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Plasticity and Heterogeneity in the Generation of Memory B Cells and Long-Lived Plasma Cells: The Influence of Germinal Center Interactions and Dynamics

Kim L. Good-Jacobson*† and Mark J. Shlomchik*†

In the humoral response, short-lived plasmablasts generate an early burst of Ab that probably plays an initial protective role. Simultaneously, another arm of the response is often triggered that leads to delayed effector function but long-term protection. This arm comprises the germinal center response and its products: long-lived memory B (Bmem) cells and plasma cells (PCs). The factors that control the differentiation of PCs and Bmem cells, as well as the composition and function of the memory compartment—how it self-renews while generating rapid secondary effector function—are poorly understood. Recent work in mice and humans is beginning to illuminate these issues. We review this progress, with emphasis on events in the germinal center, especially B–T interactions, which influence the development of memory and PC compartments and on Bmem cell heterogeneity that may underlie flexibility and self-renewal of long-lived humoral immunity. The Journal of Immunology, 2010, 185: 3117–3125.

The major function of the primary immune response is to generate a robust and appropriate effector response to any foreign stimulus. In principle, the nature of this response has evolved to successfully eliminate foreign pathogens while sparing the host excessive collateral damage. To accomplish this, the immune system must balance producing a large number of effector cells targeting foreign Ags while safeguarding host cells by appropriately regulating and ultimately turning off the response. In addition to effector cells, the primary response produces Ag-specific memory cells that remain after the infection has been cleared. These cells can be reactivated upon re-exposure to the same Ag, rapidly differentiating into effector cells. In the humoral response, memory B (Bmem) cells, together with long-lived plasma cells (PCs), enhance host protection for long periods of time.

Although we understand the basic features of a humoral response (1), we still do not know the signals that invoke Bmem cell formation or fully understand what regulates the quality and quantity of the memory and PC populations. Why do different infections or types of vaccines generate different lengths and qualities of immune protection? How is the Bmem cell population maintained in the face of recurring infections? Are there different types of memory cells geared to be functionally different? Addressing these questions and gaining a deeper understanding of B cell memory will likely lead to a better understanding of the success of vaccines to different pathogens and, therefore, will inform vaccine design in the future.

The most basic definition of a Bmem cell population is that it is Ag experienced and persists in the absence of the immunizing agent. Two other qualities often attributed to Bmem cells are that, compared with naive cells, they respond more rapidly during a secondary response and they have a higher affinity for Ag. However, the latter attribute is associated with cells that have participated in a germinal center (GC) reaction, suggesting that GC B cells are precursors to Bmem cells. Although it seems clear that GC cells can be precursors to Bmem cells, it is uncertain whether all Bmem cells have derived from GC precursors.

There are two broad types of humoral responses: T independent (TI) and T dependent (TD). Although TI responses can generate abortive GCs (2, 3), TD responses typically are associated with fully developed GCs. In the TD GC, Ag-specific B cells undergo numerous rounds of division and can undergo somatic hypermutation (SHM), class-switch recombination, and affinity selection (4–7). Therefore, the GC response endows this population of Ag-activated B cells with qualities, such as higher affinity for the Ag and a relevant Ig isotype, which should result in more efficient clearance of Ag. However, the GC itself is transient, and GC cells themselves are not known to mediate effector functions. Instead, some GC B cells differentiate into PCs or Bmem cells, both of which persist after the primary response has subsided and directly or indirectly provide effector function.

