Generation and Utilization of Diverse Memory B Cells After Flavivirus Challenges

Rachel Wong
Washington University in St. Louis

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WASHINGTON UNIVERSITY IN ST. LOUIS

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Generation and Utilization of Diverse Memory B Cells After Flavivirus Challenges

by

Rachel Wong

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The Graduate School
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requirements for the degree
of Doctor of Philosophy

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<th>Description</th>
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<tr>
<td>AID</td>
<td>activation induced cytidine deaminase</td>
</tr>
<tr>
<td>BLI</td>
<td>biolayer interferometry</td>
</tr>
<tr>
<td>BMPC</td>
<td>bone marrow plasma cell</td>
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<tr>
<td>DII</td>
<td>domain II</td>
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<tr>
<td>DIII</td>
<td>domain III</td>
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<tr>
<td>DENV</td>
<td>Dengue Virus</td>
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<tr>
<td>FL</td>
<td>fusion loop</td>
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<tr>
<td>GC</td>
<td>germinal center</td>
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<tr>
<td>GL</td>
<td>germline</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese Encephalitis Virus</td>
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<tr>
<td>LLPC</td>
<td>long-lived plasma cell</td>
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<tr>
<td>LR</td>
<td>lateral ridge</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MBC</td>
<td>memory B cell</td>
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<tr>
<td>WNV</td>
<td>West Nile Virus</td>
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<td>ZIKV</td>
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ABSTRACT OF THE DISSERTATION

Generation and Utilization of Diverse Memory B Cells After Flavivirus Challenges

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Doctor of Philosophy in Biology and Biomedical Sciences
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Professor Deepta Bhattacharya, Chair
Professor Paul Allen, Co-Chair

Over the course of an effective immune response to an infection, two distinct B cell populations are generated to provide protection against reinfection, long-lived plasma cells (LLPCs) and memory B cells (MBCs). LLPCs and MBCs originate from germinal center (GC) B cells that have undergone B cell receptor (BCR) affinity maturation through iterative rounds of somatic hypermutation, proliferation, and selection. Thus, LLPCs and MBCs can bind to their antigen with higher affinity than their naïve B cell precursors. LLPCs constitutively secrete antibodies and can provide sterilizing immunity that pre-exists subsequent infections. MBCs, on the other hand, are quiescent and provide protection only after being re-activated. Re-activation of MBCs can result either in the re-initiation of GC reactions to mold and affinity mature BCRs toward new specificities or in the differentiation into plasma cells. However, the likelihood of re-activating MBCs in the presence of pre-existing serum antibodies secreted by LLPCs is low. Thus, the etiological role of MBCs is not well defined. We previously showed that MBCs are capable of recognizing pathogen escape mutants that have evaded serum neutralization by
antibodies secreted from LLPCs, though it is unclear the breadth of diverse antigens recognized by MBCs.

Here, we demonstrate that the diversity of MBCs extends to recognizing heterologous antigens. Using mouse models of sequential heterologous flavivirus vaccinations and activation induced cytidine deaminase conditional knockout mice in which affinity maturation is ablated, we observed that MBCs respond to heterologous antigens without further affinity maturation and remodeling of their BCRs. Instead, MBCs respond by directly differentiating into plasma cells to secrete antibodies, some of which are neutralizing, targeting the secondary pathogen. Thus, the diversity of MBCs generated after a primary response dictates the response to subsequent heterologous challenges. Mechanistically, this diversity results from the continued, active recruitment of low affinity germinal center B cells into the MBC pool.

Given the different roles of LLPCs and MBCs upon secondary challenge by either a homologous or heterologous pathogen, understanding the durability of LLPC and MBC recall responses can provide insights on how to modulate length of responses. We previously showed that two members of the Broad complex, tramtrack, bric-a-brac-poxvirus, and zinc finger (BTB-POZ) family of transcription factors, ZBTB20 and ZBTB32, regulate distinct aspects of B cell memory responses. ZBTB20 regulates adjuvant-dependent survival of long-lived plasma cells, while ZBTB32 regulates the durability of recall response. Although the mechanisms of how ZBTB20 and ZBTB32 regulate responses is unclear, the N-terminal BTB-POZ domain likely recruits transcriptional repressors to different areas of DNA binding mediated by the C-terminal zinc finger domains. Thus, we hypothesized that ZBTB38, another member of the BTB-POZ family, may also regulate B cell responses. Using a mouse model in which ZBTB38 is deleted in
hematopoietic cells, we demonstrate that ZBTB38 is dispensable for both primary and recall responses to a hapten immunogen.

Taken together, we propose that the MBC diversity after a primary response is generated by the continued, but active, selection of low affinity germinal center B cells into the MBC pool, and that this diversity is directly utilized upon secondary challenges without further affinity maturation. In addition, both the primary B cell and MBC recall responses do not require expression of ZBTB38. Further work needs to be done defining the recall responses of MBCs to live viruses and cues that promote the continuous selection of low-affinity GC B cells into the MBC compartment.
Chapter 1:
Introduction

The contents of this chapter have been previously published in *Immunology*. Personal contributions include writing and editing this review in collaboration with Deepta Bhattacharya.

Protective immunity elicited by almost all vaccines and many infections is mediated by pathogen-specific B cells and antibodies. The generation of such protective cells and antibodies is mediated by a process of clonal expansion and diversification of fates, followed by a contraction to the essential components of durable immunity. For T cell-dependent responses, naïve B lymphocytes that recognize cognate antigens rapidly proliferate and differentiate into short-lived plasma cells or germinal center B cells. Short-lived plasma cells secrete low-affinity antibodies that are important for the initial defense against pathogens such as West Nile virus, and are thought to live for only several days. Germinal center B cells progressively affinity mature their B cell receptors through multiple rounds of somatic hypermutation and selection. Cells carrying affinity-enhancing mutations can then differentiate into memory B cells or long-lived plasma cells.

Long-lived plasma cells constitutively secrete affinity-matured antibodies that pre-exist subsequent infections, thereby providing sterilizing immunity. These long-lived plasma cells can live up to an entire human lifetime in the absence of cellular division. Yet their persistence varies greatly with the specific infection or vaccine for reasons that still remain unclear. Metabolic, but not transcriptional pathways appear to be the major determinants of plasma cell longevity, but how infection- or vaccine-specific properties such as antigen avidity, innate immune activation, and T cell help influence these programs remain unknown. Pathogens that evade pre-existing serum antibodies may be recognized by memory B cells, which make up a second line of defense. Memory B cells are quiescent, do not require continued antigenic stimulation for survival, and are more easily reactivated than are their naïve counterparts. The molecular and cellular details of memory B cell development have been recently reviewed.
and thus the focus of this article will be on memory B cells during recall responses in the context of real-world infections.

During a recall response, reactivated memory B cells can differentiate into antibody-secreting cells and/or germinal center B cells, depending on the responding subpopulation. In mice, memory B cell subpopulations were initially distinguished by immunoglobulin isotype expression \(^{21,22}\). These studies showed that IgG+ memory B cells preferentially generate plasma cells, whereas IgM+ cells re-initiate germinal center reactions. Subsequent studies reported that CD80 and PD-L2 expression on antigen-specific cells are functional markers that distinguish memory subsets irrespective of isotype \(^{23}\). CD80\(^+\)PD-L2\(^+\) cells are poised to differentiate into antibody-secreting cells, CD80\(^-\)PD-L2\(^-\) cells robustly differentiate into germinal center B cells, and CD80\(^-\)PD-L2\(^+\) cells possess an intermediate ability to differentiate into antibody secreting cells and re-initiate germinal center reactions. These markers mostly, but not fully correlate with IgM (enriched in the CD80\(^-\)PD-L2\(^-\)) or IgG (enriched in the CD80\(^+\)PD-L2\(^+\)) usage. Moreover, the specific isotype used by memory B cells correlates with unique transcriptional programs that support function and development \(^{24}\). It is likely that the isotype is a marker of the innate signals received by memory B cells during ontogeny rather than a causative determinant of a specific transcriptional program \(^{25}\). In humans, memory B cells are identified based on CD27 \(^{26,27}\) expression, and as in mice, their fate upon reactivation can be predicted by surface Ig expression. Similar to mouse memory B cell subpopulations, IgG\(^+\) human memory B cells are poised to differentiate into antibody secreting cells, and IgM\(^+\) memory B cells are predisposed to form germinal center-like cells ex vivo \(^{28}\). This correlation is reassuring, as mouse models allow for a deeper mechanistic understanding of cellular behavior, whereas studies in humans bring a real-world relevance to these findings. Another memory B cell subset, atypical memory B cells,
has been identified in tonsils. These cells express neither CD27 nor CD21, and express high levels of inhibitory receptor FcRL4 or FcRL5. These cells tend to be hyporesponsive to BCR stimulation, and are expanded in the blood in chronic infections like malaria and HIV. Understanding the recall behavior of each memory B cell subpopulation in different infections is important for determining how to establish protective immunity, particularly when faced with the challenge of vaccinating a population that is already partially immune to an endemic pathogen.

Here, we focus on flavivirus and Plasmodium infections as they pose some unique challenges for generating immunity. As a result, there are interesting lessons to be applied to the basic study of memory B cells. Reciprocally, principles from the cell biology of memory B cells can be potentially applied to vaccination efforts. As examples of the challenges that these globally relevant pathogens pose, infections of flavivirus-immune individuals by heterologous or heterotypic strains can result in markedly exacerbated symptoms compared to the primary challenge. Malaria, caused by Plasmodium infections, is characterized by the lack of a durable antibody response, and requires multiple exposures to develop naturally acquired immunity. For each infection, we will discuss the underlying antibody and memory B cell responses, speculate on the ideal memory B cell response that considers the challenges faced, and conclude on implications for vaccine design and remaining questions. We fully acknowledge that many aspects of this review are speculative. Yet we believe it is imperative to apply the fundamentals of memory B cell biology to contemporary, problematic infections to better guide vaccine design and future research.

1.1 Flavivirus pathogenesis, epidemiology, and immunity

Flaviviruses present a global threat to public health, especially with the recent emergence of epidemic Zika virus (ZIKV). Among many relatives, members of the Flavivirus genus
include the human pathogens ZIKV, West Nile Virus (WNV), Dengue virus (DENV), and Japanese Encephalitis virus (JEV). These viruses are mainly transmitted by mosquitoes, and for WNV and JEV, humans are a dead-end host. For the epidemic pathogens YFV, DENV, and ZIKV, viral titers in humans can reach sufficient levels that these pathogens can be re-transmitted by mosquitoes or by direct human-human contact. Most infections are asymptomatic or present mild symptoms, such as fever, arthralgia, and myalgia. However, some cases of severe symptoms, such as severe hemorrhagic fever and vascular leakage, have been reported. These severe symptoms have been largely associated with secondary DENV infections.

DENV has four serotypes, DENV1 to DENV4, all of which co-circulate in the same geographic regions. Work by Sabin in the 1950s showed that DENV infection by one serotype provided lifelong protection against homotypic infection, but not against heterotypic infections. Indeed, heterotypic infections increase the severity of symptoms when infections occur after antibodies generated from the primary challenge waned. Thus, primary DENV infection generates a durable, serotype-specific antibody response that can be harmful to the host upon heterotypic challenge. The increased severity of secondary infections is thought to be mediated by antibody-dependent enhancement (ADE), a process whereby antibodies that neutralize poorly, either due to epitope specificity or insufficient concentrations, enhance viral uptake via Fc gamma receptors on mononuclear phagocytes. In addition to the humoral contribution (ADE) to increased disease severity upon heterologous infection, there is also a cellular contribution termed original antigen sin (OAS).

The OAS hypothesis was first described as “the imprint established by the original virus infection governs the antibody response thereafter”, whereby memory B cells from the primary
infection are activated during subsequent infections. When antigenic determinants vary between strains, these memory B cells can often bind only weakly and provide poor protection to the second infection. Yet by virtue of reduced activation requirements, these ineffective recall responses dominate over primary naïve B cells. For DENV, OAS was first described after observations that while serum antibodies had varying degrees of neutralizing activity to all four DENV serotypes after heterotypic DENV infection, potent neutralization only occurred to the primary infecting serotype. Similar observations have since been made for memory T cells.

Given the overlap in the geographical prevalence of many flaviviruses, increases in travel, and a potentially exacerbated immune response to secondary infections, there is a need for understanding recall responses to heterologous flavivirus infections. To understand why non-protective antibodies dominate secondary responses, we must first discuss the different antibody epitopes in flavivirus infections.

1.1.a Non-protective and protective antibodies in response to flavivirus infections

Most flavivirus antibodies target epitopes found on the envelope (E) protein, which mediates cellular attachment and membrane fusion to the host cell. The E protein contains three different domains (DI to DIII) with both non-protective and protective epitopes. The primary, non-protective epitope on the E protein is the DII fusion loop (DII-FL), which is highly-conserved across flavivirus species. In humans, this is the immunodominant epitope, and explains why antibodies from one flavivirus infection can cross-react with other flaviviruses that have yet to be encountered. In addition, these DII-FL antibodies are frequently poorly-neutralizing. For many other types of infections, the presence of such non-neutralizing antibodies is at worst harmless, and at best protective due to engagement of alternate antibody effector mechanisms such as complement deposition and opsonization. Yet ADE renders such
antibodies pathogenic in acute secondary flavivirus infections. How these non-protective epitopes impact the cellular response in secondary infections is discussed next.

As mentioned above, in humans, antibodies generated during primary infections are mostly directed against DII-FL, and have poor neutralization ability. During acute secondary infections with a different DENV serotype, plasmablasts secrete cross-reactive, E-specific antibodies with varying degrees of ADE. These antibodies carry high levels of mutations, suggesting that they originate from memory B cells. Memory B cells established after primary and secondary infections appear to retain a greater breadth of antigen specificities (E, prM, and complex epitopes) than do the responding plasmablasts (predominately E). Thus, only a subset of memory B cells differentiates into antibody secreting cells, where the majority of antibodies are poorly neutralizing with high ADE activity. Similar findings were observed following ZIKV infection of DENV-immune patients. Vaccines to protect against flavivirus infections have thus far revealed similar problems. A licensed tetravalent Dengue vaccine, CYD-TDV, can mount a neutralizing response to all four DENV serotypes. However, a longitudinal study on the efficacy of CYD-TDV found an increased risk of severe disease in previously unexposed individuals compared to those who had previously been exposed to at least one DENV serotype through natural infection. Thus, CDY-TDV likely promotes ADE upon natural secondary DENV infection, at least in a subset of individuals who were naive prior to vaccination. These findings demonstrate the importance of engaging B cells with strongly neutralizing specificities while avoiding those that preferentially enhance infection.

While non-protective epitopes dominate the human antibody response, mouse studies have provided critical insight on protective epitopes that can potentially be targeted selectively. For example, mouse studies have shown that B cell responses are critical for protection against
flavivirus infections. Passive transfer of minute quantities of immune serum from WNV-convalescent mice into B-cell deficient animals confers protection to homologous virus infection. Analysis of mouse monoclonal antibodies has revealed that the most potent neutralizing epitopes are located on the lateral ridge epitope of DIII (DIII-LR)\(^{45, 60, 61, 62, 63}\). Interestingly, these DIII-LR specific antibodies are immunodominant in mice, whereas in humans these specificities clearly exist but are overwhelmed by DII-FL-reactive cells and antibodies\(^{38, 45, 46, 64}\). The DIII-LR epitope is only weakly conserved across flavivirus species, and thus, long-lived plasma cell DIII-LR responses provide type-specific immunity with minimal ability to promote ADE\(^ {61, 63}\). Another protective and highly neutralizing epitope is the recently described E dimer epitope (EDE), present in both DENV and ZIKV\(^ {65, 66}\). Analysis of anti-EDE antibodies isolated from DENV-infected humans found that these antibodies potently and broadly neutralized all four DENV serotypes and ZIKV\(^ {53, 65, 66}\). This epitope is present on intact, mature virions but not on recombinant E, indicating that a quaternary structure is required for antibody recognition. Because of the complexity of this epitope, an equally complex strategy will likely be required to selectively generate EDE-specific antibody responses.

\section*{1.1.1b Diversity of memory B cells and their reactivation in secondary challenges}

Interestingly, mouse studies on WNV infections and vaccinations have demonstrated a greater diversity of DIII antigenic specificities in memory B cells than in long-lived plasma cells\(^ {16}\). This fundamental difference in repertoires between memory B cells and constituent serum antibodies has also been found in humans following a range of different vaccines\(^ {15}\). The diversity of WNV-reactive memory B cells imparts them with the ability to cross-react to JEV DIII upon secondary challenges\(^ {16}\). These data suggest an ability of DIII-specific memory B cells, but not pre-existing long-lived plasma cells, to recognize DIII-LR of related flaviviruses.
upon secondary exposures. This could lead to potent cross-neutralizing responses if these memory B cells were to be selectively engaged. Several innovative potential solutions are possible. For example, Richner et al. used a modified RNA to express replication-incompetent ZIKV subviral particles carrying a mutation in the non-neutralizing DII-FL epitope. Mice challenged with this modified RNA were resistant to subsequent DENV2 challenge, whereas animals immunized with wild-type ZIKV subviral particles were highly susceptible to heterologous infections. A second potential approach involves the use of DIII subunit vaccines. Multivalent or sequential DIII-based vaccines could potentially be given to specifically elicit DIII-LR neutralizing antibodies, either from naïve B cells or pre-existing memory B cells in subjects that are already immune to at least one flavivirus strain. In seropositive individuals, responses to heterologous DIII-LR epitopes would require clonal expansion and plasma cell differentiation of pre-existing cross-reactive DIII-LR-specific IgG+ memory B cells (Figure 1). Alternatively, engagement of IgM+ memory B cells specific for DIII-LR of one strain could re-initiate germinal centers to affinity mature towards DIII-LR of the new strain (Figure 1). A real-life example where cross-reactive DIII-LR-specific antibodies were expanded in secondary infections involved ZIKV infection in DENV1-experienced individuals. DENV1 and ZIKV share two critical amino acids in DIII, and thus memory B cells recognizing this epitope were selectively expanded. Tapping the potential of pre-existing DIII-LR memory B cells may guide the best vaccination strategy to elicit potently neutralizing antibodies. Yet different considerations will need to be employed for vaccines that fail not because of their epitope specificity, but rather due to the lack of durable immunity elicited, as has been observed in malaria.
1.2 Durability of plasma cell survival and antibody responses--the Achilles heel of malaria vaccines

Malaria is caused by the *Plasmodium* parasite, and the *P. falciparum* species is the leading cause of death and disease in many developing countries, particularly in Africa \(^68\). Over half of the world’s population is at risk for malaria, where over 200 million cases of clinical malaria occur each year \(^68\). In addition, over 400,000 deaths, primarily in children, occur each year \(^68\). Protection against re-infection depends on the presence of long-lasting, neutralizing serum antibodies \(^69\); passive transfer of immune serum into actively infected individuals results in rapid recovery \(^70\). However, *Plasmodium* infections themselves do not reliably generate durable antibody responses \(^71\); not only are antibodies inefficiently generated, they are lost in the absence of consistent repeated exposure \(^72\). Given the high infection and mortality rate, especially in sub-Saharan African children, development of a highly effective vaccine is of utmost importance, but the provision of durable immunity poses a major challenge as described more below.

