Do Memory B Cells Form Secondary Germinal Centers?

Impact of Antibody Class and Quality of Memory T-Cell Help at Recall

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Antigen recall can clearly induce a germinal center (GC) reaction. What has become an issue for debate are the origins of the antigen-specific B cells that form memory-response GCs (mGCs). Using antigen labeling and adoptive transfer, memory B cells expressing different antibody class can give rise to mGCs with differing efficiency. Here, we will argue that the range of class-specific memory responses reported across multiple systems represents the spectrum of memory B-cell fate and function. While the formulation of recall immunogen and location of mGCs have an important role, we propose that effective cognate regulation is the key variable influencing recall outcome. These issues remain central to contemporary efforts of rational vaccine design.

GREAT DEBATES

What are the most interesting topics likely to come up over dinner or drinks with your colleagues? Or, more importantly, what are the topics that *don't* come up because they are a little too controversial? In ***Immune Memory and Vaccines: Great Debates***, Editors Rafi Ahmed and Shane Crotty have put together a collection of articles on such questions, written by thought leaders in these fields, with the freedom to talk about the issues as they see fit. This short, innovative format aims to bring a fresh perspective by encouraging authors to be opinionated, focus on what is most interesting and current, and avoid restating introductory material covered in many other reviews.

The Editors posed 13 interesting questions critical for our understanding of vaccines and immune memory to a broad group of experts in the field. In each case, several different perspectives are provided. Note that while each author knew that there were additional scientists addressing the same question, they did not know who these authors were, which ensured the independence of the opinions and perspectives expressed in each article. Our hope is that readers enjoy these articles and that they trigger many more conversations on these important topics.

Editors: Shane Crotty and Rafi Ahmed

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Effective vaccination primes target-specific neutralizing antibodies. Challenge with the same target antigens then progressively boosts serological reactivity with varied efficacy (Corti and Lanzavecchia 2013). The cellular evolution and molecular mechanisms that drive antibody-based protection are being scrutinized with heightened resolution in new experimental models (McHeyzer-Williams et al. 2012; Victora and Nussenzweig 2012). Subspecialized follicular helper T (T_{FH}) cells are now recognized as central cognate regulators of primary B-cell immunity with less understood of their role in recall (Crotty 2011; Qi 2016; Vinuesa et al. 2016). It remains of utmost importance to unravel and target all facets of recall regulation to optimize vaccine-driven immune protection.

In this perspective, we focus on the response to antigen recall. We discuss the results from multiple groups across various experimental models that seek to address the origins of B cells that rediversify B-cell antigen receptors (BCRs) on recall. Components of the memory-response germinal center (mGC) reaction will receive special emphasis with discussion of current thinking in cognate T_{FH} function and its role in recall. In reviewing this recent literature, we seek to identify the cellular targets and recall conditions that optimize antibody-based immune protection.

ANTIGEN-SPECIFIC B-CELL MEMORY

Early studies with hybridoma and BCR sequencing clearly showed the progressive increase in antigen-specific BCR diversification with prime-boost regime (Allen et al. 1987; Berek and Milstein 1987). Direct molecular analyses (Berek et al. 1991; Jacob et al. 1991b) supported the long-held notion that the primary-response GC (pGC) reaction promoted ongoing BCR diversification (MacLennan 1994). While antigen-specific GCs emerged in primary reactive lymphoid sites (McHeyzer-Williams et al. 1993), these pGCs resolved after only a few weeks (Jacob et al. 1991a; Weisel et al. 2016). Furthermore, early adoptive transfer studies of high-affinity memory B cells produced expansion and plasma-cell (PC) differentiation without BCR rediversification (Siekevitz et al. 1987).

Hence, selective recruitment with preferential clonal expansion could account for ongoing antibody affinity maturation without BCR rediversification at recall.