Abbreviations used in this paper: AFC, Ab-forming cell; Bcl, B cell lymphoma; BLyS, B lymphocyte stimulator; Bmem, memory B; FDC, follicular dendritic cell; GC, germinal center; NP, (4-hydroxy-3-nitrophenyl)acetyl; PC, plasma cell; PD-1, programmed death-1; PNA, peanut agglutinin; SAP, signaling lymphocytic activation molecule-associated protein; SHM, somatic hypermutation; TD, T dependent; Tg, transgenic; TI, T independent; WT, wild-type.
In contrast to the more open-ended definition given above, the classical description of B<sub>mem</sub> cells requires that they have undergone affinity maturation and selection. This led researchers to conclude that the GC environment is necessary for B<sub>mem</sub> cell formation. Unfortunately, the arguments supporting GCs as the sole location in which B<sub>mem</sub> cells are formed can be circular (reviewed in Refs. 8 and 9). Nevertheless, numerous studies demonstrated that in certain immunodeficiencies in which a functional GC response is abrogated, the B<sub>mem</sub> cell pool is altered in number, affinity maturation, and/or ability to generate secondary responses. These immunodeficiencies are also marked by a decrease in circulating Ab and long-lived PC numbers (Table 1). Therefore, although it is likely that long-lived cells can be formed in the absence of functional GCs, the GC microenvironment plays an important role in producing a classically defined, fully formed B<sub>mem</sub> cell population, as well as long-lived PCs.

The role of T cells in GC responses

Under normal circumstances, fully developed GCs require T cell function. B cell–T cell interactions occur throughout the humoral response, either at the border between the T and B cell zones or within the follicle itself, and the nature of these interactions changes over time. Several such interactions and subsequent signaling are vital in the early stages of the response. In the absence of signaling downstream of CD40 or ICOS, for example, GC initiation is disrupted. Conversely, in the absence of B cells, T cells in the follicle (T follicular helper cells [TFHs]) are also absent (38, 46). It remains unclear whether the absence of T<sub>FH1</sub> in B cell-deficient animals is due to a complete inhibition of differentiation into T<sub>FH1</sub> or whether differentiation can occur, but the phenotype of the T<sub>FH1</sub> subset requires B cells for maintenance (47). In this vein, the absence of signaling lymphocytic activation molecule-associated protein (SAP), which is expressed on T cells, directly impacted the duration of B cell–T cell interactions (48), leading to diminished T<sub>FH1</sub> development and expansion. Therefore, when the quality of signaling downstream of early B–T interactions is impaired, formation of GCs is also disrupted.

After a GC has formed, T<sub>FH1</sub> critically affect the outcome. T<sub>FH1</sub> influence B cells via two mechanisms: direct cell contact and cytokine secretion. T<sub>FH1</sub> can control the initiation and termination of humoral responses through the expression of positive and inhibitory ligands and receptors, such as CD40L, ICOS, programmed death-1 (PD-1), CD28, and CTLA-4, thereby regulating the extent of the response and possibly the quantity of effector cells (10, 11, 13–15, 17, 23, 49). T<sub>FH1</sub> also affect the quality and types of B cells produced (e.g., via the well-documented effects of T<sub>FH1</sub>-derived cytokines in influencing B cell survival, proliferation, isotype switching, and differentiation) (27, 50–53). In addition, the availability of T<sub>FH1</sub> cell help and the expression of FasL (CD178) regulate selection within the GC, affecting the affinity of resulting long-lived PCs (28, 33, 52). Although it is evident from these studies that T cells can affect GC dynamics, are they involved in the decision of GC B cells to differentiate into B<sub>mem</sub> cells or PCs?

How is B<sub>mem</sub> cell and Ab-forming cell differentiation regulated?

How is GC B cell differentiation regulated? Is there an actual fate decision per se, or is differentiation the result of an accumulation of factors that overrides a death or GC-retention signal?

One hypothesis is that a master regulator of transcription directs the fate of a B<sub>mem</sub> cell. B cell lymphoma (Bcl)-6 is required to induce the GC transcriptional program (54), and B lymphocyte-induced maturation protein-1 plays a similar role in PCs (55). However, despite extensive investigation of gene-expression profiles of human and murine B<sub>mem</sub> cells (56–59), no single deterministic transcription factor for B<sub>mem</sub> cells has come to light. In fact, unlike the comparison between naive cells and GC B cells or PCs, the transcriptomes of naive and B<sub>mem</sub> cells are very similar (56–59).

