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Development and function of *Plasmodium*-specific memory B cells during blood stage malaria infection

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Abstract

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Humoral immunity to infection depends upon two layers of protection: pre-existing antibodies expressed by long-lived plasma cells and a stable population of rapidly reactive memory B cells (MBCs). If pre-existing antibody concentrations are not sufficient to control secondary infections, MBCs are poised to respond by rapidly producing antibody-secreting cells to help neutralize the invading pathogen. Classically defined MBCs are derived in a germinal center (GC) dependent fashion and express high-affinity class-switched, somatically hypermutated B cell receptors (BCR), making these cells the gold standard target of most vaccine platforms. Recent studies have expanded upon conventional MBC paradigms and it is now recognized that phenotypically and functionally heterogeneous MBCs have been described in mice and humans. Given the complexity of these processes, the development of a system to better visualize how endogenous populations of distinct MBC subsets form in response to complex infections will better elucidate their identity and function to inform novel vaccine strategies.

In the studies presented here, we chose to focus on the development of the humoral immune response to *Plasmodium*, the causative agent of malaria. The major roadblock to an effective malaria vaccine is our limited understanding of the cellular mechanisms that lead to

long-lasting immunity against the *Plasmodium* parasite. B cells are known to be critical mediators of immunity against blood stage malaria infection but little is known about the development, function or maintenance of malaria-specific MBCs. To gain insight into the types of MBCs that form and function during primary and secondary *Plasmodium* infection, we developed a novel B cell tetramer against the *Plasmodium* protein Merozoite Surface Protein 1 (MSP1). Using this approach in mice, phenotypically and functionally heterogeneous subsets of MSP1-specific MBCs form and persist for at least a year after control of parasitemia. Long-lived murine MSP1-specific MBCs consisted of three populations: GC-dependent, somatically hypermutated IgM⁺ (defined as CD73⁺CD80⁺IgM^{high}IgD^{low}) and IgG⁺ (CD73⁺CD80⁺IgG⁺) MBC subsets and an unmutated, GC-independent IgD⁺ (CD73⁻CD80⁻IgM^{low}IgD^{high}) MBC population. Remarkably, somatically hypermutated MSP1-specific IgM⁺ MBCs outcompete IgG⁺ MBCs early during a secondary challenge resulting in the expansion of newly formed plasmablasts and increased levels of serum MSP1-specific IgM antibodies. Analyses of *Plasmodium*-specific B cells in malaria-infected individuals from endemic areas also revealed the presence of mutated IgM⁺ and IgG⁺ MBCs, substantiating the relevance of these cells in humans. Our studies provide the first glimpse of how memory B cells contribute to anti-*Plasmodium* immunity and highlight a previously unrecognized role for IgM⁺ MBCs during infection. We believe the findings from these studies will significantly enhance our understanding of how to develop a malaria vaccine that can provide long-lasting protection as well as provide novel insights into memory B cell biology in the context of infection.

TABLE OF CONTENTS

| | |
|--|------|
| List of Figures | vii |
| List of Tables | viii |
| 1 Introduction..... | 11 |
| 1.1 Generation of the innate and adaptive immune response | 11 |
| 1.2 B cell activation and memory B cell formation | 12 |
| 1.3 Memory B cell heterogeneity and function | 14 |
| 1.4 Malaria: a global health burden..... | 17 |
| 1.5 Humoral immunity to malaria | 20 |
| 1.6 Questions to address | 22 |
| 2 Plasmodium-specific memory B cell formation and function during blood stage malaria infection | 24 |
| 2.1 Introduction | 24 |
| 2.2 Results | 26 |
| 2.2.1 MSP1-specific B cells expand, differentiate, and form memory in response to blood stage malaria infection..... | 26 |
| 2.2.2 MSP1-specific B cell fates emerge early after infection and MBCs persist | 30 |
| 2.2.3 Switched and unswitched Plasmodium-specific MBCs can be found in malaria-exposed mice and humans..... | 34 |
| 2.2.4 Murine MSP1-specific MBC subsets are phenotypically and genetically distinct .. | 34 |
| 2.2.5 Secondary infection induces the rapid proliferation and differentiation of MSP1-specific MBCs..... | 37 |
| 2.2.6 The early secondary antibody response is IgM-dominant | 42 |
| 2.2.7 Secondary IgM response is not affected by challenge dose or timing..... | 44 |
| 2.2.8 IgM ⁺ MBCs generate both T-independent and T-dependent antibody secreting effectors | 46 |
| 2.3 Discussion | 46 |
| 2.4 Methods | 50 |
| 3 Mechanisms of development of Plasmodium-specific memory B cells | 54 |
| 3.1 Introduction | 54 |

| | | |
|-------|--|----|
| 3.2 | Results and Discussion | 56 |
| 3.2.1 | Maintenance of MSP1-specific B cells is CD4 ⁺ T cell dependent | 56 |
| 3.2.2 | Germinal center dependence of MSP1-specific B cells | 59 |
| 3.2.3 | IgM ⁺ and swIg ⁺ MBCs are GC-dependent. | 61 |
| 3.2.4 | Myd88 deficiency results in defective GC-dependent MBC output | 63 |
| 3.2.5 | GC-derived MBCs mediate secondary response to rechallenge | 63 |
| 3.3 | Discussion | 68 |
| 3.4 | Methods | 72 |
| 4 | Characterization of <i>Plasmodium</i> -specific memory B cells in malaria infected humans | 74 |
| 4.1 | Introduction | 74 |
| 4.2 | Results and Discussion | 74 |
| 4.2.1 | <i>Plasmodium</i> -specific MBCs are expanded in malaria exposed humans | 74 |
| 4.2.2 | <i>Plasmodium</i> -specific IgM ⁺ and IgG ⁺ MBCs display somatic hypermutation | 76 |
| 4.3 | Methods | 78 |
| 5 | Concluding Remarks | 80 |
| 6 | Glossary | 83 |
| 7 | References | 84 |

LIST OF FIGURES

| | |
|---|----|
| Figure 2.1. Detection and kinetics of MSP1 ⁺ B cells. | 27 |
| Figure 2.2. MSP1-specific B cells bind tetramer and expand in an antigen specific manner. | 28 |
| Figure 2.3. Measurement of parasitemia by flow cytometry. | 29 |
| Figure 2.4. MSP1 ⁺ B cell fates emerge early after infection and MBCs persist. | 32 |
| Figure 2.5. MSP1 ⁺ B cell fates emerge early after infection and MBCs persist. | 33 |
| Figure 2.6. MSP1 ⁺ MBCs are heterogeneous. | 35 |
| Figure 2.7. Rapid expansion of MSP1 ⁺ B cells after rechallenge. | 38 |
| Figure 2.8. Naïve MSP1 ⁺ B cells do not differentiate or secrete antibody after primary infection with 1x10 ⁷ iRBCs. | 39 |
| Figure 2.9. Rapid expansion of MSP1 ⁺ B cells after rechallenge. | 41 |
| Figure 2.10. Early secondary antibody response is IgM-dominant. | 43 |
| Figure 2.11. iRBC challenge dose or timing does not impact secondary IgM response. | 45 |
| Figure 2.12. Requirements for secondary IgM ⁺ MBC responses. | 47 |
| Figure 3.1. CD4 ⁺ T cell dependence of MSP1-specific B cells. | 58 |
| Figure 3.2. GC-dependent MSP1 ⁺ B cell formation. | 60 |
| Figure 3.3. GC-dependent MSP1 ⁺ memory B cells. | 62 |
| Figure 3.4. Myd88 deficiency affects late MSP1 ⁺ B cell differentiation. | 64 |
| Figure 3.5. GC-dependent MSP1 ⁺ MBC are also affected by Myd88 deficiency. | 65 |
| Figure 3.6. GC-dependent MBCs mediate rapid secondary response to malaria rechallenge. | 67 |
| Figure 3.7. MSP1 ⁺ IgM ⁺ MBCs cells phenotypically resemble marginal zone B cells. | 71 |
| Figure 4.1. Human <i>Plasmodium</i> -specific MBCs are phenotypically heterogeneous. | 75 |
| Figure 4.2. IgG ⁺ and IgM ⁺ <i>Pf</i> -specific MBCs are mutated. | 77 |

LIST OF TABLES

| | |
|---|-----------|
| Table 2.1. Mouse and human antibodies..... | 53 |
|---|-----------|

DEDICATION

To my Tata, for instilling in me a passion for learning and for teaching me how to be a good person.

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1 Introduction

1.1 Generation of the innate and adaptive immune response

The vertebrate immune system has evolved to continuously survey the plethora of microbes in the environment that are in constant interaction with the host organism. While many of these microbes are innocuous and can exist in symbiosis with the host, more harmful microbes termed “pathogens”, including viruses, bacteria, and parasites, are capable of causing significant damage and must be effectively eliminated by the host. The immune system must therefore quickly recognize invading pathogens and mount the appropriate response in order to eliminate it before causing excessive injury to the host. The immune system uses two arms in tandem to accomplish this: the innate and adaptive immune systems [1]. The innate immune system, comprised of sentinel cells poised at barrier interfaces of the host and the external environment, makes up the early response to pathogen invasion through recognition of conserved motifs present on the invading microbe. And though highly effective, cells of the innate immune system rely on a limited number of invariant receptors and effector functions to fight against invading pathogens. Therefore, should the invading pathogen get beyond innate immune defenses, activation of the adaptive immune system can help sustain clearance of the pathogen and provide persistent protective immunity. The adaptive immune system is comprised of lymphocytes that, through the process of somatic cell gene rearrangement, express a vast and diverse repertoire of cell surface antigen receptors. As a result of this receptor specificity, lymphocytes are capable of targeting the pathogen with much higher affinity and specificity than innate cells. The adaptive immune system also has the added benefit of forming long-lived memory populations even after pathogen clearance, allowing the host to respond much more rapidly to a secondary infection. Thus, efficient activation of the adaptive immune system is crucial to confer optimal immunity to infection that persists for the life of the host and is the gold standard for successful vaccines.

1.2 B cell activation and memory B cell formation

B cell development

More than 50 years ago the adaptive immune system was delineated into distinct functional lineages: “cellular” immunity mediated by T lymphocytes and “humoral” immunity mediated by B lymphocytes [2]. T cell development occurs in the thymus where thymocytes undergo positive and negative selection events with the goal of exporting T cells bearing a T cell receptor (TCR) that is capable of responding to foreign pathogens while remaining tolerant to self antigens. T cells are exported as either CD8⁺ cytotoxic T cells or CD4⁺ helper T cells that recognize their cognate antigen when presented in the context of peptide and major histocompatibility complex (MHC) I or MHCII, respectively, via antigen presenting cells (APCs). B cell development occurs in the bone marrow (BM) where developing cells undergo recombination of genes encoding for the heavy chain and light chain within the immunoglobulin (Ig) locus, resulting in membrane-bound surface expression of a unique B cell receptor (BCR). Similar to T cells, B cells undergo both positive and negative selection events to hopefully form B cells that will respond to foreign antigens while limiting self-antigen responsiveness that seed secondary lymphoid organs, which include the spleen and lymph nodes [3]. Upon activation, B cells can differentiate into several fates including plasma cells that actively secrete antigen-specific immunoglobulins, termed “antibodies” [4]. Secreted antibodies, depending on their isotype, can mediate different functions including clearance through direct neutralization of the pathogen, activation of the complement cascade, or through opsonization and Fc-mediated phagocytosis via interactions with other immune cells [5].

B cell activation and GC formation

Coordinating B cell responses is a highly dynamic process governed by various factors. In the naïve state, within secondary lymphoid organs, IgM⁺IgD⁺ B cells are localized in the B cell

follicle in close proximity to T cells found in the periarteriolar lymphoid sheath (PALS), commonly referred to as the T cell zone. During infection, B cells are activated by the invading pathogen through direct recognition of pathogen-derived antigens via their BCR. Upon BCR engagement, activated B cells migrate to the T cell-B cell (TB) border, begin to proliferate, and in the case of T-dependent (TD) antigens, engage in cognate interactions with CD4⁺ T cells [6]. Upon interactions with T cells, B cells undergo transcriptional changes to differentiate into short lived antibody-secreting cells (ASCs) known as plasmablasts that express predominantly low-affinity IgM⁺ antibodies. This is termed the extrafollicular antibody response as it occurs predominantly in the red pulp of the spleen and extramedullary cords of the lymph node [7]. Though low affinity, this initial wave of ASC formation and antibody secretion are important in the early control of infection [8]. B cells that continue to interact with T cells at the TB border undergo further transcriptional and phenotypic changes that drive them, along with the T cells, further into the B cell follicle to initiate the second wave of TD B cell responses, the germinal center (GC) reaction [9-11].

GCs are specialized microenvironments within lymph nodes and spleen in which B cells undergo antigen-dependent affinity driven selection to produce B cells bearing high-affinity BCRs. Within the follicle, B cells and T cells colocalize with follicular dendritic cells (FDCs) and create histologically distinct GC zones, termed the dark zone (DZ) and the light zone (LZ) [12]. Within the DZ, B cells rapidly proliferate and upregulate the mutator enzyme activation-induced cytidine deaminase (AID) which induces somatic hypermutation (SHM) of the immunoglobulin locus [13]. SHM creates random mutations within the variable region genes of the BCR with the goal of increasing its affinity to cognate antigen. Due to the random nature of the mutation process, SHM can lead to lower affinity or potentially even self reactive clones BCRs. Therefore once mutated, DZ B cells migrate via chemotactic gradients to the LZ of the GC where the mutated B cells will then test their new BCR to antigen presented by LZ resident follicular dendritic cells (FDCs). Within the LZ, newly mutated B cells will also interact with a specialized

subset of CD4⁺ T cells, termed T follicular helper (Tfh) cells, to promote the positive selection of B cells with an improved receptor. Tfh cells play a central role in mediating GC B cell selection and survival through cognate peptide:MHCII interactions with higher-affinity B cell clones preferentially receiving cognate Tfh cell help as they can present more antigen [14]. High-affinity Tfh interactions leads to the upregulation of additional signals through receptor-ligand interactions, like CD40:CD40L and ICOS:ICOSL, and cytokine signaling, like IL-21 and IL-4, which further promote the positive selection of higher-affinity B cell clones within the GC [15]. The B cells that do not receive sufficient enough help can continue to cycle back the DZ, undergo further proliferation and SHM, and return to the LZ to test their BCR again. Additional cytokines from Tfh cells, elicited by the nature of the antigen, can also promote class-switch recombination (CSR) of the BCR heavy chain constant region from IgM and IgD to the appropriate effector isotype, IgG, IgA, or IgE, which can enhance effector B cell function [16, 17]. Through several iterative rounds of mutation and selection, aided by both intrinsic and extrinsic processes, ultimately GC B cells are exported as either long lived plasma cells (LLPCs) that secrete class-switched, high-affinity antibodies or as class-switched, high-affinity, long-lived memory B cells (MBCs) that retain membrane bound expression of the matured BCR but are programmed to rapidly differentiate into high-affinity ASCs after reexposure to antigen during secondary infections [18]. The importance of appropriately coordinating these dynamic events in response to infection is substantiated by the fact that nearly all successful vaccine campaigns are based on strategies that generate durable, long-lived memory B cell and antibody responses [19] and understanding how long lived B cell memory is generated is vital to this process.

1.3 Memory B cell heterogeneity and function

The classical view of MBC formation is thought to occur via GC-dependent processes resulting in the formation of class-switched, somatically hypermutated, high-affinity cells [20].

More recently, however, this view has been challenged and it is now appreciated that heterogeneous subsets of MBCs can form. The notion of unswitched (IgM and IgD expressing) MBCs was initially proposed from observations in humans that displayed somatically hypermutated IgM⁺ BCRs on the surface of MBCs [21-23]. In support of these observations, several studies have shown IgM⁺ MBCs also form in mice. In a study by Dogan et al., they used an AID-eYFP (yellow fluorescent protein) reporter to system to irreversibly fluorescently label any B cells that expressed AID to mark cells that have passed through a GC, although it is now known that AID can also be expressed outside of a GC [24]. In response to sheep red blood cell immunization, a TD response, eYFP⁺ MBCs were comprised of both IgM⁺ and IgG⁺ subsets [25]. A follow up study by Pape et al. tracked phycoerythrin (PE)-specific B cells in response to PE protein immunization and found both PE-specific IgM⁺ and class-switched (swIg⁺) MBCs form [26]. Notably, PE⁺IgM⁺ MBCs displayed significantly fewer mutations suggesting they underwent less affinity maturation and were perhaps derived independently of a GC. These conclusions supported an even older study by Toyama et al. demonstrating that MBCs are capable of forming via GC-independent mechanisms using mice whose B cells were deficient in the transcriptional repressor Bcl6, which is required for GC formation [27, 28]. In this setting, MBCs formed but were largely IgM⁺ and displayed little to no mutation. Using BrdU pulse-labeling methods, Weisel et al. showed that IgM⁺ MBCs peaked and formed earlier than later forming IgG⁺ MBCs in response to protein immunization, likely due to the fact that IgM⁺ MBCs can form in a GC-independent manner and were able to form before later arising GC-dependent IgG MBCs. In this same study IgM⁺ MBCs still formed even after GC formation, indicating their formation is not completely GC-independent [29]. MBC isotype has also been shown to impact MBC survival and stability. In the aforementioned study by Pape et al., class-switched MBCs, which are more mutated, appear to have a shorter half-life than less mutated unswitched MBCs [26]. In a follow up study by Gitlin et al. AID-mediated SHM was found to negatively impact long-term MBC survival and this effect was independent of MBC isotype [30]. Taken together, these

findings suggest that class-switched MBCs are more mutated and GC-derived, though less stable, while unswitched MBCs are less mutated and GC-independent and can persist for longer periods of time.

The appreciation of MBC heterogeneity has also led to a better understanding of the functional capacity of MBCs during recall responses. During protein immunization and protein rechallenge, MBC populations identified by BCR isotype showed that IgG⁺ MBCs respond more rapidly to rechallenge than unswitched MBCs by generating isotype-switched antibody-secreting plasmablasts [25, 26]. Conversely, unswitched MBCs are slower to respond and preferentially form new GC B cells, likely to replenish any IgG⁺ MBCs that had terminally differentiated into ASCs upon activation. In other models using a different protein immunization, however, IgG⁺ MBCs were found to preferentially form new GC B cells [31]. Importantly, in all of the aforementioned studies, all IgM and IgD expressing MBCs have been grouped together when analyzing the formation and function of unswitched MBCs which may influence our interpretation of their specific role during immune responses and a more careful analysis of the unique contributions of distinct single IgM or IgD expressing MBCs is needed.

In addition to BCR isotype, the expression of surface markers such as CD73, CD80 and PDL2 have also been shown to identify disparate MBCs [32-35]. Increased expression of these markers correlates with MBCs that are more class-switched and more mutated, indicative of MBCs having come out of the GC. Functionally, MBCs that display higher CD80 and PDL2 expression rapidly formed antibody-secreting plasmablasts whereas cells that did not express these markers primarily formed new germinal centers [35]. These findings were independent of BCR isotype as CD80/PDL2^{high} IgM⁺ MBCs were as capable of forming early plasmablasts as CD80/PDL2^{high} IgG⁺ MBCs.

The use of protein immunization models has proven extremely insightful to our understanding of MBC formation and function. While such studies may be representative of B cell responses during an acute infection in which antigen presence is short-lived, how these

findings pertain to B cell responses in the face of more sustained antigen presence is unclear. Pathogens such as HIV, hepatitis C virus, and malaria can establish life long, chronic infections even in the presence of ongoing B cell responses and have been known to induce B cell exhaustion and the formation of functionally impaired MBCs, termed atypical MBCs [36]. Therefore a better understanding of infection-specific MBC formation and function will be essential for better vaccine development against clinically relevant global infectious diseases to combat the negative impacts they can have on protective B cell responses.

1.4 Malaria: a global health burden

Plasmodium life cycle

Malaria, caused by parasites of the *Plasmodium* genus, is a deadly infection that kills nearly 450,000 people annually, with a majority of deaths occurring in children and pregnant women in Sub-Saharan Africa [37]. In humans the primary etiological strain of malaria is *Plasmodium falciparum*, which is one of five *Plasmodium* species that can cause malaria in humans. The parasite life cycle begins when a female *Anopheles* mosquito carrying *Plasmodium* bites its host to take a blood meal and releases the parasite, in the differentiated form of a sporozoite, into the skin of the infected host. Sporozoites, which are highly motile, traverse into the circulation to make their way to the liver where they initiate the liver stage of disease. Once in the liver, sporozoites invade hepatocytes and over the course of 3-10 days, depending on the species, undergo replication and differentiation up to nearly 40,000 fold [38]. Despite the incredible activity of the parasite within the liver, the host remains unaware they are infected as the liver stage of disease is completely asymptomatic. Once replication is complete, the parasites, now differentiated into asexual merozoites, bud out of the infected hepatocyte and are released into the bloodstream and begin the second stage of the disease, the blood stage. Within the circulation, merozoites initiate a 48 hour cycle of invasion into red blood cells (RBCs), asexual replication within the RBC, RBC rupture and lysis, and release of newly formed

merozoites that are then free to initiate the process of RBC invasion again. It is during this blood stage of disease, through exponential replication of merozoites and decimation of the RBC compartment, that the infected host experiences the onset of malaria signs and symptoms including inflammation, anemia, fever, and in some cases the development of cerebral malaria, all of which can ultimately lead to death in at risk individuals. To complete the life cycle within the mammalian host, some merozoites will differentiate into male and female gametocytes that are then taken up by a mosquito during a blood meal. Within the mosquito midgut, *de novo* sporozoite formation is carried out and the parasite life cycle is fully complete and ready to be transmitted to the next host via mosquito bite [39].

Control of malaria

There have been significant multilateral efforts to combat malaria infection for those highest at risk. Public health interventions such as insecticide-coated bed nets, vector control programs, and anti-malarial drugs, like chloroquine and artemisinin, have proven effective in lowering the overall worldwide incidence of malaria infections by almost two fold over the last decade as well as reducing malaria-related deaths [37, 40]. While these methods have helped reduce the global burden of disease, growing anti-malarial drug resistance and increasing costs to sustain resource-intensive interventions in predominantly resource-poor developing countries has challenged the malaria field to provide a more cost-effective and durable solution. A protective anti-malaria vaccine likely represents the answer to this challenge in order to significantly reduce the burden of disease long term.

