

# FLICE-inhibitory Protein Is a Key Regulator of Germinal Center B Cell Apoptosis

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## Abstract

Affinity maturation of the B cell response to antigen (Ag) takes place in the germinal centers (GCs) of secondary follicles. Two sequential molecular mechanisms underpin this process. First, the B cell repertoire is diversified through hypermutation of the immunoglobulin (Ig) variable region genes. Second, mutant B cell clones with improved affinity for Ag are positively selected by Ag and CD40 ligand (L). This selection step is contingent upon “priming” of GC B cells for apoptosis. The molecular means by which B cell apoptosis is initiated and controlled in the GC remains unclear. Here, we show that GC B cell apoptosis is preceded by the rapid activation of caspase-8 at the level of CD95 death-inducing signaling complex (DISC). We found that GC B cells *ex vivo* display a preformed inactive DISC containing Fas-associated death domain-containing protein (FADD), procaspase-8, and the long isoform of cellular FADD-like IL-1 $\beta$ -converting enzyme-inhibitory protein (c-FLIP<sub>L</sub>) but not the CD95L. In culture, c-FLIP<sub>L</sub> is rapidly lost from the CD95 DISC unless GC B cells are exposed to the survival signal provided by CD40L. Our results suggest that (a) the death receptor signaling pathway is involved in the affinity maturation of antibodies, and (b) c-FLIP<sub>L</sub> plays an active role in positive selection of B cells in the GC.

Key words: human • signal transduction • cell death • affinity maturation • B lymphocytes

## Introduction

Germinal centers (GCs)<sup>1</sup> are specialized structures within secondary lymphoid tissues in which B lymphocytes undergo affinity maturation of their B cell receptors (BCRs). This process develops in two steps that take place in distinct microanatomical compartments of the GC. First, the Ab repertoire of Ag-activated B lymphocytes is diversified in the GC dark zone through the random introduction of point mutations in their Ig variable region genes. Second, somatic mutants are tested in the GC light zone for their

capacity to efficiently take up Ag and present it to helper T lymphocytes. B cells that have successfully passed this selection test have two options. They can further differentiate into memory cells or plasma cells and leave the GC, or alternatively, they can return to the dark zone to undergo another mutation/selection cycle (for reviews, see references 1, 2). Affinity maturation of the Ab response is contingent on the enhanced propensity of GC B cells to undergo apoptosis. This allows for (a) elimination of the low affinity, irrelevant, and self-reactive B cell clones, and (b) the selective rescue of the high affinity B cell clones through the delivery of survival signals by Ag and CD40 ligand (CD40L; reference 3). It has been postulated that during their clonal expansion and hypermutation phase in the GC dark zone, B cells are protected from death by secreted or cell-bound antiapoptotic factors provided by the follicular dendritic cells (FDCs; references 4, 5).

*In vitro*, isolated GC B cells spontaneously and rapidly undergo apoptosis (3). It has been proposed that they are “primed” to cell death because the equilibrium between the negative and positive apoptosis regulators in these cells

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<sup>1</sup>Abbreviations used in this paper: BCR, B cell receptor; c-FLIP, cellular FLICE-inhibitory protein; c-FLIP<sub>L</sub>, long isoform of c-FLIP; c-FLIP<sub>S</sub>, short isoform of c-FLIP;  $\Delta\psi_m$ , mitochondrial transmembrane potential; DD, death domain; DISC, death-inducing signaling complex; FADD, Fas-associated death domain-containing protein; fmk, fluoromethylketone; FLICE, FADD-like IL-1 $\beta$ -converting enzyme; FDC, follicular dendritic cell; GC, germinal center; HRP, horseradish peroxidase; L, ligand; PS, phosphatidylserine; RT, reverse transcription.

is displaced in favor of proapoptotic molecules such as Bax, p53, or c-myc (6). It is generally admitted that initiation of the apoptotic cascade in cultured GC B cells is consecutive to their removal from their supportive stromal microenvironment and from the source of Ag and T cell help. Therefore, GC B cell apoptosis has often been assimilated to a form of death promoted by trophic factor deprivation. Significant progress has been made in understanding the biochemical mechanisms that control the execution of the apoptotic program in GC B cells. It has been documented that both endonucleases (7) and cysteine proteases, including both caspases and cathepsin (5), are required for GC B cell apoptosis *in vitro*. As far as caspases are concerned, although they intervene in the execution of most apoptotic pathways, some, such as caspase-8 or -10, are more specifically involved in initiating transduction of the signal delivered by the so-called "death receptors" (8). One of the best characterized members of this proapoptotic receptor family is CD95 (Fas/APO-1). In the mature B cell compartment, the strongest expression of CD95 is found on GC B cells (9). Clustering of CD95 at the cell membrane induces recruitment of the Fas-associated death domain-containing protein (FADD) to the trimerized intracellular death domain (DD) of the receptor (10, 11). FADD in turn recruits the proenzymatic form of caspase-8/FADD-like IL-1 $\beta$ -converting enzyme (FLICE; references 12–14), thus leading to the formation of a multimolecular signaling platform named death-inducing signaling complex (DISC; reference 15). After the formation of the DISC, caspase-8 is activated by autoproteolytic cleavage and initiates a cascade of caspases (16). One of the most proximal regulators of the CD95-induced death program is FLICE-inhibitory protein (FLIP). FLIP was first identified as a virus-encoded apoptosis-inhibitory protein, but its cellular homologue (c-FLIP) also has the capacity to interfere with DISC formation (for a review, see reference 17). Alternative splicing generates two isoforms of c-FLIP: a long form (c-FLIP<sub>L</sub>) which contains a caspase-like domain but is devoid of caspase catalytic activity, and a short form (c-FLIP<sub>S</sub>) lacking the caspase-like domain.

In this study, we report that human GC B cell apoptosis relies on caspase-8 but not on caspase-9 activation. Our data show that caspase-8 is cleaved at the level of the CD95 DISC within minutes when GC B cells are cultured in the absence of exogenous stimuli. Immunoprecipitation of CD95 or FADD in freshly isolated GC B cells revealed that they display a preformed CD95 DISC that contains FADD, the zymogen form of caspase-8 and c-FLIP<sub>L</sub>, but not the CD95L. Since GC B cells express neither the transcript nor the protein for the CD95L, our results suggest that CD95 self-aggregates on these cells. GC B cells rapidly lose c-FLIP<sub>L</sub> expression as they undergo apoptosis *in vitro*. Furthermore, c-FLIP<sub>L</sub> is no longer detected in the DISC of apoptotic cells while FADD and caspase-8 are still connected to CD95. Exposure of cultured GC B cells to the survival signal provided by CD40L maintains expression of c-FLIP<sub>L</sub> and its association with the CD95 DISC. Altogether, our findings suggest that (a) death receptors such as

CD95 are involved in the commitment of GC B cells to apoptosis, and (b) c-FLIP<sub>L</sub> plays an active role in positive selection of high affinity B cell mutants during the GC reaction. The implications of these findings on the molecular mechanisms underlying the affinity maturation process are discussed.

