Humoral immunity to ehrlichial infection: identification and characterization of an IgM+ memory B cell population

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HUMORAL IMMUNITY TO EHRlichial INFECTION: IDENTIFICATION AND CHARACTERIZATION OF AN IgM⁺ MEMORY B CELL POPULATION

by

Jennifer L. Yates

A Dissertation Submitted to the University at Albany, State University of New York in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

School of Public Health
Department of Biomedical Sciences

2013
Humoral Immunity to Ehrlichial Infection:

Identification and Characterization of an IgM⁺ Memory B cell Population

By

Jennifer L. Yates

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DEDICATION

To dissertation is dedicated to my husband Chris, without whose love, support, and encouragement this would not have been possible.
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ABBREVIATIONS

AID   activation-induced cytidine deaminase
APC   antigen-presenting cell
APRIL a proliferation-inducing ligand
ASC   antibody secreting cell
Bcl-6 B cell lymphoma-6
BCR   B cell receptor
BrdU bromodeoxyuridine
BTK   Bruton’s tyrosine kinase
CMV   cytomegalovirus
CTM   complete tumor medium
DTX   diphtheria toxin
ELISA enzyme-linked immunosorbent assay
EML E. muris-like agent
FBS   fetal bovine serum
FCRL4 Fc receptor-like-4
FDC   follicular dendritic cell
g  gram
GC   germinal center
HBSS Hank’s Balanced Salt Solution
HCV   Hepatitis C Virus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>HGA</td>
<td>Human Granulocytic Anaplasmosis</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HME</td>
<td>Human Monocytic Ehrlichiosis</td>
</tr>
<tr>
<td>HVR</td>
<td>hypervariable region</td>
</tr>
<tr>
<td>IOE</td>
<td><em>Ixodes ovatus ehrlichiae</em></td>
</tr>
<tr>
<td>i.p.</td>
<td>intra-peritoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LLPC</td>
<td>long-lived plasma cell</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>lipotechoic acid</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MZ</td>
<td>marginal zone</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>OMP</td>
<td>outer membrane protein</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PNA</td>
<td>peanut agglutinin</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>swIg</td>
<td>class-switched immunoglobulin</td>
</tr>
<tr>
<td>T4SS</td>
<td>type 4 secretion system</td>
</tr>
<tr>
<td>TACI</td>
<td>transmembrane activator and calcium modulator cyclophilin</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>T&lt;sub&gt;FH&lt;/sub&gt;</td>
<td>T follicular helper cell</td>
</tr>
<tr>
<td>TD</td>
<td>T cell-dependent</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TI</td>
<td>T cell-independent</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
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</table>
ABSTRACT

Immunological memory is a fundamental concept that is key to generating and maintaining immunity to pathogens. Humoral memory resides in part in antigen-specific memory B cells, which are classically defined as class-switched, somatically mutated, long-lived cells that are highly responsive to specific antigen challenge. Despite the focus on class-switched memory B cells, several studies have validated the existence of IgM memory B cells, and have demonstrated distinct functions of IgM and IgG memory B cell subsets. Based on the expression of CD11c, we have identified a large population of IgM memory B cells using a natural model of infection by the bacterium *Ehrlichia muris*. This long-term CD11c+ IgM B cell population exhibited phenotypic characteristics of memory B cells, including expression of CD73, and PD-L2. In addition, the CD11c+ IgM memory B cells lacked expression of CD138, were largely quiescent, and accumulated somatic mutations. Although these cells did not proliferate or secrete antibody *ex vivo*, they produced antigen-specific IgM upon *in vitro* stimulation with mitogens. The CD11c+ IgM memory B cells were located in the splenic marginal zone, but were not detected in blood or other secondary lymphoid organs. *In vivo* depletion of the CD11c-positive IgM memory B cells caused a transient decrease in long-term IgM production, and abrogated the IgG recall response to specific antigen challenge. These results indicate that the IgM memory B cells were responsible for both the maintenance of serum Ig, and humoral memory. Generation of the IgM
memory B cells was independent of infectious doses tested, and required CD4 T cells, Bcl-6, and IL-21R signals. *In vivo* labeling of AID-expressing cells revealed that the IgM memory B cells were generated during acute infection, and as early as day 4 post-infection. Our findings demonstrate that T cell-dependent IgM memory B cells can play an important role in maintaining long-term immunity during bacterial infection.
CHAPTER I: Introduction
**Biology of the Ehrlichiae**

**Phylogeny**

The ehrlichiae are small, gram-negative, obligate intracellular, α-proteobacteria in the family Anaplasmataceae, order Rickettsiales. Members of the genus Ehrlichia include *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia chaffeensis*, *Ehrlichia muris*, *Ehrlichia ruminantium*, *Ixodes ovatus* ehrlichia (IOE), and a more recently ehrlichia isolate *E. muris*-like agent (EML) (Pritt et al., 2011; Rikihisa, 2010; Shibata et al., 2000). Of these species, all but *E. muris* and IOE have been shown to be infectious in humans (Allsopp et al., 2005; Buller et al., 1999; Perez et al., 2006; Thomas et al., 2009). Related genera include Anaplasma, Wolbachia, and Neorickettsia. The Anaplasmactaceae include *Anaplasma phagocytophilum*, the causative agent of Human Granulocytic Anaplasmosis (HGA) (Rikihisa, 2010).

**Genetic Characteristics**

The *E. chaffeensis* genome is greatly reduced in size compared to other free-living bacterial species. This is presumably due to the process of reductive evolution demonstrated in many intracellular bacterial species. The genome of *E. chaffeensis* is encoded on a single 1.18Mb chromosome, which includes 1,115 open reading frames (ORFs). Included are genes encoding a type 4-secretion system (T4SS), and vitamin/cofactor biosynthesis. Genes required for the biosynthesis of lipopolysaccharide (LPS) and peptidoglycan are absent (Dunning Hotopp et al., 2006; Lin and Rikihisa, 2003a). The lack of LPS and peptidoglycan not only affects membrane stability, but also prevents the activation of both toll-like
receptor (TLR)-2 and TLR4 in the mammalian host. To compensate for the loss of membrane stability due to the lack of LPS and peptidoglycan, cholesterol is acquired from the host cell and incorporated into the bacterial membrane (Lin and Rikihisa, 2003a). In addition, disulfide bond linkages have been demonstrated between major surface proteins in *Anaplasma* species that may act to stabilize the bacterial membrane, and similar linkages likely occur between the OMPs in *Ehrlichia* species (Mavromatis et al., 2006; McBride et al., 2002).

*E. chaffeensis* encodes 22 OMPs (p1- p28) encoded in a polymorphic multi-gene family that are clustered in a 29 KB locus (Ohashi et al., 2001). Together, these OMPs comprise approximately 1-2% of the bacterial genome, indicating the importance of these proteins in the life cycle of the ehrlichiae. The genomic location and organization of the OMPs are conserved among several ehrlichial species, including *E. canis, E. ewingii, E. ruminantium, and E. muris* (Crocquet-Valdes et al., 2011; Ohashi et al., 2001; van Heerden et al., 2004; Zhang et al., 2008). However, divergence does exist in the OMPs within distinct hypervariable regions (HVRs) of the OMP genes, variation which is likely responsible for evasion of the host immune system (Reddy and Streck, 1999). This notion is supported by data showing that an HVR of OMP-19 was a target of recognition of protective antibodies in *E. chaffeensis* infected mice (Li et al., 2002). *E. chaffeensis* also encodes another immunogenic 120 kDa protein, gp120. This protein is expressed on the bacterial cell membrane exclusively in the dense-core stage of the life cycle (Popov et al., 2000).
The ehrlichiae are aerobic organisms, yet they lack the genes required for the transport and use of glucose as an energy source. Instead, the ehrlichiae possess genes for amino acid transporters and enzymes sufficient for using the amino acids aspartate, proline, glutamate, glutamine, and arginine as energy sources. The ehrlichiae also contain genes required for a functional tricarboxilic acid (TCA) cycle, electron transport chain, and pyruvate metabolism (Mavromatis et al., 2006; Rikihisa, 2010).

**Life Cycle**

The life cycle of the ehrlichiae is comprised of both tick and mammalian stages. The white tailed deer is considered the natural reservoir of *E. chaffeensis* (Lockhart et al., 1997), and the Lone Star tick (*Amblyomma americanum*) is the primary vector. The cycle begins when non-infected larvae take a blood meal from an infected vertebrate reservoir host, such as the white tailed deer. After the blood meal, the larvae mature to nymphs, which can infect another reservoir host during their next blood meal. The nymphs then mature into infected adults where they can establish another infection in an additional reservoir host at their next blood meal, before laying eggs for the next cycle. Humans and other mammals can become incidental hosts of ehrlichia infection when the infected nymphaal ticks or adults take their blood meal (Paddock and Childs, 2003).

**Interactions with Host Cells**

The ehrlichiae are obligate intracellular bacteria, and as such must invade a host cell to survive and replicate. Two major forms of the ehrlichiae exist in the
mammalian host: an infectious dense-cored form, and replicative reticulate cell form (Zhang et al., 2007). The bacteria are leukotropic in mammalian hosts, preferentially infecting monocytes, macrophages, and dendritic cells (Nandi et al., 2009; Paddock and Childs, 2003). Upon invasion of the host cell, *E. chaffeensis* enters the endosomal trafficking pathway via calveolin-mediated endocytosis (Lin and Rikihisa, 2003b). The intracellular inclusions that contained *E. chaffeensis* stained with Rab5, and early endosome antigen-1 (EEA1), demonstrating that the bacteria reside in early endosomes within the host cell (Mott et al., 1999). It is within the early endosomes that the bacteria divide and form distinctive aggregates called morulae. In addition, the bacteria-containing endosomes accumulate transferrin and the transferrin receptor by fusing with endosomes containing these elements (Barnewall et al., 1997; Mott et al., 1999). *E. chaffeensis* is susceptible to intracytoplasmic iron depletion, suggesting that iron is required for the survival of the bacteria in mammalian host cells (Barnewall and Rikihisa, 1994). The ehrlichia have also developed highly evolved mechanisms to subvert destruction by the host cell. These mechanisms include inhibition of phagosome-lysosome fusion, inhibition of host cell apoptosis, and inhibition of oxidative damage by reactive oxygen species (ROS), mechanisms which all act to secure a niche within which the bacteria can replicate (Barnewall et al., 1997; Kumagai et al., 2006; Lin and Rikihisa, 2007; Zhang et al., 2004). These, and other host cell activities are likely mediated by effector proteins secreted by the ehrlichial T4SS (Bao et al., 2009; Liu et al., 2012a).
Human Monocytic Ehrlichiosis

History and Identification

Human ehrlichioses are classified as emerging infectious diseases, which became reportable to the CDC in 1997 (Paddock and Childs, 2003). Historically considered a veterinary disease, ehrlichiosis was first identified in a human patient in 1986 (Maeda et al., 1987). Although serologically related to *Ehrlichia canis*, the bacteria isolated from febrile, tick-exposed patients shared only 98.7% homology with *E. canis* by 16S ribosomal RNA analysis (Dawson et al., 1991). *Ehrlichia chaffeensis* was then identified as the causative agent of human monocytic ehrlichiosis (HME) (Anderson et al., 1991).

Human ehrlichioses are currently considered the most prevalent tick-borne zoonoses in the United States, and co-infection with other tick-transmitted agents is a common occurrence. The majority of HME cases in the United States are reported from the South-Central and Southeastern regions of the country, the primary range of the main tick vector, *Amblyomma americanum* (Paddock and Childs, 2003).

Clinical Manifestations, Diagnosis, and Treatment

Symptoms of ehrlichial infection are generally non-specific, and typically manifest within 9-10 days following a tick bite. Common symptoms consist of fever, headache, arthralgia, myalgia, and malaise. Less frequent symptoms are rash, lymphadenopathy, and confusion. Patients who are very young, very old, or immunocompromised tend to develop more severe disease that may become fatal.
Important laboratory features of ehrlichia-infected patients include leukopenia, thrombocytopenia, anemia, and elevated hepatic aminotransferases (Dumler et al., 2007).

Due to the non-specific nature of the symptoms presented during ehrlichial infection, laboratory confirmation is critical for an accurate diagnosis (Walker et al., 2004). Blood smears showing infected monocytes with characteristic bacterial inclusions (morulae) with Wright’s stain, are present in less than 10% of patients. Alternatively, PCR analysis of ehrlichial 16S ribosomal RNA in whole blood offers a highly sensitive, timely, and accurate diagnosis (Anderson et al., 1991). However, the most sensitive confirmation of ehrlichial infection, is detection of ehrlichia-specific antibodies in the serum of infected or convalescent patients (Dumler et al., 2007). Due to high antibody cross-reactivity with other rickettsial pathogens, and the time required for generating antibody responses, a combination of PCR and serology by IFA are needed for a definitive diagnosis (Kordick et al., 1999; Walker, 1998).

The ehrlichiae are susceptible to the tetracycline class of antibiotics, whose mechanism of action is to inhibit bacterial protein translation (Paddock and Childs, 2003). Because it is well tolerated by patients, doxycycline is the treatment of choice for ehrlichial infection. Response to treatment is generally rapid, and symptoms abate within 24-48 hours. Doxycycline is highly effective, and post-therapy relapse has never been reported. In addition, doxycycline may also be used
for cases with co-infection with susceptible infectious agents such as *Borrelia burgdorferi*, the causative agent of Lyme disease (Dumler et al., 2007).

**Animal Models of Disease**

Immunocompetent mice infected with *E. chaffeensis* are able to clear infection within 10-17 days post-infection, and exhibit transient pathology. In contrast, immunocompromised mice, such as the BALB/c severe combined immunodeficient (SCID) mouse, develop a persistent and fatal infection, becoming moribund within 24 days. Since mice sufficient in T and B cells were able to clear infection with *E. chaffeensis*, these studies demonstrate the importance of the adaptive immune response to these infections (Winslow et al., 1998).

Subsequently, *E. muris* and IOE have been used as mouse models of human ehrlichiosis. *E. muris* produces a persistent non-lethal infection in immunocompetent mice that resembles characteristics of HME in humans (Kawahara et al., 1993; Kawahara et al., 1996). In contrast, IOE establishes a fatal infection in immunocompetent mice, illustrating the most severe consequences of ehrlichial infection (Okada et al., 2001; Sotomayor et al., 2001).

**Host Defense**

**Cellular Immunity**

It has long been assumed that cell-mediated responses, rather than humoral responses provide immunity to intracellular pathogens. Several studies have confirmed a role for cell-mediated responses in immunity to ehrlichial infections.
Mice deficient in $\alpha/\beta$ T cells and $\gamma/\delta$ T cells were unable to clear infection with *E. chaffeensis*, in contrast to their immunocompetent counterparts (Winslow et al., 2000). Similar studies showed that mice deficient in CD4 T cells exhibit delayed clearance of *E. chaffeensis* when compared to wild-type mice (Ganta et al., 2004). The effect of the absence of T cells was more dramatic in mice infected with *E. muris*, where 80% of mice deficient in CD8 and CD4 T cells succumbed to infection (Feng and Walker, 2004). Mortality was also higher in the absence of IFN$\gamma$ and/or TNF$\alpha$, demonstrating the importance of T cells and effector cytokines in protective immunity to ehrlichial infection (Feng and Walker, 2004). Infection of mice with *E. muris* was also found to be effective at providing protective immunity to a subsequent challenge with the highly pathogenic, heterologous ehrlichia, IOE. This cross-protection was mediated in part by antigen-specific, IFN$\gamma$-producing CD4 and CD8 T cells (Ismail et al., 2004). These studies were confirmed by our laboratory, showing that IFN$\gamma$ and CD4 T cells were critical for providing protection against a sub-lethal dose of IOE, validating that Th1 responses are important for protection against ehrlichial infection (Bitsaktsis et al., 2004). In regard to T cell recognition, five immunoreactive CD4 T cell epitopes from OMP-19 of IOE have identified. These epitopes are conserved between *E. chaffeensis* and *E. muris*, and likely contribute to heterologous immunity (Nandi et al., 2007).