The multi-stage, multi-antigen nature of the *Plasmodium* life cycle and the complexity involved in acquisition of immunity to disease has proven to be a major roadblock to the development of an effective vaccine that induces a robust and durable protective immune response. A majority of vaccine efforts have been focused on eliminating the parasite during the clinically silent liver stage. The most advanced malaria vaccine to date is the RTS'S vaccine, which contains the pre-erythrocytic liver stage antigen circumsporozoite protein (CSP) but has

been shown to only provide ~30% efficacy in 5-17 month old children and to an even lesser extent in infants, resulting in breakthrough infections to blood stage disease [41, 42]. More recent efforts have also been made to generate a vaccine targeting the symptomatic blood stage of disease. Many of these approaches have employed subunit vaccines in combination with model adjuvants aimed to generate antibodies against blood stage merozoite antigens that would block parasite RBC invasion and reduce transmission [43, 44]. While these approaches hold great promise, they unfortunately have not been proven to be efficacious either [45-47]. Without a better understanding of the immune mechanisms that lead to protection an effective malaria vaccine will be difficult to generate.

In longitudinal studies of individuals living in endemic areas of malaria it is well established that even though immunity to malaria forms it is slow to develop and takes several years [48]. Even in areas of high transmission, where children are exposed to hundreds of mosquito bites annually, severe malaria disease persists typically until the age of five and susceptibility to uncomplicated febrile malaria symptoms can persist until late adolescence [49]. These findings are notable as it has been shown that it only take one or two mosquito transmission events to acquire protection from death [50]. Despite the eventual acquisition of resistance to malaria symptoms with repeated exposure, sterilizing immunity and resistance to infection is rarely achieved and adults living symptom-free in these regions are still likely to present with parasites in their blood, termed clinical immunity.

Both CD4⁺ T cells and B cells play key roles in the immune response to the blood stage of malaria infection in distinct ways. A major component of the CD4⁺ T cell mediated response is the production of a variety of pro-inflammatory cytokines, most notably the production of the Th1 associated cytokine interferon gamma (IFN γ) [51]. In both humans and mice IFN γ production, which in addition to CD4⁺ T cells can also be produced by natural killer (NK) cells and NKT cells, has been shown to be an effective mediator in parasite control by promoting infected RBC (iRBC) destruction via phagocyte activation [52, 53]. In addition to IFN γ , other inflammatory

cytokines, such as tumor necrosis factor alpha (TNF α) can also help mediate phagocyte destruction of iRBCs and confer protection in mice [54]. The production of these inflammatory cytokines, while effective against the parasite, can also cause unwarranted pathology and damage to the infected host and are counterbalanced by production of the suppressive cytokine, interleukin-10 (IL-10). In mice dual IL-10 and IFN γ producing Th1 CD4⁺ T cells have been shown to aid in parasite clearance while also helping prevent malaria-induced pathology [55]. Similar populations of IL-10 and IFN γ polyfunctional T cells have been identified in malaria-exposed children early in life [56]. The development of this T cell mediated cytokine response to both promote parasite clearance and prevent immunopathology may therefore represent the key to early protection from death in children. Continued protection, however, is thought to then primarily rely on the function of antibodies which are only efficiently acquired after many years as malaria-exposure persists [57]. A better understanding of how B cells confer protection during malaria and how to promote protective B cell populations early in life may help reduce the risk of death in young children at an earlier age.

1.5 Humoral immunity to malaria

It is well established that B cells and their antibodies contribute to parasite control during the erythrocytic stage of infection in studies from both humans and mice. In 1961, a seminal finding by Cohen et al. laid the foundation for antibodies as key effectors in blood stage malaria immunity. In this study they showed that the transfer of purified immunoglobulin G (IgG) from symptom-free malaria-immune adults living in a malaria endemic region of West Africa to children in the same region experiencing acute malaria led to a rapid reduction of parasitemia and resolution of fever. This was specific to malaria-induced IgG as IgG from non-malaria immune donors were unable to provide protection [58]. Similar findings were shown in other malaria-prevalent areas of Africa and Southeast Asia [59]. Stemming from the findings of these

studies, over the last few decades there has been significant interest in trying to learn how long-lived malaria-specific antibodies are generated.

In many instances of infection or vaccination, long-lasting protective antibody responses are generated after only one or a few exposures. For example, a single vaccination in children with the measles-mumps-rubella (MMR) vaccine can result in measles-specific antibodies that are maintained for life [60]. Conversely, the persistence of *Plasmodium*-specific antibodies in children after primary malaria infection is short-lived, lasting less than a year [61, 62]. Longitudinal studies of these malaria-endemic populations have shown that individuals living in constant exposure to malaria parasites acquire increasing levels of serum antibodies as well as long-lived plasma cells and memory B cells specific for blood stage malaria proteins the older they get, correlating with an increased ability to resist clinical malaria symptoms [61, 63, 64]. Thus the longer one remains exposed to malaria does eventually result in acquisition of longer-lived antibody responses. The notion that constant exposure is necessary to achieve longer-lived antibody responses is supported by a study carried out in West Africa in which over a 5-year period malaria transmission was reduced through insecticide control and anti-malarial drug distribution. During this period of intervention, malaria-specific antibody titers significantly decreased amongst the population [65]. Upon cessation of the interventions and recrudescence of parasitemia, antibody titers recovered rapidly. While clearly humans do eventually generate durable antibody-mediated protection from malaria, seemingly due to constant exposure to infection, the mechanisms that are either antagonized or not ever induced to generate sustained B cell responses much earlier in life remain an active area of research.

The use of rodent strains of *Plasmodium* in animal models that can be genetically manipulated has allowed researchers to pursue more mechanistic based studies of our understanding of B cell responses during blood stage malaria infection. There are four major *Plasmodium* strains that infect rodents and each one, while not perfect, resembles various aspects of the clinical manifestations observed in human malaria [66]. The importance of B cells

during blood stage malaria is easily observed in B cell deficient mice, which show an impaired ability to control parasitemia during infection [67, 68]. Since then several studies have gone to better characterize the *Plasmodium*-specific B cell response to merozoite antigens. One antigen of particular interest is Merozoite Surface Protein 1 (MSP1), a leading vaccine candidate. MSP1 is a highly abundant merozoite surface protein and is required for RBC binding and invasion [69]. Upon tethering to the RBC, MSP1 undergoes a series of proteolytic cleavage events to shed a majority of the protein leaving a C-terminal 19kD (MSP1₁₉) fragment on the merozoite surface which is required for final RBC entry, a process conserved across both rodent and human species of *Plasmodium* [70, 71]. More importantly, in studies similar to those performed by Cohen et al., passively transferred antibodies generated against the MSP1₁₉ fragment into naïve mice were capable of protecting them from a blood stage malaria challenge through the inhibition of RBC invasion by merozoites [58, 69, 72, 73].

Studies in mice using the *Plasmodium chabaudi* strain, a non-lethal, chronic model of rodent malaria that mimics uncomplicated malaria infection in adult humans, has shown there are two key phases of B cell responses specific for MSP1: an early wave of short-lived IgM⁺ antibodies made at the peak of infection and a later emerging phase of IgG⁺ antibodies [74-76]. MSP1-specific long-lived plasma cells and memory B cells were also found to form and stabilize long after a single blood stage infection. Furthermore, upon secondary *P.chabaudi* challenge mice show a more rapid MSP1-specific antibody and plasma cell response suggesting the formation of memory populations [76].

1.6 Questions to address

The process of MBCs formation is multidimensional resulting in heterogeneous populations with distinct developmental histories and functional capacities. While our classical view of MBCs as being high-affinity and class-switched remains true, MBCs that are unswitched or derived independently of GC reactions are now appreciated as well. This process of

differentiation results in MBCs that are poised to respond rapidly from terminally differentiated plasma cells or ensure a new GC reaction is made to sustain high-affinity MBC populations that can combat recurrent infections. Thus, given the diversity of MBCs, understanding what populations are best induced to fight complex infections like malaria is key to developing novel therapeutics and vaccines. While significant findings have been uncovered in both humans and mice in regards to humoral immunity to malaria, the approaches used have been limited in their ability to assess the formation, function, and developmental mechanisms of *Plasmodium*-specific B cells at the single cell level. We have developed the tools and strategies to overcome this roadblock and define what constitutes an optimal B cell response during malaria.

In the studies presented here, we have generated novel B cell tetramer reagents to provide the first glimpse of how *Plasmodium*-specific memory B cells develop and function during blood stage malaria infection. In chapter 2, we characterize *Plasmodium*-specific B cell differentiation in a relevant model of murine malaria, *Plasmodium chabaudi*. Here we show heterogeneous MBCs form and that IgM-expressing MBCs are the dominant responders during secondary infections. In chapter 3, we investigate the developmental mechanisms that lead to *Plasmodium*-specific MBC formation and find that IgM⁺ MBCs, in addition to IgG⁺ MBCs, are germinal center-derived and are programmed to mediate rapid recall responses. Lastly, in chapter 4, we corroborate our findings in mice by identifying similar heterogeneous populations of *Plasmodium*-specific MBCs in malaria-exposed humans.

2 Plasmodium-specific memory B cell formation and function during blood stage malaria infection

2.1 Introduction

Memory B cells (MBCs) induced by vaccine or infection are critical components of a protective humoral response. MBCs can persist for long periods of time and rapidly respond to subsequent infection through the production of antibody secreting cells, formation of new germinal centers (GCs) and repopulation of the memory pool [77]. Classically defined MBCs express class-switched, somatically hypermutated B cell receptors (BCRs) after undergoing a GC reaction. These cells produce high affinity antibodies within days of a secondary challenge, making them the gold standard for vaccine development. Recently, this homogenous view of MBCs has been challenged and it is now recognized that diverse MBC subsets exist in both mice and humans [25, 26, 78-80]. Given this, it is critical for vaccine development to understand how distinct MBC populations respond to infection.

Technical advances in tracking antigen-specific B cells have revealed that MBCs are heterogeneous. They have been shown to express either isotype switched or unswitched BCRs that have undergone various degrees of somatic hypermutation [26, 27, 81]. MBC subsets also exhibit varied expression of surface markers associated with T cell interactions such as CD73, CD80 and PDL2, revealing varied developmental histories and receptor ligand interactions [32-34]. Importantly, these phenotypically different MBC subsets have also been associated with functional heterogeneity, although different studies have led to different conclusions. Some studies have demonstrated that unswitched MBCs preferentially enter GCs while switched MBCs preferentially form plasmablasts [25, 26, 80, 82]. Other studies have shown instead that unswitched MBCs rapidly generate plasmablasts upon secondary challenge whereas switched MBCs preferentially re-enter GCs [31]. These are important distinctions to consider since different infections may have different requirements for humoral protection. Furthermore, the

majority of these studies depended upon adoptive transfer of individual MBC subsets and/or were performed in models of protein immunization or after *in vitro* rechallenge. It therefore remains unclear how endogenous MBC subsets respond in competition during a secondary infection.

B cells play a critical role in immune protection to the blood stage of *Plasmodium* infection. The protective role for antibody was first demonstrated via passive transfer of hyperimmune immunoglobulin from adults to parasitemic children [58], resulting in a dramatic decrease in blood stage parasitemia. Little is known, however, about the cellular source of *Plasmodium*-specific antibodies largely due to a lack of tools to analyze *Plasmodium*-specific B cells. We therefore generated B cell tetramers specific for the blood stage *Plasmodium* antigen, Merozoite Surface Protein 1 (MSP1). MSP1 is a key surface protein expressed by the parasite and is required for erythrocyte invasion [70]. Antibodies generated against the 19kD C-terminus region of MSP1 potently inhibit erythrocyte invasion and animals actively, or passively, immunized against MSP1 are protected against subsequent infection [72, 73, 83]. Furthermore, the acquisition of both IgG and IgM antibodies against the MSP1 C-terminus have been associated with the development of clinical immunity [84-88].

Tetramer enrichment techniques enabled the direct *ex vivo* visualization of rare *Plasmodium*-specific MBCs in malaria infected humans and mice. We then performed detailed analyses of MSP1⁺ MBC formation and function in the rodent model of malaria, *Plasmodium chabaudi*. Both isotype-switched and unswitched MBCs emerged early in infection and persisted for at least one year. MSP1⁺ MBCs consisted of three distinct subsets including: classically defined, somatically hypermutated, high affinity IgG⁺ MBCs, an IgM^{low}IgD^{high} population that resembled naïve B cells and a third IgM^{high}IgD^{low} MBC population that expressed somatically hypermutated BCRs that exhibit equivalent affinity to their IgG⁺ MBC counterparts. In response to various doses of malaria rechallenge, the majority of newly formed antibody-secreting cells (ASCs) were somatically hypermutated IgM⁺ cells, despite IgM⁺ MBCs being at a

numerical disadvantage at the time of challenge. Furthermore, IgM⁺ MBCs produced both IgM and IgG antibody in response to rechallenge, thereby also contributing to the IgG⁺ antibody response two days later. Collectively, these studies demonstrate that *Plasmodium*-specific IgM⁺ MBCs are high affinity, pluripotent early responders to malaria rechallenge that may provide a critical stop gap until IgG antibodies are generated and should therefore be considered in vaccine strategies.

2.2 Results

2.2.1 *MSP1-specific B cells expand, differentiate, and form memory in response to blood stage malaria infection.*

The direct *ex vivo* visualization of antigen-specific B cells during infection has been difficult to accomplish due to a lack of tools and techniques to track small population of B cells. We therefore adopted techniques used to analyze MBC development in response to protein immunization to study MBC development and function in response to blood stage malaria infection in C57BL/6 mice. To accomplish this, a phycoerythrin (PE)-conjugated B cell tetramer containing the majority of the 19kD C-terminal portion of the MSP1 protein from *P. chabaudi* was generated [89]. This reagent was used with magnetic bead-based enrichment to analyze malaria-specific B cells directly *ex vivo* throughout all phases of the immune response.

In all experiments, splenocytes were first stained with a decoy reagent and then with the MSP1 PE tetramer to exclude cells binding other components of the tetramer [89]. Anti-PE coated magnetic beads were then used to enrich both decoy-specific and MSP1-specific B cells, which were subsequently stained with antibodies for analysis by multiparameter flow cytometry. Antibody panels were based upon gating strategies developed to visualize all stages of mature B2 B cell differentiation. After excluding non-lymphocytes and doublets, Decoy⁻MSP1⁺ B cells were identified amongst B220⁺ and B220^{low}CD138⁺ cells (identifying plasmablasts) (**Fig. 2.1A, 2.1B**). In uninfected mice, there were approximately 400 MSP1⁺ B cells, while 8 days after

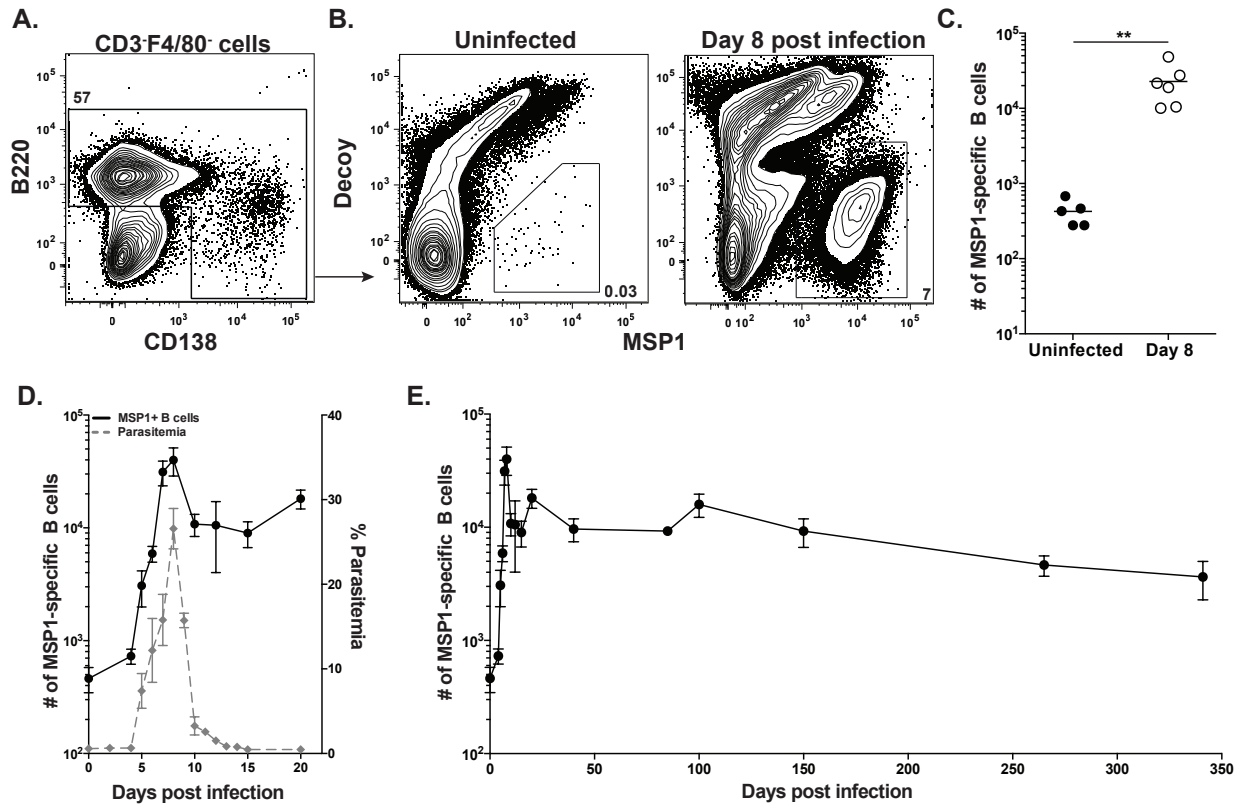


Figure 2.1. Detection and kinetics of MSP1⁺ B cells.

(A) Splenic B cells identified after excluding CD3⁺F4/80⁺ non-B cells and enrichment with MSP1 and Decoy tetramers. (B) Representative plots show MSP1⁺ B cells from (left) uninfected mice or (right) mice 8 days post-infection (p.i.). (C) Total number of MSP1⁺ B cells from uninfected or 8 days p.i. mice. Data is combined from 2 independent experiments with 5-6 mice per group. Line indicates mean **p<0.01. (D) Kinetics of MSP1⁺ B cells (left Y-axis) and percent parasitemia (right Y-axis) over 20 days (E) Total MSP1⁺ B cells over 340 days p.i. For D and E, each data point shows mean ± SEM with 3-8 mice per timepoint from at least two independent experiments.

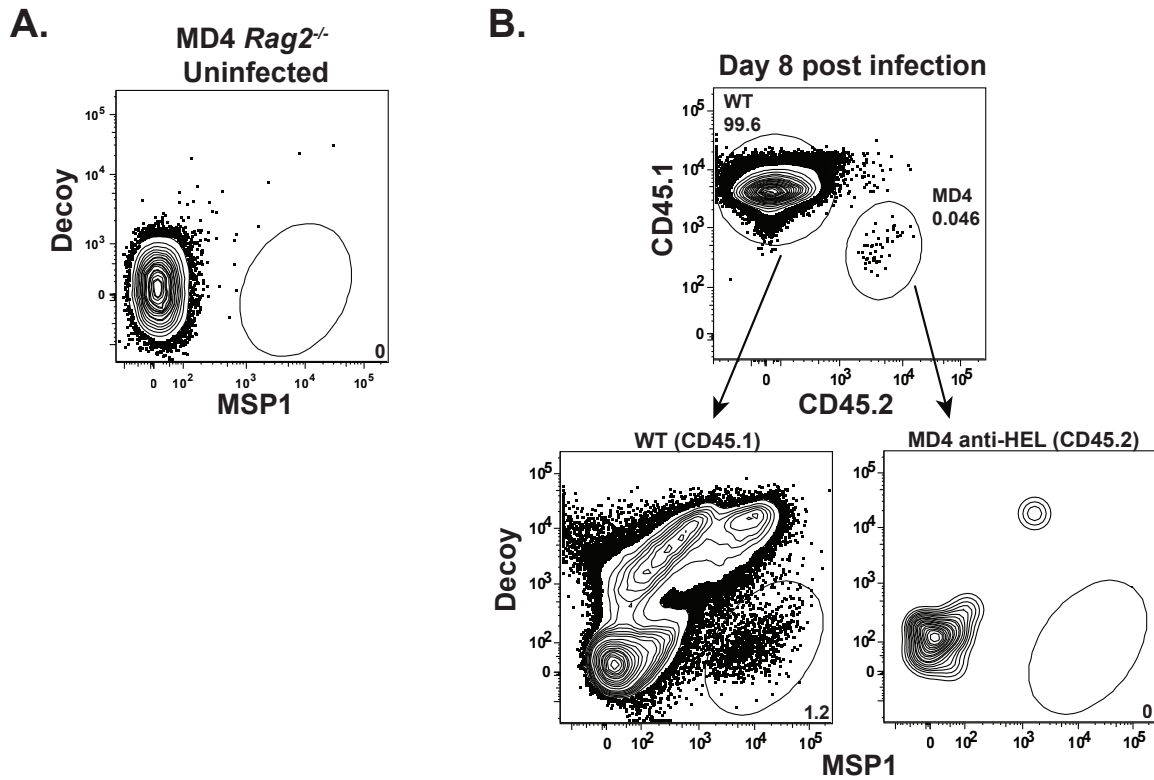


Figure 2.2. MSP1-specific B cells bind tetramer and expand in an antigen specific manner.

(A) Representative plots to identify MSP1⁺ B cells from spleens of naïve MD4-*Rag2*^{-/-} mice. (B) CD45.2⁺ MD4-*Rag2*^{-/-} splenocytes were transferred into CD45.1 WT B6 mice and infected with 1x10⁶ iRBCs the following day. 8 days post infection recipient mice spleens were enriched with Decoy and MSP1 B cell tetramers to identify MSP1⁺ B cells from either CD45.1 WT recipient cells or donor CD45.2⁺ MD4-*Rag2*^{-/-} cells.

