Memory B Cells Are Biased Towards Terminal Differentiation: A Strategy That May Prevent Repertoire Freezing

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Summary

Isolation of large numbers of surface IgD+CD38+ naive and surface IgD–CD38+ memory B cells allowed us to study the intrinsic differences between these two populations. Upon in vitro culture with IL-2 and IL-10, human CD40–activated memory B cells undergo terminal differentiation into plasma cells more readily than do naive B cells, as they give rise to five- to eightfold more plasma cells and three- to fourfold more secreted immunoglobulins. By contrast, naive B cells give rise to a larger number of nondifferentiated B blasts. Saturating concentrations of CD40 ligand, which fully inhibit naive B cell differentiation, only partially affect that of memory B cells. The propensity of memory B cells to undergo terminal plasma cell differentiation may explain the extensive extra follicular plasma cell reaction and the limited germinal center reaction observed in vivo after secondary immunizations, which contrast with primary responses in carrier-primed animals. This unique feature of memory B cells may confer two important capacities to the immune system: (a) the rapid generation of a large number of effector cells to efficiently eliminate the pathogens; and (b) the prevention of the overexpansion and chronic accumulation of one particular memory B cell clone that would freeze the available peripheral repertoire.

Materials and Methods

Abbreviations used in this paper: BCR, b cell receptor; CD40L, CD40 ligand; GC, germinal center; PC, plasma cell.
munotech, Marseille, France) and PE-conjugated anti-CD38 (Leu17; Becton Dickinson Monoclonal Center, Mountain View, CA). Antibodies used for cell purification and cell culture were anti-CD4 (Q4120) and biotinylated anti-IgD (HJ9) purchased from Sigma Chemical Co. (St. Louis, MO), anti-CD38 (T16), anti-Igκ (6E1), and anti-Igλ (C4) purchased from Immunotech, and anti-CD2, -CD3, -CD8 antibodies acquires in our own laboratory using the OKT hybridomas obtained from American Type Culture Collection (Rockville, MD). Antibodies used for immunoenzymatic stainings are described in the corresponding section. Anti-CD40 (LL48) - blocking mAb and CD40 ligand (CD40L) - transfected murine fibroblasts were produced in our laboratory (31).

Recombinant human IL-2 was purchased from Amgen Biologicals (Thousand Oaks, CA) and recombinant human IL-4 from Schering-Plough Research Institute (Kenilworth, NJ). IL-2 was used at 10 U/ml and IL-10 at 100 ng/ml in cultures. IL-2 and IL-10 were then incubated with biotinylated anti-human IgD antibodies. For naive cell purification, two rounds of positive selection were performed with a magnetic activated cell sortor (MACS®; Miltenyi Biotec, Bergisch Gladbach, Germany). For memory cell preparation, two rounds of negative magnetic beads depletion (Streptavidin-coated Dynabeads; Dynal, Oslo, Norway) were performed. Both resulting IgD- and IgD+ populations were further depleted of T cells and CD38+ (i.e., GC) B cells by incubation with anti-CD2, -CD3, -CD4, -CD8, and -CD38 antibodies followed by two rounds of depletion with anti–mouse IgG-coated magnetic beads (Dynal). This procedure lead to 98-99.5% pure naive and 95-99.5% pure memory B cell populations.

Proliferation Assays. For DNA synthesis, 2.5 × 10⁴ B cells were cultured together with 5 × 10⁵. 75 Gγ-irradiated, CD40L-transfected fibroblasts in 200 μl Iscove medium (GIBCO) complemented with 5% FCS (GIBCO) for 12 d in the presence of IL-2 and IL-10. DNA synthesis was assessed by incubation with 1 μCi of tritiated thymidine (Amersham, Les Ulis, France) during the last 8 h of culture. For cellular expansion, 1.5 × 10⁵ B cells were cultured with 5 × 10⁴ CD40L-transfected fibroblasts for 12 d in the presence of IL-2 and IL-10. Cells were harvested and counted in tripian blue (GIBCO) to exclude dead cells.