## Materials and Methods

**Reagents and Abs.** The trimeric human CD40L-leucine zipper fusion protein was provided by Dr. R. Armitage (Immunex, Seattle, WA) and was used at 500 ng/ml throughout the study. Rabbit anti-human Ig Abs coupled to polyacrylamide beads were purchased from Irvine Scientific and used at a final dilution of 1:600. Four different anti-CD95 Abs were used. The agonistic mAb 7C11 (IgM) and the antagonistic mAb ZB4 (IgG1) were used for biological assays and purchased from Immunotech. The agonistic anti-APO-1 mAb (18) was used for immunoprecipitation of the CD95 DISC. A rabbit polyclonal anti-CD95 Ab (Santa Cruz Biotechnology, Inc.) was used to reveal CD95 in the immunoprecipitates. Purified mouse myeloma proteins used as isotypic controls for the 7C11 (IgM) and anti-APO-1 (IgG3k) mAbs were obtained from Sigma-Aldrich. mAbs 7C11 and anti-APO-1 were used at 200 ng/ml and 3  $\mu$ g/ml, respectively. mAb ZB4 was used at 1  $\mu$ g/ml. The anti-FADD mAb was purchased from Transduction Laboratories and was used at 10  $\mu$ g/ml for immunoprecipitation studies. The anti-CD95L mAb G247-4 was purchased from BD PharMingen and used at a final concentration of 1  $\mu$ g/ml. The C15 mAb recognizing the p18 subunit of caspase-8 and the anti-c-FLIP mAb NF6 have been described previously (19, 20). The anti- $\beta$ -actin mAb was purchased from Sigma-Aldrich. Horseradish peroxidase (HRP)-conjugated sheep anti-mouse Abs (Amersham Pharmacia Biotech) were used for the development of the immunoblots performed on cell lysates with anti-caspase-8, c-FLIP, and FADD mAbs. For DISC analysis by Western blot analysis, isotype-specific HRP-conjugated secondary Abs (Southern Biotechnologies Associates, Inc.) were used: goat anti-mouse IgG1 (for the c-FLIP and FADD mAbs) and goat anti-mouse IgG2b for the anti-caspase-8 mAb. HRP-conjugated donkey anti-rabbit Abs were used for the Western blot analysis of CD95 in the immunoprecipitates. The broad range caspase inhibitor z-VAD-fluoromethylketone (fmk), the caspase-8-specific inhibitor z-IETD-fmk, the caspase-9-specific inhibitor z-LEHD-fmk, and the control peptide z-FA-fmk were purchased from Bachem and used at the concentrations indicated in the text.

**Cells.** Purified tonsillar B cells were isolated as described previously (21). GC and memory B cells were both isolated from the IgD<sup>-</sup> population by negative selection carried out with anti-CD44 and anti-CD38 mAbs, respectively, as described previously (9). A modified version of the protocol originally described by Feuillard et al. (22) was used to isolate highly enriched virgin B cells. In brief, GC and memory B cells were depleted after two successive rounds of rosetting performed with sheep red blood cells coated with anti-CD38 and anti-CD80 mAbs, respectively. The purity of the negatively selected virgin B cell population ranged from 70 to 85% due to the heterogeneous distribution of CD80 on memory B cells. The human T leukemia cell line H9 in which activation-induced cell death can be induced by CD3 triggering was provided by Dr. Klaus-Michael Debatin (University Children's Hospital, Ulm, Germany). H9 cells stimulated for 6 h with 10  $\mu$ g/ml of the anti-CD3 mAb OKT3 were used as a pos-

itive control for the revelation of CD95-bound CD95L in immunoprecipitation studies.

**Cultures.** All cell cultures were made in RPMI 1640 supplemented with 10% selected heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ M/ml streptomycin, and 2% Hepes (all from Life Technologies). GC B cells were seeded at a density of  $5 \times 10^6$  cells/well in 12-well plates in the presence or absence of (a) the anti-CD95 mAb 7C11 or its isotypic control, (b) soluble trimeric CD40L, and (c) immobilized anti-Ig Abs, for the times indicated in the text. To prepare CD95-sensitive B blasts, virgin B cells were seeded at the density of  $10^7$  cells in 6-well plates and stimulated for 48 h with CD40L. For secondary cultures, viable B blasts recovered by density gradient centrifugation were seeded at  $5 \times 10^6$  cells/well in 12-well plates in the presence of the anti-CD95 mAb 7C11 or its isotypic control.

**Western Blot Analysis and Immunoprecipitation.** For Western blot analysis, cells ( $5 \times 10^6$ /sample) were washed twice with cold PBS, resuspended in 100  $\mu$ l of lysis buffer (10 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM EDTA) supplemented with a protease inhibitor cocktail (Boehringer), and incubated for 15 min at 4°C. The cell-free supernatants were recovered by centrifugation of the suspension at 10,000 g for 15 min at 4°C. The protein concentration of the extracts was determined by the Lowry method (Bio-Rad Laboratories). For each sample, 30  $\mu$ g of protein was loaded on the gel, then separated by 12% SDS-PAGE, and transferred to a Hybond nitrocellulose membrane (Amersham Pharmacia Biotech). After transfer, the immunoblots were blocked by incubating with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween 20. Next, the blots were probed overnight with the appropriate dilution of the primary Abs (anti-caspase-8, c-FLIP, FADD, or  $\beta$ -actin) at 4°C and revealed with an HRP-conjugated sheep anti-mouse Ab (Amersham Pharmacia Biotech) for 1 h at room temperature. After washing, the blots were developed using the ECL chemiluminescence method (Pierce Chemical Co.) according to the manufacturer's protocol.

Immunoprecipitation of the CD95 DISC was carried out as described previously (15). In brief,  $10^7$  freshly isolated or cultured GC B cells were incubated in complete medium at 37°C for different time intervals and lysed in lysis buffer (30 mM Tris-HCl, 150 mM NaCl, 1 mM PMSF, 1% Triton X-100, 10% glycerol, and a protease inhibitor cocktail). The lysates were then supplemented with either 1  $\mu$ g/ml anti-APO-1 mAb or 10  $\mu$ g/ml anti-FADD mAb. The CD95 or FADD-associated proteins were then precipitated overnight at 4°C with protein A-Sepharose (Sigma-Aldrich). The Sepharose beads were spun down, washed, resuspended in SDS-gel sample buffer, and boiled at 95°C for 3 min. Immunoprecipitates were separated by 12% SDS-PAGE and immunoblotted with anti-CD95, FADD, caspase-8, and c-FLIP Abs.

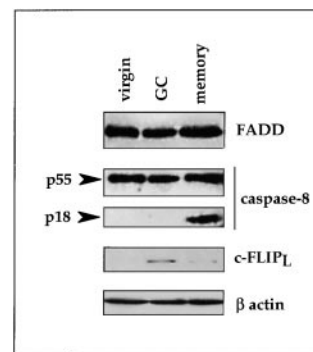
**Assays for Apoptosis.** Quantitation of apoptotic cells was made with (a) the 3,3'-dihexyloxycarbocyanine iodide (DiOC6) fluorochrome (Molecular Probes), which reveals disruption of the mitochondrial transmembrane potential ( $\Delta\psi_m$ ). In this assay, apoptotic cells are identified by their decreased  $\Delta\psi_m$  (DiOC6<sub>low</sub>). (b) Biotinylated annexin V (Boehringer) which detects the translocation of phosphatidylserine (PS) from the inner side to the outer leaflet of the plasma membrane on apoptotic cells. Staining was revealed with FITC-conjugated avidin (Immunotech) used at 2.5  $\mu$ g/ml. Immunofluorescence staining were analyzed on a FACScan™ flow cytometer using the Lysis II software (Becton Dickinson). (c) A PE-conjugated rabbit Ab specifically recognizing the active cleavage product of caspase-3 (BD PharMingen). This Ab was used at the final concentration of 1  $\mu$ g/ml.

**Cytopreparations and May-Grünwald Giemsa Coloration.** GC B cells were resuspended at  $4 \times 10^6$  cells/ml in complete medium. 50  $\mu$ l of this cell suspension was added in a cytocentrifuge chamber and centrifuged at 350 rpm for 4 min with low break. The slides were left to air dry before being fixed with methanol for 5 min at room temperature. The cytopins were incubated with a 2:3 dilution of May-Grünwald (BioLyon) solution prepared in methanol for 5 min, washed in distilled water, then incubated with a 1:9 dilution of Giemsa (RAL Products) prepared in distilled water for 10 min. The cytopins were then washed under running water, air dried, and mounted.

