fas mAb CH11 inhibited the later phases of CD40-induced B cell growth as a result of apoptotic cell death. Furthermore, Fas ligation inhibited proliferation and Ig secretion of CD40-activated B cells in response to recombinant cytokines such as interleukin (IL)-2, IL-4, and IL-10, as well as a cytokine-rich supernatant of phytohemagglutinin-activated T cells, indicating that none of those B cell tropic factors were able to prevent the Fas-induced death. Taken together, the present results show that engagement of CD40 antigen on B cells induces Fas expression and sensitizes them to Fas-mediated apoptosis. The delayed functional response to Fas ligation after CD40 activation may represent a way to limit the size of a specific B cell clone that is generated during T–B cell interactions.

To mount efficient humoral immune responses against the innumerable pathogens that a living organism may encounter, numerous specific B cell clones should be available at any given time, consequently imposing each a very limited size. The immune system must have therefore established safe systems to avoid the overrepresentation of only a few clones. Accordingly, the survival, growth, and death of cells is highly controlled in vivo through surface molecules that bind ligands which may be either soluble or membrane-bound polypeptides (1, 2). In particular, the CD40 receptor and the Fas (Apo-1/CD95) receptor represent two members of the TNF receptor superfamily that are expressed on several cell types (3–5). Although these two receptors display a comparable overall structure, their triggering results in opposite effects on cell growth. While CD40 acts as a growth/survival trigger for B cells (6), Fas principally acts as a death trigger for activated T and B cells (7, 8).

Ligation of the Fas molecule by functional antibodies or by its ligand (FasLig)[1] induces death of sensitive cells through an apoptotic process (9–12). The Fas/FasLig system has been demonstrated to participate in the activation-induced death of mature T cells that may prevent the development of autoreactive T cells and control T cell–dependent immune responses in vivo (13–15). This concept is best illustrated by the lymphoproliferative and autoimmune disease observed in *lpr* and *gld* mice that are deficient for Fas and FasLig, respectively (16–18). Interaction between FasLig expressed on cytotoxic T cells and Fas on target cells also represents a cytolysis mechanism that complements the perforin/granzyme pathway (19, 20).

When considering mature B lymphocytes, Fas is expressed at high level on B cells in germinal centers of secondary lymphoid organs, where massive B cell proliferation, somatic mutations within Ig variable region gene, and antigen-driven selection of high affinity B cells occur (21–23). Germinal center B cells are characterized by their rapid entry into spontaneous apoptosis, a process that can be prevented by CD40 ligation but accelerated by Fas triggering (22, 24). In contrast, most resting B cells express no or low level of Fas and are not prone to apoptosis (22, 23, 25). Since CD40–CD40Lig interaction appears necessary to induce the formation of germinal centers (26–28), we wondered whether CD40 activation of B lymphocytes may modulate Fas expression and function. Herein we show that CD40 engagement induces resting tonsil B cells to ex-

[1] *Abbreviations used in this paper:* CD40Lig-L cells, L cells stably transfected with the human CD40Lig; FasLig, Fas ligand; PHA-sup, supernatant of PHA-activated blood mononuclear cells.

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press high levels of Fas antigen, which ligation induces death by apoptosis. This results in inhibition of the later stages of CD40-induced B cell growth and differentiation. Thus, the function of Fas on B cells prevails over that of CD40 and is therefore likely to control the expansion of antigen-specific B cell clones.

Materials and Methods

Cytokines, Factors, and Culture Medium. Purified human rIL-4 (10^7 U/mg) and rIL-10 (2×10^7 U/mg) were from Schering-Plough Research Institute (Kenilworth, NJ) and were used at 50 U/ml and 50 ng/ml, respectively. Purified human rIL-2 (Amgen Biologicals, Thousand Oaks, CA) was used at 10 U/ml. A conditioned medium of activated mononuclear cells was produced by stimulating PBMC (2×10^6 cells/ml) with 1 μg/ml PHA (Murex Diagnostics, Dartford, UK) for 3 d and is referred to as PHA-sup. All cultures were performed in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS (GIBCO BRL, Gaithersburg, MD), and 50 μg/ml gentamycin (Schering-Plough, Levallois-Perret, France).

Antibodies. Purified anti-Fas mAb (IgM, clone CH11) and FITC-conjugated anti-Fas mAb (IgG1, clone UB2) were purchased from Immunotech (Marseille, France). IgM control mAb (for bioassays) and FITC-conjugated IgG control mAbs (for flow cytometric analyses) were from Dako (Glostrup, Denmark). FITC-conjugated anti-CD19, anti-CD20, anti-CD2, anti-CD3, and anti-CD14 were from Becton & Dickinson Co. (Mountain View, CA). The rat anti–mouse CD8α mAb (clone 53.6.72) was a gift from Dr. A. Zlotnik (DNAX Research Institute, Palo Alto, CA) and was used as culture supernatant. Purified mAbs against human κ and λ light chain Ig were from Immunotech.

