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Current Opinion in Immunology

What B cell memories are made of☆

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In many ways, memory B cells represent the ultimate outcome of humoral immunity. Many of these cells express exceptionally high affinity antigen-specific B cell receptors for antigen, and these cells are a critical source of the long-lived plasma cells that secrete protective serum antibodies to protect against secondary exposure to pathogens and other life-threatening antigens. Evidence is now emerging that not all memory B cells are created via the same cellular pathways and molecular events. Similarly, it is becoming clear that different memory B cells can take on different functions, with some producing IgM rather than IgG antibodies upon reactivation, and others preferentially producing plasma cells rather than additional waves of memory B cells. With this review, we discuss the conceptual ides and early studies surrounding early work on B cell memory, then discuss the many pathways and functional attributes of subpopulations of memory B cells and current approaches to characterize these cells directly.

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Current Opinion in Immunology 2019, **57**:58–64

This review comes from a themed issue on **Lymphocyte development and activation**

Edited by **Wasif N Khan** and **David Allman**

For a complete overview see the Issue and the Editorial

Available online 9th March 2019

https://doi.org/10.1016/j.coi.2019.01.003

Introduction

Back in graduate school, we often became engaged in conversations about various facets of immune system development and function. This was an exciting time; there was much to learn about what was known and (more importantly) what was not known, and ad hoc conversations in the lab or the hallway were often ideal for learning and throwing around ideas. One topic that often came up revolved around the question of B cell memory. Certainly, we thought, much would be known about these highly important cells. Certainly, it was understood, at least at a reasonably detailed level, how these cells were unique compared to naïve B cells, and why they are able to generate antibody responses with faster kinetics and a far greater magnitude than their naïve counterparts. And certainly, these cells could be readily identified and manipulated due to their expression of unique surface molecules.

But this was not the case. Instead, we learned that very little was known about memory B cells or why secondary humoral immune responses are faster and more protective than initial responses to a given foreign antigen. Indeed, thinking about B cell memory was a much more theoretical endeavor at that time. What is memory anyhow, we were asked. Are memory B cells really different than primary naïve B cells? Maybe memory is mainly a numbers game, meaning that faster and stronger secondary responses reflected increased numbers of antigen-specific B cells, cells that had been generated through clonal cell division during the primary response, rather than qualitatively distinct cells. More cells to respond beget a stronger response. But there had to be more to it. What about class switching and affinity maturation? At least some of these processes could be said to result in qualitative distinctions between naïve and antigen-experienced B cells. These thoughts and issues established a framework for thinking about how the fundamental attributes of antigen-experienced B cells and how these factors contribute to the mysterious process of humoral immunity and B cell memory.

Perhaps, we should also raise the question: Why consider the problem of B cell memory in the first place? As discussed previously by Hedrick [1], if a main objective of immunological research is to understand why certain infections are cleared while others are not, we must admit that we have still only scratched the surface. We would argue a similar statement can be made for the question of why some infections lead to protective immunity, while others do not. Likewise, one might ask why are some vaccines are quite effective, yielding long-lived highly protective immunity, while others are generally short-lived and not so effective. Despite our increasingly detailed understanding of immune system development and function, no clear answers have emerged for this ever-perplexing question.

Another long-standing question concerns the rapid kinetics of secondary or memory antibody responses.

☆ This work was supported by NIH grants R01-AI139123 and AI113543 to D.A and R01AI122448 to M.M.T.

One potential mechanism for this phenomenon rests on the idea that memory B cells are intrinsically able to generate antibody-secreting cells faster and with fewer inputs compared to virgin B cells. While evidence is mounting that memory T cells are able to engage transcriptional machineries needed to initiate relevant effector functions with ease relative to their inexperienced counterparts [2], the mechanistic basis for rapid secondary antibody responses remains poorly understood. A chief reason for this deficit is the previous lack of established strategies to isolate sufficient numbers of memory B cells to address this issue directly. Here a second related layer of complexity is the possibility of functional heterogeneity. Indeed, as laid out below, it would be erroneous to assume that all memory B cells react similarly to antigenic stimulation, as evidence is mounting that some memory B cells are predisposed to generate antibody secreting plasma cells, whereas others may serve mainly to produce additional memory cells. Hence our objective here is to review past and recent breakthroughs concerning B cell memory with the hope of highlighting some of the remaining knowledge gaps in this field.

