Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature: _____ _____

Caitlin Bohannon Date

B1-derived, long-lived IgM plasma cells persist in the spleen and confer long-term protection

By

Caitlin Darcy Bohannon
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science Immunology and Molecular Pathogenesis

_____

Joshy Jacob, Ph.D.
Advisor

_____ _____

Lawrence Boise, Ph.D.
Committee Member

David Steinhauer, Ph.D.
Committee Member

_____ _____

Max Cooper, M.D.
Committee Member

Ifor Williams, M.D., Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

B1-derived, long-lived IgM plasma cells persist in the spleen and confer long-term protection

By

Caitlin Darcy Bohannon
B.A., Agnes Scott College, 2008

Advisor: Joshy Jacob, Ph.D.

An abstract of A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Graduate Division of Biological and Biomedical Science
Immunology and Molecular Pathogenesis
2016

ABSTRACT

B1-derived, long-lived IgM plasma cells persist in the spleen and confer long-term protection

By Caitlin Darcy Bohannon

Long-lasting protection is a hallmark of the adaptive immune response, and long-lived plasma cells have the capacity to produce neutralizing antibodies for as long as a lifetime. These antigen-specific, long-lived IgG plasma cells develop following exposure to antigen, along with T cell help. This T cell interaction leads to the formation of germinal centers, where activated B cells undergo class-switching and affinity maturation to generate high-affinity, long-lived IgG plasma cells and memory B cells.

Here we describe an alternative pathway of plasma cell development that can occur in the absence of germinal centers, in which plasma cells are not class-switched but are somatically mutated. These activation induced cytidine deaminase-induced (AID) mutations are less frequent than in IgG plasma cells, and surprisingly they are observed within the antibody framework rather than within the antigen-binding regions (CDRs). The resulting IgM plasma cells are likely of mixed affinity, compared to the clonally selected, high-affinity IgG plasma cells.

The long-lived IgM plasma cells originate from the B1 compartment, and are distinct from natural IgM cells. Unlike long-lived IgG plasma cells, which are resident in the bone marrow, IgM plasma cells persist within the red pulp of the spleen. They develop early in the immune response - prior to germinal center formation and the development of long-lived IgG plasma cells. Further, IgM plasma cells alone are sufficient to protect against viral challenge, even in the absence of IgG and memory B and T cells. Finally, we discuss the evolution and functionality of this long-lived IgM pathway, and the potential significance of these IgM plasma cells to rational vaccine design.

B1-derived, long-lived IgM plasma cells persist in the spleen and confer long-term protection

By

Caitlin Darcy Bohannon
B.S., Agnes Scott College, 2008

Advisor: Joshy Jacob, Ph.D.

A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Graduate Division of Biological and Biomedical Science
Immunology and Molecular Pathogenesis
2016

Acknowledgments

I would like to thank all of my family and friends for supporting me throughout this endeavor - with special thanks to both my parents and my mentor, without whom none of this would be possible.

Table of Contents

Chapter 1: Introduction

The B cell repertoire

The humoral immune response is essential to host defense in its capacity to produce an almost endless diversity of antigen-specific, neutralizing antibodies. This diversity is achieved through a number of cellular processes over the course B cell development. Beginning at the earliest stage, hundreds of gene segments are encoded within each hematopoietic stem cell, which can then be assembled to create a near-infinite variety of specific antibodies[1-3]. In humans and in mice, each antibody is comprised of two heavy chains and two light chains, with both a constant and variable region. The constant region gene segment encodes the isotype of the antibody – C_μ, C_δ, C_γ, C_α, or C_ε – while the variable region encodes antigen-specificity. The variable region itself is composed of a variable (V) segment, diversity (D) segment, and joining (J) segment in the heavy chain, with the D gene segment absent from the κ and λ light chains[1-4]. The junction between the three segments forms the CDR3 region of the antibody gene, which will be discussed further as we detail somatic hypermutation. The heavy chain is reassembled first, at the pre-B stage. At this stage, the heavy μ chain complexes with Vpre-B within the B cell until the light chain is assembled[5]. Following light chain assembly, a unique, somatically rearranged IgM molecule is expressed on the surface of the immature B cell, which can then clonally expand[2,5,6]. This IgM expression is critical to the development of B cells, and deficiencies in C_μ can severely impair immune development, as is observed in μMT mice which lack B cells and are prone to infection[7].

Further diversity is induced by the imprecise joining of the V, D, and J segments and the introduction of P and N nucleotides into the sequence. Recombination events occur at the ends of each gene segment, which are capped by recombination signal sequences (RSS), with the help of RAG1 and RAG2 proteins. These proteins bind the RSS regions to form a DNA hairpin, which can then be cleaved, deleting any gene segments between the two that have been joined together[8]. This process is critical to immune development, and deficiencies in these proteins can lead to severe combined immunodeficiency (SCID) in humans and mice, with RAG-/- mice lacking B and T lymphocytes entirely[9,10]. Following cleavage of the DNA hairpin, palindromic, 'P', nucleotides are added to the cleaved ends by the DNA repair machinery[11]. Terminal deoxynucleotidyl transferase (TdT) further adds random, 'N', nucleotides[12]. The nonhomologous ends are joined and any gaps are filled by the DNA repair machinery. This process is imperfect and creates junctional diversity within the CDR3, resulting in a unique B cell clone[13-17].