Fibroblastic L Cells and Human CD40Lig Constructs. Mouse fibroblastic L cells stably transfected with the human CD32/Fc-γRII (CD32-L cells) have been previously described (29). For generation of L cells stably transfected with the human CD40Lig (CD40Lig-L cells), the CD40Lig cDNA (30) was amplified by PCR using the following primers: forward 5′-CGCTAACGTTCTCGAGCTTTAACACAGCATGATCGAA-3 and reverse 5′-CATTGTGATGCGGCCGCTCAGAGTTTGAGTAAGCCAAA-3′, introducing Xho1 and Not1 restriction sites, respectively (restriction sites are underlined). PCR amplification (Perkin-Elmer Cetus, Oakland, CA) was performed by 35 cycles of the following scheme: 1 min denaturation at 94°C, 2 min anealing at 60°C, and 3 min primer extension at 72°C. The PCR product was then digested, gel purified, and cloned into the expression plasmid pME18S-neo, which contains the neomycin resistance gene. Mouse L cells (7×10^6) were then transfected with 20 μg of the resulting expression plasmid pME18S-neo-CD40Lig using electroporation (220 V, 960 μF; Gene Pulser; Bio Rad Laboratories, Richmond, CA). After electroporation, neomycin-resistant L cells were selected in RPMI 1640, 10% FCS containing 0.5 mg/ml G418 (GIBCO), and CD40Lig-expressing cells were further isolated by three rounds of sorting on a FACStar^{plus}® (Becton Dickinson & Co.) after staining with a CD40-Fc chimeric molecule (31). For generation of a soluble CD40Lig–mouse CD8α fusion protein (CD40Lig-mCD8), the extracellular region of mouse CD8α was first amplified by reverse transcription PCR from total RNA of the mouse T cell line CTLL-2 using the following primers: forward 5′-GCTAACGTTCTCGAGGGAGAGCACACCATGGCCTCA-3′ and reverse 5′-GCTAACGTTCTGCA**GTCGAC**ATCACAGGCGAAGTCCAATCC-3′ introducing the XhoI and PstI sites, respectively (sites are underlined). The PCR amplification was performed as described above. After digestion and purification, the PCR product was cloned into the expression plasmid pME18S. By the PCR reaction, a unique SalI site was introduced directly adjacent to the 3′ end of the CD8α gene (indicated in bold). Next, the extracellular region of human CD40Lig was PCR amplified from CD40Lig cDNA using the following primers: forward 5′-GCTAACGTTGTCGAC**GTC**-AGAAGGTTGGACAAGATAGAA-3′ and reverse 5′-CATTGTGATGCGGCCGCTCAGAGTTTGAGTAAGCCAAA-3′, introducing a SalI and NotI restriction site, respectively (sites are underlined). The forward primer in addition introduced the sequence GTC (indicated in bold). PCR reaction was performed as described above, and the PCR product was digested, purified, and cloned into the plasmid pME18S-mCD8. By this procedure, the mCD8α and hCD40Lig are cloned in frame with a linker sequence of GTCGACGTC that contains both SalI and AatII sites, and coding for the peptide Val-Asp-Val. COS cells (7×10^6) were then transfected with 20 μg of the final expression plasmid pME18S-mCD8-CD40Lig using electroporation and grown in RPMI 1640, 1% Nutridoma HU (Boehringer Mannheim GmbH, Mannheim, Germany). After 4 d of culture, COS cell supernatant was harvested, centrifuged, and filtrated on a 0.2-μm filter. This expressed soluble CD40Lig-mCD8 construct was found to be functional, as determined by its specific binding on CD40-expressing human B cells after staining with FITC-conjugated rat anti-mCD8α mAb (Boehringer Mannheim) and flow cytometric analysis.

B Cell Preparations. Total normal B cells were purified from tonsils as described earlier (32). Briefly, mononuclear cells separated by standard Ficoll-Hypaque gradient were first rosetted with SRBC. Nonrosetting cells were further incubated with anti-CD2, anti-CD3, and anti-CD14 mAbs, and were then submitted to negative selection performed with magnetic beads coated with anti–mouse IgG (Dynabeads; Dynal, Oslo, Norway). Purified B cell preparations contained >98% B cells and <2% T cells and monocytes, as determined by staining with FITC-conjugated anti-CD19, anti-CD20, anti-CD2, anti-CD3, and anti-CD14 mAbs, and fluorescence analysis performed with a FACScan® (Becton Dickinson & Co.). Total tonsil B cells were further separated according to their size using a discontinuous gradient of Percoll (Pharmacia, Uppsala, Sweden) consisting of three layers of Percoll solutions in a 15-ml conical tube (4 ml of 65% [1. 085 g/ml], 4 ml of 55% [1.075 g/ml], and 2 ml of 35% [1.050 g/ml]). 5×10^7 B cells were overlaid on the top of the gradient and centrifuged for 20 min at 550 *g* at 20°C. B cells recovered at the interface of 35–55% layers were referred to as low density B cells, and cells recovered at the interface of 65–55% Percoll solutions were referred to as high density or resting B cells.

Analysis of Fas Expression. Cell-surface expression of Fas molecule was determined on freshly isolated or cultured B cells by standard direct immunofluorescence staining with FITC-conjugated anti-Fas mAb UB2 and by flow cytometric analysis with a FACScan®. Nonspecific staining was determined using a FITC-conjugated IgG_1 control mAb. Dead cells and fibroblastic L cells, used in the different culture systems, were excluded during cytometric analysis by addition of 2 μg/ml propidium iodide and according to their forward and right angle scatter parameters.

Proliferation Assay. Resting B cells were seeded in 96-well flat-bottom plates (Falcon Labware, Oxnard, CA) at 2×10^4 cells per well in the presence of 5×10^3 irradiated (7,500 rad) CD40Lig-L cells. Cytokines and antibodies were added at the

initiation of culture or at the time indicated within the text to reach a final volume of 200 μl per well. Each culture condition was performed in triplicate, and proliferation was determined by [^{3}H]TdR uptake after pulsing cells with [^{3}H]TdR (0.5 μCi per well, specific activity = 25 Ci/mmol; CEA, Saclay, France) during the last 16 h of culture. After harvesting cells on glass-fiber filters, [^{3}H]TdR incorporation was measured by standard liquid scintillation counting techniques.

Measurement of Cell Viability and Apoptosis. Resting B cells were cultured in 48-well culture plates (10^6 cells per well) over irradiated CD40Lig-L cells (5×10^4 per well). After 3 d of activation, anti-Fas mAb CH11 or IgM control mAb were added to the cultures at a final concentration of 1 μg/ml. Cell survival and apoptosis were then determined on cultured B cells collected at the time indicated in the text. Viable B cells were enumerated by Trypan blue exclusion. For detection of apoptosis, cultured B cells were gently cytospun and stained with Giemsa. For detection of DNA fragmentation, 2×10^6 cultured B cells were collected, washed with cold PBS, and centrifuged in Eppendorf tubes. Pellets were then lysed in proteinase K buffer, treated with RNase A, and samples were run on a 1.5% agarose gel containing 0.1 μg/ml ethidium bromide and visualized by UV light illumination essentially as described earlier (33).

Ig Production. Purified resting B cells (2×10^4 cells per well) were cultured on irradiated CD40Lig-L cells (5×10^3 per well) with or without cytokines or antibodies added at the onset of culture in a final volume of 200 μl. After a 14-d culture period, IgM, IgG, IgA, and IgE concentrations were measured in cell-free supernatants by using specific ELISAs (34).

Results

CD40 Engagement Induces Fas Expression on Resting Human B Cells. Human tonsillar B lymphocytes contain a subpopulation of cells expressing Fas that represent 30–40% of total B cells (Fig. 1 *A*). They are essentially low density germinal center B cells (22, 23, 35). Accordingly, Fig. 1 *A* shows that the low density tonsillar B cells obtained by Percoll gradient centrifugation contained 40–60% Fas$^+$ cells, while high density (resting) B cells contained ~5% Fas$^+$ cells (mean of five experiments).