Antigen-binding (Hayakawa et al. 1987) and single-cell assessment of BCR sequence (McHeyzer-Williams et al. 1991, 1993) provided direct access to antigen-specific B-cell memory. Only a few of these earlier studies focused on the antigen-specific recall response (Hayakawa et al. 1987; McHeyzer-Williams et al. 1991, 2000; Shapiro-Shelef et al. 2003). Direct evidence for emergent $B220^{+}CD138^{-}GL7^{+}$ antigen-binding secondary GC formation at recall in situ was most clearly observed in the absence of Blimp-1 expression (Shapiro-Shelef et al. 2003). In this context, PC differentiation was absent and the GC pathway exaggerated both in the primary and recall responses (Shapiro-Shelef et al. 2003). Hence, it appeared that secondary GC reactions could appear at recall but the origins of the antigen-specific B cells involved remain untested.

Direct sequencing of circulating antigen-specific PCs after influenza vaccine boost provided the next line of evidence for memory BCR (mBCR) rediversification (Wrarmmert et al. 2008). In these human cohorts, there were multiple antigen-specific PC expansions with evidence of intraclonal BCR diversity (Wrarmmert et al. 2008). While highly suggestive of secondary GC formation, these studies focused on the outcome of recall responses in the blood and could not resolve their presence at recall.

MEMORY GC REACTION

At this point, a variety of murine immunization/vaccination models were used to assess memory B-cell fate at recall. Noelle and colleagues (Benson et al. 2009) used phycoerythrin (PE) in alum primarily to test the earlier notion of bystander memory reactivation (Bernasconi et al. 2002). In the process, they observed no local secondary GC formation at recall with soluble antigen or in the presence of CpG adjuvant (Benson et al. 2009). Kurosaki's group (Aiba et al. 2010) focused on hapten-protein (NP-KLH) in alum using soluble recall to

show the requirement of CD4 T cells and antigen presentation by IgG1⁺ memory B cells for reactivation and antibody production. In subsequent studies (Kometani et al. 2013), this group also shows the Bach-2-dependent skewing of IgG1⁺ memory B cells into memory-response PC (mPC) differentiation at recall.

Weill, Reynaud, and colleagues (Dogan et al. 2009) used in vivo temporal fate mapping to study B-cell memory. In this model, activation-induced cytidine deaminase (AID)-expressing cells were indelibly labeled (enhanced yellow fluorescent protein [EYFP]) after priming and recall responses. SRBC immunization (classic particulate antigen) induced robust antibody and long-lasting pGC responses while adoptive transfer revealed memory B-cell function. In this model, IgM⁺ memory B cells produced mGCs and mPCs, while IgG1⁺ memory B cells only gave rise to mPCs. In this model, antibody class exerted a strong differential impact on memory B-cell fate and function.

Jenkins and colleagues (Pape et al. 2011) focused on the longevity of class-switched and IgM⁺ memory using the PE-response and complete Freund's adjuvant (CFA). PE-specific IgM⁺ memory survived at constant levels for extended periods while switched-memory decayed at a considerable rate. Nevertheless, switched-memory dominated the recall response while present but IgM⁺ memory supported more robust mGC responses on adoptive transfer. In contrast, Bachmann's group (Zabel et al. 2014) reported robust mPC formation following virus-like-particle (VLP) prime and boost after adoptive transfer with little mGC formation from either IgM⁺ or IgG⁺ memory B cells.

Pepper and colleagues (Krishnamurthy et al. 2016) tracked antigen-specific B-cell memory in a murine malaria infection. These studies revealed antigen-binding IgD⁺, IgM⁺, and switched memory compartments after infection and reinfection. Even in the presence of greater numbers of switched memory, at recall, IgM⁺ memory cells dominated expansion and robust mPC responses. Hence, mGCs are not obligatory components of all recall response conditions and the formulation of immunization and/or

the recall response will differentially impact memory-response fate at reactivation.

