

GENESIS AND MAINTENANCE OF LONG-TERM IgM+ T-BET+ B CELLS

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List of Abbreviations

Cytokines

APRIL	A proliferation-inducing factor
BAFF	B-cell activating factor
IFN- γ	Interferon gamma
IL-21	Interleukin-21

Receptors

BAFF-R	B-cell activating factor receptor
BCR	B cell receptor
CXCR3	Chemokine (C-X-C motif) receptor 3
CXCR5	C-X-C chemokine receptor type 5
Fc γ RIIb	Fc gamma receptor 2b
PD-1	Programmed cell death protein 1
TACI	Transmembrane activator and CAML interactor

Others

ABSs	Age-related B cells
AID	Activation-Induced cytidine deaminase
ASC	Antibody-secreting cells
Bcl-6	B-cell lymphoma 6 protein
BM	Bone Marrow
Blimp1	B lymphocyte-induced maturation protein-1
BrdU	Bromodeoxyuridine

CSR	Class switch recombination
eYFP	Enhanced yellow fluorescent protein
FACS	Fluorescence-activated cell sorting
FDC	Follicular Dendritic cells
FoxP3	Forkhead box P3
IA ^b	Major Histocompatibility Complex allele
IOE	<i>Ixodes ovatus</i> Ehrlichial
i.p.	Intraperitoneal
GC	Germinal center
LPS	Lipopolysaccharides
MHC II	Major Histocompatibility Class II
MMP	Mitochondrial membrane potential
MyD88	Myeloid differentiation primary response gene 88
NP-BSA	4-Hydroxy-3-nitrophenylacetyl- Bovine Serum Albumin
OMP-19	Outer membrane protein 19 (<i>Ehrlichia muris</i>)
PAX5	Paired Box 5
SHIP	Signaling Inositol Polyphosphate 5 Phosphatase
swIg	Switch Immunoglobulin
SHM	Somatic hypermutation
T-bet	T-box transcription factor
TD	T cell dependent
TI	T cell independent
T _{fh}	T follicular helper cell
TLR	Toll-like receptor
WT	Wild-type
XBP1	X-box binding protein 1

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Abstract

IgM memory cells are recognized as an important component of B cell memory, based on several studies both in mice and humans. Our studies of B cells elicited in response to ehrlichial infection identified a population of CD11c/T-bet-positive IgM memory cells and an IgM T-bet-positive bone marrow antibody-secreting cell population (ASCs). The origin of these populations was unknown, although an early T-independent spleen CD11c- and T-bet-positive IgM plasmablast population precedes both, suggesting a linear relationship. The majority of IgM memory cells detected after day 30 post-infection had undergone somatic hypermutation, indicating that they expressed activation-induced cytidine deaminase (AID). Therefore, to identify early AID-expressing precursor cells, we infected an AID-regulated tamoxifen-inducible Cre-recombinase-EYFP reporter strain. Tamoxifen administration led to labeling of both the IgM memory cells and bone marrow ASCs on day 30 and later post-infection. High frequencies of labeled cells were identified on day 30 post-infection, following tamoxifen administration on day 10 post-infection. Both IgM memory cells and IgM bone marrow ASCs were labeled when tamoxifen was administered as early as day 4 post-infection. We also identified mechanisms involved in maintenance of the IgM bone marrow ASCs and IgM⁺ memory cells, namely proliferation and Fc γ R11b respectively. BrdU studies revealed that the bone marrow IgM ASCs were maintained by proliferation, unlike the IgM memory cells. RNAseq analysis revealed a 2-fold higher expression of inhibitory Fc receptor, Fc γ R11b. Because Fc γ R11b inhibits B cell activation, we hypothesized that Fc γ R11b negatively

regulates IgM⁺ memory B cells by binding immune complexes present during low-level chronic infection. *E. muris* infection of FcγRIIb-deficient mice revealed a 3-fold expansion of the IgM⁺ memory 30 days post-infection. We further demonstrated that the expansion of the IgM⁺ memory cells was not due to increased proliferation, but a decrease of apoptosis, due to a lack of Fas expression in FcγRIIb-deficient mice. This result was mimicked in AID-deficient mice, which lack the ability to class switch to IgG and make immune complexes, revealing a role for immune complexes in regulating IgM⁺ memory. Altogether, these studies demonstrate a novel germinal center-independent pathway for the generation of two distinct long-term IgM-positive B cell populations.

Chapter 1: Introduction

There are two branches of the immune system: innate immunity and adaptive immunity (1, 2). Innate immunity is responsible for initiating the early defense against invading pathogens, and is not specific. In contrast, adaptive immunity is the malleable arm of the immune system, and is responsible for generating a pathogen-specific response (1). When fighting an infection, the ideal outcome is the establishment of a protective memory response that can rapidly clear a repeat infection. A protective memory response is composed of several parts, including pathogen-specific T and B cells, and pathogen-specific antibody (1, 2). The focus of this body of work is determining the contribution long-term B cell populations to protective memory during an intracellular bacterial infection.

B cells

In 1965 Max Cooper and Robert Good discovered B cells and demonstrated that these cells were responsible for the production of antibody. (3). After this initial study, extensive research of B cells led to a better understanding of the origin of these cells. B cell development happens primarily in the bone marrow of adult mammals and stems from the differentiation of hematopoietic precursors. The development of B cells can be reduced to three stages in the bone marrow: the pro-B cell, pre-B cell, and immature B cell stages (4-6). The progression through these stages is contingent on the rearrangement of a functional B cell receptor (BCR).

During the pro-B cell stage, the cell is actively rearranging the Ig heavy chain, one of two chains that make up the BCR. Once a functional Ig heavy chain is rearranged it is paired with a surrogate light chain; together these two chains form the pre-BCR (7). When the pre-BCR is expressed on the cell surface it signals the cell to do two things: (1) stop Ig heavy chain rearrangement and (8) begin rearrangement of the Ig light chain, the second chain in the BCR. With the rearrangement of a BCR, comprised of a function light and heavy Ig chain, that can recognize antigen, the B cell has become an immature B cell and can exit the bone marrow and circulate in the periphery (4-6). Once an immature B cell leaves the bone marrow it is referred to as a transitional B cell. Circulating transitional B cells home to the spleen, where they transition into mature naïve B cells. The exact process of how this happens remains unclear, however there is evidence to support roles for cytokines, the BCR, and competition with pre-existing B cells (9).

There are three different types of mature naïve B cells: marginal zone, follicular, and B1 cells. These different B cells can be distinguished based on the expression of certain surface markers. Follicular B cells exhibit higher expression of CD23 but lower expression of CD21, and are generally believed to enter germinal center reactions and generate high-affinity antibodies. In contrast, marginal zone B cells have low expression of CD23 but high expression of CD21 and CD1d, reside in the marginal zone of the spleen and protect against blood-borne pathogens. Further, B1 B cells express high levels of CD5, unlike other

mature B cell subsets. B1 B cells generate natural antibody and are primarily found in the peritoneal and pleural cavities; however a small population resides in the spleen.

B cell activation

B cells are the immune cells responsible for the production of antibodies of all isotypes. In order for a B cell to secrete antibody, the cells first have to be activated. B cells can be activated in two ways: via T cell independent (TI) or T cell dependent (TD) pathways. Once activated, B cells can differentiate into one of several different fates: plasmablasts, plasma cells, or memory B cells, all of which will be discussed in detail below. TI antigens can crosslink the BCR to activate B cells without T cell help. B cells activated in a TI manner have long been assumed to generate short-lived plasmablasts, but not memory B cells. However, there are a number of reports suggesting that B cell memory and long-term antibody production can be generated under TI conditions (11, 12). Both marginal zone and B1 B cells were believed to generate short-lived plasmablasts, because they were exclusively activated via TI mechanisms. One model, of *B. hermsii* infection, demonstrated that B1 B cells can generate TI B cell memory (13, 14), while other studies demonstrated that marginal zone B cells can differentiate into memory B cells following TD immunization: both challenged the notion that neither B1 nor marginal zone B cells contribute to protective memory (13, 15-17).

In contrast, TD antigens activate B cells with both a BCR signal, and additional signals from T cells. After the B cell is activated by cognate antigen, it can then receive T cell signals and proceed to clonally expand and proliferate. After this expansion, B cells can follow one of three fates: (1) become a short-lived plasmablast that secretes low-affinity IgM, (2) become a low-affinity memory B cell expressing an IgM receptor, or (3) enter a germinal center reaction. If the B cell enters a germinal center reaction, it will receive help from both T follicular cells (T_{fh}), a T cell subset that provides co-stimulatory signals, and follicular dendritic cells (FDCs) allowing the B cell to undergo class switch recombination (CSR) and somatic hypermutation (SHM). Once a B cell matures in the germinal center it can then go on to become either a long-lived plasma cell, or switched memory cell.

The first possible fate of a TD-activated B cell during infection is to become a short-lived plasmablast; these cells are responsible for the production of early antigen-specific antibody, typically IgM. Plasmablasts are among the first antibody secreting cells (ASCs) observed after secondary infection. These cells can be identified on the basis of their surface expression of CD138 and CD93, which are markers common to ASCs. Plasmablasts are programmed by the transcription of Blimp1 (18), which initiates programs essential to their differentiation and function, by suppressing Pax5, a transcription factor necessary for B cell development (19, 20). The primary function of plasmablasts is to secrete antibodies, a function driven by the transcription factor XBP1 (21).

The second possible fate of a TD B cell is to become a low-affinity IgM memory B cell. These memory B cells have none to few mutations (22). At this point the IgM⁺ memory B cells are generated outside of germinal centers. These IgM⁺ memory B cells are thought to rapidly respond upon antigen re-challenge, primarily differentiating into short-lived IgM plasmablasts. However, recent studies showed that IgM⁺ memory B cells can enter germinal centers upon challenge, suggesting multiple fates for these memory B cells (23).

The third possible fate of a TD B cell is to enter a germinal center reaction and undergo CSR and SHM. CSR and SHM are initiated by the enzyme activation-induced cytidine deaminase (AID). AID induces SHM by mutating DNA, via deamination of cytosine bases to uracil. These mutations accumulate in the DNA encoding for complementarity-determining regions (CDR) regions of antibodies, and these changes can lead to increased affinity of the antibody for an antigen. This enzyme is also responsible for CSR, the ability to switch the constant region of the Ig heavy chain. Every constant region in the heavy gene for immunoglobulin is preceded by a switch sequence. In order to undergo class switching, AID introduces mutations into the DNA encoding the switch sequences preceding the constant region the heavy chain gene for immunoglobulin (24). The accumulation of mutations in the switch sequences results in DNA breaks and repairs, which lead to recombination, and eventually to expression of a new constant region.

AID is usually induced in the germinal center when a B cell is receiving help from both T_{fh} and FDCs. Alternatively, AID is inducible through a TI mechanism via BCR and TLR co-signaling, both *in vitro* and *in vivo* (25-27). In addition to inducing both CSR and SHM, AID is required for mediating tolerance during B cell development (28, 29). Low levels of AID were also found to be essential for tolerance, possibly through a MyD88-dependent mechanism (30, 31). It has also been shown that AID can be expressed in memory B cells, and may contribute to polyreactivity (32). Although AID was originally described as essential for CSR and SHM, the enzyme has been documented as necessary for other functions, including maintenance of B cell populations (28, 31, 32).

Immunological Memory

Immunological memory is a fundamental concept for adaptive immunity, and provides the basis for vaccines. Immunological memory allows the host to recognize and eliminate pathogens the host has previously encountered. Immunological memory consists of many parts, including memory T and B cells, plasmablasts, plasma cells, and antibodies. This thesis will focus on the contributions of plasmablasts and long-lived bone marrow populations to immunological memory.

Memory B cells are quiescent populations of B cells that have matured in response to antigen stimulation. These B cells can express either IgM, or a switched BCR, and rapidly respond to repeat infection, in part due to differences in signaling potential between memory and naïve B cells (33, 34). Although the generation of switched memory was considered to be a hallmark of immunological memory, evidence has been accumulating in both human and animal studies that has revealed a role for IgM memory B cells in the maintenance of long-lived immunity (11, 35-40). Several studies have revealed that memory cells can be generated early during infection, allowing for variation in affinity and antibody isotype in the memory pool (11). Upon repeated exposure to the same antigen, memory B cells can rapidly differentiate into plasma cells, re-enter a germinal center reaction to undergo additional rounds of SHM, differentiate into long-lived plasma cells, or repopulate the memory pool (22). While memory B cells have several potential fates, plasma cells are responsible for the production of long-term protective antibody.

Plasma cells are terminally-differentiated B cells that reside primarily in the bone marrow (4). However, there are cases of plasma cells residing outside of the bone marrow, such as in inflamed tissues (41). Plasma cells are responsible for the long-term maintenance of antigen-specific antibodies post-infection or immunization. High-affinity plasma cells are primarily generated during germinal center reactions, but can also be generated via T cell-independent interactions (22, 42). Plasma cells down-regulate cell surface markers such as B220, CD19,

and surface Ig; however they express CD138 and CD93, which are markers for antibody-secreting B cells. Continually producing and secreting antibodies adds a tremendous amount of stress on the cell. To compensate, plasma cells upregulate the unfolded protein response program (UPR) to overcome endoplasmic reticulum stress associated with high protein production (21, 43). Long-lived plasma cells require signals for survival, via factors such as B cell activation factor (BAFF), and A Proliferating-Inducing Ligand (APRIL) (44-46). BAFF and APRIL are ligands for BAFF receptor (BAFF-R), Transmembrane Activator and CAML Interactor (TACI), and B-Cell Maturation Antigen (BCMA). Signaling through these receptors induces strong anti-apoptotic signals (47-49). In the bone marrow, stromal cells secrete BAFF, but dendritic cells and macrophages are the primary producers of this factor (50). Plasma cells aren't the only cells that require survival signals from BAFF and APRIL, as memory B cells also require these survival signals.

***Ehrlichia muris* Infection Model**

Our laboratory has been using a murine model of *Ehrlichia muris* infection to study the basis of long-lived protective immunity. *E. muris* is an obligate aerobic intracellular gram-negative bacteria of the order Rickettsiales (51). The bacterium does not encode genes for LPS or peptidoglycan, and therefore cannot activate the immune system through TLR4 or 2. It is a blood-borne pathogen responsible for Human Monocytic Ehrlichiosis that can be transmitted by the deer tick, *Ixodes*

scapularis (52). Ehrlichiosis is primarily seen in the South and Southeastern US. Ehrlichiosis presents clinically with flu-like symptoms, and although is not life threatening when treated properly, it can be fatal in some cases (51).

In our murine model of ehrlichiosis, infection causes splenomegaly, hematopoietic abnormalities, and disrupts the structure of the spleen, the latter leaving very few germinal centers. However, both the hematopoietic abnormalities and disrupted splenic architecture resolve after acute infection. The bacterial load peaks at day 9 post-infection, after which a low-level chronic infection is established. The infection is commonly treated with doxycycline. While *E. muris* does not cause a lethal infection in immunocompetent mice, *Ixodes ovatus* Ehrlichial (IOE), a related ehrlichiae discovered in Japan, causes a lethal infection. Our murine model of *E. muris* has led to the identification of three IgM B cell populations generated in response to infection (53-55).

During early *E. muris* infection, a CD11c⁺ B220⁺ population was identified, and was found to secrete pathogen-specific IgM (53). This population was first detected on day 4 post-infection, and receded by day 17 post-infection (53). This CD11c⁺ B220⁺ population was not detected in uninfected control mice. The population does not express germinal center markers, nor does it require CD4⁺ T cell help (53). The CD11c⁺ B220⁺ population does not express CD23, CD21, and CD5.(53). However, the cells express other markers commonly expressed by, such as CD138 and CD93.