To determine the signals required for Fas induction, resting tonsil B cells were cultured on irradiated CD40Lig-L cells and Fas expression was determined at different time points by flow cytometric analysis. As shown in Fig. 1 *B*, CD40 activation induced B cells to express Fas. Fas could be detected on the B cell surface as early as 12 h after CD40 activation, and its expression further increased with time. All B cells expressed Fas after 48 h with a maximal and stable intensity between 3 and 5 d of culture. To rule out a possible role of the fibroblastic L cell, resting B cells were cultured with a soluble human CD40Lig–mouse CD8α fusion protein (CD40Lig-mCD8). As shown in Fig. 1 *C*, culturing resting B cells for 3 d with CD40Lig-mCD8 resulted in induction of Fas expression whose intensity was further increased by cross-linking with an anti-mCD8 antibody. In keeping with this, the culture of resting B cells over untransfected L cells did not result in upregulation of Fas expression.

The CD40-dependent induction of Fas expression is not a mere indication of B cell activation inasmuch as engagement of resting B cell antigen receptor, using immobilized anti-κ and anti-λ mAbs, did not turn on Fas expression (Fig. 2), while it induced short-term DNA synthesis (data not shown).

Cross-linking Fas Molecules Inhibits the Later Stages of CD40-dependent B Cell Proliferation. To determine the function of the Fas molecule on CD40-activated B cells,

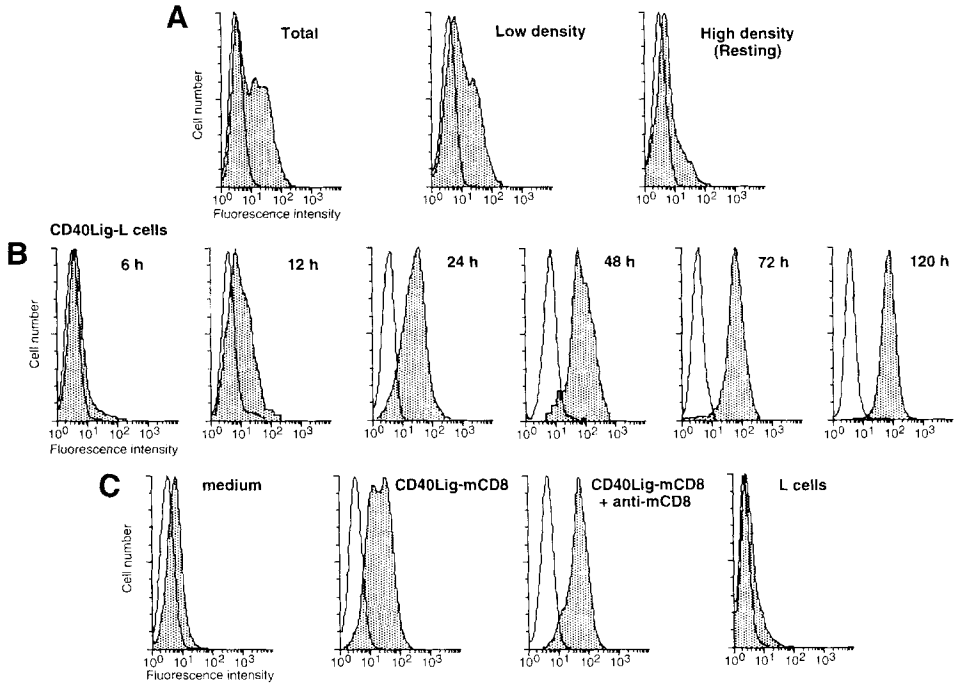

Figure 1. CD40 ligation induces Fas expression on resting tonsil B cells. (*A*) Expression of Fas on freshly isolated total, low density and high density resting tonsil B cells. (*B*) Fas expression on resting B cells cultured on irradiated CD40Lig-L cells for the time indicated. (*C*) Fas expression on resting B cells cultured for 72 h in the following conditions (*a*) in medium alone; (*b*) in the presence of soluble CD40Lig-mCD8 (20% COS supernatant) with or without anti-mCD8 mAb; or (*c*) on untransfected irradiated L cells. Cells were stained with FITC-conjugated anti-Fas mAb UB2 and Fas expression (*filled histograms*) was analyzed by flow cytometry with a logarithmic scale. Unfilled histograms represent staining with a negative control mAb.

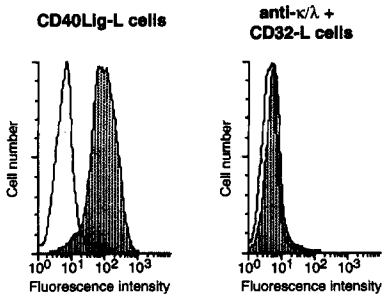

Figure 2. Triggering of the B cell antigen receptor does not induce Fas expression on resting B cells. Resting tonsil B cells were cultured either on irradiated CD40Lig-L cells or in the presence of anti-κ and anti-λ mAbs (1 μg/ml) immobilized on irradiated CD32-L cells. After 3 d of activation, Fas expression was analyzed by flow cytometry as in Fig. 1.

resting B cells were cultured on CD40Lig-L cells in the presence of the anti-Fas mAb CH11, a functional IgM mAb that induces cell death when used in a soluble form (10). Increasing concentrations of anti-Fas mAb were added at the onset of the culture, and B cell proliferation was measured by [^{3}H]TdR uptake on day 6. As shown in Fig. 3 *A*, anti-Fas mAb inhibited CD40-dependent B cell proliferation in a dose-dependent manner, with an optimum at ~0.1 μg/ml anti-Fas mAb CH11, a concentration that induces maximal growth arrest and death of sensitive cell lines (3, 10). The time kinetics of [^{3}H]TdR uptake, however, indicated that anti-Fas only weakly affected B cell proliferation during the first 3 d of culture, while the late DNA synthesis (days 4–9) was progressively blocked (Fig. 3 *B*). Fas ligation thus antagonizes the later stages of CD40-dependent B cell DNA synthesis.

