CD40 and B Cell Antigen Receptor Dual Triggering of Resting B Lymphocytes Turns on a Partial Germinal Center Phenotype

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Summary

Phenotypic alterations occur when resting human B lymphocytes become germinal center (GC) cells. These include the induction of surface CD38, CD95 (FAS/APO-1), and carboxypeptidase-M (CPM), a recently described GC marker. However, the factors that govern the in vivo induction of these surface molecules on B cells remain unknown. Here, we purified resting ($CD38^-$) human B lymphocytes from tonsils in an attempt to establish culture conditions resulting in the induction of these three GC markers. We show that interferon (IFN) α or IFN-γ, as well as antibodies against the B cell antigen receptor (BCR), could induce CD38 on resting B lymphocytes, a phenomenon further enhanced by CD40 stimulation. Concomitantly, CD95 was upregulated by CD40 ligation and, to a lesser extent, by IFN-γ. By contrast, CPM expression could be upregulated only through BCR triggering. This CPM induction was specifically enhanced by CD19 or CD40 ligation. CD40 + BCR stimulation of resting B cells with CD40 ligand–transfected fibroblastic cells in the presence of cross-linked anti-BCR monoclonal antibodies resulted in the coexpression of CD38, CD95, and CPM. As GC cells, these cells also expressed CD71, CD80 (B7.1), and CD86 (B7.2), but not CD24. However, $CD10^+$ or $CD44^-$ B cells could not be detected in these culture conditions, suggesting that yet other signals are required for the induction of these GC markers. Consistent with a GC phenotype, CD40 + BCR–stimulated cells exhibited reduced viability when cultured for 20 h in the absence of stimulus. These results first demonstrate that cotriggering of resting B cells through BCR and CD40 induces both phenotypic and functional GC features. They also show that IFN and CD19 triggering of resting B cells specifically modulate the expression of GC markers.

After antigenic challenge, a large variety of cells sequentially cooperate to develop the humoral response. APC take up the antigen, process it, and present it to specific Th cells in the context of MHC class II molecules. This cognate interaction drives Th cells to express both membrane-associated and soluble factors that control the proliferation and differentiation of B lymphocytes. In secondary lymphoid organs, this Th–B cell interaction is a prerequisite for the formation of germinal centers (GC),[1] the histological structures where B lymphocytes proliferate and mature into high-affinity memory cells (for reviews see references 1, 2).

In humans, several phenotypic alterations occur when B lymphocytes enter GC. These include both induction and disappearance of various surface molecules (CD markers) that most likely reflect B cell activation and adaptation to a new environment. Among GC markers, CD38 is a surface enzyme catalyzing the synthesis of cADP-ribose from NAD (3–7). CD38 is believed to participate in the control of GC B cell growth (4) or viability (8) and is coexpressed on GC cells with the carboxypeptidase M (CPM), a zinc-dependent protease known to cleave off basic COOH-terminal amino acids from hormonal peptides (9). In addition to these two ectoenzymes, GC cells express on their surface CD95 (FAS), a TNF receptor–related molecule that also participates in the control of GC B cell survival (10, 11).

Here, we attempted to establish culture conditions resulting in the induction of a GC phenotype on resting tonsillar B cells. This approach allowed us to show that dual triggering of CD40 and B cell antigen receptor (BCR) is required for the coexpression of CD95, CD38, and CPM. It also showed that cells coexpressing these markers are highly susceptible to spontaneous cell death. Finally, it is suggested that IFNs as well as CD19 triggering contribute to the in vivo acquisition of a GC phenotype.

[1] *Abbreviations used in this paper:* BCR, B cell antigen receptor; CPM, carboxypeptidase M; GC, germinal centers.

Materials and Methods

Cytokines. rhIL-1α and rhIL-1β (10^8 U/mg), rhIL-6 (10^7 U/mg), and rhTNF-α (2×10^7 U/mg) were purchased from Genzyme Corp. (Boston, MA). They were used at 10, 10, 200, and 2.5 ng/ml, respectively. Purified rhIL-2 (3×10^6 U/ml) (Amgen Biologicals, Thousand Oaks, CA), rhIL-3 (5×10^6 U/mg), rhIL-4 (10^7 U/mg), rhIL-5 (10^7 U/mg), rhIL-10 (10^7 U/ml), rhIL-13 (10^6 U/ml), and rhGM-CSF (2×10^6 U/mg) (Schering-Plough Research Institute, Kenilworth, NJ) were used at 20 U/ml, 10 ng/ml, 50 U/ml, 100 ng/ml, 100 ng/ml, 50 ng/ml, and 100 ng/ml, respectively. rhIFN-α (10^7 U/mg) (provided by Schering-Plough Research Institute) was used at 2,000 U/ml. rIL-12 ($2–3 \times 10^3$ U/ml) was kindly provided by Dr. A. O'Garra (DNAX Research Institute, Palo Alto, CA) and was used at 10 ng/ml. TGF-β1 and rhIL-7 were obtained from R&D Systems, Inc. (Minneapolis, MN) and were used at 0.5 and 100 ng/ml, respectively. IFN-γ (10^7 U/mg) was purchased from Amgen Biologicals and was used at 500 U/ml.

Antibodies. The monoclonal and polyclonal antibodies used for phenotypic and functional studies were obtained from the following sources: anti-CD2 and anti-CD3 mAbs used for negative selection of B cells with magnetic beads (Aster Laboratories, La Gaude, France); PE-conjugated anti-CD2 (Leu 5), anti-CD3 (Leu 4), FITC-conjugated anti-CD10 (Calla), anti-CD20 (Leu 16), and anti-CD14 (Leu M3) antibody (Becton Dickinson & Co., Mountain View, CA); FITC-conjugated anti-Bcl-2 oncoprotein (Dakopatts A/S, Glostrup, Denmark); anti-κ (6E1), anti-λ (C4) anti-CD24 (IOB3), FITC-conjugated anti-CD19 (IOB4), and biotin-conjugated anti-CD24 (IOB3) antibodies (Immunotech, Marseille, France); FITC-conjugated anti-CD44 (A3D8) antibody and biotinylated goat anti-human IgD antibody (Sigma Chemical Co., St. Louis, MO); PE-conjugated anti-CD86 (B70) (PharMingen, San Diego, CA).

The anti-CD40 (mAb 89) (12), anti-CPM (M27) (9), anti-CD21 (FIP6, FIP8, and FIP15), and anti-CD40-L (LL2) mAbs were generated and purified in our laboratory. Anti-CD38 (OKT10) antibody was purchased from American Type Culture collection (Rockville, MD) (CRL-8022) and used as ascitic fluid. Anti-CD19 (4G7) and PE-conjugated anti-CD80 (L307) were kindly provided by Drs. R. Levy and L. Lanier, respectively. All other antibodies were obtained from the Vth International Workshop on Leucocyte Typing (Boston, MA, 1993).

Chemicals. FITC-conjugated streptavidin was purchased from Immunotech.