A second IgM⁺ population present during infection was an IgM⁺ CD11c⁺ CD19⁺ B cell memory population. This population was largely quiescent, and was necessary for a re-call IgG response to antigen challenge (55). Further study of this population revealed that both CD4⁺ T cells and IL-21 are required for their generation, in contrast to the early CD11c⁺ B220⁺ plasmablasts, which are generated independent of T cells (53, 55). The IgM⁺ memory B cells express several cell surface markers identified on other memory B cells, such as PD-L2, CD80, and CD73 (55-57). The IgM⁺ memory B cells were first detected in the spleen on day 18 post-infection, and persisted indefinitely. RNAseq analysis that compared IgM⁺ memory B cells with canonical CD19⁺ B cells, revealed the differential expression of many mRNAs (**See chapter 4**). Notably, Fc γ R1Ib had a 2-fold higher expression in the IgM⁺ memory B cell population, at both the mRNA and protein level (**See chapter 4**). The fourth chapter of this work will further address the role that Fc γ R1Ib plays in controlling the IgM⁺ memory responses.

A third IgM⁺ population identified during infection was a bone marrow IgM⁺ CD138⁺ plasmablast population. This population was long-term, and was necessary for the maintenance of infection-specific IgM-producing B cells (54). The IgM produced by this population is protective against lethal IOE infection (54). These cells express high levels of CD93, consistent with the antibody secreting function of this bone marrow IgM⁺ CD138⁺ population. In this work, the

mechanisms involved in the generation and maintenance of the bone marrow CD138+ IgM+ plasmablasts was investigated.

Mouse Models

To study the mechanisms involved in maintaining the IgM+ memory B cell population, and the BM IgM+ CD138+ plasmablast population, we primarily utilized two different mouse strains: an (AID-Cre ET^{R2} x EFYP) F1, and an Fc γ RIIb-deficient strain.

The (AID-Cre ET^{R2} x EYFP) F1 mouse model has been described by *Dogan et al., 2009 (58)*. The AID-Cre ET^{R2} mouse was genetically engineered to express a tamoxifen-inducible Cre recombinase enzyme under the control of the AID promoter. The EFYP mouse encodes an enhanced yellow fluorescent protein (EYFP) preceded by a stop sequence with loxP sites flanking the ROSA26 locus. When the AID promoter is expressed, the tamoxifen-inducible Cre recombinase is sequestered in the cytoplasm; the Cre recombinase is released when tamoxifen engages the estrogen receptor (59). Once the Cre recombinase is released, it catalyzes DNA recombination so that EYFP can be expressed (59). This mouse model allows for cells expressing AID at the time of tamoxifen administration to be irreversibly marked with EYFP.

The Fc γ RIIb-deficient mouse was genetically engineered to be completely deficient for Fc γ RIIb. Any cell that would normally express Fc γ RIIb, such as B cells, macrophages, dendritic cells, monocytes, NK cells, eosinophils, and certain subsets of T cells, are all deficient for Fc γ RIIb. This mouse model is often used to study autoimmunity, because Fc γ RIIb is an inhibitory Fc γ R that negatively regulates B cell BCR signaling. Therefore, in the absence of Fc γ RIIb, B cells are hyper-active, and the mice have higher amounts of autoreactive antibodies (60, 61), although autoimmune disease does not become apparent until 7 months after birth. Given this constraint, in our studies mice were infected no later than 4 months of age so that autoimmunity would not affect our results. This mouse model was used to address the role of Fc γ RIIb in IgM memory.

Overall Summary of Work

The primary objective of the work herein was to better understand the relationships between the three IgM⁺ populations described above. In the process, we have discovered, first, that the bone marrow IgM⁺ CD138⁺ plasmablasts do not require CD4⁺ T cell help for their generation, and are maintained in part by active proliferation. Second, both the bone marrow IgM⁺ CD138⁺ plasmablasts and IgM⁺ B cell memory populations can be derived early during infection. Third, Fc γ RIIb plays a critical role in the generation and maintenance of IgM⁺ memory B cells. We also characterized the expression of T-bet in all three IgM⁺ populations, two of which also express CD11c.

Recently, there have been numerous reports of B cells co-expressing T-bet and CD11c. These cells have been found in older mice, autoimmunity models, in response to chronic infections, and in response to malaria in humans (40, 62-67). Our work expands on what is currently known about B cells expressing both T-bet and CD11c in the context of an infection. Understanding the ontogeny and maintenance of the two long-term IgM populations will provide a more comprehensive view of the role IgM contributes to protective immunity.

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Chapter 2: Materials and Methods

Mice. Sex-matched C57BL/6, B6.Cg-Gt(Rosa)26Sor^{tm3(CAG-EYFP)Hze/J}, B6.MRL-FAS^{lpr/J}, AID-Cre-ER^{T2}, B6.Cg-Gpi1^aThy1^aIgh^{a/J}, B6;129S-Fcgr2b^{tm1Ttk/J} and (MHCII)-deficient (B6.129S2-H2^{dlAb1-Ea/J}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The AID-Cre-ER^{T2} mice were generously provided by Dr. Jean-Claude Weill, ISERM, Paris, France. The UNC93b deficient mice were provided by Dr. Ann Marshak-Rothstein, University of Massachusetts Medical School. All mice were bred and maintained under microisolator conditions at the Upstate Medical University, in accordance with institutional guidelines for animal welfare.

Genotyping of (AID-Cre-ERT2 x EYFP) F1 mice. Mouse genomic DNA was extracted from tail tissue using hot sodium hydroxide, as previously described ((1)). PCR was performed using the following oligonucleotide primers: eYFP (internal positive control forward 5'-CAAATGTTGCTTGTCTGGTG-3'; internal positive control reverse 5'-TCAGTGGAATTAGTCATGCC-3'; transgene forward 5'-GGGACCATGAAGCTGCTGCCG-3'; transgene reverse 5'-GGCATTAAAGCAGCGTATCG-3'; the reactions yielded 625 and 200 bp products from the transgene and internal positive control alleles, respectively).

Infections and treatments. Mice were infected i.p. with 5×10^4 copies of *E. muris*, as previously described ((2)). CD40L blockade was performed by administration of 200 μ g of the mAb MR-1 (anti-CD40) on days 8, 10, and 12 post-infection; an irrelevant isotype-matched antibody (clone 2A3) was used as a control. Tamoxifen was dissolved in peanut oil, at a concentration of 20mg/ml, and 0.5 ml was administered, via oral gavage. To validate the effectiveness of

the CD40L blockade, NP-BSA-immunized C57BL/6 mice were administered either 200 μ g of 2A3 (rat IgG2a) or MR-1 (rat IgG2a) on days 4, 8, and 12 post-immunization.

Transfer studies. To obtain B cells for cell transfer studies, spleens from three AID-Cre-ER^{T2} x B6.Cg-Gt(Rosa)26Sor^{tm3(CAG-EYFP)Hze}/J F1 mice were harvested and pooled. T cells were depleted by magnetic bead negative selection, using a CD90.2 T cell Enrichment Kit (Stem Cell Technologies). Naïve C57BL/6 and MHCII-deficient mice were injected with 7.7×10^6 B cells intravenously (3), and the mice were infected i.p. with *E. muris*. The MHCII-deficient recipient mice were treated on day 0 and 3 post-infection with 200 μ g GK1.5 antibody, i.p., to deplete any co-transferred CD4 T cells. The mice were then infected with *E. muris*, and were administered 10 mg tamoxifen by oral gavage, on days 7 and 10 post-infection.

BrdU Incorporation studies. C57BL/6 mice, infected for at least 30 days, were administered 0.8 mg of BrdU, and were maintained thereafter on drinking water containing 0.8mg/ml BrdU and 0.5% dextrose; the water was changed daily. After ten days, spleen and bone marrow cells were analyzed for BrdU incorporation by flow cytometry. BrdU pulse was administered 0.8mg of BrdU i.p. one hour before the mice were sacrificed. Both C57BL/6 and B6;129S-Fcgr2btm1Ttk/J mice infected for 30 days, were administered 0.8mg of BrdU i.p., and maintained on BrdU water for five days. After which mice were sacrificed and analyzed for BrdU incorporation by flow cytometry. For pulse labeling experiments, infected C57BL/6 and B6;129S-Fcgr2btm1Ttk/J mice were infected and were

administered 0.8 mg of BrdU i.p. one day before sacrifice. Cell populations were analyzed by flow cytometry.

Flow cytometry and antibodies. Spleen and bone marrow cells were disaggregated using a 70 μ m cell strainer (BD Biosciences), and erythrocytes removed by hypotonic lysis, using ammonium chloride. Cells were treated with anti-CD16/32 (2.4G2) prior to incubation with the following antibodies: anti-IgM (clone II/41), BrdU (Bu20a, Biolegend), T-bet (eBio4B10, eBioscience, San Diego, CA), BAFF-R (7H22-E16), CD80 (16-10A1), CXCR4 (L276F12), CD138 (281-2), CD73 (TY/11.8), PD-L2 (TY25), CD11b (M1/70), ICOSL (HK5.3), CD40 (12-0401-81), CD62 (MEL-14), CD86 (GL-1), TACI (ebio8F10-3), Fas (15A7), BrdU (Bu20a), IgM (R6-60.2 BD Biosciences), B220 (RA3-6B2, BD), B220 (RA3-6B2; eBioscience), CD138 (281-2), and CD11c (HL3; BD Biosciences). The cells were stained at 4°C for 20 min, washed, and analyzed without fixation for marker expression. A BD Biosciences Fixation/Permeabilization Kit was used for intracellular detection of BrdU, and the eBioscience FoxP3 Transcription Factor Staining Buffer Set was used for intracellular detection of T-bet. Data were acquired on a BD Fortessa flow cytometer using Diva software (BD Biosciences), and were analyzed using FlowJo software (Tree Star, Inc.).

RT-PCR. RNA was extracted from FASCs sorted CD11c⁺ B220⁺ cells using TRIzol, following the manufacturer's protocol (Life Technologies). cDNA was generated using a Tetro cDNA Synthesis Kit (Bioline). RT-qPCR was performed using a BioRad T100 Thermo Cycler; transcripts were normalized to β -actin (Life Technologies probe Mm00607939_s1) expression. *Aicda* mRNA was detected

using a primer-probe set specified by Life Technologies (Mm01184115_m1). To generate a positive control reagent, murine *Aicda* was amplified by PCR using Phusion High-Fidelity DNA Polymerase (New England BioLabs), using the following oligonucleotide primers: CTACCTCTGCTACGTGGTGAA (forward), GCTGAGGTTAGGGTTCCATCT (reverse). The amplicon was cloned using a TOPO TA Cloning Kit (Life Technologies).

ELISAs. ELISA plates were coated with outer membrane protein-19 (OMP-19) and serum from infected C57BL/6 and B6;129S-Fcgr2b^{tm1^{Ttk}/J} mice was added to wells. IgM or IgG antigen-specific antibody was detected with anti-mouse IgM or IgG coupled to alkaline phosphatase (AP), and phosphatase substrate. Immune complexes were detected by coating ELISA plates with recombinant IgM specific for OMP-19. Serum was incubated overnight and immune complexes were detected with anti-IgG antibody coupled to AP and phosphatase substrate.

TLR9 stimulation. T-cell depleted B cell-enriched spleens from C57BL/6 and UNC93b-deficient mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and cultured with or without CpG oligodeoxynucleotides (ODN1826). Stimulation was measured by dilution of CFSE after three days in culture.

Production and purification of recombinant IgM¹. IgM heavy chain (HC) and light chain (LC) DNA was cloned from the ehrlichia OMP-19-specific B cell hybridoma Em504.1 (4), using the approach described by Tiller et al. (5). To

¹ Lisa Dishaw produced the recombinant IgM.

generate an IgM expression vector, the IgM HC constant region, containing the secretory exon, was cloned by PCR from murine cDNA by PCR using a series of overlapping oligonucleotide primers. These were designed to introduce at the C-terminus a 6X Histidine tag, separated from the HC coding sequence by a thrombin recognition site, and a recognition site for BirA (for biotinylation).

Restriction endonuclease recognition sequences (Sall and HindIII) were also introduced at the N- and C-termini, respectively, to facilitate the replacement of the IgM HC in the Ig HC expression vector described above (5). This was accomplished using a forward oligonucleotide primer 5'

GCGTCGACCAGTCAGTCCTTCCCAAATGTC 3' and the following reverse oligonucleotide primers, in successive nested PCR reactions:

Oligonucleotide #1: 5'

ATACAGGTTTTACCCAGAACCATAGCAGGTGCCGCCTGTGTC 3';

Oligonucleotide #2: 5'

GTGATGACCAGAACCGCCCTGAAAATACAGGTTTTACCCAGA 3'; and

Oligonucleotide #3: 5'

CCAAGCTTTCAGTGATGGTGATGGTGATGACCAGAACCGCC 3'.

A construct encoding the mouse J-chain was generated synthetically (DNA2.0), and was cloned in the eukaryotic expression plasmid pD609 (DNA2.0). The J-chain gene was used with the Em514.1 IgM HC and LC expression constructs to transfect HEK292T cells, using the method described by Nelson et al. (6). The transfected cells were incubated for 6 days, the culture supernatant was harvested and cleared, and the recombinant IgM was isolated by batch

purification, using Ni-NTA beads. The IgM was removed from the beads using 250mM imidazole, and was dialyzed in PBS containing 1mM dithiothreitol. Native gel electrophoresis analysis revealed that the IgM preparation was homogeneous, multimeric, and contained the J-chain. ELISA was used to verify binding of the IgM to ehrlichial outer membrane protein-19 (OMP-19).

Statistical analyses. Statistical analyses were performed using Prism (GraphPad) software. Prism was used to run unpaired T-Test, One-Way ANOVAs with multiple comparisons, and Mann-Whitney test were appropriate.

Transcript analysis and Bioinformatics. One million CD11c+ IgM+ memory cells and CD19+ B cells were sorted from C57BL/6 mice, 30 days post-infection, directly into TRIZol using the BD FACSAria III. RNA was isolated from these samples, and a library was made using a TrueSeq Stranded mRNA Prep Kit. The library was analyzed for paired sequences using the IlluminaMiSeq. Each sample had between 3.5-13.9 reads with a read length of 75-151. The data was analyzed using the RNA Express Legacy on Illumina Basespace. The sequences were aligned using the STAR aligner to mm10 *mus musculus*. Differential gene expression was determined using DESeq2.

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Chapter 3: Early Derivation of IgM Memory Cells and Bone Marrow Plasmablasts

Introduction

Memory B cells, in addition to long-lived plasma cells, provide a major component of immunological memory (1, 2). Although it has often been assumed that B cell memory is harbored in high-affinity class-switched immunoglobulin (swlg) B cells, it has become increasingly apparent that, as for T cells, the memory B cell compartment is diverse, and several different memory subsets exist (3-5). There is considerable phenotypic heterogeneity, i.e., varying surface markers and Ig expression, within populations of hapten-elicited memory cells (6), differences which may reflect different kinds of memory cell functions (7). Moreover, several studies have revealed that unswitched murine IgM B cells harbored a significant component of humoral memory (8-11). IgM memory cells have been characterized in studies of murine memory responses following immunization, and similar cells are found in humans (12, 13). IgM memory cells constitute a novel and important subset of long-lived memory B cells that may provide immunity to variant pathogens not recognized by classical high-affinity swlg memory B cells (14, 15).