Negative selection

The recognition of foreign antigen is critical to the development of immature B cells. Those that react to self-antigen are either clonally deleted or edited in the process of negative selection. This occurs in the bone marrow, where self-antigen is presented to immature B cells expressing surface IgM. Those that bind self-antigen strongly are clonally deleted - whereby apoptosis is induced in these cells. Those that bind weakly lose their IgM expression and become anergic. Other B cells are able to edit their B cell receptor to avoid negative selection[18,19]. Naïve

IgM B cells that survive the process of negative selection can then leave the bone marrow to be activated[6,18,19]. Some autoreactive cells do escape this process, but they are very rarely activated and are often of low affinity. Autoimmunity results when mature autoreactive cells are activated by self-antigen.

B cell activation and differentiation

B cell activation occurs in the secondary lymphoid organs – the spleen and the lymph nodes. Here the B cell may encounter its cognate antigen, with or without the help of T follicular helper cells. Once activated, B cells differentiate through one of two pathways[20]. The first does not require T cell help, and results in the rapid formation of short-lived, extrafollicular plasma cells. Blimp-1 is upregulated by Irf4 in these cells, leading to early differentiation into short-lived plasma cells[21,22]. These cells continuously secrete low affinity antibody within the spleen or lymph nodes, but typically do not survive for more than a couple weeks. The second pathway is initiated when activated B cells interact with cognate T helper cells, leading to the formation of the germinal center. Here, these B cells undergo rapid proliferation, affinity maturation, and differentiation into either long-lived plasma cells or memory B cells. Long-lived plasma cells, unlike short-lived plasma cells, typically secrete high affinity antibody and can survive for years or even a lifetime[23]. Memory cells do not secrete antibody, but respond to repeated antigen exposure by rapid proliferation and differentiation into high-affinity antibody-secreting plasma cells. Long-lived plasma cells are terminally differentiated B cells, and do not respond to antigen (Figure 1)[20,22,24-26].

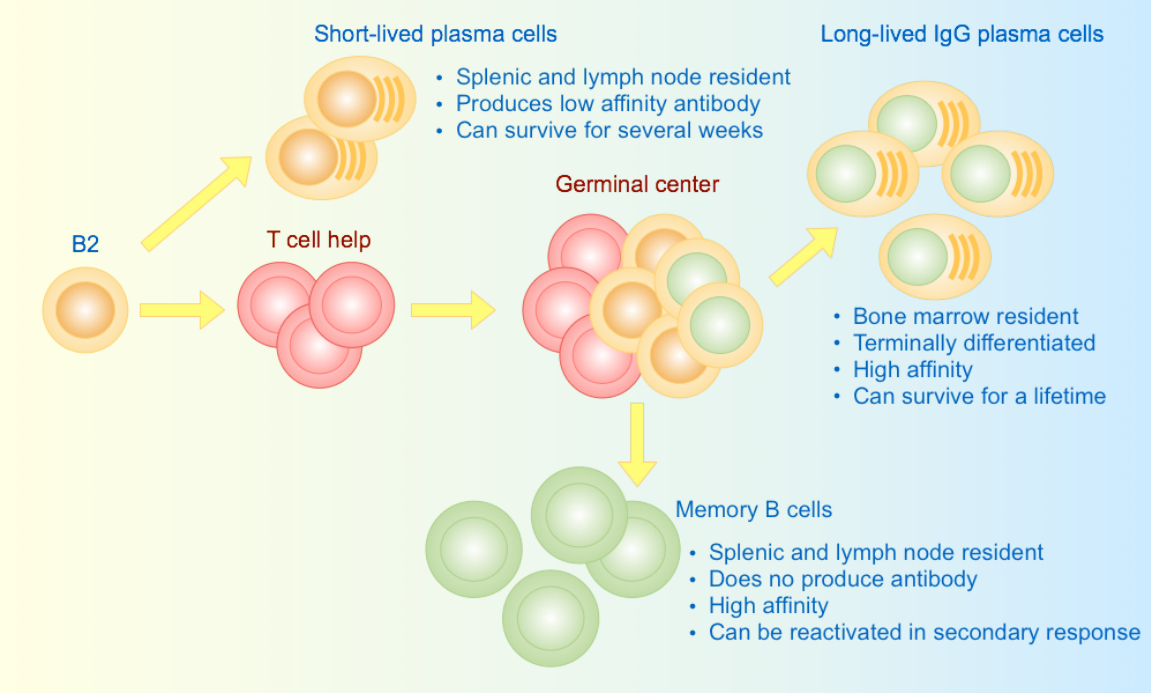


Figure 1. Model of B cell differentiation

Long-lived plasma cells, rather than memory B cells, are the key source of protective antibody[20,26]. Tedder and colleagues have shown that memory B cell depletion, via anti-CD20 antibody, does not affect serum Ig titers or long-lived plasma cell survival. Conversely, the depletion of long-lived plasma cells by disruption of adhesion molecules within the survival niche led to significantly decreased serum Ig. However, Ig levels were restored after 3 months, likely due to memory B cells differentiating into long-lived plasma cells. Cohorts did not recover when both long-lived plasma cells and memory B cells were simultaneously depleted[26].