Flow Cytometric Analysis. Antibody binding was analyzed on a FACScan® flow cytometer (Becton Dickinson & Co.). Gating was set according to forward angle light scatter parameters and propidium iodide staining to exclude subcellular particles, dead cells, and L cells from acquisition data. Data (10^4 events per sample) were subsequently processed using the LYSYS program (Becton Dickinson & Co.). The percentage of positive cells was determined by channel per channel peak substraction of the control histogram using the Consort 30 program (Becton Dickinson & Co., Immunocytometry Systems). For intracellular detection of Bcl-2 protein, cells were permeabilized by 15-min incubation at 4°C in saponine (0.5 mg/ml) before staining with anti-Bcl-2 mAb or an unrelated mAb as a negative control. Cell viability was determined by measurement of the incorporation of the DNA-binding fluorochromes Bisbenzimide HOECHST 33342 (Calbiochem-Novabiochem Corp., San Diego, CA) and propidium iodide, as previously described (13). Cells were incubated for 5 min with 10 μM HOECHST 33342 and 32 μM propidium iodide immediately before analysis with a double laser–equipped FACStar® plus (Becton Dickinson & Co.). This analysis permitted us to delineate three different cell populations: (*a*) Permeable cell membranes incorporate propidium iodide; (*b*) apoptotic cells incorporate only HOECHST 33342; (*c*) viable cells incorporate neither propidium iodide nor HOECHST 33342.

B Cell Cultures. All cultures were performed in RPMI 1640 medium enriched with 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY), 10% heat-inactivated FCS (Flow Laboratories, Irvine, Scotland), 80 μg/ml gentamicin (Gentalline; Schering-Plough), in the presence of irradiated (7,000-rad) CD32-transfected (CD32-L cells; American Type Culture Collection, 16.2 CG7) or CD40-L/CD32–double transfected murine Ltk$^-$ cells. The CD40-L/CD32–double transfected L cells were produced in our laboratory. Cultures were set up in flat-bottomed 96-well microtiter plates (100 μl final volume) in the presence of 5×10^3 transfected L cells/well and $10^4–10^5$ purified B cells. Anti-CD or anti-BCR were used at 2 μg/ml or 1:1,000 dilution of ascitic fluid throughout this study.

B Cell Purification. Tonsillar mononuclear cells were separated by standard Ficoll–Hypaque gradient method and were next submitted to E rosetting with SRBC. The following procedure was designed according to Lagresle et al. (14). Nonrosetting cells were labeled with anti–T cell (anti-CD2, -CD3, -CD4, and -CD8 mAbs), anti-monocyte (anti-CD14 mAb), and anti-CD38 (OKT10) mAbs and subsequently incubated twice with magnetic beads coated with anti–mouse IgG antibodies (Dynal, Oslo, Norway). Labeled cells were removed by applying a magnetic field for 15 min. The purity of the B cell subpopulation obtained after this procedure was >98% as estimated by FACScan® immunofluorescence labelings performed with CD2, CD3, CD4, CD19, CD38, and CD44 mAbs. For triple-labeling experiments, cells were labeled by biotinylated goat–anti-IgD antibody + tricolor–streptavidin and indirect mAb + PE-conjugated anti–mouse antibody. After saturation with mouse serum, FITC-conjugated anti-CD38 was added.

Results

CD38$^-$ Tonsillar B Cells Include Both Naive and Memory B Cells. The phenotype of highly purified tonsillar B cells was assessed using triple labeling with (*a*) anti-CD38 (FITC), (*b*) anti-IgD (Tricolor), and (*c*) PE-conjugated anti-CD20, anti-CD95 (FAS), anti-CPM, anti-CD71, anti-CD80 (B7.1), anti-CD86 (B7.2), anti-CD24, anti-CD10, or anti-CD44 mAbs. Results are shown in Fig. 1 *A*. From these labelings, two different tonsillar B cell subsets can be distinguished: (*a*) CD38$^{low/-}$ cells, which are resting and include both sIgD$^+$ naive and sIgD$^-$ memory cells (gate R1) (14–16); and (*b*) CD38^{high} cells, which correspond to GC B lymphocytes. Consequently, in this report, resting tonsillar B cells were selected by CD38 depletion (14). As expected, the resulting population (Fig. 1 *B*) typically included variable proportions of sIgD$^+$ cells. CD38$^-$-purified B cells also expressed low levels of CD95, CD80, CD86, and CD71, and no CPM, but high levels of CD24 and CD44 antigens.

IFN-α, IFN-γ, or BCR Ligation Induce CD38 Expression on Resting Tonsillar B Cells. We first examined the in vitro induction of surface CD38 expression on resting (CD38$^-$)

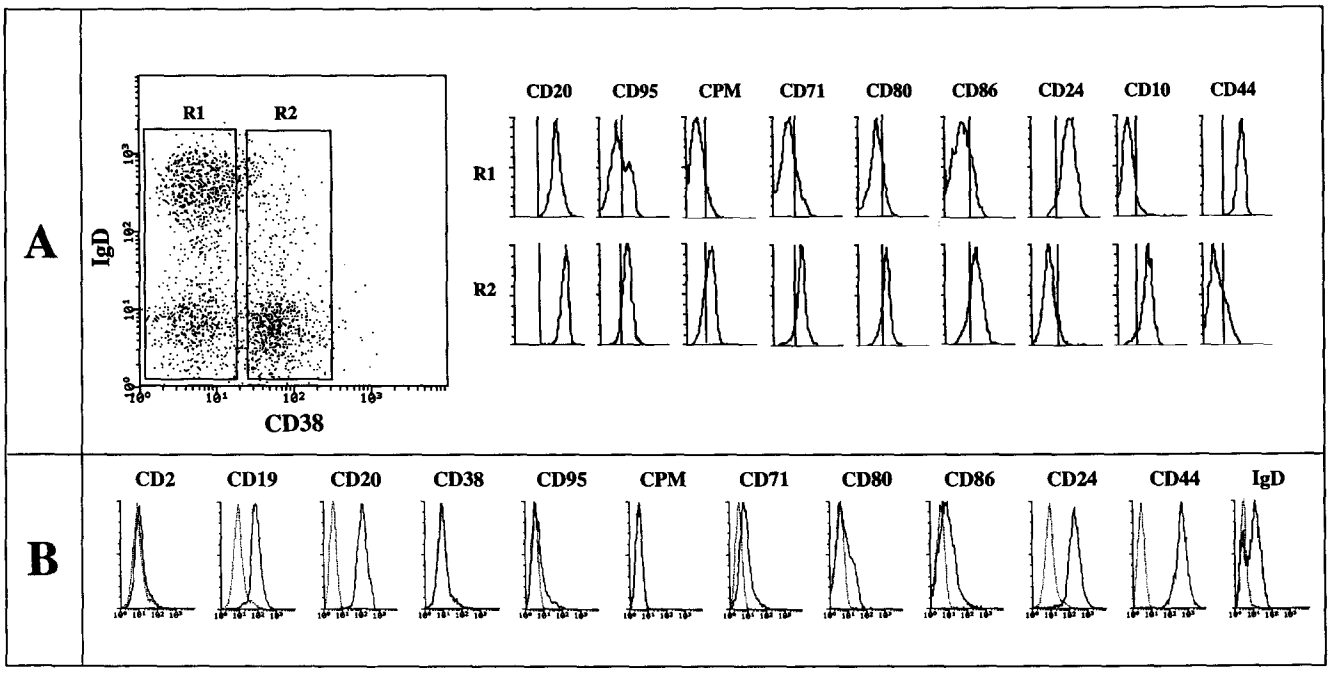

Figure 1. (*A*) Triple labeling of human tonsillar B cells. B lymphocytes were purified from tonsils and labeled as reported in Materials and Methods. Gate *R1*, resting cells; *R2*, GC cells. (*B*) Phenotype of $CD38^-$ tonsillar B lymphocytes. B cells were purified from tonsils and then labeled with anti-CD38 mAb. After adding anti-mouse Ig-coated beads, $CD38^+$ cells were depleted on a magnet. Remaining cells were phenotyped. Each staining profile (*solid line*) was superimposed onto that of the negative control (*dotted line*) performed with an isotype-matched unrelated mAb.