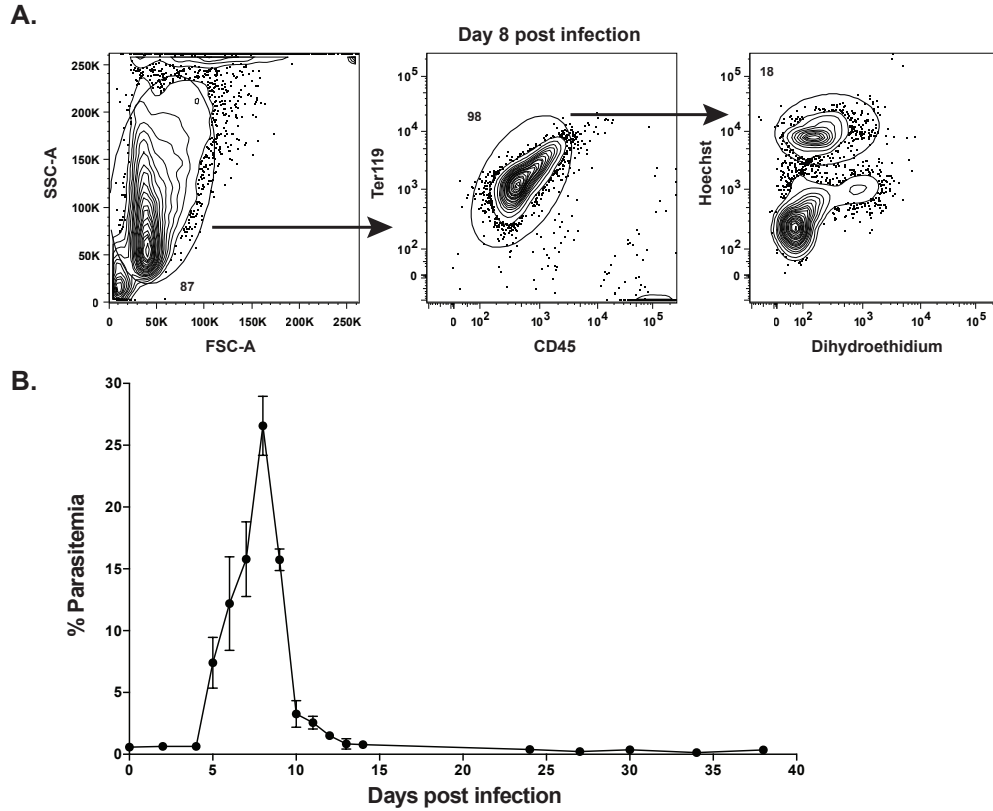


Figure 2.3. Measurement of parasitemia by flow cytometry.

(A) Representative gating scheme to identify *P.chabaudi* iRBCs gated as Ter119⁺CD45⁻ Hoechst⁺ in 1ul of blood from a WT mouse 8 days post infection. (B) Course of parasitemia in WT mice measured by flow cytometry after infection with 1×10^6 iRBCs over the course of 40 days. Each data point shows mean \pm SEM with 3-8 mice per timepoint from at least two independent experiments.

infection with 1×10^6 *P. chabaudi* iRBCs [90], the number of MSP1⁺ B cells expanded 50 fold to 23,000 cells (**Fig. 2.1B, 2.1C**). Control experiments demonstrated that B cells with BCRs specific for hen egg lysozyme (MD4 *Rag2*^{-/-} mice) did not bind the MSP1 tetramer nor were they activated non-specifically by *Plasmodium* 8 days post-infection after adoptive transfer into a congenic host (**Fig. 2.2A, 2.2B**). Thus, rare endogenous MSP1⁺ B cells that could be identified in naïve mice, expanded in an antigen-specific manner demonstrating our ability to stringently identify and analyze MSP1⁺ B cells throughout the course of *Plasmodium* infection.

Both parasitemia and MSP1⁺ B cells were quantified in the spleens of individual mice for approximately a year after infection. Parasitemia was measured in blood samples throughout the course of infection using a flow cytometry based assay [91, 92] (**Fig. 2.3A**). MSP1⁺ B cells isolated from spleens of infected mice began to expand by 4 days after infection, peaked 8 days after infection, then sharply contracted, mirroring parasitemia (**Fig. 2.1D and Fig. 2.3B**). Variations in total MSP1⁺ B cell numbers continued until day 150 although intracellular staining with the cell cycle marker Ki67 demonstrated that the vast majority (~95%) of MSP1⁺ B cells at day 100 are quiescent (**data not shown**). MSP1⁺ B cells persisted with a half-life of 221 days that resulted in a population of 3600 cells at 340 days post infection (**Fig. 2.1E**). MSP1⁺ B cells therefore expanded with ascending parasitemia, contracted and then numbers fluctuated before stabilizing and slowly declining over 350 days. Importantly, these data demonstrated that long lived, quiescent *Plasmodium*-specific B cells persisted and could be analyzed well after parasitemia is controlled.

2.2.2 *MSP1-specific B cell fates emerge early after infection and MBCs persist*

The heterogeneity of MSP1⁺ B cells was first assessed during the acute phase of the infection. Gating strategies were designed to distinguish between CD138⁺ plasmablasts (PBs), CD38⁺GL7⁺ activated precursors [33], CD38⁻GL7⁺ germinal center (GC) B cells, and expanded CD38⁺GL7⁻ MBC populations (**Fig. 2.4A**). Within 8 days of infection, multiple fates emerged

including a dominant population of MSP1⁺CD138⁺ PBs that primarily expressed IgM as measured by flow cytometry and serum ELISA consistent with previous reports [75, 93] (**Fig. 2.4A, 2.5A, 2.5D**). Several thousand MSP1⁺ B cells that retained CD38 expression, therefore resembling MBCs, were also present at day 8. The remainder of the population consisted of IgM⁺ and IgM⁻ GL7⁺CD38⁺ activated precursors which have been shown to be multipotent and capable of differentiating into GC B cells or MBCs (**Fig. 2.2A, 2.2B, 2.5B**) [33]. While GC responses were not present at day 8, they began to emerge at day 12, and expanded to a peak of about 15,000 MSP1⁺GL7⁺IgM⁻IgD⁻CD38⁻ cells at day 20, at which point numerous IgD⁻ germinal centers could also be found in the spleen by immunofluorescent microscopy (**Fig. 2.2A, 2.2B, 2.5B, 2.5C**). This was further confirmed by the presence of various sub-classes of MSP1-specific IgG⁺ antibodies measured in the serum (**Fig. 2.5D**).

To determine which of these early fates persisted into the memory phase of the response, MSP1⁺ B cells were characterized for approximately a year using similar gating strategies described above (**Fig. 2.4A, 2.4B**). Although CD138⁺ PBs initially waned between days 20 to 40, a small, consistently present CD138⁺ population re-emerged around day 85 suggesting that these were splenic plasma cells (PCs), similar to recent work demonstrating that PCs emerge after MBCs in response to protein immunization [94, 95]. These PCs persisted at all timepoints thereafter, were still present at day 340 post infection, and were predominantly IgM⁺ (**Fig. 2.4A, 2.4B, S2.5A**). Enrichment techniques also facilitated the visualization of a waning GC response. MSP1⁺ GC B cells contracted by day 40 post infection and then slowly declined before eventually disappearing around 150 days post infection. Therefore, from day 50 on, the vast majority of the MSP1⁺ cells were CD38⁺GL7⁻ MBCs that remained for at least 340 days post infection (**Fig. 2.4A, 2.4B**). These data demonstrate that well after parasite clearance and termination of the GC reaction, splenic MSP1⁺ B cells are predominantly comprised of an expanded population of CD38⁺ MBCs and a small but persistent CD138⁺ PC population.

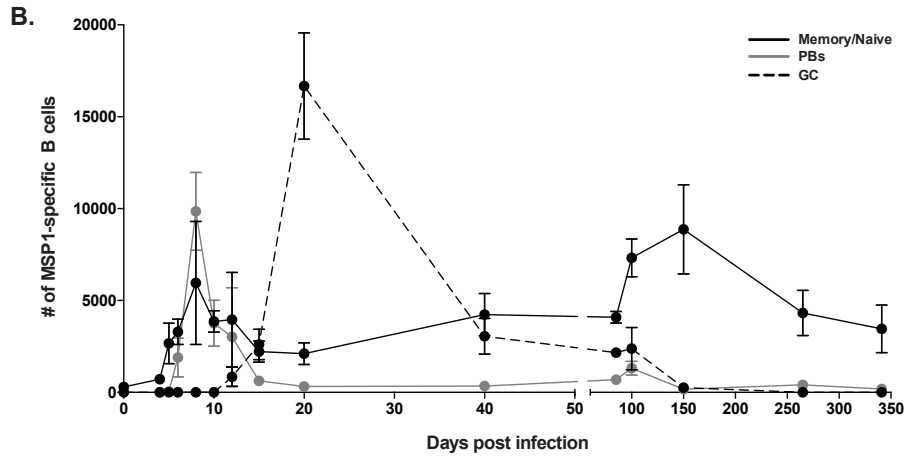
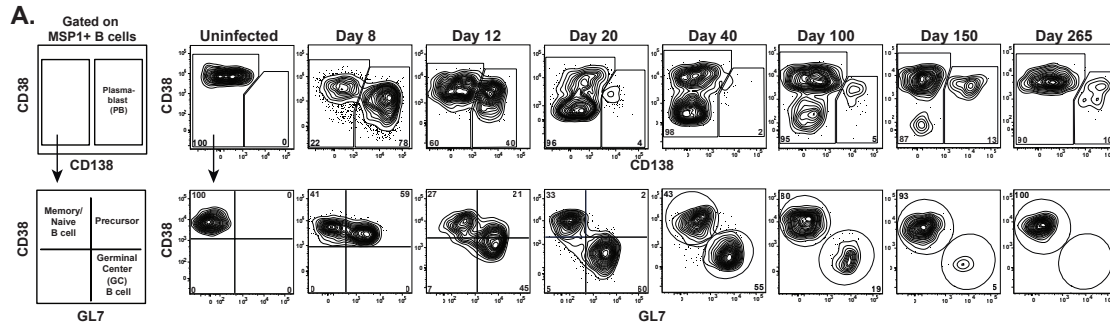


Figure 2.4. MSP1⁺ B cell fates emerge early after infection and MBCs persist.

(A) Gating scheme and representative plots of MSP1⁺ B cells. CD138⁺ cells (top row) and CD138⁻ memory/naïve cells, plasmablasts, and GC B cells (bottom row) over 265 days post infection. (B) Total MSP1⁺ MBCs/naïve, plasmablasts, and GC B cells. Each data point shows mean ± SEM with 3-8 mice per timepoint from at least 2 independent experiments. See also Figure S2.3.

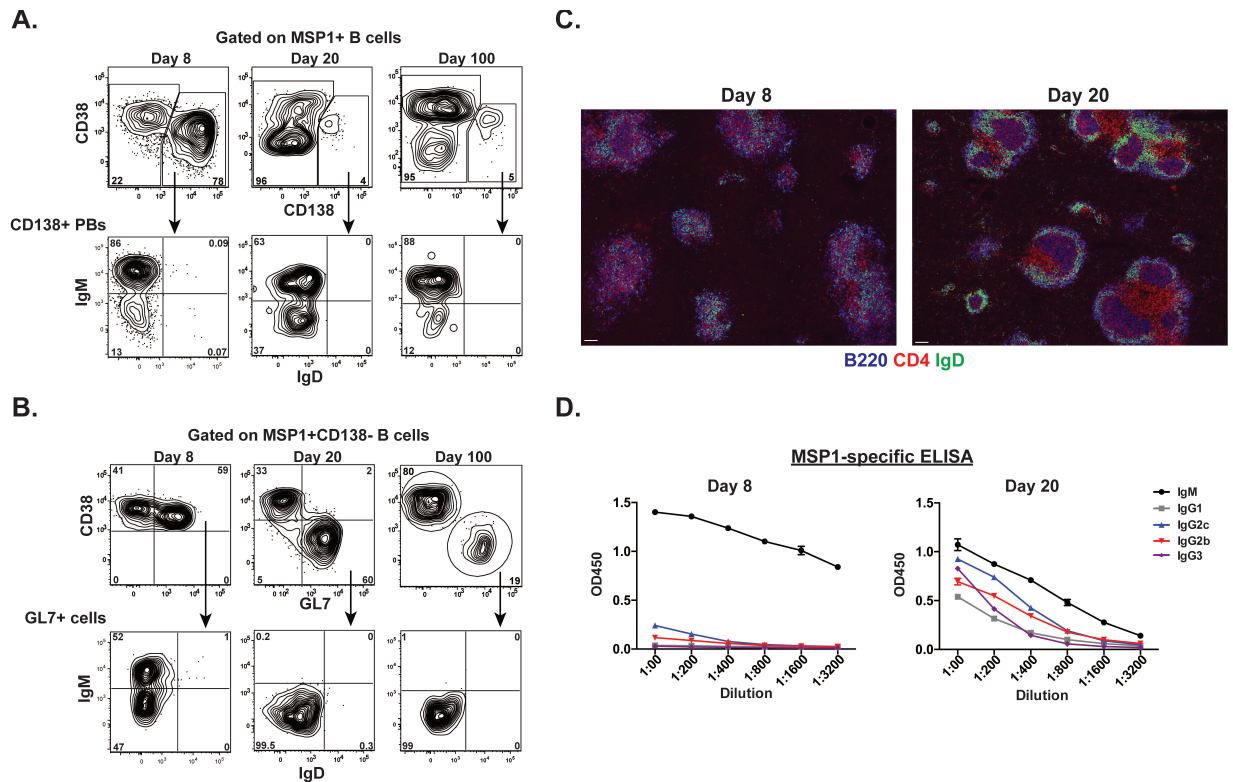


Figure 2.5. MSP1⁺ B cell fates emerge early after infection and MBCs persist.

(A) Representative plots of CD38 and CD138 expression (top row) and IgM and IgD expression (bottom row) on CD138⁺ cells of MSP1⁺ B cells on days 8, 20, and 100 post infection with 1×10^6 *Pc* iRBCs. (B) Representative plots of CD38 and GL7 expression (top row) and IgM and IgD expression (bottom row) on GL7⁺ cells of MSP1⁺CD138⁻ cells on days 8, 20, and 100 post infection with 1×10^6 *Pc* iRBCs. (C) Representative splenic sections from mice 8 or 20 days post infection with 1×10^6 *Pc* iRBCs stained with CD4 (red), B220 (blue), and IgD (green). Composite pictures generated by stitching together multiple 10x images over large area of spleen. Scale bars, 100 μ m. (D) Serum antibody analysis by ELISA for MSP1-specific IgM, IgG1, IgG2b, IgG2c, and IgG3 from individual mice on days 8 and 20 post infection with 1×10^6 *Pc* iRBCs. Each dilution point shows mean \pm SEM. Graphs represent combined data from 3 independent experiments with 3 mice per group.

2.2.3 *Switched and unswitched Plasmodium-specific MBCs can be found in malaria-exposed mice and humans*

It was next important to determine if recently defined MBC subsets that emerge after protein immunization were also present in response to infection. To interrogate the diversity of the MSP1⁺ MBCs, antibodies specific for IgM and IgD were used to identify “switched” and “unswitched” MSP1⁺ B cells. Interestingly, this staining strategy identified three distinct populations of MSP1⁺ MBCs 100 days after infection: an IgM⁺IgD⁻ isotype switched population (referred to as swIg⁺), and two unswitched subsets. One subset was phenotypically IgM^{lo}IgD^{high} (referred to as IgD⁺) while the other subset was IgM^{high}IgD^{lo} (referred to as IgM⁺) (**Fig. 2.6A**). While all three populations persisted for 340 days post infection, at the latest time points IgD⁺ MBCs stably persisted whereas both the IgM⁺ and swIg⁺ MBCs declined (**Fig. 2.6B**).

2.2.4 *Murine MSP1-specific MBC subsets are phenotypically and genetically distinct*

To further dissect the unique phenotypic and functional characteristics associated with distinct *Plasmodium*-specific MBCs, additional studies were performed in mice. Previous studies have demonstrated that MBC subsets display heterogeneous expression of surface markers associated with T cell interactions including CD73 and CD80 on both switched and unswitched MBCs [32, 34, 96]. Expression of these proteins was therefore examined on MSP1⁺ MBCs 100 days post infection. Again we found that the division of unswitched MBCs into IgM⁺ and IgD⁺ subsets largely accounted for the variability in surface marker expression. ~81% of IgM⁺ MBCs expressed CD73 and CD80 comparable to the ~96% of the swIg⁺ MBCs that expressed both markers, whereas only ~8% of IgD⁺ MBCs expressed CD73 and CD80, comparable to MSP1⁺ naïve B cells (**Fig. 2.6C**). Similar to MBC diversity generated by protein immunization, phenotypically diverse *Plasmodium*-specific IgD⁺, IgM⁺, and swIg⁺ MBC subsets develop in response to infection. Additionally, expression of CD73 and CD80 further distinguishes IgM⁺ and IgD⁺ MBCs as two distinct, unswitched populations.

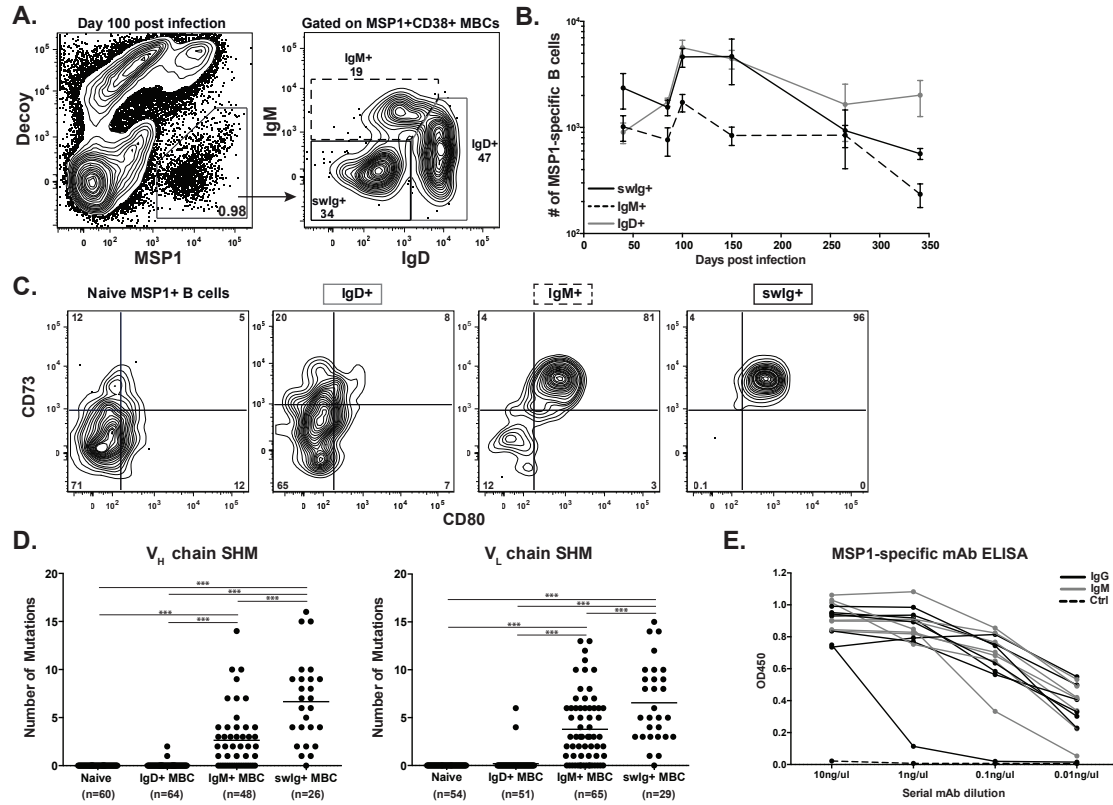


Figure 2.6. MSP1⁺ MBCs are heterogeneous.

(A) Representative plot of MSP1⁺ MBCs and isotype of MSP1⁺CD38⁺ MBCs to identify IgD⁺, IgM⁺, and swlg⁺ MBCs 100 days p.i. (B) Total number of MSP1⁺ IgD⁺, IgM⁺, and swlg⁺ MBCs from day 40 to 340 p.i. Each data point shows mean \pm SEM with 3-8 mice per timepoint from at least 2 independent experiments. (C) Representative plots of CD73 and CD80 on MSP1⁺ naïve B cells or IgD⁺, IgM⁺, and swlg⁺ MBCs 100 days p.i. (D) Number of mutations in the heavy chain (V_H) or light chain (V_L) of individual MSP1⁺ naïve B cells or CD73⁻CD80⁻IgD⁺, CD73⁺CD80⁺IgM⁺, or CD73⁺CD80⁺swlg⁺ MBCs 100 days p.i. Each dot indicates a single cell. Line indicates mean. Data combined from 3 independent experiments. ***p<0.001 (E) ELISA of serially diluted MSP1-specific IgM⁺ and swlg⁺ mAbs. Each line represents a single clone. OD₄₅₀, optical density at 450nm.

B cell expression of both CD73 and CD80 is associated with expression of activation-induced cytidine deaminase (AID) and in some cases but not all, germinal center dependence [32, 33, 81, 95]. Based on these observations, we hypothesized that our CD73⁺CD80⁺MSP1⁺IgM⁺ MBCs might represent a previously unexplained population of somatically hypermutated, unswitched MBCs identified in other immunization models [26, 81]. To test this hypothesis, we used flow cytometric sorting to isolate individual MSP1⁺ CD73⁻CD80⁻IgD⁺, CD73⁺CD80⁺IgM⁺, and CD73⁺CD80⁺swIg⁺ MBCs or MSP1⁺ naïve B cells. We sequenced and cloned individual BCRs using previously described methods [97]. The relative numbers of somatic hypermutations (SHM) in both heavy (V_H) and light (V_L) chain sequences present in individual MBC subsets were calculated after comparison to BCRs from naïve MSP1⁺ B cells, which had no mutations and were identical to germline sequences. While only 3% of IgD⁺ MBC V_H or V_L chain sequences showed SHM, 65% of V_H and 75% of V_L chain sequences of CD73⁺CD80⁺IgM⁺ cells were mutated with a mean of 3 mutations in both chains (**Fig. 2.6D**). As expected, swIg⁺ MBCs were also highly mutated (97%) and displayed significantly more mutations (mean of 8) in both V_H and V_L chains (**Fig. 2.6D**).

Since increased levels of SHM are associated with an overall increase in BCR affinity [98], we tested the affinities of IgM⁺ and swIg⁺ MBC BCRs. Individual BCR variable region sequences with varying levels of somatic hypermutation from either MSP1⁺ IgM⁺ or swIg⁺ MBC clones were therefore expressed as monoclonal antibodies (mAb) with human IgG constant (Fc) regions to prevent contributions to avidity by oligomerization. Antibodies were then used in dilution assays against MSP1 protein to compare affinity of the various mAbs by ELISA [99]. Importantly, these studies further confirm the specificity of our MSP1-tetramer techniques as 100% of expressed clones bound MSP1 protein, while the control PC-specific mAb did not (**Fig. 2.6E**). Furthermore, despite overall fewer mutations, individual BCRs from IgM⁺ MBCs showed comparable affinity for the MSP1 protein to swIg⁺ MBCs (**Fig. 2.6E**). These data therefore demonstrate that expression of CD73 and CD80 on both IgM⁺ and swIg⁺ MBCs is associated

with increased levels of SHM, resulting in similar BCR affinities. These findings raise the question of how these MBCs may respond in competition during a secondary infection.