**Humoral Immunity**

While the importance of cell mediated immunity to ehrlichial infection has been established, there is ample evidence pointing to an essential role of humoral
immunity. Passive administration of immune serum or purified ehrlichia-specific IgG, was shown to prevent signs of illness in *E. risticii* infected mice (Kaylor et al., 1991). Similarly, passive administration of immune sera or purified antibodies provided significant protection to mice infected with *E. chaffeensis* or *E. muris* (Feng and Walker, 2004; Winslow et al., 2000). Our laboratory extended these studies showing that the most effective antibodies were of the IgG2a subclass and exhibited picomolar affinity to the HVR1 region of a p28-OMP known as OMP-19 (Li et al., 2002). Together, these studies illustrate the important role humoral immunity can play in protection against intracellular bacterial infections.

Our laboratory has investigated the mechanisms by which humoral responses provide protection against ehrlichial infection. We have established that B cell deficient mice are highly susceptible to both a low-dose IOE infection, and *E. muris* infection (Bitsaktsis et al., 2007; Yager et al., 2005). B cells are also required for protection against heterologous challenge of *E. muris*-infected mice with the highly virulent IOE. This protection was largely independent of T cell help, implying that T cell-independent (TI) antibody production is an important characteristic of the immune response to ehrlichial infection (Bitsaktsis et al., 2007). Antibody-mediated clearance of IOE required Fc receptors, complement, and reactive oxygen species (ROS), consistent with a mechanism where cell-free bacteria are opsonized and lysed by complement or undergo Fc-mediated phagocytosis (Yager et al., 2005).
Mice infected with *E. muris* generate a robust OMP-19-specific IgM response during the acute phase of infection. The IgM produced is germ-line encoded and poly-reactive, as demonstrated by the ability of these antibodies to bind a variety of self and foreign antigens (Jones et al., 2012). Nearly all of this IgM was produced by a splenic, extra-follicular, TI, plasmablast population defined by the unusual B cell-specific expression of the integrin CD11c (Racine et al., 2008). During this early plasmablast response, follicular organization in the spleen was disrupted, and PNA-positive B cells were dispersed throughout the follicles, rather than in organized germinal centers. In addition, IgG production in the spleen was suppressed until 3 weeks post-infection, while lymph node IgG responses remained relatively normal (Racine et al., 2010).

Chronic *E. muris* infection is characterized by long-term production of protective IgM. The cells responsible for this IgM production were identified as CD138\textsuperscript{high} IgM\textsuperscript{high} antibody secreting cells (ASCs), residing in the bone marrow. The protective effects of IgM was evident even in the absence of class-switch recombination in AID-deficient mice, highlighting the important role IgM can play in long-term humoral immunity (Racine et al., 2011).

**Immunological Memory**

The concept of “immune memory” was first described by the Greek historian Thucydides during the plague of Athens in 430\textsubscript{B.C.}. As a survivor of the disease himself, Thucydides observed that the “same man was never attacked
“twice” (Crotty and Ahmed, 2004; Thucydides and Crawley, 1951). It is now known that immunological memory is an important outcome of the adaptive immune response and represents the ability of the immune system to recognize and respond specifically to previously encountered pathogens or antigens. This “memory” is derived from cells generated during the primary response, which are maintained in the host for long periods, in some cases for the lifetime of the animal. The cells that mediate immunological memory include memory T cells, memory B cells, and long-lived plasma cells. The protection afforded by these cells is characterized by faster, higher affinity responses to specific antigen and the continual secretion of neutralizing antibodies, respectively (Crotty and Ahmed, 2004).

Vaccination is the administration of a weakened version or subunit of a pathogen to elicit a host immune response, and is performed to provide protection in the event of a second exposure. Immunological memory is fundamental to vaccination, which is arguably the most effective strategy for the prevention of infectious disease (Zielinski et al., 2011). The most celebrated vaccination program resulted in the eradication of smallpox from the human population in 1980 (Crotty et al., 2003). Additional diseases that have been controlled by vaccines include poliomyelitis, measles, rabies, diphtheria (Corynebacterium diphtheriae), and tetanus (Clostridium tetani). In all of these cases, the respective vaccines have been successful in reducing the global burden of disease. However, major gaps still exist in the vaccination repertoire, most notably for infections that cause worldwide epidemics such as human immunodeficiency virus (HIV), tuberculosis (M.}
tuberculosis), and malaria (P. falciparum; Sallusto et al., 2010). The majority of vaccines used in the clinic thus far have been discovered empirically. Live attenuated and inactivated whole organisms, have been among the most effective of these vaccines. Our inability to create effective vaccines against highly variable and persistent pathogens underscores the need for rational vaccine design (Sallusto et al., 2010). Thus, a better understanding of memory cell generation, antigenic specificity, and maintenance of memory will aid in the development of more effective vaccines.

**Humoral Memory**

Most vaccines are thought to mediate protection via their elicitation of neutralizing antibodies (Ada, 2005). Serum antibody titers have long been used as a correlate of protection for multiple infectious diseases (Pulendran and Ahmed, 2011). Antibodies are one of the more important components of immune memory, as they are the first line of defense against invading pathogens. The long-term maintenance of serum antibody levels has been well documented. For example, studies of smallpox-vaccinated individuals demonstrated that anti-vaccinia virus titers were maintained in the serum for over 60 years (Crotty et al., 2003; Hammarlund et al., 2003). In addition, studies of the Swedish population in 1991 demonstrated that anti-polio titers were maintained in vaccinated individuals for more than 10 years, in all age groups studied (Bottiger et al., 1998). However, not all humoral memory is created equal, as demonstrated by declining anti-tetanus and diphtheria serum antibody titers in the same study groups (Bottiger et al., 1998;
Crotty and Ahmed, 2004). The mechanism(s) by which some serum antibody levels are maintained long-term is still an active area of research and is highly relevant for future vaccine design.

Long-lived plasma cells (LLPC) are the main source of protective, antigen-specific antibody production following the resolution of an immune response or infection (Slifka and Ahmed, 1998; Slifka et al., 1995). These cells are terminally differentiated, non-dividing, and incapable of self-renewal (Radbruch et al., 2006). The mechanism of LLPC generation and long-term maintenance are still a matter of debate, but it is clear that specialized niches in the lymphoid organs are required for their survival. Some LLPCs reside in secondary lymphoid organs, such as the spleen and lymph nodes, but the majority of LLPCs appear to be located in the bone marrow (Manz et al., 1998; Slifka et al., 1995). LLPC survival is dependent on specialized niches, where survival factors and cytokines such as a proliferation-inducing ligand (APRIL) and IL-6 are provided. In a model of pHox immunization, Chu et al. have shown that F4/80+ Gr-1lo eosinophils were the main source of survival factors, and were required for plasma cell retention in the bone marrow (Chu et al., 2011). Additional evidence has shown that eosinophils are also necessary for the proper differentiation of plasmablast precursors into long-lived plasma cells in the bone marrow (Chu and Berek, 2013). Despite these recent studies, the mechanisms of long-term maintenance of plasma cell populations is still a matter of debate, with regard to input from memory B cell populations,
inflammatory conditions of the host, and antigen dependence. These topics will be discussed in subsequent chapters.

The distinctive features of a memory B cell response to antigen challenge include a faster, larger, higher affinity antibody response to the recall antigen. Due to the high affinity of secondary responses, memory B cells have long been defined as those with class-switched, highly mutated B cell receptors (BCRs). The classic route for memory B cell development begins when cognate antigen binds to the BCR of a naïve B cell. This initial antigen encounter is typically mediated by subcapsular sinus (SCS) macrophages, or follicular dendritic cells (FDC) within the B cell follicle (Batista and Harwood, 2009; Cyster, 2010). Once stimulated, the B cell migrates to the B cell-T cell border of the follicle. At the edge of the follicle, the B cell competes to present processed antigen to a cognate CD4+ T cell and receive the co-stimulatory signals needed for activation. Once activated, the B cell clonally expands, forming a secondary follicle, and eventually a germinal center (GC).

Within the GC, activated B cells undergo rounds of selection and affinity maturation through somatic hypermutation. In the dark zone of the GC, rapidly dividing centroblasts undergo BCR diversification through somatic hypermutation. The process of somatic hypermutation of the immunoglobulin genes is mediated by the enzyme activation-induced cytidine deaminase (AID). The mutated centrocytes then undergo selection based on the affinity of the mutated BCR for antigen, and cognate interactions with follicular T-helper cells (T_{FH}). B cells with high affinity,
class-switched BCRs are then exported from the GC, where they differentiate into plasma cells or memory B cells (McHeyzer-Williams et al., 2011).

In addition to the canonical memory development described above, non-canonical pathways exist. Recent evidence has indicated that memory B cells are heterogenous, can be derived from both TD and TI responses, and do not necessarily bear class-switched or mutated receptors (Defrance et al., 2011; Good-Jacobson and Tarlinton, 2012; Reynaud et al., 2012). These findings underscore how little we really know about the generation and maintenance of humoral memory. The study of various memory B cell subsets will increase our understanding of humoral memory and aid in rational vaccine design (Sallusto et al., 2010).

Summary

This dissertation extends studies by our laboratory on protective antibody responses during intracellular bacterial infection, by describing the generation of long-term humoral memory. Chapter 3 will characterize a population of IgM memory B cells that were identified during chronic *E. muris* infection. The data will show that these cells exhibit many characteristics of previously described memory B cell populations, and are required for secondary responses to antigen challenge. Chapter 4 will describe the factors involved in the generation of IgM memory B cells. The data will demonstrate that the IgM memory B cells are generated in a T cell-dependent manner that also requires IL-21 and the transcriptional repressor Bcl-6. Furthermore, the data will establish that the generation of IgM memory B
cells occurs early, during the acute *E. muris* infection. Chapter 5 will speculate on the function of the IgM memory B cells during steady-state conditions. The data will show that *in vivo* depletion of CD11c-expressing cells temporarily decreases protective antigen-specific IgM titers in the serum. In addition, the data suggest that the IgM memory B cells function to slowly replenish the antigen-specific plasma cell population in the bone marrow. Finally, Chapter 6 will summarize these findings and provide a model of IgM memory B cell generation and function during *E. muris* infection.
CHAPTER II: Materials and Methods
Animals

Mice were obtained from the Jackson Laboratory (Bar Harbor, ME) or were bred in the Animal Care Facility at the Wadsworth Center under microisolator conditions. C57BL/6 mice and the following transgenic and gene-targeted strains were used: B cell-deficient (B6.129S2-Ighm<sup>tm1Cgn/J</sup>), CD11c-DTR (B6.FVB-Tg<sup>Ilgax-DTR/EGFP<sup>57Lan/J</sup>), CD73-deficient (Thompson et al., 2004), IL-21R-deficient (Ozaki et al., 2002), MHCII-deficient (B6.129S2-H<sub>2</sub><sup>dAb1-Ea/J</sup>), Bcl-6-deficient (gift from Dr. Alex Dent), ROSA-eYFP (B6.129X1-Gt(Rosa26<sup>Sor<sub>tm1(EYFP)<sub>Cos/J</sub></sup>)), and AID-Cre-Ert2 (Dogan et al., 2009). and Triple Congenic (B6.IgHaThy1aGpi1a-Ptprc2/Pep3b/Boy/J).

Antigen Challenge

Mice were administered 100 µg purified recombinant OMP-19 (Li et al., 2001; Nandi et al., 2007), with or without alum (Imject; Thermo Fisher Scientific, Waltham, MA).

Bacterial Infections

Mice were infected with *Ehrlichia muris*, via i.p. injection between 6 and 12 weeks of age, with 50,000 bacteria obtained from infected mouse splenocytes, as previously described (MacNamara et al., 2009; Stevenson et al., 2006). Mice were infected with the Wisconsin isolate (strain HM543745; Pritt et al., 2011) as a 10% spleen preparation.

Breeding and Screening of AID-Cre-Ert2 x ROSA-eYFP<sub>f</sub> Mice

AID-Cre-Ert2 mice and ROSA-eYFP mouse colonies were maintained separately as homozygous and heterozygous, respectively. Double transgenic mice were
obtained by breeding the AID-Cre-Ert2 and ROSA-eYFP parent strains. The F1 progeny were screened for the presence of both transgenes by PCR. The primers used were as follows:

**AID-Cre-Ert2**

3AID Exon2_2: CGA AAG TGG CCG AAG TCC AG  
3 cre_3_Long : AGG TTC TGC GGG AAA CCA TTT CCG  
5AIDint1end2: GTA GGT CCA GCC ATC AGC AG

**ROSA-eYFP**

WT Forward : AAG GGA GCT GCA GTG GAG TA  
WT Reverse: CCG AAA ATC TGT GGG AAG TC  
Mutant Forward: ACA TGG TCC TGC TGG AGT TC  
Mutant Reverse: GGC ATT AAA GCA GCG TAT CG

PCR conditions are as follows:

94°C 3 min; 92°C sec; 60° 1 min; 72°C 1 min; 72°C 5 min  
35 cycles

*BrdU administration and analysis*

Mice were administered BrdU (0.8 mg) by i.p. injection and were maintained on BrdU in their drinking water (0.8 mg/mL plus 10% dextrose) for 4 days. BrdU incorporation was assessed by intracellular flow cytometric analysis, using a FITC-conjugated anti-BrdU monoclonal antibody (PRB-1; eBioscience, San Diego, CA); the cells were analyzed on a FACS Calibur flow cytometer.
**ELISA**

Microtiter plates (Immulon, VWR) were adsorbed overnight with 10 μg/ml recombinant *E. muris* OMP-19 (Nandi et al., 2007). The coated plates were washed with 0.5% Tween 20 in PBS, and blocked with 20% FBS in PBS. Goat anti-mouse antibodies conjugated to alkaline phosphatase (Southern Biotec; Birmingham, AL) were used to determine the serum titers of IgM, IgG, and the subclasses of IgG. Absorbance was measured at 405 nm; endpoint titers were defined as the highest dilution that generated an $A_{405}$ value equal or greater than 0.1.

**Generation of Bacterial Stocks**

Bacterial stocks were generated by propagating *Ehrlichia muris* in RAG-deficient mice. When infected mice exhibited morbidity (ruffled fur, hunched posture, wasting), spleens were isolated, dissociated, and suspended in sucrose-phosphate-glutamate (SPG; 218 mM sucrose, 38 mM KH$_2$PO$_4$, 72 mM K$_2$PO$_4$, and 4.9 mM glutamate) buffer. Aliquots of cell suspensions were stored at -80°C until further use. The bacterial copy number was determined using quantitative probe-based PCR (Yager et al., 2005).