B cell memory as a numbers game

Let us first consider the quantitative angle. As mentioned above, a comprehensive understanding of B cell memory should incorporate quantitative as well as qualitative differences between naïve and antigen experienced B cells. So, what are some of the important quantitative metrics to consider. First, primary immunization is likely to result in greater frequencies of antigen-specific B cells. Using a clonal *in vivo* limiting dilution system, Klinman and coworkers showed that precursor frequencies for influenza hemagglutinin (HA)-specific B cells increase 10-fold after immunization of mice with inactivated virus [3•,4]. Importantly, this increase is maintained long-after primary immunization, with the antibody HA-specific repertoire becoming rather stable over extended periods [3•], suggesting that the memory B cells induced in these experiments are indeed long-lived. Antigen-induced increases in precursor frequencies for antigen-specific B cells are the result of multiple waves of clonal proliferation, both during the first few days of a primary antibody response, and subsequently via additional multiple waves of highly regulated proliferation and selection events within germinal centers (GCs).

A process that has garnered intense interest concerns the propensity for the average affinity of antibodies to increase during the course of the primary response. This process, termed affinity maturation, was originally established by the classic studies of Eisen and Siskind [5]. Affinity maturation is the combined result of the somatic hypermutation (SHM) of Ig variable regions, mediated by the cytidine deaminase Activation-induced Deaminase (or AID), and the T-cell-dependent selection of B cells based on their improved ability to compete for what remains of the immunizing antigen [6,7]. Consequently, high-affinity clonal variants of activated B cells are preferentially selected for, allowing for general increases in the affinity of resulting plasma cells and memory B cells and the antibodies they produce. Notably, AID is also an essential enzyme for class switch recombination, as AID-deficient animals experience neither SHM or class switch recombination [7].

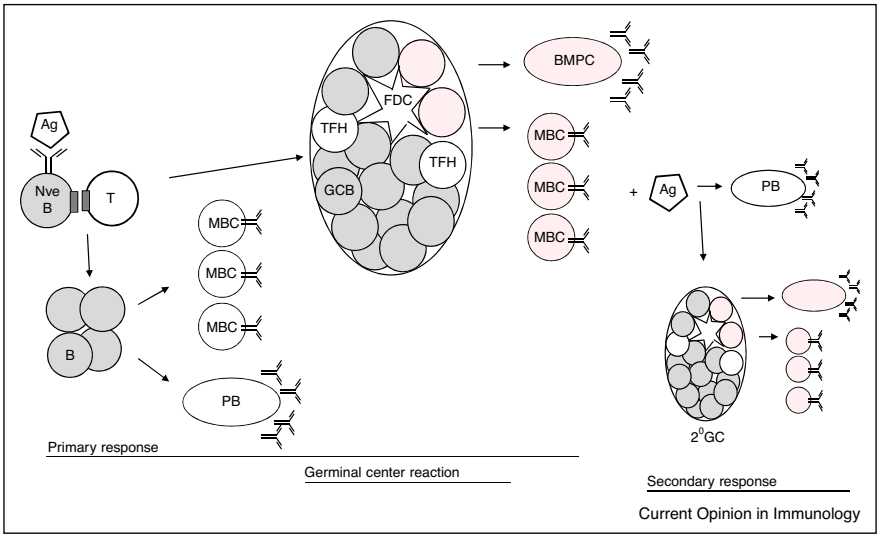
Because of the heroic studies of Jacob *et al.*, it appears that the bulk of SHM and selection events occur within GCs [8•]. Although GCs were originally thought to be seeded by small numbers of clonally related activated B cells, recent work reveals that GCs are seeded by substantial numbers of clonally diverse cells, with the additional possibility that persisting GCs may recruit additional nascent clones throughout the lifetime of an individual GC [9]. Thus, throughout the primary response activated B cells have the opportunity to exploit several mechanisms that alter key quantitative features of antigen-specific B cell populations, resulting in increased numbers of antigen-experienced cells enriched for clonal variants bearing especially high affinity antigen-specific receptors.

Therefore, we must consider at least two quantitative metrics that set antigen experienced B cells apart from their naïve counterparts: increases in numbers of cells with the potential to respond, and increases in affinity of the BCR for antigen.

