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Kinetics of Establishing the Memory B Cell Population as Revealed by CD38 Expression

Anna Ridderstad and David M. Tarlinton

In this report, we detail changes in the expression of CD38 on murine B cells during the course of a T cell-dependent immune response. CD38 is expressed on all naive B cells but is down-regulated on isotype-switched B cells from both the germinal centers (GCs) and the foci of Ab-forming cells which arise during the first weeks of the response. The down-regulation on GC B cells, however, is reversible since Ag-specific IgG1 B cells with high levels of CD38 are apparent by 2 wk postimmunization. These cells have characteristics that resemble recirculating memory B cells, in that they are small and bind low levels of peanut agglutinin. Such characteristics indicate that the restoration of CD38 levels is coincidental with the transition from GC to memory B cell. Using this observation, we plotted the development of the memory population and the demise of the GC reaction as a function of time after immunization. Our results indicate that the GC reaction ceases gradually over many weeks rather than suddenly, which corresponds with the formation of the memory B cell population. Furthermore, by segregating memory B cells and GC B cells, it was possible to assess the in vitro survival characteristics of each compared with naive B cells. These experiments demonstrated that memory B cell survival in vitro was comparable with naive B cell survival but less than the survival seen for bcl-2-transgenic B cells, whereas GC B cell survival, as expected, was very poor. Hence, by resolving murine Ag-specific memory B cells and GC B cells, we have been able to quantify the development of the memory B cell population.

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3 Abbreviations used in this paper: GC, germinal center; AFC, Ab-forming cell; KLH, keyhole limpet hemocyanin; NP, (4-hydroxy-3-nitrophenyl)acetyl; PE, phycoerythrin; AP, alkaline phosphatase; HRP, horseradish peroxidase; FSC, forward light scatter.

Memory and GC B cells can be distinguished on the basis of several physical characteristics. GC B cells are activated and as such possess the cell surface characteristics of activated B cells; they are larger, bind higher levels of the lectin peanut agglutinin (PNA) (5), and express higher levels of CD24 (heat-stable Ag) (6) and the costimulatory Ag CD86 (B7.2) than both naive and memory B cells (7). Memory B cells and GC B cells have other characteristics in common, such as mutated V genes and isotype-switched Ig heavy chains (reviewed in Ref. 2). Histology can also be used to locate Ag-specific GCs (8) and consequently determine the duration of the GC reaction. Using such an approach, Bachmann et al. (9) have observed GCs that are specific for a viral Ag for ≤100 days after primary immunization, albeit at decreasing levels. These authors postulated that this long-term GC reaction was part of the process of maintaining memory or serum Ab titers and could be unique to the particular Ag used (9). In histologic studies, however, the quantification of cell populations is difficult.

Previously we and others have reported the kinetics with which Ag-specific B cells appear and disappear during the primary response to model T cell-dependent Ags (10–15). These studies revealed that the frequency of B cells that are specific for a haptenic Ag peaked in the spleen at around day 14 and then declined rapidly during the next 5 wk before stabilizing over the next 200 to 300 days to around 1% of the level seen at day 14 (15, 16). Although not formally proven, it was assumed that the memory B cell population was established during the phase of rapid decline. Since it was not possible to distinguish between memory and GC B cells in these experiments, these data do not resolve the two possible models of generating a memory B cell population. In one model, the GC reaction ceases when Ag-specific Ab is in excess of Ag. This Ab could compete with GC B cells for Ag and consequently block access to Ag on the follicular dendritic cells and/or deliver a negative signal through FcγRII (reviewed in Ref. 17), thereby terminating the GC reaction. In this way, the memory population could be generated as a result of the synchronous dissolution of the GCs. In a second model, memory B cells are continually produced by...
the GC. During the early stages of the response, when Ag is in excess, emigres may reenter the GC for additional rounds of mutation and selection; however, at later stages, they remain outside, leading to a memory population that accumulates in an inverse relationship with the decline of the GC B cell population.