**Flow Cytometry**

Splenocytes were obtained by mechanical disruption in HBSS. The tissues were disaggregated using a 70-μm pore size nylon strainer, and erythrocytes were removed from the single-cell suspension by hypotonic lysis. For cell surface staining, single-cell suspensions (4 x 10$^6$ cells) were incubated with Fc blocking solution (1μg/ml anti-CD16/CD32; clone 2.4G2 in 10% normal goat
serum/HBSS/0.1% sodium azide), prior to staining with the mAbs. The cells were incubated on ice for 20 minutes, washed twice, and analyzed. Unstained cells were used to establish the flow cytometer voltage settings, and single-color positive controls were used to adjust compensation. Data from stained samples were acquired on a FACSCalibur flow cytometer equipped with Cell Quest software (Becton Dickinson Mountain View, CA), and were analyzed with FlowJo software (Tree Star, Inc. Ashland, OR). The antibodies used for flow cytometry included the following: BAFF-R (clone 7H22), BrdU (PRB-1) CD11b (M1/70), CD22 (2D6), CD21/35 (eBio8D9), CD23 (B3B4), CD38 (90), CD40 (1C10), CD49d (R1-2), CD95 (15A7), ICOS-L (HK5.3), IL-21R (eBio4A9), TACI (eBio8F10-3), and IgD (11-26; all from eBiociences, San Diego, CA); and B220 (RA3-6B2), CD19 (1D3), CD11c (HL3), CD62L (MEL-14), CD73 (TY/23), CD80 (16-10A1), CD86 (GL-1), CD138 (281-2), CXCR4 (2B11/CXCR4), GL7, IAβ(AF6-120.1), and IgM (R6-60.2; all from BD Biosciences, San Jose, CA).

**Flow cytometric cell sorting**

CD11c-positive IgM memory B cells were identified using PerCP-conjugated CD19 and allophycocyanin (APC)-conjugated CD11c. The CD11c-positive and -negative B cell populations were purified using a FACSaria cell sorter equipped with FACSDiva software (BD Biosciences, Santa Cruz, CA); the purity of the sorted populations was greater than 90% in all experiments.
Immunizations

Sheep red blood cells (SRBC) in Alsever’s solution (Colorado Serum Co., Denver, CO) were washed 3x with 2 volumes of HBSS, and resuspended in PBS. C57BL/6 mice were immunized with $1 \times 10^{10}$ SRBC’s intraperitoneally. C57BL/6 mice were immunized with 50 μg NP-ficoll in alum (Imject; Thermo Fisher Scientific) intraperitoneally.

Somatic Mutation Analyses

CD11c-positive and -negative B cells were purified by flow cytometric cell sorting from splenocytes, obtained from 3 mice after day 30 post-infection. Genomic DNA was isolated using DNAzol (Molecular Research Center, Cincinnati, OH).

Recombined variable regions of the immunoglobulin heavy chain from CD11c-positive and -negative samples were amplified by nested PCR (Pfu polymerase; Agilent Technologies, Santa Clara, CA) in four separate PCR reactions using genomic DNA obtained from 5,000 cells. Amplified products were pooled, sub-cloned using a TOPO PCR cloning kit (Life Technologies, Grand Island, NY), and sequenced. Ig gene sequence mutation analyses were performed only on the Vh segments. IgBlast (http://www.ncbi.nlm.nih.gov/igblast/) was used to identify Germ line V genes with the highest homology for mutation comparison; the Vh region within 4 bp of the D segment junction was not included. The following oligonucleotide primers were utilized: 1st round, 5’-ACACAGGACCTCACCATG-3’ and 5’TCACAAGAGTCCGATAGACC-3’ for 12 cycles (98 °C for 10 sec, 60 °C for 30 sec, 72 °C 1.5 min); 2nd round, 5’-GGGTGACAATGACATCCA-3’ and 5’-
GAGGAGACTGTGAGAGTGGTGCC-3’ for 18 cycles (98 °C for 10 sec, 62 °C for 30 sec, 72 °C 30 sec) (McBride et al., 2008; Reinhardt et al., 2009).

**Immunofluorescence Microscopy**

Spleens were harvested from *E. muris*-infected mice, and embedded in Optimal Cutting Temperature Compound (OCT; Sakura, Torrence, CA). The tissues were frozen in slurry of dry ice and isopentane, and 7 µm cryosections were generated. The cryosections were fixed in 100% ice cold ethanol, blocked in Fc blocking solution (1 µg/ml anti-CD16/CD32; clone 2.4G2), and stained with the following antibodies: CD19 PE-CF594 (ID3, BD Biosciences, Santa Cruz, CA), CD11c Alexa 488 (N418, BioLegend, San Diego, CA), CD169 Alexa 647 (MOMA-1, AbD Serotec, Raleigh, NC), and PNA Alexa 647 (from *Arachis hypogaea*, Life Technologies, Santa Clara, CA). Stained sections were mounted with ProLong Gold with DAPI (Life Technologies, Santa Clara, CA). Images were captured with a 20x objective on the Nikon TE2000 equipped with a CoolSNAP HQ CCD camera (Roper Scientific, Germany), and ImagePro software (MediaCybernetics, Rockville, MD). Adobe Photoshop was used to process the images for display.

**Generation of bone marrow chimeras**

C57BL/6 mice were lethally irradiated (1000 RADs, administered in 2 doses, 4 hours apart), and were reconstituted with 2 x 10^6 bone marrow cells obtained from various combinations of B cell-deficient, CD11c-DTR mice, Triple Congenic, or Bcl-6-deficient mice. The mice were allowed to recover for at least 6 weeks prior to infection.
**Toxin Administration**

Bone marrow chimeric mice were administered diphtheria toxin (DTX) from *Corynebacterium diphtheriae* (Sigma-Aldrich), at a concentration of 9 ng/g of body weight.

**Tamoxifen Administration**

Tamoxifen (10 mg; Sigma) in 500 μl peanut oil was administered to AID-Cre-Ert2 x ROSA-eYFP<sub>F1</sub> mice by oral gavage.

**Statistical analysis**

Statistical analyses were performed with a Student's t-test, or Mann-Whitney test, using Prism GraphPad Software (LaJolla, CA); a P value of < 0.05 was considered to be significant.
CHAPTER III: Identification of an IgM Memory B cell Population During Ehrlichial Infection
Acknowledgements

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Abstract

Immunological memory has been long considered to be harbored in B cells that express high-affinity class-switched IgG. IgM-positive memory B cells can also be generated following immunization, although their physiological role has been unclear. Here we show that bacterial infection elicited a relatively large population of IgM memory B cells that were uniquely identified by their surface expression of CD11c, CD73, and PD-L2. The cells also lacked expression of cell surface markers typically expressed by GC B cells, were CD138-negative, largely quiescent, and accumulated somatic mutations. The IgM memory B cells were located in the splenic marginal zone and were not detected in blood or other secondary lymphoid organs. The cells did not secrete antibody ex vivo, but they produced antigen-specific IgM upon in vitro stimulation with mitogens. In vivo depletion of the IgM memory B cells abrogated the IgG recall responses to specific antigen challenge, demonstrating that the cell population was required for humoral memory. Our findings demonstrate that IgM memory B cells can be elicited at high frequency and can play an important role in maintaining long-term immunity during bacterial infection.
**Introduction**

Immunological memory is a fundamental concept that is key to generating and maintaining immunity to pathogens, and for mediating the protection afforded by vaccines (McHeyzer-Williams and McHeyzer-Williams, 2005; McHeyzer-Williams et al., 2011). Humoral memory resides in part in antigen-specific memory B cells, which are classically defined as class-switched, somatically mutated, long-lived cells that are highly responsive to specific antigen challenge (Kaisho et al., 1997; Martin and Goodnow, 2002; McHeyzer-Williams et al., 2011; Tangye et al., 2003; Wakabayashi et al., 2002; Weiss and Rajewsky, 1990). Class-switched Ig (swIg) memory B cells are generated in germinal centers (GCs), specialized anatomic structures in secondary lymphoid organs where T cell-dependent affinity maturation and class switch recombination of the BCR occurs. Following exposure to cognate antigen, memory B cells proliferate, and differentiate into antibody secreting cells (ACSs). The resulting increase in antigen-specific serum Ig aids in the clearance of pathogens from the host (McHeyzer-Williams and McHeyzer-Williams, 2005; Yoshida et al., 2010).

Despite the focus on swIg memory B cells, several studies have shown memory B cell populations to be more diverse than originally thought (Good-Jacobson and Tarlinton, 2012). Early studies indicated that IgM-positive memory B cells could be found in both humans and mice (Klein et al., 1997; Klein et al., 1998; White and Gray, 2000; Yefenof et al., 1985; Zan-Bar et al., 1979; Zan-Bar et
al., 1978). More recent studies have validated the existence of IgM memory B cells, and have demonstrated distinct functions for both IgM and IgG memory B cell subsets. For example, Dogan et al. used an elegant model for the unbiased labeling of antigen-experienced/memory B cells, wherein activation-induced cytidine deaminase (AID)-expressing cells were permanently marked following Cre recombinase regulation of a reporter gene (Dogan et al., 2009). In this study, mice immunized with a particulate antigen, SRBCs, generated both IgG and IgM memory B cells. Following secondary encounter with antigen, the IgM memory B cells reinitiated a GC reaction, and generated swIg cells, as well as additional IgM memory B cells. In contrast, the IgG memory B cells differentiated directly into antibody secreting cells (ASCs). In other studies, Tomayko and colleagues, using a transgenic mouse model of (4-hydroxy-3-nitrophenyl)-acetyl chicken γ-globulin (NP-CGG) immunization, demonstrated the presence of several swIg and IgM memory B cell subsets that expressed different levels of the maturation markers CD80, PD-L2, and CD73 (Tomayko et al., 2010). The varied expression of cell surface markers, and the distinct ontogeny of each subset suggested functional differences between IgM and swIg memory B cells. Pape et al. utilized an antigen-based technique to purify rare antigen-specific memory B cells in a model of PE immunization, and demonstrated that both IgG and IgM memory B cells were generated following immunization. Kinetic analyses of the memory populations revealed the IgM memory subset to be longer lived than the IgG memory subset. As was also reported by Dogan et al., the swIg memory B cells gave rise to antibody
secreting cells upon antigenic challenge. In contrast to swIg memory B cells, IgM memory B cells were unresponsive to antigen challenge in immune hosts. However, upon transfer into naïve hosts, the IgM memory B cells initiated GC formation and underwent class-switch recombination when challenged with specific antigen (Taylor et al., 2012). Together, these studies have challenged traditional views of humoral memory by revealing that long-term humoral memory can be retained in IgM memory B cells. Nevertheless, questions regarding the origin, generation, and function of IgM memory B cells remain. Moreover, it has not been demonstrated whether IgM memory B cells are elicited naturally during infections. In the present study, we have identified a population of IgM memory B cells that were elicited in a murine model of human ehrlichiosis. We demonstrated that these cells can be uniquely identified by their expression of CD11c, CD73 and other cell surface markers, and that they require CD4 T cell-mediated helper functions for their generation. Furthermore, we show that these IgM memory B cells were responsible for secondary IgG responses following antigen challenge, and propose that they are correlates to IgM memory B cells identified in humans.

**Results**

**CD19hi CD11c-positive B cells are elicited during E. muris infection**

The ehrlichiae are a group of tick-borne obligate intracellular rickettsiae that cause a wide range of diseases in humans and animals. *Ehrlichia muris* causes a non-fatal acute infection in immunocompetent mice, characterized by
splenomegaly and hematological abnormalities (MacNamara et al., 2009).

Thereafter, the bacterium establishes a low-level chronic infection in immunocompetent mice, and infected mice are resistant to secondary infection. Mice infected with E. muris are also immune to lethal infection by a virulent heterologous ehrlichia (Bitsaktsis et al., 2007; Ismail et al., 2004). Our previous studies demonstrated that acute E. muris infection elicits a large population of extrafollicular T cell-independent B220lo splenic plasmablasts that can be identified in part by their unique expression of CD11c (Racine et al., 2008). The plasmablasts secrete IgM and decline in frequency and number by about day 18 post-infection. During the course of this study, we also identified a distinct CD19hi B220+ CD11c+ cell population in the spleen that emerged within approximately 30 days post-E. muris infection. The CD11c-positive B cells have been detected only in the spleen and persisted for at least 397 days (Figure 3.1a). The population ranged in frequency from 1.9 to 5.1% of splenic mononuclear cells (average 3.3%) and represented as many as 8.5 x 10⁶ cells per spleen on day 63 post-infection (Figure 3.1b).

A large proportion of antigen-specific B cells elicited during ehrlichial infections recognize an outer membrane protein-19 (OMP-19) antigen, one member of a family of OMPs encoded by the ehrlichiae (Ohashi et al., 2001). Both OMP-19-specific IgG and IgM are highly effective at eliciting protective immunity against fatal ehrlichial infection (Li et al., 2001; Nandi et al., 2007; Racine et al., 2008). We first addressed whether the CD11c-positive B cells we identified after
Figure 3.1: Identification of a long-term splenic B cell population elicited by bacterial infection

(A) Representative flow cytometry analysis of CD11c expression on CD19-positive B cells in the spleens of *E. muris*-infected, and uninfected mice. Plots shown were gated on live cells as determined by forward and side scatter. (B) Kinetic analysis of CD11c-positive B cells in *E. muris* infected mice. The frequency (top panel) and number (bottom panel) of CD11c-positive B cells among total splenocytes are shown. Each datum represents an individual mouse, and horizontal lines represent the mean. The asterisks indicate statistical significance; where * p< 0.01 and ** p < 0.001, as compared to the values obtained on day 0. (C) ELISPOT analyses of flow cytometrically-purified CD11c-positive, CD11c-negative B cells from infected mice, and CD19+ B cells from uninfected mice. The cells were stimulated with LPS (20 µg/ml), and OMP-19-specific, IgM and IgG-producing B cells, as well as IgM and IgG-secreting B cells (total IgM and IgG) were enumerated. The plots are representative of 2 experiments. (D) Representative flow cytometry analysis of CD11c-positive and –negative B cells obtained from *E. muris* infected mice. (E) Representative flow cytometry analysis of CD11c-expressing B cells from *E. muris* infected mice and from mice infected with a recently identified human isolate (Ehrlichia sp. Wisconsin HM543745) 49 days post-infection.
day 30 post-infection were capable of producing *E. muris*-specific antibodies. CD11c-positive, and -negative CD19+ B cells were purified from infected mice by flow cytometric cell sorting; the cells were then cultured with LPS, and OMP-19-specific B cells were enumerated by ELISPOT. IgM-secreting cells were detected within the CD11c-positive B cell population, and a portion of these cells produced antibodies specific for OMP-19. Some antigen-specific B cells were also detected within the CD11c-negative population from infected mice, as well as in CD19-positive B cells from uninfected mice, albeit at lower frequencies than the CD11c-positive B cells. IgG producing cells were not detected by ELISPOT among any of the B cell populations examined (Figure 3.1c). Moreover, in the absence of LPS stimulation, the CD11c-positive B cells did not secrete antibody *ex vivo* (unpublished data). Flow cytometric analyses revealed that the CD11c-positive B cells exhibited high surface expression of IgM and CD23, and low expression of CD21, consistent with a follicular B cell origin (Figure 3.1d). The cells were also larger and more granular than their CD11c-negative counterparts, a feature consistent with previous characterizations of memory B cells (Ma et al., 2006; Tangye et al., 1998). Although nearly all of the cells expressed IgM, approximately equal portions of the CD11c-positive B cell population were positive or negative for IgD expression. In addition the CD11c-positive B cells were not a canonical GC population, as they exhibited low surface expression of GL7 and high expression of CD38.
We next addressed whether a recently isolated human ehrlichial isolate (Ehrlichia sp. Wisconsin HM543745; Pritt et al., 2011) generated a similar B cell population. A phenotypically identical CD11c-positive B cell population was detected in mice infected with the related human pathogen (Figure 3.1e). Together, these data identify a long-term pathogen-specific, splenic B cell population elicited by intracellular bacterial infection.

