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## Heterogeneity of memory B cells

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### Abstract

Potential solid-organ transplant recipients broadly sensitized to HLA have long wait times, low transplant rates and poor outcomes. The new kidney allocation system has improved access to the most highly sensitized recipients; however, their long-term outcomes are unknown. Emerging data suggest that memory B cell repertoire is broader than the plasma cell repertoire, therefore, despite refinements in anti-HLA antibody detection technology, donor-specific HLA-specific memory B cells may in fact be present in some, if not most, highly sensitized recipients with no detectable donor-specific antibodies. In addition, new findings have underscored the heterogeneity in memory B cell generation, and in the signals that determine memory versus plasma cell fate during primary antigen encounter, as well as memory B cell differentiation upon antigen reencounter into plasma cells or reentry into germinal centers to subsequently emerge as higher affinity and class-switched plasma cells. Thus, heterogeneity memory B cells generation may affect the efficacy of specific immunomodulation during the recall response. We propose that the ability to quantify donor-specific B cell in transplant recipients is urgently required to provide insights into the mechanisms of sensitization and recall, and for the early detection of acute and chronic AMR.

### Introduction

One of the goals of the new kidney allocation system implemented in December 2014, is to increase transplant opportunities for difficult-to-match patients. Indeed, transplantation rates significantly increased for patients with calculated panel reactive antibody (cPRA) 99-100%, suggesting that more broadly-sensitized recipients are receiving kidney transplants (1). While early 6-month graft survival appears unchanged in these highly-sensitized recipients receiving permissive donor allografts, Hart et al. (1) cautioned that the long-term graft survival requires close monitoring as the potential impact of these high cPRA on long term grafts outcomes is unknown. Indeed, previous studies show that PRA at the time of transplant was associated with a step-wise graded association with death-censored graft failure, death with function, and the combined outcome (2). On the other hand, with the

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refinements in the anti-HLA antibody detection technology, cPRA *per se* may not imply an increased immunological risk when modern DSA assignment is used. In a recent study, donor specificity but not broadness of sensitization was noted to be associated with antibody mediated rejection and graft loss (3). Amidst this uncertainty, little is currently known about the pathophysiologic mechanisms that lead to the development of these extremely high cPRA antibodies, especially in the individuals who may have not been exposed to the breadth of HLA antigens. Emerging data suggest that the memory B cell (memB) repertoire is broader than the plasma cell repertoire (4), so that serological memory may not be equivalent to the memB repertoire. Interestingly, a relatively large retrospective study demonstrated that *past* high-sensitization status defined by either a cPRA (>50%) or peak-PRA (pPRA) (>50%) correlates with inferior graft outcomes, including increased incidence of delayed graft function, increased rejection rates and decreased graft survival (5). Furthermore, graft outcomes were inferior even in the low-sensitized group (PRA 5-50%) and in those that “converted” from a high-sensitized to low-sensitized group over time prior to transplantation. These observations raise the possibility that donor-specific memB may in fact be present in some, if not most, highly sensitized recipients of permissive donor allografts.

The diversity of the B cell repertoire supports the hypothesis that high cPRA is product of a broad repertoire of plasma cells producing antibodies that recognize specific HLA alleles or shared eplets (6, 7). The universality of this notion has however been challenged by the series of publications by Zorn and colleagues (8-11), (12) that anti-HLA serum reactivity may comprise, at least in part, polyreactive antibodies. Thus, it is possible that high cPRA may be explained by polyreactive antibodies produced by a limited repertoire of plasma cells. These antibodies may bind to antigens exposed on apoptotic cells or to denatured antigens on single HLA antigen beads used to detect HLA-specific antibodies (8, 10). Furthermore, these polyreactive antibodies, similar to HLA-specific antibodies, can activate complement to cause cell injury (10), and potentially, promote the generation of opsonins that enhance antigen uptake and presentation to donor-specific T and B cells (13, 14) or mediate the recruitment Fc-expressing cells that elicit graft injury (15-18). The potential role of polyreactive antibodies in solid organ transplantation has recently been reviewed (11) and will not be discussed further; instead we focus on discussing the latest findings on the heterogeneity in memB cells mediating the recall humoral response and potential implications to solid organ transplantation.