tonsillar B cells. To this end, resting B lymphocytes were cultured with optimal concentrations of cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IFN-α, IFN-γ, GM-CSF, or TNF-α), anti-BCR or anti-CD40 mAbs in the presence of CD32-L cells. CD38 expression was then determined at day 3 by FACS® analysis. Among the cytokines tested, only IFN-α and IFN-γ were found to consistently induce CD38 expression on resting B lymphocytes (Fig. 2 *A*). This IFN-mediated CD38 induction was observed at as early as 18 h of culture and reached near maximal levels after 24 h (Fig. 2 *A*). The IFN-α and IFN-γ-mediated induction of CD38 was a dose-dependent process with a half-maximal effect observed at ~5 and ~1 U and an optimal effect at ~1,200 and ~300 U, respectively (Fig. 2 *B*). This induction was a direct effect of IFNs on B cells because it could be seen in the absence of fibroblastic CD32-L cells (not shown). Triggering of BCR by immobilized anti-κ+λ light chain mAbs was also found to induce a moderate expression of CD38. This anti-BCR–induced CD38 expression displayed slower kinetics than that observed with IFN-α and IFN-γ with an optimal expression around day 5 (Fig. 2 *A*). At this time point, 22 ± 9% (n = 3) of cells expressed CD38. In contrast, although it is the most efficient mitogenic signal, CD40 triggering by immobilized anti-CD40 mAb (mAb 89) essentially failed to turn on CD38 expression (Fig. 2 *A*).

IFN- or BCR-induced Expression of CD38 Is Further Enhanced by CD40 Ligation. Because CD40 stimulation sensitizes B cells to anti-BCR triggering (17) and to a large variety of cytokine effects, we tested whether CD40 stimulation would affect anti-BCR- or IFN-mediated induction of CD38. To this end, resting ($CD38^-$) tonsillar B cells were stimulated with either anti-BCR (anti-κ+λ light chains) or anti-CD40 (mAb 89) antibodies cross-linked on CD32-L cells, with or without optimal concentrations of IFN-α or IFN-γ. After 3 d of culture, CD38 expression on activated B cells was determined by FACS® analysis. Results are

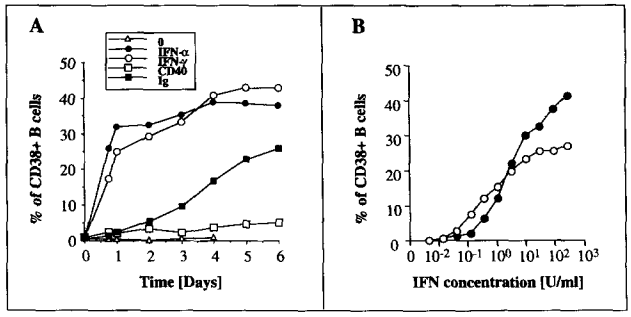

Figure 2. IFNs or BCR stimulation induce CD38 expression on the surface of resting tonsillar B cells. $CD38^-$ resting B lymphocytes were purified from tonsils as reported in Materials and Methods and seeded with $CD32^+$ fibroblasts in the presence of (*A*) medium (△), IFN-α (2,000 U) (●), IFN-γ (500 U) (○), mAb89 (2 μg/ml) (□), or anti-κ + anti-λ light chain mAbs (2 μg/ml each) (■), or in the presence of (*B*) variable concentrations of IFN-α (●) or IFN-γ (○). CD38 expression was measured by FACS®. The percentage of positive cells was determined by subtraction of the control peak. Results are representative of three independent experiments.

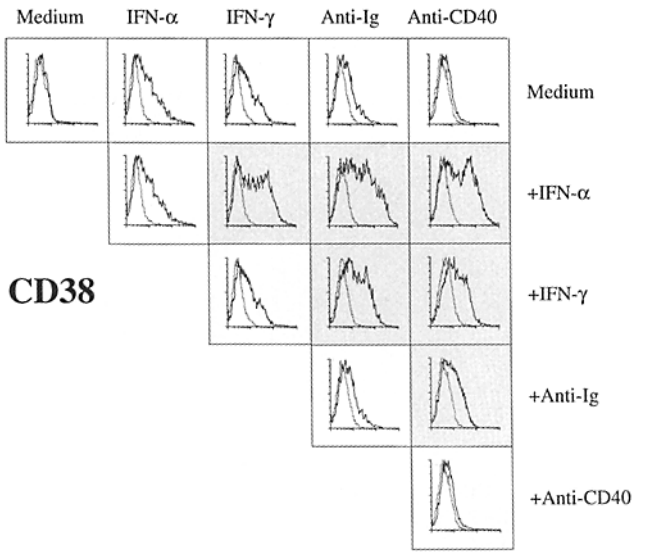

Figure 3. Double-entry table of in vitro–activated resting B cell CD38 profiles. $CD38^-$ B lymphocytes were cultured in the presence of $CD32^+$ fibroblasts with or without IFN-α, IFN-γ, anti-BCR, anti-CD40, or combinations. On day 3, cells were labeled with FITC-conjugated anti-CD38 mAb. Staining profile of CD38 (*solid line*) was superimposed onto that of FITC-conjugated isotype-matched unrelated mAb (*dashed line*). Shaded squares indicate combinations with synergistic or additive effects. These results are representative of four experiments.