**CD11c-positive B cells exhibit characteristics of memory B cells**

The longevity and specificity of the CD11c-positive B cell population suggested that the CD11c-expressing B cells were an IgM memory B cell population. To resolve this question, we first addressed whether the CD11c-positive B cells expressed cell surface markers previously shown to be associated with memory B cells. Compared with CD11c-negative B cells, CD11c-positive B cells exhibited high expression of CD38, CD73, CD80, CD95, and PD-L2, all cell surface markers previously identified on memory B cells (Anderson et al., 2007; Good-Jacobson et al., 2010; Kaji et al., 2012; Tomayko et al., 2010; Figure 3.2a).

The expression levels of these markers as measured by mean fluorescence intensity (MFI), was significantly higher on the CD11c-positive B cells than their CD11c-negative counterparts (Table 1). Most of the CD11c-positive B cells also lacked CD138 expression, consistent with the observation that they did not secrete antibodies ex vivo. Other B cell surface antigens expressed by the CD11c-positive B cells included the B-cell activating factor-R (BAFF-R), B220, CD22, CD40, CD49d, CD62L, CXCR4, IAβ, ICOS-L, IL-21R and TACI (transmembrane activator
and calcium modulator ligand interactor). Surface marker expression on the CD11c-positive B cell population was largely homogenous, with the exception of CD62L, which was expressed in a bimodal distribution. Multiple studies have demonstrated that memory lymphocytes undergo limited cell division (Ahmed and Gray, 1996; Anderson et al., 2007; Schittek and Rajewsky, 1990). Therefore, we next investigated whether the CD11c-positive B cell population was proliferating in vivo. Infected mice were administered BrdU, and were maintained on BrdU in drinking water over a 4-day period. The CD11c-positive B cell population was largely quiescent, as the majority of the population did not incorporate BrdU over the 4-day period (mean frequency of BrdU-negative cells = 86% +/- 1.4; Figure 3.2b). However, the BrdU incorporation rate of approximately 14% over 4 days, corresponds to the previously described proliferation rate of memory B cells at 1 division per month (Schittek and Rajewsky, 1990). Thus, the cell surface phenotype and cell proliferation studies provided additional support for the hypothesis that the CD11c-positive B cells were a population of relatively quiescent IgM memory B cells.

A third criterion we used to establish that the CD11c-positive B cells were memory B cells was somatic mutation, as this is a hallmark of antigen-experienced memory B cells (Klein et al., 1998). CD11c-positive B cells were purified, and recombined V(D)J regions from the relatively abundant variable heavy chain (Vh) J558 family were analyzed. The J558 family represents the largest Vh gene family, comprising nearly 40% of all such exons; this family was chosen because it
**Figure 3.2: CD11c-positive B cells exhibit characteristics of memory cells**

**(A)** Representative flow cytometry analysis of CD11c-positive and -negative CD19+ B cells during *E. muris* infection. The histograms are representative of 5-9 mice that were analyzed at several timepoints on or after day 30 post-infection. Basal cell surface staining and voltage settings were established using unstained cells; the data were omitted from the histograms for clarity. Cells obtained from uninfected mice exhibited similar cell surface marker expression as the CD11c-negative B cells obtained from infected mice (data not shown).

**(B)** BrdU incorporation studies of splenic CD11c-positive and -negative B cell from *E. muris* infected mice. The mice were administered BrdU over a 4-day period, beginning on day 63 post-infection. The splenocytes were analyzed for BrdU incorporation at day 67 post-infection; the cells were also analyzed for CD73 expression, which improved population discrimination. The data are representative of 2 experiments.

**(C)** Mutation analysis of the V region heavy chain J558 family genes from flow cytometrically-purified CD11c-negative, and –positive B cells obtained from infected mice. The segment sizes in the pie charts are proportional to the number of sequences carrying the number of mutations indicated in the periphery of the chart. The number of independent sequences analyzed is indicated in the center of the chart. The mutation frequencies, per base pair, are indicated below the pie charts. The data were compiled from two experiments. Statistical significance was determined using a two-tailed student’s T test, assuming unequal variance, by comparing the CD11c-negative and -positive B cells; the p value is indicated.
single mutation was identified in 9,396 bp from 32 clones obtained from the
CD11c-negative B cells, and 87 mutations were identified in 20,247 bp from 69
analyzed clones from the CD11c-positive B cells.
Table I: Cell Surface Marker Expression

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>CD11c-positive</th>
<th>CD11c-negative</th>
<th>Fold Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAFF-R</td>
<td>21.5 +/- 1.8</td>
<td>12.2 +/- 1.0</td>
<td>1.8</td>
</tr>
<tr>
<td>CD11b</td>
<td>246.3 +/- 57.9</td>
<td>7.9 +/- 1.0</td>
<td>31.0</td>
</tr>
<tr>
<td>CD19</td>
<td>325.6 +/- 15.4</td>
<td>213.3 +/- 13.1</td>
<td>1.5</td>
</tr>
<tr>
<td>CD22</td>
<td>102.7 +/- 48.0</td>
<td>96.6 +/- 29.7</td>
<td>1.0</td>
</tr>
<tr>
<td>CD38</td>
<td>2338.2 +/- 627.3</td>
<td>1147.7 +/- 74.2</td>
<td>1.35</td>
</tr>
<tr>
<td>CD40</td>
<td>42.8 +/- 4.3</td>
<td>32.4 +/- 3.9</td>
<td>1.3</td>
</tr>
<tr>
<td>CD49d</td>
<td>33.6 +/- 4.2</td>
<td>17.4 +/- 2.5</td>
<td>1.9</td>
</tr>
<tr>
<td>CD73</td>
<td>55.8 +/- 3.9</td>
<td>11.0 +/- 1.1</td>
<td>5.0</td>
</tr>
<tr>
<td>CD80</td>
<td>112.8 +/- 6.4</td>
<td>36.1 +/- 4.1</td>
<td>2.9</td>
</tr>
<tr>
<td>CD86</td>
<td>36.4 +/- 3.1</td>
<td>12.0 +/- 1.3</td>
<td>2.7</td>
</tr>
<tr>
<td>CD95</td>
<td>29.8 +/- 1.0</td>
<td>8.29 +/- 1.0</td>
<td>3.6</td>
</tr>
<tr>
<td>CXCR4</td>
<td>53.6 +/- 11.1</td>
<td>12.7 +/- 4.9</td>
<td>3.3</td>
</tr>
<tr>
<td>ICOS-L</td>
<td>18.8 +/- 3.9</td>
<td>10.8 +/- 2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>PD-L2</td>
<td>65.4 +/- 14.2</td>
<td>16.3 +/- 5.8</td>
<td>4.0</td>
</tr>
<tr>
<td>TACI</td>
<td>25.3 +/- 3.9</td>
<td>8.2 +/- 1.8</td>
<td>3.7</td>
</tr>
</tbody>
</table>

The data indicate the mean fluorescent intensity plus or minus standard deviation as detected by flow cytometry; the data were obtained from the studies described in Figure 2.

The asterisks indicate statistical significance, where * p<0.05, **p<0.005, ***p<0.0005
provided a representation of the hypermutation dynamics of the CD11c-positive B cell population. The majority of segments that were characterized in the CD11c-positive B cells (41 of 69 clones) contained at least one mutation, and the genes exhibited an overall mutation frequency of 4.3 \times 10^{-3} \text{ mutations/bp} (\text{Figure 3.2c}). In contrast, CD11c-negative B cell \( \mu \) chain genes contained only one mutation in 32 clones, an overall frequency of 1.1 \times 10^{-4} \text{ mutations/bp}, a frequency indistinguishable from the PCR incorporation error rate. The mutation frequencies identified in the CD11c-positive B cells are consistent with other reports of mutation frequency in IgM memory B cells (Anderson et al., 2007; Dogan et al., 2009; Pape et al., 2011).

\textit{CD11c-positive B cells reside in the splenic marginal zone and at the edge of the B cell follicles}

Lymphocyte positioning within secondary lymphoid organs is critical for their function. The splenic marginal zone (MZ) is a specialized area at the border of the periarteriolar lymphoid sheath (PALS) and B cell follicles and is populated by macrophages, dendritic cells (DCs), and MZ B cells. Due to the nature of blood flow in the spleen, cells residing in this region have unique access to blood-borne antigens and pathogens (Kraal, 1992; Martin and Kearney, 2000). Memory B cells have also been shown to localize to the MZ in both rodents and humans (Dunn-Walters et al., 1995; Liu et al., 1988; Oldfield et al., 1988; Tangye et al., 1998). \textit{In situ} analyses of spleens from mice on day 63 post-infection identified CD19-positive B cell follicles, with CD11c-positive cell staining in the red pulp (\text{Figure} 3.2c).
3.3a). CD169-positive metallophilic macrophages delineated the MZ, although in infected mice these were found in fewer numbers, and were by appearance less organized than in uninfected mice. The infected spleen also showed distinct co-localization of CD19- and CD11c-expressing cells at the edge of the B cell follicles, adjacent to CD169-positive macrophages of the splenic MZ (Figure 3.3b). In addition, cells that expressed both CD19 and CD11c were consistently found at the edges of B cell follicles, rather than in peanut agglutinin (PNA)-positive GC B cells (Figure 3.3 c, d). These data indicate that the CD11c-positive B cells are located in the splenic MZ, but not in GCs, findings that are consistent with the reported localization of memory B cells in rodents and humans.

IgG Recall Responses in Antigen-Challenged Mice

Robust, high-affinity responses to secondary antigen challenge are considered a hallmark of humoral memory. Since secondary E. muris infection is rapidly neutralized by existing antibodies, we evaluated humoral memory responses following intraperitoneal administration of recombinant OMP-19. IgG production was analyzed because we hypothesized that IgM memory B cells would undergo differentiation and class switching in GCs following antigen re-encounter, as was previously reported (Dogan et al., 2009). Mice challenged with recombinant OMP-19 delivered in alum generated reciprocal IgG titers as high as 17,000 12 days later (Figure 3.4a); IgG titers in non-challenged mice, and following primary OMP-19 immunization, were by comparison relatively low (Figure 3.4b). Analysis of IgG subclass utilization showed IgG2c to be the dominant antibody isotype
Figure 3.3: CD19-positive B cells were detected in the splenic marginal zone and at the edge of B cell follicles.

Cryosections from uninfected (A, C), and infected mice (B, D; analyzed on day 63 post-infection) were stained with Alexa 488-conjugated CD11c (green), and CF594-conjugated CD19 (red), and Alexa 647-conjugated CD169 (blue; panels A and B) or PNA-Alexa 647 (blue; panels C and D). The images show representative images of B cells identified in several randomly selected cryosections. The scale bars represent 40 μm, and yellow arrows indicate several dual CD11c-CD19-positive B cells.
elicited after antigen challenge (reciprocal IgG2c titers were detected as high as 31,200). Mice that were challenged with OMP-19 without alum also produced robust IgG2c responses, indicating that adjuvant was not necessary for IgG production. IgG1, which is not usually detected following ehrlichial infection (Racine et al., 2010), was detected after antigen challenge, suggesting that the IgG production after antigen challenge was a consequence of class switching. MHC-II-deficient mice did not generate an IgG recall response, and IL-21R-deficient mice exhibited a 7-fold decrease in OMP19-specific IgG on day 12 post-challenge, relative to challenged to wild-type mice (Figure 3.4c). These data demonstrate that mice infected with *E. muris* undergo a robust CD4 T cell- and IL-21R-dependent memory response to antigen challenge.

**CD11c-positive B cells are responsible for amnestic responses to OMP-19 challenge**

Since *E. muris*-infected mice generated a strong secondary response to OMP-19 challenge, we next addressed whether the CD11c-positive B cells were directly responsible, by specifically depleting the population *in vivo*. For these studies, we targeted CD11c-expressing B cells using CD11c-DTR transgenic mice, a mouse strain which encodes a simian diphtheria toxin receptor (DTR)-GFP fusion protein that is regulated by the CD11c promoter (Jung et al., 2002). Administration of diphtheria toxin (DTX) has been shown to efficiently deplete CD11c-expressing cells *in vivo*. To avoid possible artifacts associated with the depletion of CD11c-positive DCs, irradiation-induced bone marrow chimeric mice were generated.
Figure 3.4: Antigen challenge of E. muris-infected mice

Uninfected and infected mice were administered alum, or were challenged with 100 µg recombinant OMP-19 in alum; challenge of the infected mice was performed on day 30 post-infection. (A) Reciprocal OMP-19-specific serum IgG titers obtained on days 0, 3, 6, and 12 post-antigen challenge, determined by ELISA, are shown. (B) OMP-19-specific IgG subclass titers measured in the serum of infected mice that remained unchallenged, or had been challenged with OMP-19; OMP-19 was delivered in either alum or PBS, as indicated. (C) Serum OMP-19 specific antibody titers were determined on days 0, 6, and 12 post-challenge in MHCII-deficient, IL-21-deficient, and wild-type mice. The data in each of the panels are representative of two experiments. * p<0.05, ** p< 0.005.
using a 1:1 ratio of donor bone marrow obtained from B cell-deficient, and CD11c-DTR transgenic mice. In this experimental model, all donor-derived CD11c-expressing B cells were anticipated to be DTX-sensitive, but CD11c-negative B cells, and donor-derived DCs were expected to be DTX-resistant. The CD11c-positive B cells were indeed susceptible to the toxin, as DTX treatment eliminated these cells from infected CD11c-DTR transgenic mice within 24 hours of toxin administration (Figure 3.5a). To address whether the CD11c-positive B cells were required for the humoral recall response to antigen challenge, infected chimeric mice were administered DTX on days -1, and 6 post-OMP-19 challenge. Serum antibody titers were determined by ELISA, 6 and 12 days post-challenge. Infected chimeric mice that were administered DTX, exhibited a 5-fold reduction in OMP-19 titers relative to non-toxin-treated infected mice (Figure 3.5b). Uninfected chimeric mice that were challenged with OMP-19 exhibited modest IgG titers, and no reduction of IgG titers were observed following toxin administration. These data strongly suggested that the CD11c-positive IgM B cells were responsible for the amnestic IgG response.

DCs are known for their ability to initiate primary immune responses, and for their important role as professional APCs (Banchereau et al., 2000). In addition to these important functions, DCs also have the ability to modulate B cell responses (Cyster, 2010; Ueno et al., 2010). To rule out any possibility that the impaired humoral recall responses observed following DTX treatment were due to partial DC depletion, bone marrow chimeric mice were also generated using a 9:1 ratio of B
Figure 3.5: In vivo depletion of CD11c-positive B cells abrogated the IgG responses following antigen challenge

(A) CD11c-DTR transgenic mice were infected and administered diphtheria toxin (DTX; 9ng/g of body weight) on day 30 post-infection. Twenty-four hours later, splenocytes were analyzed by flow cytometry. The panels shown are representative of 3 mice per group. (B) Chimeric mice were generated using a 1:1 ratio of B cell-deficient and CD11c-DTR bone marrow. The chimeric mice were infected, administered DTX on day 30 post-infection, and challenged with OMP-19, as described in Figure 5. (C) Chimeric mice were generated as in B, using a 9:1 ratio of B cell deficient to CD11c-DTR bone marrow. Serum OMP-19 titers were determined by ELISA on day 12 post-challenge. The data in each of the studies shown are representative of at least two experiments; * p<0.05
A

B cell-deficient: CD11c-DTR (1:1)

B

Uninfected No DTX
Uninfected + DTX
E. muris No DTX
E. muris + DTX

C

B cell deficient: CD11c-DTR (9:1)

E. muris No DTX
E. muris + DTX
cell-deficient and CD11c-DTR transgenic bone marrow, respectively. Because in these chimeric mice the only source of B cells was from the transgenic bone marrow, all CD11c-expressing B cells were susceptible to DTX administration, and the majority of DCs were DTX-resistant. Similar to the data obtained using the equicellular chimeric mice, the 9:1 chimeric mice treated with DTX exhibited a 10-fold reduction in OMP-19-specific IgG titers, relative to untreated mice (Figure 3.5c). Therefore, it is unlikely that the ablation of the secondary IgG responses elicited following antigen challenge was due to the DTX-mediated depletion of CD11c-positive DCs. These data demonstrate that the CD11c-positive B cell population is required for IgG recall responses to antigenic challenge in *E. muris*-infected mice, supporting the conclusion that the CD11c-positive B cells are *bona fide* IgM memory B cells. The data are also consistent with the notion that IgM memory cells undergo class switching in response to antigen challenge.