2.2.5 *Secondary infection induces the rapid proliferation and differentiation of MSP1-specific MBCs.*

To understand how the MBCs described above function during a secondary infection, mice in the memory phase of the response were rechallenged with iRBCs. Of note, our experimental conditions were distinct from several previous studies that utilized adoptive transfer of individual MBC populations followed by antigen rechallenge. In intact memory mice, MBC competition for antigen and T cell help, as well as the presence of pre-existing antibodies factor into the overall response, perhaps as they would in repeatedly infected humans. To accomplish this, memory mice infected 12-16 weeks prior were left unchallenged or rechallenged with either 1×10^7 uninfected RBCs (unRBCs) or iRBCs and MSP1⁺ B cells were analyzed 3 or 5 days later. Following rechallenge with iRBCs, but not unRBCs, the total number of MSP1⁺ B cells expanded significantly on day 3 and continued to increase at day 5 compared to unchallenged memory mice (**Fig. 2.7A, 2.7B**). To ascertain whether these newly formed cells were originating from MBCs or recently formed naïve cells, naïve mice were also infected with a challenge dose of 1×10^7 iRBCs and MSP1⁺ B cells were quantified and phenotyped. In stark contrast to the logarithmic increase seen in MSP1⁺ B cells in memory mice after rechallenge, here was no significant increase in the total number of MSP1⁺ B cells in naïve mice at either 3 or 5 days after a primary infection (**Fig. 2.8A**).

We next to determined if expanded MSP1⁺ cells in rechallenged memory mice were also differentiated. Phenotypic analyses using gating strategies described above confirmed that MSP1⁺ B cells in memory mice prior to challenge consisted of both B220⁺CD138⁻ B cells (consisting primarily of MBCs and a small, waning population of GC B cells) and B220⁻CD138⁺ PCs (**Fig. 2.4 and Fig. 2.7C**). Three days after iRBC challenge, a newly formed

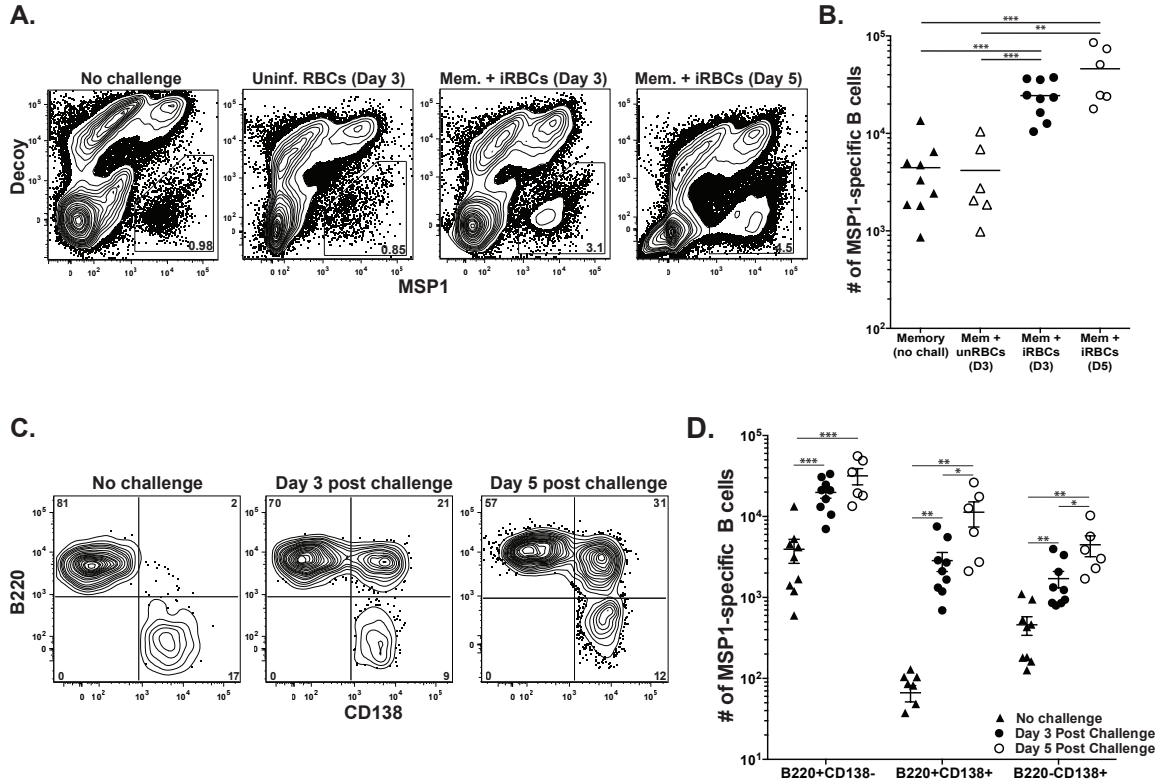


Figure 2.7. Rapid expansion of MSP1⁺ B cells after rechallenge.

(A) Representative plots identifying MSP1⁺ B cells in memory mice rechallenged with 1×10^7 unRBCs or iRBCs and analyzed 3 or 5 days later. (B) Total number of MSP1⁺ B cells in A. Data combined from 2 independent experiments with 6-9 mice per group. Line indicates mean. ** $p < 0.01$, *** $p < 0.001$ (C) Representative plots of B220 by CD138 on MSP1⁺ B cells in memory mice rechallenged with 1×10^7 iRBCs analyzed 3 or 5 days later. (D) Total number of MSP1⁺ B220⁺CD138⁻, B220⁻CD138⁺, and B220⁺CD138⁺ cells in C. Data combined from 2 independent experiments with 6-9 mice per group. Line indicates mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

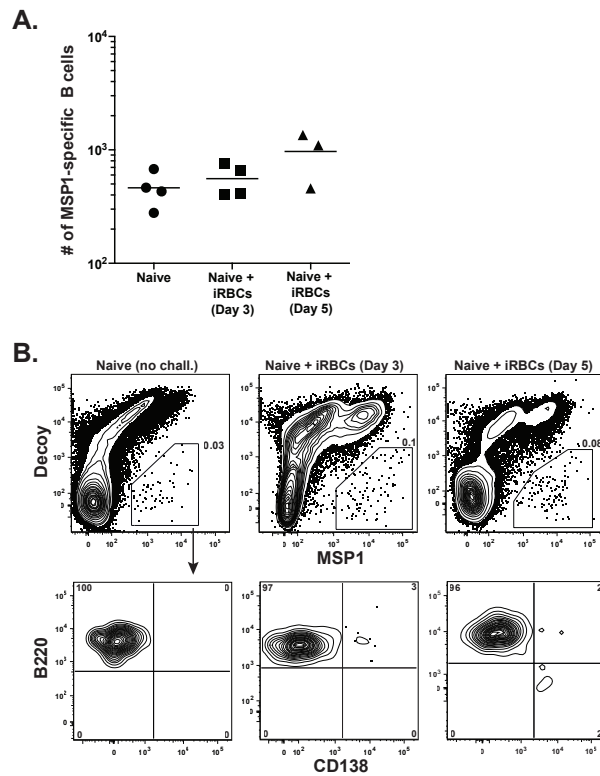


Figure 2.8. Naïve MSP1⁺ B cells do not differentiate or secrete antibody after primary infection with 1×10^7 iRBCs.

(A) Representative plots identifying MSP1⁺ B cells (top row) and B220 and CD138 expression (bottom row) in uninfected naïve mice or naïve mice 3 or 5 days post primary infection with 1×10^7 iRBCs injected i.v. (B) Total number of MSP1⁺ B cells in uninfected naïve mice or naïve mice 3 or 5 days post primary infection with 1×10^7 iRBCs injected i.v. Data is combined from 2 independent experiments with 3-5 mice per group.

MSP1⁺B220⁺CD138⁺ population emerged and remained expanded at day 5, suggesting these were the product of recently activated MBCs (**Fig. 2.7C, 2.7D**). The rapid formation of this population was unique to a memory response as we did not observe a significant B220⁺CD138⁺ population form in naïve mice three days after the same iRBC challenge (**Fig. 2.8B**). Additional quantification of the B220⁺CD138⁻ B cells and B220⁻CD138⁺ PCs revealed that these populations also increased in number after rechallenge (**Fig. 2.7D**). Together, these data demonstrate that within 3 days of rechallenge, expanded and differentiated MSP1⁺ B cells form in response to a secondary infection.

To determine what precursor populations were proliferating to produce expanded populations of MSP1⁺ B cells, Ki67 expression (which marks actively cycling cells) was compared before and after rechallenge. Prior to challenge, ~4% of MSP1⁺ B cells were Ki67⁺ (**Fig 2.9A**). Three days after rechallenge the percentage of Ki67⁺ increased to ~16% of all MSP1⁺ B cells and remained restricted to the B220⁺ B cells (B220⁻ PCs were Ki67⁻) (**Fig. 2.9A**). Detailed phenotypic analysis of the Ki67⁺ cells revealed that three separate MSP1⁺B220⁺ populations were proliferating: newly formed B220⁺CD138⁺ plasmablasts (PBs) (~30%), CD38⁺ MBCs (~50%) and CD38⁺GL7⁺ activated precursors (~20%) (**Fig. 2.9A**). Therefore, within three days, some MSP1⁺ MBCs had already proliferated and differentiated into PBs and CD38⁺GL7⁺ activated precursors, but many CD38⁺GL7⁻ MBCs were still proliferating but had not yet differentiated. The isotypes of the proliferating cells were also determined to reveal precursor relationships. Surprisingly, the majority of both Ki67⁺ PBs three days after rechallenge expressed IgM despite IgM⁺ MBCs being at a numerical disadvantage to the swIg⁺ MBCs at this timepoint (**Fig. 2.9B, Fig. 2.6B**). The activated precursors and MBCs were largely isotype switched (**Fig. 2.9B**). In contrast, very few of the MSP1⁺ IgD⁺ MBCs were proliferating. These data demonstrate that IgM⁺ MBCs rapidly respond to secondary infection and make up the majority of the early proliferating plasmablasts.

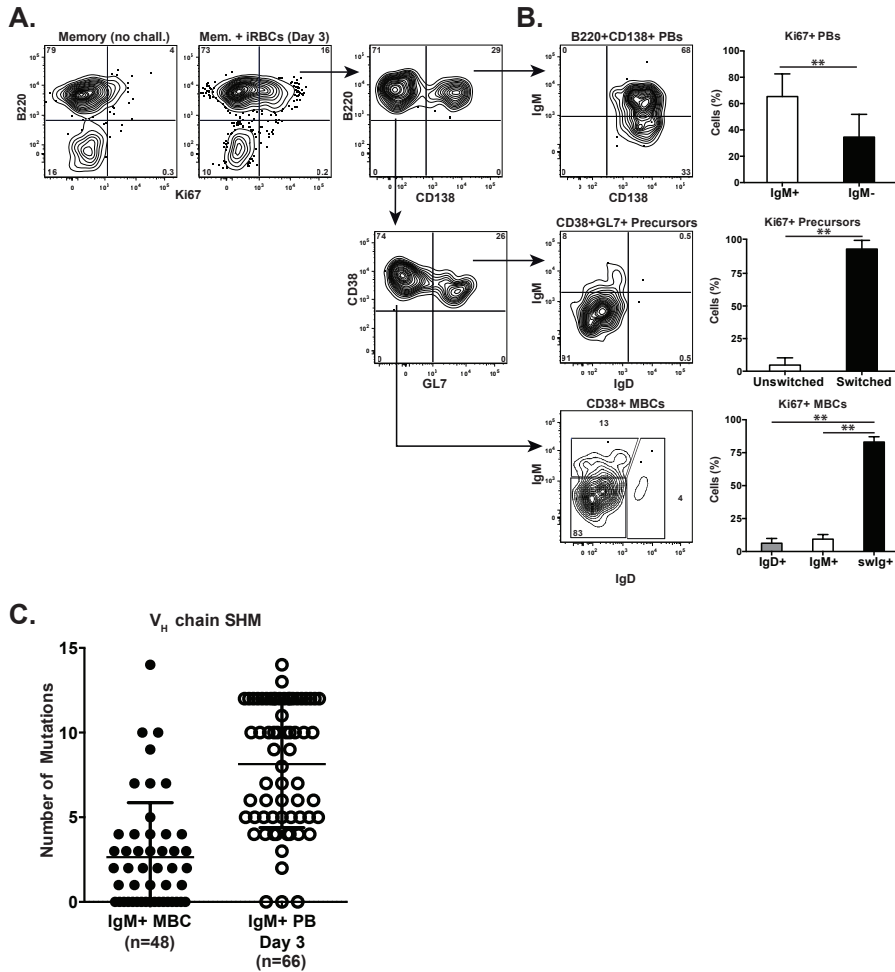


Figure 2.9. Rapid expansion of MSP1⁺ B cells after rechallenge.

(A) Columns 1 and 2: Ki67 expression on B220⁺ MSP1⁺ B cells in memory mice pre or post iRBC challenge (day 3). Column 3: representative plots of B220 and CD138 expression (top) and CD38 and GL7 expression (middle) of Ki67⁺ cells in rechallenged memory mice. (B) Representative plots of IgM and IgD expression on Ki67⁺B220⁺ PBs (top), precursors (middle), and MBCs (bottom). Adjacent graph shows percentage of indicated isotypes of each population. Bar graphs represents data combined from 2 independent experiments with 6 mice per group. Error bars show SD. **p<0.01 (C) Number of mutations in the heavy chain (V_H) of individual MSP1⁺ IgM⁺ MBCs 100 days p.i. prior to challenge and MSP1⁺IgM⁺B220⁺CD138⁺ plasmablasts from memory mice 3 days after challenge with 1x10⁷ iRBCs. Each dot indicates a single cell. Line indicates mean. Data combined from 3 independent experiments.

To further discern precursor relationships for the IgM⁺ PBs, we cloned BCRs from the IgM⁺B220⁺CD138⁺ PBs 3 days after challenge to look for somatic hypermutation. If the PBs were somatically hypermutated, it would support the idea that these cells were derived from somatically hypermutated IgM⁺ MBCs as opposed to unmutated IgD⁺ MBCs. Remarkably, 95% of newly formed IgM⁺ PB clones (mean mutation of 8) were somatically hypermutated at levels that were comparable to MSP1⁺IgM⁺ MBCs, further establishing a precursor relationship between IgM⁺ MBCs and newly formed PBs after a secondary infection (**Fig. 2.9C**).

2.2.6 *The early secondary antibody response is IgM-dominant*

We next asked what MSP1⁺ cells were differentiated antibody secreting cells (ASCs). Again, memory mice were rechallenged and intracellular staining for immunoglobulin heavy and light chain (Ig) was performed on MSP1⁺ B cells 3 or 5 days later. In memory mice analyzed prior to challenge, the only ASCs present were ~600 B220⁻CD138⁺ PCs (which represent about 5% of the total cells) (**Fig. 2.10A**). Three days after rechallenge, approximately ~3000 MSP1⁺ B cells (about 15%) were now making antibody, split between B220⁺CD138⁺ PBs and B220⁻CD138⁺ PCs (**Fig. 2.10A**). Now, approximately 70% of the MSP1⁺Ig⁺ ASCs were IgM⁺, while only about 30% of the ASCs were switched, resulting in significantly more IgM⁺ ASCs on day 3 than switched ASCs (**Fig. 2.10A, 2.10B**). Two days later, on day 5 post challenge, IgM⁺ ASCs continued to expand, but now there was also a larger, switched antibody-secreting PB pool. Interestingly, the switched PCs stayed relatively stable at all time points examined (**Fig. 2.10A, 2.10B**). To confirm that our intracellular antibody staining represented measurable changes of secreted antibody *in vivo*, MSP1-19 protein-specific ELISAs were performed on serum samples taken from individual mice before or after challenge. In conjunction with what was observed by flow cytometry, three days after infection MSP1-specific IgM antibody expression was significantly increased over pre-challenge levels while IgG antibody expression remained unchanged (**Fig. 2.10C, top row**). Two days later however, on day 5, we observed significant

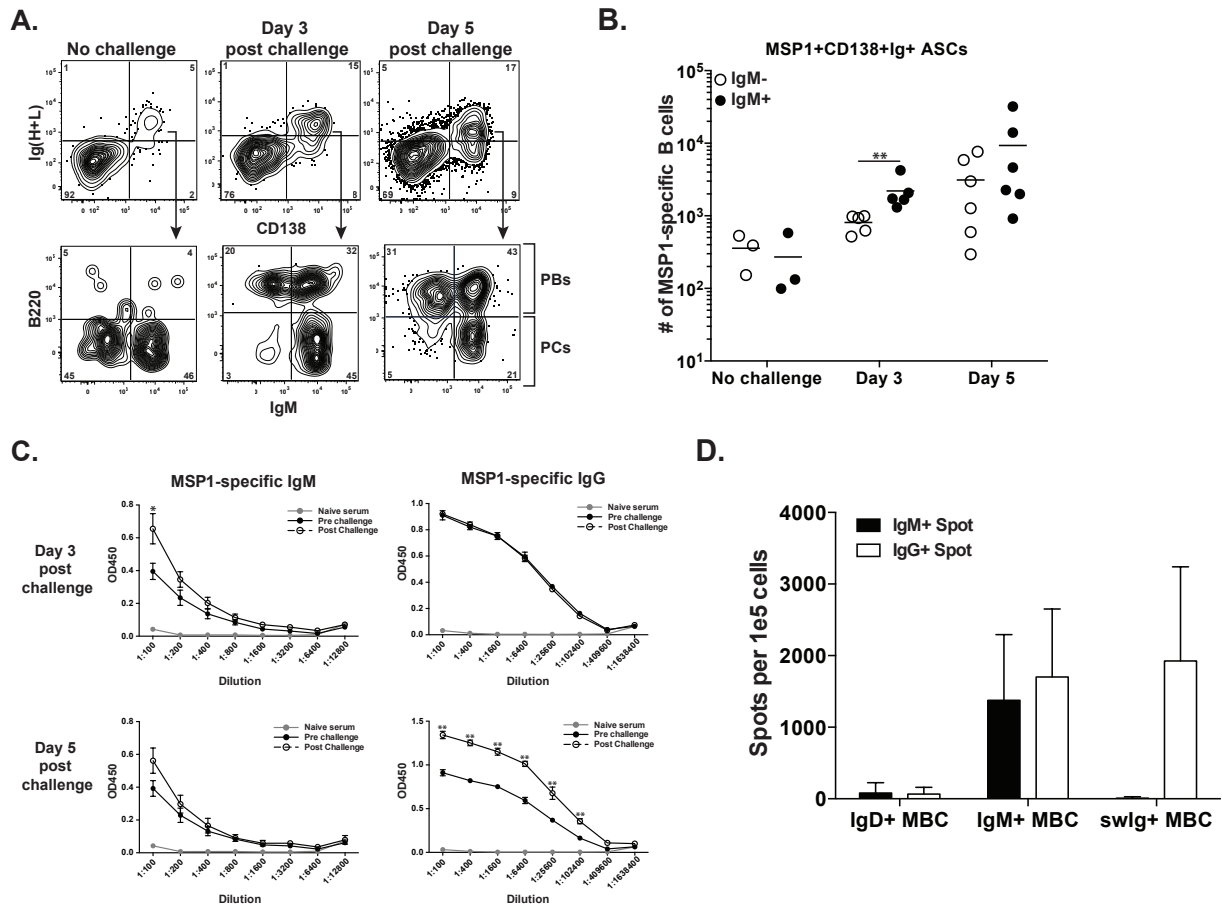


Figure 2.10. Early secondary antibody response is IgM-dominant.

(A) Representative plots of intracellular Ig(H+L) (Ig) and CD138 expression of MSP1⁺ B cells. Bottom row shows B220 by IgM expression of MSP1⁺ Ig⁺ CD138⁺ cells in memory mice pre challenge or 3 or 5 days post challenge with 1x10⁷ iRBCs. (B) Total number of all IgM⁺ and IgM⁻ MSP1⁺ CD138⁺ Ig⁺ cells in A. Data combined from 2 independent experiments with 3-6 mice per group. Line indicates mean. **p<0.01 (C) MSP1-19 IgM and IgG ELISA from serum of individual memory mice prechallenge and 3 or 5 days post challenge. OD₄₅₀, optical density at 450nm. Each dilution point shows mean ± SEM. Graphs represent combined data from 3 independent experiments with 3 mice per group. *p<0.05, **p<0.01 (D) ELISPOT on MSP1⁺ IgD⁺, IgM⁺, and swlg⁺ MBCs sorted from memory mice 2 days post challenge. Data compiled from 5 mice in 2 independent experiments. Error bars show SD. See also figure S6.

increases in MSP1-specific IgG antibodies, while IgM antibody levels remained elevated (**Fig. 2.10C, bottom row**). Since it was unclear if these switched PBs arose from swIg⁺ or IgM⁺ MBCs, we additionally sorted MBCs two days after rechallenge to look for IgM or IgG expression by ELISPOT after 2 days in culture. This approach revealed that while swIg⁺ MBCs could only form IgG⁺ ASCs, IgM⁺ MBCs formed both IgM⁺ and IgG⁺ ASCs (**Fig. 2.11D**). Collectively, these data demonstrate that the secondary response is dominated by early IgM⁺ antibody expression and later IgG⁺ antibody expression. Additionally, our findings demonstrate that IgM⁺ MBCs are capable of expressing both IgM⁺ and IgG⁺ antibodies, highlighting that the IgM⁺ MBCs are rapid, plastic responders to a secondary infection.

