Negative Selection of Human Germinal Center B Cells by Prolonged BCR Cross-linking

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Summary

The antigen receptors on T and B lymphocytes can transduce both agonist and antagonist signals leading either to activation/survival or anergy/death. The outcome of B lymphocyte antigen receptor (BCR) triggering depends upon multiple parameters which include (a) antigen concentration and valency, (b) duration of BCR occupancy, (c) receptor affinity, and (d) B cell differentiation stages. Herein, using anti-immunoglobulin κ and λ light chain antibodies, we analyzed the response of human naive, germinal center (GC) or memory B cells to BCR cross-linking regardless of heavy chain Ig isotype or intrinsic BCR specificity. We show that after CD40-activation, anti-BCR(κ+λ) can elicit an intracellular calcium flux on both GC and non-GC cells. However, prolonged BCR cross-linking induces death of CD40-activated GC B cells but enhances proliferation of naive or memory cells. Anti-κ antibody only kills κ^+ GC B cells without affecting surrounding λ^+ GC B cells, thus demonstrating that BCR-mediated killing of GC B lymphocytes is a direct effect that does not involve a paracrine mechanism. BCR-mediated killing of CD40-activated GC B cells could be partially antagonized by the addition of IL-4. Moreover, in the presence of IL-4, prestimulation through CD40 could prevent subsequent anti-Ig-mediated cell death, suggesting a specific role of this combination in selection of GC B cells. This report provides evidence that in human, susceptibility to BCR killing is regulated along peripheral B cell differentiation pathway.

In the course of their ontogeny, lymphocytes enter windows of tolerance in which they are highly susceptible to inactivation by antigen-receptor triggering (1–3). These windows are believed to represent fail-safe device for eliminating clones from the peripheral repertoire that exhibit autoreactivity. For example, when the preimmune repertoire is constituted, somatic translocation of minigenes coding for T or B cell antigen receptor variable regions may incidentally generate autoreactive immature lymphocytes. However, the newly formed repertoire is rapidly purged from self-reactive clones as immature cells pass through a differentiation stage where self-epitope ligation induces anergy and/or deletion.

After antigenic challenge, the introduction of somatic hypermutations into the variable regions of mature lymphocyte antigen receptors brings further diversity to the preexisting repertoire. As somatic mutations are introduced randomly during cell division in germinal center (GC)[1] B cells (4, 5),

it is conceivable that some of the hypermutated cells could exhibit reactivity to self-epitopes. Hence, a "second window" (1) is required for elimination of auto-reactive clones before differentiation into memory or plasma cells. Studies in mice have provided evidence that, if arising in GC, autoreactive mature B cell clones would be rapidly eliminated (6–8). These experiments are based on the principle that stimulation with large amounts of soluble antigen can mimic a situation where mature GC B cells are rendered autoreactive as a result of ongoing somatic mutations. They show that antigen specific GC B cells die by apoptosis after soluble antigen administration. Simultaneously, B lymphocytes outside the follicle do not die but rather expand and differentiate into antibody secreting cells (9). Thus, censoring of autoreactive variants can specifically take place in GC. However, it remains to be elucidated (a) whether this antigen mediated effect could be due to direct B lymphocyte antigen receptor (BCR) triggering of GC B cells; (b) whether a higher affinity for the antigen would be responsible for specific killing of B cells, and (c) whether the agonist effect of soluble antigen outside the follicle results from stimulation of naive or memory cells. To address these issues, we purified naive, GC and memory B cells from human tonsils according to the surface expression of IgD, IgG, IgA, CD38,

and CD44 antigens (10). The effects of prolonged BCR triggering (that may mimic the in vivo addition of large amounts of soluble antigen) on CD40-activated naive, GC or memory B cells were then analyzed. We show that prolonged cross-linking of CD44⁻ GC B cell antigen receptor directly induces cell death. In contrast, the same BCR triggering strongly enhances the proliferation of CD40-activated naive and memory cells. The relevance of this result is discussed in the context of the GC reaction.

Materials and Methods

Cytokines

Purified rhIL-2 (3 × 10⁶ U/ml; Amgen Biologicals, Thousand Oaks, CA) was used at 10 U/ml, rhIL-4 (10⁷ U/mg), rhIL-6 (11 μg/ml), rhIL-10 (10⁷ U/ml), (Schering-Plough Research Institute, Kenilworth, NJ) were, respectively, used at 50 U/ml, 5 ng/ml, and 100 ng/ml. IFNγ (10⁷ U/mg) was purchased from Amgen and was used at 100 U/ml. Activated T cell supernatant was prepared as follows: T cells were enriched by anti-CD14 and anti-CD19 depletion from total tonsillar cell suspension. The remaining population (>80% CD3⁺) was seeded on anti-CD3-coated plates in the presence of 1 μg/ml anti-CD28 mAb for 3 d. The supernatant included 8.8 ± 0.3 ng/ml IFN-γ and 1.4 ± 0.2 ng/ml IL-10 as determined by specific ELISA (11) and were used at 1/4 final dilution.

Antibodies

The monoclonal and polyclonal antibodies used for phenotypic and functional studies were obtained from the following sources: Aster Laboratories (La Gaude, France), anti-CD2 and anti-CD3 mAbs used for negative selection of B cells with magnetic beads; Becton-Dickinson (Mountain View, CA), FITC-conjugated anti-CD2 (Leu 5), anti-CD3 (Leu 4), FITC-conjugated anti-CD20 (Leu 16), anti-CD14 (Leu M3) antibodies; Dakopatts A/S (Glostrup, Denmark), R-phycoerythrin-conjugated F(ab)'2 fragment of rabbit Ig to human kappa or lambda light chain; Immunotech S.A. (Marseille, France), anti-κ (6E1), anti-λ (C4), anti-CD28 (CD28.2), and FITC-conjugated anti-CD19 (IOB4) antibodies; Kallestad Lab. Inc. (Austin, TX), donkey polyclonal FITC-conjugated antibody to total human Ig (Igtot); Sigma Chemical Company (St. Louis, MO), FITC-conjugated anti-CD44 (A3D8) antibody and biotinylated goat anti-human IgD antibody. The anti-CD40 (mAb 89) (12) and anti-CD40-L (CD40 ligand) (LL2) mAbs were generated and purified in the laboratory. Anti-CD38 (OKT10) antibody was purchased from American Type Culture Collection (ATCC, Gaithersburg, MD) and used as ascitic fluid. The A2 mAb was produced in the laboratory and determined as being an anti-CD44 mAb on the basis of reactivity pattern and competition studies. It was used as 1:1,000 dilution of ascitic fluid.

Chemicals

FITC-conjugated streptavidin was purchased from Immunotech.