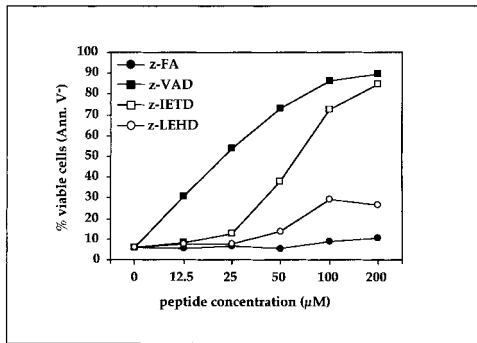
**Reverse Transcription PCR.** Isolation of total RNA was performed essentially as described by Chomczynski and Sacchi (23). For reverse transcription (RT), 1  $\mu$ g of RNA was converted into single-stranded DNA by a standard 20- $\mu$ l RT reaction using random primers P(dN)6 (Boehringer) and Superscript™ kit (RNaseH-MMLV reverse transcriptase; GIBCO BRL), according to the manufacturer's instructions. 1/10 of the total cDNA product was amplified in a 50- $\mu$ l reaction mixture using 1  $\mu$ M each of sense and antisense primers, and 1.25 U of Taq polymerase (PerkinElmer/Cetus). Expression of the  $\beta$ -actin mRNA was used as a control for RNA integrity and equal gel loading. The amplification primers for CD95L and  $\beta$ -actin were as follows: CD95L, 5'-TAAAACCGTTTGTGGGCG-3' and 5'-CTCAGCTCCTTTTTTTCAGGCG-3'; and  $\beta$ -actin, 5'-GGGTCAGAAGGATTCCTATG-3' and 5'-GGTCTCAAA-CATGATCTGGG-3'. PCR products were run on a 1.5% agarose gel, stained with ethidium bromide, and visualized by ultraviolet illumination.

## Results

**Developmental Regulation of the Expression of Active Caspase-8 and c-FLIP<sub>L</sub> in the Mature B Cell Compartment.** We have previously documented that expression of CD95 is modulated during the Ag-dependent B cell maturation process (9). Here, we have first examined whether expression of the cytoplasmic components of the death receptor signaling machinery could also be subjected to developmental regulation in the mature B cell compartment. For this purpose, Western blot analysis of FADD, caspase-8, and c-FLIP was carried out in lysates of freshly isolated virgin, GC, and memory B cells. As shown in Fig. 1, both FADD and the zymogen form of caspase-8 are constitu-



**Figure 1.** Constitutive expression of FADD, caspase-8, and c-FLIP in tonsillar B cell subsets. Equal amounts (corresponding to 30  $\mu$ g of proteins) of whole cell extracts prepared from freshly isolated virgin, GC, and memory B cells were loaded and separated on 12% SDS-PAGE. After transfer onto a nitrocellulose membrane, the blots were first probed with the anti-caspase-8 mAb, then stripped, and successively reblotted with the anti-FADD, anti-c-FLIP, and  $\beta$ -actin Abs. The proenzymatic form (55 kD) and the active enzymatic form (18 kD) of caspase-8 are indicated (arrowheads). Representative of three separate experiments.

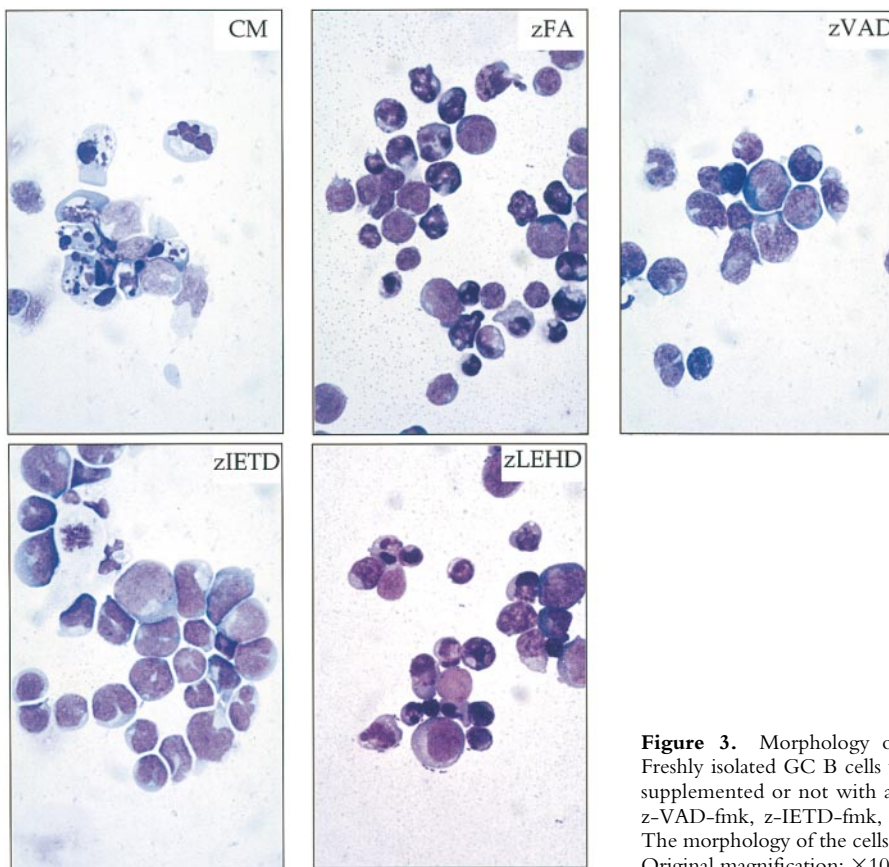


**Figure 2.** Effect of caspase inhibitors on the spontaneous apoptosis of GC B cells. Freshly isolated GC B cells were cultured for 24 h in the presence or absence of the indicated concentrations of the caspase inhibitors z-VAD-fmk (■), z-IETD-fmk (□), z-LEHD-fmk (○), or the control peptide z-FA-fmk (●). PS exposure was estimated by annexin V binding. Data are presented as means of the percent viable cells (Ann. V<sup>-</sup>) calculated from duplicate determinations. The difference between duplicate measurements never exceeded 10% of the mean values. Representative of three experiments.

tively expressed at equivalent levels in all three B cell subsets. The 18-kD active cleavage product of caspase-8 was only detected in memory B cells, suggesting a certain degree of ongoing activation of this caspase in the memory population *in vivo*. Expression of c-FLIP<sub>L</sub> was marginal in virgin and memory B cells but was significantly higher in

GC B cells. c-FLIP<sub>S</sub> was absent from all the extracts. Therefore, constitutive expression of c-FLIP<sub>L</sub> or active caspase-8 in the mature B cell compartment is restricted to GC B cells and memory B cells, respectively.