In another set of experiments, anti-Igλ and Igκ antibodies were used to trigger B cell receptor (BCR) at 2 μg/ml final concentration. Secondary cultures consisted of 1.5 × 10⁵ B cells in 1 ml Iscove medium containing IL-2 and IL-10, together with 5 × 10⁴ irradiated murine fibroblasts. Murine fibroblasts were either CD40L–transfected cells or nontransfected cells together with anti-CD40L–blocking antibody at 2 μg/ml to block the signals given by CD40L–transfected cells that could have been harvested from the primary cultures. All secondary cultures were done in triplicate. After 4 d, cultures were harvested, supernatants frozen for antibody titer assays, and cells kept for analysis.

Quantitation of CD 40L Molecules on Murine Fibroblasts. The number of CD 40L molecules expressed on transfected fibroblasts was estimated using a Q-Fikit system (Dako, Goldstrup, Denmark) immediately before establishment of cultures. In brief, cells were cultured in suspension at saturation with either an anti-CD40L mAb (IgG1, isotype) or a nonrelevant control-matched antibody for 20 min on ice. After two washes, they were incubated with FITC-conjugated sheep anti–mouse immunoglobulins at the same time as different beads suspensions, coated with a known number of mouse Igs. Cells and beads were then analyzed using a FACScan® (Becton Dickinson, Sunnyvale, CA). Means of fluorescence intensity were then plotted against the number of mouse Igs on beads and linear regression was calculated (r² = 0.998 in all experiments). The number of recognized molecules (CD40L) on stained fibroblasts was calculated using the linear regression and the fluorescence intensity of these cells, after taking account of the fluorescence of the cells stained with the control-matched antibody.

Cell Cultures with Progressive Triggering of CD 40. To assess the effect of progressive triggering of CD40 antigen on naive and memory B cells, a second two-step culture was established. Cells were grown in primary cultures as in the previous two-step culture system. After 3 d, cells were recultured under seven different conditions. As the number of CD40L molecules on transfected fibroblasts varies from one experiment to another, a fixed cell ratio, rather than a fixed number of molecules, was chosen to avoid differences in the fibroblast feeder effects. Therefore, 1.5 × 10⁵ B cells were cultured for 4 d in 1 ml Iscove medium containing IL-2 and IL-10, together with 5 × 10⁴ irradiated fibroblasts. One culture condition was established with CD40L–transfected cells whose CD40L molecules number has been determined. These cells are then diluted with nontransfected irradiated fibroblasts for other culture conditions at the ratios of 1/2, 1/4, 1/8, and 1/16. Two other cultures were also set using parental cells, with or without anti-CD40L antibody at 2 μg/ml. All secondary cultures were set in triplicates and designed as the number of CD40L molecules present in the culture per B cell.

Ig Secretion Assays. For IgA, IgG, and IgM concentrations in culture supernatants were measured using ELISA. Total Ig levels are given as the summation of these values.

Cell Sorting. Naive and memory B cells were cultured for 3 d in the presence of IL-2, IL-10, and CD40L-transfected fibroblasts. They were then harvested and recultured for an additional 4 d with IL-2, IL-10, and parental fibroblasts. After harvesting, debris and dead cells were depleted from the cultures by centrifugation on nitrozide-ficoll (Eurobio). Cells were then stained with FITC-conjugated anti-CD20 and PE-conjugated anti-CD38 antibodies. Both CD20-lowCD38high and CD20-CD38- populations were sorted using a FACStar® (Becton Dickinson Immuno cytometry Systems, San Jose, CA).

Immunoenzymatic Stainings. 7 × 10⁴ sorted cells were cytotoxic incubated on microscope slides. Some slides were stained with Giemsa-Gurr solution, whereas others were kept for immunoenzymatic staining. Human Igs were revealed by anti-human κ and λ light chain antibodies (ABB5 and N10/2 clones, respectively, IgG₁ isotypes) (Dako), whereas IgM isotype IgS were revealed by anti-human IgM mAb (145-8, IgG₁ isotype; Becton Dickinson Monoclonal Center). Enzymatic activity was developed with Fast Red substrate (Dako). All immunoenzymatically colored slides were lightly counterstained with Mayer’s hematox ylin solution.
Results