In more recent studies, Nussenzweig's group (Gitlin et al. 2016) dissected the impact of class-switch versus hypermutation on switched-memory B-cell fate. In a complex genetic model, IgG1 class-switch was driven by Cre-recombinase and dissociated from AID-dependent BCR diversification. Under these conditions, antigen-driven IgG1⁺ PC differentiation was exaggerated but had no demonstrable impact on pGC expansion. Conversely, BCR hypermutation compromised IgG1⁺ memory longevity with evidence for increased self-reactive mBCRs after pGC experience. These findings suggest that BCR diversification more than class-switch may impact memory B-cell longevity. Persistence of memory B cells after initial priming then becomes a significant component in the capacity to respond at recall. The role for memory T_{FH} (mT_{FH}) cells remains unclear and a potentially confounding variable in many of these studies.

Takemori and colleagues (Kaji et al. 2013) more directly addressed the question of mBCR redifferentiation using adoptive transfer and clonal sequence reconstruction. In this NP-CGG model driven by alum, both mutated and unmutated memory B cells could redifferentiate BCRs at transfer and recall in ways that were AID-dependent (Kaji et al. 2013). Similar studies from Shlomchik's group (Zuccarino-Catania et al. 2014) indicate that the phenotypic subdivision of B-cell memory, based on CD80 and PD-L2 regardless of antibody class, predicts differential memory-response outcome. Memory cells double positive for these markers skewed toward mPC formation, whereas the double-negative fraction produced robust mGC reactions. These latter studies indicate intrinsic memory B-cell attributes or subset-specific extrinsic reactivation difference that may further impact memory B-cell fate.

mGC B-CELL PROGRAMS AT THE SINGLE-CELL LEVEL

We too have directly addressed the question of class-switched memory B-cell fate (Wang et al. 2012; McHeyzer-Williams et al. 2015). In the

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first study, we identified a unique role for the transcription factor T-bet in the survival and reactivation of IgG2a⁺ memory B cells at recall (Wang et al. 2012). Interestingly, T-bet was also expressed in IgG1⁺ memory B cells but did not exert the same essential function. We believe these studies indicate that class-switch goes beyond the recombination event to initiate separable and divergent molecular programs within class-specific memory B-cell compartments.

In the second study, we used adoptive transfer across multiple models to show that class-switched memory B cells could drive mGCs in the adoptive host (McHeyzer-Williams et al. 2015). Hapten-carrier (NP-KLH) priming with TLR4 agonist (monophosphoryl lipid A [MPL]) generate dominantly IgG2a/2b switched-memory compartments that could form mGC reactions in either nonspecific BCR transgenic or wild-type hosts. In this models, IgM⁺ memory B cells predominantly formed mPCs. We had previously shown that local antigen-specific T_H cell responses were substantially more robust and had higher affinity with MPL than CFA or alum, suggesting a secondary role for cognate regulation (Malherbe et al. 2008). Further, we suspect that emphasis on lymph node (LN) priming rather than the spleen induced a quantitatively and qualitatively distinct memory landscape (Fazilleau et al. 2007, 2009a,b).

In these studies, we were also able to connect the cellular phenotype, gene expression and BCR repertoire of individual responding antigen-specific mGC B cells (McHeyzer-Williams et al. 2015). Taken together, we could divide single mGC B cells into four separable stages of the mGC cycle based initially on *Cd83* and *Polh* gene expression. Once “gated” on major cellular divisions for these two mRNA species, expression assorted differentially across a range of putative GC functions (depicted in Fig. 1). Importantly, there was a balance of mGC B cells across all four subsets over time and these divisions could be found within the progeny of individual clones. Hence, we could track the dynamics of mGC reactions within the clonal progeny of class-switched memory B cells.