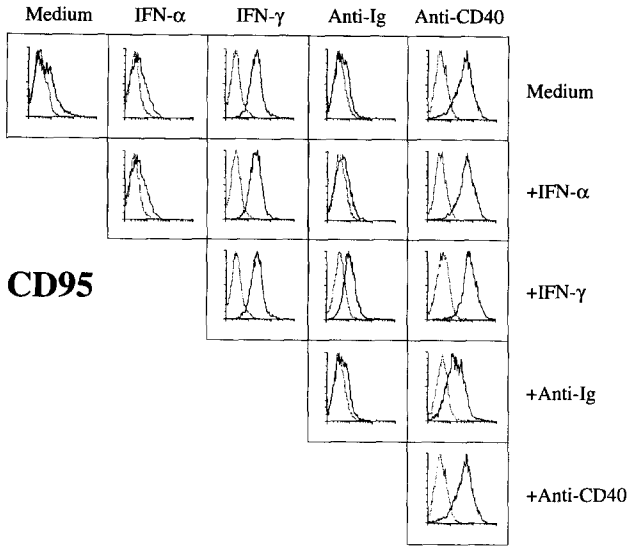

Figure 4. Double-entry table of in vitro–activated resting B cell CD95 profiles. $CD38^-$ B lymphocytes were cultured in the presence of $CD32^+$ fibroblasts with or without IFN-α, IFN-γ, anti-Ig, anti-CD40, or combinations. On day 3, cells were labeled with FITC-conjugated anti-CD95 mAb. Staining profile of CD95 (*solid line*) was superimposed onto that of FITC-conjugated isotype-matched unrelated mAb (*dotted line*).

shown as a double entry table with CD38 labeling FACS® profiles obtained for each combination (Fig. 3). CD40 stimulation significantly enhanced BCR-induced expression of CD38. Similarly, IFN-α– or IFN-γ–driven expression of CD38 could be further enhanced by CD40 stimulation. Moreover, combined stimulation of IFNs and anti-BCR or IFN-α and IFN-γ displayed synergistic effects on CD38 expression. Thus, CD40 stimulation of resting tonsillar B lymphocytes could enhance anti-BCR– or IFN-induced expression of CD38.

CD40 Triggering or IFN-γ Upregulate CD95 (FAS) on the Surface of Resting B Lymphocytes. GC B cells coexpress CD38 and CD95 antigens; thus, we examined whether CD95 expression would be upregulated in vitro on $CD38^-$ B cells. Consistent with previous studies (10, 11, 18), only anti-CD40 and IFN-γ significantly enhanced surface CD95 expression on $CD38^-$ B cells after a 3-d culture period, whereas IFN-α remained virtually inactive (Fig. 4). IFN-γ–induced CD95 expression displayed donor variability ($\Delta\%_{(IFN\text{-}\gamma)} = 53 \pm 44\%$, $n = 4$). In contrast, anti-CD40 consistently induced CD95 on the surface of resting B cells ($\Delta\%_{(mAb89)} = 88 \pm 7\%$, $n = 4$). Addition of IFN-γ to CD40-activated resting B cells did not result in significant upregulation of CD95 expression. Interestingly, anti-BCR triggering partially counteracted both IFN-γ– and CD40-induced expression of CD95. Therefore, coexpression of CD38 and CD95 (Figs. 3 and 4) can be achieved using IFN-γ and, more reproducibly, with anti-CD40 mAb plus IFN-α, IFN-γ, or anti-BCR.

CPM Expression Is Induced by BCR Triggering and Enhanced by CD19 or CD40 Ligation. We recently reported the presence of CPM on the surface of $CD38^+$ leukocytes (9). Among mature B lymphocytes, only GC cells expressed this ectoenzyme (Fig. 1). Moreover, as well as CD38 and CD95, in vivo expression of CPM appears to be an early event of B cell activation inasmuch as this enzyme is expressed on early ($sIgD^+$) unmutated GC cells (Lebecque, S., O. de Bouteille, C. Arpin, J. Banchereau, and Y.-J. Liu, manuscript in preparation). In vitro, neither CD40 stimulation nor 16 tested cytokines (see above), used alone or in combination, could induce CPM expression on $CD38^-$ B cells (Fig. 5). BCR triggering was thus the only efficient stimulus able to induce surface CPM expression on $CD38^-$ tonsillar B cells. Indeed, as shown in Fig. 5, a fraction ($13 \pm 4\%$, $n = 4$) of $CD38^-$ B lymphocytes cultured in the presence of cross-linked anti-BCR were induced to express CPM. This induction could be observed as soon as day 3 and increased up until day 6 (not shown). Anti-BCR–induced expression of CPM was significantly enhanced by anti-CD40 mAb and to a lesser extent by IFN-α (Fig. 5). In contrast, IFN-γ partially counteracted the induction of CPM. When analyzing other costimulatory antibodies, it was found that anti-CD19 mAbs were also capable of enhancing the anti-BCR–induced expression of CPM. As shown in Fig. 6, *A* and *B*, the anti-CD19 mAbs and the anti-CD40 mAbs strongly enhanced anti-BCR–induced expression of CPM, thus confirming the specific role of these molecules. Anti-CD21 mAbs stimulated slightly but reproducibly anti-BCR–induced CPM expression (Fig. 6, *A* and *B*). In contrast, antibodies specific for CD20, CD22, HLA-DR, and CD81 failed to enhance the expression of CPM on anti-BCR–activated B cells. The enhancing effect of CD40 ligand or anti-CD19 triggering was also observed for low anti-BCR concentrations

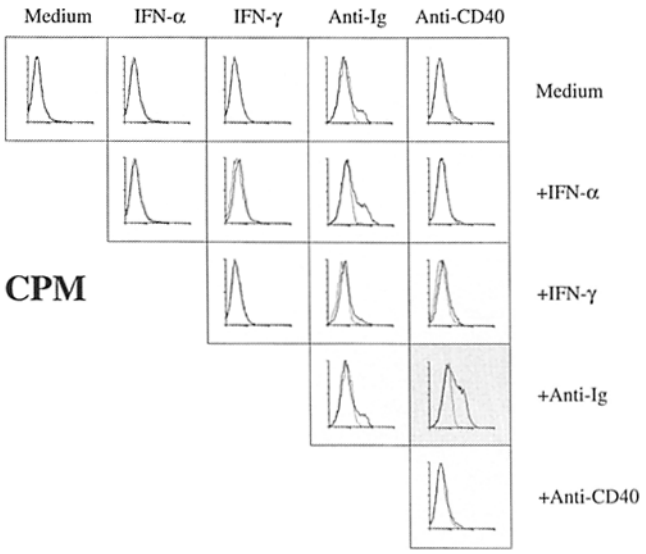

Figure 5. Double-entry table of in vitro–activated resting B cell CPM profiles. $CD38^-$ B lymphocytes were cultured in the presence of $CD32^+$ fibroblasts with or without IFN-α, IFN-γ, anti-Ig, anti-CD40, or combinations. On day 3, cells were labeled with biotin-conjugated anti-CPM mAb. Staining profile of CPM (*solid line*) was superimposed onto that of biotin-conjugated isotype-matched unrelated mAb (*dashed line*). Shaded squares indicate combinations with synergistic or additive effects.

(Fig. 6 *C*). However, the minimal anti-BCR concentration needed for day 4 CPM induction was not significantly lowered by CD40 ligand or anti-CD19 mAbs (Fig. 6 *C*). Finally, because in vivo CPM expression is restricted to $CD38^+$ leukocytes (9), CD40 + BCR– or CD19 + BCR–stimulated resting B cells were labeled with anti-CD38 and anti-CPM mAbs. As illustrated in Fig. 6 *D*, CPM^+ in vitro–activated B cells also expressed the CD38 molecule (Fig. 6 *D*). Noteably, the addition of cytokines (IL-2, IL-3, IL-4, IL-10, or IFN-α) to CD19 + BCR– or CD40 + BCR–activated B cells only displayed a marginal effect on CPM expression (not shown). Thus, CPM is a GC marker that can be induced by mitogenic concentrations of anti-BCR and specifically requires CD19 or CD40 triggering for optimal expression.