## How naïve B cells differentiate into antibody secreting cells and memory B cells

The progression of a naïve B cell into a memory and plasma cells upon soluble antigen encounter has been extensively studied in mouse models, where the fate of antigen-specific B cells in secondary lymphoid organs can be examined in detail. When the B cell receptor (BCR) on naïve B cells engages antigen in the draining lymph node, the activated B cells upregulate CCR7 and migrate to the T-B interface. At the same time, naïve CD4<sup>+</sup> T cells recognizing antigen processed and presented by dendritic cells downregulate CCR7 and upregulate CXCR5 and EBi2 that collectively direct the T cell to move to the T-B cell

border. There, these differentiated extrafollicular T follicular helper (Tfh) cells engage in cognate interactions with B cells and specialized IL-2 quenching CD4<sup>+</sup>ICOSL<sup>+</sup>CD25<sup>+</sup> dendritic cells (19, 20). This interaction results in B cell proliferation and differentiation into extrafollicular memB cells, short-lived plasmablasts (PB), or B cells that upregulate CXCR5 to migrate back into the center of the B cell follicle where they initiate a germinal center (GC) response.

The GC is divided into two compartments; the light zone comprising follicular dendritic cells (FDCs) and Tfh cells and is proximal to the marginal zone in the spleen or to the capsule in the LN, and the dark zone that is closest to the T cell zone. Within the light zone, B cells encounter antigen presented on specialized follicular dendritic cells (FDCs), and also receive survival signals from GC-Tfh cells (reviewed in (21, 22)). Survival signals include those provided by the interaction between costimulatory molecules, CD28-B7, CD40-CD154 and ICOS-ICOSL, and by cytokines such as IL-21 and BAFF. In the absence of these signals, B cells undergo apoptosis, while positively selected B cells migrate into the dark zone to undergo cell proliferation and BCR diversification through Ig somatic hypermutation (SMH) mediated by activation-induced cytidine deaminase (AID) (23, 24), (25, 26). One consequence of AID activity is damage of BCR genes and apoptosis of these B cells, while those with intact BCR exit the dark zone and reenter the light zone where their newly generated BCR are tested for binding to antigen and access to T cell help (25). Following repeated rounds of entry and exit from the dark zone (reviewed in (22)), post-GC B cells emerge as plasma cells (PC) that are ultimately responsible for persistent circulating antibodies, or as memB cells that are responsible for the recall antibody response upon antigen reencounter.

Central to the generation of protective humoral immunity is the controlled differentiation of antigen-activated B cells into short-lived effector antibody-secreting PB, long-lived PC and quiescent memB cells. The early generated PB play critical roles in containing infection, the later post-GC PC are responsible for preventing re-infection, while the quiescent memB cells generate the recall antibody response upon re-infection. Factors that determine these cell fates during primary antigen encounter have been extensively investigated, and critical roles for the affinity of the BCR for antigen, and factors derived from Tfh have been demonstrated (reviewed by (27)). The acquisition of a plasma cell over GC or memB cell phenotypes is facilitated by high BCR affinity for antigens, both in the early T:B border response and within ongoing GCs (28-30). The downstream signals of BCR engagement that determine cell fate that promote plasma cell differentiation have also been characterized. Specifically, Sciammas and colleagues (31-33) reported that a measure of BCR signaling intensity is graded expression of interferon regulatory factor-4 (IRF4). High levels of IRF4 dial up the Blimp-1 expression and promotes the plasma cell program, while shutting down the expression of Bach2 controlling germinal center B cell fate (33, 34). In contrast, more modest levels of IRF4 are required for differentiation into GC B cells (31, 33). In the late GC-dependent phase, the generation of plasma cells is also dependent on their affinity for antigen presented on FDCs, and cognate interactions with Tfh (30) (24). Krätzler et al. (35) recently provided new mechanistic details whereby PCs differentiation was shown to be induced in a discrete subset of high-affinity B cells residing within the light zone of the GC. Differentiation into PC is initiated upon engagement with intact antigen presented on FDC,

while cognate interaction with Tfh cells provided signals essential for completing the PC differentiation process and for driving the migration of maturing PCs through the dark zone and out of the GC.