Fas Engagement Induces Apoptosis of CD40-activated B Cells. To determine the mechanisms through which Fas ligation inhibits B cell proliferation, anti-Fas mAb CH11 was added at different time points after the initiation of the culture of resting B cells over CD40Lig-L cells. Results presented in Fig. 3 *C* show that addition of anti-Fas antibody after 3 d of culture prevented the subsequent increase of [^{3}H]TdR uptake measured from days 4 to 9. Addition of anti-Fas antibody on day 5 resulted in a strong and rapid blockade of the CD40-initiated DNA synthesis. Cell viability, determined by Trypan blue dye exclusion, showed the inhibition of proliferation to be mostly the consequence of Fas-induced cell death. Indeed, addition of anti-Fas mAb to B cells preactivated for 3 d with CD40Lig-L cells resulted in 50% dead cells after 24 h and 75% dead cells after 48 h of Fas ligation (mean of four experiments) (Fig. 3 *D*). Giemsa staining performed on 3-d CD40-activated B cells revealed the presence of cells exhibiting morphological features of apoptosis (chromatin condensation and fragmentation) as early as 2 h after anti-Fas treatment, and a 4-h contact with anti-Fas mAb resulted in an increased incidence of apoptotic cells (Fig. 4). Moreover, typical "ladder" patterns of internucleosomal DNA cleavage were observed on electrophoresis from DNA of CD40-preactivated B cells further cultured with anti-Fas for 2, 4, and 6 h, but not with a control antibody (Fig. 5).

Taken together, these results indicate that Fas triggering of CD40-activated B cells induces endonuclease activity and death by apoptosis.

Fas Ligation Blocks Cytokine-induced Proliferation and Ig Secretion of CD40-activated B Cells. We further wondered whether B cell tropic factors such as IL-2, IL-4, IL-10, or a PHA-sup could prevent the inhibitory effects of anti-Fas mAb CH11 on proliferation of CD40-activated B cells. To this end, resting B cells were cultured on CD40Lig-L cells in the presence of the different B cell growth factors, and [^{3}H]TdR uptake was measured during an 8-d culture period after addition of anti-Fas mAb on day 0 or 5 of the

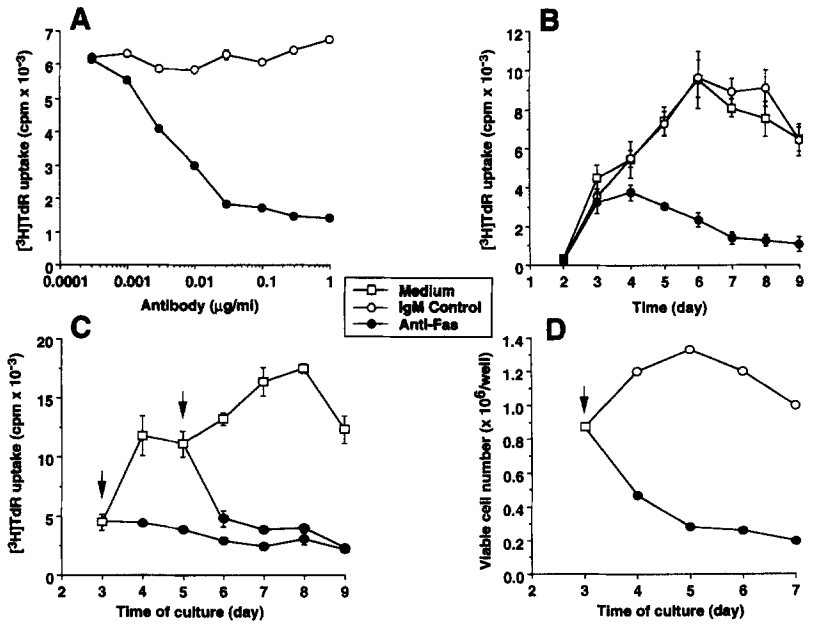

Figure 3. Fas ligation inhibits DNA synthesis and induces death of CD40-activated B cells. Resting B cells (2×10^4 per well) were cultured on irradiated CD40Lig-L cells (5×10^3 per well) and (*A*) with increasing concentrations of anti-Fas mAb CH11 or control IgM mAb added at the onset of the culture; (*B*) with medium or 0.1 μg/ml anti-Fas mAb or control mAb added on day 0; and (*C*) with or without 0.1 μg/ml anti-Fas mAb added on day 3 or 5. Proliferation was measured by [^{3}H]TdR incorporation after a pulse with [^{3}H]TdR during the last 16 h of the culture on day 6 (*A*) or at the time indicated (*B* and *C*), and results are expressed as mean ± SD of triplicate determinations. In each experiment, background of [^{3}H]TdR uptake of CD40Lig-L cells was <500 cpm. (*D*) Resting B cells (10^6 per well) were cultured on irradiated CD40Lig-L cells (5×10^4 per well). On day 3, 1 μg/ml anti-Fas mAb or control mAb was added to the culture and viable B cells were further enumerated by Trypan blue dye exclusion at the time indicated. Results represent average of duplicate determinations. Arrows in *C* and *D* indicate time of antibody addition. Data presented are representative of at least three independent experiments.