**Discussion**

Our studies have demonstrated that splenic IgM memory B cells are generated in the mouse in response to bacterial infection and that these cells are responsible for IgG production following secondary challenge. Although many researchers have sought means to monitor and enumerate memory B cells *in vivo*, definitive assessment of such a population of cells has been difficult to achieve in infected mice. Thus, our findings are significant for characterizing IgM memory B cells in a natural model of infection. Although IgM memory B cells were described
in the 1980s, and have been identified in humans, we have shown they can be elicited by bacterial infection and that they can accumulate in large numbers in the spleen. Thus, although it has been generally considered that memory B cells are normally maintained at low frequencies, our data indicate that this is not necessarily the case. Indeed, we have demonstrated on the basis of many independent criteria, that the CD11c-positive B cells we have identified in the spleen during E. muris infection are a bona fide population of IgM memory B cells. Our conclusion is based on the following definitive criteria: 1) cell surface marker expression on the CD11c-positive B cells was nearly identical to that described for memory B cells identified in other non-infectious experimental models; 2) the cell population persisted indefinitely and was largely quiescent; 3) the cells were CD138-negative and did not produce IgM or IgG spontaneously; 4) they did not express GC markers and resided in the splenic MZ, not in GCs; 5) a significant proportion of the cells contained mutated Vh genes; and 6) deletion of the cells in vivo ablated recall responses to specific antigen. It is formally possible that the CD11c-positive B cell population perform different or additional functions. For example, such functions may include their acting as non-antibody secreting, chronically-activated suppressor B cells, or APCs, although such functions are not supported by any of our data. It is also possible that only a small subset of the CD11c-positive B cells are “true” memory B cells responsible for IgG responses following antigen challenge. This explanation is unlikely correct, however, as the population was homogenous with respect to the expression of most cell surface
markers used for their characterization. Thus, although memory B cells are usually considered to be present in low frequencies, our findings indicate that bacterial infection can drive the production and maintenance of large numbers of such cells.

The observation that the CD11c-positive B cells are maintained under conditions of low-level persistent infection does not exclude them as memory B cells. In fact, memory B cells are generated in several chronic infections by pathogens such as HIV, malaria, CMV, and HCV (Aberle and Puchhammer-Stockl, 2012; Moir et al., 2008; Mouquet et al., 2011; Ndungu et al., 2009; Roughan et al., 2012; Scheid et al., 2009; Weiss et al., 2009). Although the IgM memory B cells in our studies were maintained following antibiotic treatment, we were unable to exclude the possibility that antigen persisted under these conditions and was responsible for memory B cell maintenance. Indeed, it is well established that antigen can persist for months if not years on follicular dendritic cells (Tew et al., 1980). Although there is conflicting evidence in the literature, it is possible that low antigen exposure or inflammation is required for memory cell maintenance (Ahmed and Gray, 1996; Bachmann et al., 1996; Bernasconi et al., 2002; Gray, 2002; Maruyama et al., 2000). Indeed, studies have suggested that subclinical infections may be responsible for the maintenance of memory to bacterial pathogens (Weiss et al., 2009).

Why, then, does ehrlichial infection generate such a large population of IgM memory B cells? Like memory T cells, memory B cells generated under different conditions are likely heterogeneous, depending on the inflammatory conditions
associated with infection or immunization (Good-Jacobson and Tarlinton, 2012; Jameson and Masopust, 2009). Indeed, we have described extensive spleen disorganization and/or destruction during acute ehrlichia infection that may facilitate the development of large numbers of IgM memory cells (Racine et al., 2010). We propose that ehrlichial infection drives the expansion of a particularly homogenous subset of CD11c-positive IgM memory B cells that may represent a subset of memory B cells elicited during other infections, or following distinct vaccination regimens. The particularly high frequency of the CD11c-positive B cell population does not undermine the conclusion that they are memory B cells. Rather, our findings suggest that there are significant quantitative and qualitative differences among memory B cells elicited by different pathogens.

It is possible that yet other memory B cells were generated during ehrlichial infection that we were unable to identify, including swlg memory B cells. However, the observation that DTX treatment ablated the IgG recall responses suggested that all of the IgG produced upon antigen challenge was derived from the CD11c-expressing IgM memory B cells. Indeed, we have demonstrated that IgG production and GC formation are inhibited during acute E. muris infection (Racine et al., 2010), which may explain the apparent paucity of swlg cells in our model. Nevertheless, we cannot formally eliminate the possibility that small numbers of CD11c-positive, IgM-negative, swlg memory B cells were also targeted in the DTX depletion studies. This explanation appears unlikely and does not undermine the
conclusion that the IgM memory B cells are major, if not exclusive contributors to
the IgG recall responses.

The IgM memory B cells described in our model were initially identified on
the basis of their unique expression of CD11c. Although CD11c expression is
atypical in B cells, several studies have identified CD11c expression on B cells in
both mice and humans (Ehrhardt et al., 2008; Moir et al., 2008; Postigo et al.,
1991; Racine et al., 2008; Rubtsov et al., 2011; Rubtsov et al., 2013). CD11c (α₇) is
an alpha integrin of the leukointegrin family, and is expressed as a dimer with the
common β chain CD18 (β₂), forming complement receptor 4 (CR4). CD11c can
bind a variety of ligands, including cell adhesion molecules, fibrinogen, and iC3b
(Sadhu et al., 2007). The function of CD11c on IgM memory B cells is unknown,
but as an adhesion molecule, CD11c may be partly responsible for proper
positioning of the IgM memory B cells within or adjacent to the splenic MZ. In fact,
other integrins have been shown to be responsible for proper positioning of B cells
within the MZ and for trafficking through the MZ into the white pulp (Lo et al.,
2003; Lu and Cyster, 2002). However, several other surface antigens, in addition to
CD11c and IgM, uniquely distinguish these cells from other B cell populations. We
noted several co-stimulatory and adhesion molecules that were upregulated on the
CD11c-positive B cells, as compared to CD11c-negative B cells. These markers
included CD73, CXCR4, PD-L2, TACI, and CD11b. CD73 is an ecto-enzyme with
several functions including lymphocyte adhesion, and catalysis of extracellular
AMP to adenosine (Colgan et al., 2006). CD73 is often expressed on mature
lymphocytes, and as such has been considered a B cell maturation marker (Thompson et al., 1986). Several studies have also shown CD73 expression on memory B cell populations, particularly those with somatic mutations (Anderson et al., 2007; Taylor et al., 2012; Tomayko et al., 2010). CXCR4 is a chemokine receptor responsible for cell migration towards its ligand CXCL12. This interaction is important for B cell homeostasis, plasma cell homing to the bone marrow, and GC organization (Allen et al., 2004; Hargreaves et al., 2001). The upregulation of CXCR4 on the surface of the CD11c-positive IgM memory B cells may regulate eventual homing of these cells to the bone marrow. PD-L2 (programmed death-ligand 2) is a B7 homolog, known for inhibiting T cell proliferation through its interaction with PD-1 (programmed death molecule-1). Although PD-L2 expression is typically associated with macrophages and DCs, expression has also been shown on B1 cells and to be highly up-regulated on memory B cell populations (Good-Jacobson et al., 2010; Tomayko et al., 2010; Zhong et al., 2007). TACI is a transmembrane receptor in the tumor necrosis receptor superfamily that binds both BAFF (B cell activating factor of the TNF family) and APRIL (a proliferation-inducing ligand). Signals transmitted by TACI modulate many B cell functions and survival in the periphery. Available evidence has indicated that BAFF and APRIL signals are not necessary for memory B cell survival, despite high expression of TACI on memory B cells (Benson et al., 2008), although a possible function for TACI on IgM memory B cells has not yet been evaluated. CD11b expression is characteristic of B-1 cells and may suggest a possible cellular origin for the IgM
memory B cells we have identified. The unique cell surface phenotype of the IgM memory cells we have identified suggests that it may be possible to unambiguously identify such cells during other infections, and in humans.

Several studies have described IgM memory B cells in humans (reviewed in Tangye and Good, 2007). The relationship between the IgM memory B cells identified in humans and those we and other have identified in mice is not yet known, although the CD11c-positive IgM memory B cells described here bear similarities to memory B cells described in humans. Indeed, expression of CD11c has been reported on tissue-specific Fc receptor-like protein 4 (FCLR4)-positive memory B cells in human tonsils, as well as in the blood of HIV-infected patients (Ehrhardt et al., 2008; Moir et al., 2008). CD11c expression has also been identified on a population of atypical memory B cells in malaria patients (Weiss et al., 2009), in various human B cell malignancies, and autoimmune patients (Kost et al., 2008; Molica et al., 1994; Rubtsov et al., 2011; Rubtsov et al., 2013; Schwarting et al., 1985). Thus, it will be important to determine the relationship between the various human IgM memory B cell populations and those observed in murine models, so that the generation and function of the cells may be studied systematically.

Why should humoral memory be harbored in IgM memory B cells, given that swlg memory B cells may respond more quickly, and with higher affinity to secondary challenge? Although IgM is produced early following infection, we have shown that *E. muris* infection also elicits a long-term protective IgM response
(Racine et al., 2011). IgM memory B cells may be required to maintain long-term IgM production in ehrlichial and other chronic infections, where low concentrations of foreign antigens persist. Alternatively, IgM memory B cells, but not swIg memory B cells, can initiate GC reactions and undergo affinity maturation and class-switch recombination following re-encounter with antigen (Dogan et al., 2009; Pape et al., 2011). This process may function to allow greater flexibility when the host encounters variant pathogens.

The observation that the CD11c-positive B cells are required for the class-switched antibody response to OMP-19 challenge also suggests the IgM memory cells undergo GC re-entry. However, we have not demonstrated whether the IgM memory cells re-enter GCs, as we have been unable to engraft the CD11c-positive B cells into recipient mice. Although the limited somatic mutation that occurs in IgM memory B cells may generate higher affinity B cell receptors, it is also possible that antigen affinity is less important than other attributes of IgM memory B cells. These attributes may include their increased number, specialized anatomic localization (i.e. the splenic MZ), and perhaps a better capacity for antigen presentation and cytokine production. Understanding such differences in the properties and functions of IgM memory B cells in mice and humans will facilitate strategies to generate the type of humoral memory most appropriate for particular infections. Our findings underscore the notion that immunological memory is mediated by rich and diverse memory B cell populations that together mediate host defense against the wide range of pathogens encountered in nature.
CHAPTER IV: Generation of IgM Memory B Cells During
Ehrlichial Infection
Acknowledgements

I would like to thank Tres Cookenham (Trudeau Institute; Saranac Lake, NY) for his technical assistance, and performing the experiments with the MHCII-deficient mice (Figure 4.2a). The IL-21R-deficient mice were kindly provided by Dr. Leonard Warren (National Institute of Health; Bethesda, MD), and the AID-Cre-Ert2 mice were kindly provided by Dr. Jean-Claude Weill (INSERM; Paris, France).
Abstract

Determining the signals required for eliciting quality, protective, memory B cells is essential for developing therapeutic vaccines. The mechanisms of IgG memory B cell generation are generally understood. However, little is known regarding the mechanisms of IgM memory B cell generation. Here we show that the CD11c-positive IgM memory B cells generated during ehrlichial infection are dependent on T cells, IL-21R, and Bcl-6. The number and frequency of CD11c-positive IgM memory B cells were independent of the infectious doses tested, and were not detected following immunization with typical T cell-dependent and T cell-independent antigens. Using a mouse model where AID-expressing cells are permanently labeled with eYFP, we found that the CD11c-positive IgM memory B cells were generated during the acute phase of ehrlichial infection. IgM memory B cell development began as early as day 4, with higher frequencies generated on days 7 and 10 post-infection. These data challenge traditional views of T-dependent responses and IgM memory B cell generation.
Introduction

Humoral immune responses can be classified as T cell-dependent (TD) or T cell-independent (TI). The type of humoral immune response generated is dictated by the type of antigen encountered by the host. TD antigens are predominantly protein antigens, while TI antigens are predominantly microbial ligands or multivalent ligands capable of cross-linking the BCR (Defrance et al., 2011; Vinuesa and Chang, 2013).

The classic TD response begins as a naïve follicular B cell is stimulated via a specific interaction with antigen and the BCR, and is dependent on a cognate co-stimulatory signal through T cell help (Lanzavecchia, 1985). Once the B cell interacts with a cognate T cell at the border of the T cell zone, it re-enters the follicle where a germinal center (GC) is formed (Kerfoot et al., 2011; Okada et al., 2005). The GC is a major site where B cell clonal expansion, somatic hypermutation, and class-switch recombination occurs. Somatic hypermutation, mediated by the enzyme activation-induced cytidine deaminase (AID), serves to generate high-affinity B cell clones, which then differentiate into either class-switched long-lived plasma cells or memory B cells (Allen et al., 2007).

TI antigens are able to produce antibody responses in the absence of T cell help (Vos et al., 2000). Specifically, TI type 1 antigens are able to provide a secondary signal through polyclonal activators such as lipopolysaccharide (LPS), lipotechoic acid (LTA), and peptidoglycan. These microbial ligands achieve B cell
activation through innate receptors such as toll-like receptors (TLRs). Marginal zone (MZ) and B1b cells respond to these stimuli, producing a robust extrafollicular response characterized by the production of low-affinity IgM (Vinuesa and Chang, 2013). TI type 2 antigens are typically multi-valent polysaccharide antigens that activate B cells through BCR cross-linking, and prolonged signaling which is dependent on Bruton’s Tyrosine Kinase (BTK). Classic TI-2 antigens include bacterial polysaccharides such as those from H. influenzae, N. meningitides, and S. pneumoniae. MZ and B1b cells respond to these antigens and generate early extrafollicular antibody responses characterized by the production of low affinity IgM (Vinuesa and Chang, 2013). Recently proposed is the TI type 3 response (Vinuesa and Chang, 2013), where innate cells have the ability to drive a TI antibody response. An example includes B cell helper neutrophils (N_{BH}) that have been shown to colonize the MZ and provide help to MZ B cells during a TI response (Puga et al., 2012).

Memory B cell generation was historically considered exclusively a TD, GC-derived phenomenon. Several studies have since demonstrated the existence of TI-derived memory B cells (Alugupalli et al., 2004; Foote and Kearney, 2009; Obukhanych and Nussenzweig, 2006). These cells tend to be of B1 origin and secrete unmutated antigen-specific IgM upon re-stimulation, although TI IgG memory B cells have also been described (Good-Jacobson and Tarlinton, 2012). TI memory B cells are negatively regulated by serum immunoglobulin (Ig) and must be transferred to a naïve host for reactivation (Foote and Kearney, 2009;
Obukhanych and Nussenzweig, 2006). IgM memory B cells are generally considered to be derived independently of T cells and germinal center formation (Good-Jacobson and Tarlinton, 2012). Therefore, we sought to determine what signals and mechanisms are involved in the generation of the CD11c-positive IgM memory B cells described in Chapter 3. Here we show that generation of the CD11c-positive IgM memory B cells is T-dependent, GC-dependent, and occurs early during *E. muris* infection.

