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*J Immunol* (2003) 170 (1): 261–269.

https://doi.org/10.4049/jimmunol.170.1.261

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# A Division-Linked Mechanism for the Rapid Generation of Ig-Secreting Cells from Human Memory B Cells[1]

**Stuart G. Tangye,[2]*† Danielle T. Avery,* and Philip D. Hodgkin[3]*†**

**Memory B cells, when re-exposed to Ag and T cell help, differentiate into Ig-secreting cells (ISC) at the same time as maintaining a residual pool of non-Ig-secreting cells with memory capabilities. To investigate the mechanism underlying this dual process, we followed the fate of human B cells activated in vitro with the T cell-derived signals CD40 ligand (CD40L), IL-2, and IL-10 using CFSE to monitor cell division. A substantial number of ISCs detected by ELISPOT, intracellular Ig staining, and Ig secretion could be generated from memory but not naive B cells. The proportion of ISCs increased with successive cell divisions and was markedly enhanced by IL-10 at each division. Within ISCs, two distinct populations were detected after withdrawal of CD40L. The first had acquired the plasma cell marker CD38 and continued to proliferate despite the absence of CD40L. In contrast, the second population remained CD38$^-$, ceased dividing, and underwent rapid apoptosis. The former most likely represent the immediate precursors of long-lived plasma cells, which preferentially home to the bone marrow in vivo, whereas the latter contain short-lived ISCs responsible for the initial Ab response to stimulation with Ag and T cell help. Taken together, the results point to a division-based mechanism responsible not only for regulating differentiation of short- and long-lived ISCs from memory B cells, but for preserving the memory B cell pool for reactivation upon subsequent Ag exposure.** ***The Journal of Immunology,*** **2003, 170: 261–269.**

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Mature B cells become activated in secondary lymphoid tissues after interaction with specific Ag- and T cell-derived signals. This process gives rise to a range of cell types resulting in production and secretion of protective Ig and the generation of long-lived memory cells (1, 2). The steps involved in this event are relatively well understood. After initial stimulation, some B cells undergo rapid clonal expansion and seed germinal centers (GCs),[4] where somatic hypermutation, Ig isotypes switching, and generation of high-affinity Ag-specific B cells occur (3–8). Ag-selected GC B cells then develop into memory B cells or give rise to plasma cells, which are terminally differentiated Ig-secreting cells (ISCs) (3–6, 8). A similar, if less complicated, sequence occurs when memory cells are reactivated by specific Ag. Activated memory cells rapidly proliferate and differentiate such that the memory lineage is preserved, whereas large numbers of plasma cells are concomitantly generated (1, 2, 6–11). Thus, whereas plasma cells are mitotically quiescent and terminally differentiated, memory B cells are capable of consecutive phases of stimulation, expansion, selection, and generation of effector cells (7, 8, 12–14). As a consequence, the population of memory B cells is maintained and even enhanced by frequent exposure to Ag (12, 15), giving it the attributes of a "stem" type cell for the plasma cell lineage (16).

The signals required for generating human plasma cells from GC or memory B cells in vitro have been previously investigated (17–19). In these studies, plasma cells were identified as cells containing intracellular Ig, increased expression of CD38, and concomitant down-regulation of CD20, consistent with the morphology and phenotype of plasma cells present in human bone marrow (20, 21). After initial stimulation of GC or memory B cells with CD40 ligand (CD40L), IL-2, and IL-10, a greater proportion of plasma cells was generated when these activated cells were recultured in the absence of CD40L (17–19). These findings suggested that CD40L interrupted plasma cell development while concomitantly promoting expansion of nondifferentiated B cell blasts (17–19). Thus, provision of T cell help, in the form of transiently expressed CD40L, appeared to influence the decision between the generation of plasma cells and memory B cells (22).

During the past 6 years, we and others have shown that numerous lymphocyte differentiation events alter in frequency with consecutive cell divisions (23–32), leading us to propose that division number may play an important role in key decisions associated with immune regulation (23, 25, 27). The extent to which similar division-linked regulation might apply to lymphocytes that have undergone extensive division and differentiation after Ag stimulation in vivo (i.e., memory cells) is currently unknown. Here, we have applied new quantitative analytical techniques to re-examine the generation of ISCs from human B cells after in vitro stimulation with T cell-derived signals CD40L, IL-2, and IL-10 and to dissect the differential roles played by CD40L and these cytokines in this process. Our data suggest that a division-linked process governs the formation of ISCs. Two types of ISC (CD38$^-$ and CD38$^+$) could be generated from proliferating memory B cells, with CD38$^-$ ISCs appearing to be precursors of CD38$^+$ ISCs. Strikingly, CD38$^+$ ISCs acquired increased proliferation and survival characteristics that permitted them, but not CD38$^-$ B cells, to

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Received for publication July 24, 2002. Accepted for publication October 29, 2002.

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[1] This work was supported by the National Health and Medical Research Council of Australia and the Medical Foundation of the University of Sydney, Australia. S.G.T. was the recipient of a U2000 Postdoctoral Research Fellowship awarded by the University of Sydney. P.D.H. is a Senior Research Fellow of the National Health and Medical Research Council of Australia.

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[4] Abbreviations used in this paper: GC, germinal center; ISC, Ig-secreting cell; CD40L, CD40 ligand; BrdU, 5-bromo-2′-deoxyuridine; MFI, mean fluorescence intensity.

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persist and increase in number in the absence of CD40L. Thus, the primary function of CD40L was to sustain the proliferative potential and survival of $CD38^-$ blasts rather than to prevent subsequent generation of effector ISCs. This analysis, therefore, challenges the proposal that removal of CD40L regulates differentiation of memory B cells into ISCs. Rather, our results are consistent with a stochastic division-linked mechanism for controlling the early production of protective Ig while simultaneously preserving, and even expanding, the memory B cell pool, both of which are necessary for sustained humoral immunity.

## Materials and Methods

### *Reagents*

PE-conjugated and biotinylated anti-CD38 mAbs were from Caltag (Burlingame, CA). FITC- and PE-conjugated anti-CD19, CD20, CD27, anti-5-bromo-2′-deoxyuridine (BrdU), anti-IgM, anti-IgG, anti-Ig κ L chain mAb, isotype control mAb, and rabbit polyclonal anti-active caspase-3 antisera were from BD PharMingen (San Diego, CA). Unconjugated and biotinylated goat anti-human IgM, IgG, or IgA polyclonal antisera were purchased from Southern Biotechnology (Birmingham, AL). Recombinant human CD40L expressed as membranes of the *Sf21* insect cell line infected with baculovirus vector containing human *CD40L* cDNA was a generous gift from Dr. M. Kehry (Boehringer Ingleheim, Ridgefield, CT) (33). IL-2 was purchased from Endogen (Woburn, MA), and IL-10 was provided by Dr. R. de Waal Malefyt (DNAX Research Institute, Palo Alto, CA). CFSE was purchased from Molecular Probes (Eugene, OR). BrdU was purchased from Sigma-Aldrich (St. Louis, MO).