Memory B Cells Undergo Prompt Differentiation into Plasma Cells

Using a two-step culture system, we previously demonstrated that continuous triggering of CD40 antigen on GC cells inhibits their terminal differentiation into plasma cells (PC; 37). To determine the influence of CD40L on the capacity of memory and naive B cells to generate PCs, similar culture conditions were used. Both populations were cultured for 3 d over CD40L-transfected fibroblasts in the presence of IL-2 and IL-10. Activated B cell blasts were then re-cultured for 4 d with nontransfected fibroblasts, IL-2, IL-10, and an anti-CD40L-blocking antibody to block the CD40L-transfected fibroblasts carried over from the primary culture. Although naive B cells yielded 16.4 \( \pm \) 6.6% CD20\(^{-}\)/lowCD38\(^{\text{high}}\) plasma cells (mean \( \pm \) SD, n = 7; Fig. 1B; Table 1), memory B cells yielded 62.4 \( \pm \) 11.9% plasma cells (mean \( \pm \) SD, n = 4; Fig. 1D; Table 1). Accordingly, naive B cells yielded three times more nondifferentiated CD20\(^{-}\)/lowCD38\(^{\text{low}}\) B blasts than did memory cells. Addition of CD40L during the secondary culture (Fig. 1, A and C) considerably inhibited the plasma cell differentiation of B cell blasts, generated from both naive and memory cells (Table 1).

FACS\(^{\text{in}},\) sorted CD20\(^{-}\)/lowCD38\(^{\text{high}}\) cells generated from both naive and memory B blasts display the morphology of terminally differentiated PCs (Fig. 2A), as well as an intense Igκ and Igλ light chain staining (Fig. 2C). In contrast, CD20\(^{-}\)/lowCD38\(^{\text{low}}\) populations display the morphology of blasts with a weak surface Ig expression (Fig. 2B and D). Although 50% of plasma cells generated from naive B cells contain intracytoplasmic IgM (Fig. 2E), only 20% of plasma cells generated from memory B cells expressed IgM (Fig. 2F).

High concentrations of CD40L do not completely block the terminal differentiation of memory B cells. To further understand the propensity of memory B cells to undergo plasma cell differentiation, secondary cultures of naive and memory blasts were set up in the presence of increasing density of CD40L. For that purpose, absolute numbers of CD40L molecules per fibroblast were estimated using quantitative flow cytometry and CD40L-transfected fibroblasts were gradually diluted with their parental cells (see Material and Methods). As shown in Fig. 3A, increased CD40 ligation of memory cells results in a decreased production of plasma cells and a concomitant increase of B blasts (Fig. 3B). In fact, there is a linear correlation between the log (1/CD40L available per memory blast) and the percentage of generated plasma cells (\( r^2 = 0.945, 0.966, \) and 0.983 from three experiments). Note that CD40L-transfected fibroblasts were indeed carried over from the primary cultures, as the addition of anti-CD40L antibody to the cultures with nontransfected fibroblasts further enhanced the plasma cell generation. As shown in Fig. 3C, in the absence of CD40L in the secondary culture, memory cells can generate up to eight times more PCs than do naive cells. Note that very high amounts of CD40L molecules in the secondary cultures do not completely inhibit the generation of PCs from memory cells, since up to 2 \( \times \) 10\(^5\) CD40L molecules/B blast led to the generation of 3.6 \( \times \) 10\(^5\) PCs from an initial input of 1.5 \( \times \) 10\(^5\) blasts (Fig. 3C).

### Table 1. Memory B Cells Promptly Differentiate into PC

<table>
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<tr>
<th>Secondary cultures</th>
<th>Naive cells generated from CD40L-transfected fibroblasts</th>
<th>Memory cells generated from Parental fibroblasts + anti-CD40L mAb</th>
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<tr>
<td>Percentage of CD20(^{-})/lowCD38(^{\text{high}}) cells</td>
<td>3.7 ( \pm ) 1.8 [1.1–6.7] 23.8 ( \pm ) 7.8 [15–31]</td>
<td>16.4 ( \pm ) 6.6 [5.4–25] 62.4 ( \pm ) 11.9 [50.8–79]</td>
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Mean, standard deviation, and range (brackets) of percentages of CD20\(^{-}\)/lowCD38\(^{\text{high}}\) cells generated from naive and memory B cells from seven and four experiments, respectively. Cells were cultured for 3 d in the presence of IL-2, IL-10, and CD40L-transfected fibroblasts before being seeded for 4 d in the secondary cultures together with IL-2, IL-10, and fibroblasts. The fibroblasts used in secondary cultures are listed in the table.
Increasing the number of CD40L molecules available in the cultures not only inhibited the plasma cell generation, but also the secretion of IgGs (Fig. 4A). Furthermore, in all culture conditions, memory B cells produced more total IgGs than naive B cells (Fig. 4A). With regard to secreted iso-type, although naive and memory B cells produced comparable levels of IgM (Fig. 4D), memory cells, as expected, produced considerably more IgG and IgA (Fig. 4, B and C).

