

ANTIGEN-SPECIFIC MEMORY B CELL DEVELOPMENT

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■ **Abstract** Helper T (Th) cell-regulated B cell immunity progresses in an ordered cascade of cellular development that culminates in the production of antigen-specific memory B cells. The recognition of peptide MHC class II complexes on activated antigen-presenting cells is critical for effective Th cell selection, clonal expansion, and effector Th cell function development (Phase I). Cognate effector Th cell-B cell interactions then promote the development of either short-lived plasma cells (PCs) or germinal centers (GCs) (Phase II). These GCs expand, diversify, and select high-affinity variants of antigen-specific B cells for entry into the long-lived memory B cell compartment (Phase III). Upon antigen rechallenge, memory B cells rapidly expand and differentiate into PCs under the cognate control of memory Th cells (Phase IV). We review the cellular and molecular regulators of this dynamic process with emphasis on the multiple memory B cell fates that develop in vivo.

INTRODUCTION

As outlined in 1949 by Burnet & Fenner (1), heightened reactivity to antigen recall is the central defining characteristic of adaptive immunity. It was not until 1957 that Talmage (2) and Burnet (3) postulated that it was the cells of the immune system that produced the antibodies and that they were the fundamental unit of selection for the immune response (4). Identification of cells that contained antibody (5) and the specificity of the antibody produced by individual cells (6, 7) provided experimental support for clonal selection as the driving force in adaptive immunity.

By the mid-1960s, Miller's studies of neonatal thymectomy (8) identified an essential role of the thymus for efficient adaptive immunity, and Claman's (9) transfers of bone marrow and thymus cell mixtures suggested that these cells worked together. However, it took a series of transfer studies by Mitchell & Miller (10) to establish that it was the bone marrow that produced antibody-forming cell (AFC) precursors and that the thymus-derived cells could be primed to enhance

the B cell response. Subsequent studies of the hapten-carrier effect began to probe the mechanisms of T cell–B cell collaboration (11–13) with the idea of an antigen bridge formulated by Rajewsky et al. (14). In this manner, B cell clonal selection and differentiation into plasma cells (PCs) could be controlled in an antigen-specific manner by Th cells.

These classical cellular studies provide the foundations for our understanding of cognate regulation in adaptive immunity. The role of dendritic cells (DCs) as specialized subsets of antigen-presenting cells (APCs) is now very clear, but the differential impact of the various DC subsets on adaptive immunity remains controversial. The level of functional subspecialization in the helper T cell and B cell compartments is also far greater than initially anticipated. As direct experimental access improves, we further resolve our understanding of the cellular and molecular events that shape antigen-specific immunity. Furthermore, cognate regulation begins with T cell receptor (TCR)–peptide MHC class II (pMHCII), but is modified by many molecular interactions that organize at cellular interfaces, now called the immunological synapse. We organize this review across the four major phases in Th cell–regulated development and function of antigen-specific memory B cells.

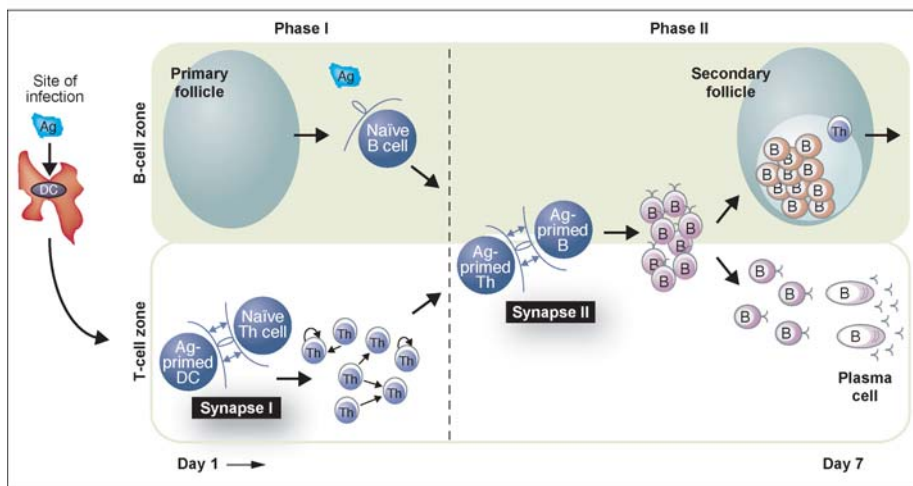
PHASE I: DEVELOPMENT OF pMHCII-SPECIFIC EFFECTOR TH CELLS

Antigen Experience in the Innate Immune System

The DC compartment is uniquely efficient at protein antigen uptake, processing and presentation (15). Conserved motifs on pathogens trigger innate activation through pattern recognition receptors (PRR), such as the IL-1R/Toll-like receptor family of receptors (16, 17). In protein vaccination strategies, immune adjuvants are needed to activate APCs through these PRR (18). Activated DCs also upregulate costimulatory molecules such as CD40, CD80, and CD86 and chemokine receptors such as CCR7, to relocate from sites of inflammation to the T cell areas of draining lymph nodes (15, 18–20). The rapid shut-down of cellular egress from lymph nodes (21) has also been attributed to local DC activity (22). Thus, antigen-experienced DCs are the critical initiators of Th cell–regulated adaptive immune responses (Figure 1: Phase I).