Memory B cells had classically been defined as class-switched B cells with non-GC phenotype; however, recent publications within the past decade have provided evidence for heterogeneity in memB cells generation. Using a new mouse model of memB cell-labeling based on cytidine deaminase expression, Dogan et al. (36) reported that following mouse immunization with sheep red blood cells, IgM<sup>+</sup> quiescent memB cells were present at 4-times the frequency of IgG1<sup>+</sup> memB cells. Subsequently, memory IgM<sup>+</sup> and IgG<sup>+</sup> B cells have been reported following immunization with other model antigens or malaria-infected erythrocytes (37, 38) (39) (40). As with antibody secreting cells, memB cells are generated in two distinct phases: the early phase that produce memB cells in a pre-GC or GC-independent manner, and a late post-GC phase. Pre-GC memB cells tend to be of low affinity and enriched for IgM<sup>+</sup> whereas the post-GC memB cells are of higher affinity and comprised both IgM<sup>+</sup> and IgG<sup>+</sup> subsets (37, 38). Similar to pre-GC B cells, lower-affinity B cells with lower mutation load in the GC are preferentially recruited into the memB cell pool while higher affinity GC B cells are PC-prone (41). Memory-prone B cells have higher expression of the transcriptional repressor Bach2, which is induced by lower levels of T cell help. Interestingly, GC B cells that lacked CXCR4 and therefore failed to enter the DZ were more likely to enter the memory compartment, suggesting that T cell help may be sufficient to drive memory differentiation without further proliferation or SMH that is necessary for PC differentiation (42). Despite these new insights, the full spectrum of signals that control the post-GC memB cell fate decision and their exit from the GC remains unclear, and their full definition is hampered by the lack of a “master” transcription factor that can be used to trace these cells *in vivo* (reviewed in(43)).

Memory B cell subsets exhibit different longevity, with the IgM<sup>+</sup> memory surviving significantly longer than the IgG<sup>+</sup> memB cells (37). Because IgG<sup>+</sup> memory cells tend to be more mutated than their IgM counterparts, whether BCR affinity or isotype switching determined memB cell persistence was recently ascertained using a novel mouse model where class-switching and SMH could be independently controlled. Gitlin et al. (44) reported that class-switching to IgG1 biased the fate choice to PC over memory fate, whereas SHM reduced the longevity of memory cells. The authors speculated that SHM reduced the longevity of memB cells by creating polyreactive specificities that were autoreactive and selected against over time, resulting in the preservation of a lower affinity, nonautoreactive memB cells.

A consequence of BCR affinity affecting B cell fate is manifest in an altered propensity to differentiate into memory or effector antibody secreting cells over the course of an immune response. Elegant pulse-chase experiments by Weisel et al. (45) showed that memB cells are generated mostly in the pre-GC and early GC period after immunization, whereas long-lived PC emerge late post-immunization. These findings of differences in affinity and kinetics of memory versus PC generation have important implications for transplant recipients. Firstly, treatment to reverse ongoing rejection process may successfully prevent the generation of long-lived plasma cells, but may not be able to prevent the earlier generation of memory

donor-specific B cells. Second, the antigen-specificity of memB cells will not be identical to that of long-lived PC and the circulating antibodies they produce. Indeed, Lavinder et al. (4) used high-resolution liquid chromatography tandem mass spectrometry proteomic analyses of serum antibodies coupled with next-generation sequencing of the V gene repertoire of peripheral B cells following tetanus toxoid booster vaccination. They showed that ~100 antibody clonotypes and that just 3 clonotypes accounting for >40% of the entire serological response. This antibody repertoire represented <5% of the peripheral memB cells that were anti-tetanus specific. Given that antibodies are used to measure B cell sensitization in pre- and post-transplant recipients, it is likely that the donor specific antibody (DSA) assays under identifies pre-sensitized patients that have developed donor-reactive memB cells but have undetectable circulating DSA. Assays that quantify the frequency of memory HLA-specific B cells (46) independently of serum anti-HLA IgG will be able to test this hypothesis. Finally, the differential longevity of memB cells suggest the possibility that higher affinity and class-switched IgG memB cells may be more quickly lost compared to lower affinity and IgM<sup>+</sup> B cells. If true, then time after sensitization may affect the quality of the recall B cell response, with those recently sensitized having the highest affinity recall response compared to those who had been sensitized a long time ago.