Naive and memory B cells proliferate equally well. We then questioned whether the poor differentiation capacity of naive B cells, as compared to that of memory B cells, may indeed reflect a reduced activation and proliferation capacity. Thus, purified naive and memory B cells were cultured over CD40L-transfected fibroblasts with IL-2 and IL-10, and proliferation was assessed by measuring thymidine incorporation, as well as viable cell numbers. As shown in Fig. 5, naive B cells proliferate at least as much as memory B cells do.

Figure 2. Morphology and intracellular Ig content. Giemsa staining of sorted CD20+CD38high B blasts (A, original magnification: 1,000) and CD20+CD38low B blasts (B, original magnification: 1,000). Red anti-Igκ + light chain staining of sorted CD20+CD38high PCs (C, original magnification: 1,000) and CD20+CD38low B blasts (D, original magnification: 1,000). Red anti-IgM staining of sorted CD20+CD38high PCs derived from naive B cells (E, original magnification: 400) and from memory B cells (F, original magnification: 400).
Memory, but Not Naive, B Cells Undergo Rapid PC Differentiation in Cultures even after Anti-BCR Triggering. Anti-Igs were shown to prevent B cell differentiation (38). Since naive B cells, but not memory B cells, were isolated by positive selection using anti-IgD, we questioned whether the difference in the differentiation capacity between naive and memory B cells could be due to the BCR triggering. Accordingly, in the first 3 d of primary cultures, 2 μg/ml of CD40L molecules/Bcell (*10^-3)

Figure 3. CD40L inhibits B cell differentiation during secondary cultures in a dose-dependent fashion. (A) Percentages of CD20^highCD38^high PCs derived from naive (closed circles) and memory (open circles) B cells. (B) Percentages of CD20^highCD38^low non-differentiated B blasts derived from naive (closed circles) and memory (open circles) B cells. (C) Total numbers of CD20^lowCD38^high PCs derived from naive (closed circles) and memory (open circles) B cells. The number of CD40L molecules per B cell in secondary cultures was measured and used as x-axis (a negative value artificially represents cultures with parental fibroblasts in the presence of a blocking antibody to CD40L at 2 μg/ml). Cell input was 1.5 × 10^5 at the beginning of secondary cultures. Each circle represents an individual value. Mean values are linked (plain and dotted lines represent naive and memory cell cultures, respectively). Standard deviations are vertical bars.

Figure 4. CD40L inhibits Ig production during secondary cultures in a dose-dependent fashion. The culture conditions are the same as described in Fig. 3. (A) Total IgG + IgA + IgM production from 10^6 cells of naive (closed circles) and of memory B cell cultures (open circles). (B) IgA production. (C) IgG production. (D) IgM production. Each circle (closed and open correspond to naive and memory cell cultures, respectively) represents an individual value. Mean values are linked (plain and dotted lines represent naive and memory cell cultures, respectively). Standard deviations are vertical bars.
anti-Igκ and 2 μg/ml of anti-Igλ antibodies were added into the cultures in the presence of CD40L, IL-2, and IL-10. At the end of the culture, cells were washed and seeded in a 4 d secondary culture with IL-2, IL-10, and different concentrations of CD40L. Fig. 6 shows that in the presence of three different CD40L concentrations (9.6 × 10⁴/cell, 4.8 × 10⁴/cell, no CD40L), 3, 6, and 13% of CD38⁺ CD20⁻ plasma cells were generated from the naive B cells. In the same culture conditions, 21, 29, and 43% of CD38⁺ CD20⁻ plasma cells were generated from the memory B cells. This experiment indicates that memory B cells, but not naïve B cells, preferentially undergo plasma cell differentiation even after BCR triggering.