Cotriggering of Resting B Cells through BCR and CD40 Induces Both Phenotypic and Functional GC Features. Triggering of resting B cells with combinations of different stimuli (CD40 ligand + anti-BCR, anti-CD19 + anti-BCR) resulted in potent induction of CPM and CD38. Thus, we tested whether these combinations could upregulate other GC markers. As shown in Fig. 7, CD40 + BCR triggering induces loss of CD24 antigen and coexpression of CD38, CD71, CD95, CD80, CD86, and CPM on resting tonsillar B cells. As a control, cells stimulated with CD40 ligand alone do not express CD38 or CPM. In contrast to CD40 + BCR–activated lymphocytes, CD19 + BCR–activated resting B cells remained $CD71^{low}$ and $CD95^-$. None of these culture conditions, however, could generate $CD10^+$ or $CD44^-$ B lymphocytes from resting tonsillar B cells, suggesting that yet other signals are required to induce a full GC phenotype. Adding IFN-α, IFN-γ, or anti-CD19 to CD40 + BCR–stimulated resting B cells did not significantly change the pattern of expression of these markers (not shown). Similarly, we could not downregulate CD44 expression or induce CD10 by adding GM-CSF (19), supernatant of an anti-CD3–activated T cell clone, anti-CD44 mAbs, or collagen type I, a natural ligand for CD44 (20) (not shown).

As GC B cells undergo spontaneous apoptotic cell death when cultured in medium (21), we asked whether the observed in vitro acquisition of a GC phenotype is associated with reduced viability. Resting B lymphocytes were isolated from tonsils and cultured for 6 d in the presence of CD40 ligand/$CD32^+$ L cells with or without anti-BCR antibodies. B cells were then harvested, washed, and fur-

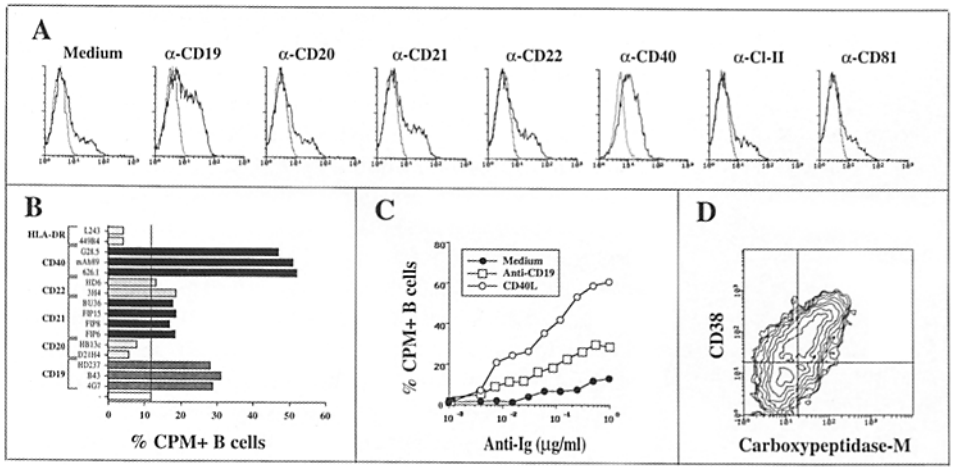

Figure 6. CPM expression on in vitro–activated $CD38^-$ B cells. (*A*) $CD38^-$ tonsillar B cells were cultured in the presence of $CD32^+$ fibroblasts with 2 μg/ml of anti-κ and anti-λ mAbs with or without anti-CD19 (4G7), anti-CD20 (MEM-97), anti-CD21 (FIP8), anti-CD22 (3H4), anti-CD40 (mAb89), anti-CD81 (1D6), or anti HLA-DR (449B4) mAbs. Cells were then labeled with biotin-conjugated anti-CPM + streptavidin–FITC. (*B*) Anti-κ+λ–activated cells were cultured with or without anti-CD19, CD20, CD21, CD22, CD40, or HLA-DR mAbs and labeled as above. The source of mAbs and the method for the determination of the percentage of positive cells are described in Materials and Methods. Results are representative of three independent experiments. (*C*) Percentage of CPM^+ cells after stimulation with suboptimal concentrations of anti-Ig. $CD38^-$ B cells were cultured for 4 d on CD40 ligand/CD32 double–transfected Ltk^- cells in the presence of variable concentrations of anti-Ig (κ+λ) (○, □, ●), 2 μg/ml anti-CD40-L (LL2) (□, ●) or 2 μg/ml anti-CD19 (□) antibodies before labeling with M27 mAb. (*D*) $CD38^-$ tonsillar B cells were cultured for 4 d in the presence of anti-CD19 + anti-Ig. Cells were then harvested and labeled with FITC-conjugated anti-CD38 mAb and streptavidin-PE + biotinylated anti-CPM mAb.

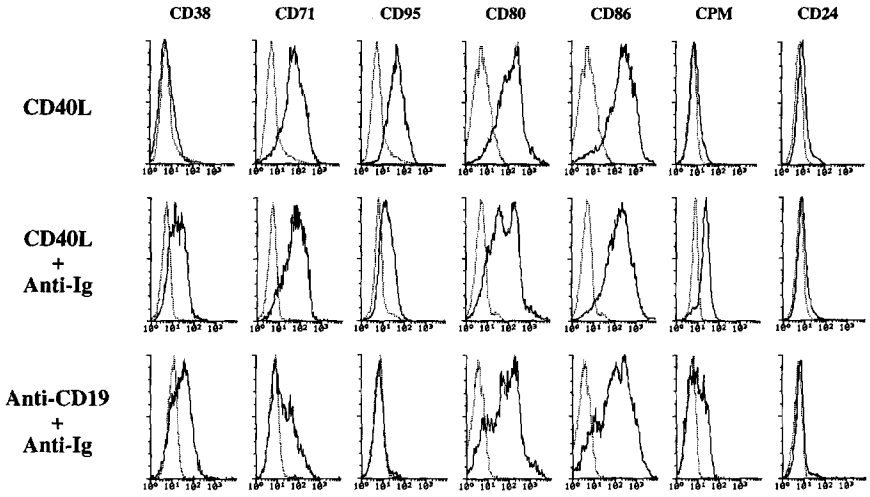

Figure 7. CD40-L + anti-Ig cotriggering induces phenotypic features of GC cells. $CD38^-$ B cells were cultured for 6 d on CD40-L/CD32-transfected fibroblasts with or without anti-κ+λ or on CD32-transfected fibroblasts in the presence of anti-κ+λ and anti-CD19 mAbs. Living cells were labeled with FITC or PE-conjugated antibodies. Staining profile (*solid line*) was superimposed onto that of a conjugated isotype-matched unrelated mAb (*dotted line*).

ther cultured in medium for an additional 20 h in the presence of anti-CD40 ligand antibody to avoid triggering by contaminating L cells. As shown in Fig. 8 *A*, when harvested, both CD40- and CD40 + BCR–stimulated resting B cells express high and comparable amounts of Bcl-2 protein. However, unlike CD40-activated lymphocytes, CD40 + BCR–triggered cells undergo rapid cell death as measured by trypan blue exclusion (Fig. 8 *B*). This spontaneous cell death can also be quantified by propidium iodide and HOECHST 33342 double labeling (13). Using this method, after 20 h in medium, only 14% of CD40 + BCR–triggered cells appear viable (Fig. 8 *C*, *right*), whereas 68% of cells that received only the CD40 triggering are viable (Fig. 8 *C*, *left*). This rapid loss of viability may be due to apoptotic cell death because a sizeable (14%) proportion of CD40 + BCR–activated cells exhibits condensed chromatin and an integral plasma membrane structure.

