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Intrinsic Differences in the Proliferation of Naive and Memory Human B Cells as a Mechanism for Enhanced Secondary Immune Responses[1]

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Humoral immune responses elicited after secondary exposure to immunizing Ag are characterized by robust and elevated reactivity of memory B cells that exceed those of naive B cells during the primary response. The mechanism underlying this difference in responsiveness of naive vs memory B cells remains unclear. We have quantitated the response of naive and memory human B cells after in vitro stimulation with T cell-derived stimuli. In response to stimulation with CD40 ligand alone or with IL-10, both IgM-expressing and Ig isotype-switched memory B cells entered their first division 20–30 h earlier than did naive B cells. In contrast, the time spent traversing subsequent divisions was similar. Consistent with previous studies, only memory cells differentiated to CD38$^+$ blasts in a manner that increased with consecutive division number. These differentiated CD38$^+$ B cells divided faster than did CD38$^-$ memory B cell blasts. Proliferation of CD40 ligand-stimulated naive B cells as well as both CD38$^+$ and CD38$^-$ cells present in cultures of memory B cells was increased by IL-10. In contrast, IL-2 enhanced proliferation of CD38$^-$ and CD38$^+$ memory B cell blasts, but not naive cells. Thus, memory B cells possess an intrinsic advantage over naive B cells in both the time to initiate a response and in the division-based rate of effector cell development. These differences help explain the accelerated Ab response exhibited by memory B cells after secondary challenge by an invading pathogen, a hallmark of immunological memory. ***The Journal of Immunology,*** **2003, 170: 686–694.**

Immune responses to T cell-dependent Ag occur within specialized areas of secondary lymphoid tissues, such as lymph nodes, tonsils, spleen, and mucosal-associated lymphoid tissue present in the gut. After exposure to a T cell-dependent Ag, naive B cells can differentiate into effector cells via one of two pathways. They can either rapidly differentiate into short-lived Ig-secreting cells producing low-affinity IgM or they can seed a germinal center, where high-affinity Ag-specific B cells are selected and develop into long-lived plasma cells or memory B cells (1–5). Once formed, memory B cells can re-enter the circulation (6–8) or remain as resident cells within discrete regions of secondary lymphoid tissue, such as marginal zone (MZ)[4] of spleen (1, 9, 10) or mucosal epithelium of tonsil (11). Human memory B cells present in these sites can be distinguished from naive B cells by a unique surface phenotype that characteristically includes expression of CD27 (7, 10) as well as up-regulation of costimulatory molecules CD80 and CD86 (10, 11) and a change in expression of homing receptors (12). Furthermore, different subsets of human memory B cells can be identified by up-regulation of IgM (6, 13) or expression of switched Ig isotypes (11, 13).

Immunological memory is characterized by a rapid and robust response after re-exposure to the original immunizing Ag (5), such that 10- to 100-fold more Ag-specific B cells become detectable in spleen and bone marrow of responding individuals, compared with the increase in naive B cells after primary exposure (14, 15). A number of different mechanisms have been proposed to explain this observation. The unique positioning of memory B cells in Ag-draining sites of secondary lymphoid tissue such as splenic MZ and tonsil mucosal epithelium, as well as enhanced expression of costimulatory molecules, may facilitate rapid presentation of Ag to specific T cells, which in turn would promote robust secondary humoral responses (1, 3, 10, 11, 16). More recently, work in a B cell receptor (BCR) transgenic system has led to the proposition that the cytoplasmic domain of surface IgG serves as the molecular determinant responsible for the characteristically heightened reactivity of memory B cells over naive B cells (17). This effect was manifested by enhanced survival of activated B cells, rather than by recruitment of a greater number of cells into the response or an increase in their rate of expansion (17). Thus, a number of intrinsic and extrinsic features of memory B cells appear to contribute to rapid secondary immune responses.

We have previously reported that a greater number of cells is generated from cultures of human memory B cells compared with naive B cells after in vitro culture with CD40 ligand (CD40L) alone or in the presence of IL-2 and/or IL-10 (51). Furthermore, the proportion of memory B cells that entered division always exceeded that of naive B cells. These experiments suggested that intrinsic differences in proliferative behavior of naive and memory B cells may contribute to enhanced responses of memory B cells in vivo. We have now studied the proliferative behavior of naive and memory B cells in greater detail with a view to explaining the difference in responsiveness of these cells. Memory B cells entered

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[4] Abbreviations used in this paper: MZ, marginal zone; BCR, B cell receptor; CD40L, CD40 ligand; SA-TC, streptavidin conjugated to TriColor; BrdU, 5-bromo-2′-deoxyuridine; *ttfd*, time to first division.

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division 20–30 h earlier than did naive B cells. Furthermore, although IL-10 enhanced proliferation of both CD40L-stimulated naive and memory B cells, IL-2 selectively promoted the expansion of memory B cells. Importantly, only memory B cells differentiated into $CD38^+$ effector cells, which acquired a unique proliferative behavior such that their rate of division exceeded that of $CD38^-$ memory B cell blasts and naive B cells by up to 2-fold. Thus, memory B cells possess an intrinsic advantage over naive B cells in both the time to initiate a response and the rate of generating rapidly dividing effector cells. These differences help explain the accelerated Ab response exhibited by memory B cells after secondary challenge by an invading pathogen, a hallmark of immunological memory.

Materials and Methods

Abs and reagents

Streptavidin conjugated to TriColor (SA-TC) and PE-conjugated and biotinylated anti-CD38 mAb were purchased from Caltag (Burlingame, CA). PE-conjugated anti-CD25, CD27, CD132 (IL-2Rγ), anti-5-bromo-2′-deoxyuridine (BrdU) mAb, isotype control mAb, and biotinylated anti-IgM, IgD, IgG, IgA, and IgE mAbs were purchased from BD PharMingen (San Diego, CA). FITC-conjugated anti-CD19, CD20, and anti-CD27 mAbs and PE-conjugated anti-CD19 mAb were purchased from BD Biosciences (San Jose, CA). IL-2 was purchased from Endogen (Woburn, MA). IL-10 was generously provided by Dr. R. de Waal Malefyt (DNAX Research Institute, Palo Alto, CA). The source of recombinant human CD40L was membranes prepared from the *Sf21* insect cell line infected with baculovirus vector containing CD40L cDNA (generously provided by Dr. M. Kehry, Boehringer Ingelheim, Ridgefield, CT) (18). CFSE was obtained from Molecular Probes (Eugene, OR) and BrdU was from Sigma-Aldrich (St. Louis, MO).