We have used a flow cytometry-based system to study the association between the expression of the membrane glycoprotein CD38 (18, 19) and the generation of B cell memory during the primary response to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) in C57BL/6 mice. First, we confirmed that CD38 expression is down-regulated on Ag-specific GC B cells (20, 21). Subsequently, we found that it was reexpressed on a progressively increasing fraction of the memory B cell population. At all times, the CD38<sup>low</sup> isotype-switched Ag-specific B cells exhibited the phenotype of activated cells and had reduced survival potential in vitro, which was typical of GC B cells. Their CD38<sup>high</sup> counterparts, however, displayed characteristics of resting memory B cells. By determining the proportion of Ag-specific IgG1<sup>+</sup> B cells that were CD38<sup>high</sup> at different times after immunization, we were able to plot the development of the memory population in relation to the decline of the GC reaction. Our results indicate that the formation of the memory population is a gradual process that occurs over many weeks.

**Materials and Methods**

**Mice, Ags, and immunization**

C57BL/6 mice that had been bred and maintained at our institute were used when they reached 8 to 12 wk of age. Hemizygous transgenic mice of the Eμ-bcl-2–22 strain (22) that had been backcrossed onto the C57BL/6 background for more than 18 generations were provided by Dr. A. W. Harris. Mice were immunized i.p. with 100 μg of alumin-precipitated NP that was conjugated to keyhole limpet hemocyanin (KLH) (NP/KLH conjugation ratio ≈ 13:1) as previously described (12).

**Preparation of cell suspensions**

Mice were killed by cervical dislocation on the stated days after immunization, spleens were removed, and single-cell suspensions were prepared as previously described (14). Briefly, spleens were forced through a metal mesh and suspended in PBS containing 1% FCS. Viable cells were counted using a light microscope and trypan blue exclusion.

**Enrichment of IgG1<sup>+</sup> spleen cells**

For cell-sorting experiments, surface IgG1<sup>+</sup> spleen cells were preenriched according to the following procedure. Live cells were separated by density centrifugation using 1.09 g/cm<sup>3</sup> of Nycodenz (Nynomed Pharma, Oslo, Norway). These were stained with rat anti-mouse IgG1 magnetic cell separation beads (Miltenyi Biotec, Bergisch Gladbach, Germany) in the presence of 10 μg/ml mouse IgM myeloma protein (MOPC-104E, Bionetics, Kensington, MD) to reduce background staining. Magnetically stained cells were isolated on a mini magnetic cell separation column (MiniMACS, Miltenyi Biotec) according to the manufacturer’s protocol. The isolated fraction was highly enriched for IgG1-expressing cells, such that between 20 and 50% of cells were positive depending upon the time point after immunization. The cells were still stainable with fluorochrome-labeled goat anti-mouse IgG1 after isolation.

**Culture conditions**

B220<sup>+</sup> spleen cells were sorted into flat-bottom microculture plates ( Falcon, Becton Dickinson, San Jose, CA) at a concentration of 10<sup>5</sup> cells/well and cultured in RPMI 1640 supplemented with 5% heat-inactivated FCS and 10<sup>–4</sup>M 2-ME. Some cultures were stimulated either with goat anti-mouse IgM (Fab<sub>′</sub>)<sub>′</sub>, fragments (Jackson ImmunoResearch, West Grove, PA) at a final concentration of 20 μg/ml or with a culture supernatant from the hybridoma FGK-45 (a gift from A. Rolink, Basel Institute of Immunology, Basel, Switzerland), which produces rat anti-mouse CD40 Abs. Both of these stimulations were performed in the presence of murine IL-4 that had been added in the form of a culture supernatant from transfected X63 cells (kindly provided by Dr. Fritz Melchers) (23). In activation experiments, cultures were harvested at days 1 and 3, and cells were analyzed on the FACScan (Becton Dickinson) (see below).

**Figure 1.** GC B cells are CD38<sup>low</sup>. Immunohistology of sequential splenic sections from C57BL/6 mice that had been immunized 14 days previously with NP<sub>−</sub>KLH. In A, C, and E, sections were stained with a rat anti-mouse B220, revealed with mouse anti-rat-IgG HRP and biotinylated PNA revealed with streptavidin-AP and fast blue. PNA<sup>−</sup> GCs are indicated by arrows, while the surrounding B220<sup>+</sup> follicles appear gray. In B, D, and F, sections were stained with rat anti-mouse B220, and a biotinylated anti-mouse CD38 was revealed with streptavidin-AP and fast blue. The B220<sup>+</sup>CD38<sup>−</sup> follicles are darkly stained, while the GCs, indicated by arrows, are a lighter gray.