*Spontaneous Apoptosis of GC B Cells In Vitro Is Associated with the Activation of Caspase-8.* It is well documented that isolated tonsillar GC B cells are committed to die unless they receive the appropriate contact-dependent rescue signals from FDCs (4) or CD40L-expressing T cells (3). To investigate the molecular mechanism underlying the increased susceptibility of GC B cells to spontaneous apoptosis *in vitro*, we first examined whether caspases are involved in this process. Three peptidic caspase inhibitors were thus tested for their ability to prevent death of GC B cells in culture: the broad range inhibitor z-VAD-fmk; the caspase-8-specific inhibitor z-IETD-fmk; and the caspase-9-specific inhibitor z-LEHD-fmk. To exclude the possible contribution of the fmk chemical group to the observed effects, the cathepsin B inhibitor z-FA-fmk was used as a negative control in these experiments. Freshly isolated GC B cells were cultured for 24 h with or without serial dilutions (ranging from 12.5 to 200 µM) of the four fmk peptides mentioned above and processed for labeling of apoptotic cells with biotinylated annexin V. As shown in Fig. 2, both z-VAD-fmk and z-IETD-fmk rescue GC B cells from apoptosis in a dose-dependent fashion. 80–90% of the cells are still viable after 24 h of culture with 200 µM z-VAD or

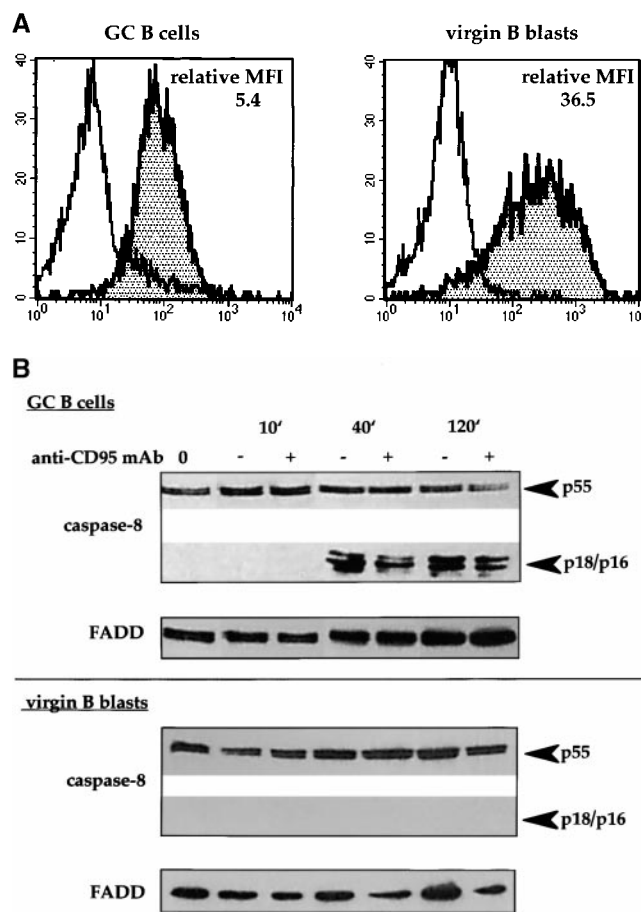


**Figure 3.** Morphology of GC B cells cultured with caspase inhibitors. Freshly isolated GC B cells were cultured for 4 h in complete medium (CM) supplemented or not with a 200 µM concentration of the caspase inhibitors z-VAD-fmk, z-IETD-fmk, z-LEHD-fmk, or the control peptide z-FA-fmk. The morphology of the cells was estimated by May-Grünwald Giemsa staining. Original magnification: ×100. Representative of three experiments.

z-IETD while the viability in control cultures drops below 10%. In contrast, the caspase-9 inhibitor z-LEHD-fmk exerted only a marginal antiapoptotic effect on GC B cells, and the control peptide z-FA-fmk failed to promote any survival of GC B cells. This finding shows that spontaneous apoptosis of GC B cells is a caspase-dependent process which relies on the activation of caspase-8 but not of caspase-9. To further document the differential implication of caspase-8 and caspase-9 in apoptosis of GC B cells in vitro, we examined the morphology of GC B cells after 4 h of culture in the presence or absence of the four fmk peptides. As illustrated by Fig. 3, many apoptotic figures, characterized by nuclear condensation and fragmentation, are seen in cultures carried out in complete medium, or in medium to which the z-FA-fmk or z-LEHD-fmk peptides were added. By contrast, most GC B cells cultured with z-VAD-fmk or z-IETD-fmk show a normal healthy morphology with a big nonfragmented nucleus.

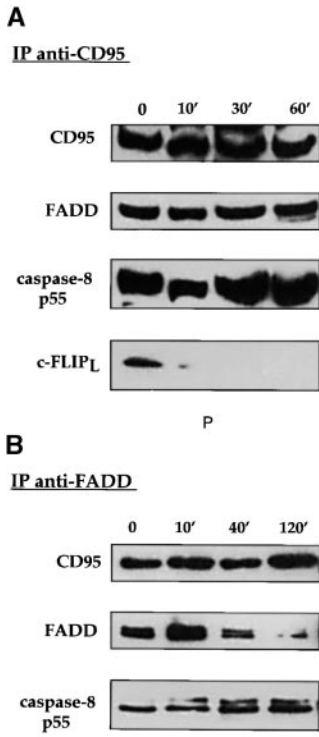
Owing to the antiapoptotic effect exerted by the caspase-8-inhibitory peptide z-IETD-fmk on cultured GC B cells, we next estimated by Western blot analysis whether spontaneous apoptosis of GC B cells in vitro was accompanied by the cleavage of caspase-8. We had found previously that CD40L-activated virgin B blasts that express high levels of CD95 are sensitive to CD95-mediated killing and activate caspase-8 in response to CD95 ligation (24). Therefore, both the density of CD95 expression and the occurrence of caspase-8 cleavage were compared in freshly isolated GC B cells and in CD40L-stimulated B blasts. For this purpose, both cell types were cultured either with the agonistic anti-CD95 mAb 7C11 or with its isotypic control. Cell lysates were prepared at different time points of the culture and probed with the anti-caspase-8 and FADD mAbs. Fig. 4 A shows that although both cell types homogeneously express CD95, its relative density of expression is approximately seven times higher on virgin B blasts than on GC B cells ex vivo. As illustrated in Fig. 4 B, caspase-8 is rapidly activated in cultured GC B cells without the need for deliberate CD95 ligation. Its active p16/p18 cleavage products appear in GC B cell lysates as early as 40 min after the onset of the cultures, independently of the addition of the agonistic anti-CD95 mAb. The spontaneous processing of caspase-8 in GC B cells is not potentiated by in vitro ligation of CD95. In spite of their higher density of CD95, no spontaneous activation of caspase-8 is observed in virgin B blasts within the time frame of the experiment. This result is consistent with our previous finding that caspase-8 cleavage in virgin B blasts is not detectable until 4 h of stimulation with an agonistic anti-CD95 Ab (24). Altogether these findings suggest that apoptosis of GC B cells in culture is associated with the rapid and spontaneous activation of caspase-8. This peculiarity is not related to the levels of expression of CD95 on GC B cells, but rather appears as an intrinsic feature of this maturational stage.

*Caspase-8 Is Activated at the Level of the CD95 DISC in GC B Cells.* Two cell types, each preferentially using one of two different CD95 signaling pathways, have been identified (25). In type I cells, cleavage of caspase-8 occurs



**Figure 4.** Spontaneous activation of caspase-8 in cultured GC B cells. (A) Viable CD40L-induced virgin B blasts and freshly isolated GC B cells were analyzed for their expression of CD95 by labeling with the anti-APO-1 (IgG3) mAb followed by PE-conjugated goat anti-mouse IgG3 Abs. The fluorescence histogram of the CD95 staining (hatched) is superimposed on that corresponding to the isotype-matched negative control Ab. The "relative" mean fluorescent intensity (MFI) corresponds to the ratio between the MFI of the CD95 fluorescence histogram and the MFI of the negative control. (B) Same cells as in A were cultured in the presence (+) or absence (-) of the agonistic anti-CD95 mAb 7C11. Cells were recovered and lysed at the indicated periods of time and equal amounts of whole cell extracts were separated on 12% SDS-PAGE. The blots were successively probed with the anti-caspase-8 and with the anti-FADD mAbs and revealed with the appropriate HRP conjugate. Representative of three experiments.