2.2.7 *Secondary IgM response is not affected by challenge dose or timing*

One potential cause for the early IgM dominant response after secondary challenge could be a high antigen load, which could somehow preferentially activate IgM⁺ MBCs. Memory mice were therefore challenged with two lower iRBC challenge doses (1×10^3 and 1×10^5) prior to MSP1⁺ B cell analysis 3 days later. Remarkably, in both lower dose challenges, the IgM⁺ ASC response still dominated the early ASC population and even more dramatically than what we had observed at the higher dose challenge (**Fig. 2.11A, 2.11B, 2.11B**). This was especially striking given the 2.5-fold numerical disadvantage of IgM⁺ MBCs compared to swIg⁺ MBCs 100 days post-challenge (**Fig. 2.11B**). While this ruled out dose dependent effects, it was also possible that the time of rechallenge influenced our results, for example if a germinal center was ongoing, which was the case for the 12-16 week rechallenge experiments. We therefore tested if the presence of an ongoing GC reaction at the time of challenge influenced the early secondary responders. Memory mice 35 weeks post infection, in which the GC reaction had ended and IgM⁺ and swIg⁺ MBCs were in equal number (**Fig. 2.4 and Fig. 2.6**), were therefore given a secondary challenge with 1×10^7 iRBCs and analyzed 3 days later. As seen in mice with an ongoing GC, IgM⁺ cells were still the predominant early antibody-expressing population (**Fig.**

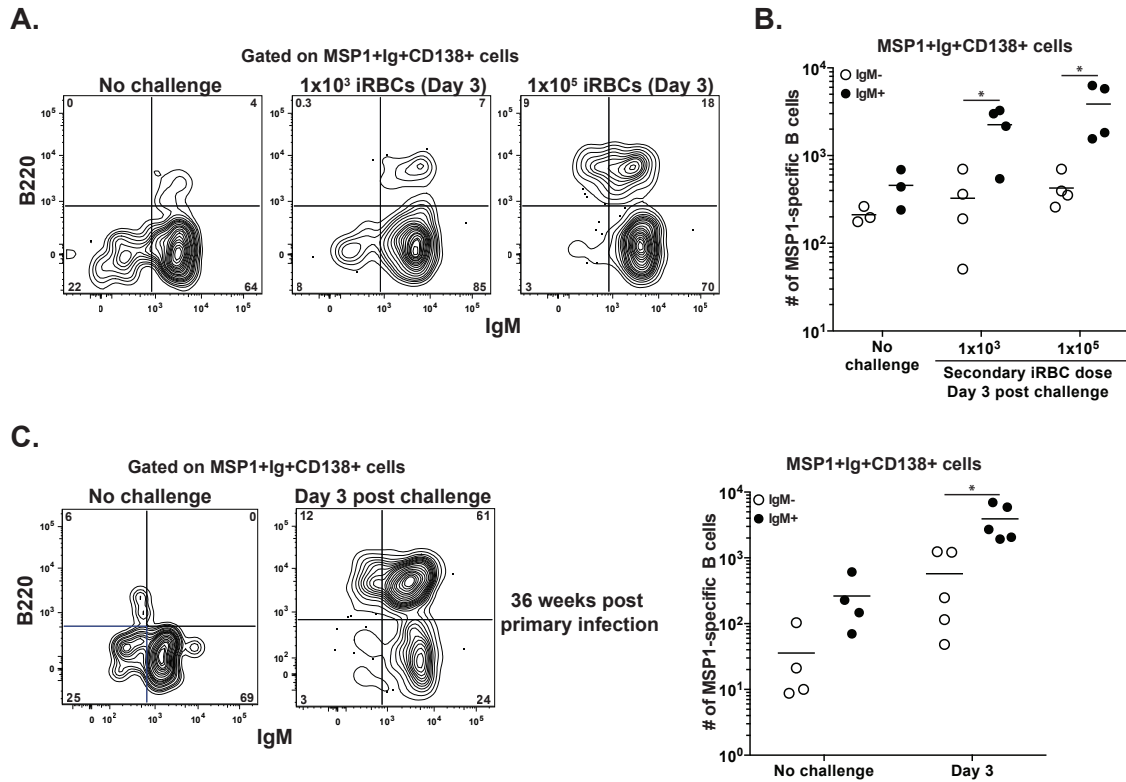


Figure 2.11. iRBC challenge dose or timing does not impact secondary IgM response

(A) Representative plots of B220 and IgM expression on MSP1⁺Ig⁺CD138⁺ cells in memory mice (12-16 weeks post primary infection) pre challenge or 3 days post challenge with 1x10³ or 1x10⁵. (B) Total number of all IgM⁺ and IgM⁻ MSP1⁺ CD138⁺Ig⁺ cells in A. Data combined from 2 independent experiments with 3-4 mice per group. Line indicates mean. *p<0.05 (C) Representative plots of B220 and IgM expression on MSP1⁺Ig⁺CD138⁺ cells (left) and total number of all IgM⁺ and IgM⁻ MSP1⁺ CD138⁺Ig⁺ cells (right) in memory mice 36 weeks p.i. prior to challenge or 3 days post challenge with 1x10⁷ iRBCs. Data combined from 2 independent experiments with 4-5 mice per group. Line indicates mean. *p<0.05

2.11C). Together these data suggest that despite variations in infectious dose, the presence or absence of a GC, or shifts in the numerical ratio of IgM⁺ to swIg⁺ MBCs, IgM⁺ MBCs can compete with swIg⁺ MBCs and are important early responders in a secondary *Plasmodium* infection.

2.2.8 IgM⁺ MBCs generate both T-independent and T-dependent antibody secreting effectors

The predominant secondary IgM⁺ memory response led us to interrogate the mechanisms of the early IgM response. We hypothesized that differences in T cell dependence could perhaps allow some populations to form faster than others. To test this, mice were treated a CD4⁺ T cell depleting antibody (clone GK1.5) for two days prior to rechallenge and formation of PBs and PCs was assessed 3 days later. Strikingly, while MSP1⁺ PBs did not form in the absence of T cell help, the PCs in the GK1.5 treated animals expanded comparably to those in a T cell replete rechallenged mouse (**Fig. 2.12A, 2.12B**). To assess the isotype of the responding T-independent ASCs, intracellular Ig staining was again performed. In mice depleted of T cells, more than 85% of the Ig⁺CD138⁺ ASCs expressed IgM⁺ (**Fig. 2.12C**). Therefore, the formation of both unswitched and switched PBs is T cell dependent yet predominantly IgM⁺ expressing PCs can still form in a T cell independent manner. These data therefore suggest that IgM⁺ MBCs can form two unique ASC populations in two mechanistically distinct ways, again highlighting their plasticity.

2.3 Discussion

Here we focused on understanding how recently described MBC subsets develop and function in response to infection with a relevant pathogen. To accomplish this, B cell tetramers were generated and enrichment techniques utilized to perform analyses of endogenous *Plasmodium*-specific B cells in malaria-infected mice. Importantly, the results presented in these studies highlight the fact that IgM⁺ and IgD⁺ MBCs are unique populations of

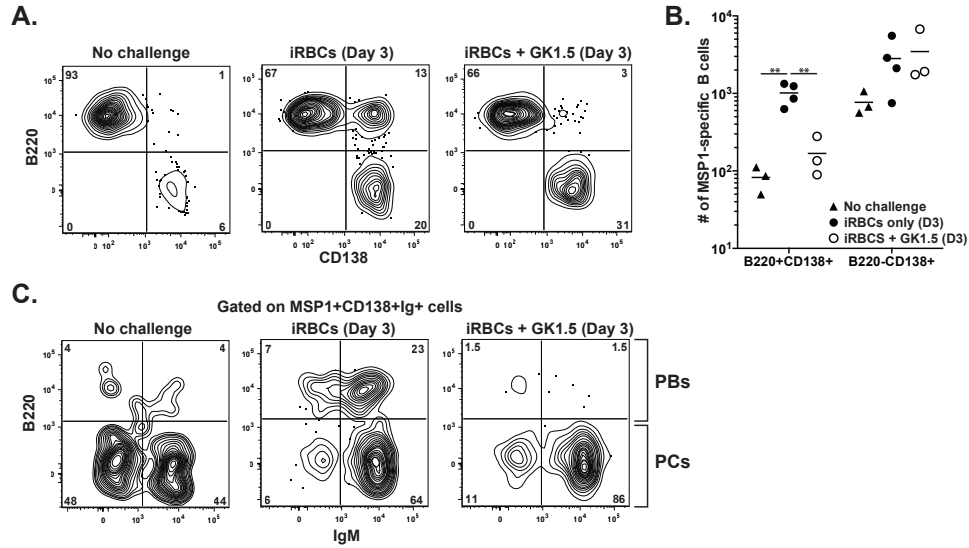


Figure 2.12. Requirements for secondary IgM⁺ MBC responses.

(A) Representative plots of MSP1⁺ B cell B220 and CD138 expression after iRBC rechallenge +/- CD4 depletion (GK1.5) (B) Total number of MSP1⁺ B220⁺CD138⁺ cells and B220⁻CD138⁺ cells in A. Data combined from 2 independent experiments with 3-4 mice per group. Line indicates mean. **p < 0.01 (C) Representative plots of B220 and IgM expression on MSP1⁺Ig⁺CD138⁺ cells in A.

cells with distinct phenotypic, functional and survival properties. Furthermore these studies emphasize that IgM⁺ MBCs are not low affinity cells that provide redundancy to IgG⁺ MBCs. On the contrary, *Plasmodium*-specific IgM⁺ MBCs express high affinity, somatically hypermutated BCRs and rapidly respond to produce antibodies prior to IgG⁺ MBCs, even in competition. Lastly, these studies reveal that a secondary memory response results in the generation of T-dependent plasmablasts and T-independent plasma cells that create multiple layers of antibody secreting cells.

In many ways, the results presented reconcile many of the disparate findings from various studies using a variety of protein immunization strategies, BCR transgenics and isolated transfer and rechallenge techniques. Dividing unswitched cells into two populations based on differential expression of IgM and IgD, revealed that IgM⁺ MBCs were far more similar in phenotype (CD73 and CD80 expression), developmental history (evidence of somatic hypermutation), affinity, survival and function (rapid plasmablast formation) to swIg⁺ MBCs than the more naïve-like IgD⁺ MBCs. Thus either isotype, as shown by Pape et al. [26] or expression of markers associated with somatic hypermutation [35] can predict MBC function, reconciling the findings of these two separate studies. While the IgD⁺ MBCs were remarkably stable, both the IgM⁺ and swIg⁺ subsets persisted with similar, and less stable kinetics as predicted by studies demonstrating a loss of somatically hypermutated B cells overtime [100]. IgD⁺ MBCs may represent a durable, expanded memory population that provides a high number of pathogen-specific clones with kinetics similar to naïve B cells.

We have also addressed how distinct antigen-specific MBC subsets respond to a secondary infection *in vivo* in competition and demonstrate a hierarchy of MBC responsiveness to secondary infection. Surprisingly, at the earliest timepoints, IgM⁺ MBCs are the dominant producers of ASCs at all doses of rechallenge and timepoints examined. By 5 days post secondary infection however, IgM antibody production did not continue to increase while switched PBs began to produce significant amounts of antibody highlighting that this dominance

is transient. Therefore unlike previous studies suggesting that IgM cells do not readily form PBs perhaps due to their low affinity [26] or form plasmablasts with similar kinetics to IgG⁺ PBs [35], in this system, IgM⁺ MBCs are high affinity, rapid, plastic early responders that appear to initiate the secondary response.

Our results may explain recent data associating the depth and breadth of *Plasmodium*-specific IgM antibodies with resistance to infection [88]. While we demonstrate that F(ab)s made from IgM⁺ MBCs are of comparable affinity to those sequenced from IgG⁺ MBCs, upon pentamerization of IgM antibodies, the IgM⁺ antibody avidity would be far greater than the IgG antibodies. Moreover, IgM antibodies are important mediators of complement mediated lysis, which is important for control of blood stage infection [101]. While the importance of IgM antibodies in *Plasmodium* infection has been shown in murine models [102], additional studies examining the importance of IgM antibodies in human malaria infection as well as the comparison of *Plasmodium*-specific IgM⁺ MBCs found in our murine system to those in malaria-exposed humans are necessary and ongoing.

Finally, these studies help to clarify long-standing controversies concerning the level of T cell dependence of secondary MBC responses [36]. Although many studies in humans and mice have demonstrated T cell-independent activation of MBCs [103-105], later studies suggested MBCs cannot be activated by bystander inflammation [82] or without the help of T cells [106]. Our results demonstrate that both T-dependent and T-independent processes contribute to a secondary MBC response and support recent studies demonstrating that IgM⁺ MBCs can be reactivated in a T-independent manner when transferred in isolation into T-cell depleted mice [35]. Specifically, secondary IgM⁺ and IgG⁺ PB formation was T-dependent, while the rapid generation of non-dividing, antibody-secreting IgM⁺ PCs was T-independent, raising many questions about the origins of these cells. It is tempting to speculate that the murine somatically hypermutated IgM⁺ MBCs identified in these studies are homologous to human IgM⁺ MBCs that can mediate T-independent IgM⁺ responses to bacterial infection [107]. In conclusion, these

studies highlight the IgM⁺ MBC as a functional, plastic, rapidly responding MBC population that should be targeted by vaccines to prevent disease.

2.4 Methods

Animals

5-8 week old female C57BL/6 and B6.SJL-Ptprc^a Pepc^b/BoyJ (CD45.1⁺) mice were used for these experiments. Mice were purchased from The Jackson Laboratory and maintained/bred under specific pathogen free conditions at the University of Washington. MD4-Rag2^{-/-} mice were provided by Dr. Marc Jenkins (University of Minnesota). All experiments were performed in accordance with the University of Washington Institutional Care and Use Committee guidelines.

Plasmodium Infection

Plasmodium chabaudi chabaudi (AS) parasites were maintained as frozen blood stocks and passaged through donor mice. Primary mouse infections were initiated by intraperitoneal (i.p.) injection of 1x10⁶ iRBCs from donor mice. Secondary mouse infections were performed 12-35 weeks after primary infection using a dose of 1x10⁷ iRBCs injected intravenously (i.v.). In some cases, when indicated, secondary challenges were given at lower doses using either 1x10³ or 1x10⁵ iRBCs injected i.v.

Tetramer Production

For murine studies, recombinant His-tagged C-terminal MSP1 protein (amino acids 4960 to 5301) from *P.chabaudi* (AS) (provided by Dr. Jean Langhorne, NIMR) was produced by *Pichia pastoris* and purified using a Ni-NTA agarose column as previously described [76]. Purified *P.chabaudi* MSP1 protein was biotinylated and tetramerized with streptavidin-PE (Prozyme) as previously described [89]. Decoy reagent to gate out non-MSP1⁺ B cells was made by conjugating SA-PE to AF647 using an AF647 protein labeling kit (ThermoFisher), washing and

removing any unbound AF647, and incubating with an excess of an irrelevant biotinylated HIS-tagged protein, similar to what has been previously described [89].

Mouse Cell Enrichment and Flow Cytometry

For murine samples, splenic cell suspensions were prepared and resuspended in 200ul in PBS containing 2% FBS and Fc block (2.4G2) and first incubated with Decoy tetramer at a concentration of 10nM at room temperature for 10 min. MSP1-PE tetramer was added at a concentration of 10nM and incubated on ice for 30 min. Cells were washed, incubated with anti-PE magnetic beads for 30 min on ice, and passed over magnetized LS columns (Miltenyi Biotec) to elute the bound cells as previously described [89]. All bound cells were stained with surface antibodies followed by intracellular antibody staining when needed (**Table 2.1**). All cells were run on the LSRII (BD) and analyzed using FlowJo software (Treestar).

Single Cell BCR Sequencing and Cloning

Single MSP1⁺ MBCs were FACS sorted using an ARIAII into 96-well plates. BCRs were amplified and sequenced from the cDNA of single cells as previously described [108], with additional IgH primers used [97]. Amplified products were cloned and generated mAbs using previously described methods [97, 108].

ELISAs

Costar 96-well EIA/RIA plates (Fisher Scientific) were coated overnight at 4°C with 10ug/ml of MSP1 protein. Plates were blocked with 2% BSA prior to sample incubation. For serum samples, plates were incubated with serially diluted serum from naïve or infected animals. For cloned mAbs, plates were incubated with serially diluted mAbs starting at 10ng/ul. Each sample was plated in duplicate. For serum samples, bound antibodies were detected using either IgM Biotin (II/41), IgG Biotin (Poly4053), IgG1 Biotin (A85-1), IgG2c Biotin (5.7), IgG2b Biotin (R12-3), or IgG3 (R40-82) followed by Streptavidin-HRP (BD). For mAbs, bound antibodies were

detected with mouse anti-human IgG-HRP (SouthernBiotech). Absorbance was measured at 450nm using an iMark Microplate Reader (Bio-Rad).

ELISPOT

96 well ELISPOT plates (Millipore) were coated overnight at 4°C with 10ug/ml of Ig(H+L) unlabeled antibody (Southern Biotech). Plates were blocked with 10% FBS in complete DMEM (Gibco). MSP1⁺ MBCs were sorted using a FACSAria (BD) from memory mice 2 days after rechallenge. Cells of each MBC population were plated onto coated ELISPOT plates and incubated at 37°C for an additional 2.5 days. Cells were washed off and secreted antibodies were detected using either IgM Biotin (II/41) or IgG Biotin (Poly4053) followed by Streptavidin-HRP (BD). Nonspecific (background) spots were determined in wells containing no cells. Spots were developed using AEC substrate (BD) and counted and analyzed using the CTL ELISPOT reader and Immunospot analysis software (Cellular Technology Limited). Number of spots detected per well were used to calculate spot frequency per 1×10^5 total cells.

Depletion of CD4⁺ T cells

For depletion of CD4⁺ T cells, GK1.5 monoclonal antibody to CD4 (rIgG2b; BioXcell) was used. One and two days prior to secondary challenge, memory mice were given an i.p. injection of 200µg GK1.5 or isotype control diluted in PBS. Efficiency of CD4⁺ T cell depletion was monitored by checking blood of mice pre-depletion, day 1 post injection and day of challenge. Depletion was found to be greater than 98% of CD4⁺ T cells as assessed by a non-GK1.5 competing anti-CD4 clone, RM4-4.

Statistical Analysis

Unpaired, two-tailed Student's t tests were applied to determine the statistical significance of the differences between groups with Prism (Graphpad) software. The p-values were considered significant when $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)).

| Specificity | Antibody | Clone | Color |
|--------------------|-----------------|--------------|-----------------------|
| Mouse | B220 | RA3-6B2 | BV711, APC eFluor 780 |
| Mouse | IgM | II/41 | APC, BV786 |
| Mouse | IgD | 11-26c.2a | BV650 |
| Mouse | CD3 | 145-2C11 | PerCpCy5.5 |
| Mouse | F4/80 | BM8 | PerCpCy5.5 |
| Mouse | CD38 | 90 | Alexa Fluor 700 |
| Mouse | CD138 | 281-2 | BV605 |
| Mouse | GL7 | GL7 | eFluor450 |
| Mouse | CD73 | Ty-23 | PE-Cy7 |
| Mouse | CD80 | 16-10A1 | FITC |
| Mouse | CD45.1 | A20 | APC eFluor 780 |
| Mouse | CD45.2 | 104 | APC |
| Mouse | Ki67 (ICS) | SolA15 | FITC, APC |
| Mouse | Ig(H+L) (ICS) | | Alexa Flour 350 |
| Human | CD19 | HIB19 | Alexa Flour 700 |
| Human | CD20 | 2H7 | PerCpCy5.5 |
| Human | CD3 | UCHT1 | BV711 |
| Human | CD14 | MOP9 | BV711 |
| Human | CD16 | 3G8 | BV711 |
| Human | IgM | MHM-88 | BV510 |
| Human | IgD | IA6-2 | PE-Cy7 |
| Human | CD27 | 0323 | APC-Cy7 |
| Human | CD21 | BL13 | FITC |

Table 2.1. Mouse and human antibodies

For murine and human samples, the following surface antibodies were used in various combinations (purchased from BD Biosciences, Ebioscience, or Biolegend). For intracellular staining (ICS), cells were fixed and permeabilized with BD Cytofix/Cytoperm and washed and stained in BD perm buffer

3 Mechanisms of development of *Plasmodium*-specific memory B cells

3.1 Introduction

The generation of effective humoral immunity is the foundation of many successful vaccines against infection [19]. B cell mediated immunity against invading foreign pathogens relies on the generation of pathogen-specific antibody-secreting plasma cells (PCs) and memory B cells (MBCs) that can persist for long periods of time [36]. While pre-existing antibodies secreted by PCs can provide immediate defense against invading pathogens, in the case of insufficient antibody levels MBCs, which maintain their BCR expression, are poised to rapidly encounter antigen, respond, and differentiate into antibody-secreting cells [25, 26, 82]. Immunity to the blood stage of malaria infection, caused by *Plasmodium* parasites, relies heavily on antibody-mediated protection from studies in both humans and mice [58, 63, 68]. We have previously shown that three heterogeneous populations of *Plasmodium*-specific MBCs are generated during blood stage malaria infection in mice [109]. Interestingly, two of three populations were somatically hypermutated, high-affinity CD73⁺CD80⁺ MBCs comprised of both class-switched (swIg⁺) and IgM-expressing cells that were rapid responders to a secondary infection. These populations were distinct from the third subset of *Plasmodium*-specific CD73⁻CD80⁻IgM^{lo}IgD⁺ (IgD⁺) MBCs, which were largely unmutated and naïve-like [109]. These data suggested distinct development histories of IgM⁺ and IgG⁺ MBCs from that of IgD⁺ MBCs. Germinal centers (GCs) are a major influence on MBC formation which aim to select for high-affinity antibody mutants [12]. Additionally, B cell engagement with pathogen-associated molecular patterns (PAMPS) on invading pathogens through toll-like receptor (TLR) recognition can also influence B cell activation and differentiation [110]. We therefore tested how these two processes influence *Plasmodium*-specific IgM⁺, swIg⁺, and IgD⁺ MBC formation, which will be important for driving their formation by vaccination.

MBC formation is complex and can result in phenotypically and functionally heterogeneous populations [36]. Activated B cells that are recruited into the GC reaction undergo multiple rounds of proliferation and somatic hypermutation (SHM), aided through continued interactions with CD4⁺ T follicular helper (Tfh) cells, with the goal of generating a higher-affinity, mutated BCR to the cognate antigen [10, 12]. Through this selection process and successful affinity maturation, B cells typically exit the GC as higher-affinity, isotype switched MBCs or plasma cells. In addition to BCR isotype, increased expression of the surface markers CD80, CD73, and PDL2 correlates with somatic hypermutation and class switching, suggesting their association with GC-derived MBCs as well [32-35]. Conversely, others have shown that activated B cells at the TB border may diverge away from GC entry and form MBCs in a GC-independent fashion, undergoing less affinity maturation and resulting in lower-affinity, largely unswitched MBCs that retain IgM and IgD expression and express lower levels of CD73 [27, 33, 81]. Functionally, MBCs that have class switched, undergone more mutations, and express more CD73, CD80, and PDL2 are faster responders upon recall, whereas unswitched MBCs which display less CD73, CD80, and PDL2 are less mutated and slower to respond, indicating MBCs that undergo GC maturation are programmed differently upon export than GC-independent MBCs [25, 26, 31, 32, 35].

In addition to a BCR, B cells also express toll-like receptors (TLR) that allow them to sense distinct pathogen associated molecular patterns (PAMPs) and integrate both BCR and TLR mediated signals to modify their function [110]. TLR signaling in B cells has been shown to influence TD B cell responses, though different studies have led to different conclusions. A study by Pasare et al. concluded that B cell-intrinsic TLR signaling was required for both IgM and IgG antibody production in a TD protein immunization model and the lack of TLR signaling impaired GC B cell formation [111]. In contrast, others have shown that B cell-specific TLR signaling to be dispensable for antibody generation using different models of TD protein immunization, but primarily looked early in the response [112]. In models of viral infection or

through the use of virus-like particles B cell-intrinsic TLR signaling has been shown to be required specifically during later GC B cell responses and for maintenance of long term IgG antibody levels [113, 114]. These findings suggest B cell TLR signaling may be important later in the immune response after a GC reaction has started and the nature of the immunization or infection, as well as timing, can influence that requirement. Furthermore, how TLR signaling effects the development of heterogeneous MBC subsets during infection remains unclear.