The most advanced approach is the RTS,S subunit vaccine containing the circumsporozoite protein (CSP). Preliminary studies revealed that vaccination in malaria-naive adults yielded robust antibody responses in which 86% were protected from subsequent *Plasmodium* challenge \(^73\). Though CSP-specific antibody titers gradually waned, these initial findings showed promise for a realistic and scalable vaccine against malaria. However, a study analyzing vaccine efficacy in the field found that only 34% of *Plasmodium* partially-immune Gambian adults given the vaccine were protected from the first round of natural infection \(^74\). Within 6 months post-RTS,S vaccination, both antibody titers and epidemiological protection waned to negligible levels \(^75, 76\). Though difficult to conclude with certainty without a side-by-
side comparison, these data suggest that the ability of *Plasmodium*-naive individuals to mount and maintain a protective response after vaccination is higher than that of *Plasmodium*-experienced individuals. These findings further suggest that natural *Plasmodium* exposures may alter the ability of B cells to respond, either directly or indirectly. Alternatively, a response against CSP may intrinsically be short-lived (discussed below). Several elegant studies have followed the B cell response over time in malaria-endemic areas, and have provided intriguing clues as to the source of the dysfunction.

1.2.a Atypical memory B cells in *Plasmodium* infected individuals

Repeated exposures to *Plasmodium* over the course of multiple wet seasons lead to a progressive increase in antibodies, but this protective humoral immunity rapidly fades during dry seasons as the mosquito population diminishes. There is also a progressive increase in the frequency of atypical memory B cells, balanced by a relative decrease in classical memory B cells. These atypical memory B cells might be the key to understanding why antibody responses are so transient following vaccination or repeated natural infections. Originally identified in HIV patients by expression of the inhibitory receptor FcRH4, these cells fail to proliferate in response to BCR ligation. Moreover, studies of the mouse age-related memory B cells, the potential analog to human atypical memory B cells, have shown markedly elevated expression of the transcription factor Zbtb32. This factor suppresses the duration of plasma cell survival following memory B cell recall responses. Thus, it might be that recall responses mounted by atypical memory B cells are inherently transient.

There remain some conflicting findings regarding the biological properties of atypical memory B cells. For example, analysis of a relatively small number of cells suggested that atypical and classical memory B cells were clonally unrelated in *Plasmodium*-immune
individuals\textsuperscript{83} whereas another larger study found these two cell types to possess overlapping immunoglobulin repertoires \textsuperscript{80}. Some studies observed Ig secretion by atypical memory B cells both \textit{in vivo} and \textit{ex vivo} \textsuperscript{83}, while others have found these cells to be completely unresponsive to stimuli \textsuperscript{80}. A recent study by Pérez-Mazlia et al. analyzed the MSP1-specific memory B cell compartment following \textit{Plasmodium} infection and found that atypical memory B cells were short-lived and had activated B cell and plasmablast gene signatures \textsuperscript{84}. Mouse studies have not resolved these issues, as age-associated memory B cells can readily respond to Toll-like receptor stimulation \textsuperscript{85,86}, whereas in some studies, human atypical memory B cells do not \textsuperscript{87}. Clearly, additional studies must be performed to explain the differences in these sets of studies. This in turn will help define whether these cells should be intentionally avoided in vaccine strategies, or whether they can be safely ignored.

A second finding that may impact the transience of immunity is the emergence of an unusual T follicular helper (Tfh) cell phenotype. Mouse studies have shown that a pro-inflammatory environment promotes T-bet expression in pre-Tfh cells, which skews toward a Th1-like phenotype \textsuperscript{79}. Similarly, human circulating Tfh-like cells from \textit{Plasmodium}-immune individuals are poised to secrete interferon gamma (IFN-\textgamma) and promote T-bet expression in atypical memory B cells \textsuperscript{79,88}. The high levels of T-bet expression in atypical memory B cells are associated with decreased BCR signaling and a switch from IgG1 to IgG3 surface expression \textsuperscript{88}. In some cases, T-bet expression limits the ability of B cells to differentiate into germinal center B cells, and ultimately results in reduced IgG antibody titers \textsuperscript{89}, though in other cases it appears that Tbet expression actually enhances serum IgG2c levels \textsuperscript{24,90}. Thus, severe malaria is associated with an inflammatory environment that results in a defective adaptive immune response. However, for those that recover from severe malaria, Th1-skewed Tfh cells drive the
gradual acquisition of T-bet that is eventually enriched in the atypical memory B cell compartment 79, 88. Thus, T-bet expression may result in impaired recall responses through its effects on atypical memory B cells.

1.2.2 Alternatives in mounting a durable anti-Plasmodium antibody response

Based on the evidence provided above, there are several aspects to consider to develop a successful vaccine that provides durable immunity to malaria. First, the outcome of the immune response changes greatly with exposures. Thus, developing vaccines by testing on naive Western populations may not be fully predictive of the response in malaria-endemic areas 87, 91. Along these lines, it may become necessary to either productively engage atypical memory B cells to 'awaken' them to produce long-lived plasma cells, or to avoid them entirely and target the classical memory populations instead (Figure 2). This latter strategy would depend on the existence of at least some non-overlapping antigen specificities between atypical and classical memory B cells. How best to awaken atypical memory B cells is far from clear, but engagement of certain pattern- or danger-associate molecular patterns might still be possible. Our own work has demonstrated that Zbtb32, which is highly expressed by age-associate memory B cells, restrains the duration of antibody production after recall responses to some challenges, such as murine cytomegalovirus and alum-adjuvanted immunogens 92. In contrast, Zbtb32 has no role in controlling recall responses to intestinal bacteria, influenza booster vaccinations, or Ribi-adjuvanted immunogens 92. Thus, selection of proper adjuvants and innate stimuli can greatly influence the survival program engaged by plasma cells and the duration of immunity 93, 94.

A second, and potentially more problematic scenario, is that inherent constraints within the human germline repertoire or pathogen evasion mechanisms prevent durable responses to certain Plasmodium antigens. Although the RTS,S vaccine response was more effective in
malaria-naïve individuals than in malaria-experienced subjects, antibody responses still faded quite rapidly relative to a number of more robust vaccines and infections. To define the underlying cause to these problems, a recent mouse study demonstrated that blood-stage parasites actively suppress the response to CSP. Other animal studies using different immunization and infection models have demonstrated that both the avidity of the antigen for its target BCR as well as the self-reactivity of the starting naïve B cell can greatly influence the duration of immunity. Studies using the model antigens demonstrated that when the starting avidity of the antigen is very high, paradoxically the duration of IgG memory B cell persistence is diminished. Additionally, high model antigen avidity promotes the expansion of short-lived plasma cells. Yet in the context of HIV infection, a minimum antigen affinity is essential to effectively recruit pathogen-specific B cells into the response. Thus, it may be that an antigen avidity 'sweet spot' is required to generate the ideal durable response to Plasmodium. Adding to this complication is that some foreign antigen-specific B cells are also somewhat self-reactive, thereby diminishing their contribution to or durability following the response. These problems can begin to be addressed by empirical antigen redesign and experimental animal studies. For example, although blood stage infection inhibits responses to CSP, responses to merozoite surface protein 1 remain intact and protect mice from subsequent infection.

Targeting naïve B cells of very rare specificities may be an alternate but effective strategy. For instance, while antibody responses to the four amino acid (NANP) repeats of CSP increase over time post-infection, this is not due to increases in affinity maturation within the germinal center. Instead, recruitment of naïve B cells with higher affinity than responding memory B cells drives these increases, as somatic hypermutations are often not affinity-enhancing. In addition, a surprising study isolated RIFIN-binding, broadly-reactive
antibodies that recognize multiple *P. falciparum* isolates\textsuperscript{108}. These highly unusual antibodies have inserted the collagen binding domain of LAIR1, an Ig superfamily inhibitory receptor, between the V and DJ segments. Somatic mutations in the LAIR1 domain abolished binding to collagen and increased binding and opsonization of infected erythrocytes. These broadly reactive antibodies were isolated from IgG+ Memory B cells, and found to be highly clonally expanded in the two donors studied. Thus, targeting high affinity naïve B cells may be a way to avoid atypical memory B cells and produce durable immunity.

There are still many outstanding questions on the association and causation between malaria severity and atypical memory B cells. Longitudinal studies analyzing the repertoire of, and clonality between, the memory B cell and plasmablast compartments after each malaria season will be important for understanding the development and contribution of atypical memory B cells. Are atypical memory B cells originally classical memory B cells that have been exhausted or are they a separate fate during germinal center reactions? Given that naturally acquired immunity is associated with an expanded atypical memory B cell pool, what is the association between protection and atypical memory B cells? In addition, there appears to be different subsets of atypical memory B cells based on T-bet expression and/or isotype expression. Do the functions of these different subsets differ in recall response? In this case, malaria is a good infection system to use to study different aspects of atypical memory B cell biology.

**1.3 Conclusion**

Decades of research in model organisms have defined the basic and essential elements of cellular and humoral immunity. Increasingly sophisticated analysis in the field has revealed properties of human responses to problematic infections, which in turn has driven a progression
of mouse models that accurately mimic these issues\textsuperscript{109}. Of course not all aspects of responses are identical between humans and mice, but our viewpoint is that evolution likely preserves the most fundamental aspects of antibody responses. Even in the cases where there are differences between mice and humans, such as in the immunodominant antigens after flavivirus infection, mouse studies have revealed critical neutralizing epitopes that may form the basis for new human vaccines. As increasing numbers of reports define new structure-based methods to elicit epitope-specific antibodies, basic studies will help guide these specificities into the correct cellular compartments to provide robust and durable humoral immunity.
1.4 References


46. Lai, C.-Y.Y. *et al.* Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II. *Journal of virology* **82**, 6631-6643 (2008).


Figure 1.1 Actual and ideal responses to heterologous flavivirus infections.
Secondary flavivirus infections in humans can result in pathogenic responses, where the immunodominant epitope is the highly conserved fusion loop on the envelope protein domain II (DII-FL). Non-neutralizing DII-FL antibodies facilitate increased viral load through a process called antibody-dependent enhancement (ADE). Ideally, the response to heterologous challenge should result in the expansion of protective antibodies and memory B cells. This can either be achieved by recruiting DIII-LR cross-reactive IgM⁺CD80⁺PD-L2⁺ memory B cells into the germinal center (GC) response to affinity mature towards the new heterologous DIII-LR epitope, or by differentiating pre-existing cross-reactive DIII-LR specific IgM⁺CD80⁺PD-L2⁺ memory B cells into long-lived plasma cells (LLPCs).
Figure 1.2 Actual and ideal response to Plasmodium.
Plasmodium infections in humans result in expanded, hyporesponsive atypical memory B cells (aMBCs), contracted classical memory B cells (cMBCs), and short-lived antibody responses. Each additional infection by Plasmodium results in further increases in aMBCs and short-lived plasma cells (SLPCs). Therefore, multiple infections are needed for immunity. Given this challenge, vaccines need to be carefully designed to induce long-lived plasma cells (LLPCs) for a robust and durable response. To mount a robust and durable response, either adjuvants that can render aMBCs responsive must be used, or memory B cells that can recognize epitopes that confer durable and protective responses must be selectively activated and expanded.
Chapter 2:

Affinity-restricted memory B cells dominate recall responses to heterologous flaviviruses.

This work was performed in collaboration with Jennifer Govero, Jennifer Uhrlaub, Haiyan Zhao, John M. Errico, Lucas D’Souza, Tyler J. Ripperger, Janko Nikolich-Zugich, Mark J. Shlomchik, Daved H. Fremont, Michael S. Diamond, and Deepta Bhattacharya. Personal contributions include the design and carrying out of selected experiments, data analysis, and writing of this work.
2.1 Abstract

Memory B cells (MBCs) can respond to heterologous antigens either by molding new specificities through secondary germinal centers (GCs) or by selecting pre-existing clones without further affinity maturation. To distinguish these mechanisms in flavivirus infections and immunizations, we studied recall responses to lateral ridge epitopes, which are potently neutralizing but poorly conserved across species. Conditional deletion of activation induced cytidine deaminase (AID) between heterologous challenges of West Nile, Japanese encephalitis, Zika, and Dengue viruses did not affect the quality and quantity of recall responses. DIII-LR-specific MBCs were contained mostly within the plasma cell-biased CD80+ subset and few GCs arose following heterologous booster vaccinations, demonstrating that recall responses are confined by pre-existing clonal diversity. Measurement of monoclonal antibody binding affinity to DIII proteins, timed AID deletion, single cell RNA-sequencing, and lineage tracing experiments suggest continuous commitment of relatively low affinity MBCs in the first weeks of the GC as a mechanism to promote diversity. Restricting this diversity through immunogen design may allow for generation of type-specific vaccines with little potential for infection enhancement of heterologous flaviviruses.
2.2 Introduction

After clearance of infections or vaccines, long-lived plasma cells (LLPCs) and memory B cells (MBCs) persist to maintain durable humoral immunity. While MBCs proliferate and differentiate into effector lineages upon antigen re-exposure, LLPCs constitutively secrete antibodies irrespective of the presence of antigen and can provide sterilizing immunity against subsequent homologous or closely related infections \(^1\), \(^2\). Because of these properties, LLPC-derived circulating antibodies can also sequester antigen that would otherwise activate MBCs \(^3\), \(^4\). From an evolutionary standpoint, it seems unlikely that MBCs serve solely as an adjunct to LLPCs to bind excess antigen, since physiological inocula are often comprised of only a small number of infectious microbes \(^5\). Instead, the most important role of MBCs may be to respond to pathogens that have antigenically changed since the first exposure, thereby evading pre-existing serum antibodies. Hapten-specific MBCs have fewer affinity-enhancing mutations than do LLPCs \(^6\), \(^7\), providing evidence that the repertoires of these two cell types do not fully overlap. Subsequent work demonstrated that pathogen-specific MBCs have distinct repertoires and distributions of epitope specificities relative to LLPCs \(^8\), \(^9\), \(^10\). These findings led us and others to propose that the diversity of MBCs enables them to combat escape mutants and heterologous viruses better than do LLPCs \(^10\), \(^11\), \(^12\).

In theory, MBCs can respond to heterologous pathogens or escape mutants in two ways. First, the diversity of this compartment may be sufficient to allow the clonal selection of cross-reactive MBCs without additional affinity maturation. Second, MBCs might re-initiate germinal centers (GCs) to shape new B cell receptors (BCRs) tailored to the second heterologous challenge. This decision is mediated principally by the subset of responding MBCs. In mice, MBC subsets can be defined by their surface expression of CD80 and programmed cell death 1
protein ligand 2 (PD-L2). CD80^+PD-L2^+ MBCs preferentially differentiate into plasma cells upon antigen re-challenge, whereas MBCs lacking CD80 and PD-L2 expression differentiate into GC B cells. Though causally unrelated to isotype, these subsets partially correlate with IgG versus IgM expression. CD80^+PD-L2^+ MBCs are enriched for IgG expression, while CD80^+PD-L2^- cells tend to express IgM. Accordingly, both in mice and humans, IgG^+ MBCs preferentially differentiate into plasma cells after re-challenge, whereas IgM^+ MBCs re-initiate GC reactions. While adoptive transfer, ex vivo culture, and transcriptional profiling experiments have established the differentiation potentials of these MBC subsets in isolation, it is challenging to define how these subsets act in concert and competition with one another in vivo, where serum antibodies and memory CD4^+ T cells may modulate recall responses to heterologous antigenic challenges. Mouse studies using model antigens have reached differing conclusions on how efficiently MBCs re-initiate GCs and undergo secondary affinity maturation, as have human influenza studies. Resolving these differences experimentally is challenging, as there have been no reports of genetic tools that can distinguish the functional importance of secondary GC reactions versus clonal selection of pre-existing diversity without also altering other components of the immune response.

Flaviviruses represent a particularly relevant system to differentiate between these possibilities. Pathogenic flaviviruses, such as West Nile (WNV), Japanese encephalitis (JEV), Dengue (DENV), and Zika (ZIKV) viruses co-circulate in similar geographic regions. Although primary flavivirus infections result in durable homotypic protection, in some instances, subsequent infections by heterologous flaviviruses can result in more severe symptoms than if the host were naïve. The increased severity of heterologous secondary infections is mediated in part by antibody dependent enhancement (ADE), and is a contributing factor as to why safe
and effective vaccines for DENV have been challenging to develop. ADE is a process by which poorly or non-neutralizing antibodies generated after the first infection enhance uptake of the second heterologous virus in mononuclear phagocytes via Fcγ receptors 25, 26, 27.