### *Cells*

Normal human spleens were obtained from trauma victims undergoing routine splenectomies (Royal Prince Alfred Hospital, Sydney, Australia) or from organ donors (Australian Red Cross Blood Service, Sydney, Australia). Mononuclear cells were prepared as previously described (28, 34) and cryopreserved in liquid nitrogen until required. Total human B cells (>98% $CD19^+$) were isolated from splenic mononuclear cells using CD19 DYNAbeads (Dynal, Oslo, Norway) (28, 34). These cells were further fractionated into naive ($CD27^-$) and memory ($CD27^+$) (35, 36) populations using CD27 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) or by cell sorting using a $FACStar^{Plus}$ (BD Biosciences, San Jose, CA) after labeling with PE-anti-CD27 mAb and collecting $CD27^-$ and $CD27^+$ B cells. The recovered naive ($CD27^-$) B cells were uniformly $IgM^+IgD^{high}$ (>98%) and contained <2% $IgG^+$ and $IgA^+$ cells, whereas memory ($CD27^+$) B cells were heterogeneous for isotype expression, containing IgM-expressing as well as isotype-switched B cells (34–36).

### *CFSE labeling and B cell cultures*

Purified naive and memory B cells were labeled with CFSE (23, 37) and cultured in 48-well plates ($4 \times 10^5$/ml; BD Labware, Franklin Lakes, NJ) with CD40L only (final membrane dilution, 1/250) or with IL-2 (50 U/ml) and/or IL-10 (100 U/ml) for 5 days. For reculture experiments, B cells were cultured for 4 days with CD40L, IL-2, and IL-10, harvested, and washed thoroughly with PBS. The cells were recultured with IL-2 and IL-10 in the absence or presence of CD40L for an additional 4 days (17). A known number of CaliBRITE beads (BD Biosciences) were added to culture wells before harvesting, and the number of viable B cells in each culture condition was calculated as a function of the ratio of beads to live cells (24, 26, 27). B cells were cultured in RPMI 1640 containing L-glutamine (Life Technologies, Grand Island, NY), 10% FCS (Life Technologies), 10 mM HEPES, pH 7.4 (Sigma-Aldrich), 0.1 mM nonessential amino acid solution (Sigma-Aldrich), 1 mM sodium pyruvate (Life Technologies), 60 μg/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml apo-transferrin (Sigma-Aldrich). All cultures were conducted at 37°C in a humidified atmosphere containing 5% $CO_2$.

### *Immunofluorescent staining*

In vitro-activated splenic B cells were harvested from culture wells and nonspecific binding sites blocked by preincubation with normal mouse IgG. Cells were then incubated on ice for 20 min with PE-anti-CD38 mAb (Caltag) and were analyzed on a FACScan flow cytometer using CellQuest software (BD Biosciences). For determining expression of active caspase-3, cells were initially labeled with biotinylated anti-CD38 mAb followed by Streptavidin-Tricolor (Caltag), fixed for 10 min with 1% formaldehyde, diluted with an equal volume of PBS containing Tween 20 (final concentration, 0.1%; ICN Pharmaceuticals, Costa Mesa, CA), and incubated overnight. The cells were then incubated with PE-anti-caspase-3 Ab. $CD38^-$ and $CD38^+$ B cells in the same divisions that were present in both the live and dead populations, as defined by scatter plots, were analyzed. Expression of intracellular Ig was similarly determined by labeling fixed and permeabilized cells with anti-IgM, IgG, or anti-Ig κ L chain mAb and analyzing $CD38^-$ and $CD38^+$ B cells.

### *BrdU analysis*

Cell cultures were pulsed with 100 μg/ml BrdU for 4–8 h, harvested, labeled with biotinylated anti-CD38 mAb and Streptavidin-Tricolor, and then fixed and permeabilized as described above. DNase I (Roche, Castle Hill, New South Wales, Australia; 50 μg/ml, prepared in 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 100 μg/ml BSA) was added at 37°C for 30 min before incubating with PE-anti-BrdU mAb (24, 25). Cultures that had not been pulsed with BrdU were similarly treated to ascertain the background binding of the specific mAb.

### *Analysis of Ig secretion*

CFSE-labeled memory B cells were cultured with CD40L, IL-2, and IL-10 for 4 days, washed, and then recultured with IL-2 and IL-10 in the presence or absence of CD40L. After 3 days, the cells were harvested and incubated with PE-conjugated anti-CD38 mAb. Activated B cells were sorted into ELISPOT plates (Multiscreen-HA plates; Millipore, Bedford, MA) that had been precoated with goat anti-human IgM, IgG, or IgA polyclonal antisera. B cells were incubated for 4 h at 37°C, after which the wells were washed three times with PBS-Tween 20, before addition of biotinylated anti-human IgG, IgA, or IgM anti-sera followed by streptavidin-conjugated alkaline phosphatase (Amersham Pharmacia, Castle Hill, New South Wales, Australia). Captured cells were visualized and enumerated after the addition of nitroblue tetrazolium/bromochloroindophenol.

After 4 days, supernatants were collected and the levels of Ig were determined. Ninety-six-well microtiter plates (Dynex Technologies, Chantilly, VA) were precoated with goat anti-human IgM, IgG, or IgA polyclonal antisera, and nonspecific binding sites were blocked with 2% FCS prepared in PBS. Culture supernatants and Ig standards were added to the wells and incubated for 2 h at 37° before the addition of biotinylated anti-human IgG, IgA, or IgM antisera. Bound Ab was detected by the addition of streptavidin-conjugated HRP (Amersham Pharmacia) and visualized with ABTS (Sigma-Aldrich; 1 mg/ml) prepared in citrate buffer (pH 4.5) containing 0.03% $H_2O_2$.

## Results

### *IL-10 promotes CD40L-stimulated memory, but not naive, B cells to differentiate into ISCs*

The role of continual stimulation with CD40L in inducing B cell differentiation was examined in cultures of naive and memory B cells distinguished on the basis of CD27 expression (35, 36). Commitment to the ISC lineage was initially assessed by acquisition of CD38, which as a marker for human bone marrow plasma cells (20, 21) has been used extensively to monitor in vitro generation of plasma cells from human GCs or memory B cells (17–19, 38–41). After stimulation with CD40L and IL-10, with or without IL-2, human $CD27^+$ memory B cells yielded 10–40 times more $CD38^+$ B cells than did $CD27^-$ naive B cells cultured under the same conditions or than did memory B cells stimulated with CD40L alone (Table I). Consistent with this finding was the demonstration of 5- to 30-fold more IgM, IgG, and IgA secreted by memory compared with naive B cells under these stimulation conditions (Table I). The $CD38^+$ cells generated in these cultures displayed reduced expression of CD20, but maintained high levels of CD39 (data not shown), thereby distinguishing them from GC B cells (17, 42).