In addition to memory B cells, bone marrow plasma cells constitutively produce class-switched antibodies that mediate long-term immunity (16-18). Switched plasma cells have long been considered to be the major source of long-term antibodies, although several studies have described long-term bone marrow IgM antibody-secreting cells (ASCs; (19, 20)). T cell-independent (TI) antigens can induce bone marrow IgM ASCs, although it has often been considered that this

response is short-lived (21, 22). Our previous studies have indicated, however, that unswitched B cells and IgM can play an important role in long-term immunity to pathogens (20, 23).

Our studies of B cells during infection have utilized a mouse model of ehrlichiosis caused by the intracellular monocyctotropic bacterial pathogen, *Ehrlichia muris*. We first identified a TI CD11c-positive splenic plasmablast population present on and about day 10 post-infection that is responsible for the initial production of antigen-specific IgM during infection (24). This day 10 CD11c-positive splenic plasmablast population produces pathogen-specific polyreactive IgM, is not found in GCs, and is generated in the absence of CD4 T cell help (24, 25). A second population of splenic CD19- and CD11c-positive B cells is elicited within 3-4 weeks post-infection, and is detected at relatively high frequencies for at least as long as one year post-infection (23). We have demonstrated that these CD19/CD11c-positive B cells are IgM memory B cells, based on a number of definitive criteria, including their expression of the integrins CD11c and CD11b, as well as many other markers previously identified on memory B cells, such as CD73, PD-L2, CD80, and CD38 (23, 26). Moreover, the cells are largely quiescent, do not reside in GCs, have undergone limited somatic mutation, and are responsible for anamnestic, memory responses following antigen challenge (23). In addition to our work, studies of B cell responses in other experimental animal models and humans have identified what are likely related memory cells (15, 27, 28). The spleen is a major reservoir of memory B cells, including IgM

memory cells (both IgD-negative and -positive) in humans (29-31). Human IgM memory cells are elicited in response to *Streptococcus pneumoniae* infection (30), malaria infection (11), and following tetanus immunization (32).

The early CD11c-positive plasmablasts, IgM memory cells that we have described also express the transcriptional factor T-bet (**Figure 3.1**). B cells that express either CD11c, T-bet, or both molecules, have been identified in both human and animals in response to immunization, infections, and in autoimmunity (33-38). The identification of CD11c-positive T-bet⁺ cells in aged autoimmune patients led to their description as Age-Related B cells (ABCs; (36, 37, 39)), although CD11c-positive T-bet⁺ B cells are now known to function in many different immunological contexts. Whether CD11c and T-bet expression define a monolithic B cell population, or a number of related but functionally distinct B cell subsets, is currently unresolved. Our studies have indicated that CD11c- and T-bet-positive B cells include both early TI plasmablasts and IgM memory cells (23, 24). The derivation of and relationship between these two subsets remained unresolved in our previous studies, however.

We have also described a third non-canonical population of IgM T-bet-positive ASCs that arises in the bone marrow of infected mice after peak infection ((20); **Figure 3.1**). These B cells express CD138, CD93, and CD44, but are CD11c-negative, and are responsible for the production of protective long-term IgM (20). Thus, ehrlichial infection generates two diverse populations of long-lived IgM-positive B cells, in the spleen and bone marrow, respectively. The phenotypic

similarity between these two populations, as well as the observation that the day
10 TI CD11c-positive

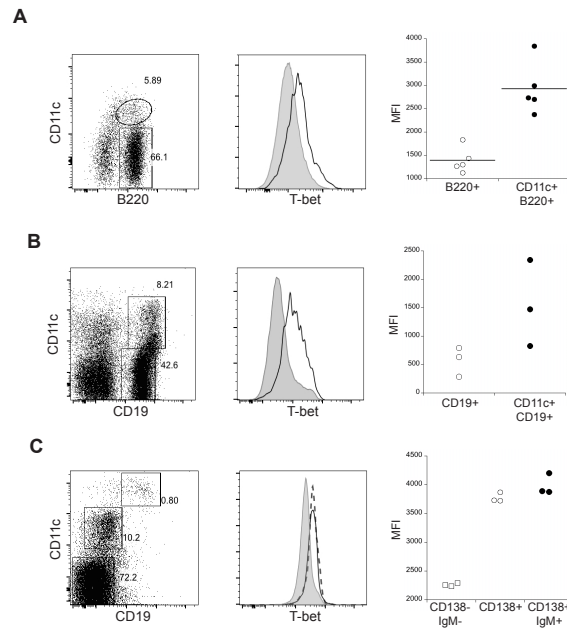


Figure 3.1. Early splenic CD11c⁺ B220⁺ plasmablasts and IgM memory population express T-bet. **(A)** Infected wild-type mice were analyzed on day 10 post-infection for intracellular T-bet expression in CD11c-negative B220⁺ B cells (rectangular gate and grey histogram) and CD11c-positive B220⁺ plasmablasts (circle gate and black histogram). Mean fluorescent intensities (MFIs) are shown for each mouse in the plot on the right side (Unpaired T-test, $p = 0.0006$). **(B)** Infected wild-type mice were analyzed 30 days post-infection for T-bet expression in the CD11c-negative CD19⁺ B cells (grey histogram) and CD11c-positive CD19⁺ IgM⁺ memory B cells (black histogram). The MFIs are shown in the plot on the right (Wilcoxon, $p = 0.2500$). **(C)** Infected wild-type mice were analyzed 30 days post-infection for T-bet expression in the CD138⁻ IgM⁻ bone marrow cells (grey histogram), the CD138⁺ bone marrow cells (grey dotted line), and the CD138⁺ IgM⁺ ASCs (black line). The MFIs are shown in the plot on the right (CD138⁻ IgM⁻ vs CD138⁺, $p < 0.0001$; CD138⁻ IgM⁻ vs CD138⁺ IgM⁺, $p < 0.001$; and CD138⁺ vs CD138⁻ IgM⁻, $p = 0.1412$).

plasmablasts precede both the IgM memory cells and bone marrow ASCs, suggested that the day 10 CD11c-positive B cells, or a yet unidentified population, are the precursors to one or both long-term populations. Here we demonstrate that both long-term IgM populations are derived from B cells elicited early following infection, at the time of the peak CD11c-positive plasmablast response. Moreover, because the bone marrow ASCs and IgM memory cells differ in their requirement for CD4 T cell help, we suggest that B cell fate is determined by the availability of signals from T_{fh} cells that are also present in abundance in the spleen during infection. Finally, we show that although the day 10 CD11c-positive plasmablasts are generated independently of CD4 T cells, they nevertheless require T cells, likely T_{fh} cells, for the induction of *Aicda* mRNA and for AID expression. These findings add to our understanding of the generation of non-canonical CD11c- and T-bet-positive B cell memory and effector cell subsets in the context of an intracellular bacterial infection.

Results

Early splenic B cells are the precursor to both splenic IgM memory B cells and IgM bone ASCs

Our previous studies showed that the majority of the IgM memory cells detected on day 30 post-infection expressed somatically-mutated receptors, indicative of AID activity (23). This observation suggested that IgM memory cell precursors could be identified on the basis of AID expression. Therefore, we utilized AID-Cre-ER^{T2} transgenic mice, described by Dogan et al. (14), to phenotypically mark IgM memory cells. The strain carries an AID promoter-regulated Cre recombinase whose activity is induced by tamoxifen; the strain was crossed to a reporter strain that carries a *Gt(Rosa26)Sor*-regulated EYFP allele containing flanking *loxP* sites (B6.Cg-Gt(ROSA)26Sor^{tm3(CAG-EYFP)Hze}/J). In B cells that express AID, tamoxifen induces Cre recombinase activity that facilitates genetic recombination and subsequent expression of EYFP, thereby permanently marking AID-expressing B cells. We first used the strain to address whether AID-expressing cells induced during acute ehrlichial infection contributed to either long-term IgM population. For these studies, mice were infected, and tamoxifen was administered 10 days later, the peak time of the early CD11c-positive splenic IgM plasmablast response that we described in our previous studies (24). When the mice were analyzed by flow cytometry on day 30 post-infection, approximately 30% of the CD11c-positive splenic IgM memory cells expressed EYFP (**Figure 3.2a**). Although AID is also expressed in GC cells, in our previous

study we demonstrated that the IgM memory cells do not express markers characteristic of GC B cells (23). Similarly,

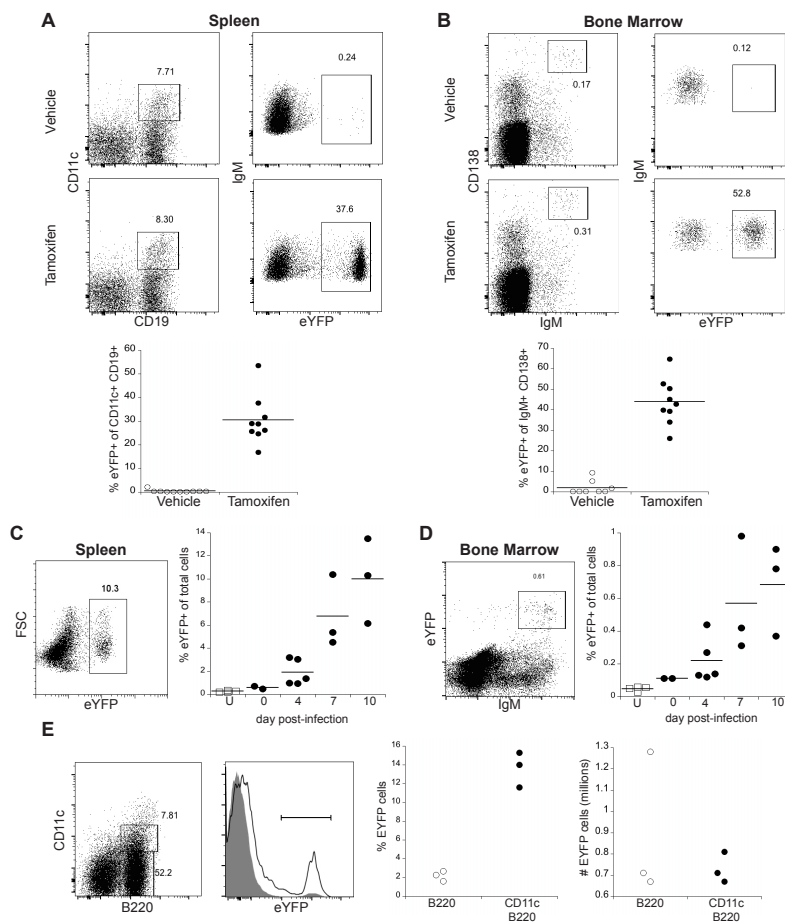


FIGURE 3.2 Splenic IgM⁺ memory B cells and CD138⁺ IgM⁺ Bone Marrow ASCs are derived early following infection. (A) Infected (AID-Cre ER^{T2} X eYFP) F₁ mice were administered tamoxifen, or a vehicle control, on day 10 post-infection, and splenic CD11c-positive CD19⁺ B cells were analyzed on day 30 post-infection for IgM and eYFP expression by flow cytometry. **(B)** Bone marrow IgM⁺ B cells from mice treated as in **A** were analyzed for eYFP expression among IgM⁺ CD138⁺ cells on 30 days post-infection. Cumulative data from mice analyzed between days 30 and 120 post-infection are shown below the flow cytometry plots in **A** and **B**. The differences between the groups in both analyses were statistically significant, as determined using a Mann-Whitney test ($p < 0.0001$). **(C and D)** Tamoxifen was administered to uninfected or infected (AID-Cre ER^{T2} X eYFP) F₁ mice, 0, 4, 7, or 10 days post-infection, and spleen **C** and bone marrow **D** were analyzed for eYFP expression on day 30 post-infection. The frequencies of eYFP⁺ cells detected in each of the mice are shown in the plots to the right of each dot plot. A multiple comparison ANOVA was used to compare data from uninfected tamoxifen-treated mice, relative to mice that had

been treated on day 0 ($p = 0.9990$), day 4 ($p = 0.6150$), day 7 ($p = 0.0054$) and day 10 ($p = 0.0002$) post-infection. Similar analyses of the bone marrow cells were performed by comparing treated uninfected mice to mice treated on day 0 ($p = 0.7289$), day 4 ($p = 0.4039$), day 7 ($p = 0.0161$), and day 10 ($p = 0.0056$) post-infection. **(E)** Infected (AID-Cre ER^{T2} X eYFP) F_1 mice were administered tamoxifen on days 4 and 7 post-infection and were analyzed on day 10 post-infection for eYFP expression in CD11c-negative B220+ (shaded histogram) and CD11c-positive B220+ cells (open histogram). Cumulative data from the analyses are shown in the plots on the right; the frequencies EYFP+ cells within each of the populations, indicated by the gate, were significantly different (Unpaired T-test, $p = 0.0005$), but total cell numbers were similar; Mann-Whitney test $p = 0.10$).

approximately 50% of the CD138-positive IgM-producing bone marrow B cells that we described previously (24) were also labeled following tamoxifen administration on day 10 (**Figure 3.2b**). EYFP-positive IgM memory cells and bone marrow ASCs were both identified at least as late as 98 days post-tamoxifen administration. Both the spleen and bone marrow had cells irreversibly marked following tamoxifen administration as early as day 4 post-infection, indicating that AID expression is induced very early after infection (**Figure 3.2c and d**). These studies indicate that early AID-expressing B cells, possibly the early CD11c-positive T-bet⁺ plasmablasts, give rise to both IgM memory cells and bone marrow IgM ASCs. Not all of the IgM memory cells or plasmablasts expressed EYFP, indicating that not all of the plasmablasts expressed *Aicda* during the window of tamoxifen administration. Our previous study demonstrated that not all of the IgM memory cells expressed mutated BCRs, so it is also possible that not all of the precursor cells transcribed *Aicda*. To identify *Aicda*-positive cells early during early infection, infected (AID-cre-ER^{T2}x EYFP) F₁ mice were administered tamoxifen on days 4 and 7 post-infection, and splenic B cells were analyzed for EYFP expression on day 11 post-infection. A higher proportion of the CD11c-positive plasmablasts expressed EYFP (approximately 13%), relative to the CD11c-negative B220⁺ B cells (approximately 2%; **Figure 3.2e**). However, EYFP-positive cells were detected in similar numbers within each population. Thus, it is possible that either one or both of the early cell populations give rise to the long-term IgM⁺ B cells observed on day 30 or later post-infection.