The type of inflammatory stimulus used for protein vaccination substantially impacts the quality of the subsequent adaptive immune response (18). Although all DCs derive from bone marrow precursors, they pre-exist antigen challenge as an array of different cellular subtypes (15, 18–20). There are three main CD11c⁺ DC populations (CD8α[−] DC, CD8α⁺ DC, and plasmacytoid DC), generally referred to as blood-derived DCs that are found in differing proportions across all secondary lymphoid tissue. In lymph nodes draining the skin, there are two further CD11c⁺ DC subtypes, the Langerhans cells and dermal DC (dDC) (15, 18–20). There are also reports of CD11c[−] monocyte-derived DCs (23) that can effectively present antigen to naive Th cells. Although the mechanisms remain poorly resolved,

A



B

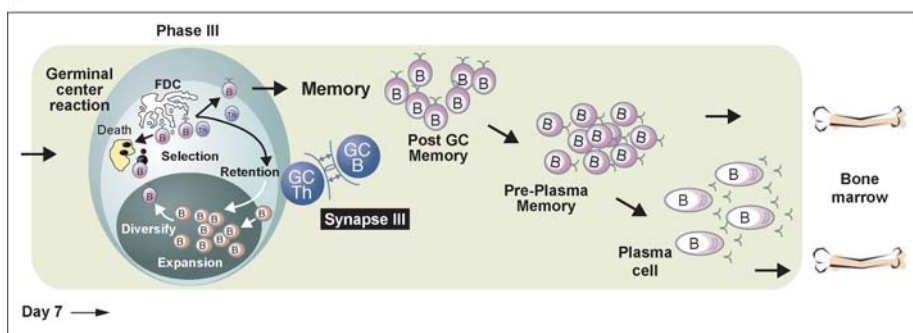


Figure 1 Th cell-regulated B cell memory development. (A) A schematic of the cellular interactions that proceed in secondary lymphoid organs toward development of antigen-specific effector Th cells (Phase I) and effector B cells (Phase II). (B) A schematic of cellular activity in the GC cycle (Phase III) that leads to the development of antigen-specific memory B cell subsets. The three main cellular products of the GC are depicted.

differential activation of DC subtypes provides one powerful means for controlling cell fate decisions in the development of adaptive immunity.

Direct Experimental Access to pMHCII⁺ DCs

The extent and dynamics of pMHCII expression and its cellular distribution and molecular context in vivo serve to define the innate to adaptive regulatory interface in vivo. Gaining experimental access to pMHCII⁺ DCs in vivo has been technically

challenging. A number of monoclonal antibodies have been generated against specific pMHC complexes from self and foreign protein antigens (24–26). These reagents produce clear and reliable signals *in vitro* and label well *in situ* for detecting self-pMHC complexes (27, 28). Studies of antigen presentation and adjuvant function *in vivo* (25, 29) were informative but suffered substantial background owing to self-peptide cross reactivity. Jenkins and colleagues (30) used peptide fused to fluorescent protein to demonstrate the capacity of pMHCII⁺ dDCs to activate naive Th cells *in vivo*. These powerful strategies can be broadly used for direct quantitative cellular and molecular assessment of antigen presentation *in vivo*.

Immune Synapse I: Cognate Priming of pMHCII-Specific Th Cells

The recognition of pMHCII by the TCR of specific Th cells is central to the development of adaptive immunity (31–33). Successful cognate interactions occur through organized rearrangement of cell surface molecules at the cellular interface, now commonly referred to as the immunological synapse (34, 35). Specific TCR-pMHCII interactions cluster toward the center of the immune synapse surrounded by complementary adhesion molecule interactions and excluding negative regulators of TCR signaling (34, 35). At least 2 h conjugate formation is necessary to promote Th cell proliferation (36) with antigen-specific signaling evident up to 10 h (37). Direct imaging provides outstanding clarity to the dynamic nature of conjugate formation (38, 39), resolving the timescale and quality of APC–T cell interactions *in vivo* (40). Thus, immune synapse formation acts as the first major checkpoint in the development of Th cell–regulated B cell immunity.

Direct Experimental Access to Antigen-Specific Effector Th Cells

Activated DC–naive Th cell conjugate formation results in extensive antigen-specific Th clonal expansion and the differentiation of effector Th cells. As with pMHCII⁺ DCs, direct experimental access to antigen-specific effector Th cells was a substantial technical barrier. The Th1/Th2 paradigm for Th cell function emerged from studies of Th cell lines propagated long-term *in vitro* (41–43). These lines and hybrids of antigen-specific Th cells served as useful models for the functional potential of Th cells and have been extraordinarily valuable for dissecting the molecular mechanisms driving functional commitment (44). Adoptive transfer of TCR transgenic Th cells has been another valuable method for accessing many facets of dynamic Th cell behavior *in vivo* (45–47). Our approach has been to retain the TCR diversity present in wild-type mice and isolate the antigen-specific compartment once expanded with antigen *in vivo* (48–52).

The first generation of the isolation strategy relied upon known V region gene expression and the modulation of cell surface molecules upon antigen-specific

activation *in vivo* (48). This strategy revealed the presence and dynamics of clonal selection based on TCR expression (53) and the impact of thymic selection on the emergence of clonal dominance (54). Functional studies directly *ex vivo* revealed a surprisingly high level of heterogeneity for cytokine production (55) and demonstrated a substantial change in Th cell physiology that accompanies antigen experience (56). These approaches laid the foundations for the pMHCII tetramer technology that was developed by John Altman and Michael McHeyzer-Williams while postdoctoral fellows with Mark Davis (49–52). This new class of reagents provided direct access to antigen-specific Th cells in a manner that was also a quantitative functional assay. The impact of this approach is most noticeable in the analysis of CD8 T cell responses and has been adapted in many forms to directly access TCR binding kinetics in the Th cell compartment (57) allowing TCR structure function connections (58) with single cell resolution (59).