Flow Cytometric Analysis

Cell labeling. Antibody binding was analyzed on a FACScan® flow cytometer (Becton-Dickinson). For viable B cell phenotype gating was set according to forward angle light scatter parameter and propidium iodide staining to exclude subcellular particles and

dead cells from acquisition data. Data were subsequently processed using the LYSYS I program (Becton-Dickinson).

Analysis of Intracellular Calcium Mobilization. The method used has been described in detail elsewhere (13). CD40 activated tonsillar B cells where loaded with indo-1 by incubation with its acetoxymethyl ester (Molecular Probes, Junction City, OR) at 1 μM concentration for 45 min (37°C). Cells were washed and kept at room temperature in the dark until analysis was performed with a FACStar® Plus (Becton-Dickinson). An argon laser was used for UV excitation. BCR stimulation was achieved by adding 20 μg/ml of anti-κ and anti-λ mAbs cross-linked by an excess of goat anti-mouse antibody (Dako A/S). Violet fluorescent emission was detected at 405 nm and the blue emission was detected at 530 nm. The ratio of Indo-1 violet to blue fluorescence was displayed as a function of elapsed time. The entire analysis was performed at 37°C with a typical flow rate of 600 cells/s.

Cell Numeration. The concentration of beads in a solution of fluorosphere for optical alignment of flow-cytometer (Immunochek, Coulter, FL) was determined (C_b). This solution was then used as an internal standard to calculate the number of viable B cell in samples. Briefly, a volume (V_c) of culture medium containing an unknown number of viable cells (N_c) was harvested. A volume (V_b) of beads solution was added before propidium-iodide addition and FACS® analysis. The number (N_c) of viable cells was then determined according to the formula: $N_c = (N_1/N_2) \cdot C_b \cdot (V_b + V_c)$ where N_1 and N_2 represent, respectively, the number of cells and beads recorded in an ungated 40,000 events acquisition data file. The number of beads (N_2) was determined after acquisition by gating FL1⁺ events. The number of viable cells recorded (N_1) was determined by gating propidium iodide negative cells. Nc was calculated as the average of five independent measurements. For Fig. 5, both Nc and the proportion of κ or λ light chain among viable B cells were determined from the same culture well.

B Cell Purification

Tonsillar mononuclear cells were separated by standard Ficoll/Hypaque gradient method and were next submitted to E rosetting with sheep red blood cells. Non-rosetting cells were depleted by using antibodies against T cells and monocytes, as previously described (14). The purity of the remaining B cell population was greater than 98% as estimated by FACScan® analysis performed with anti-CD19, CD20, CD2, CD3, and CD14 monoclonal antibodies. Separation of resting vs GC B cells was done by further incubating cells with, respectively, (a) anti-CD38 (OKT10) or (b) anti-CD44 (A2) + anti-IgD (Nordic Immun., Tilburg, The Netherlands) monoclonal antibodies before depletion with magnetic beads. The resting (CD44⁺, CD38⁻) B cell population could be further subdivided into naive or memory cells by depletion after staining with (a) anti-IgG + anti-IgA or (b) anti-IgD antibodies, respectively.

B Cell Cultures

All cultures were performed in RPMI 1640 medium supplemented with 2 mM L-glutamine (GIBCO BRL, Gaithersburg, MD), 10% heat-inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland), 80 μg/ml gentamicin (Gentalline, Schering-Plough, Levallois Perret, France), in the presence of irradiated (7,000 rad) murine Ltk⁻ cells transfected either with CD32 (CD32 L-cells, ATCC; 16.2 CG7) or CD40-L (CD40-L L-cells), or with both CD32 and CD40-L (CD32/CD40-L L-cells) as previously described (15). Cultures were set up in flat-bottomed 96-well mi-

crotiter plates (100 μl final volume) in the presence of 5 × 10³ transfected L cells/well. Anti-CD40 or anti-BCR were used at 1 μg/ml. For [³H]thymidine incorporation assays, B cells were seeded in flat-bottomed microtiter plates at a density ranging from 5 × 10⁴ (naive or memory) to 2 × 10⁵ (GC) B cells per well in a final volume of 100 μl. Incorporation lasted 16h (1 μCi(37kBq)/well) and was measured by standard liquid scintillation counting techniques.

Results

Purification of Naive, GC or Memory B Cells. Phenotypic or functional characterization of tonsillar B cells subpopulations has been reported elsewhere (16–19). Here, highly purified tonsillar B cells were subdivided into resting or GC cells. To this end, E⁻ tonsillar B lymphocytes were incubated respectively with antibodies specific for either (a) CD38 or (b) CD44 and IgD. After magnetic bead depletion, the remaining cells were essentially composed of resting (CD44⁺, CD38⁻) or GC (CD44⁻, CD38⁺) B cells, respectively. Resting cells could be further subdivided into naive and memory cells by depletion using, respectively, anti-IgG + anti-IgA or anti-IgD antibodies. The average purity of the resulting B cell subpopulations assessed by anti-CD38, CD44 or IgD stainings was 85–90% for naive cells and >95% for the two other subsets (Fig. 1). Most contaminating cells within the naive B cell subpopulation were found to be sIgM⁺sIgD⁻ lymphocytes (not shown). Consistent with previous observations (20–22), GC cells

were different from any other tonsillar B cells in that they expressed very low levels of surface Ig and CD44 antigen (Fig. 1).

CD40 Stimulation Induces GC Cells to Express CD44 and BCR. When differentiating into memory cells, GC B cells reexpress high levels of CD44 antigen and surface immunoglobulin. As CD40-ligand⁺ T cells were detected in the apical light zone of GC (23, 24), we asked whether in vitro CD40 stimulation of CD44⁻ GC B cells would result in upregulation of BCR and CD44 antigen. To this end, freshly isolated CD44⁻ GC B cells were seeded on CD40-L transfected fibroblasts. Then, CD44 or BCR expression on viable cultured cells was measured by FACS® analysis in a time course study from 6 to 120 h. CD40 stimulation of GC cells induced expression of the CD44 antigen (Fig. 2). This induction was observed as soon as 6 h and affected most viable B cells after 72 h (n = 4). CD40 triggering also induced BCR expression on GC cells (Fig. 2). This induction was observed between 48 and 72 h of culture (n = 3) and reached optimal level after 96 h. At this time point, most (87 ± 12%, n = 3) viable cells expressed BCR. Thus, CD40 stimulation of freshly isolated GC B cells induced the expression of both CD44 and BCR.