Bone marrow IgM ASCs undergo rapid turnover

Although in previous studies we characterized the early CD11c-positive plasmablasts in depth, we had not formally demonstrated that these cells were actively dividing. To address this question, we administered BrdU to *E. muris*-infected mice on day 10, and kept on BrdU water and analyzed for BrdU incorporation one-hour later. Nearly all of the CD11c-positive plasmablasts had incorporated BrdU during that time, indicating that the population consisted of actively dividing cells, i.e., they are formally plasmablasts (**Figure 3.3a**). Because the long-term IgM bone marrow ASCs we identified after day 30 post-ehrlichial infection exhibited phenotypic characteristics of both plasma cells and plasmablasts (20), it was not known whether the bone marrow IgM-positive B cells were quiescent, or were maintained by cell division. To address this question, we administered BrdU to infected mice for a 10-day period, beginning on day 30, and in some cases starting as late as 98 days post-infection. Nearly all of the bone marrow ASCs incorporated BrdU within the 10-day administration period (**Figure 3.3b**). To better define the extent to which the bone marrow ASCs were dividing, we next treated infected mice on day 55 post-infection with BrdU for one-hour. Approximately 80% of the IgM bone marrow ASCs incorporated BrdU within the one-hour labeling period (**Figure 3.3c**). Together these data reveal that the IgM bone marrow ASCs, unlike the IgM memory cells, (see (23)) undergo rapid turnover, likely due to active proliferation. Our data suggest that the IgM bone marrow plasmablasts are

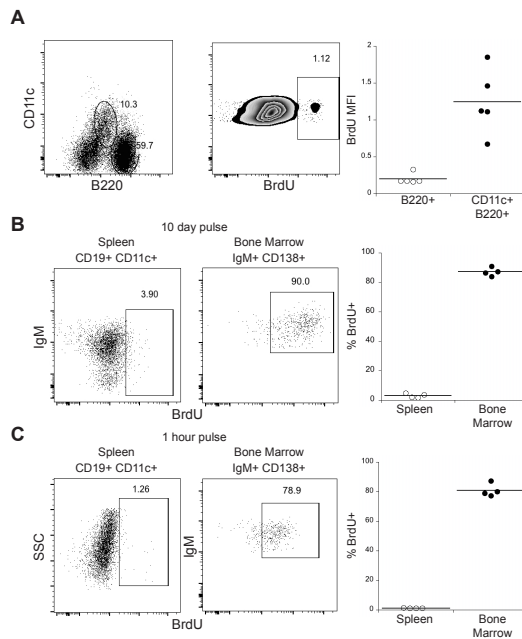


FIGURE 3.3 Bone marrow CD138+ IgM+ ASCs actively proliferate. (A) Infected mice were administered BrdU on day 10 post-infection, and maintained on BrdU drinking water for one-hour. A representative analysis of BrdU incorporation by CD11c-negative B220+ cells (grey histogram) and CD11c-positive B220+ plasmablasts (open histogram) is shown in the middle plot, and cumulative mean fluorescent intensity data are shown in the plot on right. The differences were statistically significant, as determined using an Unpaired Student's T-test (p value < 0.0008). **(B)** Infected mice were administered BrdU at day 30 post-infection, and were maintained on BrdU drinking water for ten days. CD11c-positive CD19+ spleen and CD138+ IgM+ bone marrow B cells were analyzed. The percentages of BrdU-positive cells are shown in the plot on the right; Mann-Whitney test p value=0.028. **(C)** Wild-type mice that were infected for at least 30 days were administered BrdU one hour prior to analysis, as in **B**; Mann-Whitney test p value = 0.0286.

maintained essentially indefinitely by chronic antigen stimulation, or via other mechanisms.

CD138 IgM Bone Marrow cells are generated independently of CD4 T cells.

It was unknown whether the IgM bone marrow plasmablasts we identified were generated in a TI fashion, as has been shown in related studies of bone marrow IgM B cells (19, 40). CD4 T cell help was not required for the generation of IgM bone marrow ASCs in our experimental model, because the ASCs were detected in both wild-type and MHC class II-deficient mice on day 30 post-infection (**Figure 3.4a**). The frequency of IgM bone marrow ASCs in MHC class II-deficient mice among total bone marrow cells was modestly lower; moreover, the IgM+ plasmablasts were detected at slightly higher cell numbers in the CD4-deficient mice. There was no difference in the frequency of IgM bone marrow ASCs when measured as a proportion of the frequency of total population of bone marrow CD138 ASCs. As an additional test for the requirement for CD4 T cell help, mice were administered a CD40L-blocking antibody (MR-1), on days 8, 10, and 12 post-infection. This treatment effectively inhibited T cell-dependent responses to adjuvanted NP-BSA (**Figure 3.5**). In contrast, mice treated with the anti-CD40L MAb exhibited very similar frequencies of IgM bone marrow ASCs as control mice (**Figure 3.4b**). Our data therefore identify two distinct populations of long-lived cells that are derived early following infection, likely via different mechanisms: a spleen IgM memory population that requires both CD4 T cell help and IL-21 (23), and the CD4 T cell-independent BM ASCs. These data suggest that cell fate may be determined by access to CD4 help during infection.

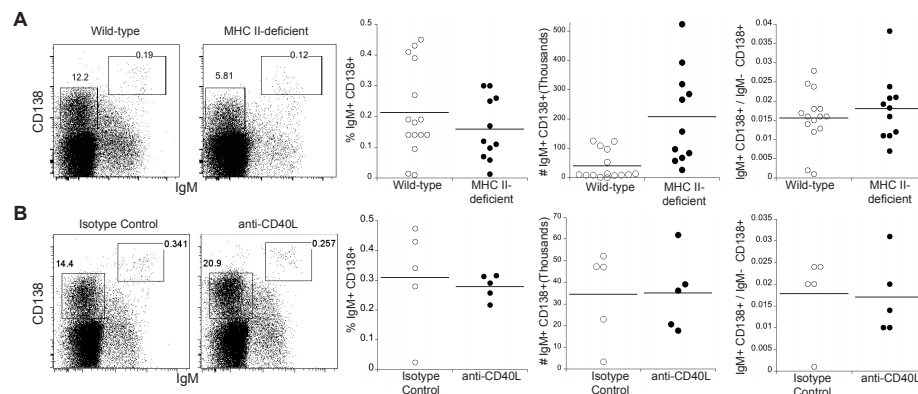


FIGURE 3.4 CD138+/IgM+ bone marrow plasmablasts were generated independently of CD4 T cell help. (A). Wild-type and MHC class II-deficient mice were infected, and bone marrow was analyzed 30 days post-infection for IgM and CD138 expression. Representative flow cytometry dot plots are shown in the left panels, indicating the IgM+ plasmablasts (upper right gates), and IgM-negative plasma cells (gate at left). Cumulative frequencies of IgM+ CD138+ cells are shown in the plot on the left; Mann-Whitney test, $p=0.32$, the number of CD138+ IgM+ cells are shown in the plot in the middle; Mann-Whitney test $p=0.0006$, and the ratio of IgM+ CD138+ cells to the IgM-negative CD138+ cells are shown on the far right; Mann-Whitney test $p=0.63$. **(B).** Infected wild-type mice were administered 200 μg of anti-CD40L, or an isotype-matched irrelevant control antibody, on 8, 10, and 12 days post-infection. The cells were analyzed, as in **A**, on 30 days post-infection. The cumulative frequencies of IgM+ CD138+ cells are shown in the plot on the left ($p=0.4206$), the number of IgM+ CD138+ cells are shown in the plot in the middle ($p = 0.0006$), and the ratio of IgM+ CD138+ cells to the IgM-negative CD138+ cells are shown in the far right plot ($p=0.61$); all statistical analyses were performed using a Mann-Whitney test. The data in each of the plots were compiled from two to three experiments.

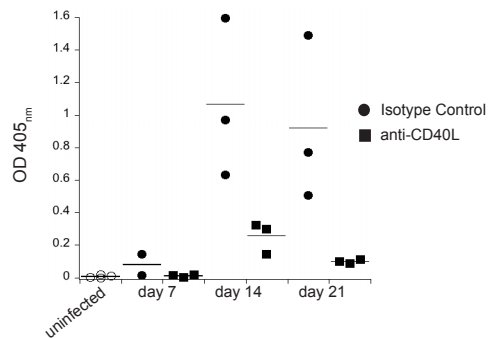


Figure 3.5. Anti-CD40L administration blocked production of NP-specific IgG. Wild-type mice were immunized with NP(26)-BSA, in alum, and were administered either 200 μ g anti-CD40L (MR-1) or an irrelevant isotype-matched antibody (2A3) on days 4, 8, and 12 post-immunization. Anti-NP IgG production was analyzed on days 7, 14, and 21 post-infection by ELISA (ANOVA, p value = 0.0005).

***Aicda* expression in early splenic plasmablasts is dependent on CD4 T cells, but not TLR9 signals**

Although we have demonstrated that CD4 T cells are not required for the generation of either the day 10 CD11c-positive plasmablasts (24), nor for the BM plasmablasts, it was possible that T cells were nevertheless required for the induction of AID expression. It is well known that CD4 T cells, in some cases in the context of innate signals, are required for the induction of AID in GC B cells (41, 42). To identify how AID expression is induced in early IgM plasmablasts, we monitored *Aicda* transcription *in vivo* using B cells from (AID-cre-ER^{T2}x EYFP) F₁ mice². In these studies, magnetic bead-enriched, T cell-depleted, naïve B cells from (AID-cre-ER^{T2}x EYFP) F₁ mice were transferred to wild-type and MHC II-deficient mice. The recipient mice were infected with *E. muris*, and tamoxifen was administered on days 7 and 10 post-infection. To eliminate any residual donor T cells, the MHC II-deficient recipient mice were administered an anti-CD4 (GK1.5) antibody on day 0 and 3 post-transfer. When the mice were analyzed 11 days post-infection, EYFP expression in CD11c-positive B220+ plasmablasts was detected in wild-type recipient mice, but not MHC II-deficient mice (**Figure 3.6a**), indicating that CD4 T cells were required for *Aicda* expression. Donor B cells that expressed IA^b were detected in MHC II-deficient recipient mice, indicating that naïve B cells were successfully transferred (**Figure 3.6a**). The frequency and number of ehrlichia-specific donor cells was low in these studies likely because B

² Experiment performed by Kevin Kenderes.

cells were obtained from naïve mice. Thus, even though the day 10 CD11c-positive T-bet+ plasmablasts

do not require CD4 T cells for their generation (20), the plasmablasts nevertheless require T cell help to induce *Aicda* expression. These data also demonstrate that the day 10 CD11c-positive plasmablasts are derived at least in part from naïve splenic precursor B cells.

Because TLRs have also been shown to be capable of inducing AID expression in B cells (43-45), we next addressed whether TLR signals played a role in the induction of AID in the day 10 CD11c-positive plasmablasts. Because the ehrlichiae do not express LPS, peptidoglycan, or flagella (46), they do not signal through any known TLRs, with the possible exception of TLR9, which recognizes CpG-containing bacterial DNA. Therefore, we addressed whether AID was expressed in *E. muris*-infected *Unc93b* gene-targeted mice (47, 48). *Unc93b*-deficient mice lack the endoplasmic reticulum protein *Unc93b* that is required for trafficking of TLRs 3, 7, 8, and 9. *Unc93b* deficiency did not impact the generation of the early plasmablast, so for these studies, *Aicda* expression was monitored by RT-PCR in FACS-purified CD11c-positive plasmablasts on day 10 post-infection. *Aicda* expression was detected in CD11c-positive plasmablasts in both wild-type and *Unc93b*-deficient mice (**Figure 3.6b**), indicating that TLR9 signaling is not required for *Aicda* expression during ehrlichial infection. In

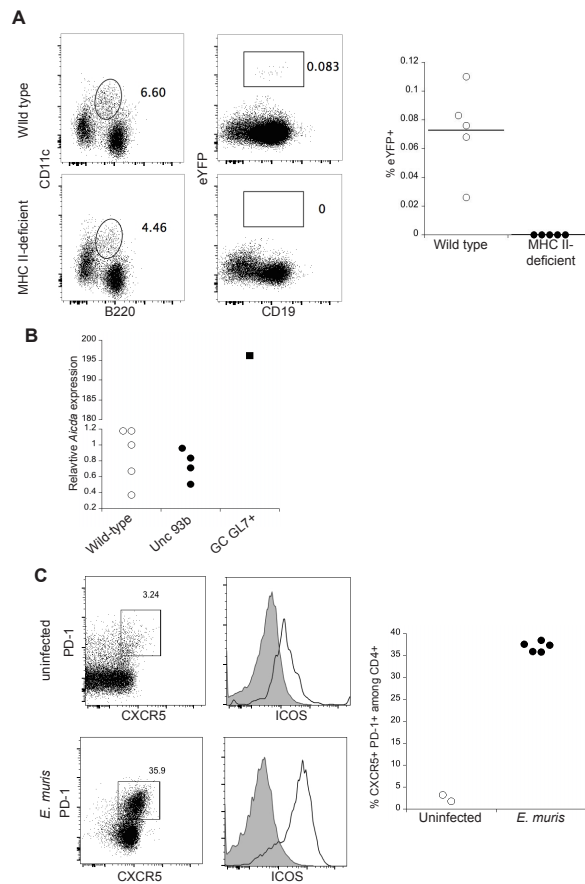


Figure 3.6 *Aicda* expression in day 10 splenic plasmablasts required CD4 T cells, but did not require TLR9 signaling. (A) Magnetic bead-purified naïve splenic B cells from (AID-Cre ER^{T2} X eYFP) F₁ mice were transferred to wild-type and MHC II-deficient mice; the recipient mice were then infected with *E. muris*, and were administered tamoxifen on days 7 and 10 post-infection. The recipient MHC II-deficient mice were treated with 200 µg of a depleting CD4 antibody (GK1.5) immediately following and three days after cell transfer, to eliminate any co-transferred donor T cells. The spleens of recipient mice were analyzed by flow cytometry on day 11 post-infection for CD11c-positive CD19⁺ donor eYFP⁺ cells. The percentages of CD11c-positive CD19⁺ eYFP⁺ cells detected in wild-type (open circles) and MHC II-deficient mice (closed circles) are shown in the plot on the right; Mann-Whitney test p value=0.0079. (B) CD11c-positive B220⁺ cells were purified by flow cytometry from the spleens of wild-type (open circles) and UNC93b-deficient (closed circles) mice. mRNA was isolated and RT-PCR was performed to quantify *Aicda* transcripts in each strain. $\Delta\Delta$ CT values were calculated, relative to β -actin transcripts. *Aicda* transcripts detected in flow cytometrically-purified GC B cells pooled from six tetanus toxin-immunized wild-type mice are shown for comparison (closed square). No statistical differences in

Aicda expression were detected between the wild-type and UNC93b-deficient mice; Mann-Whitney test $p=0.30$. **(C)** Spleen cells from uninfected and day 10 post-infection mice were analyzed for CXCR5+ PD-1+ surface expression on T_{fh} cells (gated in each dot plot). The gated T_{fh} cells were also analyzed for surface expression of ICOS (middle histograms). The percentages of T_{fh} cells among total CD4+ cells in uninfected (open circles) and *E. muris*-infected (closed circles) mice are shown in the plot on the right; Mann-Whitney test $p=0.095$.

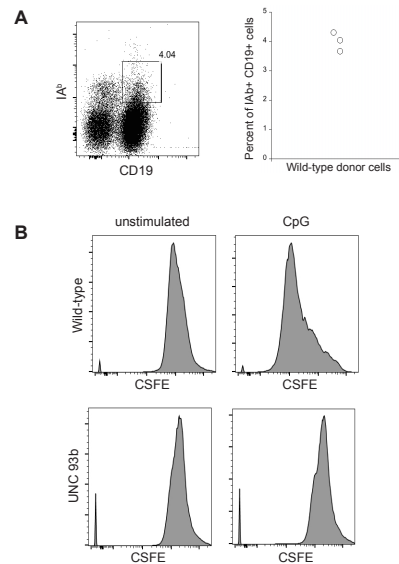


Figure 3.7 IA^b+ B cells were detected following transfer of wild type B cells to MHCII-deficient mice, and Unc 93b-deficient mice did not respond to CpG stimulation. (A) Magnetic bead-purified naïve splenic B cells from wild-type mice were transferred to MHC II-deficient mice; the recipient mice were then infected with *E. muris*. Then were treated with 200 µg of a depleting CD4 antibody (GK1.5) immediately following cell transfer, and three days later, to eliminate any co-transferred CD4 T cells. The spleens of recipient mice were analyzed by flow cytometry on day 11 post-infection for CD19+ CD11c+ IA^b+ donor cells. **(B)** T cell-depleted spleens from wild-type and Unc 93b-deficient mice were labeled with CFSE and cultured in the presence or absence of CpG (ODN 1826) for three days. CFSE dilution occurred in wild-type, but not UNC93b-deficient mice.

separate studies we confirmed that the *Unc93b*-deficient mice did not proliferate *in vitro* in response to CpG ODN(1826) (**Figure 3.7**). These data also reveal that *Aicda* expression, although sufficient to drive Cre-mediated recombination, is much lower in plasmablasts, relative to its expression in GC B cells (**Figure 3.6b**). The relatively lower *Aicda* expression in IgM memory cells may explain why class switch recombination does not occur in IgM plasmablasts or IgM memory B cells.