Cells

Normal human spleens were obtained from organ donors (Australian Red Cross Blood Service, Sydney, Australia). Mononuclear cells were prepared by slicing splenic tissue into small pieces and disrupting the capsule by forcing the tissue through a filter mesh. RBCs were lysed and the remaining cells were washed twice and cryopreserved in liquid nitrogen until required. Total B cells were isolated from mononuclear cells using the CD19 DYNAbead and DETACHABEAD system (Dynal Biotech, Oslo, Norway) (13, 19) according to the manufacturer's instructions. The resulting cell population was >98% $CD19^+$. Total B cells were then fractionated into naive ($CD27^-$) and memory ($CD27^+$) (7, 10) populations by incubating B cells with anti-CD27 mAb MACS beads and separating them on Mini or MidiMACS columns (Miltenyi Biotec, Bergisch Gladbach**,** Germany). Naive and memory B cells were also isolated by cell sorting using a FACStar^{Plus} (BD Biosciences) by labeling the total B cell population with PE-conjugated anti-CD27 mAb and collecting $CD27^-$ and $CD27^+$ B cells. IgM-expressing (nonswitched) and isotype-switched memory B cells were also isolated by cell sorting. Total B cells were labeled with PE-conjugated anti-CD27 mAb and a cocktail of biotinylated mAbs specific for IgG, IgA, and IgE (IgG/A/E) or IgM and IgD (IgM/D), followed by SA-TC (19). Gates were set to collect $CD27^+$IgG/A/E$^-$ (i.e., IgM/D-expressing, nonswitched) memory B cells and $CD27^+$IgM/D$^-$ (i.e., isotype-switched) memory B cells. The recovered B cell populations contained >98% of the respective subsets.

CFSE labeling

Purified human B cells were labeled with CFSE as previously described (13, 19–21). B cells were resuspended at 1×10^7/ml in PBS containing 0.1% BSA. CFSE, dissolved in DMSO, was added at a final concentration of 5 μM. The cells were vortexed for 10 s and then incubated at 37°C for 10 min. Labeled cells were then washed with cold PBS containing 0.1% BSA and resuspended in culture medium (see below; *B cell cultures*).

B cell cultures

CFSE-labeled naive and memory B cells (2×10^5/500 μl/well) were cultured in 48-well plates (BD Labware, Franklin Lakes, NJ) with recombinant human CD40L alone (at a predetermined optimal dilution of the membrane preparation; 1/250) or in the presence of IL-2 (50 U/ml) and/or IL-10 (100 U/ml) for different times. In some experiments, unlabeled B cells (4×10^5/ml) were cultured in flat-bottom 96-well plates (BD Labware) with CD40L, IL-2, and IL-10. Proliferation was assessed by pulsing the cultures with [^{3}H]thymidine (ICN Pharmaceuticals, Irvine, CA) after various times of activation and harvesting 4 h later. Scintillation counting was performed on a Betaplate counter (Pharmacia-LKB, Uppsala, Sweden). All B cell cultures were performed in RPMI 1640 containing L-glutamine (Life Technologies, Grand Island, NY) and supplemented with 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). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Determining time to first division

For analysis of time of entry into the first cell division, naive and memory B cells (5×10^4/125 μl/well) were cultured in flat-bottom 96-well plates (BD Labware) in the presence or absence of the mitotic inhibitor demecolcine (Sigma-Aldrich) (22) plus human CD40L alone or in the presence of IL-2 and IL-10. After various times, the cultures were pulsed with [^{3}H]thymidine and harvested 4 h later. Flow cytometric analysis of CFSE-labeled cells confirmed that treatment with demecolcine completely inhibited all cell division (data not shown). To determine the average time to first division (*ttfd*), log-normal distributions were fitted to the data with Prism software (Graphpad Software, San Diego, CA) using the technique of least squares minimization. The equation fitted was as follows: $Y = \text{Amplitude} \times (e \wedge (-0.5 \times (\ln(T/\text{Center})/\text{Width})^2)) + \text{background}$. The best fit for the "center" parameter was taken as the average *ttfd*, whereas the amplitude was assumed to be proportional to the number of cells stimulated into division.

Immunofluorescent staining and BrdU analysis

Cultured naive and memory B cells were pulsed with 100 μg/ml BrdU for 6 h. Cells were then harvested from culture wells and nonspecific binding sites blocked by preincubating with normal mouse IgG (10 μg/ml). Cells were suspended in PBS containing 0.1% BSA and 0.1% sodium azide and fixed for 20 min with 2% paraformaldehyde (Sigma-Aldrich), diluted with an equal volume of Tween 20 (final concentration 0.1%; Sigma-Aldrich), and then incubated overnight. 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-conjugated isotype control or anti-BrdU mAb on ice for 20 min. In some experiments, the expression of CD38 was determined by labeling cells with biotinylated anti-CD38 mAb before fixation and permeabilization, which was then detected with SA-TC. Cultures that had not been pulsed with BrdU were similarly harvested and treated to ascertain the background binding of the anti-BrdU mAb. Data were acquired on a FACScan flow cytometer using CellQuest software (BD Biosciences). Surface staining was measured on a logarithmic scale. Cells present in different divisions were characterized by "division slicing." Gates were drawn around each of the peaks present in histograms of CFSE-labeled B cells, representing cells in different divisions. The proportion of cells within each gate or the expression of BrdU by these cells was determined by backgating and analyzing the various divided populations, defined by CFSE dilution, using the analysis tools of CellQuest.

Results

Phenotype of $CD27^-$ and $CD27^+$ B cells

Memory B cells were distinguished from naive B cells within the $CD19^+$ population by expression of CD27 (7, 10). Naive ($CD27^-$) B cells were uniformly IgM^+IgD^{high} and contained <2% IgG^+ and IgA^+ cells. In contrast, memory ($CD27^+$) B cells were heterogeneous for isotype expression, containing IgM-only cells (Fig. 1 and Refs. 6 and 13) as well as isotype-switched B cells (Fig. 1).

Memory B cells exhibit greater proliferation than do naive B cells

Proliferation of human naive and memory B cells was initially investigated by monitoring incorporation of [^{3}H]thymidine by purified B cell subsets cultured for different periods of time with the T cell-derived stimuli CD40L, IL-2, and IL-10. Uptake of [^{3}H]thymidine by memory B cells exceeded that by naive B cells at all

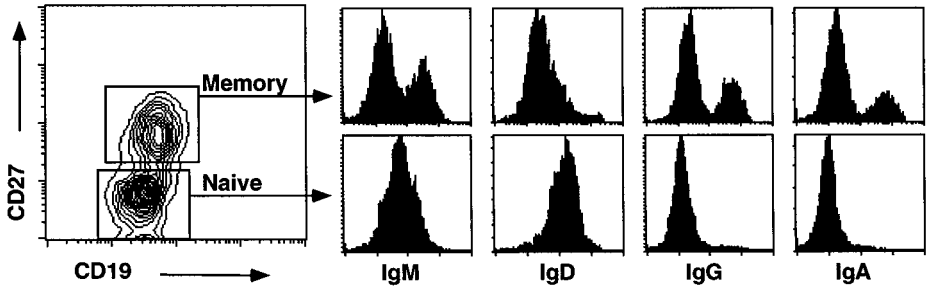

FIGURE 1. B cell subsets present in human spleen. Purified splenic B cells were incubated with FITC-conjugated anti-CD19 (*x*-axis), PE-conjugated anti-CD27 mAb (*y*-axis) and biotinylated anti-IgM, IgD, IgG, or IgA mAb, followed by SA-TC. Expression of these isotypes by $CD19^+CD27^+$ memory B cells (*upper panel*) and $CD19^+CD27^-$ naive B cells (*lower panel*) was then determined.

