Do Memory B Cells Form Secondary Germinal Centers?

Yes and No

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Memory is the defining feature of the adaptive immune system. Humoral immune memory is largely though not exclusively generated in the germinal center (GC), which spawns long-lived plasma cells that support ongoing serum antibody titers as well as “memory B cells” (MBCs) that persist in the immune host at expanded frequencies. Upon reencounter with antigen, these MBCs are reactivated and potentially can contribute to protection by further expansion, rapid differentiation to antibody-forming cells, and/or reseeding of a new round of GCs along with somatic V region mutation and selection. Here I will discuss what controls these various potential fates of MBCs and the functional significance of different types of MBC reactivation.

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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Do memory B cells (MBCs) form secondary germinal center (GC) cells? In the first case, one might think not, because the main or even exclusive function of memory has classically been seen as the generation of more immediate and higher quality effector function. GCs do not generate effector function directly, as GC B cells (GCBCs) do not secrete appreciable amounts of antibodies (Abs). Moreover, it takes most likely 1 or more weeks for GC-derived short-lived antibody-forming cell (AFC) and potentially much longer for GC-derived long-lived AFC to appear. So, from the perspective that MBCs should be dedicated to rapid effector function, it would seem counterproductive to differentiate into a GCBC.

Conversely, it could be argued that if all MBCs were to differentiate into one or another type of AFC, which are terminal effector cells, the memory compartment would not be sustainable. One or more successive strong infections would “burn out” the memory, potentially leaving the host without an adequate complement of MBCs, particularly if the effector functions, including preexisting Abs and newly elicited Abs, prevented subsequent priming of new cohorts of naïve B cells (NBCs).

HISTORY OF THE QUESTION

Indeed, this question has been the subject of investigation even before the time when it was clear that the GC was a major source of both MBCs and long-lived plasma cells (LLPCs). Siekevitz et al. (1987) transferred immune cells into irradiated recipients and then reprimed 4 days later, capturing hybridomas that reflect the plasmablast (PB) compartment another 5 days after the recipient immunization. They did see clonal expansion and AFC differentiation of already mutated cells, but did not observe another round of somatic hypermutation (SHM). Therefore, although histologic analysis was not performed, it is highly likely that under these circumstances MBCs did not seed a new round of GC responses that were able to be captured by hybridoma technology. Dell et al. reached a similar conclusion in experiments studying the

antiphosphorylcholine response after bacterial immunization (Dell et al. 1989).

On the other hand, Berek and colleagues (1987) showed that MBCs could join tertiary response in intact animals that had been rested about a year after the first two immunizations. These cells showed additional accumulation of V region mutation, consistent with reentry into a GC in either the secondary and/or tertiary responses. This seemingly different result could be attributed to either the length of rest period and/or immunization of an intact, nonirradiated animal rather than immunization after cell transfer.

MORE RECENT CLUES FROM MURINE MODEL SYSTEMS

The above studies were mainly completed in the late 1980s. Subsequently, it became clear that the GC was a primary site of somatic V region mutation (Apel and Berek 1990; Berek et al. 1991; Jacob et al. 1991; Ziegner et al. 1994), thus linking these prior results to GC entry. However, it would be some years until direct tests in mice were performed to determine the potential of MBCs to make secondary GC, AFC, or both. Hence, whereas multiple experimental approaches and systems had made it abundantly clear that persistent Ag-specific cells, many or most of which were MBCs, could contain V region mutations (McHeyzer-Williams et al. 1991; Lalor et al. 1992; Schitteck and Rajewsky 1992; Weiss et al. 1992; Smith et al. 1997), whether they underwent another round of SHM, and if so where, had not been clearly delineated. This would require either definitive lineage marking and/or transfer experiments of bona fide purified MBCs.

Dogan et al. (2009) took a lineage-marking approach using an activation-induced cytidine deaminase (AID)-ERT2-Cre knockin mouse that on administration of tamoxifen would convert a *rosa26-flox-STOP-YFP* allele to irreversibly express yellow fluorescent protein (YFP). By giving tamoxifen at key points during an ongoing immune response, and taking advantage of the fact that most activated B cells express AID, they were able to mark a substantial proportion

of cells that were responding and then “chase” these out to later time points at which it was expected that MBCs would be present. Indeed, these cells comprised a quantitatively increased proportion of B cells in immunized versus non-immunized mice, supporting the notion that MBCs were specifically labeled. In addition to allowing these investigators to confirm the existence of murine immunoglobulin M (IgM) MBCs (Dell et al. 1989; Vajdy and Lycke 1995; Toyama et al. 2002; Anderson et al. 2006, 2007), they were then able to sort live putative MBCs, transfer them, and assess their *in vivo* responses. Notably, in these studies, they separated IgM and immunoglobulin G (IgG) MBCs and saw substantial differences in the nature of their secondary responses. While IgG MBCs differentiated almost exclusively into plasmablasts, IgM MBCs appeared to join GCs, providing direct evidence that at least some types of MBCs can undergo another round of Ag-driven GC-based selection.