Using a combination of these approaches, we have recently identified a pre-existing functional division in the naive Th cell compartment (60). In these studies, half of the naive Th cell compartment of B10.BR mice was shown to express Ly6C, a GPI-anchored glycoprotein. This Ly6C^{hi} naive Th cell compartment expresses distinct pMHCII-specific TCR repertoires imprinted during thymic selection. Interestingly, the Ly6C^{hi} Th cell subset was subspecialized for PC production *in vivo* across two separate adoptive transfer models for thymus-dependent immunity. Thus, we reveal a level of cellular diversity in the naive Th cell compartment that directly impacts B cell fate *in vivo*.

Activation of Naive Antigen-Specific B Cells

Lanzavecchia (61) experimentally reconciled the idea of an antigen bridge between B cells and Th cells into two sets of signals, one involving native antigen and B cell receptor (BCR) recognition and the other processing antigen and MHC class II. More recently, Neuberger and colleagues (62) argued that native antigen may be tethered by FcR or complement receptors to cells *in vivo*. When antigen is presented by APC to B cells, there is central focusing of BCR and intracellular signaling intermediates on the B cells (62). Adhesion molecules form peripheral rings, and negative regulators, such as CD45 and CD22, are excluded from the cellular interfaces. These APC–B cell immune synapses decrease B cell activation thresholds and promote the uptake and processing of the cell-associated antigen (62). There have also been reports of soluble IL-4 *in vitro* (63) and myeloid cell-associated IL-4 production *in vivo* (64) that condition the naive B cell compartment to receive signals through MHC class II molecules. Although these signals have global effects on naive B cells as a consequence of certain immune adjuvants, they do not appear to impact the production of antigen-specific IgG1 (64). Nevertheless, adjuvant-activated cells of the innate immune system appear to have the capacity to impact early B cell fate in ways that still remain poorly understood.

PHASE II: DEVELOPMENT OF EFFECTOR B CELL RESPONSES

Immune Synapse II: Cognate Priming of Antigen-Experienced B Cells

The immune synapse between effector Th cells and antigen-experienced B cells is quantitatively and qualitatively distinct from Immune Synapse I. Besides the TCR-pMHCII requirement, there are a large and ever increasing number of cell surface molecules known to play a role in this second major checkpoint in the development of B cell memory (65). CD40 is constitutively expressed by B cells and CD40L is upregulated on activated Th cells and known to play an essential role in class switch recombination (CSR) and GC formation (66–68). Lack of OX40 (CD134) and/or OX40L (CD134L) interferes with CSR but leaves GC formation intact (65). CD27-CD70 appears to promote PC formation, whereas CD30-CD153 interactions have an inhibitory effect on CSR and limit antibody production in vivo (65). Although it is not clearly resolved when and where these specific interactions occur, many of these interactions are likely to modify B cell fate at Immune Synapse II.

Lymphotoxin (LT)- α (69), LT β (70), tumor necrosis factor receptor (TNFR)-I (71), and LT β R (72, 73) have important roles in the developmental organization of lymphoid tissue at homeostasis that indirectly impacts B cell development. B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) and their receptors, BAFFR, TACI, and BCMA, have substantial effects on B cell development (74) but are also implicated in the regulation of B cell immune responses (75, 76). CD95 and CD95L have well-characterized effects on the B cell response, with deficiencies causing lympho-proliferative disorders and autoimmune susceptibility, but they may act later during the GC reaction with impact on antigen-driven selection (77–79). Whether these TNFR/TNFR family members are expressed on separate subsets of effector Th and activated B cells, at quantitatively distinct levels, at precise times after immunization, or in precise locations within the secondary lymphoid tissues still remains to be clearly determined.

More recently, inducible costimulator ligand (ICOSL), constitutively expressed on B cells, and ICOS, on activated Th cells, have been suggested as acting upstream of CD40-CD40L interactions (80–82). Other members of this B7-CD28 family clearly indicate a temporal preference of action in vivo (83). Although CD28 signals to naive Th cells through CD80 and CD86 on activated DCs have substantial impact on activation and clonal expansion (83), their influence at Immune Synapse II is less well established. CTLA4 is another well-characterized example of a late acting effect that regulates the necessary decline in immune activation and clonal expansion (84, 85). These spatio-temporal levels of molecular control may have substantial impact on the outcome of the adaptive response effecting the quality and quantity of the primary and memory B cell response.

Class-Switch Recombination (CSR)

The magnitude and isotype spectrum of antibodies expressed and secreted by antigen-specific B cells provides the most sensitive and quantitative measure of cognate T cell help *in vivo*. Cell-associated signals, such as CD40L to CD40 (66–68), are necessary to initiate antibody isotype switch *in vivo*, with some other signals, such as BAFF-BAFFR (75), able to compensate. However, the impact of Th cell-derived cytokines has been more closely scrutinized and generally considered the more influential regulators of CSR *in vivo*.