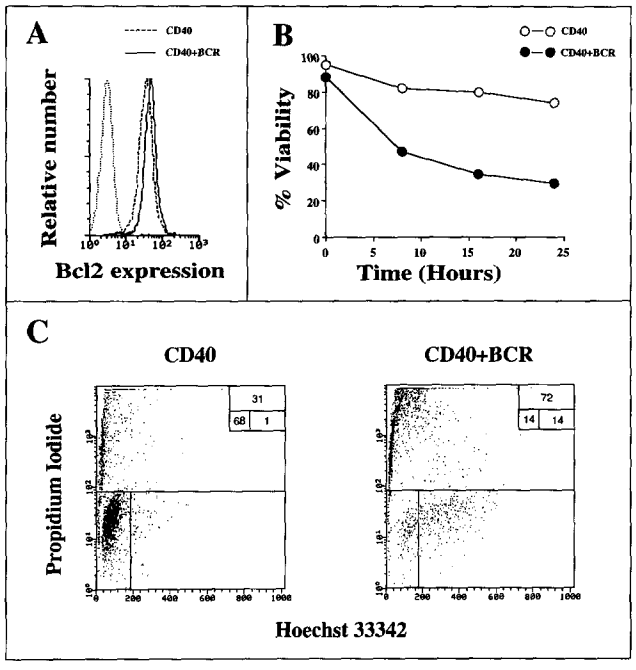

Figure 8. Cells with GC phenotype are highly susceptible to spontaneous cell death. Resting tonsillar B lymphocytes were cultured for 6 d in the presence of CD40-L/CD32–transfected L cells with or without 2 μg/ml of anti-κ + anti-λ antibodies. Cells were then harvested, washed and (*A*) labeled with Bcl-2 mAb after saponine treatment. Dashed line, CD40 activated cells; solid line: CD40 + Ig–activated cells; dotted line, isotype-matched control. Cells were further cultured in medium. (*B*) Time course measurement of viability using trypan blue. (*C*) After 20 h in secondary culture, cells were stained with propidium iodide + HOECHST 33342 as described in Materials and Methods. (*Left*) CD40-activated cells. (*Right*) CD40 + Ig–activated cells. The upper quadrant correspond to dead cells, the lower left to viable cells, the lower right to apoptotic cells. Numbers indicate the percentage of events recorded for each of these three subsets.

Discussion

Early histophysiological studies have shown that the GC is a T cell–dependent structure. Indeed, individuals with a complete T cell defect do not form GC (22). The interaction between CD40 on B cells with its ligand on Th cells recently emerged as an essential component of this Th–B cell crosstalk because a defective expression of CD40 or CD40 ligand leads to a GC formation defect (23–26). The implication of CD40–CD40 ligand interaction in the GC reaction is further illustrated by the fact that both isotype switching and the somatic mutation processes—two molecular events that take place in GC—are absent in patients displaying the nonfunctional CD40 ligand gene (27–30, D. Razanajaona, C. Van Kooten, S. Lebeque, J. M. Bridon, S. Ho, S. Smith, R. Callard, J. Banchereau, and F. Brière, manuscript in preparation). As shown in this report, in vitro CD40 stimulation of B cells leads to several phenotypic alterations that occur in the GC. Thus, in agreement with former reports (10, 11, 31, 32), CD40 stimulation induces the coexpression of CD95 (FAS), CD80 (B7.1), CD86 (B7.2), and CD71 surface molecules. In addition, CD40 triggering leads to the disappearance of CD24 antigen which, in vivo, marks B cell entry into the GC (33). When combined with certain cytokines such as IFN-α or

IFN-γ, CD40 triggering is also able to induce strong CD38 expression. This result is consistent with a previous report showing that immunoreactive IFN-α can be detected in GC tingible body macrophages of human secondary lymphoid tissues (34). A high frequency of spontaneous IFN-γ–producing T cells is also present in human tonsils (35). These IFN-γ–producing T cells are mainly located in the extrafollicular area (36), where CD40-L^+–activated T cells are also detected (37–39). Accordingly, in vivo CD40 + IFNs stimulation of antigen-specific B lymphocytes could indeed take place in human lymphoid tissues and drive the induction of CD38.

Although it has been clearly demonstrated that antigen is essential for the GC reaction, little is known concerning the site and context of the B cell–antigen encounter. In vitro experiments have shown that antigenic stimulation of B cells is critical for effective Th–B cell collaboration (40). Indeed, BCR triggering by multivalent antigen appears to be necessary for the induction of CD28 ligands (CD80 or CD86) on the B cell surface (41). These CD28 ligands are required for full Th cell proliferation and cytokine production, which, in turn, would control B cell blastogenesis. Interestingly, efficient triggering of BCR is also necessary to sensitize B cells to Th cells expressing low (physiological) levels of CD40 ligand (42). In vivo, this efficient BCR stimulation could be achieved in the presence of accessory cells like follicular dendritic cells (43) retaining the unprocessed antigen on their surface. Here we show that BCR stimulation can contribute to the acquisition of CD38 antigen. In addition, BCR triggering is the only signal tested so far that allows the induction of CPM, a surface membrane enzyme specifically expressed on GC mature B cells (9). Anti-BCR induction of CPM is further enhanced by anti-CD40 or, to a lesser extent, by anti-CD19 cotriggering. The contribution of CD19 or CD40 molecules to the induction of CPM was specific in that antibodies against six other surface molecules failed to display such an activity (see Fig. 6, *A* and *B*). Recent experiments in mice have shown that CD19 is necessary for the formation of GC in response to T cell–dependent antigens (44). CD19 is a B cell–restricted type I protein with a large extracytoplasmic portion comprising two Ig-like domains. The CD19 extracellular portion also includes a potential CD77 (globotriaosyl ceramide)–binding site (45). Together, these structural features suggest that a ligand for CD19 may exist. In addition, CD19 is a major component of the signal transduction complex associated with the CD21 (IFN-α receptor) molecule (for reviews see references 46, 47). In our hands, however, IFN-α or anti-CD21 mAbs displayed only a limited effect on CPM induction, suggesting that, if it plays a role in human GC formation, CD19 might act independently of CD21.