The majority of flavivirus antibodies recognize epitopes on the envelope (E) protein 28. The E protein is divided structurally into three domains (DI, DII, and DIII) 29. DII contains an immunodominant fusion loop (DII-FL) epitope that is recognized by poorly neutralizing antibodies and is conserved across flavivirus species 30, 31, 32, 33, 34. Antibodies that bind to the DII-FL epitope can thus recognize multiple flaviviruses and potentially mediate ADE. In contrast, DIII contains the lateral ridge (DIII-LR) epitope, which is recognized generally by flavivirus type-specific neutralizing antibodies 30, 35, 36, 37, 38, 39. Potently inhibitory antibodies that bind the DIII-LR epitope neutralize at a post-attachment step by preventing E protein dimer-trimer transitions necessary for viral fusion 36, and protect against West Nile (WNV), Japanese encephalitis (JEV), Zika (ZIKV), and Dengue (DENV) virus infections in vivo. Because of these data, the DIII-LR epitope is an attractive candidate for vaccine immunogen design.

The DIII-LR epitope is less well-conserved at the sequence level across different flavivirus species. Therefore, resultant antibody responses are less cross-reactive and do not appear to cause ADE 40, 41. DIII-LR monoclonal antibodies (mAbs) that cross-react between DENV and ZIKV have been observed, but these antibodies are rare despite their evolution from a ‘public’ germline lineage 42, 43, 44. Notwithstanding this, it remains unknown whether polyclonal MBCs generated after one flavivirus vaccination or infection respond to the DIII-LR epitope of a subsequent heterologous vaccination or infection. If pre-existing MBCs could be used as a template for new specificities through secondary GCs, this would rationalize a strategy of sequential heterotypic DIII-LR immunizations in geographic areas in which large fractions of
individuals are already immune to at least one endemic flavivirus \(^{45}\). In contrast, if recall responses were dominated by MBCs with little capacity to re-initiate new GCs, this might lead to weakly cross-neutralizing antibodies that could promote ‘original antigenic sin’ and ADE when faced with virus infections \(^{23,46,47}\). In this latter case, immunogen design is warranted to avoid pre-existing MBCs and antibodies to minimize cross-reactivity and promote virus type-specific responses.

To differentiate between these possibilities, we generated mice in which activation-induced cytidine deaminase (AID), which mediates both class switching and somatic hypermutation \(^{48}\), can be temporally deleted prior to the recall response. The lack of AID would prevent subsequent diversification or redirection of BCR specificity towards a heterologous antigenic target in secondary GCs. Nonetheless, AID deletion minimally impacted recall responses against heterologous flavivirus antigen epitopes, suggesting clonal selection from a diversity of pre-existing MBC specificities with little role for further secondary affinity maturation towards heterologous antigens. This diversity is promoted by the selection of low affinity antigen-specific B cells that are continuously recruited into the MBC compartment. Restricting the initial diversity through immunogen design may allow for flavivirus type-specific vaccines with minimized potential for infection enhancement.
2.3 Results

**MBCs do not require additional affinity maturation to respond to heterologous challenges.**

We previously demonstrated that MBCs are capable of recognizing a WNV mutant selected to escape neutralizing antibodies \(^1\)\(^,\)\(^4^9\). This mutation attenuates WNV infectivity and does not occur naturally. Thus, the mechanisms by which MBCs recognize neutralizing epitopes on naturally-occurring heterologous flaviviruses remains unexplored. To address this issue, we first developed a mouse model that allows for temporal abrogation of somatic hypermutation, and thus further affinity maturation, between primary and secondary challenges. Mice were generated with loxP sites flanking exon 2 of the *Aicda* gene (**Figure 2.1A-B**). *Aicda* encodes activation induced cytidine deaminase (AID), a protein that is critical for class switching and somatic hypermutation \(^4^8\). These mice allow us to distinguish the contributions of secondary affinity maturation of MBCs versus clonal selection of MBCs with pre-existing specificities, as the former requires expression of AID whereas the latter does not.

*Aicda^eff^* mice were crossed to a tamoxifen-inducible, hCD20-B cell specific Cre recombinase (TamCre) \(^5^0\) (**Figure 2.1A**). To confirm deletion of AID at the protein level, *Aicda^eff^* x TamCre (AID cKO) mice were immunized with sheep red blood cells, treated with tamoxifen, and then GC B cells sorted at different time points after tamoxifen treatment. AID protein expression was abolished rapidly after tamoxifen treatment and remained undetectable by immunoblot for at least seven days (**Figure 2.1C**). To confirm loss of class switching after AID deletion, AID cKO and *Aicda^eff^* (AID WT) mice were treated with tamoxifen for two weeks, vaccinated against WNV using an inactivated veterinary vaccine, which generates similar antibody responses and specificities as do live infections \(^1^0\), and IgG responses were assessed 12
days later (Figure 2.1D). As expected, AID cKO mice failed to mount an isotype-switched IgG response to WNV DIII (Figure 2.1D).

We next established a heterologous flavivirus immunization system to specifically monitor recall, but not primary antibody responses. WNV and JEV are related flaviviruses in the same serogroup and have DIII amino acid sequence similarity of approximately 75%. We thus employed Ixiaro, an alum-adjuvanted, clinically approved, inactivated JEV vaccine, which requires multiple immunizations to elicit a protective antibody response 51. Naïve mice challenged with Ixiaro generated few detectable JEV DIII-specific antibodies (Figure 2.2A). In contrast, JEV vaccination of WNV-immune mice resulted in an increase in JEV DIII-specific serum antibodies (Figure 2.2A). Thus, JEV-DIII antibodies can be elicited efficiently in secondary heterologous recall responses.

The importance of secondary affinity maturation of MBCs was determined by deleting Aicda between WNV and JEV challenges. AID WT and AID cKO mice were vaccinated with WNV and the primary response was allowed to proceed. Mice then were treated with tamoxifen 56 days later to delete AID, and then re-vaccinated with JEV (Figure 2.2B). Serum antibody titers to WNV and JEV DIII proteins were then quantified by ELISA. We also monitored responses to recombinant DIII proteins carrying point mutations that ablate the LR epitope and abolish binding by potently neutralizing mAbs. For WNV DIII, we incorporated K307E and T330I mutations (KT DIII) 35, 36, whereas for JEV DIII we introduced 4 LR mutations (E306A/S329I/A366E/K390S, JEV ESAK-DIII) based on previously solved structures 52. Proper reactivity and refolding of the JEV ESAK-DIII protein was confirmed by ELISA using the LR-specific neutralizing mAb JEV-31 37, and serum from immunized mice (Figure 2.3A). Thus, antibodies that bind WT DIII but not mutant DIII are considered DIII-LR-specific. Most
antibodies that bind both WT DIII and mutant DIII likely recognize a distinct non-LR DIII epitope, though a minority may remain DIII-LR-specific and tolerate the mutations.

Deletion of AID prior to JEV booster vaccination had no discernible effect on serum IgG or IgM titers against WNV WT DIII, WNV KT DIII, JEV WT DIII, or JEV ESAK DIII between days 14-70 of the recall response (Figure 2.2C, Figure 2.3B-C). Recall responses were primarily directed against non-LR epitopes, as most antigen-specific antibodies bound WNV KT DIII and JEV ESAK DIII (Figure 2.2C). However, the levels of JEV WT DIII-reactive antibodies were higher than those against JEV ESAK DIII (Figure 2.2D). These data suggest that although most of the response is accounted for by WNV-JEV cross-reactive non-LR antibodies, a subset of the MBC response is directed against the neutralizing DIII-LR epitopes of both WNV and JEV. These responses were not affected by AID deletion (Figure 2.2D). Accordingly, deletion of AID also did not alter the serum neutralization response to JEV (Figure 2.2E). These data demonstrate that rare pre-existing MBCs mediate cross-neutralizing responses without a requirement for further somatic hypermutation and affinity maturation upon heterologous flavivirus challenges.

We considered the possibility that additional affinity maturation is necessary for more disparate heterologous challenges. ZIKV, another member of the flavivirus genus, shares ~55% DIII amino acid sequence similarity with JEV. We tetramerized recombinant ZIKV DIII to improve avidity, immunized mice, and then followed with JEV vaccination (Figure 2.2F). Deletion of AID prior to JEV booster vaccination in ZIKV DIII-immune mice did not impact IgG or IgM antibodies against ZIKV DIII, JEV DIII, JEV ESAK DIII, or LR-mutant ZIKV DIII (ZIKV AT DIII 38) (Figure 2.2G, Figure 2.3D-E). To determine if secondary affinity maturation occurs following heterologous infections, rather than vaccinations, we utilized a sequential
DENV and ZIKV infection model (Figure 2.3F). DENV2 and ZIKV DIII share approximately 46% amino acid similarity. Analysis of ZIKV DIII-specific MBCs and LLPCs, after correcting for naïve B cell responses against ZIKV, again suggested a minimal role for secondary GC reactions and affinity maturation (Figure 2.3G). These data demonstrate that for responses against both similar and disparate flavivirus DIII-LR antigens, for both vaccines and infections, secondary affinity maturation is minimally involved. Instead, heterologous challenges promote clonal selection from pre-existing cross-reactive MBCs expressing neutralizing antibodies.

**Plasma cell-biased MBCs recognize and respond to heterologous antigens.**

We next sought to define a cellular mechanism as to why secondary affinity maturation was not engaged in heterologous recall responses. The decision to initiate new GCs or plasma cells is largely determined by the responding MBC subsets, which are distinguished using a combination of IgM, IgD, CD80, CCR6, PD-L2, and CD73 as markers (Figure 2.4) 15, 17, 53, 54. PD-L2 and CD73 expression largely overlapped with CCR6 irrespective of immunoglobulin isotype (data not shown), suggesting redundancy between these markers. Thus, we focused subsequent analyses on CCR6 and CD80 expression. Antigen-specific MBCs in WNV-vaccinated mice were identified using bacterially-expressed site-spectically biotinylated recombinant DIII assembled into tetramers with streptavidin-fluorophore conjugates (Figure 2.4). Only the isotype-switched (swIg, IgM*IgD−) CD80+CCR6+ MBC subset was enriched for WNV DIII-specificity in vaccinated mice relative to unimmunized animals (Figure 2.5A). These CD80+ MBCs preferentially generate plasma cells upon re-encounter with cognate antigen.
Secondary affinity maturation plays a minimal role in recall responses to JEV, suggesting the existence of WNV-JEV cross-reactive MBCs prior to Ixiaro vaccination. Indeed, after primary vaccination with WNV, nearly half of WNV KT DIII-reactive MBCs recognized both JEV DIII-WT and JEV ESAK DIII (Figure 2.5B). These cells likely cross-react with conserved non-LR epitopes between DIII of WNV and JEV. In contrast, only ~1% of WNV DIII-LR-specific MBCs also bound JEV DIII (Figure 2.5B). These data are consistent with previous studies reporting rare but detectable DIII-LR monoclonal antibodies that cross-react with different flavivirus species 37, 42, 43, 44. These data are also consistent with Figure 2.2D, in which the majority, but not all, of the response is directed against conserved DIII non-LR epitopes. Indeed, JEV vaccination of WNV-immune mice as in Figure 2.2A led a marked increase in WNV KT DIII-reactive antibodies (Figure 2.5C). Yet we detected only a very small frequency of WNV-DIII-specific GC B cells following JEV vaccination of WNV-immune mice (Figure 2.5D), consistent with our observations that antigen-specific MBCs are mostly contained within the CD80+ plasma cell-biased subset (Figure 2.5A). These data suggest a minimal role for secondary GCs in recall responses to heterologous neutralizing DIII-LR epitopes. Instead, recall responses are dominated by pre-existing cross-reactive MBCs.

**MBCs are actively selected from GC B cells.**

Our data demonstrate that recall responses to heterologous flaviviruses in mice are restricted by the pre-existing MBC diversity, with little capacity to shape new specificities through secondary GCs. We therefore sought to define mechanisms by which this MBC diversity is generated during the primary response. Given the results above, we focused on mechanisms by which CD80+ MBCs are selected from GCs. Diversification can be achieved potentially through
random selection of GC B cells into the MBC compartment. This would result in similar distributions of antigen-specificities in both GC and MBC compartments. However, if active selection occurred, the antigen-specificity distributions between MBCs and GC B cells might differ.

To distinguish these possibilities, wild-type mice were vaccinated against WNV and the DIII specificities in the GC B cell and MBC compartments were quantified by flow cytometry at different time points. GC B cells (CD19+GL7’CD38’IgD’) showed a skewed frequency of WNV DIII-reactive cells towards the LR epitope, although a small fraction of non-LR specific cells was present at stable frequencies throughout the experiment (Figure 2.6A). In contrast, MBCs were far less skewed towards the WNV DIII-LR epitope (Figure 2.6B), consistent with previous findings. The durable MBC population at late timepoints post-vaccination more closely mirrored the WNV DIII-LR and DIII non-LR frequencies at 1-2 weeks post-immunization than those at 3-4 weeks (Figure 2.6B). These data are consistent with a relatively early wave of MBC formation that contributes most of the durable memory population, and with the low lifespans of the highest affinity and somatically mutated MBCs. MBCs had a larger ratio of WNV DIII non-LR:DIII-LR specificities than did GC B cells at all timepoints analyzed (Figure 2.6C). To confirm that WNV DIII non-LR specific GC B cells are more predisposed towards the MBC fate, we analyzed the frequency of CD38‘CCR6+ MBC precursor cells for WNV DIII-LR and WNV DIII non-LR specific GC B cells (CD19+GL7+IgD-). CCR6 and CD38 expression mark both mature MBCs and GC precursors committed to the MBC fate. In the CD38‘CCR6+ MBC precursor population, WNV DIII non-LR-specific cells were detected at greater frequency than WNV DIII-LR specific cells at all time points analyzed (Figure 2.6D). Thus, although the frequency of WNV DIII non-LR-specific GC B cells was low, these cells were either
preferentially selected or maintained within the MBC compartment. The early emergence from the GC of WNV DIII-specific MBCs, especially those that are DIII non-LR specific, suggests that these cells likely underwent less affinity maturation and remained of low affinity, implying mechanisms as to why the MBC compartment is antigenically diverse.

**MBCs have lower avidity for their antigens than do LLPCs**

In both the hapten-response setting and in our results above, MBCs begin to exit GCs earlier than do bone marrow plasma cells \(^7\), perhaps resulting in lower overall antibody affinity. Based on the number of somatic mutations, LLPCs are thought to be selected from high affinity GC B cells \(^7,60,61,62\), whereas MBCs are thought to be selected from low affinity GC B cells due to lack of T cell help \(^63\). Thus, it is possible that an avidity threshold determines the MBC versus LLPC fate decision. A lowered avidity threshold, and therefore increased permissiveness to bind variant antigens, in turn could promote diversity in the MBC compartment. To determine if MBCs in our system have lower affinity or avidity for their antigen compared to LLPCs, we established methods to identify DIII-specific LLPCs and MBCs. Although plasma cells secrete most of their antibodies, we observed similar expression of surface Igκ as by other B cells in the bone marrow (Figure 2.7A), consistent with previous studies \(^64\). We therefore used recombinant DIII tetramers to stain and sort single antigen-specific LLPCs and MBCs (Figure 2.7A-B), cloned their V(D)J sequences into expression vectors, and then expressed and purified recombinant monoclonal antibodies (mAbs) \(^65\). The cloned mAbs from MBCs and LLPCs were specific for WNV DIII as determined by ELISA and biolayer interferometry. Analogous to the flow cytometry data, more than three times as many mAbs generated from cloned MBCs recognized WNV DIII non-LR compared to those derived from cloned LLPCs (Table 2.1).
We first tested mAbs for their ability to bind monomeric WNV WT DIII. MBC-derived mAbs bound to monomeric WNV WT DIII similarly as did mAbs from LLPCs, with modest reductions in binding seen only at the highest concentration of antibody tested (Figure 2.8A). Reversing the orientation of a subset of the antibodies and WNV WT DIII on the ELISA plate to test affinity rather than avidity also failed to reveal differences between MBC- and LLPC-derived mAbs (Figure 2.7C). To obtain data on binding kinetics of these mAbs, we performed biolayer interferometry (BLI). Affinities and on-rates again were similar between mAbs derived from MBCs and LLPCs (Figure 2.8B, Figure 2.7D). If anything, a small reduction in off-rates was observed in MBC-derived mAbs compared to LLPC-derived mAbs (Figure 2.7D). These data demonstrate that the B cell receptors (BCRs) of MBCs and of LLPCs bind monomeric DIII similarly.

Given that WNV E proteins assemble into rafts of dimers on an icosahedral virion, we considered the possibility that LLPC-derived mAbs might bind native viral structures better than MBC-derived mAbs. To evaluate this, we tested the avidity of the mAbs to WNV subviral particles (SVPs), which have similar icosahedral structures to fully infectious virions. LLPC-derived mAbs bound with higher avidity to SVPs than MBC-derived mAbs (Figure 2.8C). This result suggests that an avidity threshold for multimeric flavivirus antigens may distinguish MBC from LLPC selection in the GC and dictate the repertoire of antigens recognized.

**Absolute affinity thresholds do not segregate MBCs from LLPCs in vivo**

Our data are consistent with the existence of either an absolute affinity threshold that promotes LLPCs and excludes MBCs, or a relative threshold in which the comparatively lowest
affinity B cells within a given GC are allocated to the MBC compartment, perhaps due to the differences in the timing of their selection. To determine if an absolute affinity threshold promotes the LLPC fate and results in a diverse MBC compartment in vivo, we utilized our AicdaFlox x TamCre mouse model to freeze affinity maturation at different timepoints during the GC reaction. We first defined timepoints at which AID deletion abrogates affinity maturation but not class-switch recombination, and during which MBCs were formed but bone marrow plasma cells had not yet emerged. This is important given differences in IgM and IgG signaling that can influence plasma cell selection. At both 7 and 14 days after WNV vaccination in wild-type mice, the frequency of WNV DIII-specific GC B cells that had class switched was high (~90%, Figure 2.9A). This is consistent with recent studies showing that class-switch recombination occurs prior to GC formation. In contrast, the number of somatic mutations in WNV DIII-specific GC B cells increased each week post-vaccination (Figure 2.9B), demonstrating ongoing affinity maturation.