IL-4 drives the switch to IgG1 (86–88) and in a stepwise manner through IgG1 to IgE (89). Animals lacking IL-4 (90) or STAT-6 to transduce IL-4 signals (91, 92) switch to IgG1 with a lowered efficiency while ablating IgE production. Animals lacking the IL-21R also have lowered IgG1, but exhibit increased levels of IgE (93). Double deficiency of IL-4 and IL-21R has a broader effect on all IgG subclasses as well as IgE, suggesting a more fundamental role for IL-21 in B cell responses (93). IFN- γ is implicated in the switch to the IgG2a subclass (86) and away from IgG1 as a counter-balance to the influence of IL-4. B cell-specific ablation of TGF β RII has implicated TGF β in the induction of IgA (94). IL-2 and IL-5 also augment IgA production, but do so by preferentially supporting IgA⁺ B cells rather than promoting CSR (95, 96). A similar indirect action may be attributed to IL-6, whose absence *in vivo* (97, 98) results in the selective loss of IgG2a and IgG2b in thymus-dependent immune responses.

Hence, the induction of isotype switch recombination, the propagation and survival of isotype-switched B cells, and their development into short-lived PCs may be imprinted at first contact between antigen-specific effector Th cells and antigen-experienced B cells. The switch to downstream isotypes apparent in the GC reaction and into the memory B cell compartment may also have its origins at this early developmental stage. The isotype-switched prememory B cells may also be subject to separate and perhaps distinct levels of cellular and molecular control at this later stage in development. The long-term survival of isotype-switched memory B cells and the control of their response to antigen recall may not be related to the initial induction signals for CSR, and they remain important variables in the efficacy of long-term humoral protection.

Non-GC Development of Short-Lived Plasma Cells

In general, all rapidly emerging antigen-specific PCs in the primary response that develop without entering into GCs are thought to be short-lived (99). These PCs express and secrete germline-encoded antigen-specific antibodies (100) and have half-lives of 3–5 days *in vivo* (99). They first appear in the T cell zones and migrate to the red pulp of the spleen (101). This migration pattern *in vivo* appears to be controlled by increased responsiveness to the CXCR4 ligand CXCL12 and the decreased expression of CXCR5 and CCR7 (102). Most *in vitro* models of PC formation likely produce the equivalent of short-lived non-GC PCs. Although a

PC is considered terminally differentiated, some activated B cells can be shown to secrete antibody and still be able to divide. These cells are referred to as plasmablasts and can be considered a transient stage of PC development in vivo and in vitro.

Differential antibody isotype expression is a level of functional diversity that indicates different modes of cognate Th cell regulation. These differences in isotype emerge across all subtypes of antigen-specific PC, and the developmental impact outside antibody isotype itself remains poorly understood. What controls the expansion of particular precursors, the migration patterns of their progeny, or the potentially different requirements for survival and death across different isotype-specific PC subpopulations is still unclear.

Calame and colleagues (103) recently produced a mouse with a B cell selective ablation of *Prdm1* (encodes Blimp-1) that displayed a substantial deficit of serum antibody before intentional immunization, with little to no specific antibody production in thymus-independent and thymus-dependent immune responses (103). To ascertain whether this was a defect in antibody secretion or a defect in the development of PCs, we directly monitored the 4-hydroxy-3-nitrophenyl acetyl (NP)-specific B cell response in these animals (103). In the spleens at day 7 of the primary response, short-lived non-GC B cells were largely absent. Blimp-1 is known to promote cell cycle arrest directly through *c-myc* repression, or indirectly through upregulation of cdk inhibitors *p18* and *p21* (104, 105). Blimp-1 may also directly repress *Pax-5* to release its control over the X-box binding protein, XBP-1 (106, 107). This transcription factor, XBP-1, is also necessary for PC development in vivo (108) through its control of the unfolded protein response (109) and many secretory pathway components (107). However, it remains unclear which of these pathways more directly causes the PC developmental defect in these mice.

PHASE III: DEVELOPMENT OF MEMORY B CELLS

The Germinal Center (GC) Reaction

Secondary follicle formation is the alternate B cell fate at Immune Synapse II (Figure 1: Phase II). This massive and rapid clonal expansion displaces resting follicular B cells, creating the dynamic cellular underpinnings of the GC reaction (110, 111). There is evidence for pre-GC clonal selection based on germline encoded BCR to initiate secondary follicle formation (112, 113). Polarization of secondary follicles into a T cell proximal zone of expanding centroblasts (the dark zone) and a T cell distal zone of noncycling centrocytes (the light zone) signifies the beginning of the GC reaction (Figure 1: Phase III). The GC reaction is a cycle of cellular activity and molecular change that regulates antigen-specific clonal evolution during the development of B cell memory (114–116). Clonal expansion followed by BCR diversification and affinity-based selection results in either retention and re-entry into a second GC cycle of events, or exit from the GC and entry into the long-lived memory B cell compartment.

Somatic Hypermutation

Antigen-specific B cells that initially enter the GC cycle expand rapidly and down-regulate their germline-encoded BCR (110, 111). Clonal expansion is coupled to BCR diversification through somatic hypermutation (SHM) (117, 118). Although microdissection provided access to GC B cells, these population-based repertoire analyses generated a PCR recombination artifact *in vitro* (119). Hence, single cell resolution is necessary for these types of BCR repertoire studies. SHM has also been demonstrated outside GC (69, 120); however, in these examples it is difficult to exclude the presence of ectopic GC outside secondary lymphoid tissue (121). Thus, under normal physiological conditions and acute reaction to antigen exposure, the GC is the most likely location to support SHM.

SHM is seen through the introduction of single base-pair substitutions, with rare insertions and deletions into the variable regions of antibody gene segments. The recent discovery of the activation-induced cytidine deaminase (AID) (122, 123) represents a major breakthrough in our understanding of this diversification mechanism. However, investigators still debate whether AID acts on an RNA intermediate or directly on a DNA substrate (124, 125). Nevertheless, double-stranded DNA breaks with some sequence preference appear to be intermediates in this process (126), together with error-prone DNA polymerases, to introduce mismatched nucleotides into these lesions (127, 128). There must also be some means to subvert mismatch repair mechanisms in these GC B cells, but the genetic ablation of most known systems has no substantial influence on SHM patterns (129). The details of this multistep mechanism are of great importance to our appreciation of BCR repertoire development *in vivo*.