culture times examined (Fig. 2*a*). Furthermore, initial incorporation of [^{3}H]thymidine by memory B cells appeared to precede that by naive B cells by ~24–48 h (Fig. 2*a*). Similar results were obtained when B cells were stimulated in vitro with CD40L only (data not shown). Proliferation of naive and memory B cells was then investigated in more detail using the division-tracking dye CFSE (20, 21). This technique revealed that a greater proportion of memory B cells than naive B cells entered division, as evidenced by fewer undivided memory B cells and more memory B cells in later divisions (compare Fig. 2, *b* and *c*). The enhancement in proliferation of CD40L-stimulated memory B cells over naive B cells was observed irrespective of the presence of exogenous IL-10 alone or in combination with IL-2 (Fig. 2, *b* and *c*). CFSE-labeled B cells were then cultured with CD40L alone or with IL-10 or IL-2 plus IL-10 for up to 5 days and were harvested to calculate the proportion of cells that had undergone one or more divisions after different periods of in vitro stimulation. Naive B cells remained undivided for ~72 h (Fig. 2*d*). After this time, a small proportion of CD40L-stimulated naive B cells entered division, which then increased for the remainder of the culture period, reaching a maximum of ~20% of cells in culture. IL-10 increased the proportion of dividing naive B cells at all time points examined to a maximum of ~65% after ~100 h (Fig. 2*d*). In contrast to naive B cells, divided cells were evident in cultures of memory B cells by 72 h of in vitro stimulation (Fig. 2*e*), consistent with the earlier incorporation of [^{3}H]thymidine by memory B cells compared with that of naive B cells (Fig. 2*a*). Not only did the percentage of divided memory B cells detected rise sharply after 72 h for all culture conditions, but the proportion of them observed at each harvest time exceeded that of naive B cells. Thus, in the presence of CD40L, up to ~60% of memory B cells in culture had undergone division by 5 days, whereas adding cytokines increased this figure to >90% (compare Fig. 2, *d* and *e*). Notably, the percentage of divided naive B cells in cultures stimulated with CD40L, IL-2, and IL-10 approximated that of memory B cells stimulated with only CD40L (Fig. 2, *d* and *e*), indicating increased sensitivity of memory B cells to stimulation compared with naive B cells. IL-2 failed to increase the proliferative response of naive B cells stimulated with CD40L and IL-10, whereas proliferation of memory B cells was enhanced (Fig. 2, *b–e*). Thus, memory B cells undergo increased proliferation compared with naive B cells receiving identical stimuli.

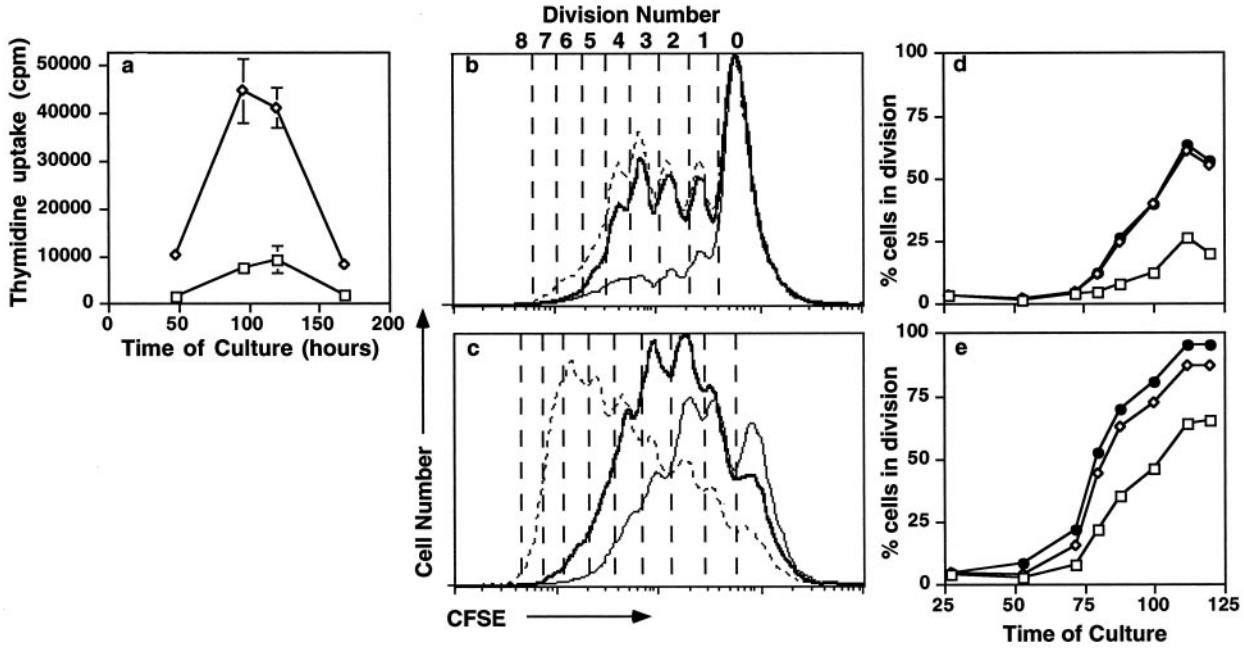

FIGURE 2. Memory B cells undergo more proliferation than naive B cells. *a*, Purified naive (□) and memory B cells (◇; 5×10^4/well) were cultured with human CD40L, IL-2, and IL-10 for 7 days. At different times, incorporation of [^{3}H]thymidine during a 4-h pulse was determined. Each point represents the mean ± SD of triplicate samples. *b–e*, CFSE-labeled naive and memory B cells (2×10^5/500 μl) were cultured for 5 days. At different times, cells were harvested, CFSE profiles were determined, and the percentage of cells that had entered division was calculated. CFSE profiles of cultured naive (*b*) and memory (*c*) B cells stimulated with CD40L alone (thin histogram), CD40L plus IL-10 (thick histogram), or CD40L plus IL-2 and IL-10 (dotted histogram) for 5 days. Percentage of naive (*d*) and memory (*e*) B cells in division after stimulation with CD40L alone (□), CD40L plus IL-10 (◇), or CD40L plus IL-2 and IL-10 (●) at the indicated period of time. These data represent results obtained from at least three independent experiments.