**Results**

*Generation of CD11c-positive B cells is Independent of the Infectious Doses Tested*

We have previously shown that infection of immunocompetent mice with *E. muris* elicits a relatively large CD11c-positive, IgM memory B cell population (Chapter 3). To determine the influence of infectious dose on the magnitude of the CD11c-positive B cell response, mice were infected with varied doses of *E. muris*. As compared to our standard dose of 5.0 x 10^4 copies of bacteria, mice infected with 2 and 4-fold fewer copies had comparable frequencies and numbers of CD11c-positive B cells in their spleen (Figure 4.1a). This data indicates that the magnitude of the CD11c-positive B cell response to ehrlichial infection is relatively insensitive to the infectious dose and is consistent with reports of IgM memory B cell generation in a model of PE immunization (Taylor et al., 2012).
Figure 4.1: Induction of CD11c-positive IgM memory B cells during E. muris infection

(A) C57BL/6 mice were infected with 1.25, 2.50, or 5.00 x 10^4 copies of E. muris. On day 30 post-infection, splenocytes were analyzed by flow cytometry. The panels shown are representative of 3 mice per group. The frequency (left panel) and total number (right panel) of CD11c-positive IgM memory B cells among total splenocytes is shown. (B) C57BL/6 mice were infected with 5.0 x 10^4 copies of E. muris, 1x10^6 SRBC’s, or 50 µg NP-Ficoll in alum. On day 30 (day 42 for NP-Ficoll) post-infection/immunization splenocytes were analyzed by flow cytometry. The panels shown are representative of multiple mice per group. The frequency (left panel) and total number (right panel) of CD11c-positive IgM memory B cells among total splenocytes is shown.
**E. muris Infection Uniquely Generates a Large Population of CD11c-positive IgM Memory B cells**

CD11c expression on B cells is a relatively unexplored phenomenon. While CD11c expression has been shown on B cells in mice and humans (Ehrhardt et al., 2008; Moir et al., 2008; Racine et al., 2008), the signals that drive CD11c expression are unknown. Therefore, we sought to determine if other immune stimuli might elicit a population of CD11c-positive IgM memory B cells similar to that of *E. muris* infection. To this end, mice were immunized with sheep red blood cells (SRBC), a classic TD antigen, or NP-ficoll, a classic TI-type 2 antigen. Both stimuli have been shown to elicit populations of IgM memory B cells in immunized mice (Dogan et al., 2009; Obukhanych and Nussenzweig, 2006). However, a no detectable increase of CD11c-positive IgM memory B cells was detected following either SRBC or NP-ficoll immunized mice (Figure 4.1b). The average frequency and number (< 1%, and < 1x10⁶ respectively) of CD11c-positive B cells for both immunizations was comparable to that of uninfected mice.

**T cells are required for the generation of CD11c-positive IgM memory B cells**

CD4 T cells are important for the development of GC-derived memory B cells, as is the cytokine IL-21 (Good-Jacobson and Shlomchik, 2010; Linterman et al., 2010; Ozaki et al., 2004; Zotos et al., 2010). Therefore, we next addressed the requirement for these factors in the generation of the CD11c-positive IgM memory B cell population. To this end, mice deficient in either CD4 T cells (MHCII-deficient) or IL-21R were infected with *E. muris*. Both strains exhibited increases
susceptibility to infection as evidenced by splenomegaly and hypercellularity of the spleen during chronic infection (data not shown). In addition, the frequency and number of total B cells in the spleens of MHCII-deficient and IL-21R-deficient mice was decreased as compared to wild-type control mice (data not shown). The frequency and number of CD11c-positive B cells as a portion of total splenic B cells was much lower in MHCII-deficient mice (Figure 4.2a). CD11c-positive IgM memory B cells were also significantly reduced as a portion of total splenic B cells by frequency and number in mice deficient for the IL-21R (Figure 4.2b). These data indicate that CD4 T cell-dependent signals are required and/or important for the generation of the CD11c-positive B cells.

CD73, or ecto-5'-nucleotidase is a cell surface enzyme capable of catalyzing the conversion of extracellular AMP to adenosine, and as such is an important molecule in many cell signaling pathways (Ernst et al., 2010). In addition to its enzymatic activity, CD73 also mediates many physiologic processes including ion and fluid transport, adaptation to hypoxia, renal function, and the facilitation of lymphocyte adhesion (Colgan et al., 2006). Several studies have shown CD73 expression on memory B cell populations (Anderson et al., 2007; Taylor et al., 2012; Tomayko et al., 2010), yet its function in memory B cells is unknown. Since CD73 is highly upregulated on the CD11c-positive IgM memory B cells, we hypothesized that CD73 may play a role in the their generation. To determine the role of CD73 in the generation of these cells, CD73-deficient mice were infected with *E. muris*. At day 76 post-infection, the frequency of CD11c-
Figure 4.2: CD4+ T cells and IL-21 signaling were required for the generation of the CD11c-positive B cell population.

(A) Flow cytometry analysis of splenic CD11c-positive IgM memory B cells from wild-type and MHCII-deficient mice analyzed on day 30 post-infection is shown. The frequencies of CD11c-positive B cells represent their percentage among total splenic B cells. Total B cell frequency in the MHCII-deficient strains was 33-41%.

(B) Flow cytometry analysis of CD11c-positive IgM memory B cells from wild-type and IL-21R-deficient mice on day 70 post-infection. The frequencies of CD11c-positive B cells represent their percentage among total splenic B cells. Total B cell frequency in the IL-21R deficient strains was 13-22%.

(C) Flow cytometry analysis of CD11c-positive IgM memory B cells from wild-type and CD73-deficient mice on days 48 and 76 post-infection. The frequencies of CD11c-positive B cells represent their percentage among total splenic B cells. Total B cell frequency in the CD73-deficient strains was 60-70%. Data was combined from two separate experiments. The data in A, and B are representative of two experiments; * p<0.05, **p< 0.005.
positive IgM memory B cells was reduced in CD73-deficient mice (Figure 4.2c). However, the total numbers of these cells in the spleen was not significantly different between WT and CD73-deficient mice. This data indicates that CD73 does not play a crucial role in the development of the CD11c-positive IgM memory B cells.

**Bcl-6 is required for the Generation of the CD11c-positive IgM Memory B Cells**

GCs are major sites of T-dependent antibody responses in secondary lymphoid organs, and it is in these specialized structures that most antibody affinity maturation occurs. Memory B cells are considered to be the product of the GC reaction, and as such, are T cell-dependent (Allen et al., 2007). IgM memory B cells may provide an exception to this conclusion, as at least a subset of these cells have been reported to undergo GC-independent development (Kruetzmann et al., 2003; Taylor et al., 2012; Weller et al., 2004), and because affinity maturation can under some conditions, occur independently of GCs (Gardby et al., 2003; Kim et al., 2006; Matsumoto et al., 1996). Since we have established the T-dependent generation of the CD11c-positive IgM memory B cells, we addressed whether these cells were also GC-derived. Bcl-6 is a transcription factor that is selectively upregulated in T follicular helper (T_{fh}) cells and GC B cells (Cattoretti et al., 1995; Liu et al., 2012b). In B cells, Bcl-6 functions to maintain an anti-apoptotic state, suppression of plasma cell differentiation, and down-regulation of BCR signaling through transcriptional repression (Basso et al., 2010; Ci et al., 2009; Saito et al., 2009; Shaffer et al., 2000). Studies have also shown that Bcl-6 expression in B cells
Figure 4.3: Germinal Center dependence of the CD11c-positive IgM memory B cell population

(A) Chimeric mice were generated using a 1:1 ratio of CD45.1 wild-type (WT) and CD45.2 Bcl-6-deficient bone marrow. The chimeric mice were infected, and splenocytes were analyzed at day 30 post-infection by flow cytometry. The panel shown is representative of 8 mice per group. The frequencies of WT and Bcl-6 deficient cells represent their percentage from a live gate.  

(B) Flow cytometry analysis of CD45.1 WT single positive and CD45.2 single positive cells based on the plot in (A). The plots shown are representative of 8 mice per group. The frequencies of CD11c-positive IgM memory B cells represent their percentage among total B cells of the single positive WT and Bcl-6-deficient gates, respectively. Data shown is representative of 2 independent experiments.
is required for proper GC formation (Dent et al., 1997). To test the GC-dependence of the CD11c-positive IgM memory B cells, chimeric mice were generated using a 1:1 ratio of bone marrow from WT congenic and transgenic (Tg) mice conditionally deficient for Bcl-6 in CD19-expressing cells. Therefore, WT CD19⁺ B cells express Bcl-6, while CD19⁺ Tg B cells are deficient in Bcl-6. At thirty days post-infection, splenocytes were analyzed based on the congenic markers CD45.1 and CD45.2, respectively. These analyses revealed unequal engraftment of cells in the spleen, where the WT CD45.1 single-positive cells comprised an average of 49.5% of total spleen cells, and the Bcl-6 CD45.2 single-positive deficient cells averaged only 2.65% of total spleen cells (Figure 4.3a). CD45.1/CD45.2 double positive cells comprised an average of 26.2% of the total spleen cells, and were excluded from the analysis. An average of 6.1% of the WT B cells were CD11c-positive, as compared to 1% of the Bcl-6 deficient B cells, a level comparable to that of uninfected mice (Figure 4.3b). The absence of CD11c-positive IgM memory B cells in the Bcl-6 deficient cells shows that B cell intrinsic expression of Bcl-6 is required for their development. These data, suggest that the CD11c-positive IgM memory B cells are generated via a GC-dependent pathway. This conclusion is supported by the up-regulation of CD73 expression on the IgM memory B cells, a marker that has been associated with GC experience (Taylor et al., 2012).

**IgM Memory B Cells are Generated Early During Ehrlichial Infection**

As previously shown in Chapter 3, CD11c-positive IgM memory B cells were detected in the spleens of *E. muris*-infected mice by day 30 post-infection
(Chapter 3; Figure 3.1a). However, we wondered at what point during ehrlichial infection the generation of these cells begins. To “track” memory B cell development during acute infection, we used a transgenic mouse model that has been described by Dogan and colleagues (Dogan et al., 2009). These transgenic mice encode a tamoxifen-inducible Cre recombinase, expression of which is driven by the Aicda promoter at one allele. When crossed with mice transgenic for the Rosa26-loxP-eYFP reporter cassette, Cre-mediated excision of the loxP terminator sequence allows permanent expression of the enhanced yellow fluorescent protein (eYFP) exclusively in AID-expressing cells. Administration of tamoxifen to these double-transgenic mice results in release of the cytoplasmic sequestration of the Cre recombinase and subsequent transcription of eYFP. Therefore, tamoxifen administration provides a permanent “snap-shot” of AID expressing cells via eYFP expression.

To determine when the generation of the CD11c-positive IgM memory B cells occurs, tamoxifen was administered at 3-day intervals to E. muris infected mice from days 4 to 13 post-infection. Analysis at day 30 an average of 66% eYFP-positive cells were detected in the CD11c-positive IgM memory B cell population. In contrast, an average of 20% eYFP-positive cells were detected CD11c-negative population on day 30 post-infection (Figure 4.4a). Tamoxifen is active in vivo for 72 hours post-administration. Therefore, these data show that 66% of the CD11c-positive IgM memory B cells were generated between 4 and 16 days post-E. muris infection.
Figure 4.4 Detection of AID-expressing Cells Generated During Acute E. muris Infection

(A) AID-Cre-Ert2 x ROSA-eYFP$_{F1}$ double-transgenic mice were infected and administered 10 mg tamoxifen on days 4, 7, 10 and 13 post-infection. Flow cytometry analysis of splenocytes was performed on day 30 post-infection. Plots shown represent cells gated on CD11c-positive and –negative B cells, and are representative of 6 mice per group. The frequencies of eYFP-positive cells represent their percentage among CD11c-positive and –negative B cells. (B) AID-Cre-Ert2 x ROSA-eYFP$_{F1}$ double transgenic mice were infected and were administered 10 mg tamoxifen on days 4, 7, or 10 post-infection. Plots shown are represent cells gated on CD11c-positive and –negative B cells and are representative of 3 mice per group. The frequencies of eYFP-positive cells represent their percentage among CD11c-positive and –negative B cells.
Next, we sought to narrow the window of IgM memory B cell generation. To this end, tamoxifen was administered to separate groups of mice to on either days 4, 7, or 10 post-infection with *E. muris*. Analysis on day 30 post-infection revealed that an average of 5.7% of the CD11c-positive IgM memory B cells were generated on day 4, 22.5% on day 7, and 31.2% on day 10 post-infection (Figure 4.4b). Together, these results indicate that the CD11c-positive IgM memory B cell population is generated during the acute phase of infection, beginning as early as day 4.

**Discussion**

Different types of antigens have the ability to activate specific helper cells, which in turn induce the antibody response best suited for pathogen clearance (Vinuesa and Chang, 2013). We have shown that the generation of the CD11c-positive IgM memory B cells is largely independent of infectious dose, and that immunization with certain TD (SRBCs) or TI (NP-ficoll) antigens was not sufficient to generate these cells, as detected by flow cytometry. We propose that ehrlichial infection drives the expansion of a particularly large subset of CD11c-positive IgM memory B cells that may represent only a portion of memory B cells elicited by other infections, or following distinct vaccination regimens. CD11c-positive IgM memory cells may be present in small numbers in other murine infection and immunization models, and may be difficult to detect by flow cytometry. In support of this theory, CD11c expression has been described on an Fc receptor-like-4
(FCRL4)-positive population of memory B cells from the tonsillar tissue of humans (Ehrhardt et al., 2008). CD11c expression, in the form of GFP fluorescence was detected on CD138+ plasmablasts in a model of NP-ficoll immunization of CD11c-DTR mice. While CD11c transcription could be detected by GFP fluorescence, detection of CD11c surface expression was not achieved by flow cytometry (Hebel et al., 2006). This observation suggests that the cell numbers of CD11c-expressing cells, or the surface expression of CD11c is too low for detection by flow cytometry.

A requirement for T cell help and GC development in the generation of IgM memory B cells remains controversial. It has been proposed that IgM memory B cells are generated in a GC-independent fashion, and function to provide long-term T-independent immunity (Taylor et al., 2012). This conclusion is based on the observations that not all memory B cells carry somatic mutations, and that IgM memory B cells can be generated in mice and humans that cannot form GCs (Anderson et al., 2007; Tangye and Good, 2007; Toyama et al., 2002; Zotos et al., 2010). Accordingly, Taylor et al., have described a population of murine CD73-negative IgM memory B cells and their precursors that develop independently of GCs (Taylor et al., 2012). In contrast, studies by Dogan et al. described an analogous IgM memory B cell population that was somatically mutated, CD73-positive, and likely GC-derived. A GC-derived population of IgM+ IgD+ CD27+ memory B cells has also been described in humans (Seifert and Kuppers, 2009). Our data has established an essential role for CD4 T cells, Bcl-6, and an important
role for IL-21 signaling in the generation of IgM memory B cells during *E. muris* infection. In addition, the presence of somatic mutations in a large portion of the IgM memory B cells we have identified is consistent with a role for GC differentiation. The apparent differences in GC-dependent generation of various IgM memory B cell subsets may be due to the particular infection or immunization model used for the study.