Antigen-Driven Affinity Maturation

Approximately one mutation is introduced into the BCR with each cell division. The variant BCR are then expressed on the surface of noncycling centrocytes that are selected on the basis of the affinity of the BCR binding to antigen (130). Follicular dendritic cells (131, 132) have been implicated in this antigen-driven selection mechanism owing to their presence at high density in the light zone of GC with Fc γ R and complement receptors that trap and display native antigen as immune complexes (IC) (133, 134). One model proposes competitive binding for antigen between germline-encoded antibody in the IC and variant antibody expressed by the newly emergent centrocyte. To test this model, Shlomchik and colleagues (135) developed an animal that can only express membrane but no secreted antibody. These animals still produce GCs with evidence for affinity maturation, indicating that there is no requirement for IC during affinity maturation. Hence, it seems clear that the processes controlling affinity maturation in the GC reaction do not require secreted antibody. As the selection mechanism must clearly use native antigen as a template, the means for presenting this antigen, its local depot, and the cells involved in regulating this process remain important open question in the field.

Immune Synapse III: Regulated Exit from the Germinal Center Reaction

The majority of centrocytes expressing variant BCR die in situ. Overexpression of antiapoptotic molecules, such as Bcl-2 or Bcl-xL, results in the accumulation of low affinity B cells in vivo, arguing for apoptosis as the clearance mechanism (79, 136). Centrocytes that express higher affinity BCR receive a productive antigen signal with costimulation that rescues them from apoptosis (137). This positive selection mechanism may be regulated in a cognate manner by GC Th cells (138, 139). GC Th cells express antigen-specific TCR (53, 140, 141) and distinct cell surface phenotype (142) and may undergo their own level of antigen-driven selection in the GC microenvironment (141). Interestingly, based on expressed TCR, not all antigen-specific Th cells enter the GC reaction. Furthermore, a GC phase in development is not necessary for the generation of Th cell memory (143), an important developmental process about which very little is known.

After positive selection in the GC cycle, the higher affinity variant centrocytes can return to the dark zone. Here, a second round of clonal expansion introduces a new set of point mutations in the progeny that will again be tested for higher affinity (144). Hence, reiterative GC cycles of expansion, diversification, and selection rapidly amplify high-affinity variants of the original antigen-specific B cell compartment.

At some stage, variant centrocytes are exported from the GC reaction to enter the memory B cell compartment (115, 145). Very little is understood of the differential signals required at this stage of development. Interestingly, in the absence of AID (123) the GC reaction contains significantly greater numbers of B cells. It is possible that delayed or distorted antigen-specific selection processes may have caused the cellular congestion in this case. GC congestion is also seen in the absence of Blimp-1 (103). The absence of this transcriptional repressor may have delayed GC exit in this model. Although the highest detectable levels of Blimp-1 are found in PCs, there are reports of small fractions of GC B cells also containing Blimp-1 protein (146). Although there are still many alternate explanations, these trends indicate the existence of B cell intrinsic signals in the regulation of prememory B cells' exit from the GC cycle.

PHASE IV: THE MEMORY B CELL RESPONSE TO ANTIGEN RECALL

Antigen-Specific Memory B Cell Subsets

Exit from the GC reaction signifies entry into the long-lived memory B cell compartment. Early studies indicate that memory B cells are persistent, long-lived, antigen-specific B cells (147), with new models that indicate this survival is not dependent on signals through the BCR on antigen-experienced B cells (148). Experimental access to the antigen-specific B cell compartment in non-BCR

transgenic animals has the longest history of the three antigen-experienced cellular subsets considered in this review. As soluble antigen is the ligand for the BCR, all approaches use variations of labeled antigen to identify antigen-specific B cells. Although panning techniques used gel-associated antigen (149, 150), flow cytometry provided the most reliable access to antigen-specific B cells (151, 152). These earliest studies by Herzenberg and colleagues (151, 152) coupled cell sorting technology and direct labeling to enrich antigen-specific B cells for adoptive transfer. These early studies helped to demonstrate that B cells with receptors for antigen were the precursors for AFCs.

This approach has been adopted by many groups (147, 153–155), with the subsequent evaluation of specificity and purity demonstrated by the frequency for AFCs or the enrichment for production of antigen-specific antibodies *in vitro*. McHeyzer-Williams et al. (156) adopted this approach to isolate NP-specific memory B cells directly *ex vivo*. We focused on the NP-specific memory response and evaluated target cell purities based on single cell cloning of memory B cells *in vitro* (156). Further, we devised the first single cell RT-PCR analysis in lymphocytes and used this technique to estimate the extent of SHM in the individual progeny of memory B cell clones (156). Subsequently, we applied single cell RT-PCR directly *ex vivo* to analyze BCR repertoires with single cell resolution (112, 157). As discussed above, we have extended this approach to Th cells using V region-based strategies (48) or pMHCII-tetramers (51) with similar applications for the study of TCR repertoire development *in vivo* (53).