BCR Triggering of CD40 Activated GC Cells Antagonizes Their Proliferation and Affects Their Viability. In view that CD40 triggering reinduces BCR expression on GC B cells, we asked what could be the outcome of BCR engagement on the proliferation of CD40-activated GC B lymphocytes. To address this issue, GC as well as naive or memory cells
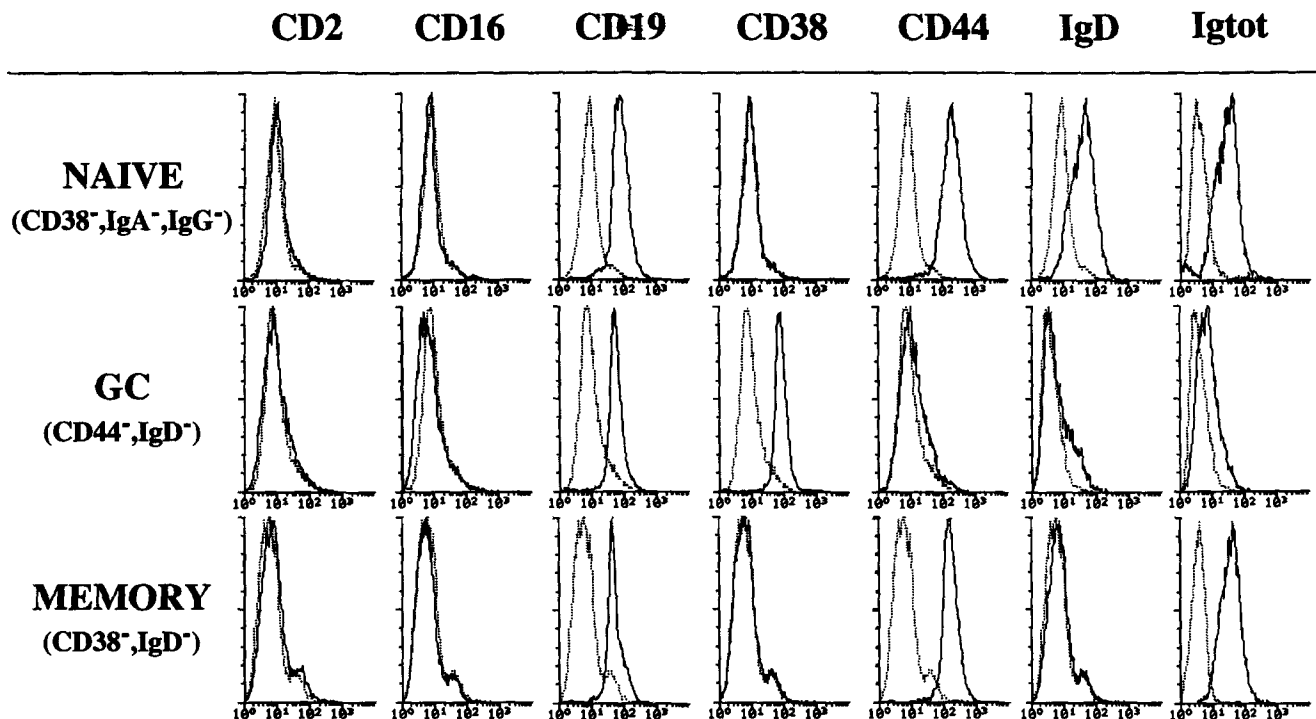
Figure 1. Phenotype of naive, GC and memory tonsillar B cells. B cells were purified from human tonsils and stained with (a) anti-CD38 + anti-IgG + anti-IgA (b) anti-CD44 + anti-IgD or (c) anti-CD38 + anti-IgD before depletion with goat anti–mouse coated beads. The resulting populations are CD19⁺ lymphocytes expressing different levels of CD38, CD44 and IgD and are referred to as naive, GC or memory B cells.

Hours / Markers	0	6	18	24	48	72	96	120
CD44								
sIg								
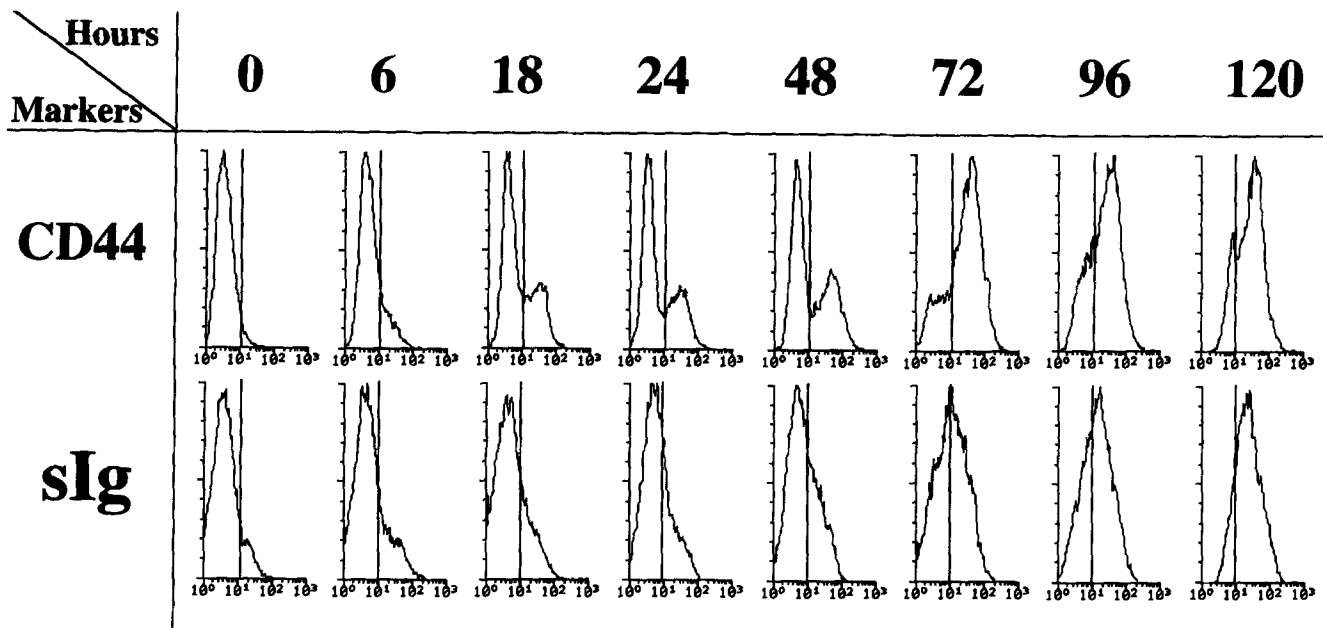
Figure 2. CD40 activation of GC cells induces expression of BCR and CD44. CD44$^-$ B cells were purified from human tonsils and seeded on CD40-L-transfected fibroblasts. Cells were then stained with FITC-conjugated anti-CD44 mAb or with anti-κ + anti-λ mAbs and R-phycoerythrin-conjugated goat anti–mouse antibody. Vertical lines represents limit for non-specific binding as measured by unrelated isotype matched antibody stainings.