A quantitative fluorescent division tracking method using CFSE (23–28) was used to examine proliferation and generation of $CD38^+$ B cells in response to T cell-derived factors within these cultures. Typically, memory B cells underwent greater proliferation than did naive B cells exposed to the same stimulus, as shown by the appearance of fewer undivided memory B cells (i.e., cells in division 0) and a greater proportion of cells reaching later divisions (Fig. 1, *a–d* and *f–i*). IL-10 enhanced proliferation of both naive

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Table I. *Effect of T cell-dependent activation on proliferation, differentiation, and Ig secretion by naive and memory B cells*[a]

| | Culture | Naive B Cells | Memory B Cells |
|---|---|---|---|
| Cell number (×10$^5$) | CD40L | 1.26 ± 0.31 | 2.17 ± 0.37 |
| | CD40L, IL-10 | 2.3 ± 0.46 | 4.0 ± 0.6 |
| | CD40L, IL-10, IL-2 | 2.7 ± 0.63 | 6.3 ± 0.72 |
| % CD38$^+$ B cells | CD40L | 3.5 ± 0.8 | 3.4 ± 0.7 |
| | CD40L, IL-10 | 2.9 ± 0.6 | 14.2 ± 1.3 |
| | CD40L, IL-10, IL-2 | 4.4 ± 0.7 | 25.5 ± 1.9 |
| CD38$^+$ B cells (×10$^4$) | CD40L | 0.4 ± 0.12 | 0.76 ± 0.17 |
| | CD40L, IL-10 | 0.5 ± 0.10 | 5.7 ± 0.94 |
| | CD40L, IL-10, IL-2 | 1.05 ± 0.35 | 16.7 ± 2.37 |
| IgM secretion (μg/mL) | CD40L | 0.34 ± 0.21 | 3.3 ± 1.6 |
| | CD40L, IL-10 | 2.29 ± 1.0 | 44.5 ± 15.5 |
| | CD40L, IL-10, IL-2 | 6.8 ± 4.2 | 32.3 ± 12.4 |
| IgG secretion (μg/mL) | CD40L | <0.05 | 0.08 ± 0.03 |
| | CD40L, IL-10 | 0.08 ± 0.06 | 2.65 ± 1.0 |
| | CD40L, IL-10, IL-2 | 0.5 ± 0.4 | 7.0 ± 2.9 |
| IgA secretion (μg/mL) | CD40L | <0.05 | 0.3 ± 0.3 |
| | CD40L, IL-10 | 0.54 ± 0.4 | 17.6 ± 7.5 |
| | CD40L, IL-10, IL-2 | 3.1 ± 2.7 | 27.6 ± 14.1 |

[a] Naive and memory B cells (2 × 10$^5$) were cultured for 5 days with CD40L alone or in the presence of IL-10 or IL-10 and IL-2. After this time, the absolute number of cells, as well as the percentage of CD38$^+$ B cells, was determined. The number of CD38$^+$ B cells was calculated by multiplying total cell number by the frequency of CD38$^+$ cells. The values represent the mean ± SEM of 11 experiments using B cells from six different donor spleens. Levels of Ig present in culture supernatants were determined by Ig H chain specific immunoassays; values represent the mean ± SEM of three to five experiments using naive and memory B cells purified from different donor spleens.

and memory B cells stimulated with CD40L, whereas IL-2 selectively increased expansion of memory B cells (Fig. 1, *a* and *f*; Table I). Consistent with these findings, the number of cells recovered from cultures of memory B cells exceeded that of naive B cells irrespective of the culture conditions (Table I).

The relationship between appearance of CD38$^+$ cells and division number was then quantitated, because several lymphocyte differentiation events, such as Ig isotype switching by naive B cells and expression of different cytokines by naive murine T cells, are known to increase in frequency with successive cell divisions (23–32). Memory B cells clearly gave rise to CD38$^+$ B cells at a much greater rate per division than did naive B cells (Fig. 1, *b–e* and *g–j*). This trend was potently enhanced by IL-10, as indicated by the fact that 20–50% of memory B cells that had undergone three or more divisions after culture with CD40L and IL-10 became CD38$^+$ (Fig. 1, *g–j*). Although IL-2 caused a 2- to 3-fold increase

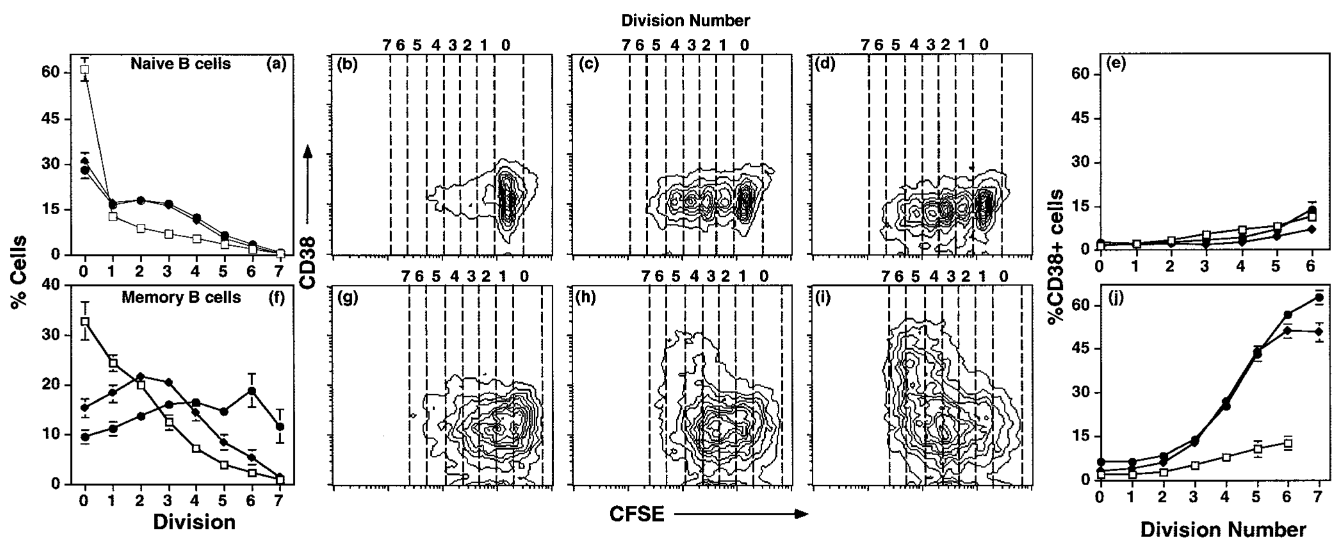


**FIGURE 1.** Distinct proliferative and differentiation responses of human naive and memory B cells. Purified, CFSE-labeled naive (*a–e*) and memory (*f–j*) B cells were cultured for 5 days. The percentages of naive (*a*) and memory (*f*) B cells in each division present in cultures stimulated with CD40L alone (□), CD40L and IL-10 (♦), or CD40L, IL-2, and IL-10 (●) was determined by division slicing. The expression of CD38 by CFSE-labeled naive and memory B cells stimulated with CD40L (*b* and *g*), CD40L and IL-10 (*c* and *h*), or CD40L, IL-2, and IL-10 (*d* and *i*) was determined by labeling with anti-CD38 mAb, followed by flow cytometric analysis. The contour plots are representative of at least ten independent experiments. The percentage of CD38$^+$ B cells in each division of naïve (*e*) or memory (*j*) B cells after culture with CD40L alone (□), CD40L and IL-10 (♦), or CD40L, IL-2, and IL-10 (●) was determined by division slicing. Each graph point represents the mean ± SEM of 11–15 experiments using cells purified from six different donor spleens.

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in the proportion and absolute number of CD38$^+$ B cells in memory B cell cultures, as well as in the amount of secreted Ig (Table I), it did not have a large effect on the rate of differentiation when measured per division (Fig. 1*j*).