To determine the kinetics of accumulation of WNV DIII-specific bone marrow plasma cells, ELISPOT assays were performed at different time points after vaccination. We found that WNV DIII-specific bone marrow plasma cells were detectable at 2 weeks post-vaccination and peaked 1 week later (Figure 2.9C), whereas MBCs emerged earlier (Figure 2.6B). We deleted AID at 1 or 2 weeks post-WNV vaccination to temporally abrogate affinity maturation and assessed the impact on MBC and LLPC numbers 8 weeks later (Figure 2.10A). The frequencies of WNV DIII-LR and DIII non-LR specific MBCs were unaffected by AID deletion at weeks 1 or 2 post-vaccination (Figure 2.10B). Cre expression alone also did not alter antibody responses to WNV WT DIII or WNV E protein (Figure 2.9D). AID deletion at 1 week after vaccination led to reduced anti-WNV DIII IgG serum antibody titers and slightly reduced numbers of DIII-
reactive LLPCs relative to control mice (Figure 2.10C, Figure 2.9E). This may indicate an affinity threshold for plasma cell selection, but these data also could be explained by a reduction in the affinity of antibodies and resulting inability to detect all antigen-specific LLPCs. Consistent with the latter mechanism, deletion of AID 2 weeks after WNV vaccination did not affect serum antibody titers or the number of WNV DIII-specific LLPCs (Figure 2.10C, Figure 2.9E). Moreover, serum antibodies bound equivalently to multivalent WNV SVPs irrespective of AID deletion (Figure 2.10D). IgM antibody responses against WNV were also unaffected by AID deletion (Figure 2.9F). Collectively, these data indicate that an in vivo absolute affinity threshold does not exist below which GC B cells are destined to become MBCs. These data are consistent with a recent study showing that AID-dependent affinity maturation is dispensable for LLPC and MBC formation 56. Notwithstanding this, our data do not exclude the existence of a relative affinity threshold that segregates MBC and LLPC specificities.

**Germline affinities of MBC antibodies are lower than those of LLPCs**

Previous studies using model antigens and BCR transgenic mice have demonstrated that MBCs and LLPCs of the same specificity have different affinities 7, 58, 60, 62, 63. Yet how differences in affinity would explain changes in specificities between MBCs and LLPCs in a polyclonal response is not immediately obvious. We first considered several affinity-independent mechanisms. One possibility is that distinct B cell subsets, such as marginal zone B cells, preferentially contribute to the MBC compartment. However, the antigen-specific precursor frequencies for DIII-LR and DIII non-LR were similar between marginal zone B cells and follicular B cells (Figure 2.11A). Furthermore, the V, D, and J genes used by our mAbs did not reveal obvious differences between the MBC and LLPC compartments (Figure 2.11B, Table 2.11C, Table 2.9E).
As repertoires of marginal zone B cells, B1 B cells, and follicular B cells are markedly distinct \(^{74}\), these data argue against a mechanism by which distinct B cell subsets contribute to MBCs versus LLPCs. A second possibility is that MBCs emerge from precursors that are more self-reactive than LLPCs. Indeed, in human studies, MBCs tend towards self-reactivity \(^{75,76}\).

However, we observed no changes in the ratios of DIII-LR and DIII non-LR during the transition from Hardy Fractions E-F (Figure 2.11C), during which self-reactivity is purged \(^{77,78,79,80,81,82}\).

Although WNV DIII-LR cells in Fraction E displayed slightly lower surface levels of IgM, a marker of anergy and self-reactivity \(^{80}\), than did WNV DIII non-LR specific cells, these lowered levels were not maintained in the recirculating mature Fraction F cells (Figure 2.11C). Thus, these avidity-independent mechanisms fail to explain how MBCs acquire specificities distinct from their LLPC counterparts.

Another possible explanation is that the starting populations of B cells destined to become MBCs are distinct from and possess lower avidities than those of LLPC precursors. In this setting, a diverse set of MBC precursors, including those with weak binding to potentially poor epitopes such as DIII non-LR, are allowed to participate in the response. Indeed, B cells with immeasurably low affinities can participate in the response and contribute to MBCs \(^{83,84,85,86}\). To test this possibility, we reverted somatic mutations in V(D)J regions from our mAbs back to their germline sequences and assessed their abilities to bind monomeric WNV WT DIII and WNV SVPs by ELISA. Germline-reverted MBC-derived mAbs bound more weakly (>10-fold) to WNV DIII and WNV SVPs than did germline-reverted LLPC-derived mAbs (Figure 2.12A). These data suggest that MBCs originate from relatively low avidity naïve B cells that are distinct from those of their LLPC counterparts.
Although MBCs have been proposed to be selected for low avidity from GCs \(^63\), this does not preclude affinity maturation of their precursors. Indeed, mutation analysis of our panel of monoclonal antibodies indicate that MBCs have similar numbers of replacement mutations in their V(D)J genes as do LLPCs (Figure 2.12B). MBC clones had in fact slightly greater numbers of silent mutations than did LLPCs, and the total number of mutations were similar between MBCs and LLPCs (Figure 2.11D). Polyclonal IgH repertoire analysis during weeks 1-4 of the response revealed reduced levels of somatic mutations in the MBC compartment relative to LLPCs, but only at weeks 3-4. For reasons that are unclear, the average number of mutations in the MBC population decreased somewhat after week 2, and the earlier week 1-2 timepoints better reflected the ultimate number of mutations observed in mAbs derived from week 8 MBCs (Figure 2.12B, 2.11E). These data provide further evidence that the durable pool of MBCs is formed early in the response \(^7\). In both the MBC and LLPC compartments, affinity-matured antibodies bound better to monomeric WNV DIII than did their germline-reverted counterparts (Figure 2.12C). The extent of affinity maturation was difficult to calculate precisely, as many germline-reverted MBC mAbs bound antigen very poorly. A similar degree of affinity maturation was observed for binding to WNV SVPs (Figure 2.12D). Taken together, these data demonstrate that although B cells destined to become MBCs may be of low relative affinity, they still participate in GC reactions and substantially and rapidly improve their ability to bind antigen.

**MBCs are continuously committed from GCs.**

MBCs and LLPCs have distinct naïve precursor populations of differing avidities and specificities, yet how these precursors remain biased toward a given fate throughout the GC is
unclear. Moreover, the selection of low affinity GC B cells into the MBC compartment is challenging to reconcile with the marked degree of MBC affinity maturation we observed in DIII-specific mAbs, resulting in many MBCs with nanomolar BCR affinities (Figure 2.8B). We considered two explanations for these observations. First, it is possible that B cells become specified and committed to the MBC fate very early in the response, but still retain GC potential and transcriptional programs, and thus are allowed to affinity mature. Second, it is possible that MBC commitment occurs continuously from GCs, which, based on BrdU pulse-chase data, likely occurs over the first few weeks of the response. Thus, as GCs improve their overall affinities over time, so too does the pool from which MBCs are selected. In this second mechanism, evidence of early MBC specification would be observed within the main body of GCs in addition to the fully-committed MBC precursors. To distinguish between these possibilities, we first performed single cell RNA sequencing of WNV DIII-specific mature GC B cells, which are marked by EphrinB1 expression (Figure 2.13A), and examined cells with both GC and MBC transcriptional signatures.

Uniform Manifold Approximation and Projection (UMAP) analysis of the data revealed five transcriptional clusters (Figure 2.14A) that segregated independently of cell cycle genes (Figure 2.13B). Clusters 0, 1, 2, and 4 contained cells expressing Aicda, Bcl6, Jchain, Cxcr4, Foxo1, and Cd86 and thus represent mature GC B cells with light zone and dark zone signatures (Figure 2.14B, Figures 2.13C-D). Cluster 3 lacked Aicda expression and instead expressed high levels of CD38, which marks EphrinB1+ MBC precursors. We further confirmed that cluster 3 is enriched in genes associated with MBC commitment (Figure 2.14C), including Zbtb32, Bach2, Il9r, Klf2, and Ccr6. This cluster expressed low levels of S1pr2 (Figure 2.14D), which is important for GC retention, but high levels of S1pr1 and Gpr183 (Figure
which are associated with GC egress. This transcriptional profile of MBC precursors argues against their potential to continue to participate in GCs and affinity mature.

The MBC genes Zbtb32, Il9r, Klf2, and Cer6 also were expressed by some cells throughout clusters 0, 1, 2, and 4 and were co-expressed with Aicda and Jchain (Figure 2.14B-C). Unlike cluster 3, clusters 0, 1, 2, and 4 express high levels of S1pr2 and low levels of Gpr183 and S1pr1, indicating GC retention, implying that these cells can still participate in GC reactions (Figure 2.14D). These data suggest that cells with intermediate levels of MBC specification and commitment might precede MBC precursor generation from the GC.

To functionally test whether committed MBC precursors continue to participate in GCs or are formed de novo from GCs, we designed a lineage tracing model. We noted that MBC precursors, but not other GC B cells, lack expression of Jchain (Figure 2.14B), a BLIMP1-dependent marker of plasma cell potential. We thus generated mice carrying an IRES-CreERT2 cassette in the 3’ UTR of Jchain and crossed them to animals with a ROSA26-loxP-stop-loxP TdTomato cassette (TdT-Jchain mice). Tamoxifen treatment of these mice revealed efficient labeling of GC B cells and LLPCs, with negligible labeling of other B cells or non-B lineages (Figure 2.13E). We reasoned that at early timepoints post-vaccination, tamoxifen treatment would preferentially mark GC B cells but leave MBC precursors mostly unlabeled. If MBCs at later timepoints remained similarly unlabeled, these data would imply that low affinity MBC precursors are committed early in the response yet continue to participate in GC reactions (Figure 2.13F). In contrast, if the percentage of labeled mature and precursor MBCs at later timepoints were substantially higher than early in the response, this would imply that MBC commitment is not complete early in the response (Figure 2.13F). To test these models, TdT-Jchain mice were vaccinated against WNV, pulsed with tamoxifen at days 5 and 6 after
vaccination, and TdTomato expression analyzed in DIII+ GCs, MBC precursors, and swIg MBCs at different time points (Figure 2.14E). Antigen-specific GCs were efficiently labeled, remaining above 60% TdTomato+ at both weeks 1 and 4 post-vaccination. In contrast, DIII+ MBC precursors were fully unlabeled at Day 7, but increased over time to approach that of GCs by Day 28 (Figure 2.14F). Because the total number of antigen-specific MBC precursor cells was very low at Day 7, we also examined the labeling frequency of polyclonal MBC precursors. This was also quite low at Day 7 (6.5%), confirming the lack of Jchain expression in these population of cells. At 28 days post vaccination, the frequency of labeled mature MBCs was similar to the frequency of labeled MBC precursors (Figure 2.14F). These data argue against prolonged participation in GCs by unlabeled and committed MBC precursors. Instead, these results suggest that MBCs are specified and selected for relatively low affinity from GCs de novo for at least the first few weeks of the response, which allows for their marked increase in affinity over time.
2.4 Discussion

Unlike LLPCs, MBCs can respond to antigen re-exposure by rapid proliferation and differentiation to either plasma cells or secondary GC B cells, the latter of which can produce new affinity-matured plasma cells and MBCs. These recall responses may be of particular importance when mutants or genetically divergent strains of pathogens arise that escape serum antibodies. Examples of such pathogens include HIV, which mutates continuously during chronic infections; influenza viruses, which undergo antigenic drifts and shifts; and flaviviruses, in which related but genetically distinct strains and serotypes co-circulate in overlapping geographic regions. For each of these pathogens, efforts are underway to design vaccine immunogens that would focus the antibody response to neutralizing epitopes. As an example, sequential immunizations are envisioned in which MBCs are gradually guided to the target antibody specificities that bind to antigens such as the CD4-binding site epitopes on HIV. This strategy assumes the ability of immunogens to engage MBCs to re-initiate GC reactions. Yet in mouse models of heterologous flavivirus challenges, we observed that the MBC compartment was largely static after the primary vaccination or infection, with little ability to generate new specificities through secondary GCs. Similar conclusions were reached in recent influenza studies in both humans and mice. Thus, the underlying MBC biology may limit the success of sequential immunization strategies. Clearly more details are needed on the cues that allow for the generation and engagement of MBC subsets that can form secondary GCs.

MBCs are diversified relative to LLPCs to ensure greater antigenic coverage than can be achieved by serum antibodies alone. Recent studies on responses to model antigens have provided some potential mechanistic explanations for this property. MBC commitment in GCs correlates with low affinity, lack of T cell help, and Bach2 expression. Reciprocally, only the
highest avidity cells in the GC receive sufficient T cell help to exit as LLPCs \(^{60, 62}\). In an alternative model, signals in the GC may preferentially promote MBC commitment early and switch to LLPC selection later in the response, thereby explaining differences in final avidity \(^{7, 98}\). Our findings on flavivirus-specific responses agree with and extend upon aspects of both these models. First, we observed an early wave of formation that contributes to most of the durable MBC population \(^{7}\). Second, we observed a lower average final avidity of MBCs relative to their LLPC counterparts \(^{6, 7, 58, 63}\). This result, along with observed differences in MBC specificities relative to GCs throughout the response, argue in favor of active selection \(^{63}\). Yet the extent of MBC affinity maturation we observed was substantial and similar to that of LLPCs. Instead, the germline precursor avidities were lower in MBCs relative to LLPCs, perhaps explaining the differences in final avidities. Unlike responses to haptens or those observed with BCR transgenics where single mutations impart large increases in avidity, polyclonal responses against protein antigens affinity mature more gradually \(^{99, 100}\). Thus, a low starting binding capacity may predispose to low final avidities and bias toward the MBC fate either through an active selection mechanism, by limiting persistence in GCs before signals switch to promote LLPCs, and/or by preferentially selecting MBC clones that improve affinities very rapidly prior to this switch.

Low relative affinities could increase the breadth of MBC specificities and contribute to recall responses in several ways. First, the low relative binding capacity of MBCs might allow for affinity maturation in reverse, in which mutations in a pathogen in fact enhance recognition by a subset of MBCs. Our previous work in fact revealed some evidence of such anticipatory memory \(^{10}\). Second, a low avidity threshold might allow for a greater clonal diversity of MBC specificities to be recruited into the primary response \(^{101}\). An implication of this mechanism is
that immunogen interactions with germline precursors can be manipulated to increase or decrease final MBC and antibody diversity. This agrees with studies that have found other B cell properties, such as proliferation, survival, and GC entry to be linked to germline affinity.\textsuperscript{57, 61, 101, 102, 103, 104, 105, 106}

For flavivirus vaccines, the best strategy might be to restrict antibody and MBC diversity to minimize weakly cross-neutralizing antibodies and ADE, as we found no evidence that such antibodies could be redeemed through secondary GC reactions. DENV and ZIKV DIII-LR cross-reactive monoclonal antibodies with ‘public’ clonotypes have been isolated from humans\textsuperscript{42, 44}, and JEV-WNV cross-reactive DIII-LR antibodies were observed both in our study and in previous work.\textsuperscript{37} Our data suggest that such cross-neutralizing antibodies are likely rare, reactive across only highly related strains, and may already exist prior to heterologous infections. As corroborating evidence, heterologous DENV and ZIKV infections do not measurably alter the pre-existing MBC pool in humans.\textsuperscript{107}

Structure-based immunogen design to minimize cross-reactive responses, even to otherwise neutralizing epitopes, may achieve flavivirus type-specific immunity while minimizing ADE. Several such efforts are underway already\textsuperscript{108, 109, 110, 111}, yet our study offers some additional insight. First, a ‘hidden’ repertoire of low affinity MBCs exists that is not necessarily reflected in serum antibodies. These MBCs potentially can cross-react with conserved non-neutralizing epitopes across flaviviruses, such as DIII-non-LR and thus yield ADE-promoting antibodies. Given that much of the target population for flavivirus vaccines is seropositive for at least one flavivirus already, special attention might be needed to avoid pre-existing MBCs via immunogen design. Second, for naïve individuals, immunogen multimerization and engineering to enhance avidity, possibly to public germline DIII-LR antibodies\textsuperscript{44}, may be an effective
strategy to limit the initial MBC diversity and focus the primary response on potently neutralizing epitopes \textsuperscript{112}. Similar strategies have shown initial promise in models of HIV vaccines \textsuperscript{103, 113, 114}, and our studies support these approaches for type-specific flavivirus immunizations.
2.5 Materials and Methods

**Mice.** All animal procedures used were approved by the Animal Care and Use Committees at Washington University and the University of Arizona. WNV and JEV infections were performed according to A-BSL3 standards. All mice were housed and bred in pathogen-free facilities. C57BL6/N mice were obtained from the National Cancer Institute. 