Memory B cells enter division earlier than do naive B cells

According to the data shown in Fig. 2, divided B cells were detectable in cultures of memory B cells earlier than in those of naive B cells, suggesting that enhanced proliferation of memory B cells may result from these cells entering division before naive B cells. To measure their *ttfd* directly, naive and memory B cells were stimulated with CD40L with or without cytokines in the presence of the mitotic inhibitory drug demecolcine. This procedure selectively measures time of entry of cells into their first S-phase (22), as demecolcine blocks all subsequent rounds of cell proliferation. Incorporation of [^{3}H]thymidine was monitored after different times of culture. Both B cell populations exhibited a broad variation in time of entry into first division (S-phase) after culture with either CD40L alone (Fig. 3*a*) or CD40L, IL-2, and IL-10 (Fig. 3*b*). Furthermore, irrespective of cell type or stimulus, the time of entry conformed accurately to a log-normal distribution, consistent with stochastic variability within the B cell population (Fig. 3, *a* and *b*). The mean of the fitted log-normal curve was taken as the mean *ttfd* for the B cell populations. In the presence of CD40L alone, the mean *ttfd* for naive and memory B cells was 85 and 57 h, respectively (Fig. 3*a*; Table I). This is consistent with the times when divided cells were first detected in cultures of CFSE-labeled

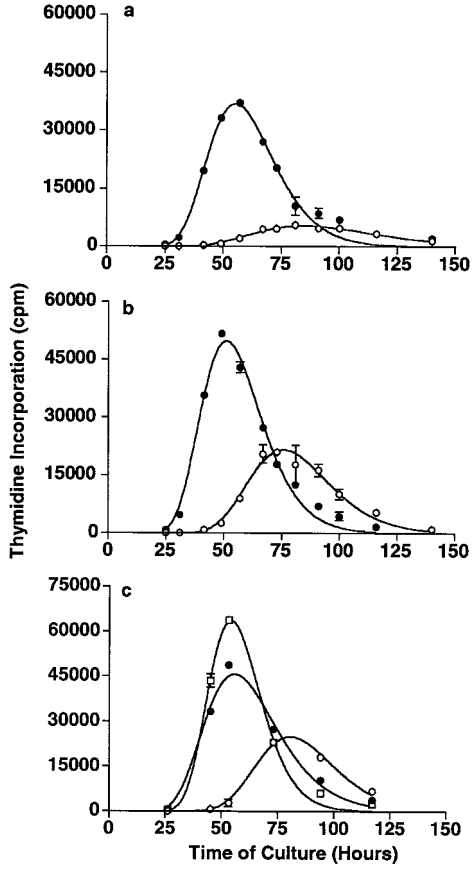

FIGURE 3. Memory B cells enter the first division earlier than naive B cells. *a* and *b*, Purified naive (○) and memory B cells (●; 5×10^4/well) were cultured with demecolcine (25 ng/ml) and CD40L alone (*a*) or CD40L, IL-2, and IL-10 (*b*). *c*, Purified naive (○), IgM-expressing memory B cells (●), or Ig isotype-switched memory B cells (□) were cultured with demecolcine (25 ng/ml) and CD40L, IL-2, and IL-10. At different times, incorporation of [^{3}H]thymidine during a 4-h pulse was determined. Each point represents the mean ± SEM of triplicate or quadruplicate samples. The results in *a* and *b* correspond to donor 7 in Table I; *c* corresponds to Experiment 2 in Table II.

naive and memory B cells (see Fig. 2, *d* and *e*). Not only did memory B cells enter division earlier than did naive B cells, but differences in the amplitude of the curves suggest that a greater number of memory B cells were being recruited into cell cycle (Table I). Addition of IL-10 (with or without IL-2) reduced the *ttfd* of naive B cells by ~10 h and increased the number of cells (i.e., amplitude of the curve) 5-fold (Fig. 3*b*; compare donors 7 and 12* cultured with CD40L ± IL-2/IL-10 in Table I). IL-10 alone (data not shown) or in combination with IL-2 had little effect on *ttfd* of memory B cells and only increased the cell number (i.e., amplitude of the curve) by a maximum of 2-fold, compared with CD40L alone (Fig. 3, *a* and *b*; Table I). Although the *ttfd* of CD40L-stimulated naive B cells was reduced by adding cytokines, memory B cells stimulated with CD40L, IL-2, and IL-10 still entered division significantly earlier ($p < 0.01$; Fig. 3*b*; Table I). By using naive and memory B cells isolated from different donors, it was clear that these results were highly reproducible (Table I).

It has been reported recently that the cytoplasmic domain of IgG caused the enhanced response of IgG-expressing transgenic B cells over IgM-expressing B cells recognizing the same Ag (17). In humans, the memory B cell population is heterogeneous, comprising cells expressing elevated levels of IgM as well as switched Ig isotypes (Fig. 1 and Refs. 6 and 13). Consequently, there may be differences in the times when each of these memory populations enters division. To test this, IgM$^+$ and isotype-switched memory B cells were isolated by cell sorting, and their *ttfd* was determined. Both memory B cell subpopulations entered division significantly earlier than did naive B cells ($p < 0.05$); however, there was no significant difference in the time at which they did so ($p > 0.05$; Fig. 3*c*; Table II). Nevertheless, for the three experiments performed, the amplitude of the curve for isotype-switched memory B cells was up to 2-fold greater than for IgM-expressing memory B cells, suggesting that a greater proportion of isotype-switched memory B cells was recruited into division than IgM-expressing memory B cells (Fig. 3*c* and data not shown). Thus, consistent with the data in Fig. 2, *d* and *e*, memory B cells, irrespective of the Ig isotypes expressed, began dividing significantly earlier than did naive B cells, and a greater number were recruited when given the same T-dependent stimulus.

Analysis of uptake of BrdU revealed differences in the kinetics of proliferative responses of naive and memory B cells, but the overall proliferative rate was similar

The increased division of memory B cells compared with naive B cells may also be associated with an elevated rate of proliferation. That is, the time to traverse each division may be less for memory B cells than naive B cells, as observed recently for murine naive and memory CD8$^+$ T cells (23). To assess this, cultures of activated naive and memory B cells were pulsed at different times with BrdU, and its incorporation after 6 h was determined as an indicator of division rate. This analysis indicated that the time when the greatest proportion of naive and memory B cells were incorporating BrdU, and therefore dividing, was 4 days and 3 days, respectively (Fig. 4, *a* and *b*). At later times, the proportion of BrdU$^+$ naive and memory B cells declined (Fig. 4, *a* and *b*). Despite this difference, the frequency of naive and memory B cells incorporating BrdU was similar during and after the peak response. The analysis was extended by measuring incorporation of BrdU by cells in different divisions. Unlike murine T and B lymphocytes, which exhibit a consistent rate of proliferation across division (24, 25), the rate of BrdU uptake by human naive and memory B cells across different divisions was heterogeneous (Fig. 4, *c* and *d*). Thus, cells in later divisions were on average dividing faster. Overall, the rate of incorporation of BrdU per division for naive and