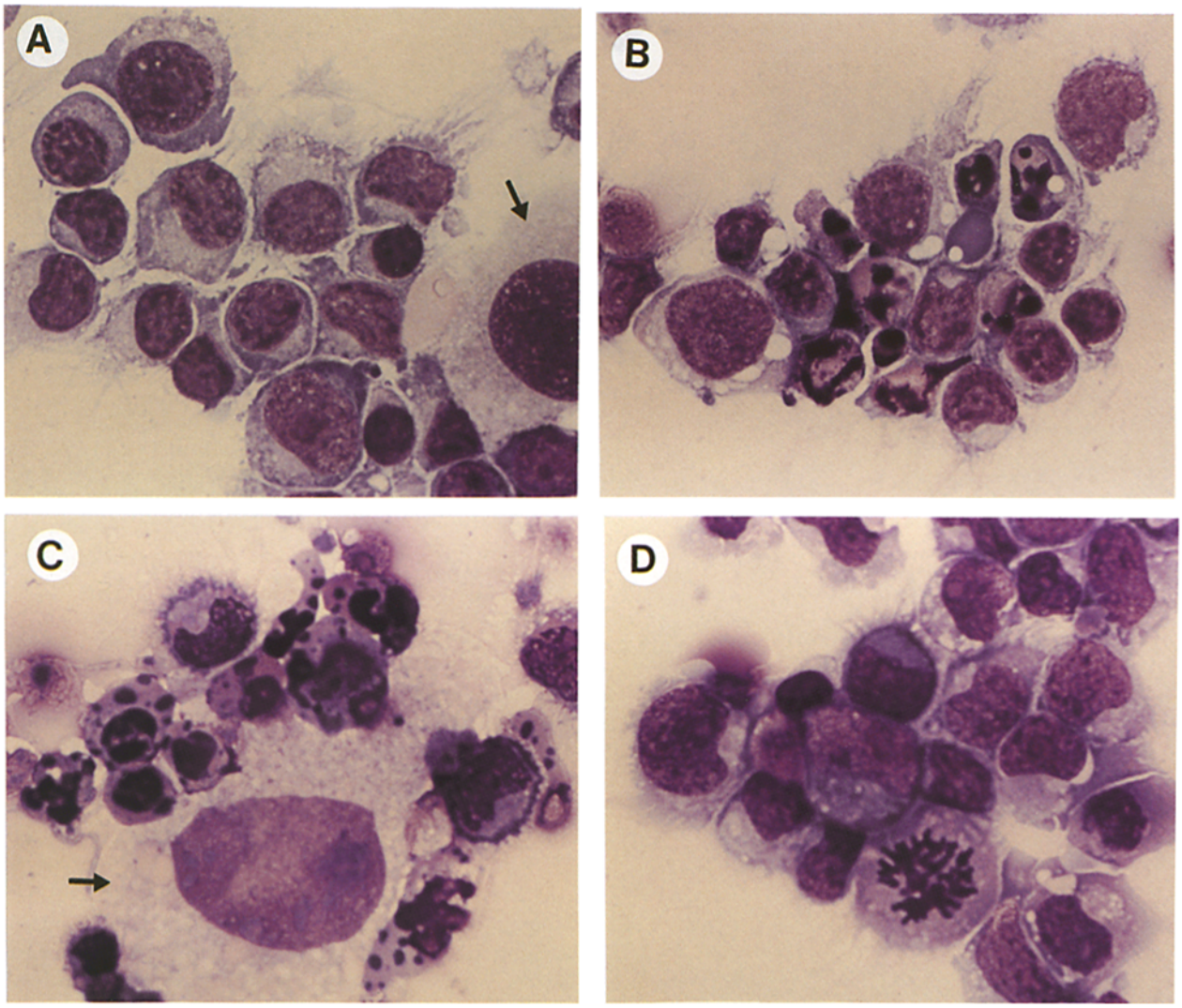

Figure 4. Fas ligation mediates apoptosis of CD40-activated B cells. Giemsa staining of resting B cells cultured for 3 d over CD40Lig-L cells (*A*) and then incubated with either 1 μg/ml anti-Fas mAb CH11 for 2 h (*B*) or 4 h (*C*), or with 1 μg/ml control mAb for 4 h (*D*). Arrows in *A* and *C* indicate a CD40Lig-L cell. ×1,000.

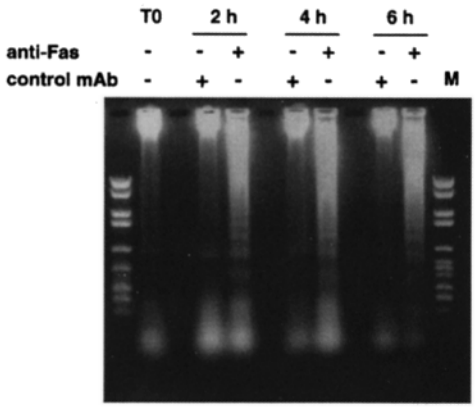

Figure 5. Fas ligation induces DNA fragmentation in CD40-activated B cells. Resting B cells were cultured for 3 d over irradiated CD40Lig-L cells. Then 1 μg/ml anti-Fas mAb CH11 or 1 μg/ml control mAb was added to the cultures. Cells were collected just before addition of antibodies (*T0*) or after 2, 4, or 6 h of treatment with antibodies. DNA from 2 × 10^6 cells was run on a 1.5% agarose gel and stained with ethidium bromide. DNA molecular weight marker (*M*) is marker VI (2,176-154 bp).

culture. As shown in Fig. 6, IL-4, IL-10, PHA-sup, and combinations of IL-10 with IL-2 or IL-4, which all increase CD40-dependent B cell proliferation, were unable to counteract the anti-Fas–induced blockade of DNA synthesis. Note that IL-2, which does not enhance the proliferation of CD40-activated B cells, was also unable to counteract the effect of anti-Fas mAb.

To further analyze whether Fas ligation affected B cell differentiation, high density B cells were cultured over CD40Lig-L cells with either PHA-sup or a combination of IL-10 and IL-2, which induces massive IgA, IgG, and IgM secretion (32), or with IL-4, which allows IgE secretion after isotype switch (36). As shown in Table 1, addition of

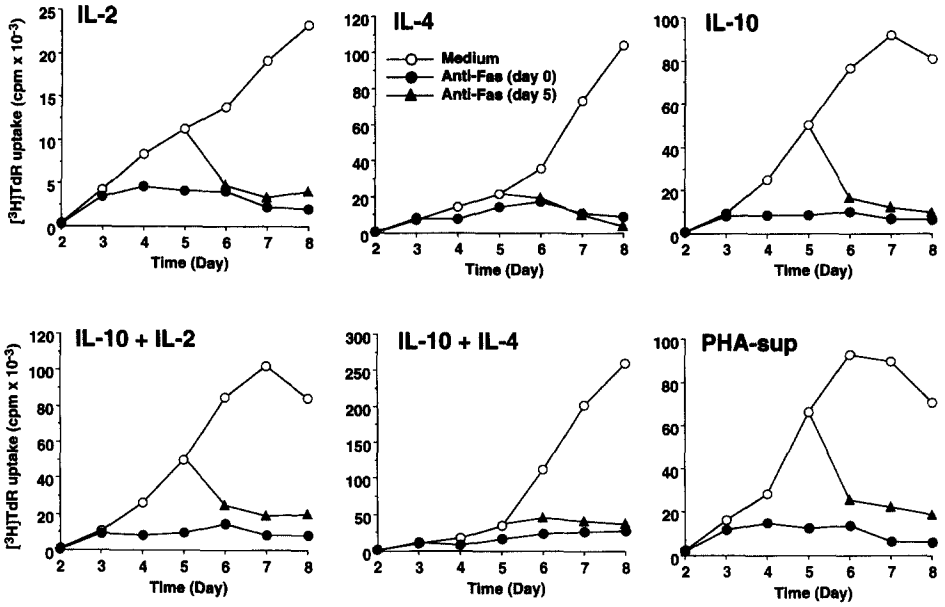

Figure 6. Fas engagement inhibits DNA synthesis of CD40-activated B cells in response to B cell growth factors. Resting B cells (2×10^4 per well) were cultured on CD40Lig-L cells (5×10^3 per well) with either IL-2 (10 U/ml), IL-4 (50 U/ml), IL-10 (50 ng/ml), IL-2 + IL-10, IL-4 + IL-10, or a PHA-sup (10% final dilution), and with or without 0.1 μg/ml anti-Fas mAb CH11 added on day 0 or 5 of the culture. Proliferation was measured by [^{3}H]TdR incorporation during the last 16 h of the culture at the time indicated. Results are expressed as means of culture triplicates. SDs are not shown and never exceeded 10% of the mean values. [^{3}H]TdR uptake of irradiated CD40Lig-L cells was <500 cpm in the different experiments. This figure is representative of three experiments.

anti-Fas to cultures strongly inhibited the cytokine-induced secretion of IgM and IgG, as well as the IL-4–induced secretion of IgE. A similar inhibition of IgA secretion was also observed (data not shown). Fas ligation therefore prevents the differentiation of CD40-activated B cells induced by IL-2 + IL-10, IL-4, and activated T cell supernatants. Note that none of these factors were found to downregulate CD40-induced Fas expression (data not shown).