were costimulated through CD40 and BCR by using anti-CD40 (mAb 89) and anti-BCR (κ+λ) mAbs cross-linked on CD32 L-cells. Proliferation was then assessed by tritiated thymidine incorporation in a time course study from day 1 to day 6. BCR stimulation of CD40 activated GC cells resulted in a reduced proliferation after a 5-d culture period (Fig. 3 B). This phenomenon was a dose-dependent process with a maximal effect obtained for 1 μg/ml of anti-BCR. At this concentration point, the average thymidine incorporation was only 11.7 ± 8.5%, $n = 3$ that of CD40-stimulated cells. This anti-proliferative effect of BCR stimulation was observed from day 1 to day 6 and was associated with an inhibition of GC B cells culture clumps formation (Fig. 4). In contrast to GC B cells, addition of anti-BCR mAbs to CD40-activated naive or memory B lymphocytes resulted in a dose-dependent enhancement of proliferation with a maximal effect obtained for 1 μg/ml of anti-BCR (Fig. 3, A and C). Thus, in this culture system, the outcome of BCR triggering depends on the stage of differentiation of the B cell subset selected; the proliferation of GC B cells, but not that of naive or memory B lymphocytes being inhibited by BCR triggering. As illustrated in Fig. 3 E, BCR cross-linking actually affected viability of CD40-activated GC B cells. Indeed, after 5 d of culture, addition of 1 μg/ml anti-BCR to CD40-activated GC B lymphocytes reduced by 60–70% the viability as measured by propidium iodide exclusion. In contrast, the viability of CD40-activated naive (Fig. 3 D) or memory (Fig. 3 F) B cells was not significantly affected by anti-BCR treatment. Comparable results could be obtained by using CD40-L/CD32 double transfected L-cells (not shown). Thus, the

inability to proliferate and survive in response to BCR triggering is a functional feature of human GC cells.

BCR Stimulation Affects the Viability of CD40-Activated GC B Cells through a Direct Effect. The BCR-mediated antiproliferative effects may either be due to direct BCR triggering or, as reported for T cells, to the production of soluble or membranous factors with paracrine antiproliferative potentials (25–28). To address this issue, the numbers of viable λ$^-$(κ$^+$) and λ$^+$(κ$^-$) GC B cells were quantified after a 5-d culture with CD40-L/CD32 transfected L cells in the presence or absence of anti-κ mAb. Fig. 5 shows that both the proportion (Fig. 5 A) and the absolute number (Fig. 5 B) of λ$^-$(κ$^+$) but not λ$^+$(κ$^-$) cells were decreased in the presence of anti-κ mAbs. Similarly, anti-λ triggering reduced the viability of κ$^-$(λ$^+$) GC B cells (not shown). Taken together, these results clearly show that the anti-BCR-induced cell death is a direct effect on triggered GC B lymphocytes that does not involve a B cell paracrine mechanism.

Addition of IL-4 to CD40-activated GC B Cells Partially Counteracts the Anti-proliferative Effect of BCR Cross-linking. We tested whether addition of cytokines could reduce the anti-proliferative effect of BCR triggering on GC B lymphocytes. GC B cells were cultured on CD40-L/CD32 double transfected L cells with or without 1 μg/ml of anti-BCR (κ+λ) antibodies and in the presence of recombinant cytokines or activated T cells supernatant. The influence of cytokine addition on the anti-proliferative effects of BCR stimulation was then assessed at day 5 (Fig. 6). Whereas IL-2, IL-10, IFN-γ, or IL-6 remained mostly ineffective, addition of IL-4 or to a lesser extent, T cell supernatant could significantly counteract the antiproliferative effect of
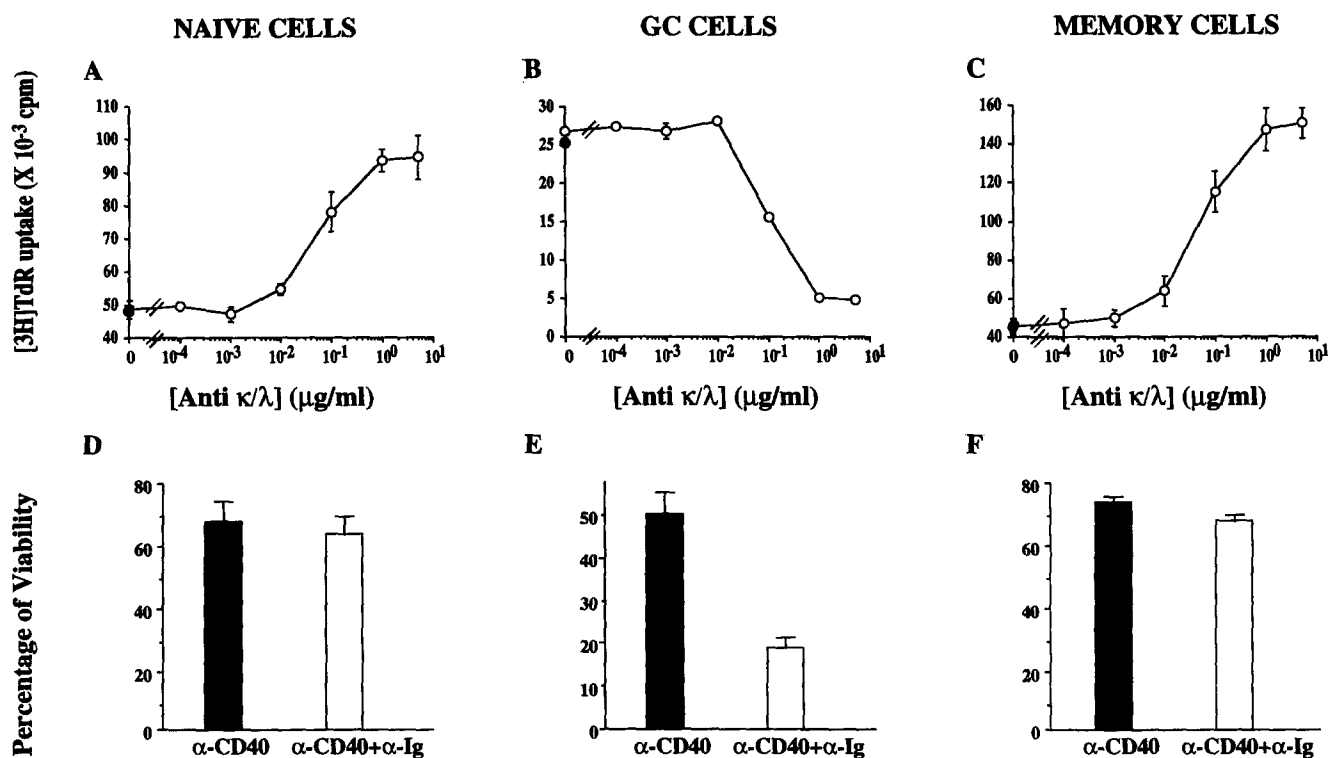
Figure 3. BCR cross-linking of CD40 activated GC cells antagonizes their proliferation and reduces their viability. Naive, GC or memory B cells were activated on CD32-transfected L-cells in the presence of 1 µg/ml anti-CD40 mAbs and increasing concentrations of anti-BCR (κ+λ) mAbs (*upper panel*). The proliferation was assessed on day 5 by [³H]thymidine incorporation assay. Black symbols show the proliferation in the presence of 1 µg/ml of unrelated mAb. The viability or these cells was measured by flow cytometry using Propidium Iodide exclusion (*lower panel*). Results are representative of three independent experiments.