**Cell viability assay**

Between 1000 and 5000 sorted cells/well were placed in Terasaki plates (Nunc, Naperville, IL) in a final volume of 10 μl medium (RPMI 1640 containing 5% FCS and 2-ME). The cultures were incubated at 37°C. At the indicated time points after the initiation of the culture, the plates were removed from the incubator, and propidium iodide (PI) was added to a final concentration of 0.5 μg/ml. Thereafter live (intact) and dead (PI-stained) cells were counted using a UV microscope. At each time point, PI was added to fresh cultures that had not previously been removed from the incubator, and between 150 and 700 cell bodies were counted.

**Immunofluorescence and flow cytometry**

NP-specific GC or memory cells, defined as IgM<sup>−</sup> IgD<sup>+</sup> NP-binding B220<sup>+</sup>, were resolved exactly as previously described (14). When a seventh parameter was needed for analysis, the cells were revealed with 11–26C (anti-IgD) biotin, 331-12 biotin, streptavidin-tricolor (Caltag, San Francisco, CA), anti-B220 phycoerythrin (PE) (RA3-6B2, Pharmingen, San Diego, CA), NP allophycocyanin, anti-IgG1 Texas Red (Southern Biotechnology Associates, Birmingham, AL), and either NIMR5/18 (anti-CD38) fluorescein or PNA fluorescein (Vector, Burlingame, CA). Dead cells were excluded with PI staining. These PI<sup>−</sup> dead cells were highly fluorescent and were therefore detectable in both the tricolor and PE channels (see Figs. 2 and 5). Ab-forming cells (AFCs) were detected using 281.2 (anti-syndecan) fluorescein or allophycocyanin. Staining was conducted as previously described (16). Cells were finally reprocessed in PBS containing FCS (3%) and PI (1 μg/ml) to exclude dead cells. Analysis and sorting were performed on a FACStar<sup>™</sup> (Becton Dickinson). B cells activated in vitro were stained with anti-B220 (RA3-6B2, Pharmingen) PE, GL-1 (anti-B7-2/CD86) fluorescein or NIMR5/18 fluorescein and analyzed on a FACScan (Becton Dickinson).
Preparation and immunohistochemical staining of splenic sections

Splenic tissues were prepared as previously described (14) and stained using either PNA biotin (Vector) or NIMR5/18 (anti-CD38) biotin followed by streptavidin-alkaline phosphatase (AP) (Southern Biotechnology Associates). Purified RA3-6B2 (anti-B220) was followed by mouse anti-rat Ig-k-horseradish peroxidase (HRP) (PharMingen). The protocol used for staining sections was as previously described (14). HRP staining was detected using the Vectastain kit (Vector), and streptavidin staining was visualized with the Vectastain AP kit III, fast blue (Vector), in the presence of levamisole.

Results

CD38 is reexpressed on memory cells after being down-regulated on GC cells

The original aim of this work was to determine whether CD38 had a role in either GC reaction or memory B cell generation. Preliminary results indicated that isotype-switched, Ag-specific B cells isolated from the spleens of C56BL/6 mice at 14 days postimmunization did not proliferate in response to stimulation with anti-CD38 Abs in the presence of IL-4 and IL-5, although IgM^-IgD^+ B cells from the same mice did proliferate (data not shown). Since the majority of Ag-specific B cells in the spleen are isotype-switched GC B cells at this time after immunization (11, 24), we assessed whether the lack of proliferation was due to the absence of CD38 surface expression. We determined that this was indeed the case, as indicated by immunohistologic staining of sequential splenic sections from mice at 14 days after primary immunization with NP13-KLH (Fig. 1, A and B) using anti-B220 in combination with either anti-CD38 or the lectin PNA (5). Strong CD38 staining of the B cell follicles was apparent, but there was virtually no overlap with the PNA^- GCs. The observation that murine GC B cells are CD38^- confirmed the recent report of Oliver et al. (21).