There are multiple subtypes of antigen-specific memory B cells. As discussed previously, antibody isotype itself defines a major developmental and functional subdivision in the memory B cell compartment. The spectrum of isotype produced depends on the quality of cognate Th cell regulation, which itself depends on the antigen, its dose, route of entry, and adjuvant context. How different isotype-specific prememory B cells are controlled at the final stages of GC exit and how they are differentially maintained in the memory B cell compartment are not clearly understood.

At a more fundamental level of function, there is general consensus for at least one other major subdivision in the memory B cell compartment. One subtype of affinity-matured memory B cells persists as nonsecreting cells that are the precursors of the rapid cellular response to antigen recall. Long-lived PCs that are terminally differentiated, affinity-matured, and home to the bone marrow are the second category of post-GC B cells that can be considered a subtype of memory B cells and that contribute to humoral or serological memory.

Post-GC and Pre-Plasma Memory B Cell Subsets

Memory response precursors have been typically defined as IgM⁻IgD⁻6B2⁺ (6B2 recognizes the B cell form of CD45) antigen-binding B cells (154, 156, 158, 159) that do not secrete antibody until expansion and differentiation into PC after rechallenge with antigen. These 6B2⁺ memory B cells express mutated receptors

and can be found in late primary and secondary responses to antigen (112, 156, 157). More recently, we have identified a separate, phenotypically distinct $6B2^-$ memory B cell compartment using NP-conjugated keyhole limpet hemocyanin (NP-KLH) and the Ribi adjuvant system (160). Both $6B2^+$ and $6B2^-$ memory subsets rapidly re-emerge upon antigen recall as IgM^-IgD^- NP-binding cells, easily distinguished from memory response PCs through lack of CD138 (Syndecan 1) expression (112, 157) (see Figure 2 for a summary of phenotype and genotype). Both subsets persist at substantial numbers for at least eight weeks postrecall and do not secrete antibody in ELISPOT assays directly *ex vivo*. Both compartments express the lambda light chain, with demonstrable levels of IgG isotype and the $Ig\beta$ BCR coreceptor (CD79b) present in at least half of the $6B2^-$ NP-binding memory B cells (160). Single cell RT-PCR analysis demonstrated the presence of somatically mutated V λ mRNA in both subsets with evidence for antigen-driven selection.

The responsiveness of each memory subset upon adoptive transfer and antigen recall, the parent-to-progeny relationship, and the capacity to produce PCs and antibody in the serum allowed us to propose a model of linear development to connect these memory response precursors and terminally differentiated PCs (160). We proposed that $6B2^+$ post-GC memory B cells were developmentally upstream of $6B2^-$ pre-plasma memory B cells that were a nonsecreting stable cell fate and developmentally upstream of memory response PCs. Our phenotypic and molecular analysis of the primary response to NP-KLH (161) identified similar subtypes of NP-specific B cells that emerged during the first weeks after initial priming and persisted up to eight weeks without rechallenge. These data were consistent with our linear development model in which the $6B2^+$ post-GC memory B cells were the main cellular product of the GC reaction that gave rise to $6B2^-$ pre-plasma memory B cells and long-lived PCs once outside the GC reaction (see Figure 1B for the schematic).

Memory B Cells with Atypical Cell Surface Phenotype

There is evidence for this atypical $6B2^-$ memory B cell compartment in studies using the NP-specific BCR transgenic quasimonoclonal (QM) mouse model (162). Wabl and colleagues (163) identified $6B2^-CD19^-CD138^-$ B cells in the peripheral blood of these animals, expressing downstream antibody isotypes that contained rearranged and mutated IgH and IgL mRNA. Noelle and colleagues (164) identified a similar $6B2^{low/-}$ antigen-experienced B cell compartment upon adoptive transfer of these QM B cells and antigen exposure *in vivo*. The low CD138 levels, nonsecreting status, and bone marrow homing propensity of this QM B cell subset (164) closely resembled the $6B2^-$ pre-plasma memory compartment described in the non-BCR transgenic model (103, 160, 161). These $6B2^{low/-}$ QM B cells were considered plasma cell precursors because they could give rise to antigen-specific PCs upon adoptive transfer, without secondary exposure to antigen (164). However, proliferation was required before differentiation into PCs, hence the distinction from PCs themselves (164). Nevertheless, the B cells that persist

	Naive T cells	Naive B cells	Day 5 Memory Response		
			NP-specific		
Cell Surface					
NP	-	-	++	++	++
IgD [11.26]	-	+++	+	+	-
λ -1 [JC5.1]	-	+	++	++	++
CD79b [HM79]	-	++	++	+++	+
B220 [6B2]	-	++	++	-	+
CD138 [281.2]	-	-	-	-	++++
CD19 [1D3]	-	+++	+++	-	++
CD22 [Cy34.1]	-	+++	++	-	-
CD24 [M1/69]	-	++	++++	++	+++
CD40 [3/23]	-	++	+	-	-
CD9 [KMC3]	-	+	+	++	+
CD43 [S7]	+++	+	-	+++	+++
CD11b [M1/70]	-	+	-	++	-
Gene					
IgI-V1 [λ -1]	-	++	++++	++++	++++
Cd79b [Ig β]	-	+++	++++	++	++
Sdc1 [Cd138]	-	-	-	-	++
Cd19	-	++	++	+	+
Cd22	-	++++	+++	++	-
Cd24a	-	+++	+++	+	+++
Tnfrsf5 [Cd40]	-	++	+	+	-
Cd9	-	-	-	++	+
prdm1 [Blimp-1]	-	-	-	-	++
xbp1	-	+	+	++	++++

Figure 2 Phenotype and genotype of NP-specific memory B cell subsets. Cells were analyzed directly by flow cytometry and isolated directly ex vivo for Affymetrix gene expression analysis. For cell surface analysis a protein signal of (–) equals an MFI of 0–20, (+) is 21–100, (++) is 101–500, (+++) is 501–1000, (++++) >1000. For Affymetrix microarray analysis an mRNA signal of (–) equals a fluorescence of 0–200, (+) is 201–1000, (++) is 1001–5000, (+++) is 5001–10000, (++++) >10000.

long-term in this model are clearly post-GC, isotype-switched, and antigen-experienced B cells, and thus may be considered cellular components of the memory B cell compartment.