There have been a number of proposals for factors that may determine B<sub>mem</sub> cell differentiation. Researchers suggested that Bcl-6 (56) or CD40 (57) can regulate B<sub>mem</sub> cell fate decisions, although these proposals have not been borne out (58). Alternatively, affinity selection, in some cases by follicular dendritic cell (FDC)-bound immune complexes, has also been suggested to determine the fate of B cells, with high-affinity cells directed to differentiate into PCs (63, 64). However, subsequent studies demonstrated that higher-affinity cells were undergoing more expansion early in the response, rather than being actively directed into the PC-differentiation pathway (65). Furthermore, in the absence of immune complexes, GC responses and B<sub>mem</sub> cells still form robustly (66); therefore, signals from immune complexes on FDCs are likely not involved in the fate decision per se.

Given the failure to find deterministic signals that direct memory cell formation from a GC precursor, it is possible that such a singular directive does not exist. Hodgkin and colleagues (67–69) hypothesized, based on a series of in vitro experiments and computer models, that a uniform population of B cells is intrinsically able to commit to any one of several different fates postactivation (such as division, differentiation, or death) based on stochastic factors, influenced by integrated external and intrinsic signals. For example, B cells stimulated with CD40 will not all behave in the same manner. Some remain undivided, some divide, and a proportion differentiate. Hodgkin and colleagues proposed that at each division, some cells commit to each of these fates, but changing intrinsic or extrinsic factors in the response only affect the probability of one event occurring over the other. In this model, the ability of a B cell to differentiate is part of its cellular make-up and can be influenced in a probabilistic sense, but not determined, by extrinsic stimuli.

In the case of the GC, we suggest that T cell-derived signals regulate the quantity and quality of the B<sub>mem</sub> cell population by influencing B cell behavior. The role of signals in producing a fully formed B<sub>mem</sub> cell population can be subdivided into those that are important for GC homeostasis and those that may influence B cell differentiation itself. Indeed, a number of recent publications investigated the effects of specific genetic and/or cellular defects on GC B cell dynamics (progression, cell death, and size), as well as differentiative outputs of GCs (i.e., B<sub>mem</sub> cells and PCs).

Dynamics of GCs and the effect on long-lived populations

In this regard, it is important to recognize that the output from the GC seems to change over time. At early stages, the reaction seems to generate a substantial population of memory phenotype B cells, which have been identified as early as day 7 postimmunization (70); this observation led to the plausible proposal that some early memory cells derive indepen-
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<td>Secondary IgG1 absent</td>
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<td>Early (days 1–3) injection: number of mutations in GC cells reduced.</td>
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<td>Decreased AFC and affinity of AFC. Class-switch was impaired.</td>
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<td>Ab was decreased, and class-switch was impaired.</td>
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<td>Increased and sustained GC, which was CD40L dependent.</td>
<td>Increased and sustained GC, which was CD40L dependent.</td>
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<td>Increased numbers of AFCs; retention of low-affinity splenic AFCs; BM AFC affinity was similar to WT.</td>
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<td>Produced normal levels of IgM and IgG1 until day 14. IgG1 levels decreased over time with low long-lived IgG1 AFCs. IgM remained strong in primary and secondary. Low-affinity IgG1 Ab produced in primary and secondary responses.</td>
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AFC, Ab-forming cell.
A variety of mutations disrupt the GC

Given the complex T–B interplay required to generate and then sustain a GC response, it is not surprising that a large number of mutations affecting molecules and cells critical to the immune system disrupt the GC reaction, to at least some degree. Although each of these mutations has its own distinct phenotype, it is interesting to consider them with respect to the timing and nature of the defects with which they are associated. One category of such mutations blocks signals and/or cellular interactions critical for the formation of GCs or the very early events involved in GC development. Mice deficient in Bcl-6, SAP, CD40, or CD40L do not form GCs (10–12, 20, 39) (Table I). $B_{\text{mem}}$ cell numbers, as well as long-lived PCs and Ab titers, are also markedly diminished in these animals, consistent with the lack of GC formation. Similarly, human patients with deficiencies in CD40, CD40L, ICOS, and SAP have dramatically decreased numbers of switched $B_{\text{mem}}$ cells. In contrast, they often have normal or slightly reduced numbers of mutated IgM$^+$CD27$^+$ B cells, with CD27 used as a marker for human memory cells (8). In mice, low-affinity $B_{\text{mem}}$ cells can be formed in the absence of Bcl-6 (39). Taken together, these data suggest that early signaling events, which occur before the formation of a functional GC, are sufficient to induce a degree of $B_{\text{mem}}$ cell formation (Table I). However, in the absence of these signals, the numbers of $B_{\text{mem}}$ cells are nonetheless markedly reduced, presumably because a GC environment in which $B_{\text{mem}}$ cell precursor expansion takes place is absent. In all of the mutants in this category, isotype-switched long-lived Ab responses were severely impaired, as expected from the lack of a GC response.