In this study we aimed to better understand the mechanisms that drive the formation of heterogeneous subsets of *Plasmodium*-specific memory B cells during blood stage malaria infection. We specifically focused on understanding the contribution of two major processes, GC dependence and TLR signaling, to MBC formation during blood stage malaria infection and secondary responses to malaria rechallenge. Our results show that while *Plasmodium*-specific MBC formation was a CD4⁺ T cell dependent process, CD73⁺CD80⁺ sIg⁺ and IgM⁺ MBCs were GC-dependent whereas CD73⁻CD80⁻IgD⁺ MBCs were primarily GC-independent. Interestingly, we also found TLR signaling to be important for the generation of GC-derived MBCs. As a result, GC-dependent MBC formation was required for the rapid secondary response to a malaria rechallenge further highlighting the important role of CD73⁺CD80⁺ MBCs. These results shed more light on the mechanisms of *Plasmodium*-specific MBC formation and a better understanding of these mechanisms is important for the targeting of vaccine strategies to promote optimal anti-malarial MBC formation.

3.2 Results and Discussion

3.2.1 Maintenance of MSP1-specific B cells is CD4⁺ T cell dependent

To initially address the mechanisms of differentiation of MSP1-specific B cell, we first investigated their dependency on CD4⁺ T cells both for their expansion and maintenance. To test this C57BL/6 (B6) wild type (WT) mice or major histocompatibility complex II deficient

(MHCII^{-/-}) mice, which lack CD4⁺ T cells [115], were infected with 1x10⁶ *Plasmodium chabaudi* (Pc) infected red blood cells (iRBCs). Both parasitemia and MSP1-specific B cells were analyzed early at the peak of infection (day 8) or at later time points after control of infection (day 20+) based on previously established parasitemia measurements in WT mice [92, 109]. The initial onset of infection, ascending parasitemia, and peak parasitemia were comparable in both WT and MHCII^{-/-} mice during the first 8 days of infection. Thereafter, while WT mice were capable of quickly controlling the infection to sub-patent levels by days 18-20 post infection (p.i.), MHCII^{-/-} mice were unable to control infection and parasitemia remained exacerbated (**Fig. 3.1A**). When analyzing the MSP1-specific B cell response we observed that MSP1⁺ B cells were equally expanded in WT and MHCII^{-/-} mice 8 days p.i., but by day 20 p.i. MSP1⁺ B cells remained expanded in WT mice whereas MHCII^{-/-} B cells had significantly contracted (**Fig. 3.1B**).

To rule out any potentially confounding effects of high antigen load on MSP1-specific B cells at later timepoints in MHCII^{-/-} mice, we also generated mixed bone marrow (BM) chimeras in which WT and MHCII^{-/-} BM cells were injected into irradiated WT mice to analyze MSP1-specific B cells in an infection controlled environment due to the presence of WT cells in the chimeric mice. Upon reconstitution, WT:MHCII^{-/-} chimeras were infected with 1x10⁶ Pc-iRBCs and MSP1-specific B cells were analyzed early (day 8) and late (day 30) after infection. Similar to what we observed in the non-chimeric setting, 8 days post infection both WT and MHCII^{-/-} MSP1⁺ B cells expanded to similar numbers whereas by day 30 while the WT cells remained expanded the MHCII^{-/-} B cells were significantly contracted (**Fig. 3.1C**). Phenotypically, at day 8, WT MSP1⁺ B cells were comprised of CD138⁺ plasma cells (PCs), CD138⁺CD38⁺GL7⁻ memory B cells (MBCs), and CD138⁺CD38⁺GL7⁺ precursors B cells, which are capable of forming germinal center (GC) B cells and MBCs [33]. Similar to WT cells, MHCII^{-/-} B cells were also capable of forming MBCs and PCs, though to a lesser extent (**Fig. 3.1D**). MHCII^{-/-} B cells were however unable to form precursor cells, indicating their generation was T-dependent. By day 30,

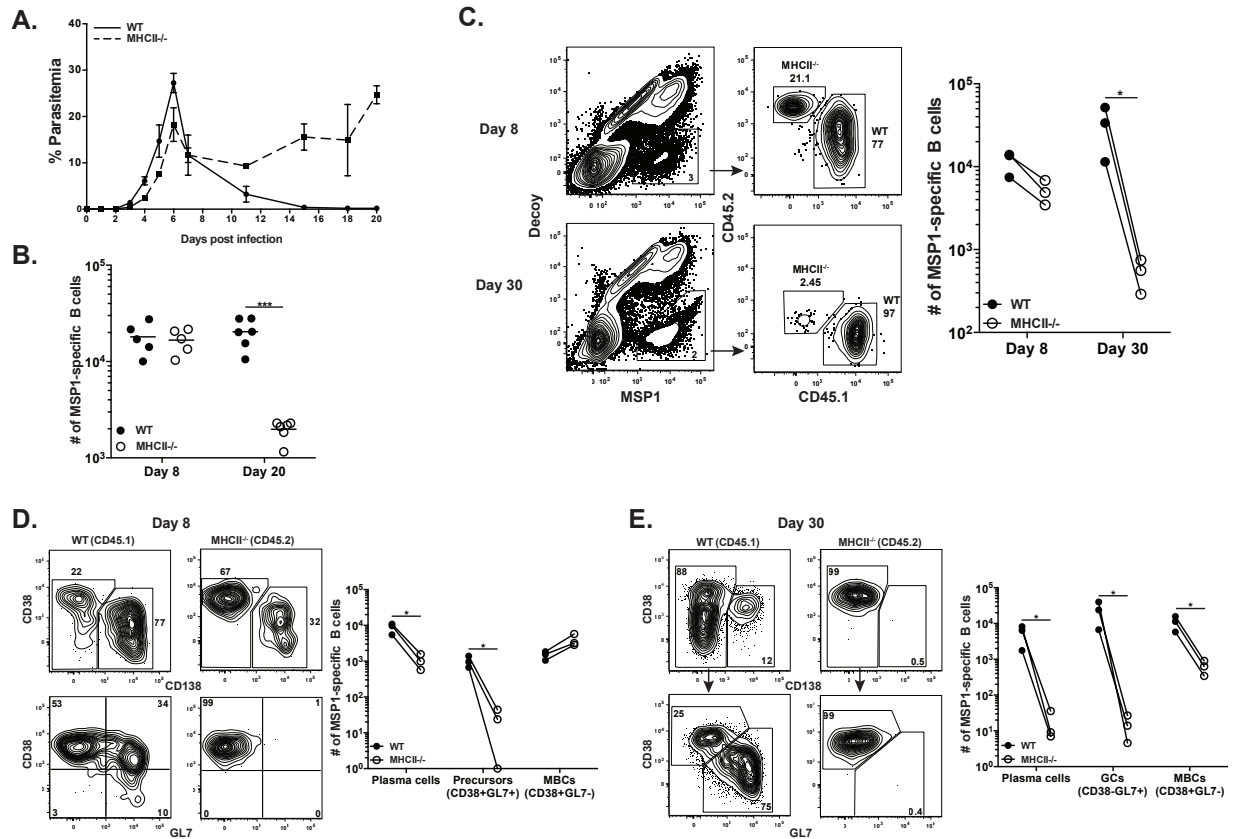


Figure 3.1. CD4⁺ T cell dependence of MSP1-specific B cells

(A) Parasitemia of WT or MHCII^{-/-} mice infected with 1×10^6 *P.chabaudi* iRBCs over 20 days.

Each data point shows mean \pm SEM (B) Total number of MSP1⁺ B cells from WT or MHCII^{-/-} on days 8 or 20 post infection (p.i.). Lines indicates mean. (C-E) Data from WT:MHCII^{-/-} mixed bone marrow chimeras showing (C) Representative plots of total MSP1⁺ B cells and percent of MSP1⁺ B cells from WT or MHCII^{-/-} cells on days 8 or 30 p.i. (left). Total number of MSP1⁺ B cells on days 8 or 50 p.i. (right) from individual WT:MHCII^{-/-} chimeric mice (right). (D)

Representative plots (left) and total number from individual WT:MHCII^{-/-} chimeric mice (right) of MSP1⁺ CD138⁺ plasma cells (top) and CD138⁻CD38⁺ MBCs, CD38⁺GL7⁺ precursor cells, and CD38⁻GL7⁺ GC B cells from WT or MHCII^{-/-} cells on day 8 p.i. (E) Representative plots (left) and total number from individual WT:MHCII^{-/-} chimeric mice (right) of MSP1⁺ CD138⁺ plasma cells (top) and CD138⁻CD38⁺ MBCs and CD38⁻GL7⁺ GC B cells from WT or MHCII^{-/-} cells on day 30 p.i.

*p<0.05, ***p<0.001

however, while WT cells were now comprised of CD138⁻CD38⁻GL7⁺ GC B cells as well as PCs, MBCs, MHCII^{-/-} B cells were devoid of all three populations of PCs, MBCs, and GCs (**Fig. 3.1E**). Taken together these data show that early MSP1-specific B cell expansion is primarily independent of MHCII, indicating T-independence, while later maintenance and subsequent GC, PC, and MBC formation requires MHCII expression, indicating CD4⁺ T cell dependence, even when parasitemia was controlled to subpatent levels.

3.2.2 Germinal center dependence of MSP1-specific B cells.

The loss of MSP1-specific B cells in the absence of CD4⁺ T cell help later during infection (**Fig. 3.1**) correlated with GC B cell formation in WT mice [109]. The observed loss of not only MSP1⁺ GC B cells, but also MSP1⁺ PCs and MBCs, led us to question the dependence on germinal center formation, which relies on CD4⁺ T cells [16], on later MSP1-specific B cell differentiation. To investigate this we generated mice with a B cell-specific deletion of *Bcl6*, a transcription factor necessary for GC B cell development [28]. To accomplish this, mice with the zinc finger-encoding exons of *Bcl6* gene flanked by loxP sites (*Bcl6^{flf}*) [116] were crossed with *Mb-1^{cre/+}* mice, deleting *Bcl6* specifically in B cells [117] (referred to as *Bcl6^{BKO}*). We then generated mixed BM chimeras with *Bcl6^{BKO}* and control *Mb-1^{cre/+}Bcl6^{+/+}* (referred to as *Bcl6^{WT}*) WT cells. Creating a B cell-specific knockout of *Bcl6* was important as CD4⁺ T follicular helper (Tfh) cells, which also rely on the upregulation of *Bcl6* for their formation and are required for GC formation would not be compromised in this chimeric setting [11, 118]. Upon reconstitution, mixed chimeras were infected with 1x10⁶ Pc-iRBCs and MSP1⁺ B cells were analyzed 10-12 weeks later. Within the chimeras, *Bcl6^{WT}* cells made up the majority of all MSP1⁺ B cells, both by percentage and number, compared to MSP1⁺*Bcl6^{BKO}* cells (**Fig. 3.2A,B**). Phenotypically MSP1⁺*Bcl6^{BKO}* B cells were unable to form GC B cells, as expected, but also showed significant defects in both PC and MBC formation as compared to *Bcl6^{WT}* B cells (**Fig. 3.2C,D**). These results suggest that not only do *Bcl6^{BKO}* B cells have impairment in GC formation but

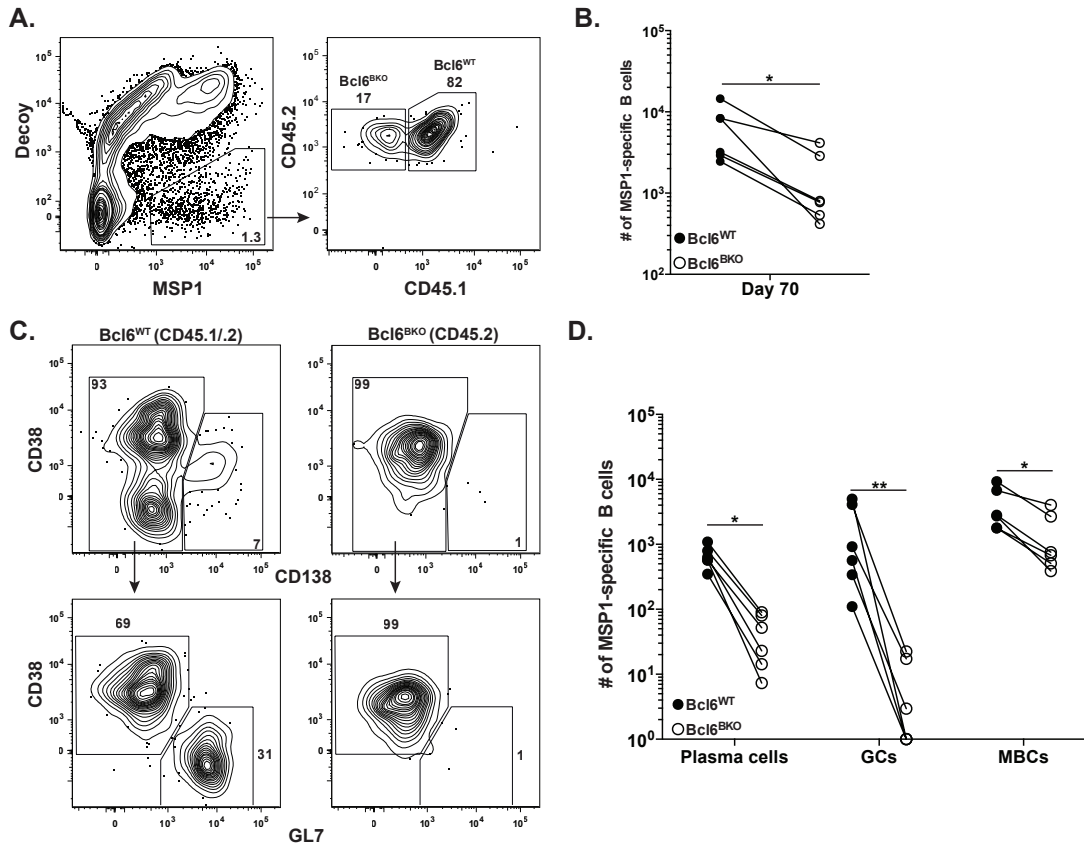


Figure 3.2. GC-dependent MSP1⁺ B cell formation

(A) Representative plots in $Bcl6^{WT}:Bcl6^{BKO}$ mixed bone marrow chimeras of MSP1⁺ B cells and percent of MSP1⁺ B cells from $Bcl6^{WT}$ or $Bcl6^{BKO}$ cells on day 70 p.i. (B) Total number of MSP1⁺ B cells from individual $Bcl6^{WT}:Bcl6^{BKO}$ chimeric mice on day 70 p.i. (C) Representative plots and (D) total number from individual $Bcl6^{WT}:Bcl6^{BKO}$ chimeric mice of MSP1⁺CD138⁺ plasma cells (top) and CD138⁻CD38⁺ MBCs and CD38⁻GL7⁺ GC B cells from $Bcl6^{WT}$ or $Bcl6^{BKO}$ cells on day 70 p.i. * $p < 0.05$, ** $p < 0.01$

subsequent GC-dependent PC and MBC formation are compromised as well, further supporting our results from the WT:MHCII^{-/-} chimeras.

3.2.3 *IgM⁺ and swlg⁺ MBCs are GC-dependent.*

To determine the impact of GC B cell ablation specifically on the types of MBC that can form, we characterized the makeup of MSP1-specific MBC subsets from infected chimeric mice derived from either Bcl6^{WT} or Bcl6^{BKO} cells. We initially focused on MBC subsets defined by their expression of IgM and IgD [26, 109]. Phenotypically Bcl6^{WT} MSP1-specific MBCs were comprised of three distinct subsets: swlg⁺ (IgM⁻IgD⁻), IgM⁺ (IgM⁺IgD^{lo}), and IgD⁺ (IgD⁺IgM^{lo}) MBCs, whereas Bcl6^{BKO} MSP1-specific MBCs were incapable of forming swlg⁺ MBCs but IgM⁺ and IgD⁺ MBCs remained present (**Fig. 3.3A**). Quantitatively, this resulted in significantly less swlg⁺ and IgM⁺ MBCs from Bcl6^{BKO} B cells as compared to Bcl6^{WT} B cells whereas equal numbers of IgD⁺ MBCs formed in both compartments (**Fig. 3.3B**). While the impact on swlg⁺ MBCs in the absence of Bcl6 was expected, the finding that a significant proportion of unswitched IgM⁺ MBCs were also affected by the loss of Bcl6 was in contrast to previous studies suggesting that unswitched MBCs are predominantly GC-independent [27, 33]. Our previous findings however revealed that when distinguishing MSP1-specific unswitched MBCs as two distinct populations (IgM⁺ and IgD⁺), IgM⁺ MBCs displayed high levels of CD73 and CD80, two markers associated with GC-dependence and expression of the mutator enzyme activation-induced cytidine deaminase (AID) [32-35, 109]. This led us to hypothesize that the loss of IgM⁺ MBCs from Bcl6^{BKO} cells was due to a lack of GC-derived CD73⁺CD80⁺IgM⁺ MBCs. Further examination of CD73 and CD80 on MSP1⁺ MBCs from Bcl6^{WT} and Bcl6^{BKO} cells showed this to be the case as the IgM⁺ MBCs found from Bcl6^{BKO} cells distinctly lacked CD73 and CD80 expression as compared to Bcl6^{WT} IgM⁺ MBCs (**Fig. 3.3C,D**). Together, these data suggest that swlg⁺ MBCs and CD73⁺CD80⁺IgM⁺ MBCs are derived from GC B cells whereas IgD⁺ MBCs are primarily GC-independent.

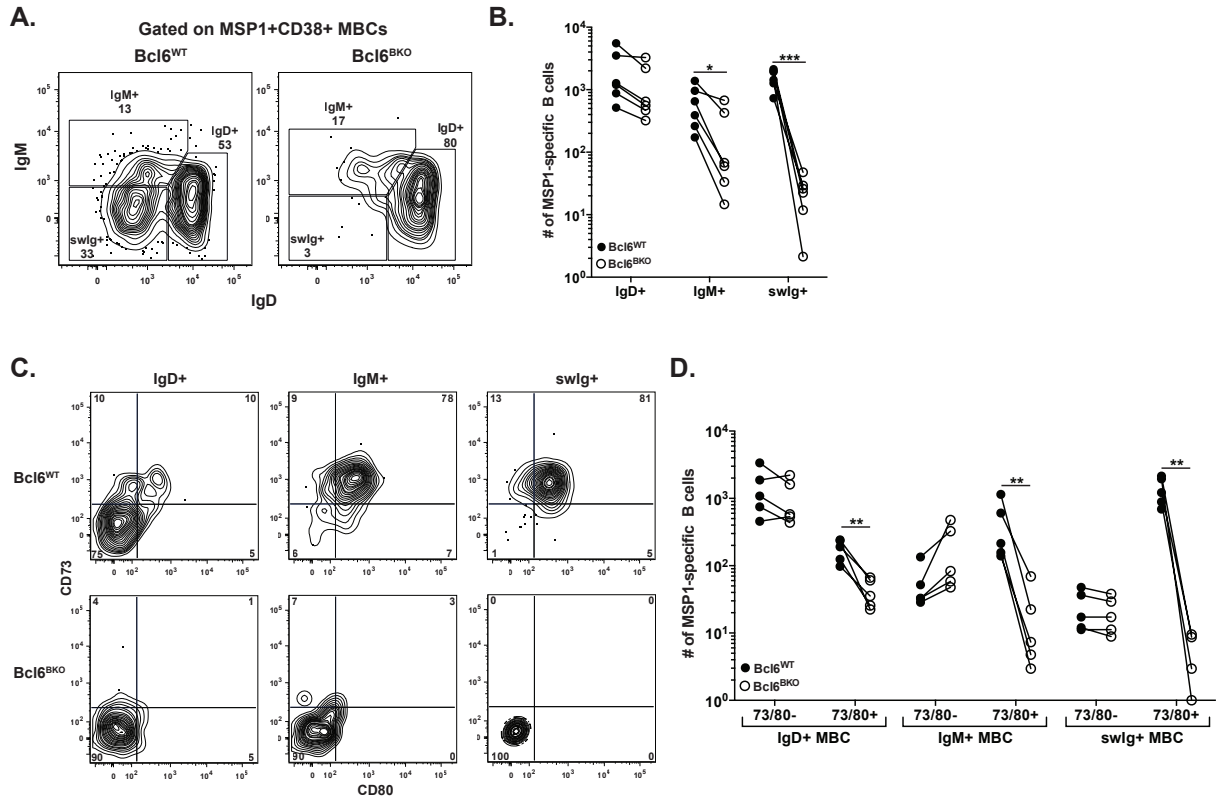


Figure 3.3. GC-dependent MSP1⁺ memory B cells

Representative plots in Bcl6^{WT}:Bcl6^{BKO} mixed bone marrow chimeras of (A) MSP1⁺CD38⁺ MBCs showing expression of IgM and IgD to identify IgD⁺, IgM⁺, and swIg⁺ MBCs from Bcl6^{WT} or Bcl6^{BKO} cells on day 70 p.i. (B) Total number of MSP1⁺CD38⁺ IgD⁺, IgM⁺, and swIg⁺ MBCs from individual Bcl6^{WT}:Bcl6^{BKO} chimeric mice on day 70 p.i. (C) Representative plots of CD73 and CD80 expression on MSP1⁺CD38⁺ IgD⁺, IgM⁺, and swIg⁺ MBCs from Bcl6^{WT} or Bcl6^{BKO} cells on day 70 p.i. (D) Total number of CD73⁺CD80⁺ or CD73⁻CD80⁻ cells from MSP1⁺CD38⁺ IgD⁺, IgM⁺, or swIg⁺ MBCs from individual Bcl6^{WT}:Bcl6^{BKO} chimeric on day 70 p.i. *p<0.05, **p<0.01

3.2.4 *Myd88* deficiency results in defective GC-dependent MBC output.

Given these findings, we next asked what additional mechanisms were influencing GC-dependent MBC output, including non-BCR mediated signals. Toll-like receptor (TLR) signaling in B cells has been previously shown to influence TD B cell responses during protein immunization, though different studies have led to different conclusions. A study by Pasare et al. concluded that B cell-intrinsic TLR signaling was required for optimal antibody production in a TD protein immunization model [111]. In contrast, others have shown that B cell-specific TLR signaling to be dispensable during TD responses [112, 119]. In models of viral infection or immunization with virus-like particles, B cell-intrinsic TLR signaling has been shown to be required for effective GC B cell responses and long term humoral immunity [113, 114], suggesting perhaps TLRs may be important later in the immune response after a GC reaction has started. Taken together these studies demonstrate that TLR signaling can impact TD B cell responses and the nature of the immunization or infection, as well as timing, can influence that requirement.