Although we have demonstrated that the generation of the IgM memory B cells was CD4 T cell-dependent and Bcl-6-dependent, the IgM memory B cells are similar in cell surface phenotype to the population of splenic CD11c<sup>lo</sup> T cell-independent plasmablasts we have previously described (Racine et al., 2008). In that earlier study, the CD11c<sup>lo</sup> plasmablasts were detected at their highest frequency and number on day 9 post-*E. muris* infection, and were no longer detected by day 18 post-infection. In contrast, the IgM memory B cells described in this study were first detected at about day 30 post-infection, after the plasmablast response had declined. Therefore, it is possible that the IgM memory cells may be derived from the plasmablasts themselves, or from early subset of precursor cells that are then “rescued” from plasmablast differentiation by T cell help. As mentioned above, Taylor et al. described a CD38<sup>+</sup> GL7<sup>+</sup> precursor population that develops as early as 2 days after PE immunization. These precursor cells were able to give rise to both GC-derived class-switched memory B cells, and to GC-independent IgM memory B cells (Taylor et al., 2012). Since AID expression in the CD11c-positive IgM memory B cells is “turned on” as early as day 4, it is possible
they are derived from a similar precursor population. The largest frequency of
eYFP-positive, CD11c-positive IgM memory B cells were detected from mice
administered tamoxifen at day 10 post-infection. As mentioned above, the
maximum frequency of the CD11c-low plasmablasts was detected at day 9 post-
infection, strengthening the argument for a relationship between the two
populations. However, future studies are required to determine the identity of the
CD11c-positive IgM memory B cell precursors. Together, these data challenge
classic views of TD responses, IgM memory B cell generation, the timing of
memory B cell development, and support the conclusion that multiple routes exist
for the development of B cell memory.
CHAPTER V: Maintenance of Serum Antibody During Chronic Ehrlichial Infection
Abstract

IgM is a class of antibody, whose role in long-term immunity has remained under-appreciated. Our laboratory has demonstrated an important role for the long-term production of IgM in protection against fatal ehrlichial challenge. Therefore, we sought to identify the role of IgM memory B cells in the maintenance of protective IgM. Deletion of CD11c-expressing cells \textit{in vivo} transiently lowered IgM memory B cells, and antigen-specific IgM in the serum of chronically infected mice. Further analyses revealed a role for both IgM memory B cells, and CD138$^{\text{high}}$ IgM$^{\text{high}}$ ASCs in the maintenance of IgM during chronic infection. We propose that IgM memory B cells contribute to long-term IgM production by populating the plasma cell compartment in the bone marrow under steady-state conditions.
Introduction

IgM is a class of antibody that is commonly associated with the early phase of an immune response to infection or immunization (Boes, 2000). In the serum, IgM is present as a pentameric structure, which gives each antibody molecule 10 antigen-binding sites (Eskeland and Christensen, 1975). IgM binds to antigen with relatively low affinity; however, the valency of IgM results in high avidity interactions. IgM can mediate pathogen clearance by several distinct mechanisms. These mechanisms include complement activation, neutralization, opsonization, and Fc-receptor-mediated uptake (Casadevall, 1998). As a multimeric structure, IgM is a particularly strong activator of complement. The complement-fixing activity of IgM is well demonstrated by the fact that one molecule of IgM is capable of activating complement and lysing an erythrocyte. In contrast, at least 1000 IgG molecules are required for the same function (Cooper et al., 1983).

IgM plays an important role in host defense against a variety of pathogens (Racine and Winslow, 2009). A protective role for IgM has been demonstrated in immunity to extracellular bacterial pathogens, such as Borrelia and Streptococcus species (Briles et al., 1981; Brown et al., 2002; Connolly and Benach, 2001; Connolly et al., 2004). IgM-mediated protection has also been demonstrated in immunity to intracellular bacterial infections such as Nocardia brasiliensis, and Franciscella tularensis (Cole et al., 2009; Salinas-Carmona and Perez-Rivera, 2004). Studies in our laboratory have also demonstrated a protective role for IgM during E.
muris infection. We have shown that AID-deficient mice, which are incapable of
class-switch recombination, are protected against lethal challenge with the
heterologous ehrlichiae, known as IOE. The absence of IgG in AID-deficient mice
indicated that IgM production is sufficient for protection against a lethal challenge
with IOE (Racine et al., 2011).

IgM is often regarded as a source of transient immunity, elicited prior to the
T-dependent production of high-affinity IgG (Boes, 2000). As such, protection
provided by the long-term production of IgM has often been overlooked (Racine
and Winslow, 2009). Persistent IgM production was described in a model of F.
tularensis live vaccine strain (LVS), where protective IgM was detected up to 70
days post-immunization (Cole et al., 2009). Similarly, studies in our laboratory
have demonstrated long-term production of poly-reactive IgM up to 396 days post-
infection with E. muris (Jones et al., 2012). In addition, our laboratory identified a
population of bone marrow resident, CD138^high IgM^high ASCs that were responsible
for this long-term production of OMP-19-specific IgM (Racine et al., 2011).

In our model of E. muris infection, antibodies are required for the
maintenance of immunity to ehrlichial infection (Bitsaktsis et al., 2007; Yager et al.,
2005). Therefore, we hypothesized that IgM memory B cells may contribute to
protection through the maintenance of serum Ig during chronic infection. In the
present study we show that CD11c-expressing B cells are responsible for the
maintenance of antigen-specific IgM during E. muris infection, and suggest that T-

dependent IgM memory B cells contribute to the maintenance of plasma cell populations in the bone marrow.

Results

**CD11c-positive IgM memory B cells are Responsible for the Long-Term Maintenance of Antigen-specific IgM**

As was demonstrated in Chapter 3, we have identified a CD11c-positive population of IgM memory B cells that are responsible for the amnestic response to secondary antigen challenge. However, we were also interested in what protective role, if any, these cells may play under steady-state conditions. As with the studies in Chapter 3, we targeted CD11c-expressing B cells during chronic *E. muris* infection using mice chimeric for CD11c-DTR and B cell-deficient bone marrow. As previously shown ([Chapter 3; Figure 3.5](#)), DTX administration effectively ablated the CD11c-positive IgM memory B cells within 24 hours post-administration. To determine what effect deletion of the CD11c-positive IgM memory B cells may have on serum antibody titers, a single dose of diphtheria toxin (DTX) was administered to chimeric mice at day 30 post-infection. Analysis of serum IgM revealed significantly reduced OMP-19 IgM titers in DTX-treated mice by day 7 post-administration, as compared to untreated mice ([Figure 5.1a](#)). In contrast, OMP-19-specific IgG titers remained unaffected in DTX-treated mice (data not shown). However, by day 14 post-DTX administration, OMP-19 IgM titers had
Figure 5.1: In vivo depletion of CD11c-positive IgM memory B cells temporarily reduces antigen-specific IgM

(A) Chimeric mice were generated using a 1:1 ratio of B cell-deficient and CD11c-DTR bone marrow. The chimeric mice were infected and administered DTX (9 ng/g of body weight) on day 30 post-infection. Serum was collected on days 0, 7 and 14 post-DTX administration. Serum OMP-19 IgM titers were determined by ELISA. (B) Frequency and total number of CD11c-positive IgM memory B cells among total splenocytes at day 14 post-DTX administration.
returned levels similar to that of untreated mice. In addition, the frequency and number of CD11c-positive IgM memory B cells in the spleen had also returned to untreated frequencies by day 14 post-treatment (**Figure 5.1b**). These data suggest that under steady-state conditions, the CD11c-positive IgM memory B cells may play a protective role through the maintenance of antigen-specific IgM. The data also suggest that the CD11c-positive IgM memory B cells continue to be generated during chronic ehrlichial infection.

**DTX-administration Specifically Deletes CD11c-expressing Transgenic B Cells**

Since the frequency and number of CD11c-positive IgM memory B cells had returned to untreated frequencies by day 14 post-DTX administration, we analyzed an earlier time-point following DTX administration. To demonstrate specificity of DTX ablation, we generated mice chimeric for wild-type (WT), and CD11c-DTR transgenic bone marrow. IgM\(^a\) and IgM\(^b\) allotype markers allowed for the specific labeling of WT and CD11c-DTR-derived B cells, respectively. Analysis of splenocytes at day 4 post-DTX treatment revealed significantly reduced frequencies and numbers of DTX susceptible, IgM\(^b\) transgenic CD11c-positive IgM memory B cells (**Figure 5.2**). In contrast, the frequency and number of IgM\(^a\) WT CD11c-positive IgM memory B cells remained constant following DTX administration, demonstrating the specificity of DTX-mediated deletion of CD11c-expressing cells. The data show that the CD11c-positive IgM memory B cells were reduced at day 4 post-DTX treatment, suggesting that the loss of the IgM memory B cells contributes to the reduced OMP19-specific IgM titers at day 7 post-DTX ablation.
Figure 5.2: Specific depletion of transgenic CD11c-positive IgM memory B cells in the spleen of infected mice

(A) Chimeric mice were generated using a 1:1 ratio of IgMa wild-type and IgMb CD11c-DTR bone marrow. The chimeric mice were infected and were administered DTX (9 ng/g of body weight) on day 30 post-infection. Flow cytometry analysis was performed on total splenocytes 4 days following DTX administration. Plots shown were gated on total IgMa or IgMb-positive B cells. (B) Frequency and total number of CD11c-positive IgMa or IgMb memory B cells among total B cells at day 4 post-DTX administration. * p<0.05, **p< 0.005.
IgM-positive B cells are Reduced in the Bone Marrow of DTX-treated Mice

As discussed in Chapter 3, the IgM memory B cells do not express the plasma cell marker CD138 or actively secrete antibody ex vivo. How then would depletion of the IgM memory B cells affect serum antibody titers? Previous studies in our laboratory identified a population of CD138$^{hi}$ IgM$^{high}$ ASCs that are responsible for the production of OMP-19-specific IgM in the bone marrow (Racine et al., 2011). To determine the cellular mechanism of the reduced serum IgM, we analyzed the frequency and number of B cell populations in the bone marrow of chimeric mice following DTX administration. On day 4 post-DTX ablation, the frequency and number of IgM$^{b}$-positive transgenic cells was significantly reduced in the bone marrow (Figure 5.3a). As seen in the spleen, the frequency and number of IgM$^{a}$-positive WT B cells in the bone marrow was unaffected by DTX-mediated ablation, confirming DTR-specific ablation of CD11c-expressing cells. To assess the possibility that antibody-secreting cells (ASC) were affected by DTX-mediated depletion, we analyzed the bone marrow for the frequencies of these cells. The frequency of CD138$^{high}$ IgM$^{high}$ transgenic ASCs as a portion of total IgM$^{b}$-positive B cells in the bone marrow was significantly increased (Figure 5.3b). Overall however, the total number of transgenic, CD138$^{high}$ IgM$^{high}$ ASCs was significantly decreased following DTX-mediated depletion. These analyses confirmed a specific decrease in the frequency and number of IgM$^{b}$-positive B cells in the bone marrow, including the total number of CD138$^{high}$ IgM$^{high}$ ASCs. The reduction in the total number of these ASCs is likely the cause of
Figure 5.3: Specific depletion of transgenic IgM-positive B cells in the bone marrow of infected mice

(A) Chimeric mice were generated using a 1:1 ratio of IgM<sup>a</sup> wild-type and IgM<sup>b</sup> CD11c-DTR bone marrow. The chimeric mice were infected and administered DTX (9 ng/g of body weight) on day 30 post-infection. Flow cytometry analysis was performed on total bone marrow cells 4 days following DTX administration. Plots shown were gated on total total bone marrow cells. (B) Frequency and total number of IgM<sup>a</sup> or IgM<sup>b</sup> B cells in the bone marrow on day 4 post-DTX administration. (C) Frequency and number of CD138<sup>high</sup> IgM<sup>high</sup> bone marrow cells among total IgM<sup>a</sup> or IgM<sup>b</sup> cells in the bone marrow. * p<0.05, **p< 0.005.
reduced serum OMP-19-specific IgM following DTX-mediated depletion of CD11c-expressing B cells.

To determine whether the CD138<sup>high</sup> IgM<sup>high</sup> ASCs were directly affected by DTX administration, we analyzed chimeric mice 24 hours post-DTX administration. GFP (CD11c-DTR) expression was detected on a portion of the IgM-positive transgenic B cells in the bone marrow and was ablated 24 hours following DTX administration (Figure 5.4). This data shows direct depletion of IgM-positive cells in the bone marrow, a portion of which express CD138 (data not shown). Together, these data suggest that DTX-mediated depletion of CD11c-expressing B cells affects protective OMP19-specific IgM titers by reducing CD138<sup>high</sup> IgM<sup>high</sup> ASCs in the bone marrow of chronically infected mice.

**Mutated B Cells Migrate to the Bone Marrow**

Current evidence indicates that bone marrow resident long-lived plasma cells are generated in TD immune responses (Chu and Berek, 2013). Memory B cells that have been activated in the secondary lymphoid organs can differentiate to ASCs with migratory potential for the bone marrow. Once in the bone marrow, recent ASC emigrants compete for survival niches in the bone marrow, which allows terminal differentiation into resident long-lived plasma cells (Chu and Berek, 2013). To determine when the bone marrow CD138<sup>high</sup> IgM<sup>high</sup> ASCs were generated, we analyzed the AID-Cre-Ert2 x ROSA-eYFP<sub>F1</sub> double-transgenic mice described in Chapter 4. Briefly, administration of tamoxifen to the double-transgenic mice allows AID-expressing cells to be irreversibly marked fluorescently.
Figure 5.4: GFP-labeling of CD11c-expressing cells in the bone marrow of *E. muris* infected mice

(A) Chimeric mice were generated using a 1:1 ratio of B cell-deficient and CD11c-DTR bone marrow. The chimeric mice were infected and administered DTX (9 ng/g of body weight) on day 30 post-infection. Flow cytometry analysis was performed on total bone marrow cells 24 hours following DTX administration. Plots shown were gated on total bone marrow cells and represent 7 mice per group.
A

Untreated

DTX-treated

GFP (CD11c) 0.46 1 0.046 0.092

IgM 91 7.56 93.3 4.67
by eYFP. On day 30 post-infection with *E. muris*, we analyzed the bone marrow of double-transgenic mice that had been administered tamoxifen on days 4, 7 or 10 post-infection. We detected both IgM-positive eYFP+, and IgM-negative eYFP+ in the bone marrow of tamoxifen-treated mice (Figure 5.5a). Similar to the IgM memory B cells in the spleen, eYFP expression in the bone marrow suggests that plasma cells are generated as early as day 4 post-infection. The frequency and number of IgM-positive eYFP+ B cells generated during acute infection increased from an average of 0.09% and $3 \times 10^4$ on day 4 to an average of 0.59% and $1.75 \times 10^4$ on day 10 (Figure 5.5b). This difference in the generation of IgM-positive eYFP+ cells between day 4 and day 10 is approximately 6-fold. The population of IgM-negative eYFP+ (presumably IgG-positive B cells) detected in the bone marrow was also generated as early as day 4. However, in contrast to the IgM-positive eYFP+ population, the frequency and number of IgG eYFP+ cells generated during acute infection remained constant from day 4 to 10 post-infection, and an average of approximately 0.2% and $5.6 \times 10^4$ cells, respectively. Together, these data show that bone marrow-resident plasma cells are generated early during infection, and that IgM and IgG plasma cells are generated at similar times during acute infection. The early AID expression in these plasma cells also suggests that they contain mutated receptors, and are likely generated via TD mechanisms.
**Figure 5.5: eYFP-labeling of AID-expressing cells in the bone marrow of E. muris-infected mice**

**(A)** AID-Cre-Ert2 x ROSA-eYFP double transgenic mice were infected and administered 10 mg tamoxifen on days 4, 7, or 10 post-infection. Flow cytometry analysis of bone marrow cells was performed on day 30 post-infection. Plots shown were gated on total bone marrow cells and represent 3 mice per group. **(B)** Frequencies and total number of eYFP-positive IgM-positive, or eYFP-positive IgM-negative (IgG) among total bone marrow cells.
Discussion

Two major theories exist to explain the maintenance of long-term antibody production following infection or vaccination. The first theory posits that antigen-specific plasma cells are maintained in the bone marrow by continual differentiation and migration of activated memory B cells. The signals to induce the activation of memory B cells may be: 1) antigen-specific stimulation by chronic infection or long-term antigen depots; or 2) polyclonal activation by microbial ligands, such as LPS, or bystander activation (Bernasconi et al., 2002; Lanzavecchia and Sallusto, 2007). The second theory posits that antigen-specific, long-lived plasma cells are maintained indefinitely in specialized niches within the bone marrow and secondary lymphoid organs (Ahuja et al., 2008; Manz et al., 2005; Radbruch et al., 2006; Slifka and Ahmed, 1998). These two theories of plasma cell maintenance are competing in the literature but may not be mutually exclusive. As has been shown with the development of memory B cells the mechanism of plasma cell maintenance may differ depending on the infection or immune stimulus.