*Removing CD40L has distinct effects on the generation and survival of CD38$^-$ and CD38$^+$ B cells*

Previous studies had suggested that B cells stimulated with CD40L, IL-2, and IL-10 were maintained in an undifferentiated ("memory") state until CD40L was removed, when differentiation into CD38$^{+/+}$CD20$^{+/-}$ plasma cells occurred (17–19). However, according to the data shown in Table I and Fig. 1, generation of CD38$^+$ B cells, as well as Ig secretion, clearly occurred in cultures from which CD40L had not been removed. In an attempt to resolve this apparent discrepancy, the two-step culture protocol used in previous experiments by other investigators (17–19) was combined with the CFSE-based quantitative technique designed to monitor division-linked differentiation in terms of both absolute and relative cell numbers. CFSE-labeled memory B cells were cultured with CD40L, IL-2, and IL-10 for 4 days, when the proportion and absolute number of CD38$^-$ and CD38$^+$ B cells were determined. Cells were then recultured for 4 days with IL-2 and IL-10 in the absence or presence of CD40L, and the characteristics of CD38$^+$ and CD38$^-$ B cells were assessed.

The greatest proportion of CD38$^+$ cells was generated in secondary cultures containing IL-2 and IL-10 only (Fig. 2*a*), in agreement with the previous findings (17–19). However, when total number rather than percentage of cells was calculated, 2-fold more CD38$^+$ B cells was now observed in the presence, compared with the absence, of CD40L (Fig. 2*b*). For CD38$^-$ B cells, the situation was different with the total number, as well as the proportion, of cells being greatly increased in cultures containing CD40L (Fig. 2, *e* and *f*). By comparing cell numbers at the beginning and end of secondary cultures, a net increase (2.5- to 5-fold) in CD38$^+$ B cells was observed irrespective of the presence of CD40L (Fig. 2*b*). By contrast, there was a net loss of CD38$^-$ B cells after CD40L withdrawal (Fig. 2*f*). This loss was strikingly evident in contour plots of CFSE vs CD38 expression on B cells obtained from primary and secondary cultures. After primary culture, ~10% of cells expressed CD38 (Fig. 3*a*). CD40L preserved the population of CD38$^-$ B cells present in the later divisions (Fig. 3*b*), whereas its withdrawal resulted in a reduction in the proportion of these cells and a concomitant increase in the proportion of CD38$^+$ B cells (Fig. 3*c*). Therefore, CD40L does not inhibit the generation of CD38$^+$ B cells and, in addition, plays an important role in maintaining CD38$^-$ B cell blasts.

*CD38$^+$ B cells acquire CD40L-independent survival and growth characteristics*

The relative increase in CD38$^+$ and loss of CD38$^-$ B cells in the absence of CD40L could result from one or more mechanisms. First, CD38$^+$ B cells may increase in number by proliferating more than CD38$^-$ B cells. Second, CD38$^-$ B cells may have been more prone to apoptosis. Third, CD38$^-$ B cells may have rapidly differentiated into CD38$^+$ cells (17–19). Each of these possibilities was then examined in turn. By comparing CFSE intensities of CD38$^+$ B cells in primary and secondary cultures, it was apparent that proliferation of these cells during secondary culture was similar irrespective of the presence of CD40L (Fig. 3). In contrast, CD38$^-$ B cells showed little evidence of proliferation when recultured without CD40L (compare Fig. 3, *b* and *c*). To assess the proliferative characteristics of CD38$^-$ and CD38$^+$ B cells in more detail, incorporation of BrdU after a short exposure was measured. Both populations incorporated BrdU after primary culture, but the percentage of BrdU$^+$CD38$^+$ B cells was ~2-fold greater than that of BrdU$^+$CD38$^-$ B cells (Fig. 2, *c* and *g*), confirming CD38$^+$ B cells to be a more actively dividing population (mechanism 1). Furthermore, although reculture with CD40L sustained proliferation of both CD38$^-$ and CD38$^+$ populations, removal of CD40L resulted in a much greater reduction in the percentage of

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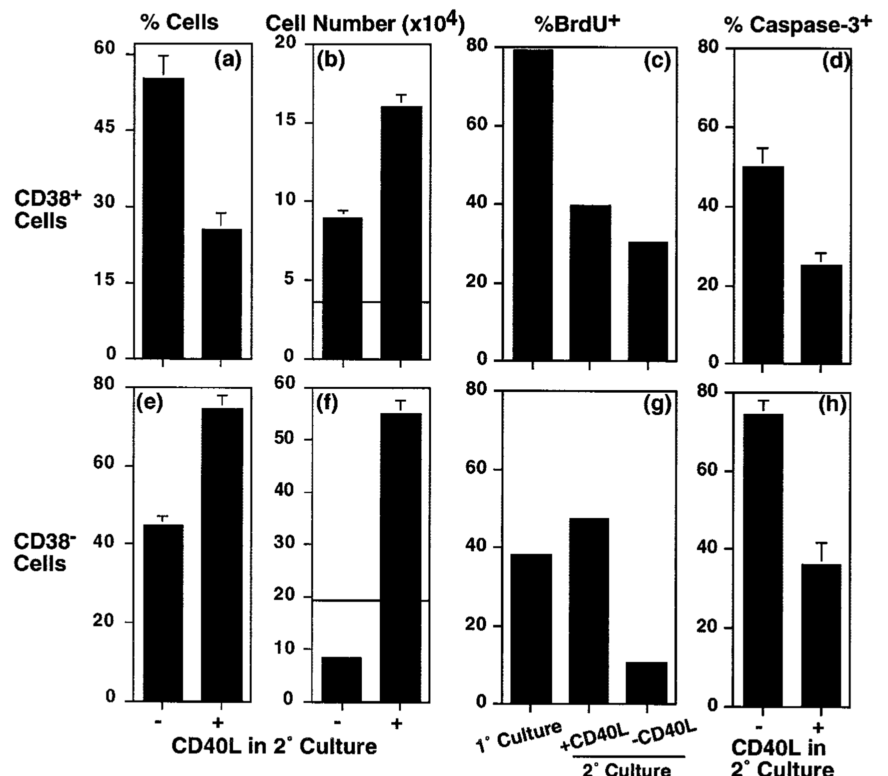


**FIGURE 2.** Acquisition of enhanced survival and proliferation characteristics by in vitro-generated CD38$^+$ B cells. CFSE-labeled memory B cells were cultured with CD40L, IL-2, and IL-10 for 4 days, and then they were washed and placed into secondary (2°) culture with IL-2 and IL-10 in the absence (−) or presence (+) of CD40L, and the characteristics of CD38$^+$ (*a–d*) and CD38$^-$ (e–*h*) B cells were determined. The overall percentage (*a* and *e*) and total number (*b* and *f*) of these B cells, as well as the percentage expressing active caspase-3 (*d* and *h*) were determined after an additional 4 days. For active caspase-3 expression, both live and dead CD38$^+$ and CD38$^-$ B cells (determined by scatter plots) were analyzed. Each value represents the mean ± SEM of five or six independent experiments. The horizontal lines in *b* and *f* indicate the number of CD38$^+$ and CD38$^-$ B cells present at the beginning of the secondary culture. The incorporation of BrdU by CD38$^+$ (*c*) and CD38$^-$ (*g*) B cells after the primary (1°) culture, and a 2-day 2° culture with IL-2 and IL-10 in the presence or absence of CD40L was determined by gating on CD38$^-$ and CD38$^+$ cells. These results are representative of two separate experiments.