Another category of mutations is at least somewhat permissive for GC formation but seems to block GC progression or maintenance. These mutants that affect late GC responses tend to show differential regulation of $B_{\text{mem}}$ cells and PCs, with generally normal or even increased numbers of memory cells but decreased numbers of PCs (Table I). For example, phospholipase Cγ2 (41) and CD21/35 (42, 72) are vital for GC B cell survival but not formation. Similarly, mice expressing mutant CD19 alleles form GCs, but these GCs fail to mature (45), and there are significant defects in the long-term Ab response. Although it was originally thought that ICOS deficiency completely blocked the GC response (13–15, 17, 18), ICOS seems to be in the middle of these two categories. More detailed research showed that GCs do form in the absence of this molecule, albeit to a much lesser degree; ICOS blockade induced during the GC response leads to a reduction in $V_{\text{m}}$ mutations in $B_{\text{mem}}$ cells but not the total numbers of memory cells themselves (16, 19). Importantly, total Ab responses and particularly high-affinity Ab were reduced by such treatment. ICOS may act, in part, to sustain CD40L expression by T cells, which, in turn, is required to maintain CD40 expression on B cells (16). In the absence of signaling through receptors of T cell-derived cytokines, GCs can form, but their frequency is decreased in the late response. In the case of IL-4, there was a 3-fold reduction in the frequency of GC B cells in mesenteric lymph nodes 2 wk postinfection with an intestinal parasite (25), whereas in lymph nodes responding to (4-hydroxy-3-nitrophenyl)acetyl (NP)-OVA infection, there was a decrease in the frequency of GC B cells in IL-4-deficient animals only at days 14 and 21 (26). IL-21 acts directly on GC B cells to maintain Bcl-6 expression; without it, the GCs dissipate later in the response (28, 29). Still other molecules are important in the maintenance of even later stages of the GC response, suggesting that constant B–T interactions are important for maintenance of GC, although the proteins responsible change as the GC evolves. Our recent research demonstrated that the absence of PD-1 resulted in increased apoptosis in the late GC, as well as smaller GCs at these time points (24). Although the numbers of cells of a Tfh phenotype increased during the late GC response in the absence of PD-1 signals, they produced less IL-4 and IL-21 directly ex vivo. This correlated with a decrease in survival of GC B cells, similar to the IL-4Rα and IL-21 gene-targeted mice (24, 25, 28, 29). Therefore, B and T cells interact to direct the formation of GC responses, as well as the maintenance of the Tfh phenotype (28, 29) and, as a result, the maintenance of the late GC response.

Quality of $B_{\text{mem}}$ cells and quantity and quality of PCs are determined in the late GC response

Although numbers of $B_{\text{mem}}$ cells are not greatly reduced in the face of mutations that block GC progression, the affinity of the resultant memory and/or PC populations can be affected, depending on the mutant (Table I). The process that controls selective enrichment of high-affinity cells in the GC population and their subsequent recruitment into the long-lived populations could be influenced by several factors. In the absence of competition between cells of varying affinities, low-affinity GC B cells are intrinsically more prone to undergo apoptosis compared with high-affinity B cells (73). The presence of competing high-affinity cells also suppresses the response of low-affinity cells, although the mechanisms underlying this are unknown (74). When the balance between pro- and antiapoptotic molecules downstream of the BCR is artificially tipped toward survival, such as in B cell leukemia-xL transgenic (Tg) and Bim-deficient mice, there is an accumulation of low-affinity $B_{\text{mem}}$ cells (6, 35). In contrast, in Fas-deficient mice, B cells continue dividing within the GC, undergoing more rounds of SHM than usually occur in normal mice, resulting in a more mutated $B_{\text{mem}}$ cell population (33, 34). Administering
CD40L-blocking Ab in the late GC response suppresses this accumulation, suggesting that extension of the GC response itself was responsible for the alteration of the memory population, rather than a direct effect of the absence of Fas signaling in B\textsubscript{mem} cells (33).