We have shown that depletion of CD11c-expressing B cells is sufficient to reduce OMP-19-specific IgM titers in the serum of chronically infected mice by day 7 post-DTX administration. This data suggests that that the cellular source (CD138\textsuperscript{high} IgM\textsuperscript{high} ASCs) of persistent IgM production (Racine et al., 2011) was eliminated. This is entirely plausible, since the half-life of serum IgM is only 28 hours in mice (Vieira and Rajewsky, 1988). The reduction of CD138\textsuperscript{high} IgM\textsuperscript{high} ASCs may have been accomplished by two routes: 1) direct depletion by DTX; or 2)
depletion of a precursor population, such as the IgM memory B cells. Both scenarios are possible and may occur simultaneously. While it has been demonstrated that the IgM memory B cells do express CD11c, it is also probable that CD11c-expressing antibody-secreting cells are deleted by DTX administration. This is a likely scenario, as GFP expression was detected in IgM-positive B cells in the bone marrow. In addition, the GFP-expressing IgM-positive B cells were deleted from the bone marrow, indicating that they were DTX-sensitive. This observation is similar to the studies of Hebel et al., where CD138+ ASCs in the bone marrow of CD11c-DTR mice were GFP-positive, and deleted in vivo by DTX administration (Hebel et al., 2006). Therefore, we propose that a portion of the IgM-positive and CD138^{high} IgM^{high} ASCs were depleted along with the IgM memory B cells following DTX administration. In addition, the absence of IgM memory B cells may compound the DTX depletion of bone marrow ASCs by preventing the differentiation and recruitment of ASCs to the bone marrow.

How then, did the OMP-19-specific titers return? As described above, memory B cells are thought to maintain long-term serum Ig levels by regular differentiation into ASCs in the bone marrow (Bernasconi et al., 2002). Therefore, we hypothesize that the IgM memory B cells were restored by the proliferation of residual cells and, in turn, re-populated the IgM-positive and CD138^{high} IgM^{high} ASC compartments in the bone marrow. Our theory is supported by the observation that both the IgM memory B cells and antigen-specific serum IgM are restored by day 14 post-DTX administration. The eYFP-labeling of B cells in the bone marrow
following tamoxifen administration during acute infection also implies a
differentiation pathway congruent with the IgM memory B cells. In addition, the
IgM memory B cells express high levels of CXCR4, suggesting the ability of these
cells to traffic to the bone marrow (Hargreaves et al., 2001). The high level of
CXCR4 expression on the surface of the IgM memory B cells supports this theory,
since CXCR4 expression is required for homing and retention of plasma cells in the
bone marrow (Hargreaves et al., 2001). In turn, CXCL12, the ligand for CXCR4 is
produced by stromal cells in the bone marrow, where it plays a crucial role in the
transmigration of plasmablasts across the endothelium and retention in the
parenchyma (Egawa et al., 2001; Ma et al., 1999; Peled et al., 1999; Tokoyoda et
al., 2004).

It is unlikely that the CD138<sup>high</sup> IgM<sup>high</sup> ASC population was self-renewed,
since plasma cells are considered non-dividing, terminally differentiated, and non-
responsive to antigen (Crotty and Ahmed, 2004; Radbruch et al., 2006). Future
studies such as the transfer of the IgM memory B cells into naive mice would allow
us to track their fate <i>in vivo</i>, including differentiation into the CD138<sup>high</sup> IgM<sup>high</sup> ASC
population. Understanding the way in which the long-lived plasma cell
compartment is maintained during a natural model of infection is important for
both basic B cell biology and the design of durable vaccines.
Chapter VI: SUMMARY
IgM Memory B cell Generation, Maintenance, and Function During Chronic Ehrlichial Infection

Based on the data discussed in the preceding chapters, we have developed a model of IgM memory B cell generation, maintenance, and function during chronic ehrlichial infection (Figure 6.1). First, naïve B cells are activated by antigen and unknown innate signals provided by the *E. muris* bacterium. Development of the IgM memory B cells, and expression of AID begins during acute infection, as early as day 4 post-*E. muris* infection. We found this developmental process to be dependent on CD4 T cells, IL-21 signals, and Bcl-6, suggesting the IgM memory B cells are derived via a GC-dependent pathway. By day 30 post-infection, a large population of CD11c-positive IgM memory B cells are detected in the spleen, where they persist through homeostatic proliferation for the life of the animal. The IgM memory B cells are maintained within the marginal zone of the spleen, and at the edges of the B cell follicles.

Under steady-state conditions, the IgM memory B cells likely have the capacity to migrate to the bone marrow and differentiate into IgM-secreting plasma cells. The contribution of the IgM memory B cells to the pool of ASCs in the bone marrow was suggested by reduced OMP-19-specific serum IgM following DTX-mediated depletion of CD11c-expressing cells. In addition, the total number of IgM-secreting, CD138\(^\text{high}\) IgM\(^{\text{high}}\) bone marrow ASCs was reduced 4 days following DTX treatment. Therefore, the maintenance of protective IgM in the serum of
Figure 6.1: Model of IgM Memory B cell Generation, Maintenance, and Function

During Chronic Ehrlichial Infection

(1) A naïve B cell is activated and receives signals to differentiate into a precursor population. (2) AID is expressed, and somatic hypermutation of the BCR begins. The AID-expressing precursor population moves into the germinal center, were T_{Fh}, and IL-21 promote the differentiation of precursor cells into IgM memory B cells. (3) The IgM memory B cells reside in the MZ of the spleen where they are capable of self-renewal by homeostatic proliferation. (4) During steady-state conditions of chronic infection, a portion of the IgM memory B cells differentiate into plasmablasts and migrate to the bone marrow. After finding a survival niche in the bone marrow, the newly emigrated cells terminally differentiate into long-term IgM secreting cells. (5) In the event of exposure to a recall antigen, the IgM memory B cells re-enter the GC. After receiving help from T_{Fh} cells and IL-21 signals, the IgM memory B cells class-switch to IgG and differentiate into ASCs. Presumably, the class-switched memory cells can then populate the IgG memory B cell and long-lived plasma cell compartments.

Abbreviations: activation-induced cytidine deaminase (AID); antibody-secreting cell (ASC); B cell lymphoma-6 (Bcl-6); marginal zone (MZ); T follicular helper cell (T_{Fh}).
chronically infected mice represents one important function of the CD11c-positive IgM memory B cells.

Another important function of a memory B cell is to respond to a re-exposure to cognate antigen. In the event of a secondary exposure to antigen, the IgM memory B cells re-enter a GC reaction, where they class switch and secrete high-affinity IgG. DTX-mediated ablation of the CD11c-positive IgM memory B cells prior to secondary challenge abrogated the IgG recall response. This data has lead us conclude that the CD11c-positive IgM memory B cells are required for secondary responses to antigen challenge in chronically infected mice. In addition, we have established that the recall response to antigen challenge requires CD4 T cell help, and IL-21R signals, suggesting a role for germinal center reactions in the secondary response. Therefore, the production of high-affinity class-switched ASCs antibody in response to antigen challenge is a second important function of the CD11c-positive IgM memory B cells.

**Future Directions**

The studies in this dissertation have described a previously unappreciated role for IgM memory B cells in host defense during a natural model of ehrlichial infection. The proposed studies represent additional research meant to further our understanding of IgM memory B cells and humoral immunity to infection.
**Identify early precursors of IgM memory B cells**

In the context of rational vaccine design, it is important to identify the pathway in which the IgM memory B cells are generated. We have established that the IgM memory B cells are generated via a TD, GC-dependent pathway. However, eYFP-labeling of AID-expressing cells has revealed that the IgM memory cells begin their generation as early as day 4, far earlier than they can be detected by flow cytometry on day 30 post-infection. Studies by Taylor et al. describe a population of CD38⁺ GL7⁺ cells in a model of PE immunization, that are precursors to both IgM memory B cells and GC B cells (Taylor et al., 2012). We would like to identify whether a similar population of such cells gives rise to the IgM memory B cells in our model of ehrlichial infection. Analysis of eYFP-labeled AID-expressing cells in AID-Cre-ERT2 x ROSA-eYFP double-transgenic mice starting at day 4 post-infection will allow the identification and characterization of such precursor populations.

Previous studies in our laboratory have identified an extrafollicular population of TI CD11clo plasmablasts that is responsible for producing OMP-19-specific IgM during acute infection (Racine et al., 2011). As mentioned in previous chapters, the CD11clo plasmablasts are detected in the spleen beginning on day 9 and decline by day 18 post-infection. Since the plasmablast population, and the IgM memory B cell population we detect by day 30 both express CD11c, it is plausible that these populations are related in some way. We proposed in Chapter 3 that T cell help provided to the CD11clo plasmablasts during acute infection may “rescue” a portion of these cells and promote differentiation into the CD11c-
positive IgM memory B cell population. We will use two approaches to test this hypothesis. First, we will attempt to transfer labeled CD11c<sup>lo</sup> plasmablasts into analogously infected mice. On day 30 post-infection, splenocytes will be analyzed by flow cytometry for the presence of labeled IgM memory B cells. Alternatively, analysis of eYFP-labeled cells from AID-Cre-ERT2 x ROSA-eYFP double-transgenic mice between day 9 and 18 post-infection will allow the possible identification of AID-expressing CD11c<sup>lo</sup> plasmablasts. Such labeling would strongly suggest a common developmental link for the two populations.

**Track differentiation of the IgM memory B cells during chronic infection and secondary challenge**

DTX-mediated deletion of the CD11c-expressing IgM memory B cells has allowed us to indirectly assess their function *in vivo*. However, direct evidence of the functions of the IgM memory B cell population would be invaluable for our studies. To this end, we will attempt transfer of a purified IgM memory B cell population into congenic mice of the same day post-infection. Analysis of recipient mice under steady-state conditions will allow us to determine the fate of the IgM memory B cells during chronic infection, and if they do in fact migrate to the bone marrow and differentiate into long-term ASCs. Similarly, analysis of recipient mice after secondary challenge will allow us to track the differentiation of the IgM memory B cells into GC B cells, and finally into high-affinity, class-switched ASCs and memory B cell populations. Once these systems are in place, many important
questions about the fate of IgM memory B cells during chronic infection can be studied in vivo.

**Protection against fatal ehrlichial challenge**

Previous studies have shown that mice infected with *E. muris* are protected from a lethal challenge with the heterologous ehrlichiae, IOE (Bitsaktsis et al., 2004; Ismail et al., 2004; Racine et al., 2011). In Chapter 3, we demonstrated that IgM memory B cells generated during *E. muris* infection were required for the secondary response generated to antigen challenge. Therefore, we would like to test the requirement of the IgM memory B cell population for the protection against lethal IOE challenge. These studies will be performed with chimeric mice, as described in Chapter 3. Briefly, chimeric mice will be generated with a 1:1 ratio of B cell-deficient and CD11c-DTR bone marrow. The chimeric mice will be infected with *E. muris*, and the IgM memory B cells will be deleted with a single dose of DTX on day 30 post-infection. Twenty-four hours following DTX-mediated depletion, the chimeric mice will be challenged with IOE. The mice will then be monitored for symptoms of morbidity, including ruffled fur, hunched posture, and wasting.

*Why is humoral memory maintained in low-affinity IgM B cells?*

Humoral memory is characterized by a larger, faster, higher affinity response to the immunizing antigen or infectious agent. Why then, would humoral memory be maintained in lower affinity, IgM memory B cells? Dogan et al. identified IgM memory B cells in their model of eYFP labeling of antigen-experienced cells
(Dogan et al., 2009). Upon antigen challenge, IgG memory B cells differentiated directly into IgG-producing plasma cells. In contrast, the IgM memory B cells re-entered the GC pathway, generated swlg cells, as well as additional IgM memory B cells. Based on these studies, Dogan and colleagues proposed a model where humoral memory exists in “layers”. According to this theory, the IgG memory B cells function to provide a quick, first line of defense against an invading pathogen. Simultaneously, the IgM memory B cells provide the host with flexibility to the secondary challenge. With the ability to re-initiate a GC, IgM memory B cells have the unique ability to adapt to variant pathogens by altering BCR affinity in the GC. This theory has major implications for vaccine design against highly variable pathogens such as HIV and influenza.

Our model of humoral immunity to ehrlichial infection provides a unique model with which to test the “layer” hypothesis of humoral memory. The ehrlichiae encode a family of as many as 22 variant OMPs that are distinct, yet antigenically related (Ohashi et al., 1998). Much of the variability present in the OMPs is found in three distinct hypervariable regions (HVRs). We have previously shown that the OMP-19-specific IgG elicited by both E. chaffeensis and E. muris almost exclusively recognize HVR1 within OMP19 (Li et al., 2001; unpublished data). The multiple OMPs and peptides within HVR1 provide sources of variable antigens with which to test this hypothesis. To evaluate the ability of the IgM memory B cells to respond with high affinity to variant ehrlichial antigens, we will challenge E. muris-infected mice or OMP19 immunized mice with variant OMPs and peptides that bind IgM
but do not bind IgG in the immune sera during primary *E. muris* infection. OMP-19 immunization does not generate a population of IgM memory B cells (data not shown). Therefore, a high affinity IgG response to the variant OMP antigens in infected rather than immunized mice would provide support for the hypothesis that IgM memory B cells are uniquely poised to respond to antigenic variants.

**Significance**

The studies described in this dissertation reveal novel aspects of humoral memory generated in response to intracellular bacterial infection. In Chapter 3, we identified and characterized a population of CD11c-positive IgM memory B cells generated by *E. muris* infection. We established that these IgM memory B cells were essential for generating a robust IgG recall response to secondary antigen challenge – a hallmark of humoral memory. Our study, and others, have challenged the belief that memory B cells must be class-switched and of high affinity. Chapter 4 provides additional significance to our study, demonstrating that the IgM memory B cells were generated via TD mechanisms. Additional evidence, such as the requirement for Bcl-6 and the presence of somatic mutations, suggests that these IgM memory B cells are GC-derived. These findings conflict with current views on the TI generation of IgM memory B cells and suggest added complexity to the mechanisms by which humoral memory is generated.
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