Qualitative distinctions and GC-independent pathways

So what are some of the qualitative distinctions that can be made between memory and naïve B cells and among different populations of memory B cells? One process that cannot and should not be ignored is heavy chain class switching. Classic models of humoral immunity focus heavily on T cell driven GCs as the chief source of IgG^+ memory B cells and long-lived IgG-secreting plasma cells (see Figure 1). Certainly, the unique mutational and selection events within GCs together with the AID-dependent switching of subclones from IgM to IgG are a central feature of effective antibody responses. However, it is important to recall that CSR may not be strictly reliant on the T cell-dependent GC microenvironment. Indeed, a substantial fraction of the IgA antibodies produced in the gut arise independently of T cells and GCs [10]. Moreover, whereas it is relatively easy to induce isolated splenic B cells to undergo CSR *in vitro*, inducing SHM *in vitro* in these cells has been far more challenging, raising the possibility that unknown GC-restricted AID cofactors are needed for SHM but not CSR. However, in this regard, we should also emphasize that SHM may also occur outside of GCs in unique cellular foci that arise during robust inflammatory responses such as in the

Figure 1



Schematic of generation of B cell memory in a T-dependent response. The T-dependent B cell response is initiated when a naïve B cell (Nve B) recognizes antigen via the B cell receptor and receives cognate $CD4^+$ T cell (T) help. These B–T interactions occur near the B–T boarder in the spleen, lymph node and other secondary lymphoid organs. B cells proliferate and expand, give rise to extrafollicular foci of antibody producing short-lived plasmablasts (PB) that are critical for immediate protection and to germinal center independent memory B cells (MBC). Some activated B cells migrate to the B cell follicle and form germinal center (GC) reactions. Here, B cells proliferate, undergo B cell receptor somatic hypermutation and interact with T follicular helper cells (T_{FH}) and follicular dendritic cells (FDC). Most die of apoptosis, but some are selected into the bone marrow-destined plasma cell (BMPC) or MBC pools. BMPC are long-lived and secret antibody that is a first line of protection upon antigen re-exposure. MBCs generated before and during the GC reaction are diverse in phenotype and function. MBCs are poised to respond with a rapid and effective secondary response to new infection with the same or structurally related pathogen. Some MBC differentiate into antibody-forming plasmablasts while others form new secondary GCs that in turn generate new specificities of BMPC and MBCs.

response against *Salmonella* and in chronic responses to self-antigens [11,12].

Additional recent work has expanded our view to include clearly GC-independent processes with substantial relevance to memory B cell induction and long-lived immunity. In a landmark paper, Alugupalli and Gerstein showed that B1 B cells can mediate long-term protection against the spirochete *Borrelia hermsii* [13,14•], an activity likely mediated by long-lived IgM^+ memory B cells that arise without GC induction. Hence these observations imply additional and relevant pathways beyond the extrafollicular GC-independent pathway diagrammed in Figure 1. More recently Pape *et al.* characterized IgM^+ memory B cells induced to a foreign complex protein antigen [15•], and other workers have shown unequivocally that memory B cells can arise independently of GCs [16•,17]. Indeed, by applying a clever BrdU pulse-chase approach to B cell receptor transgenic mice, Weisel *et al.* uncovered evidence that the bulk of memory B cells arise before or coincident with initiation of GC responses [18•]. It will be interesting to see whether this idea applies to polyclonal repertoires and whether the timing of memory B cell formation can be altered with different antigens, immunization routes, and adjuvants.

Direct isolation of memory B cells; from inference to direct characterization

Before the flow cytometer took hold as a central instrument, cellular immunologists used monoclonal antibodies with antibody-mediated complement lysis to define subpopulations of lymphocytes. For instance, Bruce and Sprent used antibody-mediated complement lysis to define unique cell surface antigens expressed by naïve and memory B cells. This work led to the idea that, unlike naïve B cells, memory B cells lack surface expression of a surface antigen identified with the antibody called J11d [19]. This conclusion stemmed from data showing that, unlike primary responses, memory activity was maintained in B cell populations after treating B cells with J11d plus complement before transfer into adoptive hosts. It is now known that J11d is one of several monoclonal antibodies specific for the surface glycoprotein Heat-stable Antigen (or CD24). Klinman *et al.*, using flow cytometry, extend these observations by showing that the majority of $J11d^{low}$ splenic B cells mounted robust secondary responses but poor primary responses, while remarkably $J11d^{hi}$ B cells did the converse [20]. These observations lead to the prediction that memory B cell pools are fed by a distinct subset of naïve B cells that function as memory B cell precursors. Importantly, whereas this latter idea never really took hold, it did

emphasize the need to develop strategies to examine and manipulate memory B cells directly.