at the level of the DISC within minutes of CD95 ligation, before the loss of  $\Delta\Psi_m$ . In type II cells, DISC formation is strongly reduced, and activation of caspase-8 is delayed and mainly occurs downstream of the mitochondria, consecutive to  $\Delta\Psi_m$  disruption. Although mitochondria are perturbed in both cell types, only type II cells depend on the proapoptotic function of the mitochondria to execute the death program initiated by ligation of CD95. Two observations suggested that caspase-8 could be activated through its association with a death receptor signaling complex in GC B cells. First, the p16/p18 cleavage products of caspase-8 are detectable very early after the onset of GC B cell cultures. Second, the caspase-8 inhibitory peptide z-IETD prevented not only PS exposure but also the mito-

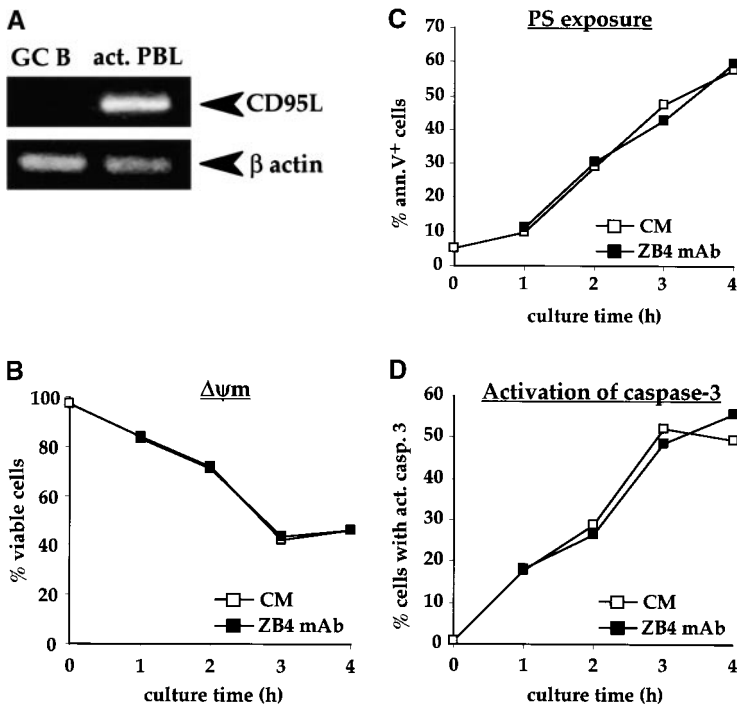


**Figure 5.** Composition of the CD95 DISC in freshly isolated and cultured GC B cells. (A) CD95 or (B) FADD was immunoprecipitated (IP) from  $10^7$  freshly isolated GC B cells or at different times after setting the same cells in culture in complete medium as described in Materials and Methods. The immunoprecipitates were washed, subjected to 10% SDS PAGE, and successively probed with the anti-caspase-8, FADD, CD95, and c-FLIP Abs. Representative of three experiments.

chondrial alterations in cultured GC B cells (data not shown). These findings led us to test the hypothesis that caspase-8 could be activated by association with the CD95 DISC. For this purpose, the proteins associated with either CD95 or FADD were immunoprecipitated in freshly isolated GC B cells or in GC B cells cultured in complete medium without exogenous stimuli for different lengths of

time. Both CD95 and FADD immunoprecipitates were analyzed by Western blotting using monoclonal or polyclonal Abs directed against the components of the DISC (CD95, FADD, and caspase-8) or against the natural antagonist of the death receptor signaling pathway c-FLIP. As illustrated by Fig. 5 A, FADD, the proform of caspase-8, and c-FLIP<sub>L</sub> are found in the CD95 immunoprecipitates obtained from freshly isolated GC B cells. c-FLIP<sub>S</sub> was below the threshold of detection in all experiments performed. Upon culturing in complete medium, CD95 remains associated with FADD and caspase-8 but there is already a strong reduction of c-FLIP<sub>L</sub> expression in the CD95 immunoprecipitates at the earliest time point (10 min). From 30 min of culture onwards, c-FLIP<sub>L</sub> is no longer associated with the CD95 DISC. Expression of c-FLIP in the FADD immunoprecipitates could not be assessed because both the anti-FADD and anti-c-FLIP mAbs are of the same isotype. However, examination of the FADD immunoprecipitates (Fig. 5 B) confirmed that FADD, CD95, and procaspase-8 are readily associated in GC B cells ex vivo. Altogether, these results provide several pieces of information. First, GC B cells contain a preformed CD95 DISC which includes FADD, the zymogen form of caspase-8, and the natural CD95 antagonist c-FLIP<sub>L</sub>. Second, caspase-8 is activated at the level of the CD95 DISC during spontaneous GC B cell apoptosis. Third, c-FLIP<sub>L</sub> disappears from the multimolecular CD95 signaling complex within minutes of in vitro culturing.

*Activation of CD95 in GC B Cells Occurs in a Ligand-independent Fashion.* Next we examined whether clustering of CD95 and the subsequent formation of a DISC in GC B cells was consecutive to the binding of CD95L. It has been demonstrated that upon prolonged stimulation through



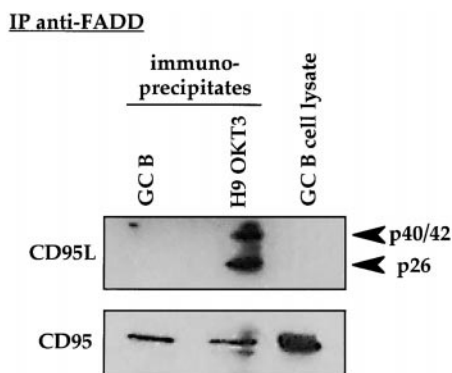
**Figure 6.** GC B cells do not produce CD95L. (A) RT and PCR amplification of the CD95L and  $\beta$ -actin transcripts were performed on RNA extracts from freshly isolated GC B cells and from PBLs which had been successively stimulated for 72 h with PHA (5  $\mu$ g/ml) and 6 h with PMA (10 ng/ml) and ionomycin (0.5  $\mu$ g/ml) (act. PBL). (B–D) Freshly isolated GC B cells were cultured in complete medium (CM) in the presence or absence of the antagonistic anti-CD95 mAb ZB4 (1  $\mu$ g/ml). GC B cells were assessed after 1, 2, 3, and 4 h of culture for their mitochondrial transmembrane potential (B), externalization of PS (C), and activation of caspase-3 (D) using a PE-conjugated anti-active caspase-3 Ab. The results are expressed as means of the (B) percent viable cells, (C) annexin V<sup>+</sup> cells, and (D) cells with activated caspase-3, calculated from duplicate determinations. The difference between duplicate measurements never exceeded 10% of the mean values. Representative of three distinct experiments.

their Ag receptor, T cells can express and release CD95L that promotes their autocrine suicide (26). Two types of experiments were undertaken to test whether the aggregation of CD95 on isolated GC B cells was consecutive to their endogenous production of CD95L. First, we examined by RT-PCR the expression of the CD95L transcript in freshly isolated GC B cells. As shown in Fig. 6 A, the CD95L message was not detectable in GC B cells, whereas it was expressed by PHA-activated PBLs which had been restimulated for 6 h with PMA and ionomycin. Second, we estimated whether blockade of the CD95-CD95L interaction by the antagonistic anti-CD95 mAb ZB4 could prevent apoptosis of GC B cells cultured for 1, 2, 3, and 4 h in the absence of exogenous stimuli. Data shown in Fig. 6, B-D, indicate that mAb ZB4 failed to prevent the  $\Delta\psi_m$  drop, PS exposure, and activation of caspase-3 in cultured GC B cells. This suggests that aggregation of CD95 on GC B cells is not because of an autocrine production loop of CD95L. To test the possible involvement of exogenous CD95L in multimerization of CD95, we next examined whether CD95 was physically bound to its ligand in GC B cells. For this purpose, we determined whether CD95L was part of the CD95 DISC in GC B cells. The proteins associated with FADD were thus immunoprecipitated in freshly isolated GC B cells and in the human leukemic T cells H9 which had been treated for 6 h with an anti-CD3 mAb. The H9 cell line was chosen as a positive control since ligation of the TCR-CD3 complex on these cells promotes their apoptosis via the endogenous release of CD95L and its subsequent binding to CD95 (27). As shown in Fig. 7, both CD95 and CD95L are coprecipitated with FADD in CD3-activated H9 cells. Two bands migrating respectively as a 40/42-kD species and as a 26-kD species are revealed by the anti-CD95L mAb in the H9 immunoprecipitates. These two bands are likely to represent the membrane-bound and the soluble form of CD95L, re-