In parallel studies, our group had used microarray gene expression analysis of MBCs generated in a transfer system to discover genes differentially expressed between MBCs and NBCs (Tomayko et al. 2008). This led to identification of several MBC-specific surface markers. Among these markers, CD80, PD-L2, and CD73 were shown to divide both IgM and IgG MBCs into distinct subsets (Tomayko et al. 2010). We focused on the subsets defined by CD80 and PD-L2, which included cells expressing neither marker (double-negative [DN]), cells expressing PD-L2 only (single-positive [SP]), and cells expressing both markers (double-positive [DP]). These subsets differed in their extent of isotype switching and somatic V region mutations (DP had the most and DN the least). SHM and isotype switch are indelible DNA marks established during the proliferative phase of the response; hence, that they were segregated in frequency among the subsets strongly suggests that there is limited, if any, interchange of cells between the subsets.

To investigate the functional potential of these MBCs subsets, we purified subsets of MBCs of identical genotype and specificity and transferred them into recipient mice that

had B cells that could not recognize the nominal Ag, then immunized the recipients. To control for effects of Ig isotype of the B-cell antigen receptor (BCR), as suggested by the Dogan et al. (2009) study, we first limited our studies to IgM MBCs. DP IgM MBCs generated a very rapid and robust early IgG AFC response, which was 10–20-fold higher than that of DN MBCs. NBCs did not make a detectable IgG response at the early time point (day 3.5). However, DP IgM MBCs did not engender a later GC response. Conversely, DN IgM MBCs could reseed a secondary GC response with almost equal efficiency to NBCs. SP IgM MBCs gave intermediate responses in both assays. Focusing only on IgG MBCs, of which there were only sufficient SP and DP to do the transfer studies, similar results were obtained. Most notably, the responses of IgG or IgM SP MBCs were indistinguishable, suggesting that for this group of cells isotype was not a determining factor for function. Moreover, the finding that there were very disparate outcomes among IgM MBCs, depending on to which subset they belonged, further argued that BCR isotype by itself was not exclusively decisive in controlling the outcome of MBCs restimulation *in vivo*.

Nonetheless, the “Ig class” model proposed by Dogan et al. (2009) and the “subset” model proposed by Zuccarino-Catania et al. (2014) do not fundamentally disagree, as shown in Figure 1, although the latter model provides a more precise explanation. Since IgG MBCs are predominantly SP and DP types, with SP being a minority, in total they would be expected to favor substantially early AFC responses over making new GC reactions. Conversely, IgM MBCs have a dominant population of DN cells that would reseed GCs, something that was readily observed by Dogan et al. (2009). Since the latter paper never tried to subdivide the IgM MBCs they did not detect the further heterogeneity, but their conclusions were not in conflict with subsequent work. Further, the agreement of these two studies suggests that either non-transfer (Dogan) or transfer (Zuccarino-Catania) systems should reveal similar biology.

More recently, McHeyzer-Williams et al. (2015) addressed the same question using NP

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Figure 1. Comparison of the immunoglobulin (Ig) class and subset models that correlate memory B cells (MBCs) phenotype with functional potential in a secondary response. (*Top*) Two circles show IgM and IgG MBCs and their proposed direct correlation with germinal center (GC) and plasmablast (PB) generation, respectively. Also shown in the pie chart, although not known at the time of the original proposal (and therefore shown in faded colors), is the different composition of these two Ig classes in terms of MBCs subsets as defined by PD-L2 and CD80 expression (see text), namely, double-negative (DN) (green), single-positive (SP) (purple), and double-positive (DP) (yellow). (*Bottom*) Three circles show the three subsets and depict their functional potential as reported by Zuccarino-Catania et al. (2014). Conversely, their differential compositions of IgM (blue) and IgG (red) MBCs are shown in the pie chart. Because IgM MBCs are dominated by DN, they will conspicuously make GCs, whereas because IgG MBCs are dominated by DP, they will tend to make more PB responses. However, as shown at the *bottom*, the more precise distinctions are seen by the subset segregation, regardless of Ig isotype.