Table I. *Memory B cells enter division earlier than naive B cells, irrespective of stimulation*[a]

	B Cells	Center (*ttfd*)[b]	Amplitude[c]	Width[d]
CD40L				
Donor 7	Naive	84.8 ± 1.0	5400 ± 146.9	0.304 ± 0.0127
	Memory	55.2 ± 0.35	36,912 ± 788	0.256 ± 0.0069
	Difference	29.6[e]	6.83[f]	
Donor 12*	Naive	85.4 ± 1.3	9118 ± 393.9	0.283 ± 0.0166
	Memory	59.1 ± 0.66	24,067 ± 717.1	0.2906 ± 0.0124
	Difference	26.4	2.64	
CD40L, IL-2, IL-10				
Donor 7	Naive	75.8 ± 0.80	21,651 ± 833	0.2285 ± 0.011
	Memory	51.1 ± 0.31	49,822 ± 910	0.2557 ± 0.006
	Difference	24.7	2.3	
Donor 8	Naive	76.9 ± 0.7	11,349 ± 312	0.22[g]
	Memory	60.3 ± 0.46	23,004 ± 634	0.22
	Difference	16.6	2.03	
Donor 10	Naive	95.8 ± 3.3	1447 ± 147	0.25[g]
	Memory	61.6 ± 1.36	8183 ± 610	0.25
	Difference	34.2	5.6	
Donor 12	Naive	73.3 ± 1.1	13,655 ± 562	0.2634 ± 0.013
	Memory	59.3 ± 0.73	14,187 ± 426	0.319 ± 0.012
	Difference	14.0	1.04	
Donor 12*	Naive	75.1 ± 0.92	45,457 ± 2226	0.213 ± 0.0125
	Memory	57.0 ± 0.40	47,217 ± 977	0.259 ± 0.074
	Difference	18.1	1.04	
Average	Naive	79.38 ± 4.1		
	Memory	57.74 ± 1.8		
	Difference	21.64 ± 3.7	2.4 ± 0.8	

[a] Naive and memory B cells (5×10^4/125 μl/well) purified from different donors were cultured with the indicated stimulus for 6 days. At different times, cultures were pulsed with [^{3}H]thymidine and harvested 4 h later. The data were fitted to a log-normal distribution using Prism software. This experiment was repeated using B cells from donor 12 on a separate occasion (*) to demonstrate reproducibility. All values represent the mean ± SE.
[b] Center represents the average *ttfd.*
[c] Amplitude represents the height of the curve.
[d] Width is the SD of the fitted log-normal curve given in "log-time."
[e] Difference for the *ttfd* represents the number of hours that memory B cells enter division earlier than naive B cells.
[f] Difference for the amplitude represents ratio of memory to naive B cells.
[g] No errors are given because the width had to be made constant to ensure accurate fitting of the log-normal curve.

memory B cells was comparable (Fig. 4, *c* and *d*). Investigating proliferation by this method further demonstrated that IL-10 altered the average division rate for both cell populations, whereas IL-2 further increased proliferation of memory B cells only (Figs. 4, *a–d*).

Memory B cells that differentiate into CD38$^+$ blasts undergo rapid cell division

After stimulation with CD40L, in the absence or presence of IL-10 and IL-2, memory B cells, but not naive B cells, yield a population of differentiated cells identified by expression of CD38 (Fig. 5*a*). This population of cells is enriched for Ig-secreting cells (51). Importantly, the appearance of these cells increases with cell division (Fig. 5*a*) and they acquire an accelerated rate of proliferation (51). To determine the contribution of differentiated CD38$^+$ B cells to the proliferative response of activated memory B cells, the proliferative characteristics of CD38$^-$ B cell blasts and differentiated CD38$^+$ B cells were examined in more detail by measuring incorporation of BrdU after a 6-h pulse. Although only a small proportion (<5%) of CD38$^+$ B cells was generated after culture of memory B cells with CD40L alone, 25–30% more of these cells incorporated BrdU than did CD38$^-$ B cells (Fig. 5*b*, *top panel*). Addition of IL-10 alone (Fig. 5*b*, *middle panel*) or in combination with IL-2 (Fig. 5*b*, *bottom panel*) to cultures of CD40L-stimulated memory B cells increased the proportion of CD38$^+$ B cells, as well as the proportion of BrdU$^+$ B cells within both CD38$^-$ and CD38$^+$ populations. Despite this increase, a greater proportion of CD38$^+$ B cells continued to incorporate BrdU than CD38$^-$ B cells in these cultures (Fig. 5*b*).

Due to the heterogeneous pattern of BrdU incorporation by total naive and memory B cells present in different divisions, incorporation of BrdU by CD38$^-$ and CD38$^+$ B cells generated in cultures of memory B cells containing CD40L and IL-10 in the absence and

Table II. *IgM-expressing and Ig isotype-switched memory B cells enter division earlier than naive B cells*[a]

	CD40L, IL-2, IL-10			
B Cells	Expt. 1	Expt. 2	Expt. 3	Mean ± SEM
Naive	85.6 ± 4.0	79.9 ± 0.45	88.55 ± 1.8	84.72 ± 2.3
IgM memory	62.0 ± 1.20	55.7 ± 0.76	76.52 ± 0.71	64.77 ± 6.1
Switched memory	53.3 ± 1.10	54.1 ± 0.45	75.17 ± 0.50	60.86 ± 7.2

[a] Naive, IgM memory and isotype-switched memory B cells (5×10^4/125 μl/well) purified from different donors were cultured with CD40L, IL-2, and IL-10 for 5 days. At different times, the cultures were pulsed with [^{3}H]thymidine and harvested 4 h later. The data were fitted to a log-normal distribution using Prism software. The values are the geometric means of the log-normal curves ± SE and represent the average *ttfd.*