Diamond and colleagues (165) developed a peptide tetramer-based method for detecting peptide-specific B cells *in vivo*. They demonstrate a substantial number of 6B2⁻ and CD19⁻ peptide-binding cells in immunized BALB/c mice (165). Specificity is demonstrated for peptide-binding cells after sorting and culture by ELISPOT; however, CD138 is not used to exclude peptide-binding PCs, and phenotype (either 6B2 or CD19 separation) is not used before assay of function. However, this group demonstrates the presence of CD79b mRNA in both 6B2⁺ and 6B2⁻ putative B cell subsets. Cambier and colleagues (166) also noted 6B2^{low/-} cells accumulate to ~50% of Id⁻ B cells in aged 3–83 μ δ BCR transgenic mice. Although CD45 is expressed by the Id⁻ B cells, the high molecular weight isoform detected by 6B2 was absent (166). Using this same antibody (>95% of naive human B cells labeled with 6B2), Bleesing & Fleisher (167) demonstrated that most CD27⁺ memory B cells in human peripheral blood (168, 169) had lost 6B2 binding (167). The 6B2 distribution was similar for IgM and downstream non-IgM CD27⁺ memory B cells (167). Thus, there is evidence for the pre-plasma memory B cells as a persistent antigen-experienced B cell in both mouse and human.

Pre-Plasma Memory B Cells and the Multiple Myeloma Progenitor

One may reasonably speculate that if pre-plasma memory B cells comprise a substantial fraction of B cell memory then they should be represented among the many different varieties of mature B cell leukemias and lymphomas. The myelomagenic progenitor in multiple myeloma (MM) may be one such candidate (164, 170). MM is an incurable malignancy characterized by accumulations of clonal PCs in the bone marrow (171, 172). Although disease pathology is largely due to the dysregulated turnover and overgrowth of long-lived PCs, populations of antigen-experienced, somatically mutated non-PC B cell progenitors appear to underlie disease etiology (170, 173). Accumulating evidence implicates a circulating post-GC B cell subtype that expresses low molecular weight isoforms of CD45, bimodal expression of CD19, low to negative CD20, CD21, and CD22, and no expression of CD138 (170, 173, 174). These putative progenitor cells also express elevated levels of the adhesion molecule CD11b, not typically found on conventional B cells in human or mouse (170) or on naive, post-GC memory B cells or PCs in mouse, but found at high levels on pre-plasma memory B cells (160, 161) (see Figure 2). Thus, the MM progenitor may represent a malignant transformation of cells from the pre-plasma memory B cell compartment.

Monocytes Masquerading as Memory B Cells

Bell & Gray (175) recently raised the possibility that the pre-plasma memory B cells were actually monocytes that had captured specific antibody, masquerading

as antigen-binding 6B2⁻ memory B cells. These studies used a three-color flow cytometric strategy without assessing CD79b expression on their putative 6B2⁻ B cell compartment. Using immunized serum transfer in a variety of experimental situations, FcγR ablated animals (RIII and common γ chain), and PE-specific memory responses, we found little evidence of monocytes that interfered with our specific analysis (103; L. McHeyzer-Williams & M. McHeyzer-Williams, unpublished data). Conversely, we found 6B2⁻ pre-plasma memory B cells that also expressed CD79b in both FcγR-knockout mice and the PE-memory response (L. McHeyzer-Williams & M. McHeyzer-Williams, unpublished). One may be misled by background staining of non-B cells when relying on antigen binding alone to identify memory B cells. Also, obtaining purified populations of B cells in the absence of typical B cell-associated markers is difficult, as we found in our original studies. There were many CD79b⁻ cells within the 6B2⁻ fraction of NP-binding events that we also inadvertently selected for our original studies. However, our functional assays outside of flow cytometry (BCR repertoire studies and adoptive transfer) detected the presence of B cell activity within these putative memory B cell populations, even without the CD79b selection that we now use for all our isolation procedures involving the 6B2⁻ pre-plasma memory compartment.

Extended Phenotype and Genotype of Memory B Cell Subsets

Figure 2 summarizes the cell surface phenotype of the two memory response precursors, compared quantitatively with naive B cells and memory response PCs. The first section of phenotype (Figure 2, upper 6 rows) emphasizes the main selection criterion to use when purifying the NP-specific memory B cell subsets. We have now expanded the molecular analyses of both NP-specific memory B cell subsets to quantify more clearly the levels of the most critical and only reliable B cell lineage marker, rearranged immunoglobulin genes. Affymetrix microarray assessment direct ex vivo (Figure 2, lower half) and Q-PCR confirmation of lambda light chain expression (data not shown) from equivalent numbers of 6B2⁺ post-GC memory cells and 6B2⁻ pre-plasma memory B cells indicate equivalent amounts of mRNA in these putative memory populations. Although surface-expressed CD79b protein is even higher in the 6B2⁻ memory subset (Figure 2, upper panel), CD79b mRNA levels are present in all subsets but lower in the pre-plasma memory cells (Figure 2, lower panel). These microarray data for CD79b were also confirmed using Q-PCR (data not shown). A series of other typical and atypical cell surface molecules found to change across the proposed cellular development program, have mRNA levels that are broadly concordant with the protein expression (Figure 2).