Aicda expression in IgM memory cells was CD4 T cell-dependent, so we next investigated whether CD4 T_{fh} cells underwent expansion during early *E. muris* infection. We observed a major expansion of CXCR5⁺ PD-1⁺ T_{fh} cells on day 10 post-infection, relative to uninfected mice (**Figure 3.6c**). Nearly all of the T_{fh} cells exhibited high expression of ICOS, relative to PD-1/CXCR5 double-negative CD4 T cells. Thus, although the day 10 plasmablasts are generated in the absence of CD4 T cells, this occurs in the presence of a vigorous T_{fh} cell response, which is in part required for induction of *Aicda* in the B cells.

Persistent *Aicda* expression in IgM memory cells

We also addressed whether *Aicda* was expressed in long-term IgM memory B cells and bone marrow plasmablasts, by administering tamoxifen to infected (AID-Cre-ER^{T2}x EYFP) F₁ mice at least 30 days post-infection. Spleen IgM memory cells from 4 of 6 mice exhibited EYFP expression 12 days following tamoxifen administration (**Figure 3.8**; approximately 30% of the IgM memory

cells were EYFP-positive within the mice that expressed *Aicda*). In contrast, lower percentages of EYFP+ cells were detected among the bone marrow plasmablasts. Although we cannot completely exclude the possibility that some memory cells had recently emigrated from GCs, where they expressed AID, it is unlikely that 30% of the cells had emigrated during the time tamoxifen was administered. These data indicate that *Aicda*-expression can be maintained in IgM memory cells during low-level chronic ehrlichial infection, in the absence of class switching.

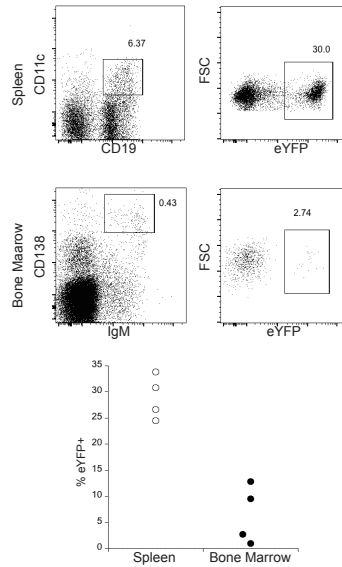


Figure 3.8 *Aicda* was constitutively expressed in IgM memory B cells. (AID-Cre ER^{T2} X eYFP) F₁ mice that had been infected for at least 30 days, were administered tamoxifen, and 12 days later the frequencies of EYFP-expressing cells among the CD11c-positive CD19+ and IgM+ CD138+ B cell populations were quantitated in the spleen and bone marrow. Cumulative data is shown in the plot at the bottom. Two mice did not exhibit any EYFP expression, and were eliminated from the plot. The differences in EYFP expression between the spleen and bone marrow cells were statistically significant; Mann-Whitney test p=0.028.

Discussion

Our findings reveal that both long-term IgM memory cells and bone marrow plasmablasts are derived from precursor cells present early during ehrlichial infection, well before the generation of canonical class-switched memory B cells that develop in GCs (49, 50). Our studies therefore highlight what is likely a novel pathway of memory development that is important for the generation and maintenance of long-term unswitched memory cells. Although we have demonstrated that the long-term IgM-positive B cells in our model are derived early during infection, as early as day 4 post-infection, we have not yet resolved whether the memory cells and plasmablasts are generated independently, or via a single pathway. Given their phenotypic similarities, it is possible that both long-lived B cell populations are derived from the early splenic CD11c-positive T-bet+ plasmablasts that we have described (24). Indeed, nearly all of the ehrlichial-specific IgM detected on day 10 post-infection was secreted by the early CD11c-positive plasmablasts, not CD11c-negative B cells (24, 51). However, plasmablasts are by definition short-lived cells, so an alternative explanation is that the long-term IgM-positive memory cells are derived independently, perhaps from CD11c-negative follicular B cells. Although EYFP-positive cells were found at much higher frequencies among CD11c-positive plasmablasts, EYFP+ CD11c-negative B cells were found in similar numbers as the CD11c-positive plasmablasts. Ongoing studies will help to resolve the origin(s) of the long-term IgM B cells. These findings nevertheless highlight a novel pathway for the generation of long-term IgM memory and antibody-secreting plasmablasts in the context of a TI response to infection.

Although the day 10 CD11c-positive T-bet⁺ plasmablasts do not require CD4 T cell help for their generation, the signals required for the subsequent differentiation of the plasmablasts to either IgM B cell memory cells or BM ASCs are not yet known. However, the IgM memory cells require CD4 T cells (and IL-21) for their generation (23), whereas the BM plasmablasts do not, suggesting that the availability of T cell help may be a key factor in what drives the development of B cell memory versus long-lived BM plasmablasts. In this regard, we have observed a large population of splenic T_{fh} cells that are present at the time of the early CD11c-positive plasmablast response. Given the magnitude of the T_{fh} cell response, it suggests that T cell help is not limited by cell number, and it is possible that the fate of the B cells may be determined instead by their physical location. In such a model, extrafollicular B cells that fail to interact with T_{fh} cells may exit the spleen and migrate to the bone marrow, whereas B cells that enter follicles may elicit T cell signals that drive IgM memory cell development. Both populations likely differentiate independently of GCs, which are suppressed during early ehrlichial infection (52). Although we have not yet formally resolved the requirements for GCs in our experimental model, other studies of non-canonical memory B cells suggest IgM memory cells follow a GC-independent pathway (4, 5, 53). Alternately, affinity may be a factor in the fate decision; higher affinity B cells may elicit more T cell help, thereby promoting IgM memory B cell development, whereas cells that fail to elicit sufficient T cell help may follow a default pathway to become bone marrow plasmablasts.

Our demonstration that the bone marrow IgM plasmablasts are generated independently of T cells is consistent with other studies that have shown that TI responses can elicit long-term antibody production and/or protection (21, 22, 40). Those studies demonstrated that long-term IgM production could be maintained indefinitely, via two different mechanisms. One mechanism is that IgM is maintained by the continual recruitment of short-lived plasma cells (21, 22). Alternatively, it has been proposed that TI pathogens can induce long-lived plasma cells (40). Our studies suggest that long-term IgM production can be maintained indefinitely by proliferation, although this process may also involve the elicitation of new bone marrow plasmablasts from IgM memory cells. The factors that drive proliferation of the IgM bone marrow plasmablasts are unknown. *E. muris* establishes a low-level chronic infection, although it is unknown if sufficient antigen can be derived from these intracellular pathogens to maintain B cell proliferation in the bone marrow. Alternatively, low-level inflammation in the bone marrow may drive the production of factors that support plasmablast/plasma cell maintenance and proliferation (54).

Our studies also allowed us to address the source of the signals that drive *Aicda* expression in early splenic CD11c-positive T-bet⁺ plasmablasts. Although these cells are generated in the absence of CD4 T cell help, we show that T cell signals are nevertheless required to induce *Aicda* transcription. We propose *Aicda* transcription is driven by interactions with T_{fh} cells that are abundant in the

spleen at that time, perhaps via classical interactions involving CD40:CD40L. Unlike what has been described in other studies (42, 43, 45, 55), *Aicda* transcription did not require TLR9, and unlikely involves other TLRs, because the ehrlichiae lack classical TLR ligands. Other innate signals may substitute during ehrlichial infections, although the nature of these signals and/or receptors is unknown. The observation that *Aicda* expression was maintained, apparently indefinitely, in the IgM memory B cell population, suggests that the same or different factors that elicit *Aicda* expression on day 10 post-infection are maintained in the IgM memory cells, perhaps as a consequence of low level inflammation. The consequences of long-term AID expression in IgM memory B cells is unknown, but it has been suggested that chronic low level AID expression in memory B cells can promote polyreactivity, self-reactivity, and elimination (56).

Our studies also shed light on the origin and function of CD11c-positive T-bet⁺ B cells, which are now emerging as an important B cell subset involved in both host defense and autoimmunity. Although it is unclear whether CD11c and/or T-bet define a single or multiple functionally distinct B cell subsets, our previous and current findings support the hypothesis that such B cells include IgM memory B cells (23). Although some CD11c-positive T-bet⁺ B cells are generated in response to TLR signaling (36, 38), our data indicate that these signals are not required, although other innate signals likely substitute for TLRs. T-bet activity may also be responsible for maintaining persistent *Aicda* expression.

Our studies highlight a novel pathway for the development of both IgM memory cells and long-term bone marrow plasmablasts. We have shown that IgM production is maintained indefinitely following *E. muris* infection, and it is likely that both non-switched populations are important for maintenance of humoral memory. It will be important to address whether similar mechanisms contribute to long-term immunity in humans after either infection or vaccination.

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**Chapter 4: IgM+ memory B cell population are regulated
by Fc γ RIIb induced apoptosis**

Introduction

The generation of B cell memory is important for the maintenance of long-term immunity (1-3). While it has been well established that class-switched memory B cells harbor a significant amount of long-term immunity, recently, there has been mounting evidence to support a role for IgM memory B cells (4). Our own work has identified a CD11c⁺ T-bet⁺ IgM⁺ memory B cell population that arises in response to *Ehrlichia muris* infection (5). The IgM⁺ memory B cell population is required for recall IgG responses, is relatively quiescent, and expresses memory surface markers identified in other memory models of B cells, such as CD73, CD80, and PD-L2 (5, 6). The IgM⁺ memory B cell population also required both CD4⁺ T cells and the cytokine IL-21 for their generation (5). While our studies have extensively characterized the IgM⁺ memory population, and explored mechanisms surrounding the generation of the IgM⁺ memory B cell population, our understanding of the mechanisms that maintain this population remain unclear.

To understand more about the mechanisms involved in the maintenance and genesis of the IgM⁺ memory B cell population, we compared the IgM⁺ memory B cell population to canonical CD19⁺ B cells, using RNAseq (**Figure 4.1a**). Our analysis revealed the upregulation of multiple genes, but the 2-fold increase in Fc γ RIIb mRNA in the IgM⁺ memory B cell population was of particular interest. Fc γ RIIb is an inhibitory Fc γ R receptor that recognizes immune complexes, but not monomeric IgG (7). It is the only Fc γ R expressed on B cells, although other

immune cells express the receptor, including dendritic cells, macrophages, monocytes, and neutrophils (8, 9). Fc γ RIIb is known to inhibit BCR signaling and to trigger apoptosis when the receptor is crosslinked (10). In fact, it was demonstrated that Fc γ RIIb was necessary for quorum sensing of IgG to regulate IgM B cell population homeostasis (11). This was not the first case made for the involvement of Fc γ RIIb in the regulation of lymphocyte populations. There have been reports of Fc γ RIIb regulating memory T cells and plasma cell survival (12, 13). Given the increased expression on the IgM⁺ memory B cell population in our model, we sought to determine what role, if any, Fc γ RIIb plays in the maintenance and/or generation of our IgM⁺ memory B cell population.

Here we show that Fc γ RIIb-deficient mice have a 3-fold increase in the IgM⁺ memory B cells. This increase is not because of a change in the rate of proliferation either during ontogeny, or after the population reaches homeostasis. Rather, the increased population size is likely be due to the loss of FAS expression on the IgM⁺ memory B cell population, which, in turn, decreases the susceptibility to apoptosis. We propose that infection-specific immune complexes signal through both the B cell receptor (BCR) and Fc γ RIIb to regulate the homeostasis of the IgM⁺ memory B cell population, by inducing apoptosis, via a FAS dependent mechanism.

Results

Fc γ RIIb-deficiency leads to a 3-fold increase in IgM⁺ memory B cells

Previously, we identified a splenic IgM⁺ memory B cell population that responds to *E. muris* infection. This population expresses both CD11c and T-bet, and shares similar characteristics to other CD11c⁺ T-bet B cell populations described in the literature (14-17). To further understand the mechanisms involved in the maintenance and genesis of the IgM⁺ memory B cell population, both the IgM⁺ memory population and CD19⁺ canonical B cells were purified. Both populations were processed for RNA extraction, and prepared for analysis by RNAseq. Analysis of the mRNA transcripts revealed multiple genes were upregulated including, *Itgam* (CD11b), *Tbx21* (T-bet), *Itgax* (CD11c), and *Fcgr2b* (Fc γ RIIb) in the IgM⁺ memory B cell population as compared to CD19⁺ canonical B cells³ (**Figure 4.1a**). We decided to further explore the increase in mRNA expression of Fc γ RIIb, by examining the surface expression of Fc γ RIIb. Analysis of the IgM⁺ memory B cell population revealed that the 2-fold increase in Fc γ RIIb mRNA transcripts was correlated with a 2-fold increase in Fc γ RIIb surface expression (**Figure 4.1b**).

To determine whether Fc γ RIIb played a role in the generation and/or maintenance of the IgM⁺ memory B cells, both wild-type and Fc γ RIIb-deficient mice were infected with *E. muris*, and splenic B cells were analyzed on 30 days post-infection. The results revealed that Fc γ RIIb-deficiency led to a 3-fold

³ Russell Levack preformed the analysis of RNAseq data.

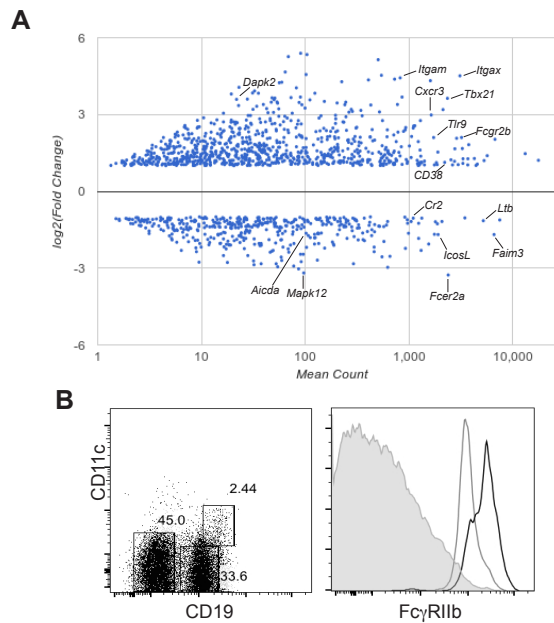


Figure 4.1 Fc γ RIIb is upregulated on the IgM $^{+}$ memory B cell population. (A) RNAseq revealed a 2-fold increase in Fc γ RIIb mRNA expression among other genes. **(B)** Spleen cells from day 30 post-infection were analyzed for Fc γ RIIb expression on the IgM $^{+}$ memory B cells (black line), canonical CD19 $^{+}$ B cells (grey line), and on CD11c-negative CD19-negative cells (grey histogram).

increase in the size of IgM⁺ memory B cell population; this increase was not due to a decrease in the number of canonical CD19⁺ B cells, but to an increase in the number of IgM⁺ memory B cells (**Figure 4.2a**). In addition to an increase in the IgM⁺ memory B cell population, the Fc γ RIIb-deficient mice had an increase in both serum IgM and IgG antibodies specific to *E. muris* infection (**Figure 4.2b**). This increase in antibody titer did not correlate with a decrease in bacterial load (**Figure 4.2c**). The Fc γ RIIb-deficient mouse used for these studies lacked expression on all cells that would normally express Fc γ RIIb, including B cells, macrophages, and dendritic cells. It is possible that the complete deficiency in Fc γ RIIb affected the function of macrophages and dendritic cells, observations that may explain the higher bacterial load. Together, these data suggests that Fc γ RIIb is involved in a mechanism that negatively regulates the IgM⁺ memory B cell population.