In considering how to look for memory B cells, it had become increasingly reasonable to propose that individual memory B cells possess intrinsic functional capabilities distinct from naïve B cells and beyond the expression of different immunoglobulin heavy chain isotypes. Starting with hypothesized specialized memory functions, several investigators compared expression of proteins important to these functions between naïve and antigen experienced B cells in mice. Similar studies were done in humans, where expression of CD27, which marks somatically mutated B cells, is commonly employed as a memory marker [21•]. The results of these studies revealed memory B cells in mice express different levels of markers of activation, survival, B cell receptor signaling modulation, adhesion and migration such CD80, CD86, CD95, CD62L, CD73, and FcRH4 [22–24].

In a complementary approach to determining functional capabilities of memory cells, other groups compared global gene expression between naïve and memory B cells [25,26,27•]. In mice, splenic naïve and memory B cells were isolated before and after immunization with haptenated carrier antigen. As rigorously defined memory markers were lacking at this stage, memory cells were defined as antigen-specific cells that had responded to antigen that had persisted for $\geq$10 weeks post immunization. These results revealed that, overall, gene expression between naïve and memory B cells is remarkably similar. However, consistent differences were seen in expression of genes encoding B7 family members, cell signaling molecules, adhesion and migration molecules and stem cell related genes. By drilling down on these data, it has become clear that memory B cells can be distinguished from antigen inexperienced B cells through their expression of an appreciable array of surface proteins. These include the immune modulators PD-L1 and PD-L2, the adenosine receptor A2a, the TACI cytokine receptor, the leukemia inhibitory factor receptor, and once again and the adenosine-producing ecto-enzyme CD73 and the B7 family members CD80 and CD86.

Memory B cell subsets and what they may do

As mentioned above it has long been recognized that memory cells can differ from one another with regard to expression of IgM versus IgG or another heavy chain class, and these cells may differ with respect to any one of a variety of functional attributes [28,29,30•]. The notion that memory B cells may experience differential expression of CD80, CD73, and/or other surface markers raises the question of whether any potential unique subpopulation of these cells would also possess unique functions. Indeed, CD80, PD-L2, and CD73 are each expressed heterogeneously among memory cells, and together define at least five phenotypic subsets of memory B cells [31]. These subsets are independent of immunoglobulin isotype expression, as immunoglobulin class switched and unswitched B cell receptors are found in each group. The five subsets are CD73-single positive, CD73/CD80/PD-L2 triple negative, CD80-PD-L2+, CD73/CD80/PD-L2 triple positive and CD80+PD−L2+CD73−. These cells differ in abundance of class-switched cells, proliferative histories, BCR mutational content and BAFF-dependence and function in the secondary response, forming a spectrum from more naïve-like to more memory-like cells. Moreover, these cells appear to form at different times after immunization [18•]. More naïve-like cells preferentially give rise to GCs upon antigen re-challenge, while the more memory-like rapidly differentiate into antibody-forming plasma cells [32•]. These memory subsets and markers have since been applied to studies of the memory response to an array of microorganisms [33–35]. It is quite possible, perhaps even likely, that we will come to learn that different types of infections can result in the induction and maintenance of different types of memory B cells with differential expression of functionally relevant surface proteins.

Memory B cell lifespan

Are all memory B cells long-lived? Whereas classic models predict that memory B cells are exceptionally long-lived, the currently available data suggest that while some antigen-specific memory B cell populations are exceptionally stable [18•,29,36], others are not. For instance, populations of hapten-specific IgM and class-switched memory B cells induced with a standard hapten–protein conjugate are both exceptionally stable [37]. Indeed, in these experiments neither population exhibited a detectable decay rate over nearly one year. Of note, in these studies these cells were exclusively PD-L2+ CD73+ CD80+. By contrast, Pape *et al.* found that IgM^+ memory B cells induced by the protein antigen phycoerythrin are exceptionally long-lived, in their studies populations of Ag-specific IgG^+ cells decayed with kinetics reminiscent of naïve B cell populations [15•]. Why class-switched B cells in the later studies failed to achieve a maximal lifespan is unclear, to say the least, however it should be noted that switched memory B cells induced via malaria or sheep red blood cells also exhibit readily measured decay rates [30•,34].