*AiCda* mice were generated by injecting targeted C57BL/6N embryonic stem line (HEPD0615_4_B08, *AiCda*-tm1a(EUCOMM)Hmgm, European Conditional Mouse Mutagenesis Program) into B6 albino (Jackson Laboratory) mice by the Transgenic Knockout Microinjection Core facility at Washington University in St. Louis. Pups were first crossed to ROSA26::FLP2 (Jackson Laboratory, Stock No: 008463) mice to remove the LacZ and neomycin resistance sequences, and then to hCD20-TamCre mice to generate *AiCda* mice. Mice were then maintained as either *AiCda* or *AiCda* × hCD20-TamCre and littermates were used as controls. The following primers were used for genotyping the *AiCda* allele: common forward 5′- AGCCCCTCAGCCCTTTAATC-3′, wild-type reverse 5′- AGCTGGTGTTGTGTGCGAAG-3′, *AiCda* targeted 5′- TCGTGGTATCGTTATGCGCC-3′. JchainCreERT2 mice were generated by microinjecting Cas9-gRNA ribonucleoparticles into C57BL6/J zygotes alongside an IRES-CreERT2 donor cassette targeting the 3′ UTR of Jchain. Targeted mice were crossed to C57BL6/N mice and then to LoxP-Stop-LoxP (LSL)-TdTomato mice (Jackson Laboratory, Stock No: 007914). Mice heterozygous for Jchain and LSL-TdTomato were used. Tamoxifen treatment consisted of placing mice on tamoxifen for two weeks. For timed deletions, mice were also gavaged for two consecutive days with 50 ug of tamoxifen dissolved in corn oil per gram of mouse weight.
**DIII refolding and biotinylation**

To generate biotinylated DIII, DIII sequences were cloned downstream of a modified pET21 expression vector containing 6x Histidine tag, AviTag (GLNDIFEAQKIEWH), and Thrombin cut site 36. WNV, ZIKV and JEV DIII proteins were refolded by oxidative refolding as previously described 30, 37, 38. Briefly, BL21(DE3) E. coli cells were used for autoinduction of DIII cloned into the pET21 expression vector. The DIII protein was refolded from inclusion bodies by oxidative refolding and purified by size exclusion. AviTagged DIII protein were biotinylated using the NIH Tetramer Core Facility biotinylation protocol (https://tetramer.yerkes.emory.edu/support/protocols#9).

**Flow cytometry.**

Single cell suspensions were prepared from bone marrow or spleen. Erythrocytes were lysed using an ammonium chloride-potassium solution, and lymphocytes were isolated with Hisopaque-1119 (Sigma-Aldrich) and density gradient centrifugation. Cells were resuspended in PBS with 5% adult bovine serum and 2 mM EDTA prior to staining with antibodies and labeled DIII-tetramers. The following antibodies were purchased from Biolegend: 6D5 (CD19)-Alexa Fluor 700; GL7-FITC -PerCP-Cy5.5, -PE, or Pacific Blue; 90 (CD38)-BV510 or -APC-Cy7; 281-2 (CD138)-PE or -APC; RMM-1 (IgM)-APC; 11-26c.2a (IgD)-PerCP-Cy5.5, -PE-Dazzle594, -BV510, or -BV605; 16-10A1 (CD80)-PE, -Alexa Fluor 488, or -Brilliant Violet 421; TY25 (PD-L2)-Brilliant Violet 421 or -PE-Dazzle594; TY/11.8 (CD73)-APC-Cy7; B3B4 (CD23)-BV510; and RA3-6B2 (B220)-FITC. The following antibodies were purchased from eBioscience: 11/41 (IgM)-PerCP-e710 and 4E3 (CD21/35)-FITC. Ephrin-B1-biotin was purchased from R&D. The following antibodies were purchased from Bio X Cell: M1/70
(CD11b), 2C11 (CD3), GK1.5 (CD4), 53-6.7 (CD8), and TER119. Labelled DIII tetramers were generated by incubating biotinylated DIII with labelled streptavidin (Brilliant Violet 421, Brilliant Violet 605, PE, or APC, all from Biolegend) at a 1:4 molar ratio, where 1/10th of the total streptavidin was added to DIII every 10 minutes. DIII tetramers were diluted in PBS to 130 ug of DIII/mL, and cells were stained at 2.6 ug/mL of DIII tetramer per 10^7 cells for 20 min on ice. Antigen-specific MBCs and GC B cells were enriched by depleting non-B cells, IgM+ and IgD+ splenocytes cells by cellular panning. Splenocytes were stained with rat anti-CD4, CD8, CD11b, CD3, Ter119, IgM, and IgD on ice for 20 min, wash, and then incubated on plates pre-coated with 1 ug/mL of mouse anti-rat IgG (3053-01, Southern Biotech) for 20 min at 4°C. The non-adherent cell fraction was collected, washed, and then stained with MBC and GC B cell surface markers (CD19, GL7, CD38, CD80, CCR6 and DIII tetramers). Antigen-specific bone marrow plasma cells were subjected to CD138 enrichment prior to DIII-tetramer staining. Total bone marrow cells were stained with 2 uL of anti-CD138 APC, cells washed and then stained with anti-PE magnetic beads (0.5 uL/10^7 cells, Miltenyi Biotec). Positive enrichment of CD138-expressing cells was performed using MACS LS columns (Miltenyi Biotec), where enriched cells were stained with 2.6 ug/mL of DIII tetramers.

**ELISA.**

ELISA plates (9018, Corning) were coated overnight at 4°C in 0.1 M sodium bicarbonate buffer, pH 9.5 containing 5 ug/mL of the appropriate domain III antibody, WNV E protein, or 4G2 mAb. All other incubation steps were performed at room temperature for 1 hour. Wash steps were performed between each step using PBS + 0.05% Tween-20. Plates were blocked with PBS + 2% BSA followed by serial dilutions of serum. Plates coated with 4G2 were further
incubated with media containing WNV SVPs. Serum was probed with 0.1 ug/mL of biotinylated anti-mouse IgG (715-065-151, Jackson ImmunoResearch Laboratories) or 0.14 ug/mL of biotinylated anti-mouse IgM (115-065-075, Jackson ImmunoResearch Laboratories) and then detected with streptavidin conjugated horseradish peroxidase (554066, BD biosciences). MAbs were probed with 0.8 ug/mL of peroxidase conjugated anti-human IgG (709-035-149, Jackson ImmunoResearch). Plates were developed using TMB (J61325, Alfa Aesar) and neutralized with 2N H₂SO₄. Optical density (OD) values were measured at 450 nm. Serum endpoint titer was defined as the inverse dilution factor that is three standard deviations above background using variable slope measurements and Prism software (GraphPad Software). OD values from mAbs were normalized to the OD values of humanized E16 (NR-31082, BEI Resources) at each dilution.

**Neutralization Assays.**

Serum was heat-inactivated at 56°C for 30 min. Serial dilutions of serum or mAbs were incubated with 100 FFU of WNV-NY99 or JEV SA14-14-2 for 1 h at room temperature. Serum/mAbs-virus complexes were added to a monolayer of Vero cells in a 96-well plate and incubated at 37°C with 5% CO₂ for 1 h. Cells were overlaid with 1% (w/v) carboxymethycellulose in MEM supplemented with 2% FBS, 1x Pen-Strep, and 1x GlutaMax. Plates were harvest 24 (WNV) or 36 (JEV) h later and fixed with 1% PFA in PBS. Plates were incubated with 500 ng/mL of hE16 (for WNV) or JEV-31 (Fernandez, E. et al., mBio, 2018) diluted in PBS + 0.1% saponin+0.1% BSA. hE16 was detected using 0.8 ug/mL of peroxidase conjugated anti-human IgG, whereas JEV-31 was detected using 100 ng/mL biotinylated anti-
mouse IgG followed by streptavidin-HRP. Foci were visualized using TrueBlue peroxidase substrate (50-78-02, KPL) and enumerated on the CTL ImmunoSpot S6 Analyzer.

**ELISpot Assays.**

ELISpot plates (MSHAN4510, Millipore Sigma) were coated with 10 ug/mL of anti-mouse Ig kappa (559749, BD biosciences) in PBS overnight at 4°C. Plates were blocked with complete DMEM for 1 h. Bone marrow plasma cells were enriched on CD138 as described above and plated in triplicates. Cells were incubated at 37°C with 5% CO₂ for 16 h. Plates were washed with PBS followed by PBS+0.05% Tween-20, incubated with 5 ug/mL of biotinylated WNV WT or KT DIII diluted in PBS, 2% adult bovine serum and 0.05% Tween-20 and incubated at room temperatures for 1 h. Plates were rinsed with PBS + 0.05% Tween-20 and then incubated with streptavidin HRP diluted in PBS, 2% adult bovine serum, and 0.05% Tween-20 at room temperatures for 1 h. Wells were washed one last time with PBS + 0.05% Tween-20 and PBS. Spots were developed using TrueBlue peroxidase substrate and enumerated on the CTL ImmunoSpot S6 Analyzer. The total number of DIII-specific bone marrow plasma cells was calculated by multiplying the number of enumerated spots by the plating dilution factor. The value was then divided by the total number of bone marrow cells.

**WNV SVP production.**

Lenti-X 293T cells were transfected with WNV prM-E construct using GeneJuice transfection reagent. Supernatants were collected 48 h later, filtered using a 0.2 um filter, aliquotted, flash frozen, and stored at -80°C.
**Immunizations.**

Mice were administered two doses of inactivated WNV Innovator vaccine (Valley Vet Supply) by intraperitoneal injection on days 1 and 2 $^{10}$. Biotinylated ZIKV WT $^{38}$ and A310/T335 (LR mutant) DIII were tetramerized with streptavidin at a 1:4 molar ratio, where 50 µg of tetramer was mixed with 100 µL of AddaVax vaccine (Invivogen) and then administered to mice by intraperitoneal injection.

**MAb generation and purification.**

Antigen-specific MBCs were sorted using a FACSaria II and cultured as previously described $^{10}$. Briefly, each well of a 96-well plate were seeded with 30,000 mitomycin C-treated BAFF + CD40L + NIH 3T3 cells $^{10}$, one day prior to seeding individual MBCs. Sorted MBCs were cocultured with the feeder cells in the conditioned 3T3 complete DMEM medium (DMEM, 10% FBS, 1x penicillin-streptomycin, 1 mM sodium pyruvate, 1x nonessential amino acids, and 2 mM GlutaMax) containing 1 µg/ml pokeweed mitogen lectin (Sigma-Aldrich), 10 µg/ml lipopolysaccharide (from *E. coli* 0.111:B4; Sigma-Aldrich), 1 µg/ml phosphorothioated murine CpG (5′-TCCATGACGTTCTGATGCT-3′; Integrated DNA Technologies), 16 ng/ml mIL-2, 10 ng/ml mIL-6, 17 ng/ml mIL-10 (PeproTech), 10 mM HEPES, and 50 µM β-mercaptoethanol). Supernatant was collected after 6 days of culture, and DIII specificity assessed by ELISA. V(D)J sequences were amplified as described in (Tiller, 2009) and cloned into modified heavy (Addgene plasmid # 8079) and light chain (Addgene plasmid # 80796) expression vectors $^{116}$. The modified heavy chain expression contains a G4S linker sequence and an AviTag sequence at the 3’ end of the hIgG1 sequence. Single antigen-specific LLPCs from the bone marrow were sorted into catch buffer containing 0.1M Tris pH 8.0 and RNase inhibitor $^{117}$ and flash frozen.
V(D)J sequences were isolated according to a previously published protocol. Briefly, cDNA was generated, two rounds of nested PCRs performed to amplify the V(D)J sequences, and then V(D)J sequences cloned into the modified heavy and light chain expression vectors.

Lenti-X 293T cells (Clontech) used for transfections were maintained in complete DMEM containing 10% ultra-low IgG FBS (ThermoFisher), 1x penicillin-streptomycin, 1 mM sodium pyruvate, 1x nonessential amino acids, and 2 mM GlutaMax. Transfections were performed using GeneJuice (EMD Millipore Novagen). Somaically hypermutated mAbs were produced by co-transfecting heavy chain construct, light chain construct, and a BirA ligase construct. Germline reverted mAbs were produced by co-transfecting heavy and light chain constructs. Fresh media was supplemented every other day. Six days after transfection, supernatants were collected, cellular debris filtered, and then mixed with Protein G IgG Binding Buffer (Thermo Scientific) at a 1:1 ratio. MAbs were purified from the supernatant by Protein G (GE Life sciences) affinity chromatography. Purified antibodies were concentrated and buffer exchanged with PBS containing 0.1% sodium azide using an Amicon® Ultra-15 Centrifugal Filter Unit (30 kDa membrane).

**BLI Binding Assays.**

The binding affinity of affinity matured mAbs to WNV WT DIII was assessed by BLI using an Octet Red384. The buffer used contained 150 mM NaCl, 10 mM HEPES, 3 mM EDTA, 0.005% Tween20, and 1% BSA, pH 7.4. Biotinylated mAb was loaded onto streptavidin biosensors (ForteBio) at 5 ug/mL for 3 minutes. The association and dissociation of antibody to WNV WT
DIII was measured at 30°C. Data were analyzed using the ForteBio DataAnalysis 11.0 software, and fitted to a 1:1 binding model.

Single cell RNA-seq.

WNV DIII-specific GC B cells (CD19⁺GL7⁺EphrinB1⁺IgD⁻) were sorted using a FACS AriaII and prepared and processed according to 10x Genomics instructions for Single Cell Protocols Cell Preparation Guide and Chromium Single Cell V(D)J Reagents kit. Sequencing files were processed using the CellRanger program (10x Genomics), where minimum read cutoffs were kept at default settings. Sequences were further processed and visualized using Seurat. Cells containing more than 5% mitochondrial genes were discarded. UMAP clusters of cells were determined based on global gene expression or on cell cycle genes.

GC mutation analysis.

WNV DIII-specific GC B cells (CD19⁺GL7⁺IgD⁻CD38⁻) from WT mice. were sorted 7, 14, 21, and 28 days after WNV vaccination. RNA was using the NucleoSpin RNA XS kit (Macherey-Nagel) per manufacturer’s instructions. cDNA synthesis was performed as previously described. IgG immunoglobulin transcripts were amplified with the following primers: msVHstdseq1 5’-

ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGGATTCGAGGTGCAGCTGCAGG
AGTCTGG-3’

and commonCgstdseq2 5’-

GTGACTGGAGTTCCAGACGTTGCTCTTCCGATCTCARKGGATRRRCHGATGGGG-3’.

A final amplification with P5 forward Stdseq1 and P7 reverse Stdseq index primers were used as previously published. Samples were pooled, purified, and then sequence dusing Illumina
Miseq v3 2x250 platform using previously published 121. Forward and reverse reads were paired using the default settings of PEAR 122. Paired reads were further analyzed using Migmap (https://github.com/mikessh/migmap) where full length VDJ sequences were merged and corrected for PCR errors. Somatic hypermutation information was extracted using the Migmap post-analysis command.

**Immunoblotting.**

*Aicda<sup>−/−</sup>* TamCre and *Aicda<sup>−/−</sup>* mice were immunized with 8x10<sup>8</sup> sheep red blood cells (Lampire) by intraperitoneal injection. GC B cells were sorted on a FACS Aria II at different timepoints after immunization. Cells were lysed in Laemmli buffer containing 2% SDS and 5% 2-mercaptoethanol and stored at -20°C until analysis. Cell lysates were separated by 10% SDS-PAGE and transferred to PVDF membrane (Roche). The membranes were probed with 20 ug/mL of anti-AID monoclonal antibody (mAID-2, eBioscience). The signal was detected using a Luminata HRP substrate (Millipore). Signal from the horseradish peroxidase was quenched by incubating the membrane with 30% H<sub>2</sub>O<sub>2</sub> for 10 min. The membrane then was probed with anti-ERK2 (Santa Cruz, C-14) antibody as a loading control. The signal was detected using a Luminata HRP substrate.

**Statistics.**

All statistical tests were conducted using Graphpad Prism and are detailed in each figure legend.
2.6 Acknowledgments

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2.7 Author Contributions

2.8 References


31. Lai, C.-Y.Y. et al. Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II. Journal of virology 82, 6631-6643 (2008).


Figure 2.1 Validation of Aicda f/f mice.

(A) Targeting construct for Aicda. (B) PCR genotyping of the Aicda allele to confirm targeting and absence of neomycin resistance cassette. (C) Aicda f/f x TamCre mice were immunized with sheep red blood cells and treated with tamoxifen starting on day 7 after immunization. CD19^+GL7^+ cells were sorted at different days after tamoxifen and immunoblotted for AID. ERK2 level (probed with anti-ERK2 antibody) was used as the loading control. (D) Aicda f/f x TamCre mice were treated with tamoxifen for two weeks and then immunized with the inactivated WNV vaccine. Serum was collected 12 days later to confirm absence of anti-WNV WT DIII IgG antibodies. Mean values ± SEM are shown.
Figure 2.2 MBCs do not require additional affinity maturation to respond to heterologous flavivirus challenges.

(A) WNV-vaccinated and naïve mice were bled before (D0) and 14 days after JEV vaccination. Serum IgG endpoint titers against JEV WT DIII were calculated as the reciprocal serum dilution that was three standard deviations above background. Endpoint titers were calculated as the reciprocal serum dilution that was three standard deviations above background. Each symbol represents an individual mouse. **p < 0.01; paired student’s t-test. Data comes from one experiment. (B) Schematic representation of the experimental setup for WNV vaccination and JEV recall responses. (C) Serum from WNV-immune Aicda f/f x TamCre (AID cKO) and Aicda f/f (AID WT) was collected 70 days after JEV vaccination. Levels of WNV DIII- and JEV DIII-specific IgG were measured by ELISA. Mean endpoint titers ± standard error of the mean (SEM) are shown. Each symbol represents an individual mouse. (D) IgG levels, shown as OD 450nm values for 1:50 diluted serum, collected in (C), for binding to JEV WT and JEV ESAK DIII. Each symbol connected by lines represents paired values from an individual mouse. Data are pooled from two independent experiments. (E)
Neutralizing antibody titers (1/IC50) to JEV were measured by a focus reduction neutralization test (FRNT) from serum collected in (C). Mean ± SEM are shown; each symbol represents an individual mouse. (F) Schematic representation of the experimental setup for ZIKV DIII immunization and subsequent JEV recall responses. (G) Serum from ZIKV DIII-immunized AID cKO and AID WT was collected 28 days after JEV vaccination. Levels of ZIKV DIII and JEV DIII specific IgG antibodies were measured by ELISA. Mean endpoint titers ± SEM are shown. Each symbol represents an individual mouse. Data are pooled from two independent experiments.
Figure 2.3 IgM responses after heterologous JEV vaccination.