Taken together, these results indicate that neither IL-2, IL-4, IL-10, nor activated T cell supernatants can antagonize the Fas-induced apoptosis of CD40-activated B cells.

Discussion

The present study demonstrates that CD40 triggering readily turns on Fas expression on resting B cells and that further Fas ligation prevails over CD40 engagement, since B cells that undergo the dual triggering enter into apoptosis.

This study focused attention on the analysis of Fas$^-$ B cells in culture that have been negatively selected by density gradient centrifugation because positive selection (e.g., using antibodies to surface IgD for selecting Fas$^-$ naive B cells) could have induced subsequent alteration of Fas expression and B cell death/survival (37, 38). Maximal Fas expression, reached after 48–72 h of activation, requires an optimal ligation of the CD40 molecule that can be achieved either by CD40Lig stably expressed on fibroblastic L cells or by cross-linking a soluble CD40Lig–CD8 fusion protein with anti-CD8 mAb. Accordingly, an anti-CD40 antibody had to be cross-linked with CD32-L cells (CD40 system) (39) for inducing Fas expression on resting B cells (data not shown). In keeping with this, all the experiments presented herein with the CD40Lig-L cells have been reproduced using the CD40 system (data not shown). Consistent with the expression of CD40Lig on activated T cells (31, 40, 41), anti-CD3 activated T cells were also

Table 1. *Fas Ligation Inhibits Cytokine-dependent Ig Secretion of CD40-activated B Cells*

Cytokine	IgM (ng/ml)			IgG (ng/ml)			IgE (ng/ml)		
	Medium	IgM control	Anti-Fas	Medium	IgM control	Anti-Fas	Medium	IgM control	Anti-Fas
None	69	53	<40	80	97	<40	<0.15	<0.15	<0.15
IL-4	206	157	<40	208	224	<40	10.5	8.2	<0.15
IL-10 + IL-2	1,790	1,557	<40	1,060	1,127	<40	<0.15	<0.15	<0.15
PHA-sup	1,253	1,181	45	3,860	3,281	50	<0.15	<0.15	<0.15

Resting tonsil B cells (2×10^4 per well) were cultured over irradiated CD40Lig-L cells (5×10^3 per well) without or with IL-4 (50 U/ml), IL-10 (50 ng/ml) plus IL-2 (10 U/ml), or PHA-sup (10%), as well as in the absence or presence of 0.1 μg/ml anti-Fas mAb CH11 or an IgM control mAb. After 14 d of culture, cell-free supernatants were collected, and the concentrations of IgM, IgG, and IgE were measured by specific ELISAs. Results are expressed as the mean of triplicate determinations, and they are representative of three independent experiments. SDs were <10% in all cases.

found to readily induce Fas expression on B cells (data not shown).

While considerable Fas expression could be observed after 24–48 h of CD40 activation, a functional response to Fas triggering could only be seen after 3 d of activation, since B cells costimulated through both CD40 and Fas display reduced growth and the first signs of cell death only after 3 d of culture. Such a delayed establishment of Fas sensitivity has been previously described for normal B cells activated with pokeweed mitogen (42), for leukemic B cells activated with a combination of *Staphylococcus aureus* particles and IL-2 (43), and for mitogen-activated T cells (44, 45). The cell susceptibility to Fas-mediated killing was previously associated with an inverse expression of the anti-apoptotic intracellular Bcl-2 protein (43, 46, 47). Though a slight decrease in Bcl-2 protein expression was observed in B cells activated for more than 3 d through their CD40 (data not shown), this does not permit us to account for the susceptibility of B cells to Fas ligation. This may be explained by the numerous genes that appear to be involved in the control of cell survival (48, 49). Thus, a thorough analysis aimed at understanding the establishment of B cell susceptibility to Fas ligation will require the availability of antibodies specific for these gene products because the death/survival decision depends on the balance between these proteins.

An efficient T cell–dependent humoral immune response against one specific antigen is initiated when APCs and B cells present the processed antigen to T cells in an MHC-restricted fashion. T cells activated through their TCR/CD3 complex are induced to express CD40Lig and to secrete cytokines that in turn induce CD40-dependent B cell proliferation (6, 50). TCR engagement, however, can induce FasLig expression on T cells (13–15, 51), and the present results clearly demonstrate that CD40 engagement induces Fas expression on B cells, and further ligation of this inhibits CD40-dependent DNA synthesis. Thus, simultaneous induction of the CD40/CD40Lig and Fas/FasLig systems may result in aborted humoral immune responses. Several possibilities should be considered to explain how B cells can survive, grow, and differentiate when they receive signals from activated T cells. (*a*) Activated $CD4^+$ T cells may not always express FasLig. In keeping with this, murine Th2 cells, which represent the preferential helpers of humoral responses, were shown to express no or low level of FasLig, while Th1 cells represent potent effectors of Fas-mediated cytotoxicity (52, 53). (*b*) T cells may deliver additional signals that may counteract FasLig/Fas-induced B cell death. Inasmuch as efficient T cell help required different T/B cell interactions (54), such cell-surface antigen interactions have to be considered since the experiments reported herein do not support a possible effect of T cell cytokines. (*c*) B cell antigen receptor signals may limit sensitivity to Fas ligation, thus favoring expansion and differentiation of antigen-specific B cells and preventing expansion of bystander activated B cells. This was recently described in an analysis of mouse B cell susceptibility to Fas-dependent, Th1-mediated cytotoxicity measured in 4-h chromium release assays (55). Our preliminary data indicate that surface Ig triggering on resting B cells downregulates CD40-induced Fas expression and partially prevents further Fas-induced cell death (data not shown). (*d*) Functional Fas/FasLig interaction may represent a late event in the T/B cell dialog. While Fas is expressed early on activated B cells, their sensitivity towards Fas-induced death is acquired only after prolonged activation (reference 42 and our present results). Thus, during T cell encounter, an antigen-selected B cell (e.g., in the germinal center light zone) will undergo CD40 activation that leads to clonal expansion and seeding of daughter B cells that will eventually differentiate into plasma blasts and/or memory B cells (56). The initial B cell of the clone will then be eliminated by apoptosis after a prolonged Fas engagement. The CD40-induced upregulation of Fas expression, associated to a delayed functional FasLig responsiveness, may therefore represent a way to limit the expansion of a given B cell clone. A dysfunction of this Fas system will result in the generation of a predominant clone that will compete with the multiple B cell clones required for appropriate humoral responses. Complementary findings on the upregulation of functional Fas expression on B lymphocytes activated through their CD40 antigen have been reported in two recent studies (57, 58).