The availability of T cell help provides further extrinsic modulation of selection. In the absence of signals through IL-21 (28, 29), there is an accumulation of low-affinity B\textsubscript{mem} cells. In contrast, in the absence of PD-1 (24) or complement receptor 2 (43) signaling, the affinity of the B\textsubscript{mem} cell pool seems to be relatively unaffected, but the remaining long-lived PCs are of higher affinity than the wild-type (WT) controls. In the case of PD-1 deficiency, this could be related to the partial reduction of IL-4 and IL-21 found at late GC time points (24). Although the results from IL-21- and PD-1-deficient animals may seem contradictory, we hypothesize that the difference can be explained by the differing degrees of T cell help available in the two systems (Fig. 1). In the complete absence of IL-21, low-affinity cells are produced in the initial phase of the response. As a result of the lack of IL-21 signals, Bcl-6 expression is not maintained in GC B cells, and they exit from the GC too early to produce substantial numbers of high-affinity cells. As a result, low-affinity post-GC (memory phenotype) cells accumulate; in fact, there is an excess of such cells (28). This could reflect more that the process was terminated prematurely rather than a direct effect on selection per se. In contrast, in the absence of PD-1 signaling, IL-4 and IL-21 production was reduced, but not absent; this was most evident at later time points (24). When low- and high-affinity cells are formed, but there is reduced T cell help, competition for T cell help could result in high-affinity cells outcompeting the low-affinity cells. This idea is supported by data showing that when competition with high-affinity cells is reduced, even very low-affinity cells are capable of forming GCs and entering the B\textsubscript{mem} cell pool (75). Because PCs are the main output of the late GC reaction, the net result is seen within the PC compartment, which is smaller and particularly lacking in lower-affinity PCs when PD-1 signals do not occur (24). Therefore, it is possible that the levels of cytokines, and possibly other undefined T cell-derived signals, can affect the quality of the long-lived populations by affecting selection of high-affinity GC precursors.

Integrating these findings, we propose a model for how a population of B\textsubscript{mem} cells is formed. After each division, an activated B cell can continue dividing, or it can exit the cell cycle and differentiate into a memory cell or PC. This can occur within or outside of a GC. However, this fate decision is regulated in a stochastic fashion by the microenvironment, with some signals [e.g., those that downregulate Pax5 (76)] favoring PC over memory cell commitment, or vice versa. Within the GC, cells expand and mutate; although low- and high-affinity cells may differentiate into B\textsubscript{mem} cells, competition for T cell help favors survival of the fittest, presumably the high-affinity cells (Fig. 1). Therefore, T cells and their products influence the magnitude and quality of the response, as well as the ratio of B\textsubscript{mem} cells/PCs, in part depending on when during the GC response individual mediators and mutations exert their effects (Fig. 1).

Subsets of B\textsubscript{mem} cells

Defining subsets of B\textsubscript{mem} cells requires the phenotypic, rather than functional, identification of B cell memory, something that until recently has mainly been pursued using cells from human tissues or blood. Studies of human B\textsubscript{mem} cells have long used CD27 as a marker of B\textsubscript{mem} cells, although it subsequently was found that there is a small subset of CD27\textsuperscript{+}, isotype-switched B cells (77–79). The notion that CD27 marked human B\textsubscript{mem} cells derived initially from the finding that this subset contained the vast majority of isotype-switched and somatically mutated B cells, which, strictly speaking, only indicates that the cells have undergone prior activation.