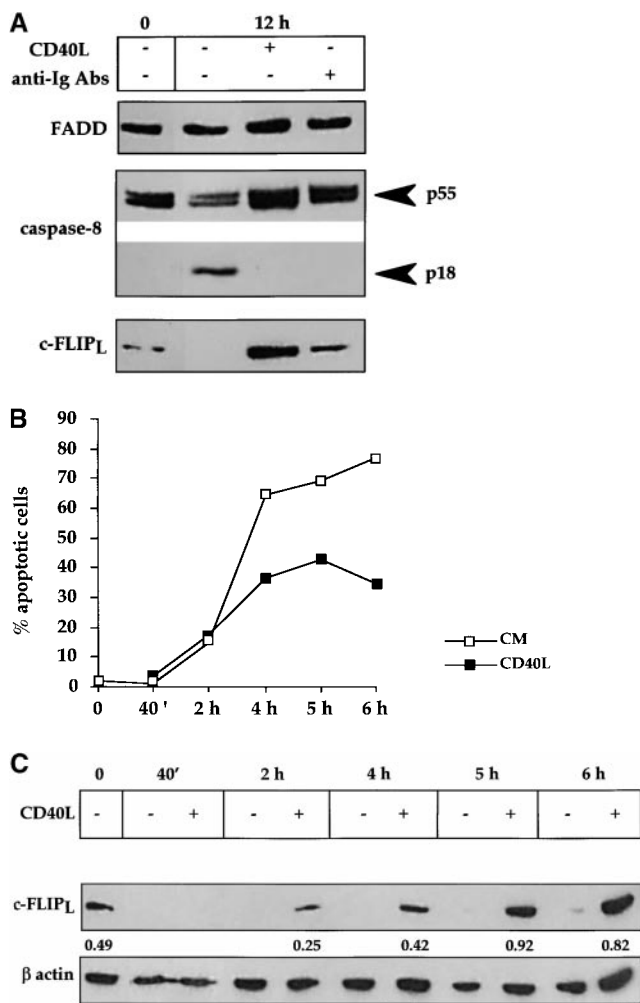
spectively. They were also found in OKT3-treated Jurkat T cells (data not shown). In contrast, despite the fact that CD95 was efficiently coprecipitated with FADD in GC B cells, no signal was obtained with the anti-CD95L mAb in these immunoprecipitates. In agreement with our observation that GC B cells lack the CD95L transcript, whole cell lysates prepared from GC B cells were also negative for the expression of the CD95L protein. Altogether, these data suggest that CD95L is not responsible for oligomerization of CD95 and formation of a DISC in GC B cells.

*The CD40- and BCR-mediated Rescue of GC B Cells Is Correlated with the Upregulation of c-FLIP<sub>L</sub>.* It has been shown previously that engagement of CD40 or BCR protects GC B cells from spontaneous apoptosis in vitro and promotes expression of the bcl-2 protein (28). This finding supported the concept that reinduction of antiapoptotic members of the bcl-2 family was instrumental in positive selection of mutated B cell clones which express a high affinity BCR for the relevant Ag. Since we had found that entry of a GC B cell into apoptosis is correlated with downregulation of c-FLIP<sub>L</sub>, we next investigated whether the antiapoptotic signals provided by surrogate Ag or CD40L could prevent the loss of c-FLIP<sub>L</sub> in cultured GC B cells. For this purpose, Western blot analysis of c-FLIP<sub>L</sub>, FADD, and caspase-8 was carried out in lysates of GC B cells cultured for 12 h in the presence or absence of either trimeric CD40L or immobilized anti-Ig Abs. As expected, CD40L and anti-Ig Abs inhibit both PS exposure and the activation of caspase-3 in GC B cell cultures (data not shown). As shown in Fig. 8 A, the p18 cleavage product of caspase-8 was clearly detectable in cultures of GC B cells carried out in complete medium, whereas expression of c-FLIP<sub>L</sub> was lost. In contrast, the p18 form of active caspase-8 was undetectable in both CD40L- and anti-Ig-treated cells. Furthermore, inhibition of caspase-8 processing promoted by ligation of CD40 or BCR was tightly correlated with the upregulation of c-FLIP<sub>L</sub>. To further document that c-FLIP<sub>L</sub> is instrumental in promoting survival in CD40L-treated cells, time course experiments were carried out in which both PS exposure and expression of c-FLIP<sub>L</sub> were simultaneously monitored in cultured GC B cells stimulated or not with CD40L. As illustrated by Fig. 8 B, the antiapoptotic effect of CD40L on GC B cells, as estimated by the reduction of the proportion of cells binding annexin V, was not detectable before 4 h of culture. In contrast, reexpression of c-FLIP<sub>L</sub> revealed by Western blot analysis (Fig. 8 C) was already found within 2 h of CD40L stimulation and thus preceded the inhibition of PS exposure promoted by CD40L. Altogether, these findings suggest that c-FLIP<sub>L</sub> contributes to the survival signal provided by surrogate Ag or CD40L.

*CD40L Prevents Dissociation of c-FLIP<sub>L</sub> from the CD95 DISC.* To investigate whether rescue signals control association of c-FLIP<sub>L</sub> with the CD95 DISC, we analyzed the composition of the CD95 signaling complex in CD40L-activated GC B cells. CD40L was preferred to surrogate Ag for these experiments since it consistently provided a stronger survival signal to GC B cells than anti-Ig

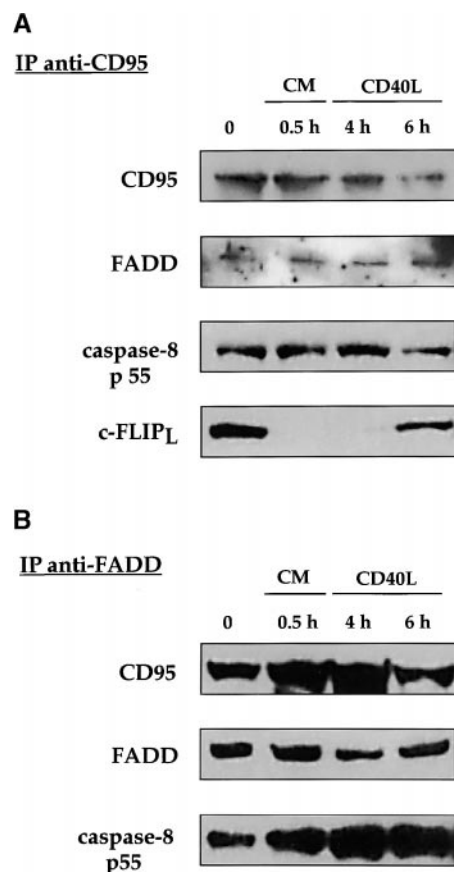


**Figure 7.** CD95L is not responsible for oligomerization of CD95 in GC B cells. FADD was immunoprecipitated (IP) from  $10^7$  freshly isolated GC B cells or  $10^7$  H9 cells previously stimulated for 6 h with the anti-CD3 mAb OKT3 (left two lanes). The immunoprecipitates were washed, subjected to 10% SDS-PAGE, and successively probed with the anti-CD95L and anti-CD95 Abs. Whole cell lysates prepared from the same batch of GC B cells were run on the same gel and probed with the anti-CD95 and anti-CD95L Abs (right lane). Representative of three experiments.