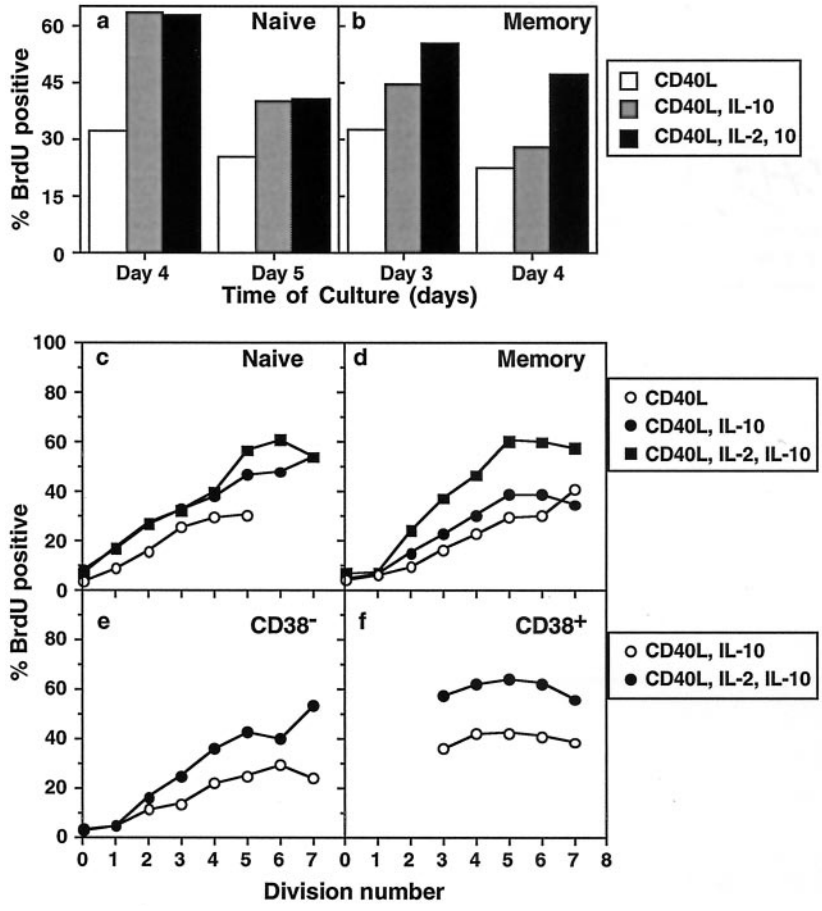

FIGURE 4. Rate of division of activated naive and memory B cells. Purified, CFSE-labeled naive and memory B cells were cultured with CD40L alone, CD40L and IL-10, or CD40L plus IL-2 and IL-10. The cells were pulsed with 100 μg/ml BrdU for 6 h before harvesting. *a–d*, Incorporation of BrdU by naive and memory B cells stimulated with CD40L, CD40L and IL-10, or CD40L, IL-2, and IL-10 harvested after the 6-h pulse at the indicated culture times (*a* and *b*) or in different divisions after 4 days of culture (*c* and *d*). *e* and *f*, Cultures of stimulated memory B cells were first labeled with anti-CD38 mAb before detection of incorporated BrdU. Uptake of BrdU by $CD38^-$ and $CD38^+$ B cells generated in the presence of CD40L and IL-10 or CD40L, IL-2, and IL-10 in different divisions was determined by gating on $CD38^-$ (*e*) and $CD38^+$ (*f*) cells. These results are representative of data from two to three independent experiments using cells from different donors.

presence of IL-2 was analyzed on a per division basis. This revealed that $CD38^-$ B cells behaved like total naive and memory B cells; i.e., proliferation was heterogeneous across division (Fig. 4*e*). In contrast, $CD38^+$ B cells exhibited a uniformly high rate of proliferation across divisions in which these cells were detected (i.e., after division 3), and this rate exceeded that of $CD38^-$ B cells at all divisions (Fig. 4*f*). IL-2 increased the average division rate of $CD38^-$ and $CD38^+$ B cells cultured with CD40L and IL-10 (Fig.

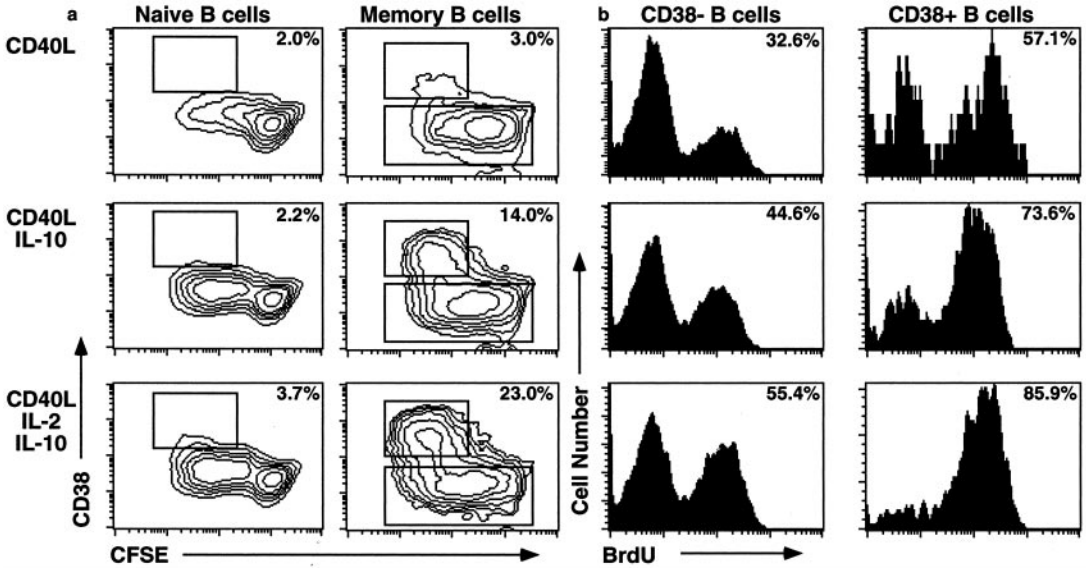

FIGURE 5. $CD38^+$ B cells generated in vitro from memory B cells divide rapidly. *a*, Purified CFSE-labeled naive and memory B cells were cultured with CD40L alone (*top row*), CD40L plus IL-10 (*middle row*), or CD40L plus IL-2 and IL-10 (*bottom row*) for 3–4 days, after which time the percentage of $CD38^+$ B cells was determined. *b*, Cultures of memory B cells were pulsed with 100 μg/ml BrdU for 6 h before harvesting. The uptake of BrdU by $CD38^-$ and $CD38^+$ B cells generated from memory B cells in vitro after culture with CD40L alone (*top row*), CD40L and IL-10 (*middle row*), or CD40L, IL-2, and IL-10 (*bottom row*) was determined by gating on $CD38^-$ and $CD38^+$ cells, as shown in *a*. These results are representative of three experiments. The values represent the percentage of $CD38^+$ (*a*) and $BrdU^+$ (*b*) cells present in the different cultures.