The original single cell RT-PCR repertoire analysis of the pre-plasma memory compartment (160, 161) was criticized (175) as having a low success rate for obtaining sequence (60%, 30%, 15% across PC, post-GC, and pre-plasma memory for Vλ mRNA, respectively). Using CD79b as an extra selection criterion (compared with the initial study) and a redesigned single cell RT-PCR, we can now

amplify rearranged lambda light chain mRNA from 45%–50% of single memory B cells from either population (data not shown). The PCs from the same D5 memory timepoint still achieve 60% efficiency in these experiments and represent the higher end of efficiency for the current system.

Thus, regardless of the unique cell surface phenotype expressed, we believe that the current flow strategy focuses on NP-specific memory B cell populations. The schematic presented in Figure 2 should allow purification of these separate cellular compartments through application of the extended phenotype and evaluation through genotypic population and single cell analysis.

Long-Lived Plasma Cells as a Memory B Cell Compartment

Long-lived antibody-secreting B cells can also be considered part of the memory B cell compartment. The long-lived PCs secrete isotype-switched antibody and display evidence of SHM with affinity-increasing mutation patterns, but they do not self-replenish through turnover (114, 176, 177). This post-GC antigen-specific B cell compartment appears during the second week after initial antigen exposure (112) and preferentially homes to the bone marrow for growth factor support of stromal cells (178). On the basis of gene ablation studies, investigators have concluded that these cells use a variety of redistribution mechanisms, such as upregulation of CXCR4 and $\alpha 4 \beta 1$ integrin binding to its ligand VCAM-1 (102), to get to the bone marrow, where they can persist for the life of the animal (99, 179–183). In the bone marrow, long-lived PCs need signals through the TNFR family member, BCMA for survival (184). Investigators have also proposed that a pre-plasma cell precursor (164) or memory cells themselves (185) produce PCs in a non-antigen-dependent manner as a means of maintaining serum antibody levels for extended periods.

The extended longevity of the long-lived PC can be demonstrated using BrdU incorporation and adoptive transfer (180–183). The extinguished gene programs associated with PC development (106, 107, 186) support a terminally differentiated end stage cell that needs to arrest cell cycle progression (187) and that will not be reactivated upon antigen recall. Nevertheless, on the basis of the evidence from a GC phase in development, of the extended longevity of these PCs, and of the continued production of high-affinity antibody, we consider these cells to belong to the memory B cell compartment.

Immune Synapse IV: Cognate Regulation of Memory B Cell Responses

Persistent high-affinity serum antibody provides the first layer of protection against antigen rechallenge. Although binding to antigen is a clearance mechanism, it may also serve to increase the efficiency of antigen presentation to memory B cells through rapid IC formation and binding to FcR or complement receptors on cells of the innate system. In this manner, antibody may amplify the sensitivity of the memory B cell response to antigen recall. Memory B cells to thymus-dependent

antigens require cognate Th cell regulation (110, 111). Although the details on memory Th cells remain poorly resolved, the quality of immune synapse formation between memory Th cells and memory B cells is distinct from any of the preceding exchanges. The level of cellular heterogeneity on both sides of this interaction can substantially impact the developmental choices available to the memory responders.

Blimp-1 Impacts Memory B Cell Development

As previously discussed, in the absence of Blimp-1, primary response GCs are oversized (103). Surprisingly, by day 14 after priming, there is also an obvious deficit in the development of 6B2⁺ pre-plasma memory B cells. Although the phenotype in the spleen was somewhat variable, the propensity of the pre-plasma memory B cell to home to the bone marrow (160, 161) displayed a clear and dramatic quantitative developmental phenotype (103). Furthermore, long-lived PCs that dominate at day 14 of the primary response were also missing in the absence of Blimp-1. Thus, the oversized GCs may reflect the accumulation of cells whose differentiation was blocked, consistent with the proposed linear development model.

Upon reimmunization, there was an exaggerated and equivalent expansion of NP-binding B cells in the presence or absence of Blimp-1 (103). Hence, by this criterion, memory B cells had developed in the absence of Blimp-1 during the primary response GC reaction. However, the developmental progression was again dramatically blocked at the 6B2⁺ post-GC memory B cell stage. Many of the NP-specific 6B2⁺ B cells also expressed the GC marker GL7 (tenfold greater than wild type), but there were negligible numbers of 6B2⁺ pre-plasma memory B cells or memory response PCs (103). The bone marrow was also devoid of pre-plasma memory cells after antigen recall. Thus, the primary response GC produced B cells with the capability of rapid expansion upon antigen recall, but no ability to produce pre-plasma memory B cells or memory response PCs.

CONCLUDING REMARKS

Humoral immunity considers specific serum antibody as the effector element of long-term B cell responses. In this review, we emphasize memory B cells and their subspecialized activities as the key regulators of the response to antigen recall. Th cell-regulated memory B cell development is an emergent cellular behavior regulated through an ordered series of cellular and molecular changes *in vivo*. We now have experimental access to the cells involved at each stage of this process to probe the molecular regulators of these critical cell fate decisions *in vivo*. Cellular heterogeneity at all stages is extensive. Direct quantitative cellular and molecular analysis offers new standards for evaluating vaccine efficacy, while transformed equivalents of memory B cell subtypes provide new targets for therapeutic intervention in disease.

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