### Heterogeneity of the memory B cell recall response

A faster, higher-titer and class-switched antibody response are hallmarks of a recall response upon reencounter with antigen. Heterogeneity in memB cell generation suggests that these B cells will have different fates upon antigen re-encounter, with some favoring plasmablast differentiation, while others developing into GC B cells that can undergo rounds of proliferation and SMH to generate new, higher-affinity and class-switched memory cells and antibody-secreting cells. The heterogeneity in cellular response during antigen recall is well documented experimentally, although the rules that predict their cell fates in the recall response remain to be clarified. Seminal studies by Dogan et al. (36) demonstrated that the adoptive transfer of memIgM<sup>+</sup> B cells and boosted with SRBC differentiated into mGC cells, whereas the IgG1<sup>+</sup> memB cells gave rise primarily to mPCs. Likewise, Jenkins and colleagues (37) reported that the IgG<sup>+</sup> memB cells predominated over IgM<sup>+</sup> memB cells in the recall response when challenged soon (d320) after primary immunization. These IgG<sup>+</sup> memB cells preferentially differentiated into antibody-secreting cells. The lack of a memIgM<sup>+</sup> B cell response was due to the presence of circulating antibodies that outcompeted for access to antigen, the low affinity BCR expressed on IgM<sup>+</sup> memB cells. Upon adoptive transfer, the IgM<sup>+</sup> memB responded to antigen by differentiating into GC B cells that underwent additional rounds of class-switching and affinity maturation to generate a high- affinity IgG response. In contrast, when recall was performed at d450 post-primary immunization, at a time when circulating antigen-specific IgG titers and the frequency of IgG<sup>+</sup> memB cells had significantly declined, IgM<sup>+</sup> B cells were the primary cells responding by differentiating into GC cells.

The phenotype memB that predicts PC or GC fate has been further refined by recent studies. Shlomchik and colleagues (39, 45, 47, 48) reported that irrespective of IgM or IgG expression, memB cells that express CD80, PD-L2 and CD73 were mostly likely to produce PC, while those that lack these markers were more likely to produce GC cells. Since most of

the IgM<sup>+</sup> memB cells lack CD80 and CD73, while IgG<sup>+</sup> memory cells expressed CD80 and CD73, these observations are not inconsistent with those of Jenkins and colleagues. More recently, Krishnamurthy et al. (40) investigated the memB cell and recall response in mice infected with malaria. Three sets of memB cells were detected; somatically hypermutated IgM<sup>+</sup> and IgG<sup>+</sup>, and an unmutated IgD<sup>+</sup> subsets, with their longevity corresponding to their levels of somatic hypermutation. Challenge with malaria-infected erythrocytes, resulted in the early (d3) proliferation of high-affinity IgM<sup>+</sup> memB cells and differentiation into PC in both a T cell-dependent and independent manner. Notably the more highly mutated IgM<sup>+</sup> memory that responded to secondary challenge expressed CD80 and CD73 expression. Finally, the sequestration of malaria antigens within the infected cells may have contributed to the ability of the higher affinity IgM<sup>+</sup> memB cells to respond even in the presence of IgG<sup>+</sup> memB cells and circulating IgG.

Teleologically, the ability of memB cells to generate GC progeny that further diversify their BCR would be essential for protective immunity towards rapidly evolving pathogens. Supporting this notion, McHeyzer-Williams and colleagues (49, 50) have investigated the memB cell response following prime and boost with NP conjugated with keyhole limpet hemocyanin adjuvanted with monophosphoryl lipid A. They showed that the boost resulted in IgM<sup>+</sup> memory cells that favored differentiation into PC, whereas the IgG<sup>+</sup> memB cells preferentially differentiated into GC B cells that underwent BCR diversification and acquired a GC transcription program. Taken collectively, it appears that both IgM and IgG memory can participate in the recall response, with different propensities to PC or GC differentiation depending on their BCR isotype, affinity for antigen and differentiation state. More recently, Asrir et al. (51) reported that the location and access to the type of Tfh can also determine memB cell fate. Circulating memory Tfh promoted more secondary germinal centers, whereas the resident memory Tfh cells that localized close to memB cells in the B cell follicle promoted plasma cell differentiation. Those variables in turn dictated the kinetics of antibody production in the recall, and also the functional properties of the secreted antibodies.

## Concluding remarks

How do these basic findings on heterogeneity of memB cells impact our understanding of the antibody response in sensitized patients after transplantation? When incompatible allografts are transplanted into recipients with pre-existing DSA, it is reasonable to assume that they will also harbor donor-specific memB and memory T cells. These individuals are more likely to subsequently develop a recall DSA response post-transplantation that mediates acute or chronic antibody-mediated rejection, or mixed AMR plus TCMR rejection (52-54). Whether the *de novo* DSA response is the result of memB cells differentiating directly into PCs or reentering a GC response to subsequently generate PCs may affect the efficacy to immunosuppression. Our recent studies show that the donor-MHC B response following cardiac transplantation in sensitized mouse recipients is T cell-dependent and skewed towards a PC response (55, 56). Thus, co-stimulation blockade with CTLA-4Ig was effective at preventing the development of a recall DSA response, a finding consistent with the clinical observations of significantly low rates of DSA development in transplant recipients receiving belatacept, despite high rates of acute rejection (57). In contrast,