**Figure 8.** *c*-FLIP<sub>L</sub> is upregulated in CD40L- or anti-Ig-stimulated GC B cells. (A) Freshly isolated GC B cells (0) were cultured for 12 h in complete medium with (+) or without (-) soluble trimeric CD40L or immobilized anti-Ig Abs. Cell lysates were separated on 10% SDS-PAGE and analyzed sequentially by Western blot for expression of FADD, caspase-8, and *c*-FLIP. (B and C) GC B cells were cultured for the indicated periods of time with or without soluble trimeric CD40L and processed for analysis of PS exposure (B) or for analysis of *c*-FLIP expression by Western blot. CM, complete medium. (C) In B, results are expressed as means of the percent apoptotic cells (annexin V<sup>+</sup>) calculated from duplicate determinations. The difference between duplicate measurements never exceeded 10% of the mean values. The blot shown in C was subjected to densitometry scanning analysis. The relative intensity of *c*-FLIP<sub>L</sub> expression at each time point (indicated below the *c*-FLIP<sub>L</sub> blot) was estimated by the ratio between the intensity of the *c*-FLIP<sub>L</sub> and β-actin bands. Representative of three separate experiments.

Abs. Immunoprecipitations with anti-CD95 and anti-FADD Abs were thus carried out on (a) freshly isolated GC B cells, (b) GC B cells cultured for 30 min in complete medium, and (c) GC B cells stimulated for 4 and 6 h by trimeric CD40L. As shown in Fig. 9 A, *c*-FLIP<sub>L</sub> is no longer detectable in the CD95 DISC by 30 min of culture of GC B cells in complete medium but reassociates with the CD95 signaling complex after 6 h of CD40L stimulation. By contrast, FADD and the proform of caspase-8 remain within the DISC whatever the culture conditions. This



**Figure 9.** Composition of the CD95 DISC in CD40L-stimulated GC B cells. (A) CD95 or (B) FADD was immunoprecipitated (IP) from 10<sup>7</sup> freshly isolated GC B cells (0), or from the same cells cultured in complete medium (CM) in the absence (30 min) or the presence (4 and 6 h) of trimeric CD40L. The immunoprecipitates were washed, separated on 10% SDS-PAGE, and analyzed sequentially by Western blot for expression of *c*-FLIP, caspase-8, FADD, and CD95. Representative of three experiments.

contention is supported by the results of both the CD95 and FADD (Fig. 9 B) immunoprecipitation experiments. Altogether, this finding suggests that the CD40L-mediated rescue of GC B cells is correlated with the persistence of *c*-FLIP<sub>L</sub> within the CD95 DISC.

## Discussion

In this study, we show that the “commitment” of GC B cells to programmed cell death which underlies the affinity maturation process of the Ab response, involves CD95. Furthermore, our data indicate that the cytoplasmic death receptor antagonist *c*-FLIP<sub>L</sub> contributes to the positive selection of mutant B cell clones with high Ag-binding affinity in the GC. The experimental evidence that supports this conclusion is discussed below.

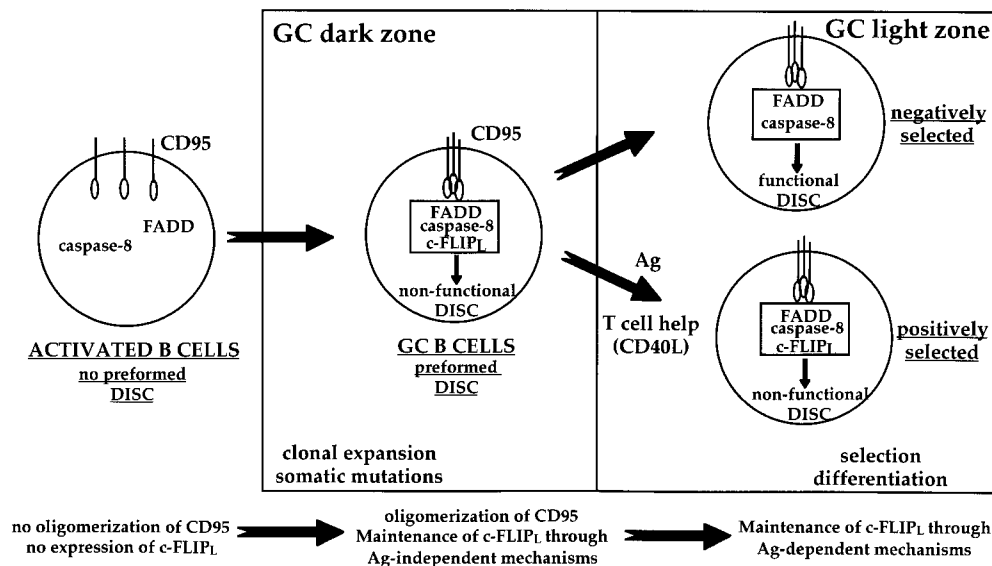
To begin with, our data suggest that spontaneous apoptosis of GC B cells *in vitro* presents striking similarities with the CD95-induced death of type I cells. First, caspase-8 which is one of the most proximal transduction elements of



the apoptotic signal initiated by death receptors (12–14) is proteolytically cleaved within minutes of *in vitro* culturing to release its active p16/p18 products. Second, we directly demonstrate by immunoprecipitation experiments conducted both with anti-CD95 and anti-FADD Abs that procaspase-8 is constitutively complexed with FADD and CD95 in freshly isolated GC B cells. Third, blockade of caspase-8 activity but not caspase-9 by specific inhibitory peptides prevents spontaneous apoptosis of GC B cells in culture. This suggests that formation of the multimolecular complex Cytc/Apaf-1/procaspase-9 (also called apoptosome) downstream of the mitochondria is not mandatory for execution of the death program in GC B cells. On the other hand, the crucial importance of the mitochondrial death pathway in the GC reaction has been highlighted by the observation that expression of a *bcl-2* or a *bcl-xL* transgene inhibits apoptosis in the GC (29) and impairs the process of affinity maturation (30, 31). One explanation that would reconcile our present observations with the latter reports is that other mitochondrial apoptogenic proteins such as AIF, whose release from the mitochondria is also prevented by *bcl-2* (32), collaborate with caspases to promote GC B cell apoptosis. Alternatively, it is also possible that the antiapoptotic members of the Bcl-2 family intervene at later stages of the rescue process to secure the long-term survival of the selected high affinity mutant B cell clones. In support of this hypothesis is the observation that the CD40-mediated rescue of GC B cells from apoptosis is effective hours before these cells reexpress significant levels of the Bcl-2 protein (33). In this scenario, c-FLIP<sub>L</sub> would promote rapid but transient protection while the later induction of Bcl-2 or Bcl-xL would confer a durable apoptosis-resistant phenotype.