Unlike CD19 + BCR stimulation, CD40 + BCR cotriggering drives resting B lymphocytes to exhibit seven GC phenotypic features, including the lack of surface CD24 and the presence of markers like CD80, CD86, CD71, CD95, and CD38. The latter could possibly result from anti-BCR–induced endogenous IFN-α production (48). However, CD40 + BCR stimulation could not generate $CD10^+$ or $CD44^-$ cells, thus suggesting that other stimuli are required for modulating these markers. It should be noted that, in vivo, CD40–CD40 ligand contact is short lasting and tightly regulated. Indeed, within hours after contact, CD40 is released from APC—presumably through proteolitic cleavage (49)—whereas CD40 ligand is internalized in T cells (50). Thus, two step culture experiments using CD40 + BCR–prestimulated B cells are in progress to identify the factors responsible for the generation of $CD44^-$ or $CD10^+$ B cells. Interestingly, CD40 + BCR–activated B cells undergo spontaneous cell death in medium that is reminiscent of the apoptosis of GC B cells. This in vitro–induced cell death is likely to be the result of continuous triggering of BCR, and is observed on cells exhibiting high levels of Bcl-2 protein. The mechanisms responsible for this cell death induction are unknown and could be dependent upon the expression of other survival/death genes. Because lymphocytes undergoing in vivo somatic hypermutation are highly susceptible to apoptotic cell death (21), we are presently investigating whether CD40 + BCR–stimulated naive B cells undergo somatic mutation in culture. In conclusion, our results imply that the acquisition of a GC phenotype requires efficient cotriggering of BCR and CD40. They also show that IFNs or CD19 triggering could contribute to the induction of GC markers in humans. Finally, consistent with earlier reports on freshly isolated GC cells, in vitro acquisition of a GC phenotype renders resting B lymphocytes highly susceptible to spontaneous cell death.

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References

1. Nossal, G.J. 1994. Differentiation of the secondary B-lymphocyte repertoire: the germinal center reaction. *Immunol. Rev.* 137:173–183.
2. MacLennan, I.C. 1994. Germinal centers. *Annu. Rev. Immunol.* 12:117–139.
3. Gelman, L., P. Deterre, H. Gouy, L. Boumsell, P. Debre, and G. Bismuth. 1993. The lymphocyte surface antigen CD38 acts as a nicotinamide adenine dinucleotide glycohydrolase in human T lymphocytes. *Eur. J. Immunol.* 23:3361–3364.
4. Howard, M., J.C. Grimaldi, J.F. Bazan, F.E. Lund, A.L. Santos, R.M. Parkhouse, T.F. Walseth, and H.C. Lee. 1993. Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science (Wash. DC).* 262:1056–1059.
5. Nishina, H., K. Inageda, K. Takahashi, S. Hoshino, K. Ikeda, and T. Katada. 1994. Cell surface antigen CD38 identified as ecto-enzyme of NAD glycohydrolase has hyaluronate-binding activity. *Biochem. Biophys. Res. Commun.* 203:1318–1323.
6. Summerhill, R.J., D.G. Jackson, and A. Galione. 1993. Human lymphocyte antigen CD38 catalyzes the production of cyclic ADP-ribose. *FEBS Lett.* 335:231–233.
7. Takasawa, S., A. Tohgo, N. Noguchi, T. Koguma, K. Nata, T. Sugimoto, H. Yonekura, and H. Okamoto. 1993. Synthesis and hydrolysis of cyclic ADP-ribose by human leukocyte antigen CD38 and inhibition of the hydrolysis by ATP. *J. Biol. Chem.* 268:26052–26054.
8. Zupo, S., E. Rugari, M. Dono, G. Taborelli, F. Malavasi, and M. Ferrarini. 1994. CD38 signaling by agonistic monoclonal antibody prevents apoptosis of human germinal center B cells. *Eur. J. Immunol.* 24:1218–1222.
9. De Saint-Vis, B., L. Cupillard, D. Pandrau-Garcia, S. Ho, N. Renard, G. Grouard, V. Duvert, X. Thomas, J. Galizzi, J. Banchereau, and S. Saeland. 1995. Distribution of carboxypeptidase-M on lymphoid and myeloid cells parallels the other zinc-dependent proteases CD10 and CD13. *Blood.* 86: 1098–1105.
10. Garrone, P., E.-M. Neidhardt, E. Garcia, L. Galibert, C. Van Kooten, and J. Banchereau. 1995. Fas ligation induces apoptosis of CD40-activated human B lymphocytes. *J. Exp. Med.* 182:1265–1273.
11. 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.
12. Vallé, A., C.E. Zuber, T. Defrance, O. Djossou, M. De Rie, and J. Banchereau. 1989. Activation of human B lymphocytes through CD40 and interleukin 4. *Eur. J. Immunol.* 19: 1463–1467.
13. Dive, C., C.D. Gregory, D.J. Phipps, D.L. Evans, A.E. Milner, and A.H. Wyllie. 1992. Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim. Biophys. Acta.* 1133:275–285.
14. Lagresle, C., C. Bella, and T. Defrance. 1993. Phenotypic and functional heterogeneity of the IgD^- B cell compartment: identification of two major tonsillar B cell subsets. *Int. Immunol.* 5:1259–1268.
15. Pascual, V., Y.-J. Liu, A. Magalski, O. de Bouteiller, J. Banchereau, and J.D. Capra. 1994. Analysis of somatic mutation in five B cell subsets of human tonsil. *J. Exp. Med.* 180: 329–339.
16. 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.
17. Wheeler, K., J.D. Pound, J. Gordon, and R. Jefferis. 1993. Engagement of CD40 lowers the threshold for activation of resting B cells via antigen receptor. *Eur. J. Immunol.* 23:1165–1168.
18. Sumimoto, S., T. Ishigami, Y. Horiguchi, S. Yonehara, S. Kanazashi, T. Heike, K. Katamura, and M. Mayumi. 1994. Anti-Fas antibody induces different types of cell death in the human histiocytic cell line, U937, and the human B cell line, B104: the role of single-strand DNA breaks and poly (ADP-ribosyl)ation in cell death. *Cell. Immunol.* 153:184–193.
19. Connelly, J., R. Chambless, D. Holiday, K. Chittenden, and A. Johnson. 1993. Upregulation of neutral endopeptidase (CALLA) in human neutrophils by granulocyte-macrophage colony-stimulating factor. *J. Leukocyte Biol.* 53:685–690.
20. Wayner, E.A., and W.G. Carter. 1987. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique alpha and common beta subunits. *J. Cell. Biol.* 105:1873–1884.
21. Liu, Y.J., D.E. Joshua, G.T. Williams, C.A. Smith, J. Gordon, and I.C. MacLennan. 1989. Mechanism of antigen-driven selection in germinal centres. *Nature (Lond.).* 342:929–931.
22. Kroese, F.G., W. Timens, and P. Nieuwenhuis. 1990. Germinal center reaction and B lymphocytes: morphology and function. *Curr. Top. Pathol.* 84:103–148.
23. Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani. 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity.* 1:167–178.
24. 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.
25. Renshaw, B.R., W.C. Fanslow, III, R.J. Armitage, K.A. Campbell, D. Liggitt, B. Wright, B.L. Davison, and C.R. Maliszewski. 1994. Humoral immune responses in CD40 ligand–deficient mice. *J. Exp. Med.* 180:1889–1900.
26. Notarangelo, L.D., M. Duse, and A.G. Ugazio. 1992. Immunodeficiency with hyper-IgM (HIM). *Immunodefic. Rev.* 3: 101–121.
27. Allen, R.C., R.J. Armitage, M.E. Conley, H. Rosenblatt, N.A. Jenkins, N.G. Copeland, M.A. Bedell, S. Edelhoff, C.M. Disteche, D.K. Simoneaux, et al. 1993. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science (Wash. DC).* 259:990–993.
28. Aruffo, A., M. Farrington, D. Hollenbaugh, X. Li, A. Milatovich, S. Nonoyama, J. Bajorath, L.S. Grosmaire, R. Stenkamp, M. Neubauer, et al. 1993. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell.* 72:291–300.
29. DiSanto, J.P., J.Y. Bonnefoy, J.F. Gauchat, A. Fischer, and G. de Saint Basile. 1993. CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM. *Nature (Lond.).* 361:541–543.
30. Korthauer, U., D. Graf, H.W. Mages, F. Briere, M. Padayachee, S. Malcolm, A.G. Ugazio, L.D. Notarangelo, R.J. Levinsky, and R.A. Kroczek. 1993. Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with