immunization in a nontransfer system. They did not have available lineage marking as did Dogan et al. (2009) but instead tracked cells lacking both IgM and IgD after secondary immunization and found them in the GC. The proportion of such cells among GCBC was not reported, as the IgM Ag-specific cells were not quantitated, although other studies showed that IgM MBCs and GCBC are plentiful (Shinall et al. 2000; Wolniak et al. 2006; Dogan et al. 2009; Pape et al. 2011; Zuccarino-Catania et al. 2014). While these data were interpreted as demonstrating the IgG MBCs could readily populate GC on

restimulation—in contrast to prior reports (Dogan et al. 2009; Pape et al. 2011; Zuccarino-Catania et al. 2014)—without either lineage tracing or cell transfer, it would be difficult to exclude that IgM MBCs underwent rapid switching once restimulated. Indeed, McHeyzer-Williams et al. (2015) show expression of AID in the GCBC and argue that further SHM was induced; Zuccarino-Catania et al. (2014) found highly efficient class switching of IgM DP MBCs as they differentiated to plasmablasts within 3.5 days of restimulation (Zuccarino-Catania et al. 2014), further supporting this possibility.

RECENT WORK ON INFLUENZA AND MALARIA IN MICE

Pathogen infection can change parameters of both memory development as well as response (Onodera et al. 2016). Therefore, it is important to determine whether hapten or model Ag responses—as studied by many laboratories including our own—are modified in any way by specific infections. MBC behaviors that are intrinsically different among subsets or between MBCs and NBCs are difficult to assess in the context of other extrinsic changes as would be seen in an immune animal (e.g., preexisting Ab as well as primed CD4 and CD8 T cells). While transfer systems can control for such variables, it has been asserted that intrinsic behaviors are influenced by the presence of other factors in immune animals and that competition between IgM and IgG MBCs renders IgM MBCs effete (Pape et al. 2011). Nonetheless, bulk splenocytes or purified B cells from immune animals have been transferred to recipients where they have been shown to mediate protective secondary IgG antibody responses, in either a T-dependent or -independent fashion, depending on the circumstances and immunogen (Weisel et al. 2010; Onodera et al. 2016).

In general, because of very low cell numbers, it has been difficult to isolate enough pathogen-specific MBCs to then transfer them, although Onodera et al. (2016) recently did isolate high-affinity-specific MBCs for transfer experiments. Even so, MBCs in these experiments were recovered from twice-vaccinated, rather than post-infection, animals. Very small numbers of IgM⁺ MBCs were transferred and only AFC responses were assessed. Also very recently, Ag-specific MBCs have been identified in mice that were infected with malaria (Krishnamurthy et al. 2016). These MBCs consisted of both switched and unswitched cells, and among the IgM MBCs, heterogeneity was observed for expression of previously identified murine MBC markers CD73 and CD80. They used infected RBCs as immunogens and thereby circumvented Ab-mediated Ag clearance to effectively re-immunize intact animals without the need for B-cell transfer. The vast majority of the response

at days 3 and 5 were from MBCs, as inferred by comparison to nonimmune animals. By determining that many responding cells remained IgM they were able to show that much of the early response indeed derived from IgM MBCs, although they were in the presence of IgG MBCs. Though they did show that some MBCs begin to adopt a potentially pre-GC phenotype (i.e., express the GC marker GL7 but do not downregulate CD38 as would occur in mature GCs), they did not directly assess MBC contribution to GCs. Overall, these results in a malaria system and without cell transfer tended to agree with Zuccarino-Catania et al. (2014) that IgM MBCs can contribute to rapid AFC responses provided that they belong to subsets expressing CD80. They further establish that this is the case whether MBCs are transferred or left in original hosts, with or without competing IgG MBCs.

BACK TO THE HUMAN

It is naturally much more difficult to show that MBC responses involve reseeding of GCs with established MBCs in the human. However, a few seminal studies provide important clues that this is likely the case. Wrarmert et al. (2008) evaluated responses to drifted influenza viruses by isolated responding plasmablasts in the peripheral blood of vaccinated individuals and found that they likely derived from preexisting MBCs. However, these MBCs had undergone an additional round of SHM, accumulating numerous mutations that had been selected to bind the newly introduced hemagglutinin of the drifted vaccine virus. This mutation, selection, and differentiation into plasmablasts occurred within 7 days, suggesting either a rapid new GC response and/or extra-GC mutation during the differentiation of the plasmablasts (William et al. 2002; Di Niro et al. 2015). Similar findings were subsequently obtained during the 2009 pandemic virus response, this time with natural infection, in which broadly neutralizing antibodies were elicited among the responding plasmablasts, again because of another round of mutation of MBCs (Wrarmert et al. 2011; Li et al. 2012).