(A) Validation of JEV ESAK DIII by ELISA. JEV DIII-LR specific mAb JEV-31 was used to confirm loss of the LR epitope. Wild-type mice were immunized with JEV ESAK DIII, and immune serum was used to probe for binding to JEV WT and ESAK DIII. Data from 1 experiment is shown. Mean values ± SEM are shown. (B) Serum from WNV-immune Aicda f/f x TamCre (AID cKO) and Aicda f/f (AID WT) was collected before, 14 and 28 days after JEV vaccination. Levels of JEV WT DIII and JEV ESAK DIII specific IgG antibodies were measured by ELISA. Endpoint titers were calculated as the reciprocal serum dilution that was three standard deviations above background. Mean endpoint titers ± SEM are shown. Each symbol represents an individual mouse where matched samples are connected by a line. Data is from one experiment. (C) Serum from WNV-immune AID WT and AID cKO mice was collected ten weeks after JEV vaccination. JEV WT and JEV ESAK DIII specific IgM levels are shown as the OD450nm values from serum diluted 1:50. Each symbol represents an individual mouse. Mean ± SEM are shown. Data are pooled from two independent experiments. (D) Serum from ZIKV DIII immunized AID WT and AID cKO was collected
before and 28 days after JEV vaccination. Levels of JEV WT DIII and JEV ESAK DIII specific IgG antibodies were measured by ELISA. Endpoint titers were calculated as the reciprocal serum dilution that was three standard deviations above background. Mean endpoint titers ± SEM are shown. Each symbol represents an individual mouse where matched samples are connected by a line. Data are pooled from two independent experiments. (E) Serum from ZIKV-immune AID WT and AID cKO mice was collected four weeks after JEV vaccination. JEV WT and JEV ESAK DIII specific IgM levels are shown as the OD\textsubscript{450nm} values from serum diluted 1:50. Data are pooled from two independent experiments. (F) Schematic representation of DENV and ZIKV recall responses. (G) The frequency of ZIKV DIII-specific MBCs and LLPCs were quantified by flow cytometry and ELISPOT, respectively. Mice challenged with only ZIKV were used to delineate the naïve B cell responses. AID WT (2° only) is calculated by subtracting the average WT primary response value from the AID WT (1° and 2°) values. Mean values ± SEM are shown; each symbol represents one mouse. Data are pooled from two independent experiments.
Figure 2.4 Gating strategy for memory B cell subsets.
Gating strategy for MBC subsets based on CD80 and CCR6 expression. Cells are gated on CD19^+GL7^- and IgM^+IgD^+, IgM^+IgD^-, or swlg (IgM^+IgD^-) expression. Representative data from 2 experiments are shown.
Figure 2.5 Plasma cell-biased MBCs recognize and respond to heterologous antigens.

(A) Mice were vaccinated with inactivated WNV vaccine and the number of DIII-specific cells in different MBC subsets was calculated by flow cytometry 8-12 weeks later. WNV-vaccinated mice are compared to naïve animals. Mean ± SEM are shown; each symbol represents an individual mouse. Data are pooled from two independent experiments. *p < 0.05; student’s two-tailed t test for each subset. (B) Representative flow cytometry plots showing the cross-reactivity of WNV DIII-LR and WNV DIII non-LR cells to JEV DIII-LR and JEV DIII non-LR cells. WNV DIII-binding cells were enriched from 5 pooled spleens from WNV-vaccinated mice and then probed for JEV WT and ESAK DIII reactivity. (C) Sera from WNV-immune mice, collected in Figure 1A, were assessed for WNV WT and WNV KT DIII binding before (D0) and 14 days post JEV vaccination. Endpoint titers were calculated as the reciprocal serum dilution that was three standard deviations above background. Each symbol represents an individual mouse. **p < 0.01; matched two-way ANOVA. Data comes from one experiment. (D) The frequency of WNV DIII-specific GC B cells (CD19⁺GL7⁺IgD⁻CD38⁻) 7 and 14 days after JEV vaccination of WNV-immune mice was quantified by flow cytometry. A representative plot of DIII-specific GC B cells is shown on the left and quantified on the right. Mean ± SEM are shown; each symbol represents an individual mouse. Data comes from one experiment.
**Figure 2.6 MBCs are actively selected from GC B cells.**

(A) Mice were immunized with WNV inactivated vaccine and DIII-specificities in GC and MBC compartments were enumerated by flow cytometry at days 7, 14, 21, 28 and 56+. A representative plot of DIII-specific GC B cells is shown on the left and quantified on the right. Mean frequencies ± SEM are shown; each symbol represents an individual mouse. *** p < 0.001, **** p < 0.0001; paired student’s t-test. Data are pooled from two independent experiments. (B) The frequencies of WNV DIII-LR and WNV DIII non-LR binders in sw1g CD80+CCR6+ MBCs were quantified by flow cytometry. A representative plot of DIII-specific MBCs is shown on the left and quantified on the right. Mean values ± SEM are shown; each symbol represents an individual mouse. *** p < 0.001, **** p < 0.0001; paired student’s t-test. Data are pooled from two independent experiments. (C) Ratios of WNV DIII non-LR to WNV DIII LR-specific cells were quantified for GC B cells and MBCs using values in (A) and (B). Mean values ± SEM are shown. *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; paired student’s t-test. (D) The frequency of CD38+CCR6+ expressing MBC precursor cells within WNV DIII-LR and WNV DIII non-LR specific GC B cells (CD19+GL7+IgD-) was quantified by flow cytometry. The gating strategy is shown on the top.
with a representative CD38+CCR6+ gate shown for DIII-LR cells. Mean values ± SEM are shown; each symbol represents an individual mouse. Data are pooled from two independent experiments.
Figure 2.7 Isolation of mAbs from MBCs and LLPCs.

(A) Gating strategy for WNV DIII-specific LLPCs. LLPCs were first assessed for surface B cell receptor expression by analyzing surface kappa expression. LLPCs in the bone marrow were enriched for CD138 expression followed by WNV WT DIII staining with two different tetramers. (B) Wild-type mice were immunized with inactivated WNV vaccine, and DIII-specific MBCs and LLPCs were sorted at least 8 weeks after vaccination. Gating strategy for CD19^+^GL7^-^WNV DIII-specific MBCs. Splenocytes were depleted of CD3, CD4, CD8, CD11b, Ter119, IgM and IgG prior to sorting. (C) Reverse orientation ELISA of a subset of DIII-specific mAbs to measure monovalent affinities. ELISA plates were coated with a fixed concentration of mAbs and incubated with increasing concentrations of WNV WT DIII. Mean values ± SEM are shown. (D) Example binding curves for representative MBC and LLPC mAbs (left, middle) generated by biolayer interferometry with increasing concentrations of WNV WT DIII. Binding curves were fit to a 1:1 binding model using ForteBio’s analysis software. The panel below each binding curves shows steady-state analysis results (K_D, equilibrium), plotted as the binding response (nm) versus concentration of DIII, also shows binding saturation. A Scatchard plot, shown as an insert in the steady-state analysis panels, suggests single binding affinity. The binding association and dissociation constants were calculated and plotted on the right. Mean ± SEM are shown; each symbol represents one mAb.
Figure 2.8 MBCs have lower avidity for their antigens than do LLPCs.

(A) Monoclonal Abs from WNV-immune mice were isolated from MBCs and LLPCs, and WNV DIII reactivity was confirmed by ELISA. MAb binding curves for WNV WT DIII were performed using ELISA. Mean ± SEM are shown. *p < 0.05; 2-way ANOVA. (B) Binding affinity (K_D, kinetic) to WNV WT DIII for a subset of mAbs was determined by biolayer interferometry. Mean ± SEM are shown; each symbol represents one mAbs. n.s. indicates p > 0.05; Mann-Whitney test. (C) MAb binding curves for WNV subviral particles (SVPs) were performed by ELISA. Mean ± SEM are shown. ****p < 0.0001; 2-way ANOVA.
Figure 2.9 Deletion of AID during GC reactions does not impair class switching or differentiation of MBCs and LLPCs. (A) Frequencies of isotype switched (IgM→IgD) GC B cells (CD19+GL7+) 7 and 14 days after immunization of wild-type mice with inactivated WNV vaccine as measured by flow
Mean values ± SEM are shown. Each symbol represents one mouse. Data are pooled from two independent experiments. (B) WT mice were immunized with inactivated WNV vaccine. WNV DIII+ GC B cells (CD19+GL7+IgD-) were sorted at different time points and IgH repertoire analysis was performed by MiSeq. The total number of replacement mutations for IgH is shown for each week. (C) WT mice were immunized with inactivated WNV vaccine, and the number of DIII-specific bone marrow plasma cells were enumerated by ELISPOT at different time points. Representative wells are shown on the left and quantified on the right. Mean values ± SEM are shown. Each symbol represents one mouse. Data are pooled from two independent experiments. (D) Schematic of experiments to test for Cre toxicity. Sera were collected 28 days after vaccination and probed for anti-WNV WT DIII and E protein IgG antibodies. Mean values ± SEM are shown. (E) WNV DIII-specific LLPC numbers after WNV vaccination and AID deletion were enumerated by ELISPOT. Mean values ± SEM are shown. Each symbol represents one mouse. Data are pooled from two independent experiments. (F) Serum from mice after WNV vaccination and AID deletion was collected 8 weeks post-immunization, and IgM antibodies were probed for WNV E reactivity by ELISA. Mean values ± SEM are shown. Data are pooled from two independent experiments.
Figure 2.10 Absolute affinity thresholds do not segregate MBCs from LLPCs in vivo.

(A) Experimental setup to temporally ablate AID during ongoing GC reactions. (B) Frequencies of WNV DIII-LR and WNV DIII non-LR specific MBCs were quantified by flow cytometry for each AID genotype and deletion group. Mean values ± SEM are shown; each symbol represents an individual mouse. Data are pooled from two independent experiments. (C) Serum IgG endpoint titers for WNV WT and WNV KT DIII were calculated by ELISA. Mean values ± SEM are shown. Each symbol represents an individual mouse. Data are pooled from two independent experiments. **p < 0.01 and ***p < 0.001; matched 2-way ANOVA. (D) Serum from week 1 AID deletion was assessed for WNV SVP binding by ELISA. Mean values ± SEM are shown.
Figure 2.11 WNV DIII-LR and WNV DIII non-LR naïve and precursor cells are not selected from distinct B cell subsets.

(A) Spleens from naïve mice were enriched for WNV WT and KT DIII tetramers binding.
Frequencies of antigen-specific cells in the follicular (FoB) and marginal zone (MZ) B cell compartments were enumerated by flow cytometry. A representative flow cytometry plot is shown on the left and quantified on the right. Mean values ± SEM are shown. (B) The frequency is shown of V, D, and J immunoglobulin heavy chain gene usage (top) or light chain V and J gene usage (bottom) from mAbs derived from MBCs and LLPCs. (C) Frequencies of WNV DIII-LR and WNV DIII non-LR-specific cells in Hardy Fractions E (Fr. E) and F (Fr. F) were enumerated by flow cytometry. A representative plot is on the left and quantification is shown in the middle. The IgM geometric mean fluorescence intensities (gMFI) of WNV DIII-LR and WNV DIII-non LR specific cells in Hardy Fractions E and F are quantified on the right. Mean values ± SEM are shown. *p < 0.05; matched 2-way ANOVA. (D) The number of silent and total mutations in the heavy and light chains of the isolated panels of mAbs are plotted. Mean ± SEM are shown; each symbol represents one mAb. *p < 0.05; Mann-Whitney test. (E) WT mice were immunized with inactivated WNV vaccine. WNV DIII+ MBCs (CD19+GL7+IgD−IgM+CD80+CCR6+) and WNV DIII+ bone marrow PCs (CD138hiIgM+) were sorted at different time points and IgH repertoire analysis was performed by MiSeq. The total number of non-silent mutations for IgH is shown for each week. Mean ± SEM are shown. ****p < 0.0001; Kruskal-Wallis test.
Figure 2.12 Germline affinities of MBC-derived antibodies are lower than those from LLPCs.

(A) MAbs isolated in Figure 4 were reverted to their germline sequences, and binding curves against monomeric WNV WT DIII and SVPs were measured by ELISA. Mean values ± SEM are shown. ***p < 0.001 and ****p < 0.0001; 2-way ANOVA. (B) The total number of replacement mutations in the heavy and light chains of the isolated panels of mAbs are plotted. Mean ± SEM are shown; each symbol represents one mAb. (C, D) ELISA binding curves for monomeric WNV WT DIII (C) and WNV SVPs (D) are compared for germline-reverted and somatically-mutated mAbs from MBCs and LLPCs. Mean ± SEM are shown. ****p < 0.0001; two-way ANOVA.
Figure 2.13 Single cell RNA sequencing and lineage tracing validation.  
(A) Gating strategy to sort mature GC B cells one week after WNV vaccination of wild type mice. Splenocytes were pre-enriched for GL7 expression. (B) UMAP plot indicating cell cycle
stage for each cluster. (C) UMAP plot showing expression level of Bcl6. (D) UMAP plots showing expression of light zone (Cd86) and dark zone (Foxo1 and Cxcr4) genes. (E) Jchain expression in GC B cells was confirmed by crossing Jchain to LSL-TdTomato mice and administering tamoxifen for two weeks. TdTomato expression was quantified for B cells (CD19\(^+\) in the spleen or B220\(^+\) in the bone marrow), plasma cells (PC, CD138\(^+\)), non-B cells (B220\(^-\)CD138\(^-\) in bone marrow, CD19\(^-\)GL7\(^-\)CD138\(^-\) in spleen), and GC B cells (CD19\(^+\)GL7\(^+\)). Mean values ± SEM are shown. (F) Expected results for TdT-Jchain mice if MBCs are committed early and continue to participate in GCs or if there is continuous selection of MBCs.
Figure 2.14 MBCs are continuously selected from GCs.
(A) DIII-specific GC B cells (CD19^+GL7^-IgD^-EphrinB1^+) were isolated one week after WNV
vaccination, subjected to single cell RNA-sequencing and analyzed using Seurat. UMAP plot is shown of DIII-specific GC B cells with each uniquely colored cluster segregated by differential transcriptional profiles. (B) UMAP plots highlighting the gene expression level per cell for GC genes Aicda and Jchain. (C) UMAP plots highlighting the gene expression level per cell for MBC genes. (D) UMAP plots highlighting the gene expression level for GC egress (Gpr183 and S1pr1) and retention (S1pr2) genes. (E) Schematic representation of GC lineage tracing experiment. (F) A representative histogram for TdTomato expression in polyclonal MBC precursors (CD19⁺GL7⁺IgD⁻IgM⁻CD38⁻EfnB1⁺) is shown (left). The frequency of TdTomato expression in the DIII⁺ and polyclonal MBC precursor, DIII⁺ GC B cells (CD19⁺GL7⁺IgD⁺CD38⁻EfnB1⁺) and DIII⁺ swIg MBC populations is quantified by flow cytometry (right). Mean ± SEM are shown. *p < 0.05; paired Student’s t test for each population.
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<td>IGHD1-1</td>
<td>IGHJ2</td>
<td>IGKV6-25</td>
<td>IGKJ5</td>
</tr>
<tr>
<td>725B4</td>
<td>DIII non-LR</td>
<td>IGHV1-82</td>
<td>IGHD2-1</td>
<td>IGHJ2</td>
<td>IGKV9-124</td>
<td>IGKJ4</td>
</tr>
<tr>
<td>626C1</td>
<td>DIII non-LR</td>
<td>IGHV1-26</td>
<td>IGHD2-4</td>
<td>IGHJ2</td>
<td>IGKV3-2</td>
<td>IGKJ1</td>
</tr>
</tbody>
</table>

**Table 2.1.** V, D, and J gene usages for mAbs derived from MBCs and LLPCs.  
MAbs isolated in Figure 2.8 were subject to V(D)J gene usage analysis for both the heavy (H) and light (K) chains. IMGT nomenclature is used.
Chapter 3:

Defining the role of ZBTB38 in B cell responses

This work was performed in collaboration with Deepta Bhattacharya. Personal contributions include the design and carrying out of selected experiments, data analysis, and writing of this work.
3.1 Abstract

Long-term B cell mediated immunity is provided by long-lived plasma cells (LLPCs) and memory B cells (MBCs). LLPCs secrete antibodies to provide sterilizing immunity whereas MBCs provide protection only after reactivation and differentiation into plasma cells. LLPCs and MBCs primarily differentiate from germinal center (GC) B cells. Thus, these three B cell compartments are critical for generating durable immunity. Members of the broad complex, tram track, bric-a-brac and zinc finger (BTB-ZF) family of transcription factors include BCL-6, ZBTB20, and ZBTB32. These transcription factors regulate key aspects of B cell responses. BCL-6 is essential for GC formation. ZBTB20 regulates the durability of LLPC responses in an adjuvant dependent manner. ZBTB32 is important for restricting the durability of memory B cell recall responses. ZBTB38 is another member of the BTB-ZF family, whose functional importance in hematopoiesis and B cells has not been characterized. Here, we demonstrate that ZBTB38 is dispensable for the development of many mature hematopoietic lineages as well as for both primary and secondary B cell responses to a hapten antigen.
3.2 Introduction

Humoral immunity to an infection or vaccination is mediated by long-lived plasma cells (LLPCs) and memory B cells (MBCs), which have distinct antigen specificities and therefore mediate different aspects of immunity \(^1\). LLPCs constitutively secrete antibodies, and are important for providing protection against re-infection by the same pathogen. Memory B cells, on the other hand, can only provide protection after re-activation by a cognate antigen by rapidly differentiating into plasma cells. Recent studies have identified the broad complex, tram track, bric-a-brac and zinc finger (BTB-ZF) family of transcription factors as key regulators in B cell development. BTB-ZF family members are involved in many cellular processes by binding DNA through its variable number of C-terminal zinc finger domains and recruiting SMRT co-repressors and histone deacetylases to its N-terminal BTB/POZ domain \(^2, 3, 4, 5, 6\). Notable members include BCL-6, ZBTB20, and ZBTB32, where each member regulates distinct aspects of B cell mediated immunity. BCL-6 is important for germinal center (GC) formation \(^7\), ZBTB20 promotes durable immunity in an adjuvant dependent manner \(^8, 9\), and ZBTB32 restricts memory B cell recall responses \(^10, 11\). Thus, understanding the role of additional BTB-POZ members in regulating B cell responses may provide further insight into how the durability of B cell memory responses are established.