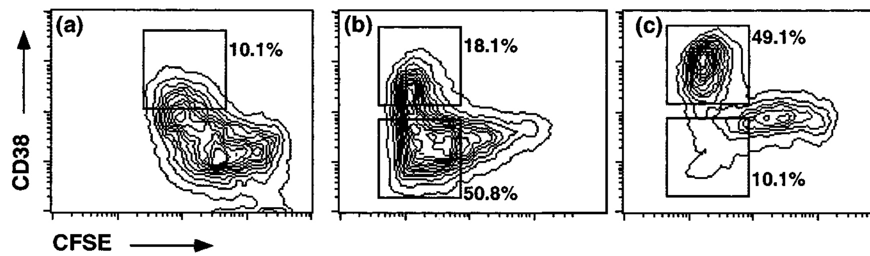


**FIGURE 3.** Proliferating CD38$^-$ memory B cells are preferentially lost from culture after withdrawal of CD40L. Populations of activated memory B cells defined by CFSE dilution and CD38 expression were determined at the end of the primary culture with CD40L, IL-2, and IL-10 (*a*), and after a 4-day secondary culture with IL-2 and IL-10 in the presence (*b*) and absence (*c*) of CD40L. The values represent the percentage of cells that were CD38$^+$ and the percentage of cells with the same division history that remained CD38$^-$. The contour plots are representative of six independent experiments.

BrdU$^+$CD38$^-$, compared with CD38$^+$ B cells (Fig. 2, *c* and *g*), consistent with the CFSE profiles of the two populations shown in Fig. 3. To investigate whether the selective loss of CD38$^-$ B cells in the absence of CD40L (Fig. 2*f*) resulted from cell death (mechanism 2), apoptosis was quantified by assessing acquisition of active caspase-3 (43). No significant difference in the proportion of apoptotic CD38$^-$ and CD38$^+$ B cells was detected after reculture with CD40L ($p > 0.05$; Fig. 2, *d* and *h*). In contrast, removal of CD40L was associated with a greater increase in apoptotic CD38$^-$ vs CD38$^+$ B cells ($p < 0.001$; Fig. 2, *d* and *h*). Thus, during the differentiation process, CD38$^+$ B cells acquire the capacity to both proliferate and survive without continued stimulation by CD40L, whereas CD38$^-$ B cell blasts require sustained T cell help for their expansion and survival.

The increase in number of CD38$^+$ B cells after removal of CD40L (Fig. 2*b*) may also result from differentiation of CD38$^-$ blasts into CD38$^+$ B cells (mechanism 3). To investigate this, memory B cells were cultured with CD40L, IL-2, and IL-10, and CD38$^-$ B cells present in later divisions were then isolated by cell sorting (Fig. 4, *a* and *b*) and recultured with IL-2 and IL-10 in the absence or presence of CD40L. As shown in Fig. 4, *c* and *d*, after a 2-day reculture, 38.1% and 17.0% of cells harvested from these cultures were CD38$^+$ in the absence and presence of CD40L, respectively, consistent with the notion that the CD38$^-$ B cell population

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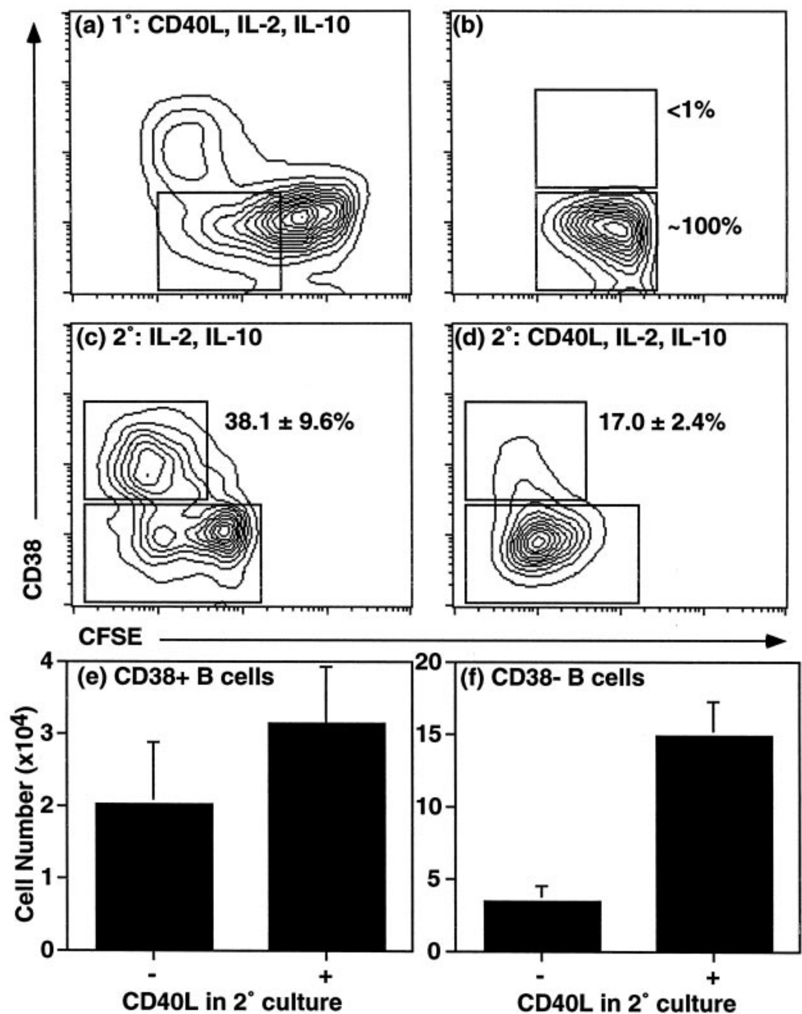


**FIGURE 4.** CD38$^-$ B cells contain precursors of CD38$^+$ B cells. *a*, CFSE-labeled memory B cells were cultured with CD40L, IL-2, and IL-10 for 5 days. The population of divided CD38$^-$ B cells was then isolated by sorting using the indicated region. Contour plots of CFSE vs CD38 expression were determined immediately after sorting (*b*) or after 2 days of secondary culture with IL-2 and IL-10 in the absence (*c*) or presence (*d*) of CD40L. The values represent the percentage of CD38$^+$ B cells present at the beginning (*b*) and end (*c* and *d*) of the secondary cultures. The absolute numbers of CD38$^+$ (*e*) and CD38$^-$ (*f*) B cells present after the secondary culture were determined. For these experiments, the average number of cells recultured after sorting was $1.7 \times 10^5$. The values represent the mean ± SEM of four independent experiments using memory B cells isolated from different donors.

contains precursors of $CD38^+$ cells. Importantly, the absolute number of $CD38^+$ B cells generated in these secondary cultures (Fig. 4*e*), as well as the extent of their proliferation (indicated by reduced CFSE intensity; compare Fig. 4, *c* and *d*) was similar irrespective of the presence of CD40L. This reinforces the notion that CD40L does not prevent the generation of $CD38^+$ cells. Moreover, no loss of CD38 occurred during secondary culture of sorted $CD38^+$ cells (data not shown). In contrast, there were 5-fold fewer $CD38^-$ B cells recovered in the absence, compared with the presence, of CD40L (Fig. 4*f*), which was consistent with the data shown in Fig. 2. Furthermore, the isolated $CD38^-$ B cells underwent limited proliferation in the absence of CD40L, whereas CD40L promoted continued division of these cells (Fig. 4, *b–d*). Taken together, these results confirm that, although the relative proportion of $CD38^+$ B cells increases when CD40L is removed, CD40L does not inhibit the generation of these cells, but rather is required for survival of $CD38^-$ B cells. Consequently, continued proliferation of $CD38^-$ B cells in secondary cultures containing CD40L would also indicate that it is unlikely that $CD38^+$ B cells are generated due to CD40L being consumed in these cultures. The preferential survival of $CD38^+$ B cells in the absence of CD40L is therefore due to a combination of greater proliferation by the $CD38^+$ cells per se (mechanism 1), enhanced apoptosis on the part of the $CD38^-$ subset (mechanism 2), and differentiation of some $CD38^-$ blasts into $CD38^+$ B cells (mechanism 3).