BCR-triggering on GC cells. Indeed, in the presence of IL-4, triggering of GC B cells only reduced by 20% the CD40-mediated thymidine uptake. However, none of the cytokines tested could drive GC cells to respond positively to BCR stimulation. As a control, addition of anti-BCR on CD40-activated resting cells displayed an agonist effect on the proliferation whatever the cytokines used (not shown). Thus, although IL-4 or T cell supernatants could reduce the anti-proliferative effects of BCR triggering on GC B cells, none of the cytokines tested nor T cell supernatant could reverse this effect.

CD40 + IL-4 Preactivation of GC B Cells Prevents the Antiproliferative Effect of BCR Triggering. Because optimal expression of BCR is observed on CD44⁻ GC B cells after 3 d of stimulation through CD40 (Fig. 2), we asked whether death-promoting effect of BCR triggering could also be observed on GC B lymphocytes after full reexpression of BCR. GC cells were preactivated for 3 d on CD40-L-transfected L cells with or without IL-4. B cells were then harvested, washed and reseeded on CD40-L/CD32 double transfected L-cells, in the presence or absence of anti-Ig mAbs and IL-4. As shown in Fig. 7, whereas preactivation with CD40-L alone did not prevent subsequent anti-Ig-mediated cell death (Fig. 7 *A*), preincubation with CD40-L plus IL-4 markedly inhibited the anti-Ig-mediated GC B cell death (Fig. 7 *B*). Here again, the reduction in viability

of CD40-activated GC B cells was associated with disruption of cellular clumps (not shown). Thus, CD40 stimulation of GC B cells induced reexpression of BCR, the triggering of which displayed anti-proliferative effects. However, preactivation with CD40-L and IL-4 protects GC B cells from subsequent anti-Ig-mediated cell death.

BCR Ligation on CD40-activated Cells Elicits a Rise in Intracellular Free Calcium Concentration. Considering that anti-BCR mediated growth inhibition can be associated or not with changes in cytosolic-free calcium concentration (29), we asked whether intracellular Ca^{2+} signals would be elicited by BCR stimulation of CD40-activated normal GC cells. Thus, (CD44⁻IgD⁻) GC or (CD38⁻) resting tonsillar B cells were purified and cultured for 4 d on CD40-L⁺ fibroblasts. At this time point, both GC and resting cells expressed surface immunoglobulins as measured by FACS® labeling (Fig. 8, *upper panel*). Cells were then harvested, washed, further cultured in fresh medium for 12 h in the presence of anti-CD40-ligand antibody to avoid triggering by contaminating L cells, and then loaded with Indo-1 dye before BCR cross-linking and measurement of intracellular calcium flux by flow cytometry. As illustrated, calcium fluxes of comparable duration were observed 15–20 s after BCR ligation of both CD40-activated GC or resting B cells. However, the amplitude of the calcium flux elicited on GC cells was slightly and reproducibly lower than that
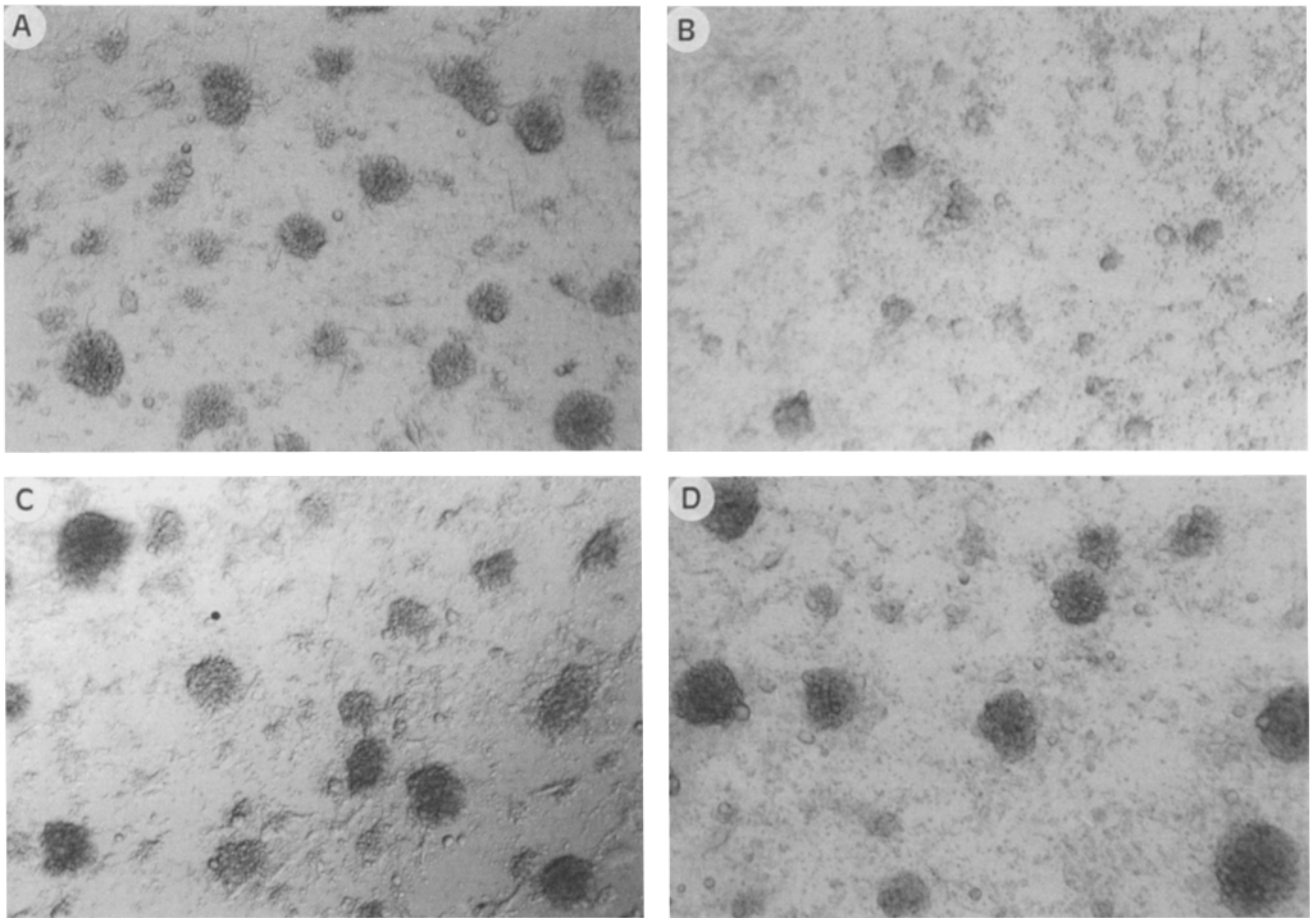
Figure 4. Anti-BCR treatment precludes to CD40-activated GC B cells clumps formation. CD44$^-$ GC (*A* and *B*) or CD38$^-$ resting (*C* and *D*) B cells were cultured for 5 d on CD32-transfected fibroblasts in the presence of 1 μg/ml anti-CD40 mAb, with (*B* and *D*) or without (*A* and *C*) 1 μg/ml anti-BCR(κ+λ) antibodies. ×100.