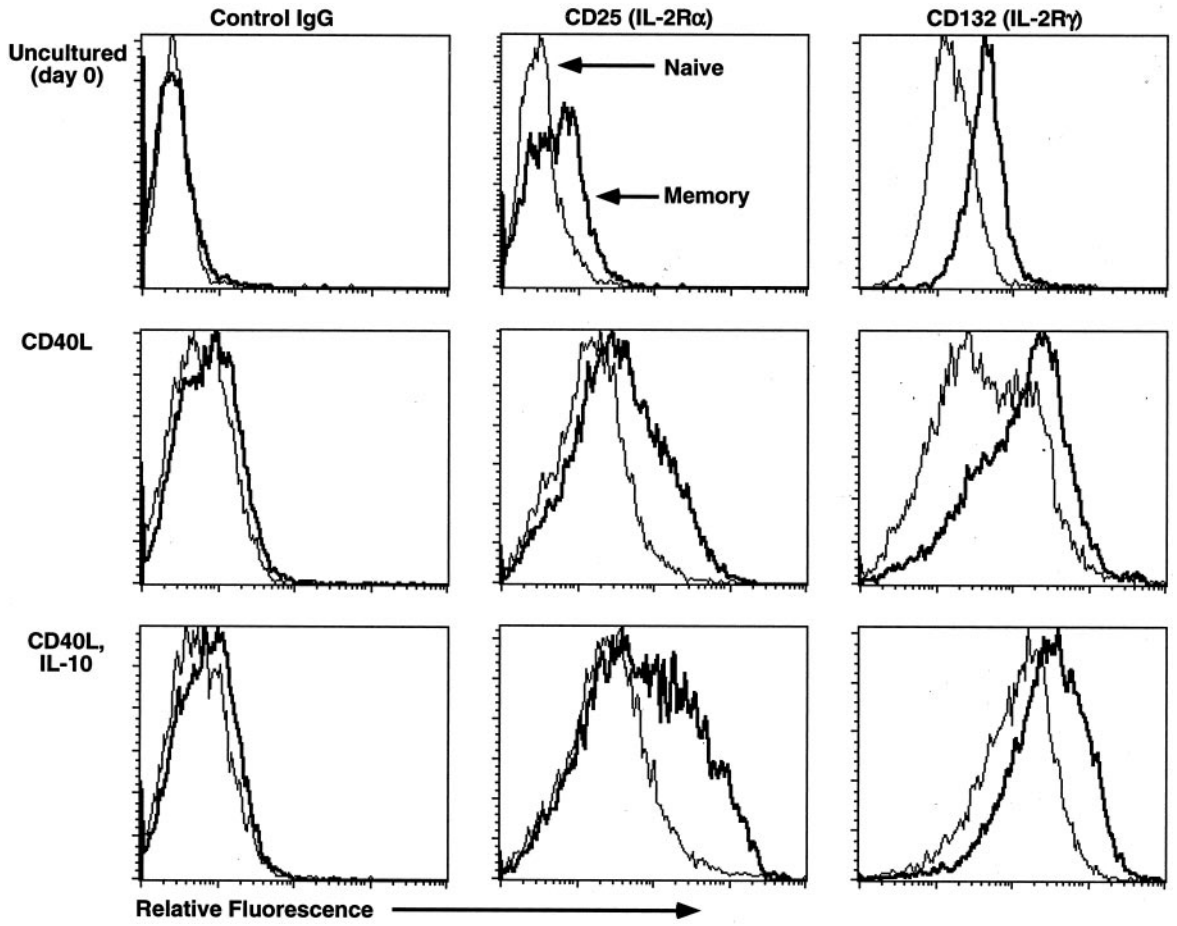

FIGURE 6. Differential expression of the IL-2R complex on human B cells. Naive (thin histogram) and memory B cells (thick histogram) were incubated with PE-conjugated control mAb or mAb specific for CD25 (IL-2Rα) or CD132 (IL-2Rγ) before culture (*top panel*) or after 5 days of culture with CD40L alone (*middle panel*) or CD40L and IL-10 (*lower panel*). These results are representative of three independent experiments.

4, *e* and *f*). Thus, during the differentiation process, $CD38^+$ B cells acquire the capacity to proliferate more rapidly than nondifferentiated $CD38^-$ B cells present in the same culture.

Differential expression of IL-2R components by naive and memory B cells

The data presented in the preceding sections demonstrated that IL-2 selectively enhanced proliferation of memory B cells activated with CD40L and IL-10, yet had no effect on naive B cells (Figs. 2 and 4). This may reflect differential expression of the IL-2R complex on naive and memory B cells. To investigate this, expression of CD25 and CD132, the α- and γ-chains of the IL-2R complex, respectively, by naive and memory B cells before and after culture was determined. Before culture, CD25 was absent from naive B cells but was detected on 25–50% of memory B cells (Fig. 6, *top panel*). Similarly, expression of CD132 was up to 4-fold higher on memory B cells than on naive B cells (Fig. 6, *top panel*). After culture with CD40L alone (Fig. 6, *middle panel*) or in combination with IL-10 (Fig. 6, *lower panel*), CD25 and CD132 expression was increased on both B cell populations; however, expression remained higher on a greater proportion of memory B cells compared with naive B cells (Fig. 6). Thus, differential expression of the IL-2R complex may contribute to the selective effect of IL-2 on memory B cell proliferation.

Discussion

The ability of memory B cells to generate a robust, prompt, and efficient Ab response to secondary challenge by an invading pathogen is a hallmark of immunological memory. Indeed, in vivo and in vitro studies have demonstrated that memory B cells differentiate into Ig-secreting effector cells more rapidly than do naive B cells (1, 2, 26, 51). However, the mechanisms underlying this difference have not been fully elucidated. Here, we extended our initial studies demonstrating differences between proliferation and differentiation of naive and memory human B cells (51) and revealed additional unique features of the activation programs of these B cell populations. The proliferative capacity of naive and memory B cells was found to be distinct when given an identical stimulus. On average, a greater proportion of memory B cells entered division 20–30 h earlier and underwent further rounds of cell division than did naive B cells, although the subsequent rate of proliferation of each cell type was comparable (Table I; Figs. 2–4). Importantly, both IgM-expressing and Ig isotype-switched memory B cells behaved in a similar way (Tables II; Fig. 3*c*), indicating that the isotype-switching process per se does not provide switched B cells with an enhanced capacity to respond to stimulation. Rather, selection of high-affinity, Ag-specific B cells within germinal centers, which subsequently mature into memory B cells, is likely to be responsible for altering the response capabilities of memory B cells, compared with naive B cells.