### *Ig secretion increases with cell division, however CD38 does not exclusively identify ISCs*

Traditionally, plasma cells have been identified in vivo by a $CD38^{++}CD20^{\pm}$ phenotype, expression of intracellular Ig, and spontaneous production of Ig (20, 21, 44–46). Accordingly, many studies on the generation of $CD38^+$ B cells from GC or memory B cells in vitro have led to the suggestion that a direct correlation exists between increased numbers of $CD38^+$ "plasma" cells and the appearance of increased levels of secreted Ig (17, 38–41). Therefore, it has been assumed to be appropriate to equate in vitro-derived $CD38^+$ B cells with plasma cells. However, those previous studies did not compare Ig secretion by in vitro-generated $CD38^+$ B cells with that by $CD38^-$ B cells present in the same cultures to ascertain whether Ig secretion is indeed restricted to $CD38^+$ B cells. To do so, we extended the findings shown in Table I and Fig. 1 by examining the ability of activated B cells defined by division history and CD38 expression to secrete Ig. Memory B cells stimulated in secondary cultures with CD40L and cytokines were sorted into early divisions (population 1) and late divisions comprising either $CD38^-$ (population 2) or $CD38^+$ B cells (population 3; Fig. 5*a*). Undivided B cells or B cells that had undergone only a few divisions (population 1) secreted very little Ig (<5% were ISCs in ELISPOT assays; Fig. 5*b*). However, B cells sorted from later divisions produced significantly more Ig than did B cells in population 1, demonstrating that the frequency of ISCs increased with division number. Surprisingly, the overall frequency of ISCs in $CD38^-$ (population 2) and $CD38^+$ (population 3) populations of divided B cells was similar, although the relative proportion of cells from population 3 secreting IgG and IgA appeared to increase compared with population 2 (Fig. 5*b*). A similar trend was observed when Ig secretion by populations of sorted B cells was measured in the supernatants of secondary cultures containing CD40L, IL-2 and IL-10. Thus, although B cells corresponding to populations 2 and 3 secreted comparable amounts of IgM, 2.5-fold more IgG and IgA were detected in supernatants of $CD38^+$ B cells (population 3) compared with $CD38^-$ B cells (population 2; Fig. 5*c*). These results were also obtained when secondary cultures were performed in the presence of the cytokines only (data not shown).

Expression of intracellular Ig by the different populations of activated B cells followed a similar pattern. Thus, there was a significant increase in the level of intracellular IgM, IgG, and Ig $\kappa$ L chain expressed by B cells as they differentiated from population 1 into population 2 and then 3 (Fig. 6) when measured in terms of mean fluorescence intensity (MFI). Moreover, a significantly higher proportion of $CD38^+$ B cells (population 3) expressed intracellular IgG and Ig $\kappa$ L chain than did $CD38^-$ B cells in populations 1 and 2 (Table II). The increase in expression of intracellular Ig by B cells as they underwent division and differentiation to express CD38 (Fig. 6) paralleled the increase in Ig secreted by these different populations of B cells (Fig. 5), thereby confirming the important role of cell division in differentiation of ISCs from memory B cells and demonstrating that CD38 expression does not exclusively identify ISCs.

### *CD40L does not inhibit Ig secretion by stimulated B cells*

The experiments described so far indicated that withdrawal of CD40L is not a critical factor leading to differentiation of $CD38^+$ "plasma" cells from $CD38^-$ precursors (Figs. 2 and 4). However, because CD38 expression did not appear to be an absolute marker

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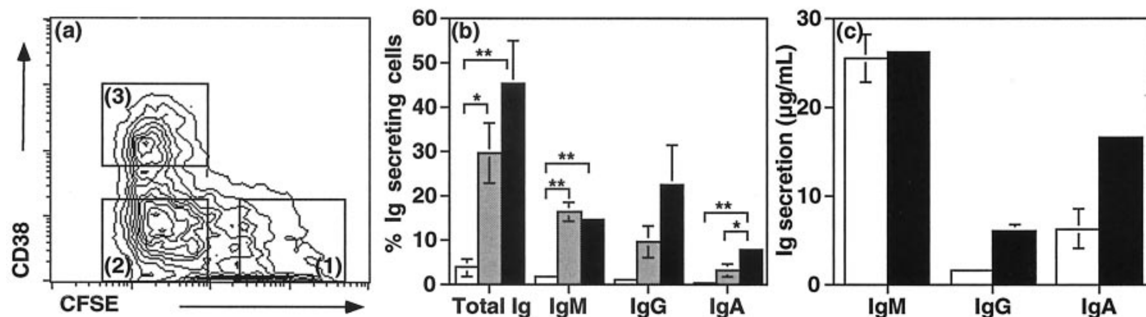


**FIGURE 5.** Ig secretion increases with cell division. CFSE-labeled memory B cells were cultured with CD40L, IL-2, and IL-10 for 4–5 days and then were washed and recultured for an additional 3 days under the same conditions. *a*, B cell populations defined by division history and expression of CD38. Population 1, Undivided B cells or B cells in early divisions; population 2, B cells in late divisions that were $CD38^-$; population 3, B cells in late divisions that were $CD38^+$. *b*, B cells in populations 1 (open bars), 2 (gray bars), and 3 (filled bars) were sorted into quadruplicate wells of microtiter plates precoated with Ig H chain specific Ab to determine the proportion of cells secreting total Ig, IgM, IgG, or IgA by ELISPOT assay. Each value represents the mean ± SEM of three experiments using memory B cells from different donors. *, $p < 0.05$; **, $p < 0.01$. *c*, After 5 days of culture with CD40L, IL-2, and IL-10, memory B cells were sorted into populations 2 (open bars) and 3 (filled bars) and were recultured for an additional 2 days with CD40L, IL-2, and IL-10. Secretion of IgM, IgG, and IgA was then determined by Ig H chain specific immunoassays. The values are the means of duplicate cultures and represent three to four different experiments.