To address the impact of TLR signaling on MSP1-specific MBC development during malaria infection, mixed bone marrow chimeras were generated with WT and myeloid differentiation primary-response protein 88 deficient (*Myd88*^{-/-}) BM, a critical TLR signaling adaptor [110]. Upon reconstitution, mixed WT:*Myd88*^{-/-} chimeras were infected with 1x10⁶ Pc iRBCs and MSP1-specific B cells were analyzed 70 days later. At this timepoint, WT MSP1⁺ B cells were at a numerical advantage to MSP1⁺ *Myd88*^{-/-} B cells suggesting TLR deficiency impairs MSP1⁺ B cell maintenance (**Fig. 3.4A**). Phenotypically, *Myd88*^{-/-} B cells displayed an impaired ability to form PC, GC B cell, and MBC populations as compared to WT counterparts (**Fig. 3.4B**). To determine the impact on specific subsets of MBCs, we further analyzed MSP1⁺ WT and *Myd88*^{-/-} MBCs and found that IgM⁺ and swlg⁺ MBC formation was impaired while IgD⁺ MBC formation was not impacted (**Fig. 3.5A, 3.5B**). This same impact was observed when

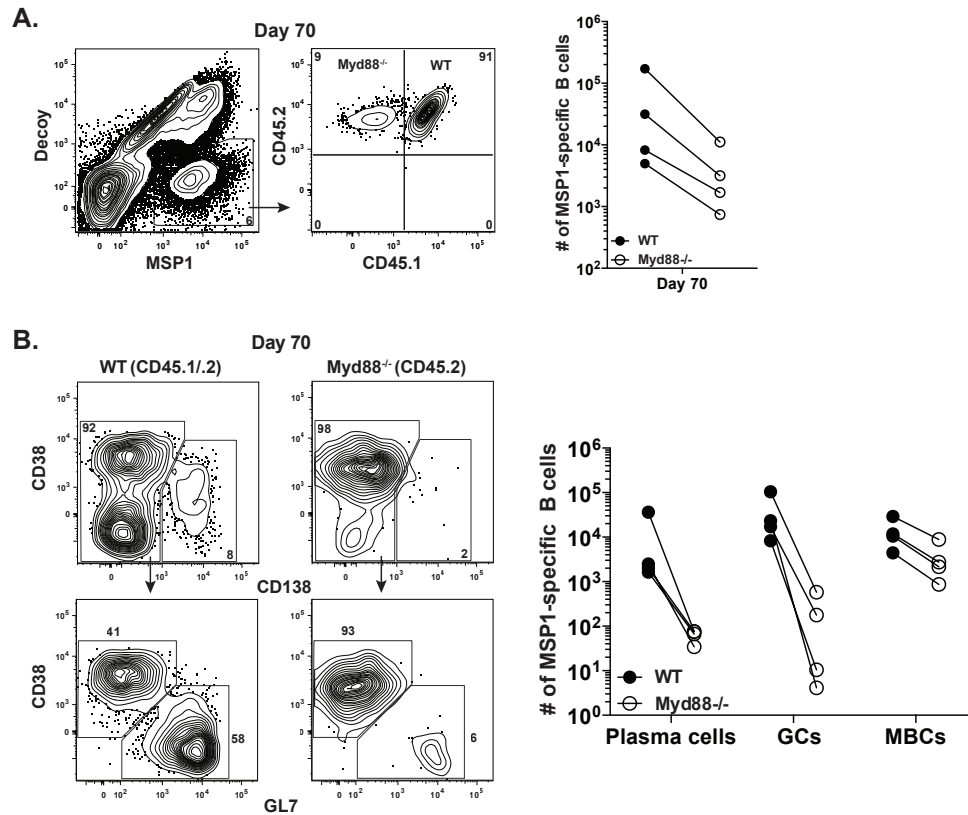


Figure 3.4. Myd88 deficiency affects late MSP1⁺ B cell differentiation

Data from WT:Myd88^{-/-} mixed bone marrow chimeras showing (A) Representative plots of total MSP1⁺ B cells and percent of MSP1⁺ B cells from WT or Myd88^{-/-} cells on day 70 p.i. (left). Total number of MSP1⁺ B cells on day 70 p.i. (right) from individual WT:Myd88^{-/-} chimeric mice (right). (B) Representative plots (left) and total number (right) from individual WT:Myd88^{-/-} chimeric mice of MSP1⁺CD138⁺ plasma cells, CD138⁻CD38⁺ MBCs, and CD38⁻GL7⁺ GC B cells from WT or Myd88^{-/-} cells on day 70 p.i.

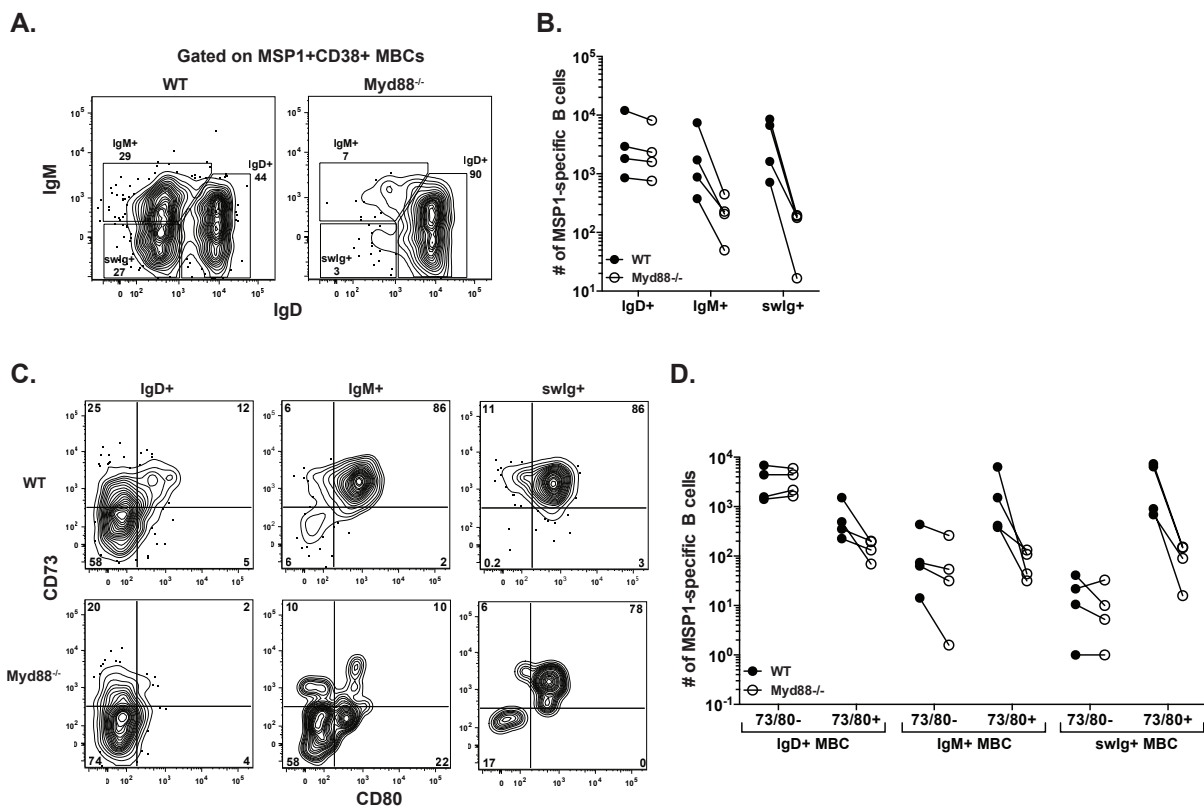


Figure 3.5. GC-dependent MSP1⁺ MBC are also affected by Myd88 deficiency

Representative plots in WT:Myd88^{-/-} mixed bone marrow chimeras of (A) MSP1⁺CD38⁺ MBCs showing expression of IgM and IgD to identify IgD⁺, IgM⁺, and swlg⁺ MBCs from WT or Myd88^{-/-} cells on day 70 p.i. (B) Total number of MSP1⁺CD38⁺ IgD⁺, IgM⁺, and swlg⁺ MBCs from individual WT:Myd88^{-/-} chimeric mice on day 70 p.i. (C) Representative plots of CD73 and CD80 expression on MSP1⁺CD38⁺ IgD⁺, IgM⁺, and swlg⁺ MBCs from WT or Myd88^{-/-} cells on day 70 p.i. (D) Total number of CD73⁺CD80⁺ or CD73⁻CD80⁻ cells from MSP1⁺CD38⁺ IgD⁺, IgM⁺, or swlg⁺ MBCs from individual WT:Myd88^{-/-} chimeric on day 70 p.i.

MBCs were analyzed for their expression of CD73 and CD80 (**Fig. 3.5C, 3.5D**). Together these data suggest that TLR signaling required for later B cell differentiation. Furthermore, the specific impact observed later during infection correlated with populations previously found to be GC-dependent, indicating the importance of TLR signaling within the GC to generate both GC-dependent MBCs and PCs. Importantly, similar analyses will need to be performed at more early timepoints during infection to determine the specific impact of Myd88 deficiency. This will allow us to determine if the lack of TLR signaling impairs the MSP1-specific B cells response prior to GC formation or if the impact is specific to later timepoints when GC B cells have already formed.

3.2.5 *GC-derived MBCs mediate secondary response to rechallenge.*

We next wanted to assess the contribution of GC-derived MBCs to a secondary challenge. Previously it was observed that within three days after malaria rechallenge, MSP1-specific MBCs expanded rapidly to form two distinct populations of CD138+ antibody secreting populations: B220+ plasmablasts and B220- plasma cells [109]. To test whether GC-derived memory B cell populations were mediating early recall responses, previously infected mixed Bcl6^{WT}:Bcl6^{BKO} chimeric mice were given a secondary malaria challenge and MSP1-specific B cells were analyzed three days later. As observed before, MSP1⁺Bcl6^{WT} B cells were capable of undergoing a recall response within three days of challenge, and formed both expanded B220+ plasmablasts and B220- plasma cells. Additionally, the isotype of the secondary response of Bcl6^{WT} B cells was IgM-dominant. In contrast, MSP1⁺Bcl6^{BKO} B cells were unable to mediate a similar response (**Fig. 3.6A, 3.6B, 3.6C**). Taken together these findings suggest that GC-derived MBCs are required for a rapid secondary response and further support the notion that the GC-derived IgM⁺ MBCs and not IgD⁺ MBCs is the unswitched MBC population responsible for the early IgM-dominant secondary response.

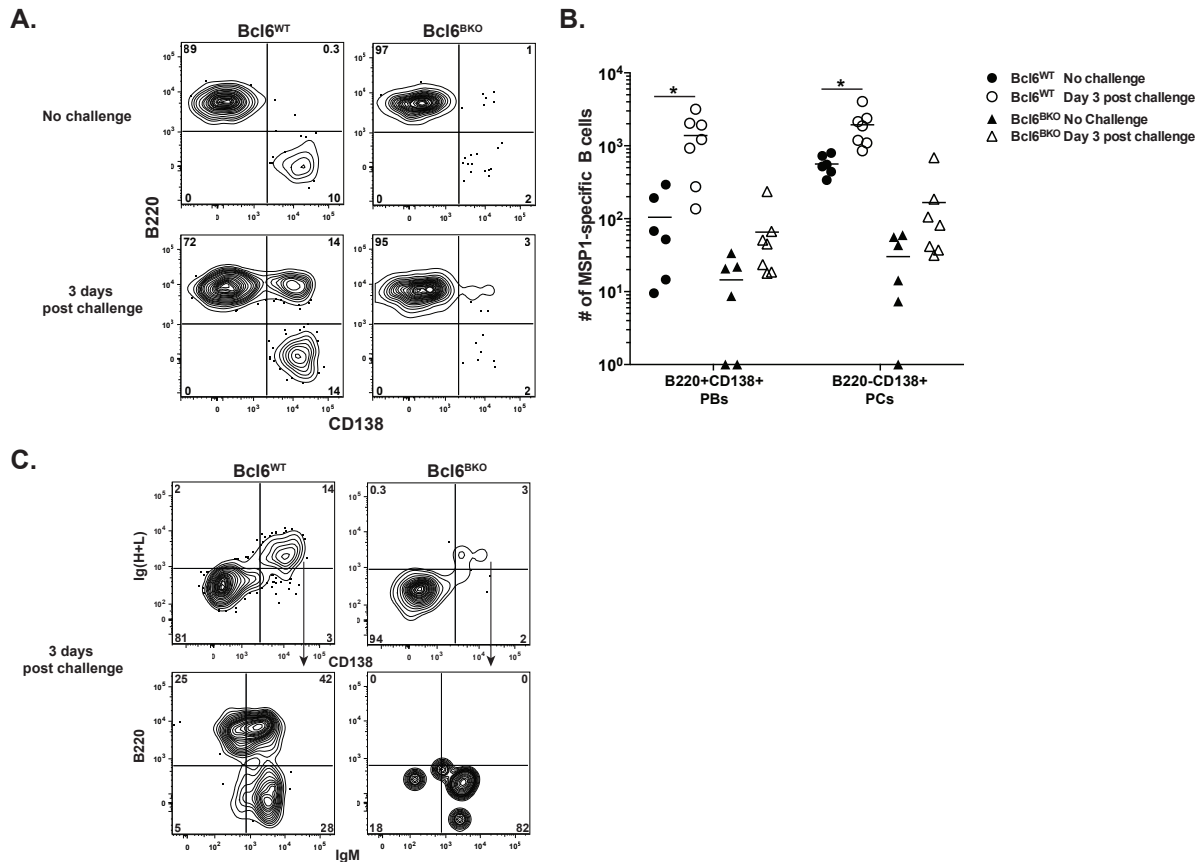


Figure 3.6. GC-dependent MBCs mediate rapid secondary response to malaria rechallenge.

(A) Representative plots of B220 by CD138 on MSP1⁺ B cells from *Bcl6*^{WT} or *Bcl6*^{BKO} cells and (B) Total number of MSP1⁺ B220⁺CD138⁺ PBs and B220⁻CD138⁺ PCs and (C) Representative plots of intracellular Ig(H+L) (Ig) and CD138 expression of MSP1⁺ B cells (top) and B220 by IgM expression of MSP1⁺Ig⁺CD138⁺ cells (bottom) from *Bcl6*^{WT} or *Bcl6*^{BKO} cells in unchallenged *Bcl6*^{WT}:*Bcl6*^{BKO} chimeric mice or *Bcl6*^{WT}:*Bcl6*^{BKO} chimeric mice rechallenged with 1x10⁷ iRBCs analyzed 3 days later. *p<0.05

3.3 Discussion

Here we have shown that heterogeneous subsets of *Plasmodium*-specific MBCs form via distinct mechanisms after blood stage malaria infection in mice. Unswitched IgD⁺ MBCs were primarily GC-independent while swIg⁺ MBCs were GC-dependent, which was in line with previous findings [27, 33, 81]. Interestingly, however, not all unswitched MBCs were GC-independent but IgM⁺ MBCs that express CD73 and CD80 were also GC-dependent. These results are supported by previous findings that MSP1-specific CD73⁺CD80⁺IgM⁺ MBCs display somatically hypermutated, high-affinity BCRs, two attributes of MBCs more likely acquired as a result of being in the GC reaction [109]. While the expectation that unswitched MBCs are generally thought to be GC-independent, by recognizing MSP1-specific unswitched MBCs as two distinct subsets of IgM and IgD expressing cells we have revealed a novel population of IgM⁺ GC-dependent MBCs.

One goal of the GC is to mature the entering B cells into higher-affinity cells upon export through iterative cycles of BCR mutation, class-switch recombination (CSR), proliferation, and selection [13]. The finding of a high-affinity, somatically hypermutated GC-derived MBC that remains IgM⁺ warrants further investigation into why some cells remain as IgM⁺ and others switch. Temporally, a study by Weisel et al. observed that IgM⁺ MBCs formed prior to IgG⁺ MBCs in response to hapten-protein immunization [29]. Within the GC, B cells that are able to present higher affinity peptide:MHCII complexes receive enhanced T cell help and move through the GC reaction faster [120]. Additional signals between GC B cells and Tfh cells beyond peptide:MHCII interactions can also significantly impact GC B cell longevity and high-affinity B cell output, such as receptor-ligand pair interactions, through CD40 or ICOS [15], and cytokine signaling, such as IL-21 or IL-4 [121, 122]. As a result, precursor BCR affinity of the B cell that enters into the GC could play a significant role in the nature of Tfh help it receives. It may be that the B cells that are ultimately exported as matured IgM⁺ MBCs were already of a

certain affinity prior to GC, were able to get strong T cell help, and did not require extensive SHM or class-switch recombination (CSR) to form a high-affinity MBC and were exported earlier. GC B cells that were of lower affinity would then spend more time within the GC, undergoing more rounds of proliferation, selection, and T cell help to then be later exported as class-switched MBCs. In our previous study we observed that blood stage malaria infection induces a GC reaction that lasted for up to ~150 days [109]. This is in contrast to protein immunization which induces a much shorter GC reaction [33]. These kinetics may be significant as a recent study by Weinstein et al. showed that over time as the GC reaction matures, GC Tfh cells also mature phenotypically and transcriptionally to provide stronger T cell help to B cells through IL-4 and CD40 ligand interactions potentially bringing lower affinity B cells into the later response [123].

Interestingly both GC-derived IgM⁺ and swIg⁺ MBCs were also diminished in B cells lacking Myd88, signifying TLR signaling may contribute to the programming of T cell dependent and GC-dependent MBC output. A study by Meyer-Bahlburgh et al. showed expression of Myd88 is highly increased on GC B cells while IRAK-M, a negative regulator of TLRs, is decreased [119]. In support of this finding, BCR signaling is largely inhibited in GC B cells through Shp1 phosphatase activity making the primary role of the BCR to capture antigen for MHCII presentation to Tfh cells [14, 124]. Thus, enhanced TLR signaling in GC B cells may serve to compensate for any mitigated BCR-mediated signals, though how the integration of these various signals would impact GC B cell programming and subsequent MSP1-specific MBC output will need to be further explored.

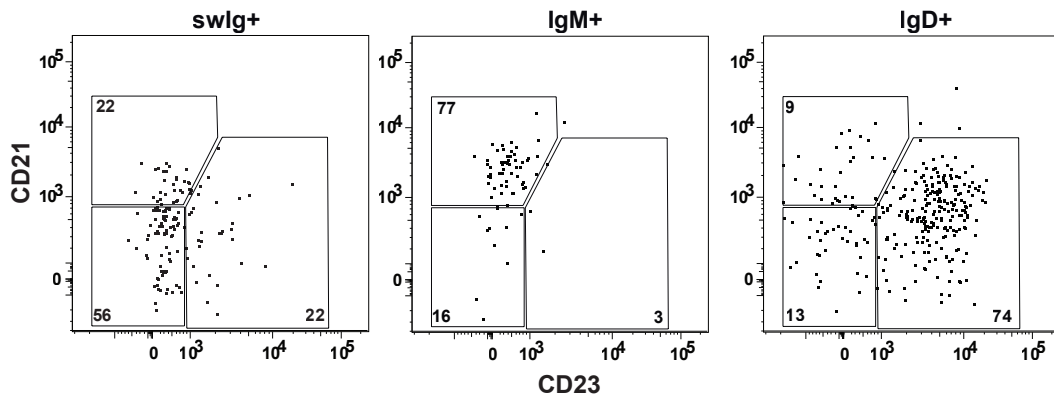
The importance of generating a GC-dependent MBCs is further supported by the loss of a rapid MSP1-specific B cell response during rechallenge by Bcl6^{BKO} MBCs. This finding is in line with evidence that more affinity matured MBCs are more likely to respond faster during recall [25, 26, 31, 35]. The IgM-dominant secondary response also leads to ask what it is about the programming of an IgM⁺ MBC that makes them a more rapid responder than swIg⁺ MBCs

during secondary infection. Given that malaria is an infection of red blood cells, B cells positioned within the marginal zone (MZ) of the spleen would allow for rapid antigen capture [125]. MZ B cells are defined by their expression of the complement receptor CD21 (CR2), the nonclassical major histocompatibility complex (MHC) class I molecule CD1d, and low levels of the Fc receptor, CD23. We observed that ~77% of MSP1⁺IgM⁺ MBCs are CD21⁺CD23⁻ and expressed higher levels of CD1d, indicative of a MZ B cell phenotype (**Fig. 3.7**). MZ B cells are also known to express high levels of TLRs and are specialized in capturing complement-opsonins via CD21 [126]. Combining these attributes to an affinity matured GC-dependent IgM⁺ MBC could lead to a program of a more rapidly responsive cell upon recall.

Targeting the production of high-affinity IgM⁺ MBCs by vaccination could prove highly effective against an infection like *Plasmodium* due to IgM's ability pentamerize upon secretion and as an efficient complement mediator [127]. Once within the GC, given the multiple signals B cells can integrate both intrinsically and extrinsically, it will be important to interrogate the specific factors that determine whether MSP1-specific MBCs remain as IgM⁺ or undergo class switching and how those signals program the MBC for the appropriate recall response. Additionally, further studies are warranted to better understand the types of *Plasmodium*-specific CD4⁺ T cells that promote GC-dependent MBC output since interactions with CD4⁺ T cells are necessary for the production of these GC matured populations, both for primary and secondary responses [109]. A vaccine strategy that ultimately generates high-affinity CD4⁺ T cell and MBC populations may be the key to conferring long-term protection to humans at highest risk for malaria infection.

A.

Day 100: Gated on MSP1+CD38+ MBCs



B.

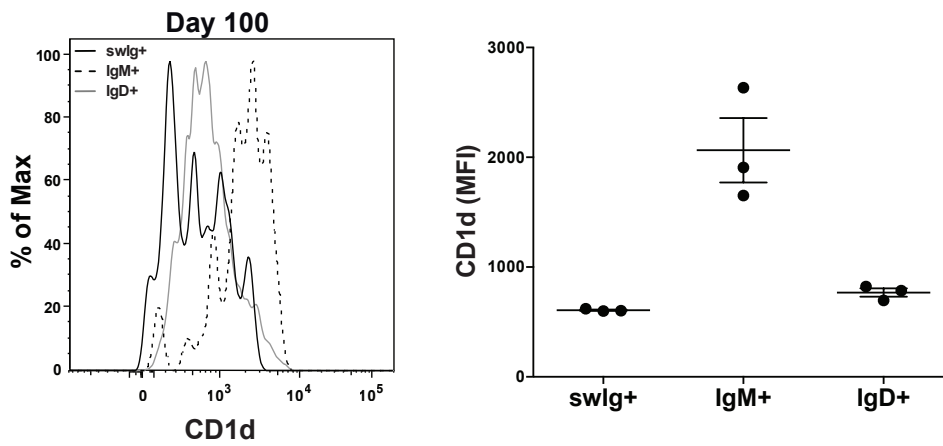


Figure 3.7. MSP1⁺IgM⁺ MBCs cells phenotypically resemble marginal zone B cells

(A) Representative plots of CD21 and CD23 expression and (B) CD1d MFI expression on MSP1⁺CD38⁺ IgD⁺, IgM⁺, and swlg⁺ MBCs 100 days p.i.