**Discussion**

This paper describes the striking differentiation ability of memory versus that of naïve B cells. This correlates with previous histophysiological observations in vivo showing that secondary antigenic challenge in carrier-primed rats leads to a massive extrafollicular PC reaction and a poor follicular GC reaction in the spleen. In contrast, only small extrafollicular PC reactions, but large GC reactions develop upon primary immunization (Fig. 7; 39, 40). Likewise, in mice infected with reoviruses, adoptively transferred memory B cells give rise to a large extrafollicular PC reaction, but a small GC reaction; in contrast, transferred naïve cells generate a large GC reaction (41). Thus, the differences in the capacity of memory versus naïve B cells to differentiate is an intrinsic property of the B cells, rather than of the microenvironments. The propensity of memory B cells to undergo rapid differentiation into effector cells may confer two important properties to the immune system. First, it allows the rapid generation of large numbers of effector cells, whose products (antibodies) efficiently eliminate pathogens. This novel feature of memory B cells, together with their low threshold for activation, and their ability to home to the antigen draining sites and to directly present antigen to T cells, may all contribute to the velocity of secondary antibody responses. Second, it prevents the overexpansion and accumulation of a particular memory B cell clone that would otherwise overload the immune system and freeze the available Ig repertoire (26). Since PCs have a relatively short lifespan and do not proliferate in response to further stimulations (42–44), the majority of memory B cells will undergo clonal exhaustion by differentiating into effector cells during secondary immune responses. Interestingly, T memory cells show a similar tendency not to expand and overload the whole immune system, as towards the end of a primary immune response specific T blasts are rapidly eliminated (45–48).

The finding that CD40L inhibits the differentiation of both activated memory and naïve B cells, complements the previous observations made with GC B cells (37) or total B cells isolated from blood and tonsils (49, 50). Thus, CD40L represents a differentiation suppressor during not only the
Figure 7. Memory B cells are biased towards terminal plasma cell differentiation in vivo and in vitro. Naive B cells predominantly give rise to germinal center reaction within a rat spleen after primary immunization with DNP-KLH in KLH-primed animals (A, original magnification: 40; B, original magnification: 200). The rats were given BrdU in their drinking water for 48 h before they were killed. Red stains BrdU, blue stains DNP-binding cells, brown stains total B cells. MZ, marginal zone; PALS, periarteriolar lymphoid sheath. Consistent with this in vivo finding, human naive B cells predominantly give rise to proliferating B blasts upon activation in vitro (C). Memory B cells predominantly give rise to plasma cell reaction along the outer edges of the periarteriolar lymphoid sheath and within the red pulp of a rat spleen after 2 d of secondary immunization with DNP-KLH (D and E). The rats have received BrdU in their drinking water for 48 h before killing. DNP-specific plasma blasts are cells with strong blue cytoplasmic staining. Consistent with this in vivo finding, human memory B cells predominantly give rise to plasma cells upon activation in vitro (F). The figures on immunohistology are derived from Y.-J. Liu and I.C.M. MacLennan (40).
GC, but also the extrafollicular reactions (51). Indeed, CD40L-expressing T cells have been reported by immunohistology, both within the GCs and the extrafollicular T zones (52–54).

Although CD40L inhibits the PC differentiation of naive, GC, and memory B cells, a fraction of the memory cell subset seems to be resistant to this effect. Differential effects of CD40L on mature B cell subsets have already been noticed. For instance, CD40 triggering is an important survival but a minor proliferative signal for GC cells (55–57), whereas it provides a strong and long-term proliferative signal to resting naive and memory B cells (58–61). The molecular mechanisms underlying the propensity of memory B cells to undergo terminal differentiation are still unknown. CD40 triggering on human GCs and resting mature B cells results in the activation of different protein kinases (62, 63). Further comparative studies of CD40 signaling pathways in naive, GC, and memory B cells should now be carried on to explain how mature B cells change their responses to CD40 triggering at different stages of their immunopoiesis.

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