Having observed that GC B cells did not express CD38 on the cell surface, we subsequently addressed the kinetics of this change in expression by examining the level of CD38 on Ag-specific B cells at specific times during the immune response. This examination a 7-parameter flow cytometry system, an example of which is shown in Figure 2. Ag-specific B cells, defined as IgM^-IgD^-B220^ high IgG1^- NP-binding, were gated and analyzed for CD38 expression. At days 6 and 12 postimmunization, NP-specific IgG1^-B220^ high cells are mainly GC cells (6, 12), and the majority of those GC cells had down-regulated CD38 expression relative to the level on naive B cells. However, at day 34 postimmunization, we found a dichotomy in CD38 expression among NP-specific IgG1^- B cells. Indeed, at successively later time points, an increasing fraction of NP-specific B cells were CD38^high. Figure 3 illustrates the decrease in the number of splenic NP-specific IgG1^- cells with time (Fig. 3A) as well as the simultaneous increase in the proportion which CD38^high (Fig. 3B). A quantification of these data is given in Table I. After day 85, the percentage of NP-binding IgG1^- cells that were CD38^high plateaued at <100%, indicating the persistence of the GC B cell phenotype (see below).

CD38^- B cells retain characteristics of activated cells

The persistence of B cells with a GC phenotype for many weeks after primary immunization could represent either the persistence of GCs themselves or the generation of two phenotypically distinct types of B cells. To examine this, we sorted NP-specific IgG1^-CD38^- cells from the spleens of mice at various times after immunization and determined the proportion that were CD38^high. As shown in Figure 4, a similar fraction of CD38^- cells from days 34 to 85 postimmunization were CD38^high as were found in the spleens of mice at day 34 postimmunization (Fig. 3B). These data suggest that CD38^- B cells isolated from the spleens of mice at various times after immunization retain characteristics of activated cells.
types of memory B cells. To distinguish between these possibilities, we examined a number of characteristics of the CD38<sup>bright</sup> and CD38<sup>low</sup> Ag-specific B cells. Comparing the forward light scatter (FSC) distribution of the CD38<sup>bright</sup> and CD38<sup>low</sup> cell populations at 21, 29, and 35 days postimmunization revealed that CD38<sup>low</sup> cells always had a higher FSC distribution; this higher distribution is indicative of a larger cell size and compatible with these cells being activated (Fig. 4). Furthermore, 7-parameter staining protocols using either PNA or anti-CD38 were used in parallel at certain time points. This allowed a direct comparison of these two methods in determining the proportion of NP-binding IgG<sub>1</sub> B cells that were of GC or memory phenotype. Both approaches gave essentially the same result (Fig. 5), validating the use of the CD38<sup>low</sup> phenotype as a marker of GC B cells. Furthermore, these CD38<sup>low</sup> cells showed diminished proliferation in response to CD40-mediated stimulation in vitro (data not shown), which is typical of GC B cells and in agreement with the findings of Oliver et al. (21).

Survival potential of CD38<sup>bright</sup> and CD38<sup>low</sup> Ag-specific B cells in vitro

The reduced proliferation of CD38<sup>low</sup> Ag-specific B cells in response to CD40-mediated stimulation could result from either an intrinsic difference in the responsiveness of the two cell types or the reduced survival of CD38<sup>low</sup> B cells in culture. This latter possibility was tested by performing in vitro survival experiments. Naive B220<sup>+</sup>, CD38<sup>bright</sup>, and CD38<sup>low</sup> Ag-specific B cells (IgM<sup>-</sup>IgD<sup>-</sup>IgG1<sup>+</sup>NP<sup>+</sup>) were sorted from the spleens of C57BL/6 mice around 30 days postimmunization and cultured without deliberate stimulation. The fraction of viable cells in each culture was determined after 24 and 48 h on the basis of PI exclusion. In two independent experiments, CD38<sup>bright</sup> NP-binding IgG<sub>1</sub> B cells were found to have in vitro survival characteristics that were essentially the same as those seen in naive B220<sup>+</sup> B cells. The survival potential of CD38<sup>low</sup> NP-binding IgG<sub>1</sub> B cells, on the other hand, was significantly reduced (Fig. 6). It is likely that this difference reflects the reduced survival potential of CD38<sup>low</sup> IgG<sub>1</sub> B cells and is not a result of the cell-sorting procedure, since naive B cells stained with anti-B220 only or with anti-B220 plus anti-CD38 showed identical survival without stimulation in vitro. As an additional control, purified B cells expressing a bcl-2 transgene were cultured without stimulation and were found to survive much longer than any of the other sorted populations. The reduced survival potential of CD38<sup>low</sup> Ag-specific B cells at this time point after immunization is a further indication that these cells are in an activated state and is consistent with the phenotype revealed by FACS (Fig. 4).