hyper-IgM. *Nature (Lond.).* 361:539–541.
31. Ranheim, E.A., and T.J. Kipps. 1993. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. *J. Exp. Med.* 177:925–935.
32. Azuma, M., D. Ito, H. Yagita, K. Okumura, J.H. Phillips, L.L. Lanier, and C. Somoza. 1993. B70 antigen is a second ligand for CTLA-4 and CD28. *Nature (Lond.).* 366:76–79.
33. Ling, N., I. MacLennan, and D. Mason. 1987. B cell and plasma cell antigens: new and previously defined clusters. *In* Leukocyte Typing III. A.J. McMichael, editor. 302–335.
34. Khan, N.U., K.A. Pulford, M.A. Farquharson, A. Howatson, C. Stewart, R. Jackson, A.M. McNicol, and A.K. Foulis. 1989. The distribution of immunoreactive interferon-alpha in normal human tissues. *Immunology.* 66:201–226.
35. Quiding, M., G. Granstrom, I. Nordstrom, B. Ferrua, J. Holmgren, and C. Czerkinsky. 1993. High frequency of spontaneous interferon-gamma-producing cells in human tonsils: role of local accessory cells and soluble factors. *Clin. Exp. Immunol.* 91:157–163.
36. Hoefakker, S., E.H. van't Erve, C. Deen, A.J. Van den Eertwegh, W.J. Boersma, W.R. Notten, and E. Claassen. 1993. Immunohistochemical detection of co-localizing cytokine and antibody producing cells in the extrafollicular area of human palatine tonsils. *Clin. Exp. Immunol.* 93:223–228.
37. Lederman, S., M.J. Yellin, G. Inghirami, J.J. Lee, D.M. Knowles, and L. Chess. 1992. Molecular interactions mediating T-B lymphocyte collaboration in human lymphoid follicles. Roles of T cell-B-cell-activating molecule (5c8 antigen) and CD40 in contact-dependent help. *J. Immunol.* 149:3817–3826.
38. Van den Eertwegh, A.J., R.J. Noelle, M. Roy, D.M. Shepherd, A. Aruffo, J.A. Ledbetter, W.J. Boersma, and E. Claassen. 1993. In vivo CD40–gp39 interactions are essential for thymus-dependent humoral immunity. I. In vivo expression of CD40 ligand, cytokines, and antibody production delineates sites of cognate T–B cell interactions. *J. Exp. Med.* 178: 1555–1565.
39. Casamayor-Palleja, M., M. Kahn, and I.C.M. MacLennan. 1995. A subset of $CD4^+$ memory T cells contains preformed CD40 ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor complex. *J. Exp. Med.* 181:1293–1301.
40. Cooke, M.P., A.W. Heath, K.M. Shokat, Y. Zeng, F.D. Finkelman, P.S. Linsley, M. Howard, and C.C. Goodnow. 1994. Immunoglobulin signal transduction guides the specificity of B cell–T cell interactions and is blocked in tolerant self-reactive B cells. *J. Exp. Med.* 179:425–438.
41. Ho, W.Y., M.P. Cooke, C.C. Goodnow, and M.M. Davis. 1994. Resting and anergic B cells are defective in CD28-dependent costimulation of naive $CD4^+$ T cells. *J. Exp. Med.* 179:1539–1549.
42. Poudrier, J., and T. Owens. 1994. Co-stimulation by anti-immunoglobulin is required for B cell activation by $CD40\text{-}L^{low}$ T cells. *Eur. J. Immunol.* 24:2993–2999.
43. Kosco-Vilbois, M.H., D. Gray, D. Scheidegger, and M. Julius. 1993. Follicular dendritic cells help resting B cells to become effective antigen-presenting cells: induction of B7/BB1 and upregulation of major histocompatibility complex class II molecules. *J. Exp. Med.* 178:2055–2066.
44. Rickert, R.C., K. Rajewsky, and J. Roes. 1995. Impairment of T-cell dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature (Lond.).* 376:352–355.
45. Maloney, M.D., and C.A. Lingwood. 1994. CD19 has a potential CD77 (globotriaosyl ceramide)-binding site with sequence similarity to verotoxin B subunits: implications of molecular mimicry for B cell adhesion and enterohemorrhagic *Escherichia coli* pathogenesis. *J. Exp. Med.* 180:191–201.
46. Fearon, D.T. 1993. The CD19-CR2-TAPA-1 complex, CD45 and signaling by the antigen receptor of B lymphocytes. *Curr. Opin. Immunol.* 5:341–348.
47. Tedder, T.F., L.J. Zhou, and P. Engel. 1994. The CD19/CD21 signal transduction complex of B lymphocytes. *Immunol. Today.* 15:437–442.
48. Weigent, D.A., M.P. Langdord, E.M. Smith, J.E. Ballock, and G.J. Stanton. 1981. Human B lymphocytes produce leucocyte interferon after interaction with foreign cells. *Infect. Immun.* 32:508–515.
49. van Kooten, C., C. Gaillard, J.P. Galizzi, P. Hermann, F. Fossiez, J. Banchereau, and D. Blanchard. 1994. B cells regulate expression of CD40 ligand on activated T cells by lowering the mRNA level and through the release of soluble CD40. *Eur. J. Immunol.* 24:787–792.
50. Yellin, M.J., K. Sippel, G. Inghirami, L.R. Covey, J.J. Lee, J. Sinning, E.A. Clark, L. Chess, and S. Lederman. 1994. CD40 molecules induce down-modulation and endocytosis of T cell surface T cell-B cell activating molecule/CD40-L. Potential role in regulating helper effector function. *J. Immunol.* 152:598–608.