3.4 Methods

Mice

Female C57BL/6 (CD45.2), B6.SJL-Ptprc^a Pepc^b/BoyJ (CD45.1⁺), B6.129S2-*Ciita*^{tm1Ccum/J} (MHCII^{-/-}) mice were purchased from The Jackson Laboratory. MB1^{cre/+} mice were provided by Dr. David Rawlings (Seattle Children's Research Institute) and crossed to Bcl6^{flox/flox} mice, provided by Dr. Alexander Dent (Indiana University), to generate MB1^{cre/+}Bcl6^{flox/flox} mice and MB1^{cre/+}Bcl6^{+/+} mice. Myd88^{-/-} mice were provided by Dr. Michael Gale (University of Washington). All mice were maintained/bred under specific pathogen free conditions at the University of Washington and all experiments were performed in accordance with the University of Washington Institutional Care and Use Committee guidelines.

Bone Marrow Chimeras

Bone marrow cells were collected from the tibia, femur, humeri, and sternum and labeled with anti-Thy1.2 (30-H12, Ebioscience) and NK1.1 (PK136, Ebioscience). Cells were resuspended and incubated with low toxicity rabbit complement (Cedarlane Laboratories). After complement lysis, cells were washed with media containing 10% fetal calf serum. Recipient mice were lethally irradiated (1000 rads) and injected with 5x10⁶ total bone marrow cells with WT cells mixed with MHCII^{-/-} or Myd88^{-/-} cells mixed in equal portions and given antibiotic treated water for 6 weeks. For Bcl6^{WT}:Bcl6^{BKO} chimeras, MB1^{cre/+}Bcl6^{+/+} and MB1^{cre/+}Bcl6^{flox/flox} cells were mixed in equal proportion and injected into irradiated recipients.

Tetramers

Purified recombinant His-tagged C-terminal MSP1 protein (amino acids 4960 to 5301) [76] was biotinylated and tetramerized with streptavidin-PE (Prozyme) as previously described [89, 109]. Decoy reagent to gate out non-MSP1⁺ B cells was made by conjugating SA-PE to AF647 using an AF647 protein labeling kit (ThermoFisher), washing and removing any unbound AF647, and incubating with an excess of an irrelevant biotinylated HIS-tagged protein [89, 109].

Mouse Cell Enrichment and Flow Cytometry

Splenic cell suspensions were prepared and resuspended in 200ul in PBS containing 2% FBS and Fc block (2.4G2) and first incubated with Decoy tetramer at a concentration of 10nM at room temperature for 10 min. MSP1-PE tetramer was added at a concentration of 10nM and incubated on ice for 30 min. Cells were washed, incubated with anti-PE magnetic beads for 30 min on ice, and passed over magnetized LS columns (Miltenyi Biotec) to elute the bound cells as previously described [89, 109]. All bound cells were stained with surface antibodies followed by intracellular antibody staining when needed (**Table 2.1**). All cells were run on the LSR II (BD) and analyzed using FlowJo software (Treestar).

Parasitemia by flow cytometry

Parasitemia was measured by flow cytometry by staining 1ul of blood with Ter119 APC eFluor780 (eBioscience), CD45 APC (BD), Hoechst33342 (Sigma), and Dihydroethidium (Sigma) [109].

4 Characterization of *Plasmodium*-specific memory B cells in malaria infected humans

4.1 Introduction

CD27-expressing MBCs make up approximately 40% of circulating B cells in healthy human peripheral blood. Of these circulating MBCs, ~16% remain unswitched [23, 107]. Although IgG⁺ *Pf*-specific MBCs have been detected by ELISPOT [128] in individuals exposed to both high [63, 129] and low [130-132] malaria transmission, it is unknown if *P. falciparum* infection induces antigen-specific unswitched MBCs as well. The persistence of heterogeneous MBCs after malaria infection in mice led us to ask if similar populations of *P. falciparum* (*Pf*)-specific MBCs also form in exposed individuals residing in a malaria endemic area. To address the question of whether *Pf*-specific MBCs form in malaria-exposed humans, antigen-specific enrichment experiments were performed on peripheral blood mononuclear cells (PBMCs) collected from *P.falciparum*-infected Malian subjects during the malaria season [133] or malaria-naïve U.S. subjects.

4.2 Results and Discussion

4.2.1 *Plasmodium*-specific MBCs are expanded in malaria exposed humans

To enhance the sensitivity of *Plasmodium*-specific B cell detection (less than 20 million PBMC were available in some samples), we generated B cell tetramers using the C-terminal region of MSP1 and apical membrane antigen 1 (AMA1) from the human *P. falciparum* (3D7) strain and B cells specific for either antigen will be termed *Pf*-specific. In Malian subjects, we found that approximately 40% of the *Pf*-specific B cells in blood were CD21⁺CD27⁺ MBCs in keeping with expected frequencies of total MBCs in human blood [21, 134, 135] (**Fig. 4.1A**). Furthermore, there was a 6 fold increase in the total number of *P. falciparum*-specific B cells and a 60-fold increase amongst CD27⁺CD21⁺ MBCs compared to uninfected US control PBMC samples (**Fig. 4.1B**). We further characterized the *P. falciparum*-specific CD27⁺ MBCs from Malian samples

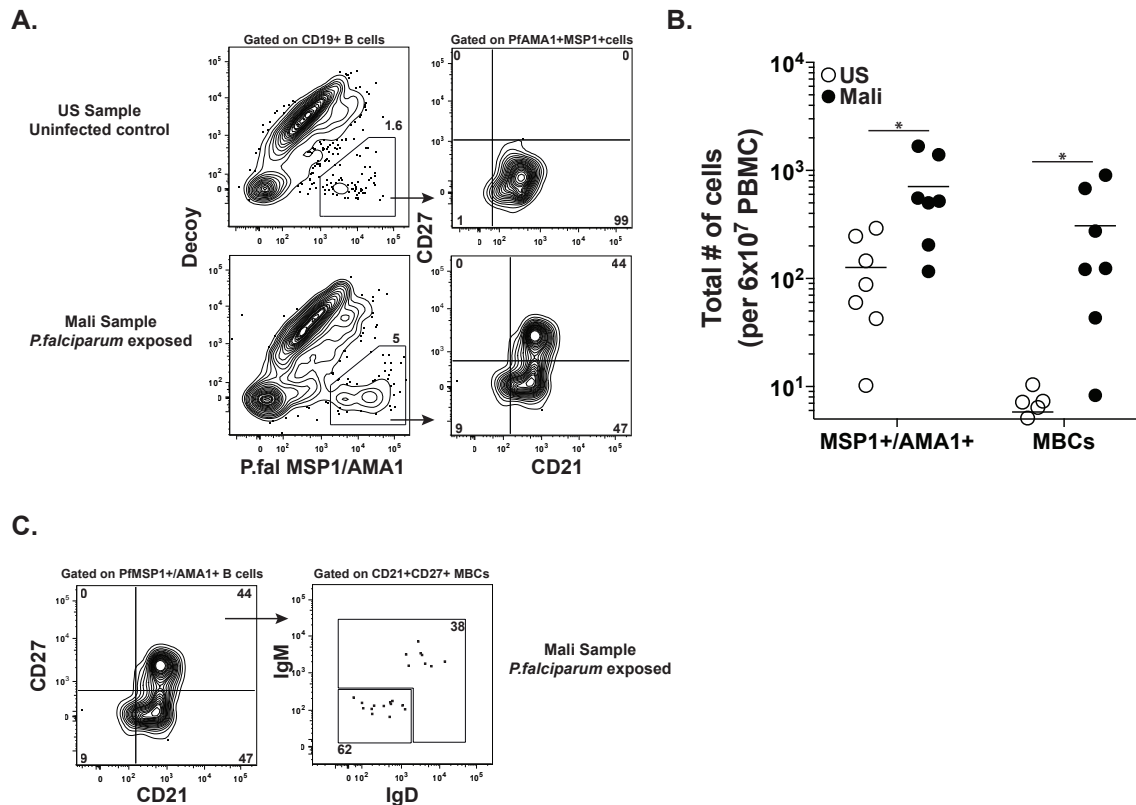


Figure 4.1. Human *Plasmodium*-specific MBCs are phenotypically heterogeneous.

CD19⁺ human B cells identified in US and Mali PBMC after excluding CD3⁺CD14⁺CD16⁺ non-B cells and enrichment with PfMSP1, PfAMA1 and Decoy tetramers. (A) Representative plots of PfMSP1/AMA1⁺ B cells and CD21/CD27 expression. (B) Total PfMSP1/AMA1⁺ B cells and MBCs per 6×10^7 PBMC in US and Mali PBMC. Data is combined from 3 independent experiments with 7 samples per group. Line indicates mean. * $p < 0.05$ (C) Representative plot of IgM and IgD expression on CD27⁺CD21⁺ memory B cells from Mali sample.

for their expression of BCR isotype and found that they were comprised of both switched and unswitched cells (**Fig. 4.1C**). Thus, heterogeneous populations of *Plasmodium*-specific MBCs are expanded in both mice and humans.

4.2.2 *Plasmodium*-specific IgM⁺ and IgG⁺ MBCs display somatic hypermutation

To further establish correlations between our murine studies, we next wanted to determine whether MBCs from malaria infected humans were somatically hypermutated. To do this, *Pf*-specific B cells were enriched from PBMC of nine malaria-exposed Malian humans and single CD27⁺CD21⁺IgM⁺ and CD27⁺CD21⁺IgG⁺ MBCs were individually sorted. We sequenced and cloned the BCRs using previously established methods [97] (**Fig. 4.2A**). Individual *Pf*-specific MBC BCR sequences were then compared to germline sequences to assess relative numbers of mutation. Across all 9 individuals, *P. falciparum*-specific MBCs in humans displayed SHM with IgG⁺ MBCs containing a mean of 8 mutations in the heavy chain (V_H) and IgM⁺ MBCs containing a mean of 3 V_H mutations. (**Fig. 4.2B and Fig. 2.6**).

The initial findings presented here show that *Plasmodium*-specific B cells can be identified in peripheral blood of humans. Malaria-exposed humans display an expanded population of malaria-specific B cells that are comprised of both switched and unswitched populations that were mutated, much like was observed of *Plasmodium*-specific MBCs generated in mice. While these findings validate studies that have shown *Pf*-specific populations are present in humans as seen by ELISPOT assays [63], our approach lets us interrogate the B cell response at the single cell level. Single cell sorting methods allows us to analyze not only the genetic features of the MBC BCR but enables us to express monoclonal antibodies (mAbs) from these cells. Future works will take advantage of mAbs generated to test antigen-specificity, antigen affinity, and for use in functional assays, such as parasite growth inhibition assays or complement-mediated lysis assays. These approaches will allow us to better understand how

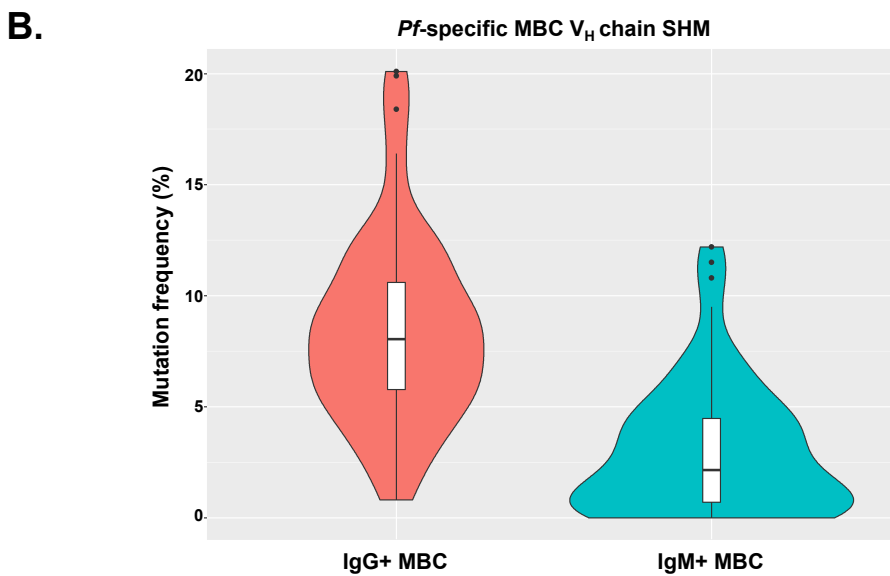
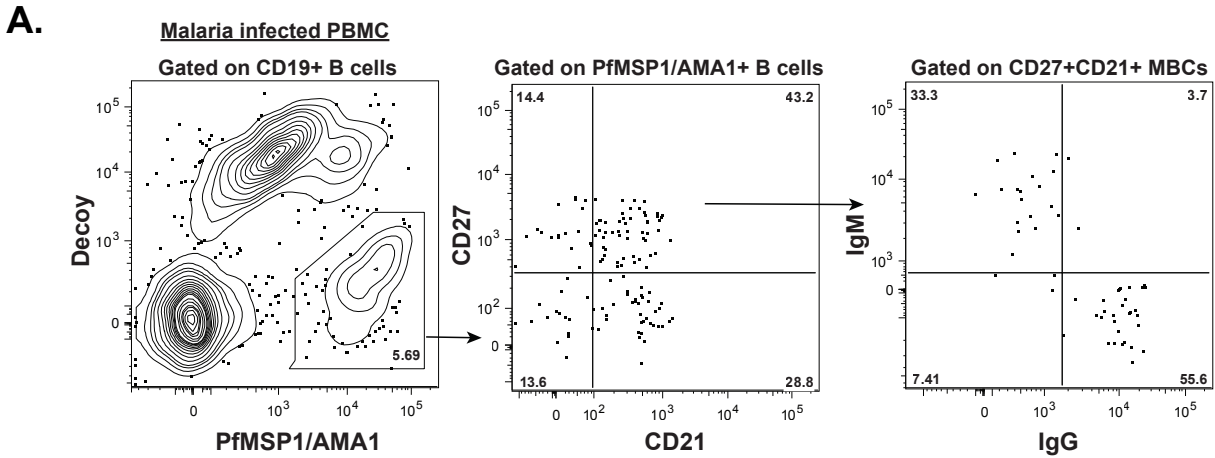


Figure 4.2. IgG⁺ and IgM⁺ *Pf*-specific MBCs are mutated.

(A) Representative gating strategy from one individual to identify and single cell sort

PfMSP1/AMA1⁺CD21⁺CD27⁺ IgM⁺ and IgG⁺ MBCs in malaria-exposed Mali PBMC. (B) Violin plot of number of mutations in the heavy chain (V_H) of individual *Pf*-specific IgG⁺ and IgM⁺ MBCs averaged across nine Mali individuals.

human MBCs develop and function in malaria-infected humans and provide insight into what populations of B cells are most protective against the *Plasmodium* parasite.

4.3 Methods

Human PBMC samples

Deidentified *Plasmodium*-infected PBMC samples are from a previously described cohort in Mali previously described. [133]. Uninfected control PBMC are from healthy U.S. adult donors enrolled in NIH protocol #99-CC-0168. Demographic and travel history data were not available from the anonymous U.S. donors, but prior *P.falciparum* exposure is unlikely

Tetramer Production and B cell enrichment

AMA1 protein from *P.falciparum* (3D7) (provided by Dr. Julian Rayner, Wellcome Trust Sanger Institute) and MSP1-19 protein from *P.falciparum* (3D7) (provided by Dr. Anthony Holder, Francis Crick Institute) were biotinylated and tetramerized as described above in section 2.4. Decoy reagent to gate out non-MSP1⁺ B cells was made by conjugating SA-PE to AF647 using an AF647 protein labeling kit (ThermoFisher), washing and removing any unbound AF647, and incubating with an excess of an irrelevant biotinylated HIS-tagged protein, similar to what has been previously described [89]. Frozen PBMC were thawed and resuspended in 200ul in PBS containing 2% FBS and stained and enriched using Decoy, PfAMA1 and PfMSP1 tetramers as described in section 2.4. Cells were washed, incubated with anti-PE magnetic beads for 30 min on ice, and passed over magnetized LS columns (Miltenyi Biotec) to elute the tetramer bound cells. All bound cells were stained with surface antibodies listed in **Table 2.1**. All cells were run on the LSRII (BD) and analyzed using FlowJo software

Single Cell BCR Sequencing and Cloning

Single *Pf*-specific MBCs were FACS sorted using an ARIAll into 96-well plates. BCRs were amplified and sequenced from the cDNA of single cells as previously described [136].

Assemblies were blasted against the IMGT human database, VDJ alleles were recorded as was the percent homology to database reference alleles. For analysis with respect to VDJ segment analysis and mutation rate, only functional sequences were used (no out-of-frame or stop codon bcrs) and removed any sequence that didn't include all CDRs or had any ambiguous base calls. All graphs were made in R using ggplot2.

5 Concluding Remarks

Even though a variety of anti-malarial vaccine candidates against both blood stage and liver stage have been tested, generating protective immunity against malaria has been difficult to achieve. While the RTS'S vaccine against the liver stage of disease has proven to be effective, its impact is short lived and often leads to breakthrough blood stage malaria infection. Additional data generated in our lab has shown that breakthrough to blood stage disease can negatively impact pre-existing liver stage immunity (Keitany et al., unpublished data). An ideal malaria vaccine should therefore target multiple stages of the malaria life cycle to confer cross-stage immunity and avoid the detrimental effects of merozoite breakthrough. What has remained a difficult endeavor, however, is understanding what components of the humoral immune response are important during blood stage infection. Carrying out our studies in a murine model in which protective immunity to the blood stage of malaria is induced after a single infection represented the best way to determine how endogenous malaria-specific B cells form and function. It was our hope that this approach would shed new light on what constitutes optimal B cell immunity against malaria infection to better inform vaccine design.

Much of our understanding of MBC formation and function has been largely derived from studies using model protein antigens that have led to how we delineate MBC subsets. Paradigmatically, class-switched MBCs are primarily GC-derived and mediate rapid recall responses whereas unswitched MBCs are thought to be GC-independent and are slower to respond. Whether these approaches represent the endogenous B cell response to pathogen invasion remains unclear. We have developed novel tetramer reagents to identify rare populations of *Plasmodium*-specific B cells to provide the first glimpse of how MBCs develop and function during blood stage malaria infection. In doing so, we have uncovered a previously unrecognized population of GC-derived, somatically hypermutated, high-affinity IgM⁺ MBCs that dominate the early secondary response to infection.

IgM antibodies have often been categorized as low-affinity effectors that are secreted early during immune responses or through natural secretion mechanisms by B1 B cells and have been often overlooked as effective mediators of protection during infection [127, 137]. A number of studies, however, have shown IgM to be a key mediator of protection in a variety of infectious settings, including viral, bacterial, and parasitic disease [138-140]. The potency of IgM is highlighted by the fact that it is secreted predominantly in pentameric and hexameric forms [141] and is a much more potent mediator of complement activation and complement-mediated lysis (CML) than monomeric IgG [142, 143]. Therefore, the effector function of IgM would prove especially beneficial against the blood stage of malaria infection to aid in the clearance of infected RBCs. Our findings in chapters 2 highlight that there is a preference to generate high-affinity *Plasmodium*-specific IgM memory B cells in response to infection that has not been typically observed in studies using protein immunization. Because we have the ability to clone and generate monoclonal, high-affinity antibody specificities from these MSP1-specific IgM⁺ MBCs, we can better understand their protective and functional capacities. Recently, we have developed a method to purify large amounts of murine IgM and are currently testing different clones for their ability to protect against homologous *P.chabaudi* rechallenges after passive transfer as well as functional capacities to test their capacity to block parasite RBC invasion and destroy iRBCs in a complement-mediated manner. These findings could also lead to the use of IgM antibody treatment as a potential malaria therapeutic

Understanding protection will be one critical facet of determining the relevance of malaria-specific IgM⁺ antibodies. Another key question moving forward is to determine whether standard protein/adjuvant vaccine strategies are capable of generating these types of populations or does the *Plasmodium* parasite generate a unique environment that promotes the formation of this distinct population of IgM-expressing MBCs. Our findings in chapter 3 provide some insight into these processes with the notable finding that IgM⁺ MBCs are predominantly GC-derived. There are likely additional transcriptional and regulatory mechanisms that are also

at play that may inform how to best target the generation of IgM⁺ MBCs by vaccination. To this end, ongoing studies using next-generation sequencing technologies to visualize the transcriptome of distinct MSP1-specific MBC subsets that form after *P. chabaudi* infection are underway. Incorporating these findings into more powerful vaccine platforms like nanoparticles, which can be highly customizable to incorporate immunodominant proteins, immune modulators, and adjuvants to trigger specific immune pathways [144], may be a way to drive more durable and protective anti-malarial immunity that would represent what we observe during natural infection.

Lastly, these ongoing and future studies are further warranted and supported by our ability to visualize similar populations of IgM⁺ and IgG⁺ MBCs in malaria infected humans as shown in chapter 4. Studies of specific ethnic groups in malaria endemic regions show a correlation between increased resistance to infection and the presence of *Plasmodium*-specific IgM antibody levels [88], suggesting measurements of *Plasmodium*-specific IgM can be used as a biomarker of protection for future studies. This also gives us hope that the generation of these MBCs, if deemed protective, can be harnessed in humans by way of vaccination.

The data shown here shift current paradigms of how MBCs function and highlight the importance of analyzing the endogenous response of B cells during infection to uncovering novel MBC programs. Together these findings advance our understanding of malaria-specific B cell immunity and memory B cell biology to infection and have important implications for vaccine development.

6 Glossary

AMA1: apical membrane antigen 1
ASC: antibody-secreting cell
BCR: B cell receptor
BM: bone marrow
CSR: class-switch recombination
CML: complement-mediated lysis
GC: germinal center
Ig: immunoglobulin
LLPC: long-lived plasma cell
MBC: memory B cell
MHC: major histocompatibility complex
MSP1: merozoite surface protein 1
MZ: marginal zone
PB: plasmablast
PC: plasma cell
PE: phycoerythrin
RBC: red blood cell
SHM: somatic hypermutation
TB: T cell-B cell
TD: T-dependent
TI: T-independent
Tfh: T-follicular helper cell
TLR: toll-like receptor
TCR: T cell receptor
WT: wild type

7 References

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