reversing an established recall DSA response that is mediated predominantly by PC may be more amenable to drugs that deplete PC, such as bortezomib or carfilzomib (58, 59), whereas a recall response that is dependent on GC output may additionally be responsive to drugs that inhibit the GC response. In addition, the notion that memB cells have different longevity depending on the mutation load suggests that recently sensitized recipients may mount a recall DSA response that has a more rapid kinetics and higher affinity, whereas when the sensitizing event occurred many decades ago, a functional recall response may require reentry into a GC response and thus be more sensitive to immunosuppression that prevents the reactivation of Tfh and lower-affinity memB cells. Donor specific memory B cells that reenter a germinal center may also switch from non-C1q binding to C1q-binding IgG producing cells, and thus produce IgG antibodies that not only mediate pathology via Fc-receptor binding but also through complement activation. Finally, the observation that circulating antibodies may shape the quality of the recall response, raises the possibility that plasmapheresis used to remove DSA in preparation of transplantation in sensitized recipients, may inadvertently promote a more vigorous recall response by allowing antigen to access memB cells with low affinity for alloantigen.

Testing the behavior of alloreactive B cells, with either naïve or memory phenotype, in transplant recipients or those waiting to receive a transplant are hampered by the need to study developing B cell responses in the secondary lymphoid structures, concerns that the blood does not fully capture the full repertoire of memB cells, and a lack of an assay to quantify the frequency of donor-specific B cells. Reports that memB cells specific for vaccine antigens can be detected in the blood, and that their frequency tracks with time post-antigen exposure (60, 61), suggest that an assay that accurately quantifies circulating donor-specific memB cells is feasible (46, 62). Such an assay will likely complement our ability to quantify DSA by providing an earlier diagnosis of acute or chronic AMR, as well as in identifying successful desensitization protocols and treatments of AMR.

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## Abbreviations

<b>AID</b>	activation-induced cytidine deaminase
<b>BCR</b>	B cell receptor
<b>BAF</b>	B cell activation factor
<b>CPRA</b>	calculated panel reactive antibody
<b>CTLA4</b>	cytotoxic T-lymphocyte-associated protein 4
<b>DSA</b>	donor-specific antibodies
<b>FDC</b>	follicular dendritic cells
<b>GC</b>	germinal center

<b>HLA</b>	human leukocyte antigen
<b>IRF-4</b>	interferon regulatory factor-4
<b>MemB</b>	memory B cells
<b>PB</b>	plasmablasts
<b>PC</b>	plasma cells
<b>SHM</b>	somatic hypermutation
<b>Tfh</b>	T follicular helper

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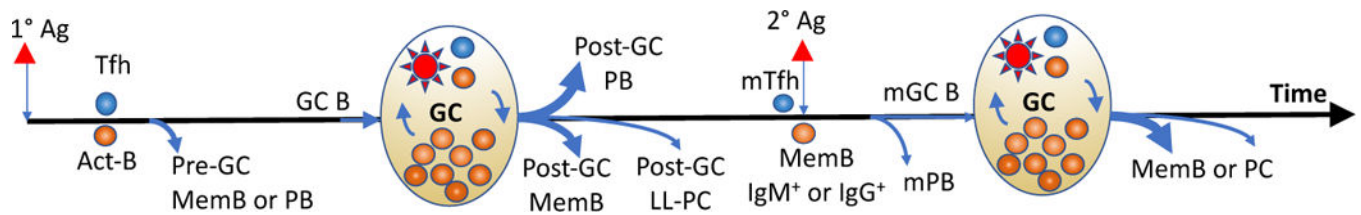
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**Figure 1.**

Summary of B cell differentiation after primary (1°) or secondary (2°) antigen (Ag) encounter. Primary antigen encounter generates two waves of memory B cells (MemB) and antibody secreting cells. The early wave is prior to and independent of the germinal center (GC) response, and generates pre-GC MemB cells as well as short-lived plasmablasts (PB). The later wave generates a post-GC MemB and long-lived plasma cells (LL-PC). Upon a secondary Ag encounter, IgM<sup>+</sup> and IgG<sup>+</sup> memory B cells have distinct abilities to differentiate directly into secondary PB (mPB) or GC cells that undergo further B cell receptor diversification to ultimately emerge as secondary MemB or PCs. Width of blue lines indicates relative quantities of cell output.