Why are memory B cells long-lived in certain scenarios but not others? One possibility is that to avoid death antigen-experienced B cells must gain access to limiting and poorly defined survival signals found only in unique anatomical microenvironments. This idea has been popularized with respect to how plasma cells avoid apoptosis [38,39]. However, given that many memory B cells appear to recirculate throughout the lymphatic system [40], this idea may not apply to memory B cells. A related possibility is that memory cells rely on different signals for survival than their naïve counterparts. Indeed, while

naïve cells require stimulation with BLyS/BAFF for survival, memory B cells as a whole are relatively independent of such stimulation. Further, reliance on BAFF stimulation differs between IgM^+ and IgG^+ memory cells [41•].

An alternative idea that is slowly gaining traction is that genetic programs needed to avoid apoptosis are implemented very early during B cell activation and are influenced by the degree of antigen receptor signaling. In a recent paper Pape *et al.* noted that high-avidity interactions between antigen and the B cell receptor result in abbreviated survival of the resulting class-switched memory B cells [42•]. It should also be noted that additional signals, emanating from pattern recognition receptors including Toll-like Receptors (TLRs) on B cells, may also influence these decisions. In this regard, Wang *et al.* observed that mice lacking the transcription factor ZBTB20 were unable to generate long-lived plasma cells when immunized with a standard hapten–protein conjugate in the adjuvant alum. Yet, when ZBTB20-deficient mice were immunized with the same antigen mixed with ligands for TLR2 and TLR4 the result was the ready induction of long-lived plasma cells [43]. Whether these researchers would have observed parallel results for memory B cells in ZBTB20-deficient mice is unknown at the moment, but it is tempting to speculate that the lifespan of memory B cells and plasma cells is influenced by early and perhaps overlapping signaling cascades activated early during B cell differentiation. Nonetheless, it is becoming clear that the lifespan of cells within memory B cell populations can vary, and this may reflect differences in an array of variables including the degree of antigen-receptor signaling that occurred during the early phases of B cell activation and/or the constellation of cytokines available to these cells at this time.

Why different types of memory B cells?

B cell memory was once defined simply by the functional outcome of re-exposure to infection or immunization, but now we are increasingly able to identify the specific cells responsible for memory and to define their unique functional capabilities. Memory is a composite function comprised of standing antibody titers and the plasma cells that produce them, memory T cells and numerous subsets of memory B cells with distinct phenotypic and functional properties. Memory B cells are not simply expanded populations of antigen-specific naïve cells, although their increased frequencies compared with their precursors probably does play a role in their effectiveness. Memory B cells have mRNA and protein expression differences that are distinct from their naïve and germinal center precursors. These and additional observations support the notion that memory B cells acquire unique capabilities for activation, differentiation, signaling, migration, homing, and self-renewal.

The B cell memory response occurs in overlapping waves. Antibodies of the same specificities generated in the primary response, produced by bone marrow plasma cells, are the first line of defense against pathogen. Next, there is a rapid production of even more antibody, generated after the differentiation of particular memory B cell subsets into plasmablasts. At the same time, other subsets of memory B cells initiate secondary GC reactions with the capacity to generate new antibody, plasma cells and memory B cells with higher specificities for the offending pathogen. Thus, the diversity of memory B cell subsets may enable a rapid response to re-infection with familiar pathogen as well as the flexibility required to prime an effective response to a novel but similar pathogen. This later GC-dependent pathway may be particularly important for protection from viruses such as influenza and other pathogens that are known to mutate frequently.

Given the abundant diversity in phenotype and function of memory B cell subsets, it is reasonable to speculate that memory B cell subsets differ in ways that have not yet been defined. It is intriguing to consider that specific subsets of memory cells are stem cell like in the sense that they possess the ability to self-renew and replenish the memory compartment when needed. It also seems highly probable that memory subsets differ in their propensity to traffic through, maintain residence, or survive in different tissues. Beyond effector functions, it is plausible that some memory subsets function in a regulatory capacity, preventing pathogenic autoimmunity. Clearly, there is much yet to be learned about memory B cells. We look forward to the future when we will understand more about these cells, their unique functional properties and the molecular mechanisms by which they achieve them.

Conflict of interest statement

Nothing declared.

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