These mGC B-cell subsets were also found in the pGC compartment and the proposed

stages consistent with earlier dynamic imaging studies (Victora and Nussenzweig 2012). The iterative pGC cycles of selection followed by BCR diversification drive rapid clonal evolution under the cognate guidance of GC-localized T_{FH} cells (Crotty 2011; McHeyzer-Williams et al. 2012; Victora and Nussenzweig 2012; Vinuesa et al. 2016). Dynamic imaging provided evidence of B-cell antigen scanning on follicular dendritic cells (FDCs), cognate contact with GC-T_{FH} cells, light–dark zone recycling, and robust local proliferation (Allen et al. 2007; Schwickert et al. 2007). Elegant photolabeling studies tracked zonal movement in real time (Victora et al. 2010), enabling many of the central attributes of the pGC to be measured directly for the first time (Victora and Nussenzweig 2012). Finally, the elegant use of the “confetti” mice with temporally controlled clonal labeling capacity (Tas et al. 2016) provided a new means to access BCR diversification in real time. Hence, we propose essentially similar functions for the mGC cycle, but suggest that distinct precursors are produced at priming and differentially reactivated at recall.

FOLLICULAR HELPER T CELLS

Extending to GC-T_{FH} studies, the clonal dynamics and regional movements of GC-T_{FH} cells (Shulman et al. 2013), their costimulatory effect, and impact on clonal selection have also been dynamically tracked (Shulman et al. 2014; Liu et al. 2015; Qi 2016). Although the events associated with the mGC cycle are reminiscent of pGC workings, the intrinsic programs and regulatory requirements of switched-memory B cells may vary substantially at recall.

Antibody class may also impact pGC persistence and/or memory B-cell production after initial priming. There have been earlier reports of distinct T_{FH} cell subsets associated with pGC B cells of a different class (Reinhardt et al. 2009). Combinations of TLR agonists with nanoparticle delivery of antigen promoted efficient class-switch and extended local LN response (Kasturi et al. 2011). Expressing the intracellular IgG tail on naïve B cells promoted excessive clonal bursts (Martin and Goodnow 2002). Similar

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Figure 1. The memory-response germinal center (mGC) cycle. The zonal distribution of GC B cells depicted progressing through an ongoing cyclic series of cellular and molecular events. Typically, the light zone (LZ) is associated antigen-dependent selection, and the dark zone (DZ) involves more proliferation and B-cell antigen receptor (BCR) diversification. Single-cell RT-qPCR-based analysis of gene expression within antigen-specific mGC B cells assorted cells into four major subsets providing trajectory predictions based on gene expression that are depicted as stages 1–4 in the schematic above (McHeyzer-Williams et al. 2015). Antigen scanning on follicular dendritic cells (FDCs) can be considered stage 1 followed by antigen presentation to antigen-specific GC follicular helper T (GC-T_{FH}) cells as stage 2. DZ reentry follows as one consequence of productive cognate contact accompanied by extensive proliferation and upregulation of the somatic hypermutation machinery for BCR diversification as stage 3. There appears to be a prolonged phase after DZ reentry and before mutated mGC clonal progeny appear to express changes associated with LZ reentry that signifies stage 4 and begins another cycle.

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findings indicate intrinsic differences in signaling for IgG switched B cells (Wakabayashi et al. 2002; Horikawa et al. 2007; Waisman et al. 2007). There is also evidence for alterations in biophysical properties of IgG1 BCRs (Liu et al. 2010). It is difficult to predict how these changes may impact pGC selection. Hence, the quality of the initial priming event substantially impacts the recall response spectrum permitting a range of outcomes imprinted at the outset.

Selection of preferred responding clones amplifies effective BCR specificities (Schwickert et al. 2011) with some impact on the bifurcation of early differentiation (Schwickert et al. 2007, 2009). Under the cognate guidance T_{FH} cells, some antigen-specific B cells undergo class-

switch recombination (CSR) and PC differentiation, whereas others rapidly expand and organize to form the pGC reaction (Qi et al. 2008). Recent studies suggest the GC- T_{FH} cells undergo further changes that progressively impact pGC B-cell selection (Weinstein et al. 2016). These earliest events may have long-term consequences in shaping the memory potential at recall through establishment of the appropriate mT_{FH} compartments.