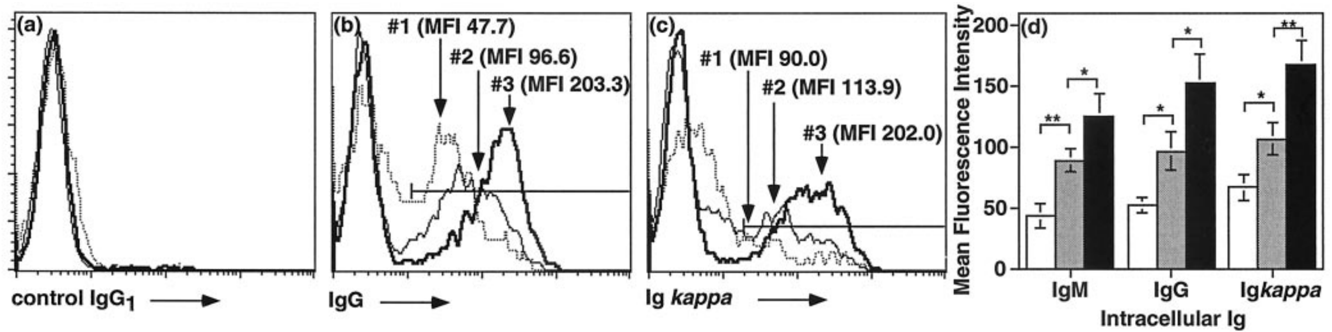


**FIGURE 6.** Accumulation of intracellular Ig by CD38$^+$ B cells. CFSE-labeled memory B cells were cultured with CD40L, IL-2, and IL-10 for 4 days and then were washed and recultured under the same conditions. Expression of intracellular IgM, IgG, and Ig $\kappa$ L chain by B cells was determined after 4 days of secondary culture by intracellular staining and flow cytometric analysis. *a–c*, Histograms of cells present in populations 1 (dotted line), 2 (solid line), and 3 (bold line) incubated with an isotype control (*a*), anti-human IgG (*b*), or anti-human Ig $\kappa$ L chain mAb (*c*). The values represent the MFI of the positive population of B cells present in this experiment (representative of six independent experiments), defined by the indicated marker. *d*, MFI of IgM, IgG, and Ig $\kappa$ L chain expressed by positive memory B cells in populations 1 (open bars), 2 (gray bars), and 3 (filled bars). The MFI was calculated according to the histograms presented in *b* and *c*. Each value represents the mean ± SEM of six independent experiments. *, $p < 0.05$; **, $p < 0.01$.

for ISCs (Fig. 5), it remained possible that removing CD40L might lead to an increase in the number of ISCs, if not CD38$^+$ B cells. To investigate this possibility, the amount of secreted Ig present in supernatants of 4-day secondary cultures was measured because such an approach makes no assumptions regarding a phenotype of ISCs. The prediction was that if CD40L does indeed maintain activated B cells in an undifferentiated (memory) state (17–19), a reduction in total Ig secretion would be observed in the presence of CD40L. When the cultures were performed without CD40L, large amounts of IgM, IgG, and IgA were produced (Table III). After addition of CD40L, no decrease in Ig secretion was observed. On the contrary, an increase in each isotype was detected (Table III), consistent with our previous conclusion that withdrawal of CD40L does not trigger differentiation to ISCs.

Ig secretion by B cells sorted into populations 2 and 3 (Fig. 5*a*) and placed in secondary cultures containing IL-2 and IL-10 with or without CD40L was also determined by ELISPOT. In the presence of CD40L, 29.9 ± 6.7% of CD38$^-$ population 2 B cells and 45.5 ± 9.5% of CD38$^+$ population 3 cells were found to secrete Ig (Fig. 5*b*). When secondary cultures lacked CD40L, 25.1 ± 1.8% and 31.9 ± 2.0% (mean ± SEM, $n = 3$) of population 2 and 3 B cells, respectively, secreted Ig, thereby demonstrating that CD40L does not inhibit Ig secretion on a per cell basis.

## Discussion

Understanding the mechanisms of lymphocyte differentiation is of vital importance to immune regulation. Here, we applied quantitative analytical methods to investigate the differentiation of activated human B cells into ISCs, with a particular emphasis on the role played by CD40L in this process. As previously reported (7, 8, 18), CD27$^+$ memory B cells differentiated at a much greater rate than did CD27$^-$ naive B cells into CD38$^+$ cells (Table I; Fig. 1), which are usually considered to be synonymous with plasma cells (17, 38–41). When differentiation of CD40L-activated memory B cells was studied in relation to cell division, an increasing proportion of these cells was shown to differentiate at each division to become rapidly proliferating CD38$^+$ B cells (Fig. 1). By linking differentiation to cell division, it became feasible to characterize the distinct effects of IL-10 and IL-2 on CD40L-activated memory B cells. IL-10 was clearly a differentiation factor for memory B cells, because the number of CD38$^+$ B cells generated per division in its absence was low. In contrast, IL-2 appeared to be a growth factor for activated memory cells irrespective of whether they expressed CD38 or not. Thus, although it did not affect the division-based rate of differentiation, IL-2 did increase B cell recovery (Table I; Fig. 1).

The signals required for sustaining division and survival changed once memory B cells had differentiated into CD38$^+$ B cells. In particular, removal of CD40L did not prevent expansion of CD38$^+$ B cells (Figs. 2–4), although it did arrest proliferation of the CD38$^-$ population. Consequently, CD38$^+$ B cells rapidly became the dominant ISCs after withdrawal of CD40L (Figs. 2 and 4). In contrast, <50% of CD38$^+$ B cells secrete Ig (Fig. 5). When coupled with their high proliferation rate, it would appear that in vitro-generated CD38$^+$ cells represent precursors of terminally

Table II. *Expression of intracellular Ig increases with cell division and differentiation*[a]

| B Cells | Expression of Intracellular Ig (MFI) | |
|---|---|---|
| | % IgG$^+$ | % Ig $\kappa$ L chain$^+$ |
| Population 1 | 32.8 ± 4.7 | 23.3 ± 2.5 |
| Population 2 | 41.1 ± 6.1 | 42.0 ± 2.5 (**)[b] |
| Population 3 | 55.9 ± 5.8 (*)[c](**)[d] | 53.5 ± 1.6 (**)[c](**)[d] |

[a] CFSE-labelled memory B cells were cultured for 4 days with CD40L, IL-2, and IL-10, and were harvested, washed, and recultured under the same conditions. The percentage of B cells corresponding to populations 1, 2, and 3 (see Fig. 5*a*) expressing intracellular Ig was determined by labeling the cells with anti-CD38 mAb, fixing, and permeabilizing the cells and then labeling with mAb specific for IgG or Ig $\kappa$. The values represent MFI ± SEM of six different experiments.
[b] Level of significance between expression by populations 1 and 2.
[c] Level of significance between expression by populations 2 and 3.
[d] Level of significance between expression by populations 1 and 3.
*, $p < 0.01$; **, $p < 0.001$.

Table III. *Effect of CD40L on the secretion of Ig by recultured memory human B cells*[a]

| | 2° Culture | Ig Secretion (μg/mL) | | |
|---|---|---|---|---|
| | | IgM | IgG | IgA |
| Expt. 1 | IL-2, IL-10 | 9.2 | 7.3 | 25.3 |
| | CD40L, IL-2, IL-10 | 30.2 | 28.1 | 48.8 |
| Expt. 2 | IL-2, IL-10 | 69.7 | 14.8 | 27.0 |
| | CD40L, IL-2, IL-10 | 75.2 | 14.1 | 31.6 |
| Expt. 3 | IL-2, IL-10 | 22.6 | 2.9 | 31.4 |
| | CD40L, IL-2, IL-10 | 31.1 | 5.0 | 41.5 |
| Expt. 4 | IL-2, IL-10 | 32.7 | 3.7 | 10.9 |
| | CD40L, IL-2, IL-10 | 88.3 | 6.7 | 24.0 |

[a] Human memory B cells were cultured for 4 days with CD40L, IL-2, and IL-10. The cells were then harvested, washed, and recultured with IL-2 and IL-10 in either the absence or presence of CD40L for an additional 4 days. After this time, the levels of secreted IgM, IgG, and IgA present in the culture supernatants were determined by Ig heavy chain specific immunoassays.