produced on resting B cells ($n = 3$) (Fig. 8). Thus, anti-BCR cross-linking elicits a rise in intracellular Ca^{2+} in both CD40-activated GC and non-GC cells. As previously reported for malignant mature human B cells (30), differences further downstream in signal transduction pathway seem to be decisive for the outcome death vs proliferation.

Discussion

Using anti-κ and anti-λ light chain antibodies, we developed a culture system where naive, GC and memory human B cells perceive comparable BCR-stimulation regardless of heavy chain Ig isotype or intrinsic BCR specificities. We show that a prolonged BCR cross-linking on CD40-activated GC cells antagonizes their proliferation and in-
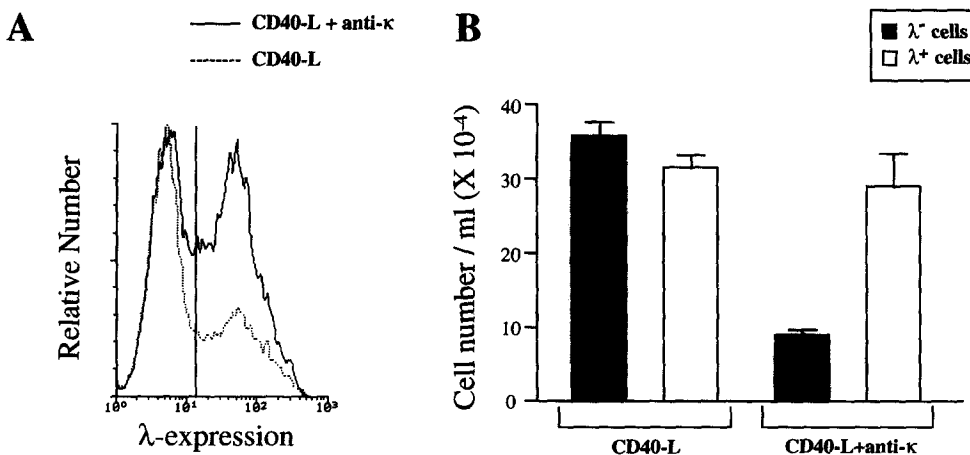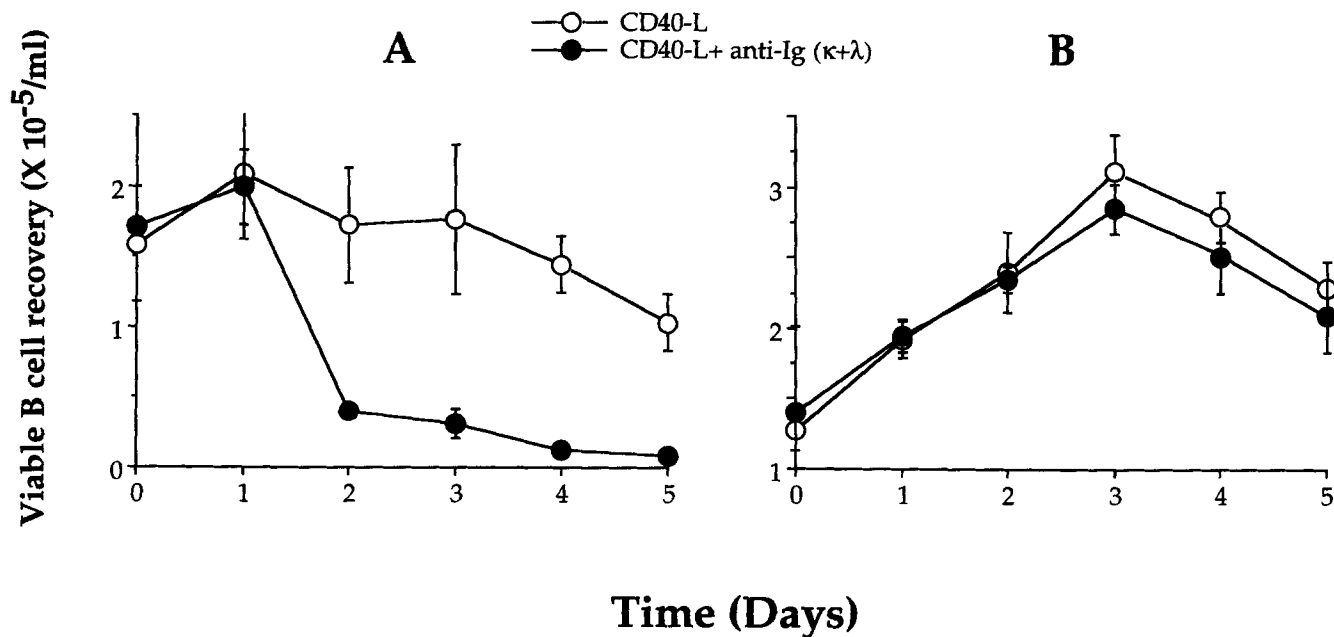