In Figure 2, we depict the spectrum of outcomes plausible for the recall response to a T_H -cell-dependent memory response. Accordingly, cognate T-cell help is depicted as an obligatory initial event and memory B cells of different antibody class are not separated. We propose

Figure 2. Reprogramming B-cell memory. In this model, we depict the predicted potential associated with a memory follicular helper T (T_{FH})-driven memory B-cell response to recall. Here, we suggest that the memory B cell is the dominant presenter of recall antigen and that its earliest requirement for ongoing reactivity is cognate contact with antigen-specific memory T_{FH} cells. In this scenario, we propose that both IgM and class-switched memory can be precursors of this recall response. Major outcomes depicted then include initial activation and expansion of responding clones and bifurcation into differentiation pathways of memory B-cell replenishment and memory-response plasma-cell (mPC) formation that do not require B-cell antigen receptor (BCR) rediversification. These outcomes expand/replenish the precursor pool and provide immediate effector function. The third outcome is the de novo formation of the memory-response germinal center (mGC) reaction, which can then give rise to further affinity maturation with higher-affinity memory B cells and mPCs for long-lived, further-matured compartments of memory.

that recall outcomes can be considered across the full spatiotemporal spectrum as depicted. Initial memory responders can expand to self-replenish and differentiate into mPCs as early robust outcomes. mGC formation provides a secondary niche to drive BCR rediversification with cognate GC-mT_{FH} cells as the central presumptive regulator. These mGCs would produce higher-affinity post-mGC memory B cells and mPC than their earlier counterparts.

THE CONTEMPORARY VACCINE EFFORT

The tracking of memory B-cell responses in humans has undergone explosive activity in recent years. Antigen-labeling and judicious timing after antigen exposure coupled to deep sequencing enables dissection of antigen-specific BCR repertoires to complex antigens in humans. The range of studies in this arena is expansive and will not be reviewed here. However, it is pertinent to highlight the recent direction of research into HIV vaccines in which viral escape is driven by effective neutralizing antibody responses. Landmark studies by the Haynes group (Liao et al. 2013; Gao et al. 2014) outlined the coevolution between host immunity and viral escape that eventually produced broad neutralization. These studies highlighted the need to understand and direct ongoing clonal evolution with prime-boost vaccines.

In HIV infection, the problem seemed even more complicated such that the germline precursors of broadly neutralizing antibodies had negligible binding to the original neutralizing epitope. Now through antigen redesign (Jardine et al. 2016) and sequential immunization (Briney et al. 2016; Escolano et al. 2016; Sok et al. 2016; Tian et al. 2016), germline precursors can be targeted in humanized mouse models to drive broadly neutralizing outcomes after effective affinity maturation. This most recent example highlights the immediate and current need to understand the rules that govern clonal evolution within the GC reaction. Antigen design will permit initiation of appropriate memory B-cell and mT_{FH} compartments. Targeted recall responses that promote and permit BCR rediversification to redirect BCR binding to a

related but altered vaccine target promotes the appropriate evolution of broadly neutralizing reactivity within a series of mGC reactions.

CONCLUSIONS

Overall, we argue that there is ample evidence that antigen recall promotes a secondary GC reaction. Initial and subsequent studies of human peripheral blood and local analyses of model immune responses indicates that already matured mBCRs can rediversify through involvement in secondary GC reactions. Across multiple models, the capacity of IgM⁺ and class-switched memory B cells varies depending on the form of antigen, adjuvant used and location of the priming event. It is also likely that the quality and quantity of cognate mT_{FH} compartments influence these outcomes in ways that remain central but unresolved.

Finally, we propose that the intention of the recall determines the strategy to be used. In the first scenario, priming with expectation of viral escape, flexibility in the recall response may be what is required. Hence, the less-mutated and potentially broadly focused IgM mBCR repertoire may be beneficial to target for the recall. Alternatively, if the highest affinity to the same target antigen is needed for long-term high-affinity neutralizing protection, then affinity-matured class-switched memory B cells may be the best target for the recall. In both scenarios, the requisite cognate mT_{FH} compartment will be needed to induce the mGC reaction. To this end, it remains critical to unravel the rules of cellular engagement and molecular mechanisms driving IgM⁺ and class-switched memory B-cell clonal evolution in the mGC reaction at recall.

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