Because we observed an expansion of the IgM⁺ memory B cell population in Fc γ RIIb-deficient mice, we next investigated whether challenging infected Fc γ RIIb-deficient mice with OMP-19 would further increase the IgM⁺ memory B cell population. Analysis of the spleen of both wild-type and Fc γ RIIb-deficient 12 days post-challenge revealed no expansion in the IgM⁺ memory B cell population, beyond what is seen after primary infection (**Figure 4.2d**). Analysis of serum post-challenge showed no increase in antigen-specific IgM; however there was a significant increase in IgG post-challenge in the Fc γ RIIb-deficient mice as

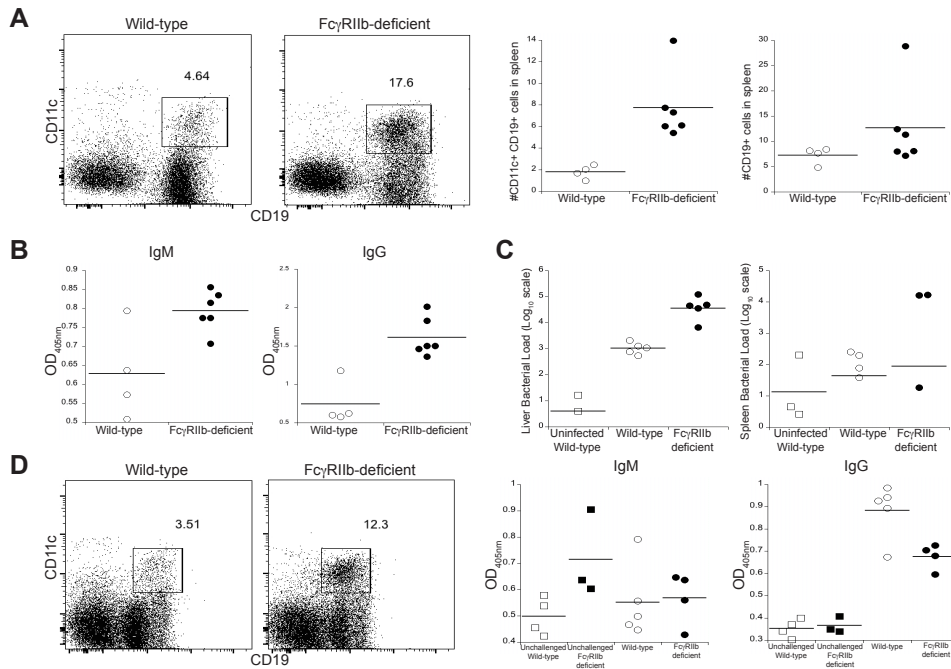


Figure 4.2 Fc γ RIIb-deficiency leads to a 3-fold increase in the IgM+ memory B cell population. (A) Representative flow plots of 30 days post-infection wild-type and Fc γ RIIb-deficient mice. Graphs quantifying both the number of CD11c+ CD19+ (unpaired T-test $p = 0.0064$) and CD19+ cells (unpaired T-test $p = 0.2398$) in the spleen of both wild-type and Fc γ RIIb-deficient mice are on the right. (B) Graphs depicting the OD reading of IgM (unpaired T test $p = 0.0175$) and IgG (unpaired T-test $p = 0.0010$) antibody specific for *E. muris* infection in wild-type and Fc γ RIIb-deficient mice at 30 days post-infection. (C) Bacterial load analysis of uninfected wild-type, wild-type, and Fc γ RIIb-deficient mice in both the liver and spleen of mice 30 days post-infection. A one way ANOVA with multiple comparisons determined there was a significant difference between uninfected wild-type and infected wild-type mice ($p = 0.0001$), uninfected wild-type and infected Fc γ RIIb-deficient mice ($p = 0.0001$), and between infected wild-type and infected Fc γ RIIb-deficient mice ($p = 0.0005$) in the liver. A one way ANOVA with multiple comparisons determined there was a significant difference between uninfected wild-type and infected wild-type mice ($p = 0.8662$), uninfected wild-type and infected Fc γ RIIb-deficient mice ($p = 0.7043$), and between infected wild-type and infected Fc γ RIIb-deficient mice ($p = 0.9489$) in the spleen (D) Representative flow plots of the spleen of both wild-type and Fc γ RIIb-deficient post-challenge analyzed for IgM+ memory B cell population. Graphs on the right depict the OD reading of IgM and IgG antibody specific for *E. muris* in both infected and challenged wild-type and Fc γ RIIb-deficient mice. A one-way ANOVA with multiple comparisons determined there was no significant differences in IgM OD between infected wild-type and challenged wild-type mice ($p = 0.9153$), infected Fc γ RIIb-deficient or challenged Fc γ RIIb-deficient mice ($p = 0.4269$). A

one-way ANOVA with multiple comparisons determined there was a significant difference in IgG OD between infected wild-type and challenged wild-type ($p < 0.0001$), infected Fc γ R11b-deficient and challenged Fc γ R11b-deficient ($p = 0.0014$), and challenged wild-type and Fc γ R11b-deficient ($p = 0.0106$).

compared to controls (**Figure 4.2d**). Together these data suggest that the mechanism through which Fc γ RIIb regulates the IgM⁺ memory population happens during an early stage of development.

The IgM⁺ memory B cell population expands after peak of infection

One possible explanation for the expansion in the size of the IgM⁺ memory B cell population is an increase in the rate of proliferation. To address this question, infected wild-type and Fc γ RIIb-deficient mice were administered 0.8mg of BrdU, i.p., and maintained on BrdU water for five days. Analysis of BrdU incorporation among the IgM⁺ memory B cell population revealed no difference in the incorporation of BrdU between wild-type and Fc γ RIIb-deficient mice (**Figure 4.3a**). Since an increase in proliferation did not account for the expansion in the IgM⁺ memory B cell population, we next investigated the possibility that a change to the CD11c⁺ B220⁺ plasmablasts generated during early infection could account for the increase in the IgM⁺ memory B cell population at 30 days post-infection.

To address the above hypothesis, both wild-type and Fc γ RIIb-deficient mice were infected, and the spleen was examined on days 8, 10, and 12 post-infection, our data revealed that there was no increase in the early CD11c⁺ B220⁺ splenic plasmablasts (**Figure 4.3b**). We concluded that the increase in the IgM⁺ memory B cell population seen at day 30 post-infection was not due to a change in the early CD11c⁺ B220⁺ population.

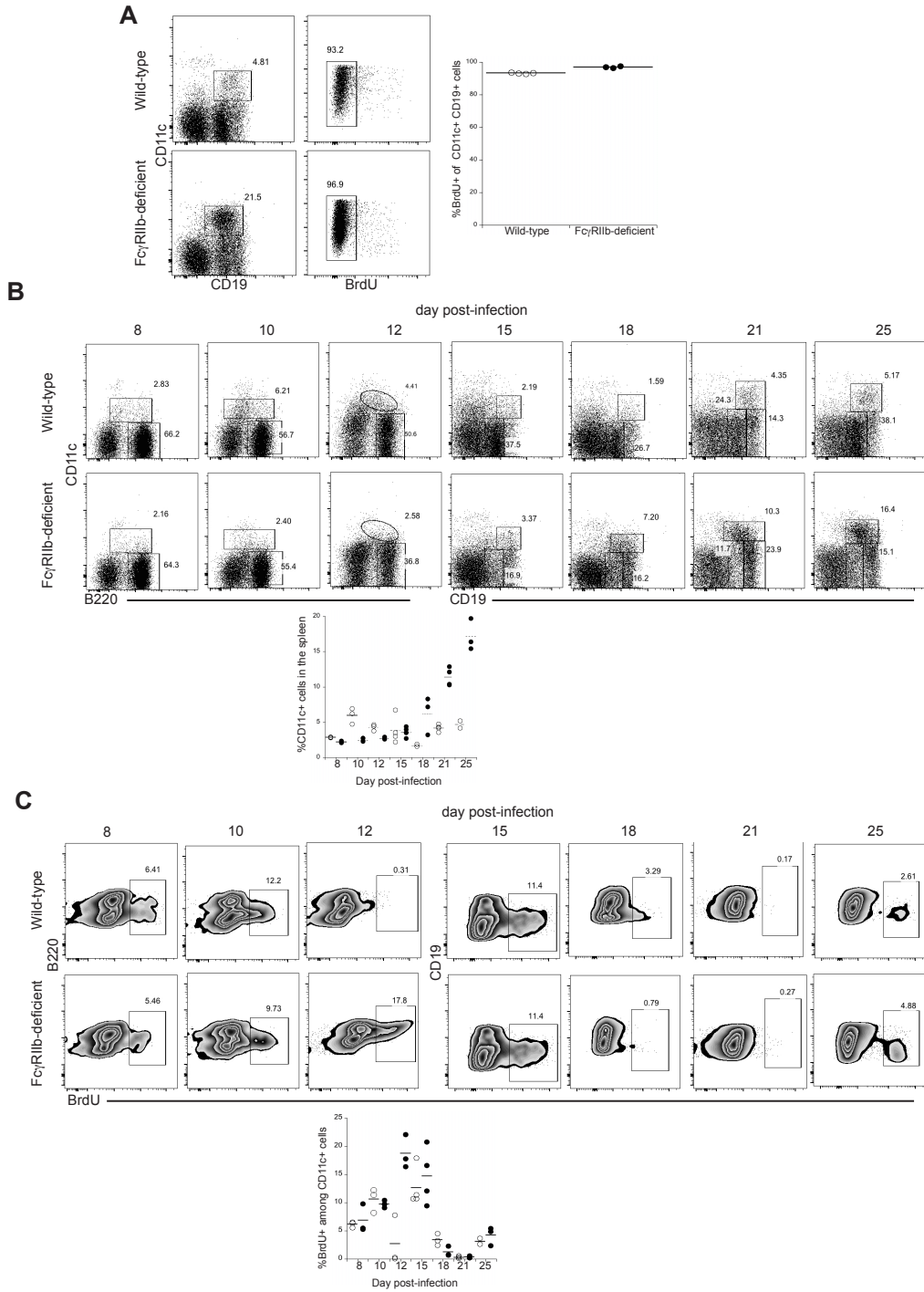


Figure 4.3 Expansion of IgM⁺ memory B cell happens after the peak of infection. (A) Thirty days post-infection both wild-type and FcγRIIb-deficient were administered BrdU i.p. followed by administration of BrdU water for five days after which BrdU incorporation was accessed. Representative flow cytometry plots depicting BrdU incorporation are shown (unpaired T-test $p < 0.0001$). Graph on right shows the percentage of BrdU-negative cells among the

CD11c+ CD19+ IgM memory B cell population in both wild-type and Fc γ R11b-deficient mice. **(B)** Representative flow cytometry plots demonstrate the CD11c+ B220+ early splenic plasmablasts and CD11c+ CD19+ of both wild-type and Fc γ R11b-deficient mice. A graph depicting the percentage of CD11c+ cells in the spleen on day 8, 10, 12, 15, 18, 21, and 25 post infection is shown below the dot plots. **(C)** Representative flow cytometry plots BrdU incorporation in CD11c+ B220+ or CD11c+ CD19+ cells after 24-hour pulse. A graph depicting the percentage of BrdU+ among CD11c+ cells in the spleen is shown below the zebra plots.

Next, we hypothesized that the expansion of the IgM⁺ memory B cells happens later during infection. To address this question, infected wild-type and Fc γ RIIb-deficient mice were examined for the generation of IgM⁺ memory B cells on day 15, 18, 21, and 25 post-infection. This analysis revealed an increase in the IgM⁺ memory B cell population in the Fc γ RIIb-deficient mice, beginning on day 18 post-infection that increased until the IgM⁺ memory B cell population was roughly three-fold the size of the population in wild-type mice by day 25 post-infection (**Figure 4.3b and c**). These data suggest that Fc γ RIIb plays a role in regulating the IgM⁺ memory B cell population.

Although there was no increase in proliferation in the IgM⁺ memory B cell population at day 30 post-infection, it was possible that there were changes in proliferation during the ontogeny of the IgM⁺ memory B cell population, when the expansion of the memory population was first observed. To address this hypothesis, both wild-type and Fc γ RIIb-deficient mice were administered 0.8 mg of BrdU 24 hours before sacrifice. Analysis of BrdU incorporation over the course of days 8-25 post-infection revealed there was no significant difference in the incorporation of BrdU between wild-type and Fc γ RIIb-deficient mice at any time point, except day 12 (**Figure 4.3d**). These data reveal that the mechanism by which Fc γ RIIb is regulating the IgM⁺ memory B cell population is not via increased proliferation.

Expansion of the IgM⁺ memory B cell population is B cell intrinsic and requires immune complexes

Next, we wanted to address whether the expansion of the IgM⁺ memory B cell population was B cell intrinsic or extrinsic. To test hypothesis, naïve Fc γ RIIb-deficient IgM^b-allotype B cells were transferred into wild-type (IgM^a-allotype) recipient mice; the recipient mice were infected following transfer. If the expansion of the population is B cell intrinsic, due to the loss of Fc γ RIIb on B cells, then the IgM^b-positive Fc γ RIIb-deficient donor cells will undergo characteristic high expansion into the IgM⁺ memory B cells. If the expansion of the population is B cell extrinsic, due to the loss of Fc γ RIIb on cells other than B cells, then the IgM^b-positive donor cells will differentiate into the IgM⁺ memory B cells in a similar manner as wild-type recipient cells. Analysis of the spleen 30 days post-infection, revealed that 45.9% of the IgM^b-positive donor B cells differentiated as IgM⁺ memory B cells, whereas only 14.6% of the recipient B cells were detected within the IgM memory cell population (**Figure 4.4a**). These data demonstrates that the expansion of the IgM⁺ memory B cell population in Fc γ RIIb-deficient mice is B cell intrinsic.