CD38 expression on plasma cells in vivo

We subsequently asked whether the down-regulation of CD38 seen on GC B cells also occurred on the AFCs that develop during the response to T cell-dependent Ags. Splenic AFCs were identified on the basis of syndecan (CD138) expression (6, 12) at 8 days postimmunization with NP<sub>13</sub>-KLH, at which time the vast majority were contained within extrafollicular foci (11, 25). CD38 was observed to be down-regulated on all AFCs (syndecan<sup>+</sup>) relative to its level on naive B cells (IgM<sup>+</sup>IgD<sup>-</sup>syndecan<sup>-</sup>) (Fig. 7). Interestingly, the level of CD38 expression on AFCs appeared to decrease in parallel with the level of surface IgM and IgD. This observation is consistent with the hypothesis that CD38 down-regulation is associated with Ig isotype switch (21). Thus, in the early stages of the immune response to T cell-dependent Ags, CD38 down-regulation is common to both the intra- and extrafollicular pathways of B cell differentiation.

In vitro stimulation of B cells leads to down-regulation of CD38

An analysis of Ag-specific B cells indicated that CD38 down-regulation was a normal outcome of T cell-dependent B cell activation. Therefore, we determined whether CD38 down-regulation could be mimicked by in vitro stimulation. B220<sup>+</sup> splenic B cells were purified by FACS and stimulated with anti-IgM or anti-CD40 Abs in the presence and absence (data not shown) of IL-4. At the

Table I. CD38<sup>+</sup> Ag-specific memory B cells represent a relatively constant fraction of splenocytes despite a proportional increase

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<th>Days Postimmunization</th>
<th>NP&lt;sup&gt;-&lt;/sup&gt;Ig&lt;sub&gt;1&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt; (%) of Spleen</th>
<th>CD38&lt;sup&gt;+&lt;/sup&gt; (%) of NP&lt;sup&gt;-&lt;/sup&gt;Ig&lt;sub&gt;1&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</th>
<th>NP&lt;sup&gt;-&lt;/sup&gt;Ig&lt;sub&gt;1&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;CD38&lt;sup&gt;+&lt;/sup&gt; (%) of Spleen</th>
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initiation of culture, all B220+ cells expressed CD38 (data not shown). After 1 day, CD86 (B7-2) was up-regulated on B cells that had been stimulated with either anti-CD40 or anti-IgM, whereas there was no change in CD38 expression (data not shown). At day 3 of culture, however, both anti-CD40 and anti-IgM stimulated B cells had down-regulated surface CD38 compared with unstimulated B cells (Fig. 8). Thus, both B cell receptor crosslinking and stimulation through CD40 induced the down-regulation of CD38, indicating that the down-regulation seen in vivo was a consequence of B cell activation and was not due to changes in the environment of the B cells. In repeated experiments, stimulation through CD40 induced a less marked down-regulation of CD38 than did anti-IgM stimulation, indicating that these two stimuli have a somewhat different impact on CD38 expression. Stimulating B cells with anti-CD40 or anti-IgM in the absence of IL-4 resulted in a modulation of CD38 expression that was identical with that seen in the presence of IL-4 (data not shown), indicating that IL-4 is not critical to this process.