As opposed to the DISC formed upon *in vitro* ligation of CD95 in sensitive activated peripheral T cells (20) or CD40L-activated virgin B blasts (24), only the full-length forms of caspase-8 and c-FLIP<sub>L</sub> are found within the CD95 DISC of freshly isolated GC B cells. Therefore, although caspase-8 is connected to CD95 and FADD in such a DISC, its autoproteolytic cleavage is prevented, thus rendering this signaling complex nonfunctional. Several pieces of evidence suggest that it is c-FLIP<sub>L</sub> that precludes activation of caspase-8 at the level of the CD95 DISC in GC B cells. First, from all three B cell subsets examined, only GC B cells constitutively express significant levels of the c-FLIP<sub>L</sub> protein. Furthermore, appearance of the active cleavage products of caspase-8 in cultured GC B cells is inversely correlated with the expression of c-FLIP<sub>L</sub>. Altogether, these observations are compatible with the hypothesis that execution of the cell death program in GC B cells is prevented as long as c-FLIP<sub>L</sub> remains associated with the CD95 DISC. The molecular mechanism whereby c-FLIP<sub>L</sub> exerts its antiapoptotic function is not entirely clear. However, in most CD95-resistant cells, c-FLIP forms stable complexes with CD95, FADD, and caspase-8 (20, 34). Our present findings are in line with these observations and support the notion that association of caspase-8 and c-FLIP<sub>L</sub> within the same DISC blocks the autoproteolytic cleavage of caspase-8.

Removal of GC B cells from their microenvironment results in the rapid exclusion of c-FLIP<sub>L</sub> from the CD95 DISC since it is no longer coprecipitated with CD95 after only 30 min of *in vitro* culturing. As illustrated by the hypothetical model depicted in Fig. 10, we postulate that c-FLIP<sub>L</sub> expression in GC B cells should be maintained under two circumstances. The first is when B cells have to be



**Figure 10.** Hypothetical model for the role of c-FLIP<sub>L</sub> in affinity maturation of the Ab response. This model proposes that the CD95 signaling pathway plays a central role in the selection of the B cell repertoire in the GC. Our data are compatible with the hypothesis that CD95 is oligomerized in GC B cells and constitutively connected with the proximal elements of its signaling pathway. Association of c-FLIP<sub>L</sub> in the CD95 DISC prevents B cells from undergoing apoptosis while they expand and somatically mutate in the dark zone of the GC. The existence of a preformed CD95 DISC in GC B cells would allow for rapid elimination of unwanted B cells (low affinity B cell mutants) when they are selected on the basis of their ability to capture Ag and present

it to T helper cells in the light zone of the GC. We postulate that non-Ag-derived signals (possibly provided by the FDCs) maintain the expression of c-FLIP<sub>L</sub> during diversification of the B cell repertoire in the dark zone. These signals are no longer available as GC B cells move to the light zone where selection occurs. After the loss of c-FLIP<sub>L</sub> expression, the CD95 DISC becomes operational and promotes cell death unless GC B cells are signaled through their Ag receptor and cognate interaction with T helper cells.

protected from apoptosis while they expand and mutate their Ig genes in the dark zone of the GC. T cell help is minimal if present at all in this microanatomical compartment of the GC. Furthermore, all mutated B cell clones must be preserved at this stage, independently of the affinity of their BCR. Thus, we favor the hypothesis that c-FLIP<sub>L</sub> expression in centroblasts of the dark zone is maintained through non-Ag-dependent mechanisms. The second is when B cell variants which the BCR displays an improved affinity for the Ag which initiated the response are positively selected by Ag and CD40L. We assume that, to ensure selectivity of the rescue process, the Ag-independent signals which promoted c-FLIP<sub>L</sub> expression in the dark zone must no longer be available when positive selection occurs in the GC light zone. Therefore c-FLIP<sub>L</sub> in the GC plays the role of a molecular switch turning the preformed CD95 signaling complex on or off.

The conclusion that c-FLIP<sub>L</sub> behaves as an early mediator of the antiapoptotic signal delivered by CD40L is supported by our observation that reexpression of c-FLIP<sub>L</sub> precedes by at least 2 h the restoration of the integrity of the plasma membrane in CD40L-treated GC B cells. The early loss of c-FLIP<sub>L</sub> reactivity in the cell lysates of cultured GC B cells (Fig. 8 C) suggests that its disappearance from the CD95 immunoprecipitates is not merely the consequence of its dissociation from the DISC. It is not yet clear how c-FLIP<sub>L</sub> expression can become extinct in GC B cells but the rapidity of the phenomenon evokes the possibility of a conformational change or degradation of the c-FLIP<sub>L</sub> protein. At face value, our hypothesis that c-FLIP<sub>L</sub> initiates the survival signal delivered by CD40L is difficult to reconcile with the kinetics of reexpression of c-FLIP<sub>L</sub> in CD40L-activated B cells and of caspase-8 cleavage in untreated GC B cells. We show that caspase-8 is rapidly activated (within 40 min) in untreated GC B cell cultures while c-FLIP<sub>L</sub> is hardly detectable before 2 h of CD40L stimulation (that is, later than the first signs of caspase-8 cleavage). One possibility would be that completion of the apoptotic program in GC B cells requires sustained activation of caspase-8. In other words, the amounts of active caspase-8 produced before the CD40L-mediated reinduction of c-FLIP<sub>L</sub> may not be sufficient to irreversibly engage GC B cells to die. Alternatively, it is also possible that relatively low levels of c-FLIP<sub>L</sub> expression (which would fall below the thresholds of detection of the Western blot analysis) are sufficient to block caspase-8 activation in GC B cells. In any case, further experiments will be required to nail down the precise function and mode of action of c-FLIP<sub>L</sub> during GC B cell apoptosis.

Our finding that CD95L is neither endogenously produced by GC B cells nor associated with the CD95 DISC suggests that oligomerization of CD95 in GC B cells occurs in a ligand-independent fashion. Self-aggregation of death receptors and subsequent activation of the apoptotic machinery are common when these molecules are overexpressed. This has been documented in particular for the TNF-receptor 1/TNF-R1 (35). In fact, it has recently been shown that several members of the TNF receptor

family including CD95 and TNF-R1 are constitutively expressed as oligomers rather than as monomers on the cell surface (36, 37). These oligomerized death receptors could be maintained in a silent state by cytoplasmic regulators. At least, two distinct molecules have been described to fulfill this function. The first is a protein called silencer of death domain (SODD) which binds the DD of TNF-R1 and DR3/TNF-R-mediated apoptosis-mediating protein (TRAMP) (38). The second, designated sentrin (39), can associate with the DD of both TNF-R1 and CD95. Both proteins prevent spontaneous transduction of the apoptotic signal when they are bound to the DD of these receptors. The release of such a silencer molecule from CD95 as B cells penetrate the follicles to participate in the GC reaction could allow for recruitment and association of the cytoplasmic components of the DISC.

The role of CD95 in the process of affinity maturation that we postulate in this study is in contradiction to the study of Smith et al. showing that the selection of high affinity mutants in the GC is not affected in *lpr* mice immunized with a T cell-dependent Ag (40). A scenario that would be consistent both with our data and with the latter study is that the CD95 deficiency is compensated for by another death receptor in *lpr* mice. Because our immunoprecipitation studies revealed that caspase-8 is associated with FADD in GC B cells, DR3/TRAMP (41), TNF-R1 (42), and the TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 (43, 44), which all rely on FADD to transduce their death signal, stand as possible candidates. Unfortunately, this question cannot be addressed in mice with targeted disruption of the FADD gene in the lymphoid compartment since these animals lack mature B cells in the periphery (45). Finally, the regulation of the B cell's life and death in the GC is likely to be a multifactorial process. It has already been shown that FDCs can interfere with distal steps of the GC cell death pathway by silencing a nuclear endonuclease activity (7) and by neutralizing a cathepsin activity (5). Our present findings indicate that the microenvironment of the GC also regulates B cell survival at a death receptor-proximal level by maintaining the expression of antagonists such as c-FLIP<sub>L</sub>.

I dedicate this article to the memory of my father, Robert De-france.

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