Because the expansion of the IgM⁺ memory B cell population was due to the loss of Fc γ RIIb on the B cells, we hypothesis that infection-specific immune complexes could play a role in the Fc γ RIIb-dependent regulation of the IgM⁺ memory B cells by inducing signaling likely through both the BCR and Fc γ RIIb

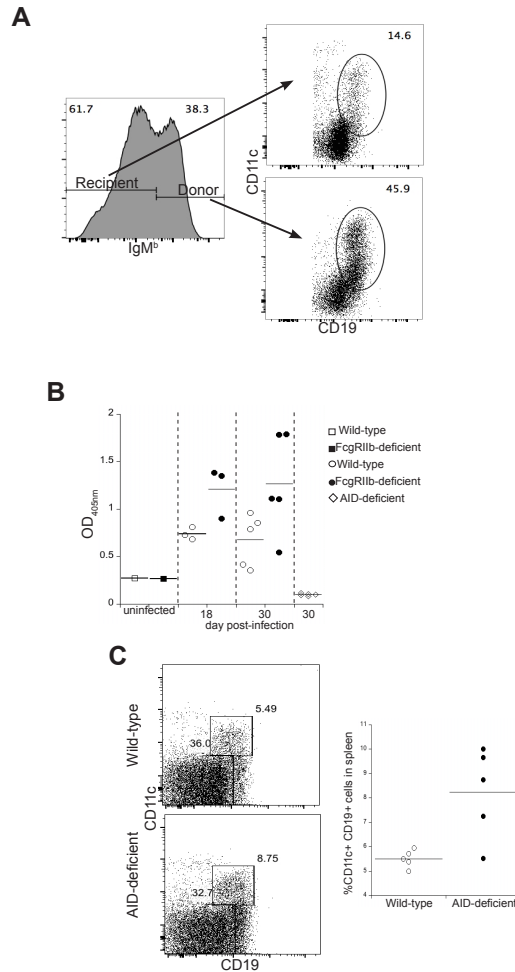


Figure 4.4 Expansion of IgM^b memory B cell population requires immune complexes. (A) Representative flow cytometry flow plots depicting IgM^b-positive donor B cells and IgM^b-negative recipient B cells that differentiated into IgM memory B cells. (B) ELISA for the detection of *E. muris* specific immune complexes in uninfected wild-type and FcγRIIb-deficient mice and infected wild-type, FcγRIIb-deficient, and AID-deficient mice. (C) Both wild-type and AID-deficient were infected and the spleens were analyzed on day 30 post-infection for the frequency of IgM^b memory B cells. A graph depicting the percentage of CD11c⁺ CD19⁺ B cells is on right (unpaired T-test p = 0.0121).

simultaneously. To determine if infection-specific immune complexes were present during infection,

serum was collected from infected wild-type, Fc γ R11b-deficient, and AID-deficient mice. The serum was analyzed using an ELISA designed to detect infection-specific immune complexes. Only wild-type and Fc γ R11b-deficient mice at days 18 and 30 post-infection tested positive for infection-specific immune complexes; however, immune complexes were detected at a higher levels in the serum from Fc γ R11b-deficient mice (**Figure 4.4b**). These data reveal that infection specific immune complexes are present during infection, and may play a role in the mechanism whereby Fc γ R11b regulates IgM⁺ memory B cell expansion or homeostasis.

Given the presence of immune complexes, we hypothesized that if infection-specific immune complexes are necessary for the mechanism by which Fc γ R11b works to control the IgM⁺ memory B cell population, then mice that are unable to produce immune complexes would also exhibit an expansion of the IgM⁺ memory B cell population. To test this hypothesis, we used AID-deficient mice, which lack a functional copy of AID making these mice unable to class switch and by extension unable to produce immune complexes, The AID-deficient mice were infected and spleens were analyzed for IgM⁺ memory B cells at 30 days post-infection. The infected AID-deficient mice exhibited a 2-fold expansion of the IgM⁺ memory B cell population relative to wild-type mice (**Figure 4.4c**). These

data suggest that immune complexes play an important role in the mechanism by which Fc γ RIIb regulates the IgM⁺ memory B cell population. However, the absence of immune complexes did not completely replicate the phenotype seen in Fc γ RIIb-deficient mice suggesting other mechanisms are also involved.

Fc γ RIIb-deficient IgM⁺ memory B cells lacked surface expression of FAS, BAFF-R, TACI, and CD40

Because we observed an expansion of the IgM⁺ memory B cell population in the Fc γ RIIb-deficient mice, we next monitored the cell surface marker phenotype of IgM⁺ memory B cell population to address whether other changes occurred in Fc γ RIIb-deficient mice. Using a panel of surface markers defined by Yates et al. 2013, both wild-type and Fc γ RIIb-deficient mice were stained for various surface markers on the IgM⁺ memory B cells (5). The IgM⁺ memory B cell population in the Fc γ RIIb-deficient mice expressed most of the markers at the same MFI seen in the wild-type IgM⁺ memory B cell population, including CD73, CD11b, PD-L2, CD86, and CD80 (**Figure 4.5 and Table 4.1**). However, the Fc γ RIIb-deficient IgM⁺ memory B cell population exhibited a lower expression of CD19, and very low to no expression of CXCR4, BAFF-R, TACI, CD40, and FAS (**Figure 4.5 and table 4.1**). The loss of expression in these additional receptors suggests that the Fc γ RIIb deficiency affects the expression of several other cell surface receptors and other factors. Exactly how the loss of these receptors affects the maintenance of the IgM⁺ memory population needs to be further explored.

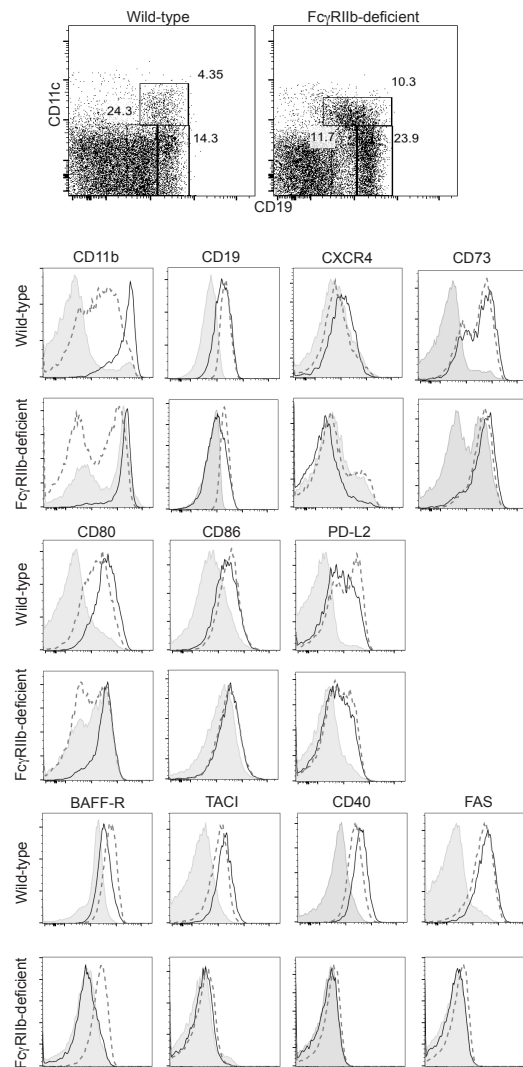


Figure 4.5 Cell surface phenotype of the Fc γ RIIb-deficient IgM+ memory B cells. Both infected wild-type and Fc γ RIIb-deficient mice were analyzed for the expression of the indicated markers. Representative flow cytometry plots depict the expression level of each surface marker for CD19+ canonical B cells (grey histograms), CD19^{hi} B cells (grey dotted lines), and the IgM+ memory B cell population (black lines).

Table 4.1 MFI values of surface markers examined on the IgM+ memory B cell population.

Surface markers	CD11c+ CD19+			CD19+			CD19hi		
	Wild-type	FcγRIIb-deficient	Fold Change	Wild-type	FcγRIIb-deficient	Fold Change	Wild-type	FcγRIIb-deficient	Fold Change
<i>CD11b</i>	25321	19812	1	20850	20380	0.98	25321	19812	0.78
<i>CD38</i>	25285	12340	0.49	7416	10609	1.43	19058	12733	0.67
<i>CD40</i>	4227	311	0.07	727	356	0.49	2726	388	0.14
<i>CXCR4</i>	1650	1746	1.06	1468	1746	1.19	1516	6823	4.50
<i>CD80</i>	5241	3539	0.68	668	1793	2.69	3692	3462	0.94
<i>CD86</i>	2983	4393	1.47	1115	3944	3.54	3568	4458	1.25
<i>BAFF-R</i>	4093	1009	0.25	1606	2075	1.29	6243	2577	0.41
<i>CD19</i>	8436	5641	0.67	2521	6533	2.59	9582	8704	0.91
<i>CD73</i>	7885	7205	0.91	6763	5206	0.77	6241	5562	0.89
<i>PD-L2</i>	1320	976	0.74	232	422	1.82	3063	2401	0.78
<i>FAS</i>	4061	240	0.06	338	307	0.91	3106	353	0.11
<i>ICOS-L</i>	340	251	0.74	343	298	0.87	369	295	0.80
<i>TACI</i>	2023	322	0.16	311	355	1.14	1311	370	0.28
<i>CD80</i>	5241	3539	0.68	668	1793	2.69	3692	3462	0.94
<i>CD86</i>	2983	4393	1.47	1115	3944	3.54	3568	4458	1.25

All MFI values are mean values of 4 wild-type and 4 FcγRIIb-deficient mice at day 21 post-infection.

As part of our studies of IgM⁺ memory B cells in wild-type and Fc γ RIIb-deficient mice, we also observed a population of CD19^{hi} CD11c-negative B cells. This population expressed several markers in common with the IgM⁺ memory B cell population, including CD80, CD86, CD73, PD-L2, FAS, TACI, BAFF-R, and CD40 (**Figure 4.5 and Table 4.1**). The CD19^{hi} CD11c-negative B cell population did not express CD11b, however, it is possible that the CD19^{hi} CD11c-negative B cells are another memory B cell population, or a precursor to the IgM⁺ memory B cell population, or IgM⁺ memory B cells that down regulated CD11c. Further study is required to determine the origin of this population.

The loss of FAS could explain the expansion of the IgM⁺ memory B cell population in Fc γ RIIb-deficient mice by leading to a decrease in apoptosis. Altogether these data suggest that infection-specific immune complexes engage Fc γ RIIb, which induces apoptosis through a FAS-dependent mechanism to regulate the homeostasis of the IgM⁺ memory B cell population.

Discussion

This work highlights a role for Fc γ RIIb in the regulation of IgM⁺ memory B cells during *E. muris* infection. Our studies suggest that the 2-fold increase in expression of Fc γ RIIb on the IgM⁺ memory B cells (**Figure 4.1**) is essential for IgM⁺ memory B cell homeostasis, which likely occurs by sensing infection-specific immune complexes through both the BCR and Fc γ RIIb, and triggering apoptosis. RNAseq analysis revealed not only an increase in Fc γ RIIb, but also multiple other genes including *Itgax* (CD11c), *Itgam* (CD11b), *CXCR3*, and *Tbx21* (T-bet). The IgM⁺ memory B cells that we described here bear a striking resemblance to CD11c⁺ CD21^{low} B cells described in other studies, including studies of HIV, Hepatitis C and B virus infections, SLE, and COVID; suggesting a commonality among these CD11c⁺ B cells (14, 16-21). Understanding the required for the maintenance of the IgM⁺ memory B cell population described here will aid in our understanding of the role these cells play in other diseases.

In the absence of Fc γ RIIb, there was a 3-fold increase in IgM⁺ memory B cells. This expansion was due to an increase in the number of IgM⁺ memory cells, and not a decrease in the number of canonical B cells, which suggests that Fc γ RIIb plays a specific role in IgM⁺ memory B cell regulation. When infected Fc γ RIIb-deficient mice were challenged, there wasn't a further expansion of the IgM⁺ memory B cells beyond what is seen in primary infection, which suggests that the

mechanism that by which Fc γ RIIb regulates the IgM⁺ memory B cells most likely happens during the ontogeny of the population.

We also addressed the signals involved in the expansion of the IgM⁺ memory B cells. One possible explanation for this expansion is an increase in the rate of proliferation, which was ruled out, because no differences were found in the incorporation of proliferation between Fc γ RIIb-deficient and wild-type IgM⁺ memory B cells at any time point examined, ranging from day 8 to day 30 post-infection. A second explanation for the expansion of the IgM⁺ memory B cell population is that infection-specific immune complexes signal through both Fc γ RIIb and BCR to regulate the homeostasis of the IgM⁺ memory B cell population. The BCR is likely required for the recognition of infection-specific immune complexes, which ensures that the mechanism is specific for IgM⁺ memory B cell population. The detection of the infection-specific immune complexes in infected mice, and the expansion the IgM⁺ memory B cell population in AID-deficient mice makes this explanation feasible.

We also addressed whether the loss of Fc γ RIIb affected the expression of other known surface markers, by examining markers in the IgM⁺ memory B cell population (5). Analysis of the surface markers in the Fc γ RIIb-deficient IgM⁺ memory B cell population revealed a decrease in the expression of FAS, BAFF-R, TACI, and CD40. BAFF-R and TACI are receptors for BAFF, which is a pro-survival signal for B cells. With the decrease in the expression of these receptors,

it may have been expected that there would be a decrease in the size of the IgM⁺ memory B cell population in FcγRIIb-deficient mice; instead, we observed the opposite. The decrease in the expression of BAFF-R and TACI is likely accompanied by a decrease in the binding of BAFF, and by extension, a decrease in pro-survival signals (22-24). The decrease in pro-survival signals from BAFF-R and TACI is likely balanced by lack of expression of FAS, a cell death receptor that strongly induces apoptosis. The induction of apoptosis through FcγRIIb has been demonstrated to both require FAS expression and the disruption of mitochondrial membrane potential (MMP) (25). The loss in FAS expression most likely leads to a reduction in apoptosis, accounting for the expansion in the IgM⁺ memory B cell population in the FcγRIIb-deficient mice. However, in addition to antagonizing BCR signaling, it has been demonstrated that Fas ligand (FAS-L) can be induced in response to microbial polysaccharide through FcγRIIb signaling (26). Therefore, it is formally possible that FcγRIIb and Fas:FASL signaling have other functions during infection besides the regulation of the IgM⁺ memory B cell population, such as aiding bacterial clearance, which is a known function of FASL.

Our studies highlight a novel pathway for the regulation of IgM⁺ memory B cells through the sensing of infection-specific immune complexes via FcγRIIb and BCR, which likely leads to the induction of apoptosis, thereby regulating the size of the IgM⁺ memory B cell population. Although we demonstrated that in the absence of FcγRIIb, the IgM⁺ memory B cell population expands to three times

what is seen in wild-type controls, the exact mechanism by which this expansion occurs will require further study.

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Chapter 5: Discussion

Class-switched high-affinity B cells have been well established as a component of long-term immunity (18, 74, 99). It was only recently acknowledged that unswitched IgM memory B cells played a role in maintenance of long-term immunity (40, 79, 80, 117). Prior to that time, it was thought that IgM B cells are derived early during infection and primarily exist to differentiate into short-lived ASCs (118). However, it has been demonstrated that IgM memory cells can re-enter germinal centers and undergo additional rounds of somatic mutation (23). This body of work explored the mechanisms involved in the generation and maintenance of two long-term IgM⁺ populations that contributed to long-term immunity. Our murine model of *E. muris* generated two robust long-term IgM⁺ populations, which made studying the mechanisms surrounding the role of IgM⁺ in immunological memory ideal (54, 55). Our laboratory has identified and characterized the two long-term IgM⁺ populations: a splenic IgM⁺ memory B cell population and a bone marrow CD138⁺ IgM⁺ ASC population (54, 55). While we had characterized these two populations in previous studies, the mechanisms involved in the ontogeny and maintenance of these long-term IgM populations have been addressed here.

Our murine *E. muris* infection model provides a scenario where long-term immunity is largely IgM-driven. Early infection is characterized by the appearance of a T cell independent (TI) CD11c⁺ B220⁺ T-bet⁺ plasmablast population responsible for the production of infection-specific IgM that controls bacterial load (53). After the peak of infection, two long-term IgM⁺ populations are established,

one in the bone marrow and the other in the spleen. The population that resides in the bone marrow are the CD138⁺ IgM⁺ T-bet⁺ ASCs, which are responsible for the long-term production of antigen-specific IgM (54). The bone marrow CD138⁺ IgM⁺ ASCs can be generated without a CD40L:CD40 interaction, or CD4⁺ T cell help. The IgM⁺ CD11c⁺ T-bet⁺ memory B cell population resides in the spleen after the peak of infection, and requires both CD4⁺ T cell help, and IL-21 for its generation (55). We identified a large population of T_{fh} cells present during infection that most likely provides the CD4⁺ T help the IgM⁺ memory B cells require (**see Chapter 3**). The similarities between these three B cell populations, and the timing of their appearance, suggest that the early CD11c⁺ B220⁺ plasmablasts are the precursors to either the bone marrow CD138⁺ IgM⁺ ASCs, or the IgM⁺ memory B cell population, or perhaps both.