ZBTB38, also known as CIBZ (CtBP-interacting BTB zinc finger protein) is another member of the BTB-ZF family, and can function either as a transcriptional repressor or activator \(^12\). ZBTB38 transcriptional regulation occurs by binding primarily to sequence-specific, methylated CpG sequences and recruiting transcriptional repressors or activators \(^13, 14, 15\). ZBTB38 has been shown to repress overall transcription by inhibiting expression of MCM10, a component of the pre-replication complex \(^16\). Additionally, ZBTB38 can, directly or indirectly,
regulate cell cycle progression, cellular differentiation, and apoptosis. Given the ability of ZBTB38 to regulate cell cycle progression and anti-apoptosis, we hypothesized that ZBTB38 is important for regulating GC reactions, which require a balance between proliferation and apoptosis. Quantitative real-time PCR of different B cell subsets revealed elevated expression of ZBTB38 in GC B cells and plasma cells compared to naïve B cells. Here, we demonstrate that ZBTB38 deficiency does not impair primary or secondary B cell responses to a hapten immunogen.
3.3 Results

Generation and validation of ZBTB38 floxed mice.

To assess the role of ZBTB38 in vivo, we generated Zbtb38 floxed mice by targeting exon 3 using CRISPR technology (Figure 3.1A). NdeI and EcoRI restriction sites were introduced near the 3’ LoxP and 5’ LoxP sites to screen for successful integration of the plasmid. Correct targeting of exon 3 was confirmed by PCR and restriction enzyme digestions, where genomic DNA sequences flanking exon 3 was amplified followed by single and double restriction enzyme digests with NdeI and EcoRI (Figure 3.1B). Wild-type, targeted, and deleted Zbtb38 mice can be differentiated using a set of three primers that flank the 5’ and 3’ LoxP sequences (Figure 3.1C). To confirm deletion of Zbtb38 exon 3 at the genomic level, Zbtb38 f/f mice were crossed to mice expressing CMV-Cre to obtain germline ZBTB38 deletion. Genomic DNA from Zbtb38 f/f x CMV-Cre was amplified where deletion was confirmed by a 4 kb reduction in PCR size (Figure 3.1D). ZBTB38 germline deletion did not result in gross developmental abnormalities. No differences in the number or size of ZBTB38 deficient and wild-type mice were observed (data not shown).

ZBTB38 deficiency does alter the development of hematopoietic cells

ZBTB38 gene expression in multiple cell types was analyzed using available RNA-sequencing data assembled by the ImmGen Consortium. A subset of cell types expressing DESeq2 normalized ZBTB38 levels greater than 800 are shown in Figure 3.2A. Given the high expression of ZBTB38 in hematopoietic stem cells (HSCs), and downstream lineages such as B and T cells, we crossed Zbtb38 f/f mice to VavCre mice (Zbtb38 f/f x VavCre, ZBTB38 KO), which result in ZBTB38 deletion in hematopoietic cells.
We first assessed if ZBTB38 deficiency alters the development of hematopoietic lineages under physiological conditions by quantifying the frequencies of different cell populations by flow cytometry. We first focused on hematopoietic stem cells (HSCs, cKit+Sca1+Flk2−CD27+) and progenitors with varying degrees of lineage commitment in the bone marrow. HSCs differentiate into multipotent progenitors (MPPs, cKit+Sca1+Flk2−CD27+) that can give rise to both the myeloid and lymphoid lineages. MPPs can then differentiate into common myeloid progenitors (CMPs, cKit+Sca1+Flk2+FcyR−) or common lymphoid progenitors (CLPs, cKit−/Sca1−CD27+FcyR−Flk2−IL7Rα+) or common lymphoid progenitors (CLPs, cKit−/Sca1−CD27+FcyR−Flk2−IL7Rα+) or common lymphoid progenitors (CLPs, cKit−/Sca1−CD27+FcyR−Flk2−IL7Rα+) or common lymphoid progenitors (CLPs, cKit−/Sca1−CD27+FcyR−Flk2−IL7Rα+) or common lymphoid progenitors (CLPs, cKit−/Sca1−CD27+FcyR−Flk2−IL7Rα+) or common lymphoid progenitors (CLPs, cKit−/Sca1−CD27+FcyR−Flk2−IL7Rα+) or common lymphoid progenitors (CLPs, cKit−/Sca1−CD27+FcyR−Flk2−IL7Rα+)

23, 24. CLPs give rise to B, T, natural killer (NK), and innate like cells (ILCs). CMPs give rise to basophils, eosinophils, mast cells, dendritic cells as well as granulocyte monocyte progenitors (GMPs, cKit+Sca1−Flk2−FcyR+) or common lymphoid progenitors (CLPs, cKit−/Sca1−CD27+FcyR−Flk2−IL7Rα+). GMPs can then give rise to neutrophils and monocytes. We identified no statistically significant differences in the frequencies of these progenitor populations between ZBTB38 WT and KO mice (Figure 3.2B). Although ZBTB38 was not required for the maintenance and differentiation of hematopoietic progenitors, it does not preclude the possibility that ZBTB38 is required for the development of mature lineages.

To assess if mature hematopoietic lineages require ZBTB38 for their development, we analyzed the frequencies of different peripheral blood mononuclear cell (PBMCs) populations. We observed no differences between ZBTB38 WT and KO mice in the frequencies of B cells (B220+), NK cells (NK1.1+), T (CD4+ or CD8+ B220−NK1.1−CD11b+), monocytes (CD11b+Ly6ChLy6G−), or neutrophils (CD11b+Ly6C+Ly6G+) (Figure 3.2C). Thus, ZBTB38 is not required for the development or maintenance of these hematopoietic lineages. We next reasoned that perhaps ZBTB38 functional importance occurs after cellular activation.
ZBTB38 is highly expressed in germinal center B cells.

Further analysis of ImmGen’s RNA-sequencing data identified highest expression of ZBTB38 in splenic, short-lived plasma cells (SLPCs, data not shown). However, ImmGen’s microarray data identified highest expression of ZBTB38 in GC B cells (data not shown). Thus, we decided to confirm ZBTB38 expression in GC B cells and SLPCs by quantitative real-time PCR. Wild-type mice were immunized with alhydrogel-adjuvanted 4-hydroxy-3-nitrophenyl-acetyl (NP) conjugated to ovalbumin (OVA) and naïve B cells (CD19^+CD138^-), NP-specific dark (CXCR4^+) and light zone (CD86^+) GC B cells (CD19^+GL7^+IgD^-), and NP-specific SLPCs (CD138^+) were sorted 11 days after immunization. RNA was extracted from sorted cells and quantitative real-time PCR performed to quantify ZBTB38 transcript levels. GC B cells and SLPCs contained 9- or 3-fold, respectively, higher expression of ZBTB38 compared to naïve B cells (Figure 3.3). No differences in ZBTB38 expression were observed between light and dark zone GC B cells. These data suggest that ZBTB38 might be functionally involved in primary antibody responses.

ZBTB38 deficiency does not impair primary B cell responses.

To determine if ZBTB38 has a functional role in primary B cell responses, we first examined GC reactions. We immunized ZBTB38 WT and KO mice with NP-OVA and quantified the frequency of NP-specific GC B cells 2 weeks later, which corresponds with peak GC reactions. We observed no differences in the frequencies of NP-specific GC B cells between ZBTB38 WT and KO mice (Figure 3.4A), indicating that ZBTB38 is not required for the expansion of NP-specific GC B cells. We next wondered if ZBTB38 is important for plasma cell differentiation or antibody secretion. Although only SLPCs were assessed for ZBTB38
expression, given the transcriptional similarity between SLPCs and LLPCs \(^{26}\), it is possible that ZBTB38 may also potentially regulate LLPC differentiation or function. Additionally, although ZBTB38 deficiency does not alter antigen specific GC B cell expansion, it may impair plasma cell differentiation or antibody secretion.

To assess both SLPC and LLPC function, ZBTB38 WT and KO mice were immunized with alhydrogel-adjuvanted NP-OVA and NP-specific serum antibodies were quantified over time by ELISA. The levels of NP-specific antibodies, captured using NP\(_{16}\), were similar between ZBTB38 WT and KO mice at all time points measured (Figure 3.4B). To specifically quantify the level of high affinity antibodies in the serum by ELISA, low density antigen (NP\(_4\)) was used to probe for antibody binding. Low density NP (NP\(_4\)) is used to capture antibodies with slow off-rates, which is correlated with antigen high affinity. This contrasts with high density NP (NP\(_{16}\)), which can capture antibodies with slower off rates due to decreased distance between NP molecules. Unlike the total number of NP-specific antibodies over time, which plateaued two weeks after immunization, the number of high affinity antibodies increased steadily over time and plateaued four weeks after immunization (Figure 3.4C). No difference in the quantity of high affinity antibodies was observed between ZBTB38 WT and KO mice (Figure 3.4C). Furthermore, the extent of affinity maturation, quantified as the ratio of NP\(_4\) to NP\(_{16}\) endpoint titers, was similar between ZBTB38 WT and KO mice (Figure 3.4D). Possible explanations for the similar serum antibody levels in ZBTB38 WT and KO mice include similar frequencies for antigen-specific LLPCs or compensatory increased antibody secretion from fewer LLPCs.

To differentiate between these two possibilities, the frequency of NP-specific LLPCs was quantified by flow cytometry 8 weeks after alhydrogel-adjuvanted NP-OVA immunization of
ZBTB38 WT and KO mice. The frequency of NP-specific LLPCs was similar between ZBTB38 WT and KO mice, suggesting that ZBTB38 does not have a functional role in plasma cells. Our data thus far demonstrate that ZBTB38 is not required for either GC expansion or plasma cell differentiation and function. We next speculated that perhaps the high expression of ZBTB38 in GC B cells is important for initiating a transcriptional program required for subsequent memory B cell differentiation or function.

**ZBTB38 deficiency does not alter recall responses.**

To determine if ZBTB38 expression is required for MBC differentiation, the frequency of NP-specific memory B cells (CD19^+^GL7^+^IgM^+^IgD^-^CD86^+^CCR6^-^) was quantified in ZBTB38 WT and KO mice 8 weeks after immunization with alhydrogel-adjuvanted NP-OVA. No difference in the frequency of NP-specific MBCs was observed (Figure 3.5A). Thus, ZBTB38 is not required for the differentiation of memory B cells, but may still be required for MBC reactivation. To determine if ZBTB38 deficiency alters recall responses, splenocytes from ZBTB38 WT and KO mice were adoptively transferred into allotype-distinct naïve IgH^a^ hosts and mice challenged with soluble NP-OVA 24 hours later. Donor IgH^b^ NP-specific antibodies originating from ZBTB38 WT and KO mice were tracked over time. NP-specific antibody titers were not altered with ZBTB38 deficiency (Figure 3.5B). Thus, ZBTB38 is also dispensable for secondary B cell responses.
3.4 Discussion

BTB-ZF family members such as BCL-6, ZBTB32, and ZBTB20, have demonstrated critical roles in regulating different aspects of B cell responses. Our data demonstrate that ZBTB38, another member of the BTB-POZ family, is dispensable for maintaining homeostasis of lymphoid and myeloid hematopoietic lineages. In addition, ZBTB38 is also dispensable for both primary and recall B cell responses to a hapten antigen. While hapten immunogens are useful in studying B cell responses, they are not representative of B cell responses to protein antigens. Thus, future studies should assess the role of ZBTB38 in regulating B cell responses to protein antigens or pathogens. Not only is ZBTB38 highly expressed in different hematopoietic lineages, it is also highly expressed in neurons. This suggests that ZBTB38 may have a role in neuronal function.

Various models of ZBTB38 deletion or knockdown have resulted in impaired cellular processes in neuronal injuries or tumors. For instance, increasing ZBTB38 expression reduces apoptosis and promotes autophagy in a spinal cord injury model, and results in increased neuronal repair \(^{21, 27, 28}\). In contrast, ZBTB38 expression has been shown to promote proliferation and differentiation of a neuroblastoma cell line \(^{29}\). These differences in the functional roles of ZBTB38 may be attributed varying sensitivity of different neurons to oxidative stress \(^{30}\). ZBTB38, along with USP9X, a deubiquitinase, is required to limit basal reactive oxidative species (ROS) levels and the response to oxidative stress \(^{31}\). Given the high levels of ZBTB38 in neurons, perhaps ZBTB38 is involved with balancing neuronal death with recovery after various challenges. To further understand the functional importance of ZBTB38 in various neurons, Zbtb38 f/f mice may be crossed to different neuron specific Cre mice and
different neuronal function assessed. Neuronal insult, such as injury, stroke, or cancer may be necessary to identify processes regulated by ZBTB38.

Genome-wide association studies in humans have identified single nucleotide polymorphisms (SNPs) in ZBTB38 that are associated with shorter stature in Chinese populations but taller stature in Korean populations. However, when Zbtb38 f/f mice were crossed to CMV-Cre expressing mice, germline deletion of ZBTB38 did not result in observable differences in the length or weight of the mice (data not shown). Further characterization of how SNPs influence ZBTB38 function, and identifying the location of SNPs, may provide further explanation of why certain polymorphisms are associated with increased height while other SNPs are associated with decreased height.

Together, our data indicate that ZBTB38 is likely not important for the development or steady-state maintenance of hematopoietic lineages. In addition, primary and recall B cell responses to a hapten antigen do not require ZBTB38. Of course, it remains possible that defects exist in rare subsets of lineages that we did not analyze. Future studies focused on other cell types can utilize the novel Zbtb38 f/f mice that we have generated.
3.5 Methods

Mice.

All animal procedures used on mice were approved by the Animal Care and Use Committees at Washington University and at the University of Arizona. All mice were housed and bred in pathogen-free facilities. C57BL6/N mice were obtained from the National Cancer Institute. B6.Cg-Igh\(^a\)Thyl\(^a\)Gpil\(^a\) (IgH\(^a\)) mice were obtained from Charles River Laboratories. ZBTB38\(^f\) embryonic stem cells were generated at the Genome Engineering and iPSC center at Washington University School of Medicine and injected into B6 albino (Jackson Laboratory, stock no. 000058) mice by the Transgenic Knockout Microinjection Core facility at Washington University in St. Louis. ZBTB38 f/f mice were crossed to CMV-Cre (Jackson Laboratory, stock no. 006054) or VavCre (Jackson Laboratory, stock no. 008610) and maintained as ZBTB38 f/f or ZBTB38 x CMV- or Vav-Cre where littermates were used as controls. The following primers were used for genotyping the Zbtb38 allele: 5’LoxP forward 5’-TCTGAGTTCAAGGCCAGCTT-3’, 5’LoxP reverse 5’-TCTCCAAGCAGAAAGGGGTGT-3’, and 3’LoxP reverse 5’-GGGTCGTTAGAGGGATTCAGC-3’.

Immunizations.

Mice were immunized intraperitoneally with 100 µg NP-OVA (Biosearch), adjuvanted with Alhydrogel (Invivogen). NP-APC used for staining was made by conjugating allophycocyanin (Sigma-Aldrich) with 4-hydroxy-3-nitrophenylacetyl-O-succinimide ester (Biosearch Technologies).
RNA extraction, cDNA synthesis, and qRT-PCR.

Total RNA was extracted with TRIzol (Life technologies) and cDNA synthesized using Superscript III Reverse transcription kit with random hexamers (Life Technologies) according to manufacturer’s instructions. qRT-PCR was performed using SYBR Green PCR master mix (Applied Biosystems) on a Prism 7000 Sequence Detection System (Applied Biosystems). The primers used for Zbtb38 are: forward 5’- AGAACCAAGGATTTCGGACTG-3’ and reverse 5’-GATGGAGAGTACTGTGTCGACTG-3’. Zbtb38 transcript levels were normalized to 18S ribosomal RNA, forward 5’-CGGCTACCACATCCAAGGA-3’ and reverse 5’-GCTGGAATTACCGCGGCT-3’ 36.

ELISA.

ELISA plates (9018, Corning) were coated overnight at 4°C in 0.1 M sodium bicarbonate buffer, pH 9.5 containing 5 ug/mL of NP16- or NP4-BSA (Bioresearch Technologies). All other incubation steps were performed at room temperature for 1 hour. Wash steps were performed between each step using PBS + 0.05% Tween-20. Plates were blocked with PBS + 2% BSA followed by serial dilutions of serum. Serum was probed with 0.1 ug/mL of biotinylated anti-mouse IgG (715-065-151, Jackson ImmunoResearch Laboratories) and then detected with streptavidin conjugated horseradish peroxidase (554066, BD biosciences). Plates were developed using TMB (Dako, S1599) and neutralized with 2N H2SO4. Optical density (OD) values were measured at 450 nm. Serum endpoint titer was defined as the inverse dilution factor that is three standard deviations above background using one-phase decay measurements and Prism software (GraphPad Software).
Adoptive transfer for recall responses.