In conclusion, CD40 triggering turns on Fas expression on B cells that undergo apoptosis after subsequent Fas engagement. The Fas system may thus represent a safeguard system that operates not only on T cells, but also at the B cell level, and may permit to limit the B cell clone size during T cell–dependent humoral immune responses.

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References

1. Raff, M.C. 1992. Social controls on cell survival and cell death. *Nature (Lond.).* 356:397–400.
2. Cohen, J.J. 1991. Programmed cell death in the immune system. *Adv. Immunol.* 50:55–85.
3. Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S.I. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell.* 66:233–243.
4. Stamenkovic, I., E.A. Clark, and B. Seed. 1989. A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *EMBO (Eur. Mol. Biol. Organ.). J.* 8:1403–1410.
5. Smith, C.A., T. Farrah, and R.G. Goodwin. 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death. *Cell.* 76:959–962.
6. Banchereau, J., F. Bazan, D. Blanchard, F. Brière, J.-P. Galizzi, C. van Kooten, Y.-J. Liu, F. Rousset, and S. Saeland. 1994. The CD40 antigen and its ligand. *Annu. Rev. Immunol.* 12:881–922.
7. Nagata, S., and P. Golstein. 1995. The Fas death factor. *Science (Wash. DC).* 267:1449–1456.
8. Krammer, P.H., J. Dhein, H. Walczak, I. Behrmann, S. Mariani, B. Matiba, M. Fath, P.T. Daniel, E. Knipping, M.O. Westendorp et al. 1994. The role of APO-1-mediated apoptosis in the immune system. *Immunol. Rev.* 142:175–191.
9. Trauth, B.C., C. Klas, A.M. Peters, S. Matzku, P. Moler, W. Falk, K.M. Debatin, and P.H. Krammer. 1989. Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science (Wash. DC).* 245:301–305.
10. Yonehara, S., A. Ishii, and M. Yonehara. 1989. A cell-killing monoclonal antibody (anti-FAS) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* 169:1747–1756.
11. Suda, T., T. Takahashi, P. Golstein, and S. Nagata. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell.* 75:1169–1178.
12. Takahashi, T., M. Tanaka, J. Inazawa, T. Abe, T. Suda, and S. Nagata. 1994. Human Fas ligand: gene structure, chromosomal location and species specificity. *Int. Immunol.* 6:1567–1574.
13. Dhein, J., H. Walczak, C. Bäumler, K.-M. Debatin, and P.H. Krammer. 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature (Lond.).* 373:438–441.
14. Brunner, T., R.J. Mogil, D. LaFace, N.J. Yoo, A. Mahboubi, F. Echeverri, S.J. Martin, W.R. Force, D.H. Lynch, C.F. Ware, and D.R. Green. 1995. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature (Lond.).* 373:441–444.
15. Ju, S.-T., D.J. Panka, H. Cui, R. Ettinger, M. El-Khatib, D.H. Sherr, B.Z. Stanger, and A. Marshak-Rothstein. 1995. Fas (CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature (Lond.).* 373:444–448.
16. Cohen, P.L., and R.A. Eisenberg. 1991. *Lpr* and *gld*: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu. Rev. Immunol.* 9:243–269.
17. Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature (Lond.).* 356:314–317.
18. Takahashi, T., M. Tanaka, C.I. Brannan, N.A. Jenkins, N.G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell.* 76:969–976.
19. Kägi, D., F. Vignaux, B. Lederman, K. Bürki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science (Wash. DC).* 265:528–530.
20. Rouvier, E., M.-F. Luciani, and P. Golstein. 1993. Fas involvement in Ca^{2+}-independent T cell–mediated cytotoxicity. *J. Exp. Med.* 177:195–200.
21. MacLennan, I.C.M. 1994. Germinal centers. *Annu. Rev. Immunol.* 12:117–139.
22. Liu, Y.-J., C. Barthelemy, O. de Bouteiller, C. Arpin, I. Durand, and J. Banchereau. 1995. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid upregulation of B7.1 and B7.2. *Immunity.* 2:239–248.
23. Yoshino, T., E. Kondo, L. Cao, K. Takahashi, K. Hayashi, S. Nomura, and T. Akagi. 1994. Inverse expression of bcl-2 protein and Fas antigen in lymphoblasts in peripheral lymph nodes and activated peripheral blood T and B lymphocytes. *Blood.* 83:1856–1861.
24. Liu, Y.J., D.E. Joshua, G.T. Williams, C.A. Smith, J. Gordon, and I.C.M. MacLennan. 1989. Mechanisms of antigen-driven selection in germinal centres. *Nature (Lond.).* 342:929–931.
25. Miyawaki, T., T. Uehara, R. Nibu, T. Tsuji, A. Yachie, S. Yonehara, and N. Taniguchi. 1992. Differential expression of apoptosis-related Fas antigen on lymphocyte subpopulations in human peripheral blood. *J. Immunol.* 149:3753–3758.
26. Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani. 1994. The immune response in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity.* 1:167–178.
27. Foy, T.M., J.D. Laman, J.A. Ledbetter, A. Aruffo, E. Claassen, and R.J. Noelle. 1994. gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. *J. Exp. Med.* 180:157–163.
28. Xu, J., T.M. Foy, J.D. Laman, E.A. Elliott, J.J. Dunn, T.J. Waldschmidt, J. Elsemore, R.J. Noelle, and R.A. Flavell. 1994. Mice deficient for the CD40 ligand. *Immunity.* 1:423–431.
29. Peltz, G.A., M.L. Trounstine, and K.W. Moore. 1988. Cloned and expressed human Fc receptor for IgG mediates anti-CD3 dependent lymphoproliferation. *J. Immunol.* 141:1891–1896.
30. Cocks, B.G., R. de Waal-Malefyt, J.-P. Galizzi, J.E. de Vries, and G. Aversa. 1993. IL-13 induces proliferation and differentiation of human B cells activated by the CD40 ligand. *Int. Immunol.* 5:657–663.
31. Hermann, P., D. Blanchard, B. de Saint-Vis, F. Fossiez, C. Gaillard, B. Vanbervliet, F. Brière, J. Banchereau, and J.-P. Galizzi. 1993. Expression of a 32 kD ligand for the CD40 antigen on activated human T lymphocytes. *Eur. J. Immunol.* 23:961–964.
32. Fluckiger, A.-C., P. Garrone, I. Durand, J.P. Galizzi, and J. Banchereau. 1993. IL-10 upregulates functional high affinity IL-2 receptors on normal and leukemic B lymphocytes. *J. Exp. Med.* 178:1473–1481.
33. Smith, C.A., G.T. Williams, R. Kingston, E.J. Jenkison, and J.J.T. Owen. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature (Lond.).* 337:181–184.