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differentiated plasma cells. The proportion of cells within this population that secrete Ig would be expected to increase with further differentiation, consistent with the recent finding that plasmablasts must first exit the cell cycle for terminal differentiation into nondividing high-rate Ig-secreting plasma cells to occur (47).

At face value, our findings do not support the hypothesis that removal of CD40L is the molecular trigger for facilitating plasma cell generation from memory B cells (17–19, 22). Theoretically, CD40L could have been consumed in our cultures at a rate equal to its removal from the cultures reported in earlier studies. However, such an explanation can be excluded on the grounds that the absolute number of $CD38^+$ B cells and ISCs generated in secondary cultures, as well as the level of Ig secreted, was always greater in the presence of CD40L (Figs. 2 and 4; Table III). Furthermore, because $CD38^-$ B cells require CD40L to survive, a reduction in the levels of CD40L during in vitro culture due to consumption would be expected to compromise survival of these cells. However, because large numbers of viable $CD38^-$ B cells were generated in the presence of CD40L (Fig. 2, *f* and *h*), this did not appear to occur. Thus, the increased proportion, as opposed to absolute numbers, of $CD38^+$ B cells observed after removal of CD40L (Figs. 2 and 4) (17–19) is likely to have resulted from a combination of the accelerated cell cycle arrest and death of $CD38^-$ B cell blasts, relatively greater proliferation of $CD38^+$ vs $CD38^-$ B cells, and differentiation of $CD38^-$ B cells to $CD38^+$ B cells (Figs. 2–4). In fact, close examination of the findings originally presented by Arpin et al. (see Fig. 1*c* of Ref. 17) reveals data very similar to ours—withdrawal of CD40L from secondary cultures caused a ~10-fold reduction in the number of $CD38^-$ $CD20^+$ B cells, but had very little effect on the number of $CD38^+$ B cells present.

The finding here that both $CD38^-$ and $CD38^+$ B cells with the same division history were ISCs (Fig. 5) also contrasted with previous data indicating that $CD38^+$ B cells contained more intracellular Ig than did $CD38^-$ B cells (17, 18). Because withdrawal of CD40L greatly compromised survival of $CD38^-$ B cell blasts (population 2; Figs. 2–4), the population of $CD38^-$ B cells examined previously may well have been largely composed of undivided B cells (population 1), which secrete and contain very little Ig (Figs. 5 and 6). Thus, comparison of secretion and expression of Ig by differentially divided B cells may explain this discrepancy. Overall, the major difference between the two models appears to be explicable in terms of alternative interpretations of similar results, combined with the use, in our case, of CFSE to resolve B cells into discrete populations based on division history rather than phenotype alone. Importantly, our interpretation receives support from other previous studies showing that B cells produced high levels of Ig in the continual presence of CD40L (41, 48) and that proliferation was necessary for generating ISCs from precursor cells (49, 50).

The detection of CD40L-independent $CD38^+$ and CD40L-dependent $CD38^-$ ISCs in vitro is novel, as is the demonstration that acquisition of CD38 is not an absolute prerequisite for Ig production. It is likely that the two populations of cells are related in a linear manner, with $CD38^-$ ISCs subsequently acquiring CD38 together with altered survival and stimulation requirements. This is consistent with the observation that isolated $CD38^-$ B cell blasts yielded $CD38^+$ B cells (Fig. 4). Alternatively, they may represent a different lineage of ISC. Additional experiments to resolve this question will require the identification of a reliable marker for the $CD38^-$ ISC population. Nevertheless, if the changing sensitivity of activated memory B cells to CD40L observed in vitro can be extrapolated to the in vivo situation, it is possible that $CD38^-$ and $CD38^+$ ISCs represent short-lived and long-lived ISCs, respectively (14, 50–53). Thus, acquisition of CD38 expression may correlate with selection into a population of T cell stimulation-independent, rapidly proliferating plasma cell precursors, which contribute to an initial expansion of the selected population of ISCs (9, 10, 47, 49, 54). The rapidly dividing $CD38^+$ ISCs presumably then acquire altered homing characteristics, resulting in their migration to sites including bone marrow (55, 56), where they undergo terminal differentiation to yield long-lived quiescent $CD38^+$ plasma cells (38, 45, 52, 53, 57, 58). In this way, Ig produced by the selected ISCs will be sustained for long periods even after Ag clearance (2, 14). In contrast, the $CD38^-$ ISCs, being CD40L dependent, will only survive and contribute Ig as long as Ag and T cell help are both available. Given that stimulation of activated T cells requires Ag presentation by memory B cells (1, 42), successful Ag clearance would act as a feedback mechanism limiting memory B cell expansion and further development of both short- and long-lived ISCs, although limited differentiation of the $CD38^+$ ISCs already formed would continue. In vivo studies have revealed that the number of Ag-specific B cells present at the conclusion of a secondary immune response is reduced 10- to 50-fold compared with the peak of the response, indicative of apoptosis of the majority of memory blasts after Ag clearance (12). The surviving memory blasts most likely revert to a quiescent state to serve as the memory cell pool, which can be reactivated upon subsequent Ag exposure (12, 15, 16, 59). Our in vitro findings that $CD38^-$ blasts undergo rapid apoptosis after removal of CD40L (Figs. 2 and 4) are in accord with these in vivo observations and suggest that a small number of surviving non-Ig-secreting $CD38^-$ blasts may contribute to this pool of long-lived memory B cells.

The findings here of an increase in differentiation of memory B cells upon consecutive cell divisions to $CD38^+$ B cells with enhanced proliferation and survival characteristics is reminiscent of other important lymphocyte differentiation events, including Ig isotype switching (23, 24, 26–28), cytokine production (25, 29, 30), and changes in expression of cell surface molecules (23, 30–32, 34). The transcription factors B lymphocyte-induced maturation protein-1 (60) and X-box-binding protein (61) facilitate development of plasma cells, while B cell-specific activator protein (62) and B cell lymphoma 6 (16, 63) repress this event. An attractive hypothesis for the features of B cell differentiation described here is that cell division alters the level of expression of key transcriptional regulators that affect the rate of differentiation, perhaps by controlling expression of a key master switch protein (11). By regulating the proportion of ISC precursors formed per division, a balance between the numbers of memory cells and plasma cells can be achieved. It is reasonable to conclude that the rate of division-linked differentiation has evolved to deal efficiently with pathogens through rapid development of short- and long-lived ISCs, while ensuring preservation and enhancement of the memory pool for protection against future exposure. Consequently, exploiting mechanisms involved in regulating the generation of ISCs and preserving memory B cells in vivo, for example by modulating IL-10, B lymphocyte-induced maturation protein-1, or bcl-6, may be advantageous for the development of vaccines designed to maximize protective humoral immunity.

## Acknowledgments

We thank Drs. Marilyn Kehry and Brian Castle for human CD40L, Dr. Rene de Waal Malefyt for human IL-10, the Australian Red Cross Blood Service for providing human spleens, Joseph Webster for assistance with cell sorting, Prof. Tony Basten for meticulous and valuable review of this manuscript, and Gabriella O'Neill for assistance with preparation of this manuscript.

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