Splenocytes from NP-immunized $ZBTB38^{ff}$ or $ZBTB38^{ff} \times VavCre$ mice were isolated and processed into single cell suspension. Erythrocytes lysed using an ammonium chloride-potassium solution, and lymphocytes isolated by using a Hisopaque-1119 (Sigma-Aldrich) density gradient. Cells were washed twice prior to transfer. 10% of cells were retained for cellular analysis whereas the remaining 90% of cells were transferred into one non-irradiated $IgH^{\mu}$ recipient mice by intravenous injection. A recall response was elicited by intravenously challenging mice with soluble NP-OVA 24 hours later.

Flow cytometry.

Single cell suspensions were prepared from bone marrow or spleen, erythrocytes lysed using an ammonium chloride-potassium solution, and lymphocytes isolated by using a Hisopaque-1119 (Sigma-Aldrich) density gradient. Cells were resuspended in PBS with 5% adult bovine serum and 2 mM EDTA prior to staining with antibodies and NP-APC. The following antibodies were purchased from Biolegend: 6D5 (CD19)-Alexa Fluor 700; GL7-FITC; 281-2 (CD138)-PE; RMM-1 (IgM)-APC; 11-26c.2a (IgD)-PerCP-Cy5.5 or -Brilliant Violet 605; 16-10A1 (CD80)-PE; RA3-6B2 (B220)-FITC, -Pacific Blue, or APC-Cy7; PO3 (CD86)-FITC; PK136 (NK-1.1)-PerCP-Cy5.5; M1/70 (CD11b)-Pacific Blue; HK1.4 (Ly-6C)-Brilliant Violet 510; 1A8 (Ly-6G)-Brilliant Violet 605; A7R34 (IL-7R)-Brilliant Violet 421; and E13-16.7 (Ly-6A/E)-PE. The following antibodies were purchased from eBioscience: 11/41 (IgM)-PerCP-e710; 11-26c (IgD)-FITC; 2B11 (CXCR4)-PerCP-e710; 2B8 (c-Kit)-PE-Cy7; and LG.7F9 (CD27)-APC. The following antibodies were purchased from BD Pharmingen: 53-6.7 (CD8a)-PE; RM4-5 (CD4)-PE-Cy7; A2F10.1 (CD135)-PE-CF594; and 93 (CD16/CD32)-PerCP-Cy5.5.
Cells were stained on ice for 20 minutes. Germinal center B cells were enriched by staining cells with GL7-PE followed with anti-PE magnetic beads (0.5 uL/10^7 cells, Miltenyi Biotec). Positive enrichment of GL7-expressing cells was performed using MACS LS columns (Miltenyi Biotec).
3.7 Acknowledgements

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3.7 References


Figure 3.1 *Zbtb38* targeting strategy and confirmation of deletion.

(A) Targeting construct of *Zbtb38* exon 3. NdeI and EcoRI restriction sites were introduced to allow for screening of plasmid integration. (B) PCR showing that exon 3 is correctly targeted. Lane 1 is the undigested PCR product, lane 2 is a NdeI digest (expected band sizes of 5.8 kb and 445 bp), lane 3 is a EcoRI digest (expected band sizes of 2.6 kb, 2 kb, and 1.6 kb), and lane 4 is a NdeI and EcoRI double digest (expected band sizes of 445 bp, 2.1 kb, 2 kb, and 1.6 kb). (C) PCR showing that *Zbtb38* can be deleted upon Cre activity. Primers used are the same ones used to amplify the genomic DNA in (B). (D) Genotyping strategy to identify if a mouse has the WT, floxed, or deleted *Zbtb38* allele.
Figure 3.2 ZBTB38 deficiency does not alter the development of hematopoietic cells. (A) ZBTB38 expression values in select cell subsets were extracted from ImmGen’s RNA-seq SKYLINE and grouped based on cell type. Cell types shown have a ZBTB38 expression value, normalized by DESeq2, of 800 or greater. (B) The frequencies of hematopoietic stem cells (HSCs, cKit+Sca1+Flk2−CD27+), multi-potent progenitors (MPPs, cKit+Sca1+Flk2+CD27+), common myeloid progenitors (CMPs, cKit+Sca1−Flk2+FcγR+), granulocyte monoocyte progenitors (GMPs, cKit+Sca1−Flk2+FcγR−), and common lymphoid progenitors (CLPs, cKit−/loSca1−CD27+FcγR−Flk2+IL7Rα+) in the bone marrow from ZBTB38 WT and KO mice were quantified by flow cytometry. Mean ± SEM are shown; each symbol represents an individual mouse. Statistical significance was determined by unpaired student’s 2-tailed t-test; n.s. indicates no significance (p > 0.05). Data shown are from one experiment. (C) The frequencies of B cells (B220+), natural killer (NK, NK1.1+) cells, CD4+ and CD8+ T cells (B220−CD11b−NK.1+), monocytes (CD11b−Ly6C+Ly6G−), and neutrophils (CD11b−Ly6C−Ly6G+) of peripheral blood mononuclear cells (PBMCs) from ZBTB38 WT and KO mice were quantified by flow cytometry. Mean ± SEM are shown; each symbol represents an individual mouse. Statistical significance was determined by unpaired student’s 2-tailed t-test; n.s. indicates no significance (p > 0.05). Data shown are from one experiment.
Figure 3.3 ZBTB38 expression level in select B cell subsets.
Naïve B cells, antigen-specific dark (DZ, CXCR4+) and light (LZ, CD86+) zone germinal center B cells (GCBC, CD19+GL7-IgD−), and splenic plasma cells (SpPC, CD138+) were sorted and Zbtb38 RNA levels quantified by quantitative real time PCR. Zbtb38 expression level was first normalized to 18S expression level followed by normalization to naïve B cells. Mean ± SEM are shown; each symbol represents an individual mouse. Data shown are from one experiment.
Figure 3.4 ZBTB38 deficiency does not impair primary B cell responses.

(A) ZBTB38 WT and KO mice were immunized with NP-OVA and the frequency of NP-specific germinal center B cells two weeks post immunization was quantified by flow cytometry. Mean ± SEM are shown; each symbol represents an individual mouse. Statistical significance was calculated by unpaired student’s two-tailed t-test; n.s. = not significant (p > 0.05).

(B, C) ZBTB38 WT and KO mice were immunized with NP-OVA and total serum antibody titers (B) and high affinity serum antibody titers (C) to NP were quantified by ELISA. Endpoint titers are calculated as the reciprocal serum dilution that was three standard deviations above background. Mean ± SEM are shown; each symbol represents an individual mouse. Statistical significance was calculated by Mann-Whitney test; n.s. = not significant (p > 0.05).

(D) Affinity maturation of the antibodies was calculated as the ratio of endpoint titers to NP<sub>4</sub> : NP<sub>16</sub> and plotted at each time point for ZBTB38 WT and ZBTB38 KO mice. Mean ± SEM are shown; each symbol represents an individual mouse.

(E) The frequency of NP-specific long-lived plasma cells was calculated by flow cytometry. The long-lived plasma cells were analyzed 8 weeks post-NP immunization. Mean ± SEM are shown; each symbol represents an individual mouse. Statistical significance was calculated by unpaired student’s two-tailed t-test; n.s. = not significant (p > 0.05). Data shown are from one experiment.
Figure 3.5 Recall responses to NP do not require ZBTB38.

(A) The frequency of NP-specific memory B cells (CD19^+GL7^+IgM^+IgD^-CD80^+CCR6^+) was quantified by flow cytometer in ZBTB38 WT and KO mice 8 weeks after NP-OVA immunization. Mean ± SEM are shown; each symbol represents an individual mouse. Statistical significance was calculated by unpaired student’s two-tailed t-test; n.s. = not significant (p > 0.05). Data shown is from one experiment. (B) Splenocytes from ZBTB38 WT and ZBTB38 KO mice were adoptively transferred into IgH^a naïve hosts and challenged with soluble NP-OVA one day after transfer. Donor (IgH^b) antibody responses were calculated as the endpoint titer against high density NP. Mean ± SEM are shown; each symbol represents an individual mouse. Statistical significance was calculated by Mann-Whitney test; n.s. = not significant (p > 0.05). Data shown is from one experiment.
Chapter 4:

Discussion
In this study, we characterized the generation and utilization of diverse memory B cells in their native environment following heterologous flavivirus challenges, and thus provide an etiological explanation for the existence of memory B cells. A primary flavivirus challenge generates flavivirus-specific long-lived plasma cells (LLPCs) but broadly reactive memory B cells (MBCs). These broadly reactive MBCs respond to a secondary heterologous challenge without undergoing further affinity maturation. Therefore, we propose that MBCs have been evolutionarily selected for in order to provide anticipatory protection against antigens that have evaded neutralization by serum antibodies and heterologous pathogen. This in turns provides more rapid protection against the secondary pathogen compared to a naïve B cell response. While our study contributes to a better understanding of how MBCs are generated and utilized, future work should clarify whether GC-biased MBCs can even be engaged during recall responses and to mechanistically define cues that promote the differentiation of MBCs from GC B cells.

Analysis of MBC subsets in WNV vaccinated mice identified DIII-specificity exclusively in the CD80+, plasma cell-biased MBC subset (Figure 2.5A). Thus, this observation cannot definitively exclude the possibility that the lack of GCs after a secondary heterologous challenge is due to the absence of GC-biased MBCs. Preliminary analysis of MBC subsets in WNV infected mice identified the existence of both plasma cell-biased and GC-biased MBCs. Future work using WNV infection followed by JEV infection is necessary to more conclusively show that MBCs generated after a primary flavivirus infection respond to heterologous infection by directly differentiating into plasma cells to provide protection. Although we utilized DENV and ZIKV sequential infections to further support that MBCs do not require further affinity maturation to respond to heterologous antigens (Figure 2.3G), we did not analyze the
distribution of DENV-specificity in different MBC subsets prior to ZIKV infection. Thus, it is unclear if the DENV infection generated GC-biased MBCs that could be potentially engaged upon secondary challenge. Should a primary infection result in the engagement of GC-biased MBCs upon a heterologous secondary infection, further studies clarifying signals that promote the differentiation of GC-biased versus plasma cell-biased MBCs, as well as their antigen specificities, are necessary. Such signals may originate from the cytokine milieu and/or strength of BCR signaling.

Irrespective of the ability of GC-biased MBCs to be engaged during secondary recall responses, it remains to be seen how MBCs respond to a tertiary flavivirus challenge. Secondary flavivirus challenges result in the activation of cross-reactive MBCs, of which a minority binds the neutralizing LR epitope. Thus, how do neutralizing and non-neutralizing antibodies derived from cross-reactive MBCs impact tertiary heterologous challenges? Will ADE of the tertiary challenge occur? Will original antigenic sin occur by further activating cross-reactive MBCs that responded to the secondary heterologous challenge, or will a tertiary heterologous challenge activate a different subset of cross-reactive MBCs generated after the primary challenge? To address these questions, the MBC and antibody specificities after a tertiary challenge need to be assessed. This is important for better understanding how the breadth of MBCs is utilized upon sequential challenges.

In addition to understanding how MBCs respond to tertiary heterologous challenges, it is important to understand how disparate antigens have to be in order to bypass MBC reactivation and initiate naïve B cells responses. Our data indicate that sequential challenges between flaviviruses that are approximately 55% and 75% similar at the DIII amino acid sequence level do not require additional affinity maturation to generate cross-reactive antibodies (Figure 2.2).
Thus, it remains to be seen if flaviviruses that are more disparate can still initiate cross-reactive responses. To address this, future experiments secondary challenges utilizing flaviviruses that are more or less disparate than the primary challenge are necessary. For instance, what is the response between a St. Louis Encephalitis Virus (SLEV) primary challenge and a Dengue virus (DENV) secondary challenge, where SLEV and DENV share approximately 40% DIII sequence identity?

Thus far, we have focused on the utilization of MBCs upon recall challenge. However, it is still unclear what cues promote the MBC fate. Two mechanisms for MBC differentiation have been proposed. One is the temporal switch in which GCs go from supporting MBC fate during early reactions to supporting LLPC fate during late reactions\(^1\). The other is the selection of low affinity GC B cells into the MBC compartment due to weaker T cell help, and thus elevated levels of Bach2\(^2\). While these two mechanisms are not completely mutually exclusive, especially during early GC reactions, it is unclear how GC environments change over time, and how that in turn impacts GC B cell selection, affinity maturation, and differentiation. Here, we demonstrate that a continuous, but active, selection of relatively low affinity germinal centers into the MBC compartment likely promotes the diversity of MBCs (Figure 2.14F). The active selection conclusion is based on the significant enrichment of MBCs for DIII-nonLR specificity compared to their GC B cell precursors (Figure 2.6C). In addition, DIII-nonLR specific GC B cells were enriched for MBC precursor surface markers (Figure 2.6D). Thus, DIII-nonLR specificity can perhaps be used as a surrogate marker for an ultimate MBC fate, and thus aid in identifying signals that promote the MBC fate.

Additionally, it is necessary to have a better understanding of how DIII-nonLR and DIII-LR specific cells differ, and why DIII-nonLR specificity is predominately found in MBCs and
not LLPCs. One hypothesis is that DIII-nonLR specific cells perhaps have different transcriptional or chromatin-accessibility signatures relative to DIII-LR specific cells. In this case, single cell RNA- and ATAC-sequencing over time may provide clues to why DIII-nonLR specific cells are predisposed towards the MBC fate. We would expect that DIII-nonLR specific cells have a greater MBC signature than DIII-LR specific cells. Alternatively, perhaps DIII-nonLR specific GC B cells are constantly of lower affinity compared to DIII-LR specific GC B cells. Bulk VDJ sequencing of the heavy chain from WNV DIII-LR and -nonLR specific GC B cells over time identified a reduced mutational load in DIII-nonLR specific cells (data not shown). However, analysis of DIII-LR and DIII-nonLR MBC-derived mAbs did not reveal binding differences to WNV WT DIII. Thus, it is inconclusive if DIII-nonLR specific GC B cells are of consistently lower affinity or not. To test this, monoclonal antibodies from GC B cells over time need to be produced. Or over time, DIII-specific GC B cells from WNV vaccinated Nur77 reporter mice can be analyzed for GFP expression, which corresponds to strength of BCR signaling. Yet another hypothesis for why DIII-nonLR specific cells are enriched in the MBC compartment is that DIII-LR and -nonLR specific cells are differentially sensitive to BCR stimulation. Analysis of Hardy Fractions E and F revealed a slight reduction in surface IgM levels in Fraction E DIII-LR cells compared to DIII-nonLR cells, although this reduction was not maintained in the recirculating mature Fraction F population. Given that lower surface IgM levels correlate with anergy, and that anergic B cells can still participate in GC reactions ³, perhaps DIII-LR specific cells require a higher activation threshold for differentiation. To test this, calcium flux assays can be performed using different concentrations of DIII. What we would expect is that DIII-LR specific cells would require a higher concentration of DIII to generate a calcium flux compared to DIII-nonLR specific cells.
Although our study focuses on flaviviruses infections, it can be applied to different infections. In the context of flaviviruses, we propose that the diversity of MBCs may negatively impact the host response towards heterologous challenges due to the increased likelihood of generating poorly neutralizing but broadly reactive antibodies, which in turn may promote ADE. The inability of MBCs to remodel their specificity towards new antigens by re-initiating GC reactions indicates that the primary response is critical for defining secondary responses. Thus, vaccine efforts should focus on two aspects: 1) limit the diversity of MBCs and 2) focus responses towards neutralizing epitopes that are type specific. However, for rapidly mutating pathogens such as influenza, a diverse MBC compartment may be beneficial.

The constantly evolving nature of influenza, especially at the hemagglutinin (HA) head, means that neutralizing anti-HA head antibody responses to one influenza strain is unlikely to neutralize another strain. Thus, current vaccine efforts have focused on mounting antibody responses against the conserved HA stem region. Careful analysis of newly generated and pre-existing MBCs after secondary influenza exposure revealed the inability of stem-reactive MBCs to affinity mature. These findings corroborate our findings where MBCs do not undergo further affinity maturation after re-activation. In the context of influenza infections, we hypothesize that MBC diversity may be beneficial to the host since it increases the likelihood of recognizing variant antigens, especially at the conserved hemagglutinin (HA) stem region. Thus, we propose that influenza vaccine strategies should focus on generating a broadly reactive primary MBC pool that has the potential to recognize multiple variants, either at the HA head or stem. Immunogen design, such as hyperglycosylation of the HA head, may aid in focusing the MBC response towards stem epitopes by increasing the accessibility of the HA stem during a primary exposure.
Our findings can also be applied to another problematic pathogen, HIV. HIV also constantly evolves over time where broadly neutralizing antibodies are associated with high levels of somatic hypermutation. Current vaccine strategies focus on guiding the B cell response through sequential immunizations with variant antigens or by targeting public clonotypes. These strategies rely on the re-initiation of GCs by MBCs to further somatically hypermutate their BCRs. However, our data indicate that MBCs are unable to further affinity mature. Therefore, sequential activation of MBCs in hopes of re-initiating GCs and further somatically hypermutate BCRs to generate broadly neutralizing antibodies (bnAbs) is likely an ineffective strategy. Instead, perhaps modifying the timing of antigenic challenges to ensure persistent GCs is a more effective strategy at generating bnAbs.

The data from this study further expand the etiological role of MBCs to not only recognizing pathogen escape variants but also heterologous antigens. Our data suggest that the diversity of MBCs results from the continued active selection of lower affinity GC B cells into the MBC compartment. Furthermore, the diversity of MBCs generated after a primary challenge is broad enough that further molding of the BCR towards new specificities via GC reactions is unnecessary after a secondary heterologous exposure. Remaining questions include identifying the cues that are necessary to promote the preferential differentiation of broadly reactive GC B cells into the MBC compartment as well as determining if GC-biased MBCs can be activated upon recall in the presence of antigen-specific antibodies. A better understanding of these processes has different implications for vaccine development for multiple pathogens, such as flaviviruses, influenza, and HIV.
4.1 References