34. Rousset, F., E. Garcia, and J. Banchereau. 1991. Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. *J. Exp. Med.* 173:705–710.
35. Möller, P., C. Henne, F. Leithäuser, A. Eichelmann, A. Schmidt, S. Brüderlein, J. Dhein, and P.H. Krammer. 1993. Coregulation of the APO-1 antigen with intercellular adhesion molecule-1 (CD54) in tonsillar B cells and coordinate expression in follicular center B cells and in follicle center and mediastinal B-cell lymphomas. *Blood.* 81:2067–2075.
36. Gascan, H., J.-F. Gauchat, M.-G. Roncarolo, H. Yssel, H. Spits, and J.E. de Vries. 1991. Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by interleukin 4 and a signal provided by activated $CD4^+$ T cell clones. *J. Exp. Med.* 173:747–750.
37. Nossal, G.J.V. 1994. Negative selection of lymphocytes. *Cell.* 76:229–239.
38. Von Boehmer, H. 1994. Positive selection of lymphocytes. *Cell.* 76:219–227.
39. Banchereau, J., and F. Rousset. 1991. Growing human B lymphocytes in the CD40 system. *Nature (Lond.).* 353:678–679.
40. Armitage, R.J., W.C. Fanslow, L. Strockbine, T.A. Sato, K.N. Clifford, B.M. MacDuff, D.M. Anderson, S.D. Gimpel, T. Davis-Smith, C.R. Maliszewski et al. 1992. Molecular and biological characterization of a murine ligand for CD40. *Nature (Lond.).* 357:80–82.
41. Lane, P., A. Traunecker, S. Hubele, S. Inui, A. Lanzavecchia, and D. Gray. 1992. Activated human T cells express a ligand for the human B cell-associated antigen CD40 which participates in T cell-dependent activation of B lymphocytes. *Eur. J. Immunol.* 22:2573–2578.
42. Daniel, P.T., and P.H. Krammer. 1994. Activation induces sensitivity toward APO-1 (CD95)-mediated apoptosis in human B cells. *J. Immunol.* 152:5624–5632.
43. Mapara, M.Y., R. Bargou, C. Zugck, H. Döhner, F. Ustaoglu, R.R. Jonker, P.H. Krammer, and B. Dörken. 1993. APO-1 mediated apoptosis or proliferation in human chronic B lymphocytic leukemia: correlation with bcl-2 oncogene expression. *Eur. J. Immunol.* 23:702–708.
44. Owen-Schaub, L.B., S. Yonehara, W.L. Crump III, and E. Grimm. 1992. DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by Fas antigen engagement. *Cell. Immunol.* 140:197–205.
45. Klas, C., K.-M. Debatin, R.R. Jonker, and P.H. Krammer. 1993. Activation interferes with the APO-1 pathway in mature human T cells. *Int. Immunol.* 5:625–630.
46. Iwai, K., T. Miyawaki, T. Takizawa, A. Konno, K. Ohta, A. Yachie, H. Seki, and N. Taniguchi. 1994. Differential expression of bcl-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils. *Blood.* 84:1201–1208.
47. Itoh, N., Y. Tsujimoto, and S. Nagata. 1993. Effect of bcl-2 on Fas antigen-mediated cell death. *J. Immunol.* 151:621–627.
48. Williams, G.T., and C.A. Smith. 1993. Molecular regulation of apoptosis: genetic controls on cell death. *Cell.* 74:777–779.
49. Oltvai, Z.N., and S.J. Korsmeyer. 1994. Checkpoints of dueling dimers foil death wishes. *Cell.* 79:189–192.
50. Durie, F.H., T.M. Foy, S.R. Masters, J.D. Laman, and R.J. Noelle. 1994. The role of CD40 in the regulation of humoral and cell-mediated immunity. *Immunol. Today.* 15:406–410.
51. Vignaux, F., E. Vivier, B. Malissen, V. Depraetere, S. Nagata, and P. Golstein. 1995. TCR/CD3 coupling to Fas-based cytotoxicity. *J. Exp. Med.* 181:781–786.
52. Ramsdell, F., M.S. Seaman, R.E. Miller, K.S. Picha, M.K. Kennedy, and D.H. Lynch. 1994. Differential ability of Th1 and Th2 T cells to express Fas ligand and to undergo activation-induced cell death. *Int. Immunol.* 6:1545–1553.
53. Stalder, T., S. Hahn, and P. Erb. 1994. Fas antigen is the major target molecule for CD4+ T cell-mediated cytotoxicity. *J. Immunol.* 152:1127–1133.
54. Clark, E.A., and J.A. Ledbetter. 1994. How B and T cells talk to each other. *Nature (Lond.).* 367:425–428.
55. Rothstein, T.L., J.K.M. Wang, D.J. Panka, L.C. Foote, Z. Wang, B. Stanger, H. Cui, S.-T. Ju, and A. Marshak-Rothstein. 1995. Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells. *Nature (Lond.).* 374:163–165.
56. Arpin, C., J. Dechanet, C. van Kooten, P. Merville, G. Grouard, F. Brière, J. Banchereau, and Y.-J. Liu. 1995. In vitro generation of memory B cells and plasma cells. *Science (Wash. DC).* 268:720–722.
57. Schattner, E., K.B. Elkon, D.-H. Yoo, J. Tumang, P. Krammer, M.K. Crow, and S.M. Friedman. 1995. CD40 ligation induces Apo-1/Fas expression on human B lymphocytes and facilitates apoptosis through the Apo-1/Fas pathway. *J. Exp. Med.* 182:1557–1565.
58. Lagresle, C., C. Bella, P.T. Daniel, P.H. Krammer, and T. Defrance. 1995. Regulation of germinal center B cell differentiation. Role of the human APO-1/Fas (CD95) molecule. *J. Immunol.* 154:5746–5756.