**Discussion**

In this report we have demonstrated that memory B cells can be distinguished from their GC precursors on the basis of the level of expression of CD38, and that this distinction can be used to monitor the development of the Ag-specific memory B cell compartment. That the CD38low and CD38 high phenotypes are indicative of GC and memory B cells, respectively, was confirmed by several criteria: Ag-specific isotype-switched CD38low cells were found to have cell surface characteristics that were similar to GC B cells.
such as the expression of high levels of B220 (6) and the ability to bind high levels of PNA. Additionally, the CD38 low cells were larger than their CD38 high counterparts as determined by FSC but had poor survival in vitro, both of which are characteristic of GC B cells. CD38 down-regulation, however, is not an exclusive property of GC B cells, since a similar alteration in expression was observed on the AFCs found in the spleen during the early stages of the immune response.

Previous studies on the formation of the memory B cell compartment determined that an immune response could be transferred adoptively to a naive recipient at 2 wk postimmunization with a hapten-carrier conjugate (3, 4), and that this response resided primarily in the PNA-binding fraction (4). After 4 wk, immunity was predominantly seen among the IgD^2 PNA-nonbinding (4) fraction. This observation presumably reflects the formation of a recirculating memory compartment during this interval. Another measure of the generation of a memory compartment is provided by a study in which the frequency of proliferating, isotype-switched, Ag-specific B cells was determined at various times after immunization with a protein Ag (10). It was found that the initial 4 wk of the response was a period of extensive proliferation of isotype-switched Ag-specific B cells; after this, only a small fraction of such cells was proliferating. The end of the period of extensive B cell proliferation presumably corresponds to the formation of the stable, resting memory B cell population. It is of interest that a small but detectable fraction of Ag-specific B cells was proliferating at up to 140 days postimmunization, a phenomenon also observed in an anti-hapten response (8) and an anti-viral response (9). In these latter cases, the proliferating cells were localized by histology. Bachmann and colleagues suggested that the persisting GC B cells were a consequence of the particular Ag used, a viral-coat protein (9). The results presented in this report indicate that such a phenomenon is not unique to viral Ags and may be a more general property of the B cell response to Ag. Using the reappearance of CD38 on IgG1^1 NP-binding B cells as a marker of the memory phenotype, we have determined that memory B cells become a discernible population by day 21. From this...
point on, the proportion of Ag-specific B cells displaying a memory phenotype continues to increase, although the fraction of total spleen that these cells represent changes little (Fig. 3 and Table I). Interestingly, this time interval coincides with that seen in a study showing that the ablation of CD4+ T cells before day 21 resulted in a failure to generate adoptively transferable B cell memory in an anti-hapten response, while development of memory was unaffected after this time point (26).

A comparison of the kinetics of memory B cell generation with that of the decline of GC B cells gives some insight into the manner in which memory is generated. Our data, showing that GC and memory B cells coexist, indicate that the generation of memory is not a sudden event triggered by an environmental signal such as the titer of Ag-specific Ig. The memory population is formed reasonably early in the response and then maintained at an approximately constant level while the GC population continues to decline. The question arises as to how the size of the memory population remains unchanged despite an ongoing GC reaction. That is, why is the output of the persistent GCs not reflected in an increase in the size of the memory compartment? There are three possible explanations as to why this might occur. The first is that the GC and memory populations are in equilibrium, so that any input into the memory population is balanced by an equal output that can take the form of death, recirculation back into the GC, or migration out of the spleen. The second possibility is that the output of these GCs is not apparent in the memory population because they have a different fate, such as becoming AFCs. The third explanation is that these are not true GCs in the sense of continuing the process of affinity maturation, but rather are structures associated with, for example, the maintenance of B cell memory. While our data do not address these possibilities directly, some observations can be made. For example, the level of V gene somatic mutation seen among secondary response B cells is equal to that found 21 days into the primary response (15, 27), suggesting that the composition of the memory compartment is fixed at more or less this time point. Thus, the GCs that persist beyond this point either do not contain somatically mutating B cells or their output does not enter the memory population. Do these cells give rise to high affinity AFCs in the bone marrow? Again the answer would appear to be no, since such AFCs are apparent by about day 10 of the response and are all high affinity by day 21, (6, 15) precluding the need for further affinity maturation. Although little is known concerning the life span of bone marrow AFCs after primary immunization, existing evidence suggests that such cells live for extensive periods without replenishment (15, 28). Collectively, these data would argue against the persistent GCs in the spleen contributing to either the memory or the long-term AFC populations. Of the possibilities raised previously, the last is that these structures are important in the maintenance of B cell memory as has been suggested on the basis of histologic studies in the rat (8) and mouse (9). The application of the cell separation techniques outlined in this report will assist in resolving this important question.