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A series of elegant experiments by Dorner and colleagues addressed similar issues in humans, this time using tetanus toxoid vaccination (Frolich et al. 2010; Giesecke et al. 2014). They also found that at least some of the plasmablast response consisted of cells that likely came from MBCs and that had undergone additional rounds of SHM. They also found plasmablasts that apparently expanded from MBCs without additional mutation. These findings in many ways echo the classic studies in mice showing the potential for both pathways, as well as more recent studies suggesting that, in mice, subsets of MBCs contain different potentials for either new GC or plasmablast responses. However, in neither the influenza nor tetanus toxoid studies could the actual site of additional V region diversification be pinpointed. Since it is now clear that SHM and AID expression are not restricted to the GC (William et al. 2002; Marshall et al. 2011; Di Niro et al. 2015), it remains unclear where these newly diversified plasmablasts that derive from MBCs are being generated in humans. However, in any case, it is clear that at least some MBCs can undergo second rounds of mutation and selection.

THEORETICAL CONSIDERATIONS

It appears from both human and murine studies that, under certain circumstances, MBCs can reenter GCs or at least reenter a pathway that will generate and select additional V region diversity. What is conceptually most interesting is the recent appreciation that there are some MBCs that are committed to quick effector function, without much regenerative potential and others that are capable of creating new and additionally mutated and selected crops of MBCs clones, allowing for stepwise evolution and maintenance of the MBCs compartment. This cellular division of labor does not seem to apply to NBCs. For example, the most abundant follicular type of NBC (as well as marginal zone [MZ] NBC) can generate early extrafollicular responses, GCs, and, subsequently, both MBCs and long-lived PCs (Jacob and Kelsoe 1992; Song and Cerny 2003). Hence, these cells are less specialized and more flexible than MBCs.

Such a setup could make teleological sense. Most NBCs are constantly replenished and are unbiased with respect to prior Ag experience. Exceptions that prove the rule may be B1 B cells that are generated early during development and with a restricted repertoire that may be biased via evolution to certain conserved pathogen epitopes (i.e., innate-like cells) (Hardy and Hayakawa 1991; Hardy 1992). MBCs are a more unique and precious resource, having collected information about pathogen experience that is likely to be useful in the future. The next encounter with a pathogen may involve the identical pathogen, or an antigenically varied version. The optimal system needs to anticipate both possibilities as well as the next round (i.e., even a tertiary infection). Having an expanded population with the highest degree of SHM and affinity maturation that is committed to quick differentiation on reencounter (e.g., DP MBCs) makes clear sense. This differentiation is terminal and if it were the only mode of response, it could exhaust the MBCs compartment and leave the host relatively unprotected from subsequent challenge. Furthermore, some pathogens evolve, either seasonally to escape preexisting Ab (such as influenza) or even during the course of an infection as in retroviruses like HIV. In these cases, previously mutated MBCs may not optimally recognize the newly mutated pathogens and, if they differentiated directly to AFCs, they would produce low-affinity Ab. For these two eventualities, it makes sense to include a second layer in the MBCs compartment that proliferates and makes new MBCs while it undergoes another (perhaps more brief) round of SHM and affinity selection to direct the response to the modified epitopes of the evolved pathogen.

CONCLUSION

So, in the context of MBC subsets, it can be said that both concepts about MBC potential are correct: they both do and do not reenter GCs. Yet, a number of salient questions remain. Are the MBC subsets that we have defined independent of Ig isotype, or does this isotype add yet another layer of regulation? Not enough work

has been performed to resolve this in multiple systems. Are these MBC subsets homogeneous, or is there yet more heterogeneity and hence functional potential to be discovered? And what about tissue-resident MBCs; they clearly exist but to date in murine systems not enough cells have been recovered to allow robust functional studies. The parallels between murine and human systems are clear but not yet fully defined either. Most significantly, are there markers in humans that define similar subsets as have been seen in multiple murine models? CD80 seems somewhat similar, but PD-L2 is not heterogeneously expressed among human CD27⁺ MBCs (unpublished data). Nonetheless, quite a bit of heterogeneity has been found among human MBCs (Sanz et al. 2008), but the links to functional potential are only partly known (Weisel and Shlomchik 2017). Most intriguingly, although it is known that cross-reactive influenza virus immunization can elicit in 7 days a plasmablast reaction that already contains a substantial additional V region mutation that redirects previously existing MBCs toward the new immunogen, the question is where does that response take place? It may seem very quick for a new GC to form, begin GCBC V region mutation and selection, and then create full-fledged plasmablasts that circulate in blood. It will not be straightforward to definitively address this question in humans, as tissues are not readily available but perhaps nonhuman primate models will help us answer this and other related questions beyond whether or not MBCs can reseed secondary GCs.

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