The work described in the third chapter demonstrated that both the bone marrow CD138⁺ IgM⁺ ASCs and the IgM⁺ memory B cells were derived early during infection. This was shown using the AID-Cre ERT2 X EYFP F1 mouse model. The irreversible labeling of the cells expressing AID with eYFP identified two populations of eYFP-expressing B cells: the TI CD11c⁺ B220⁺ plasmablasts, and a CD11c-negative B220⁺ B cell population. We concluded that it is likely that the early CD11c⁺ B220⁺ plasmablasts most likely are the precursor to both the bone marrow CD138⁺ IgM⁺ ASCs and the IgM⁺ memory B cell population. However, we have not definitely proven that the CD11c⁺ B220⁺ plasmablasts contribute to both the long-term IgM⁺ populations or whether only the CD11c⁺

B220+ plasmablasts are precursors. It is formally possible, for example that a CD11c-negative B220+ B cell population contributes to one or both of the long-term IgM+ populations. Transfer of highly purified eYFP+ CD11c-negative B220+ or eYFP+ CD11c+ B220+ populations to mice matched for infection date, and examination of both the spleen and bone marrow at 30 days post-infection will delineate the contribution of each donor population to the two long-term IgM+ populations. Each of these scenarios presents a different interpretation of how long-term IgM+ populations are generated.

If only the CD11c+ B220+ plasmablasts contribute to both long-term IgM+ populations, it would be a novel finding because plasmablasts are generally accepted as terminally differentiated cells that do not have the ability to give rise to different B cell fates. To our knowledge, this would be the first example. If both CD11c-negative B220+ and CD11c+ B220+ populations contribute to both the long-term IgM+ populations, it would be novel for the same reasons stated above, as commitment to the plasmablast fate is often considered terminal. However, a contribution of the CD11c-negative B cell population is likely, because these B cells do not appear to be antibody-secreting cells, and therefore more likely to be able to differentiate into other effector B cell lineages. If the CD11c-negative B cell and CD11c+ B220+ plasmablast populations each contribute to one of the long-term IgM+ populations, this would also be an intriguing finding, because it would suggest that there are multiple pathways for the generation of long-term IgM+ memory cells. If this latter possibility is correct,

it would be highly likely that the CD11c+ B220+ plasmablast population is a precursor to the bone marrow CD138+ IgM+ ASCs, because both of these populations exhibit similar characteristics, such as their ability to produce infection-specific IgM, and their expression of certain cell surface markers, i.e. CD138 and IgM. If that holds true, then the CD11c-negative population is likely the precursor to the IgM+ memory B cell population. The contribution of the CD11c-negative B cells provides an area of research where questions regarding whether the population is infection-specific, maintained by active proliferation, or expresses any of the markers present on IgM+ memory B cell population can be explored.

The third chapter also focused on the mechanisms surrounding the expression of AID in our model. It was determined that the early TI CD11c+ B220+ plasmablast population required CD4+ T cell help, but not TLR signaling to express AID (**see chapter 3**). The IgM+ memory B cell population was also demonstrated to express AID past the resolution of the infection, as long as 200 days post-infection, in the absence of antigen challenge (**see chapter 3**). The continued expression of AID in the IgM+ memory population is not without precedence, as it was shown that continued expression of AID can lead to a decrease in polyreactivity and more antibody specificity over time (32). It is possible that the continued expression of AID in the IgM+ memory B cells has a similar function in our IgM+ memory B cell population. To address this hypothesis, assessing the Ig heavy chain of the IgM+ memory B cell population over the course of time for the

accumulation of mutations would aid in our understanding of the reason for the continued expression of AID. This process must be tightly regulated, because continued expression of AID has been demonstrated to lead to the development of lymphomas, although this does not occur in our experimental model (119).

This work also addressed the mechanisms responsible for the maintenance of the two long-term IgM⁺ populations. It was revealed that the bone marrow CD138⁺ IgM⁺ ASCs are maintained at least in part by proliferation, however there are likely other mechanisms used to maintain these cells. To further explore those mechanisms, sectioning bone would provide a more comprehensive look at the cells that are in close contact with the CD138⁺ IgM⁺ ASCs, and provide clues to what other mechanisms maintain this population. For the IgM⁺ memory B cell population, RNAseq analysis revealed a 2-fold increase in Fc γ RIIb, which was also confirmed at the protein level (**see chapter 4**). The IgM⁺ memory B cell population expanded 3-fold in the absence of Fc γ RIIb which suggested Fc γ RIIb was critical to the regulation of the IgM⁺ memory B cells. Further research revealed that Fc γ RIIb regulates IgM⁺ memory B cell population through the sensing of infection-specific immune complexes, which most likely triggers apoptosis, thereby regulating the expansion of the population. The mechanism that triggers apoptosis has not been determined, but it can be addressed using an apoptosis dye and a dye for determining mitochondrial membrane potential (MMP). Cells positive for the apoptosis dye, Annexin V, and negative for the MMP dye, TMRE, would be undergoing

apoptosis. Fc γ RIIb-deficient IgM⁺ memory B cells should have fewer cells positive for the apoptosis dye and be negative for the MMP dye indicating a lack of apoptosis. Such a finding would demonstrate that apoptosis is a critical process required for the regulation of the IgM⁺ memory B cell population.

The Fc γ RIIb-deficient studies identified a population of CD19^{hi} CD11c-negative cells that also expands in the absence of Fc γ RIIb. This CD19^{hi} CD11c-negative population shared expression of several surface markers with the IgM memory B cells, including CD73, CD86, CD80, FAS, and PD-L2 (**see chapter 4**). However, there was a lower expression of CD11b, BAFF-R, TACI, and CD40 (**see chapter 4**). The many similarities suggested relatedness between the two populations. These differences could be a defining characteristic of a yet-to-be identified B cell memory population generated during *E. muris* infection. It is also formally possible that the CD19^{hi} CD11c-negative population could be a precursor to the IgM memory population that has not fully differentiated. However, it remains to be determined whether the CD19^{hi} CD11c-negative population is specific to infection, which could be assessed with an ELISpot. Sorting both the CD19^{hi} CD11c-negative population and the IgM⁺ memory B cell population for RNAseq analysis will provide a more in-depth information about the differences and similarities between the two populations.

Altogether, the work described in this thesis highlights a novel pathway for the development of long-term IgM⁺ populations (**Figure 5.1**). During *E. muris*

infection, we propose that the long-term IgM⁺ populations are formed outside of the germinal center, in B cell follicles. In early infection, the TI CD11c⁺ B220⁺ T-bet⁺ plasmablasts population secretes antigen-specific IgM that controls the bacterial infection (53). The CD11c⁺ B220⁺ T-bet⁺ plasmablasts population likely differentiates into the CD138⁺ IgM⁺ T-bet⁺ BM ASCs, in the absence of CD4⁺ T cell help, or into the IgM⁺ T-bet⁺ IgM⁺ memory B cell population with CD4⁺ T cell help (likely from T_{fh} cells). Although, the AID-Cre eYFP model used to draw this conclusion also identified a population of CD11c-negative eYFP⁺ B cells that could potentially differentiate into either the IgM⁺ memory B cells, or the CD138⁺ IgM⁺ BM ASCs, or both. Once in the bone marrow, the CD138⁺ IgM⁺ BM ASCs were maintained in at least in part by active proliferation, where they continue to secrete IgM. The IgM⁺ memory B cell population does not proliferate, but we have described a mechanism of regulation involving infection-specific immune complexes engaging Fc γ RIIb, probably along with the BCR to induce apoptosis in a FAS-dependent manner. This model provides an alternative to the traditional model of germinal center-derived high-affinity class switched B cell memory, where low-affinity IgM B cell memory derived early during infection does not greatly contribute to the maintenance of long-term immunity.

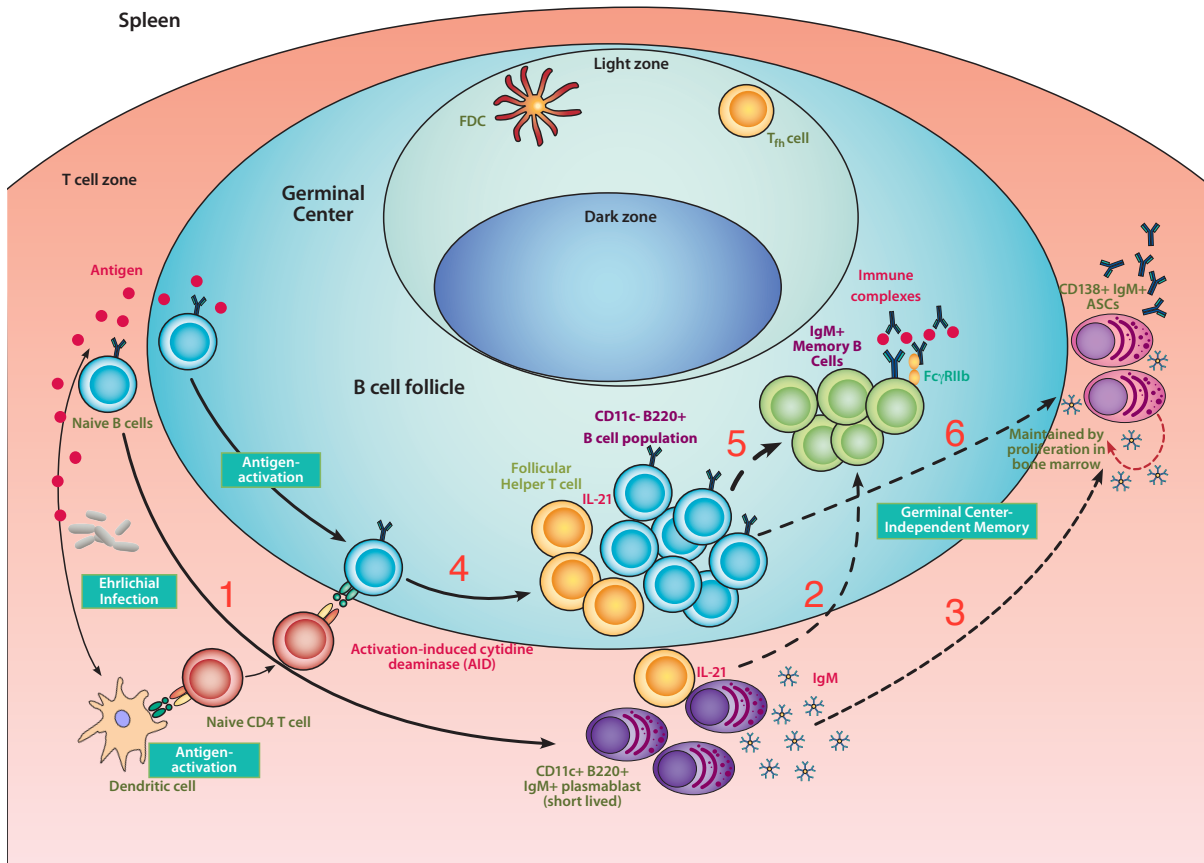


Figure 5.1 Model of long-term IgM generation and maintenance. It is proposed that, in response to *E. muris* infection, antigen stimulates naive B cells to become the CD11c⁺ B220⁺ IgM⁺ plasmablasts (1). The CD11c⁺ B220⁺ plasmablasts are possibly the precursors to the IgM⁺ memory B cell population (8), or the CD138⁺ IgM⁺ ASCs (3). Our studies did not exclude the possibility that another population of B cells that do not receive signals to differentiate into CD11c⁺ B220⁺ plasmablasts respond to *E. muris* infection (4). It is formally possible that CD11c⁻ B220⁺ cells can be stimulated to differentiate into either IgM⁺ memory B cells (5) or CD138⁺ IgM⁺ ASCs (6). We have also demonstrated that the bone marrow CD138⁺ IgM⁺ ASCs are maintained at least in part by active proliferation. Maintenance of the IgM⁺ memory B cell population is not dependent on proliferation, but rather Fc γ RIIb. Fc γ RIIb senses infection-specific immune complexes by triggering apoptosis through a FAS-dependent manner to regulate the homeostasis of the IgM⁺ memory B cells.

Recently, there have been reports of B cells co-expressing T-bet and CD11c (62, 89). However, studies of different chronic infections or diseases such as HIV, Hepatitis C, malaria, and SLE have documented reports of B cells that express CD11c and T-bet (40, 63, 64, 110). These reports describe B cell populations with a similar phenotype to the IgM⁺ memory B cell population described within this work. The function of CD11c⁺ T-bet⁺ B cells likely depends on the context in which the B cells were generated. For instance in the case of chronic infection, the generation of the IgM⁺ CD11c⁺ T-bet⁺ B cells is advantageous, providing antibodies that help control the infection. When the IgM⁺ CD11c⁺ T-bet⁺ memory B cells are present during autoimmune disorders, the population probably contributes to disease pathology by producing autoreactive antibodies. The phenotype of the lymphoma cells in Hairy cell Leukemia (HCL) is remarkably similar to the IgM⁺ CD11c⁺ T-bet⁺ memory B cells, others and we have described. These IgM⁺ CD11c⁺ T-bet⁺ memory B cells are also found in healthy humans suggesting that this population is more common than we may have initially thought.

Our model of *E. muris* has provided an opportunity to study the generation and maintenance of long-term IgM⁺ T-bet⁺ populations. These studies will not only further our understanding of the populations within *E. muris* infection, but it will assist in our understanding of how B cells with a similar phenotype in other models contribute to disease. The more we understand about IgM⁺ CD11c⁺ T-

bet+ B cell populations, the better equipped we will be in treating diseases wherein these populations arise.

Table I. CD11c+ B cell populations identified in various studies

Studies	Observations	References
Hepatitis C Virus/ Hepatitis B Virus	IgM ⁺ CD21 ^{low} memory B cells found in patients with MC	Charles 2011; Visentini 2012, 2015; Chang 2016
HIV	IgM ⁺ CD21 ^{low} Tissue-like memory found in viremic patients	Moir 2004, 2008
Health Humans	IgM ⁺ FcRH4 ⁺ non-germinal center memory B cells found in the tonsils	Ehrhardt 2008, 2010
Malaria	IgM ⁺ memory B cells with high affinity BCRs	Krishnamurty 2016
HCL and HCL-variant	IgM ⁺ B cells expressing CD11c	Wang 2011; Matutes 2015; Tabata 2016; Stetler-Stevenson 2011; Arons 2009; Cornet 2014
Health Humans	IgM ⁺ CD21 ^{low} FcRL5 ⁺ Tissue-like memory B cells	Li 2016
CVID	IgM ⁺ CD21 ^{low} B cells associated with splenomegaly and granulomatous	Warnatz 2002, Isnardi 2010; Rakhmanov 2009
ABCs	IgM ⁺ CD21 ^{low/-} B cells produce autoreactive Abs	Rubtsov 2011, 2013, 2015; Rubtsova 2015; Hao 2011
mCMV, LCMV, vaccinia	CD11c ⁺ T-bet ⁺ B cells respond to viral infections ¹	Rubtsova 2013
Human IgM memory	IgM ⁺ CD11c ⁺ memory cells found at higher frequencies than switched memory B cells	Vasquez 2015
SLE	IgM ^{low} CD21 ^{low} B cells a present in the periphery of patients	Wehr 2004

¹ Authors did not examine CD21 or IgM expression, however the B cell population shared other characteristics in common with the populations described here.

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