Figure 5. Anti-BCR-mediated cell death is a direct effect on triggered GC B lymphocytes. (*A*) CD44$^-$ GC cells were cultured for 5 d on CD40-L/CD32 double transfected L cells in the presence (*solid line*) or absence (*dotted line*) of 1 μg/ml anti-κ antibody before staining with R-phycoerythrin conjugated anti-λ antibody. (*B*) Anti-λ staining was coupled to cell numeration (as reported in Materials and Methods) and the absolute number of λ$^-$ and λ$^+$ was then determined.
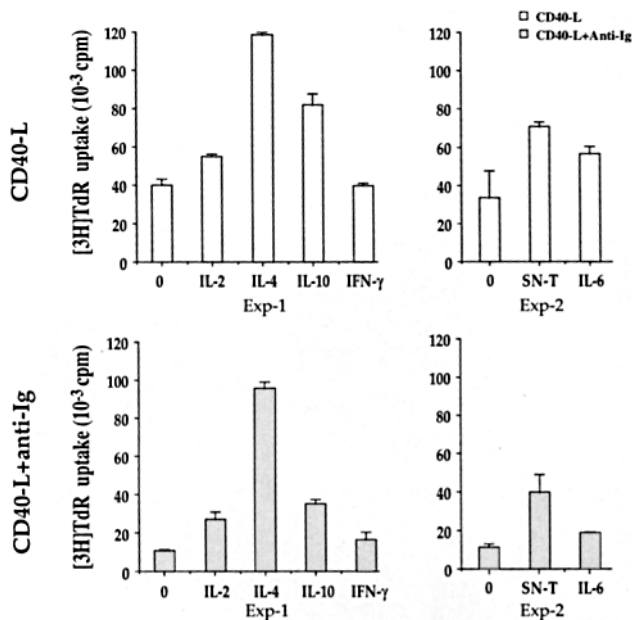
Figure 6. Antiproliferative effect of BCR cross-linking is modulated but not reversed by cytokines. CD44⁻ GC B cells were cultured on CD40-L/CD32 double transfected L cells in the presence (*bottom*) or absence (*top*) of anti-BCR (κ+λ) mAbs with or without recombinant IL-2, IL-4, IL-6, IL-10, IFNγ or activated T cell sup. On day 4, proliferation was assessed by [³H]thymidine incorporation assay.

induction whereby auto-reactive B cell clones, incidentally generated by somatic mutations, could be deleted through prolonged triggering by auto-antigens. The present study further characterizes some features of BCR-mediated GC B cell death: (*a*) The cell-killing is a direct effect of BCR-triggering as anti-κ mAb only kills κ⁺ GC B cells without affecting the surrounding λ⁺ GC B cells (and vice-versa). Thus, antigen receptor mediated death of GC B cells is different from that of T lymphocytes which relies on the production of soluble/membrane-bound factor (FAS-ligand) with autocrine and paracrine activity (25–28). In addition, we failed to detect FAS-ligand mRNA by PCR in BCR and CD40-stimulated GC B cells (not shown). These results are consistent with recent studies showing that GC B cells from lpr (FAS deficient) mice are fully susceptible to soluble antigen-induced cell death (6). (*b*) In this report, the cell-killing effect of BCR-stimulation is observed with cross-linked anti-BCR mAbs thus suggesting that membrane-bound or multivalent auto-antigens in GC may also elicit clonal deletion of self-reactive clones. However, BCR-mediated GC cell death is not due to hypercross-linking of GC cell BCR as previously reported for murine mature B cells (31), because the same BCR stimulation did not kill naive or memory B cells. (*c*) BCR-mediated killing of GC B cells could be modulated but not reversed by the addition of cytokines. Consistent with previous reports showing that CD40 + IL-4 costimulation reverse BCR-mediated apoptosis of mature B cells (31, 32), in the presence of IL-4, BCR triggering only marginally affected viability or proliferation of CD40-activated GC B cells. In addition, CD40-L + IL-4 preincubation protected GC B cells from

duces cell death. In contrast, the same BCR stimulation of CD40-activated naive and memory B cells enhances their proliferation. These results confirm previous in vivo findings showing that GC represents a window for tolerance

Figure 7. Preactivation with CD40-ligand and IL-4 prevents death promoting effect of BCR triggering. CD44⁻ GC B cells were cultured for 4 d on CD40-L/CD32 double transfected L cells in the presence (*B*) or absence (*A*) of IL-4. Cultured cells were then harvested and reseeded for 5 d on CD40-L/CD32 double transfected L cells in the presence of Anti-BCR (κ+λ) (*black symbols*) or unrelated mAb (*white symbols*). Number of viable B cells was estimated by propidium iodide exclusion as described in Materials and Methods.

A   CD44⁻ GC B-cells   **B**   CD38⁻ resting B-cells
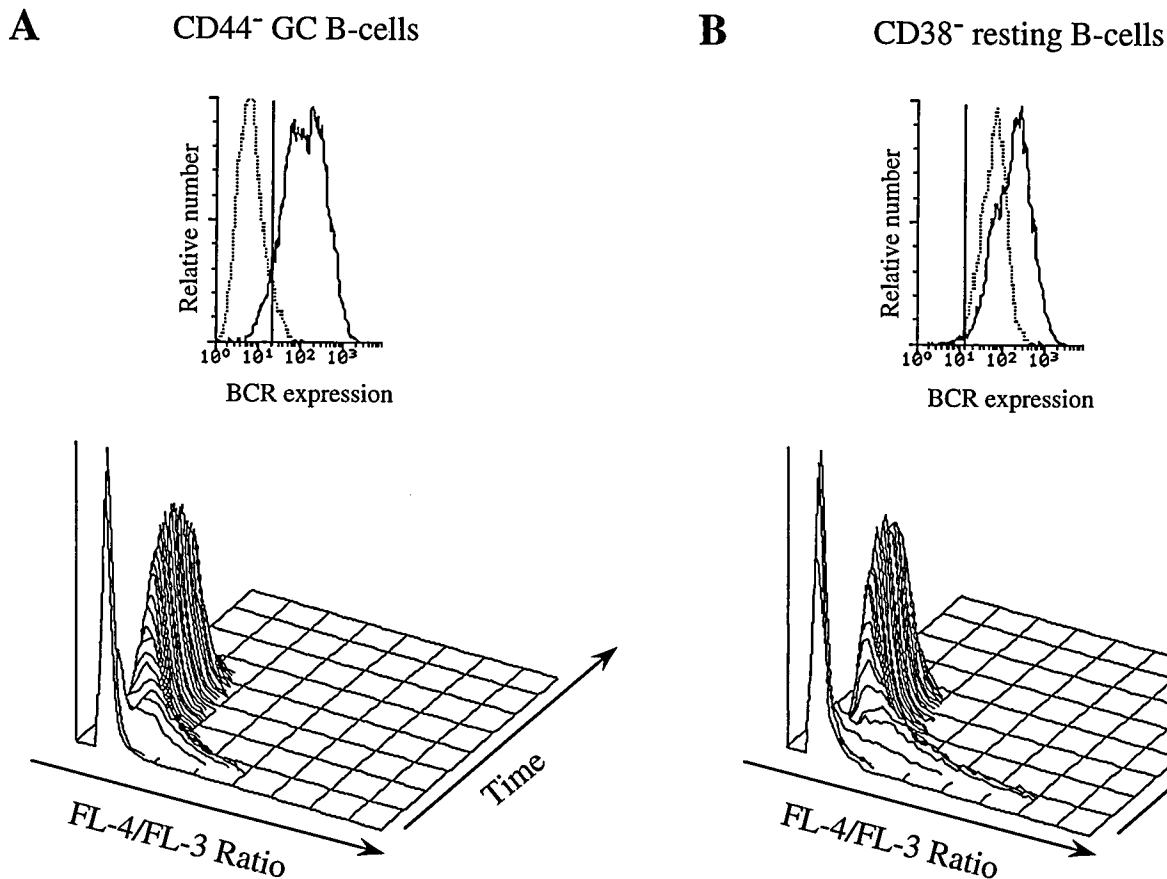
Figure 8. BCR-triggering induces calcium flux on CD40-activated CD44⁻ GC B cells. CD44⁻ GC (*A*) and CD38⁻ resting (*B*) B cells were purified from human tonsils and cultured for 4 d on CD40-L-transfected fibroblasts. The calcium flux produced by cross-linked anti-BCR (κ+λ) mAbs was then assessed by flow cytometry. (*Top*) BCR expression before (*dotted line*) and after (*solid line*) CD40 stimulation as measured by FITC-conjugated donkey anti–human Ig. (*Bottom*) BCR induced calcium flux by CD40-activated cells as measured by flow-cytometry using indo-1 dye. These results are representative of three independent experiments.