Naive and memory B cells also showed differences in sensitivity to cytokines. IL-10 significantly reduced the *ttfd* of naive B cells. In contrast, the magnitude of the CD40L-mediated response of memory B cells leading to initial entry into cell division was only slightly affected by IL-10 (Fig. 3; Table I). Despite this enhancement in the presence of IL-10, naive B cells were still considerably

slower than memory B cells to start dividing, and fewer naive B cells underwent proliferation. Thus, memory B cells appear to be more sensitive to stimuli and require less stimulation to initiate a maximal response. This conclusion was further supported by the observation that the proportion of naive B cells undergoing division in response to stimulation with CD40L, IL-2, and IL-10 approximated that of memory B cells stimulated with CD40L alone (Fig. 2, *d* and *e*). Consequently, entry of naive B cells into their first division may be subjected to increased layers of regulation compared with memory B cells, which respond more rapidly and to a smaller number of stimuli. This trend can explain previous findings demonstrating that memory B cells have a lower threshold for activation than do naive B cells, responding to reduced concentrations of specific Ag and T cell help (27). Similarly, in vivo-activated B cells, which are likely to contain a population of memory B cells, respond to concentrations of CD40L below the threshold needed for stimulating naive B cells (28). Moreover, IL-2 selectively increased proliferation of memory, but not naive, B cells after culture with CD40L and IL-10 (Figs. 2 and 4). Consistent with this, expression of CD25 and CD132 was ~2-fold greater on memory than on naive B cells, and in vitro activation up-regulated these receptor components further on memory than on naive B cells (Fig. 6). The synergistic effect of IL-2 and IL-10 on growth and differentiation of human B cells has previously been attributed to the ability of IL-10 to increase expression of CD25 on CD40-activated B cells (29). Taken with our results, the B cells observed in this previous study to up-regulate CD25 were presumably memory B cells present in the population of total tonsillar B cells used (29). Our results predict that differentiation of naive B cells into memory B cells in vivo will alter the proliferative behavior of the resulting memory B cells in terms of time to enter division, magnitude of the response, and the growth factors capable of affecting this response. These novel growth characteristics acquired in vivo by memory B cells provide an explanation for the more rapid Ab response that occurs after re-exposure to an invading pathogen.

A recent study in a BCR transgenic model suggested the molecular determinant for enhanced secondary responses in vivo is the cytoplasmic domain of IgG expressed by isotype-switched memory B cells (17). The mechanism whereby Ag-specific signaling through a BCR comprising this motif enhanced the response of memory B cells was by reducing cell death, rather than increasing the number of cells recruited into the response or increasing the rate of cell division (17). Although these results appear to contrast with ours, we did observe that the proliferation rate of activated naive and memory B cells was comparable once the cells had commenced dividing (Fig. 4). In an attempt to resolve this issue, we conducted an experiment that cannot be performed in mice, due to lack of appropriate cell surface markers in which responses by naive (i.e., IgM^+) and IgM-expressing memory B cells were compared. The data revealed that the *ttfd* of IgM-expressing memory B cells was comparable to switched memory B cells and significantly shorter than naive B cells (Fig. 3; Table II). Thus, factors other than, or in addition to, the cytoplasmic domain of switched Ig isotypes may account for differences in the magnitude of responses exhibited by naive and memory B cells. However, because the need for specific Ag was bypassed in our culture system by providing B cells directly with T cell help in the form of CD40L, the apparent discrepancies between the data for murine and human memory B cells may also be explained by involvement of the IgG cytoplasmic domain when B cells receive signals concomitantly via CD40 and the BCR.

Some of the characteristics identified here for human memory B cells are similar to $IgM^{high}IgD^{low}CD21^{++}CD23^-$ B cells present in the MZ of mouse spleen (16, 30–34). These MZ B cells exhibited a greater degree of activation than did naive B cells when proliferation, differentiation into Ig-secreting cells, and intracellular signaling in response to LPS and BCR ligation in vitro or Ag exposure in vivo were assessed (16, 30–33). Notably, the differences we observed in CFSE profiles between human naive and memory B cells (Fig. 2) were also observed for murine naive follicular and MZ B cells stimulated with anti-CD40 mAb (16), suggesting that murine MZ B cells enter division earlier than do follicular B cells. It has recently been demonstrated that murine memory $CD8^+$ T cells began dividing ~15 h earlier than did naive $CD8^+$ T cells in vivo in response to the same immunizing Ag (23). Thus, accelerated entry into division may represent a common mechanism for elevated responses of memory lymphocytes belonging to B and T cell lineages, as well as MZ B cells. Another mechanism by which human memory and murine MZ B cells respond more rapidly than naive B cells may involve differential expression of molecules involved in delivering activating signals. Expression of IgM, CD19, CD21, CD40, costimulatory molecules (CD80, CD86), and adhesion molecules is greater on memory and MZ B cells compared with naive B cells (Fig. 1 and Refs. 10, 12, 13, 16, 31, and 35). It has been found that coligating CD40 and CD21 or surface Ig enhances B cell proliferation (36–38), and interactions between B and T cells or follicular dendritic cells via adhesion molecules promotes B cell survival, activation, proliferation, and differentiation (39–43). Similarly, costimulation of anti-CD3 mAb-activated human T cells with anti-CD28 mAb rapidly up-regulates and sustains CD40L expression, which subsequently heightens B cell responses (44, 45). Thus, after interactions with Ag, cytokines, T cells, and dendritic cells within secondary lymphoid tissues, the response time of human memory and murine MZ B cells may be further reduced due to the potentially lower sensitivity of these cells to signals delivered via coreceptors, as well as enhanced "help" memory B cells may receive from costimulated T cells.

Naive and memory B cells differed in their ability to differentiate into $CD38^+$ effector cells that proliferated at a rate exceeding that of $CD38^-$ memory B cells as well as naive B cells (Fig. 5). Importantly, the $CD38^+$ B cell subset contained a population of Ig-secreting cells (51). These acquired differences suggest that distinct molecular mechanisms, such as the differential expression of transcription factors, regulate proliferation of $CD38^-$ and $CD38^+$ B cell blasts generated from activated memory B cells in a manner analogous to regulating B cell differentiation (46, 47). Recent studies have indicated that bcl-6, a transcriptional repressor expressed by germinal center B cells, maintains B cells in a nondifferentiated state by suppressing expression of the transcription factor Blimp-1 (46). Similarly, differentiation of B cells into plasma cells is accompanied by increased expression of Blimp-1, which down-regulates the cell cycle regulatory protein c-*myc*, contributing to the reduced proliferation of plasma cells (47, 48). Curiously, plasma cells coexpressing bcl-6 and the proliferation Ag Ki-67 have been identified in situ (49, 50). Our data suggest that the rapidly proliferating $CD38^+$ B cells generated from memory B cells in vitro may correspond to these plasmablasts observed in vivo. The sustained expression of bcl-6 in developing plasmablasts may delay the Blimp-1-mediated down-regulation of c-*myc* and thus facilitate the generation of large numbers of Ag-specific, Ig-secreting cells from proliferating precursors before their terminal differentiation into nondividing plasma cells (46). Thus, although Blimp-1 is expressed by plasmablasts, its effects may only manifest after this proliferative burst and subsequent down-regulation of bcl-6. In conclusion, human memory B cells possess intrinsic proliferative and differentiation characteristics over naive B cells that facilitate

their early entry into cell division and differentiation into a rapidly dividing population of Ig-secreting cells. These characteristics, along with their unique positioning within Ag-draining sites of lymphoid tissue (1, 9–11), may contribute to the accelerated and efficient Ab response exhibited by memory B cells after secondary challenge by an invading pathogen.

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