While the poor survival of the GC B cells in vitro was expected (29), it was interesting to find that purified memory B cells survived only as well as naive B cells. The reason why immunologic memory is long lived is still not clearly understood. There is debate over whether the persistence of memory is intrinsic to the memory B cells or is the result of the interaction of these cells with some component of their environment. Various groups have reported the up-regulation of the cell survival-promoting genes bcl-2 and bcl1-Xi in memory B cells (30, 31), suggesting that their expression could confer a degree of longevity to memory B cells that is not available to GC B cells. While this appears to be the case (Fig. 6), memory B cells themselves have no particular survival advantage over naive B cells. Gray and Skarwell (32) found that transferred memory was short lived in the absence of Ag. Similarly, it has been reported that the Ab-mediated depletion of nerve growth factor in a mouse resulted in the almost immediate loss of memory phenotype B cells (33). Both these latter experiments argue that factors extrinsic to the memory B cells are important in the maintenance of memory. Our data support this concept, since memory B cells survive to the same extent as naive B cells but not nearly as well as B cells constitutively expressing high levels of Bel-2.

The requirement of extrinsic signals for the maintenance of B cell memory and the observation of persistent GC phenotype B cells reported here and elsewhere (8, 9) may consequently be directly linked.

Our analysis of CD38 expression on mouse B cells during an immune response to a T cell-dependent Ag allows a direct comparison between mouse and human B cells. At first inspection, it would appear that CD38 expression is regulated in a diametrically opposite manner in these two systems. Human naive B cells are CD38- and become CD38+ upon both entry into the GC and after differentiation into AFCs, while memory B cells are again CD38- (see Ref. 20). If human B cell types are grouped according to levels of CD38 expression, then naive and memory B cells constitute one group and GC B cells and AFCs constitute another. Interestingly, the same groupings can be made for mouse B cells, although their CD38 levels are inverted. The inconsistent expression pattern on human and mouse B cells raises questions about the role of CD38 in B cell activation and differentiation. CD38 is an ectoenzyme that catalyzes both the conversion of nicotinamide adenine dinucleotide to cyclic ADP-ribose, which can mobilize Ca2+, and also the hydrolysis of cyclic ADP-ribose to form adenosine diphosphoribose (34, 35). Abs to CD38 induce a number of effects on B lymphocytes including proliferation, protection from apoptosis, and the inhibition of B lymphopoiiesis (19, 35). How these activities relate to the in vivo role of CD38 is currently unclear, although there has been some speculation that the down-regulation may result from the internalization of CD38 along with the B cell receptor (36). A consequence of this internalization may be that the second messengers generated by the enzymatic activity of CD38 could operate intracellularly. One final point to note concerning the low level of CD38 on mouse GC B cells is the support this lends to the recent observation of neoteny by GC lymphocytes (37, 38). These investigators noted that GC B cells display a number of phenotypic characteristics that are otherwise restricted to immature lymphocytes. The level of CD38 found on pro-B and pre-B cells is lower than that found on naive recirculating B cells and is similar to that of GC B cells (Ref. 18 and our unpublished observations).

In conclusion, our results have shown that CD38 is selectively down-regulated on splenic GC B cells and AFCs. However, it is reexpressed on B cells that are derived from the GC, namely memory B cells. On the basis of this observation, we have shown that the memory B cell compartment is formed during wk 3 of the response to a nonreplicating hapten-protein Ag, but that B cells with a GC phenotype persist for many weeks after the initial immunization. The resolution of GC and memory B cells is an important step in determining the criteria necessary for the transition from one differentiation state to another.

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