subsequent anti-Ig-mediated cell death. This observation confirms the hypothesis that CD40-L + IL-4 may play a particular role in selection of centrocytes in GC (33). (*d*) BCR triggering induced comparable calcium fluxes in CD40-activated GC or non-GC cells, thus indicating that the proximal events of BCR signal transduction, e.g., activation of PLC-γ2, are still operative in CD40-activated GC B lymphocytes (34) and that, as reported for follicular lymphomas, the fate of BCR-triggered cells depends upon other components of BCR-associated transduction machinery (30).

Unlike naive or memory cells, GC cells are unable to survive and proliferate in response to prolonged BCR triggering. The molecular basis for this discrepancy remains largely unknown: a possibility could be that, after in vivo antigenic stimulation, some changes occur in the signal transduction apparatus associated with membrane immunoglobulin of cells entering GC so that further in vitro or in vivo prolonged BCR stimulation would lead to cell death. Alternatively, as proposed by Goodnow et al. (3), the fate of BCR-triggered cells could result from an interplay between BCR-mediated activation of cell death and concur-

rent stimulation by survival factors eventually produced by stromal elements. The latter hypothesis is substantiated by the finding that in vivo as well as in vitro acquisition of a GC phenotype abrogates autonomous production of IL-6 by B lymphocytes without affecting B cells IL-6 responsiveness (18, 35). This result suggests that when entering windows of selection, B cells can delegate the production of trophic factors to other cellular components of their microenvironment that consequently would exert a control on B cell activation/survival. Although addition of IL-6 did not protect CD40-activated GC cells from anti-Ig-induced cell death, it is still possible that CD40-activated GC B cells are killed by in vitro prolonged BCR triggering because they fail to produce survival factors which are normally produced by CD40-activated naive or memory cells. We are presently investigating this point.

In GC, stimulation through BCR can lead either to deletion of auto-reactive cells or to positive selection of high-affinity mutants (4, 36, 37). How can then GC B lymphocytes discriminate between foreign- and self-antigen? Such a discrimination could be a quantitative issue. As a matter of fact, the signal delivered by foreign-antigens trapped on

the surface of follicular dendritic cells might be shorter than that delivered by high doses of ubiquitous auto-antigen. This assumption is illustrated by the fact that short-term anti-BCR triggering may protect GC B cells from apoptosis (38) whereas prolonged stimulation leads to death (7, 8, this report). Alternatively, there might be a context proper to foreign-antigen recognition by GC B cells. Foreign-antigen are retained in the form of antigen/antibody/C3 complexes on follicular dendritic cells (39 for review). GC B-lymphocytes recognizing the foreign-antigen may therefore perceive cosignals from Fc (40) or complement (C3) receptors (41–44) leading to the modulation of BCR stimulation and possibly influencing antigenic selection. Finally, GC B cells with a sufficient affinity for the foreign antigen may pick it up from FDCs and present it to antigen-specific T-lymphocytes (45, 46). Unlike auto-reactive B lymphocytes, these B cells could thereby perceive additional survival signals that would allow positive selection to occur.

When differentiating into $CD38^-sIgD^-$ memory cells, GC B lymphocytes recover the ability to proliferate in response to BCR-triggering. Both site and mechanisms by which this switch occurs remain unknown. As suggested above, it is possible that recovery of BCR proliferative potential corresponds to the reexpression of high levels of survival genes. However, within our hands, CD40-stimulation of $CD44^-$ GC B cells induces high levels of Bcl-2

expression (47 and not shown) but does not prevent BCR-mediated cell death. In line with this observation, BCR stimulation of HF1, a GC-like $Bcl-2^+$ human B cell lymphoma, elicits both intracellular calcium flux and apoptotic cell death (30, 48). In addition, consistent with our results, overexpression of Bcl-2 in mice does not alter antigenic selection in GC (49). Unlike CD40 stimulation, costimulation with IL-4 and CD40 prevents subsequent anti-Ig-mediated cell death. This result is in line with previous report showing that apoptosis mediated by Ig hypercross-linking of peripheral B cells is reversed by CD40 + IL-4 costimulation (31). As suggested by others (32), production of CD40-L (23, 24) and IL-4 (50) by antigen-specific T cells in the light zone of GC could play a particular role in the prevention of apoptosis and differentiation toward memory.

As a conclusion, the present report shows that a functional characteristic of human GC B cells lies in their inability to proliferate and survive in response to prolonged in vitro BCR triggering thus further illustrating the role of GC in the induction of peripheral tolerance. As susceptibility to BCR-mediated killing is regulated along B cell differentiation pathway in secondary lymphoid organs, tonsillar B cell subsets provide a unique comparative model for studying the molecular basis of BCR downstream events leading to either proliferation or cell death.

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