Heterogeneity within the B\textsubscript{mem} cell pool

Although, in general, B cell memory has been referred to in monolithic terms, the B\textsubscript{mem} cell population is actually phenotypically and functionally heterogeneous. The identification of such diversity within the B\textsubscript{mem} cell pool raises additional interesting questions about the signals that influence B\textsubscript{mem} cell formation. How would variations within the memory compartment arise? In fact, there are almost no data that speak to this question. However, we speculate that in much the same way that stochastic interactions with environmental cues could dictate GC cell fates in general, diverse experiences and timing could result in variation of B\textsubscript{mem} cell properties. Specifically, it is likely that the specific nature of the components of the primary response alter the types of B\textsubscript{mem} cells produced. These alterations may include the type and amount of the infectious agent, route of infection, number of precursor cells, and types of T\textsubscript{H} cells (and thus, cytokines).
memory cells. The presence of mutated IgM memory cells in mice has long been known (84), but these cells are understudied. We showed that such cells are common in immune mice that received small numbers of NP-specific B cells with the B1-8 knockin H chain; such IgM memory cells retained some dependence on B lymphocyte stimulator (BLyS) for survival, unlike their IgG1-expressing counterparts, indicating functional heterogeneity (85). Recently, the existence of murine IgM memory cells was confirmed by use of an AID-Cre YFP-reporter mouse, which demonstrated that IgM and switched Ag-experienced B cells are formed in response to immunization with sheep RBCs or NP-chicken gamma globulin (71). Transfer of IgM+YFP+ (putative memory) cells induced GC formation in a secondary response, whereas transfer of isotype-switched YFP+ cells induced a predominantly plasmablast response, leading the investigators to conclude that the heterogeneity seen in Ig isotypes directly relates to different functions during a secondary response. Whether this was purely a correlative finding or whether the isotype differences were mechanistically responsible for the different responses of IgM and IgG Bmem cells was not determined; however, differences in IgM and IgG1 cytoplasmic tails were hypothesized to account for the rapid differentiation into Ab-forming cells by Bmem cells (86).

In addition to differences between switched and unswitched isotypes, further heterogeneity lies in functional differences of memory cells with different (IgG, IgA, and IgE) isotypes. The importance of producing different isotypes lies in the ability of the immune system to effectively respond appropriately to a multitude of foreign Ags and at different anatomical sites, such as by producing IgA in mucosal tissues. Interestingly, studies of human cells show that multiple subsets of TFHs, as defined by differential production of cytokines, can be induced in the presence of the same cytokine milieu (87). Furthermore, isolation and study of B cell–T cell conjugates demonstrated that the same response could induce TFH subsets, as defined by distinct patterns of cytokine production, which, in turn induced B cells with diverse, corresponding isotypes (26). Although more work is required to explore the presence, timing, and location of TFH subsets during an immune response, it is possible that they play a role in producing diversity of the Bmem cell pool, inducing switching of most B cells to one isotype, as well as producing some B cells of other isotypes, allowing for plasticity during the secondary response.

**Phenotypic differences within the memory compartment**

Comparisons of human naive, IgM, and switched Bmem cells demonstrated that IgM and switched Bmem cells are genotypically and phenotypically similar and behave similarly in culture to various stimuli (56). Although they express many of the same proteins, the abundance of some of these proteins does differ,
In addition to parameters that affect GC dynamics, other fine-tuning mechanisms, such as selection of affinity variants and control of Bmem cell numbers postexit of the cell cycle, exist to shape the Bmem cell repertoire. These processes all converge to form an optimized, yet phenotypically and functionally diverse, Bmem cell population.

Conclusions
To survive, an organism must be able to respond to multiple pathogens and appropriately adapt to variations in Ag stimulation and availability and type of T cell help. Therefore, heterogeneity in the Bmem cell pool would be adaptive with respect to the variety of different infectious agents, recurring infections, and/or evolution of viruses over time. Furthermore, to provide protection over the lifetime of the host, the Bmem cell pool must also persist in the face of what could be many antigenic challenges. Diversity that includes some cells with more self-renewal capacity or ability to re-enter the GC reaction and others with a greater tendency to quickly differentiate into plasmablasts would be one way to ensure this. The next phase of investigation into Bmem cell development may include testing of whether different infections, through different dynamics of the response and/or different innate immune signals, affect the make-up of the Bmem cell pool. If so, it may help to explain why some vaccinations and infections induce memory over the lifetime of the host, whereas others do not.

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