Germinal center formation

Germinal center formation follows activation of mature B cells by their cognate T helper cell. These activated B cells rapidly proliferate within the B cell follicle. Proliferating B cells, or centroblasts, populate the dark zone of the germinal center, nearest the T cell zone. Gradually, around day 4, they differentiate into centrocytes, which proliferate less as they move away from T cell help into the light zone Germinal centers reach their peak around 7-10 days after initiation, and class-switched, high-affinity plasma cells and memory B cells begin exiting the germinal centers around this time[22].

Bcl-6 upregulation in B cells is also critical to germinal center formation, and Bcl-6 deficient mice are unable to develop functional germinal centers, or class-switched memory populations[27]. Germinal center-derived plasma cells do not differentiate as rapidly as extrafollicular plasma cells due to Bcl-6 upregulation by Irf8, which temporarily suppresses Blimp-1 expression. Conversely, Blimp-1 expression later represses Bcl-6, as germinal center B cells differentiate into plasma cells[6,28-32]. These transcription factors are key to regulating B cell fate.

The CD40:CD40L interaction between B and T cells is also key to initiating germinal center formation, and disruption of this interaction by anti-CD40L antibody leads to depletion of class-switched plasma cells and memory B cells, and a significant reduction in IgG titers[33,34]. Interestingly, work in our lab has shown that disruption of the germinal center does not affect long-lived IgM plasma cells, while others have similarly shown that germinal center is not required for the formation of unswitched memory B cells[35]. However, CD40L

deficiency in humans leads to hyper-IgM syndrome, and those patients with this disorder are prone to recurring infections[36]. CD40L is also essential to the induction of class switch recombination (CSR) and somatic hypermutation (SHM)[36,37].

Class switch recombination

Class-switching directs antibody synthesis from IgM and IgD on the B cell surface to secreted IgG, IgA, and IgE isotypes[38,39]. This process occurs through activation-induced cytidine deanimase (AID), which is expressed in activated, germinal center B cells[40]. AID, with the help of uracil-DNA glycosylase (UNG1) and apurinic endonuclease (APE1), creates an abasic site, or nick, within the switch regions of the DNA. This allows for the replacement of C_μ with an upstream constant region of a different isotype.[41,42] This was demonstrated rather elegantly through the induction of AID expression in fibroblasts with artificial switch regions, which was sufficient to drive CSR at a level comparable to germinal center B cells[43]. Recombination deletes the intervening DNA, so this process is irreversible. CSR does not change the specificity of the antibody[44]. Deficiencies in AID or UNG1, like those in CD40L, can also result in hyper-IgM syndrome, and an increased susceptibility to pathogens[45,46].

Following SHM and clonal selection, the resulting IgG, IgA, and IgE are higher affinity than IgM, and their smaller size allows them to diffuse to body tissues. High affinity IgG is the primary isotype found circulating in the blood and extracellular fluid shortly after the initiation of the immune response, and dominates the humoral memory response. IgG is also the most stable isotype,

with a half life of 4-8 days in mice and 20 days in humans, while IgM antibodies only have a half life of 2 days or 5-8 days, in mice and humans respectively[47,48]. IgG is also critical for induction of antibody-dependent cellular cytotoxicity (ADCC)[47]. However, some IgM is still present during this later response. IgM, though lower affinity, can multiplex to form a pentamer or hexamer of high avidity, effectively neutralizing many pathogens[49-51]. The high valency allows IgM to efficiently bind antigen with repeating epitopes, such as a viral particles or bacterial surface carbohydrates and phospholipids[51]. The pentameric structure is also key in activating the complement system, and in the clearance of these pathogens[49]. In addition to these roles, IgM has also been shown to enhance IgG production, making it essential to both the pre- and post-germinal center immune response[52-54].

Somatic hypermutation, clonal selection, and affinity maturation

Somatic hypermutation, clonal selection, and affinity maturation typically take place in the germinal centers[34,55,56]. SHM is the process by which point mutations are introduced within the immunoglobulin variable gene, further diversifying the antibody repertoire. Like CSR, SHM occurs through the actions of AID. AID expression alone is able to induce SHM in late stage B cell hybridomas, which would not normally undergo SHM, demonstrating that AID is sufficient to drive SHM[57]. Like in CSR, AID deaminates cytosine residues - leaving a uracil in its in place, which is then excised by UNG1 and APE1 to form an abasic site. Unlike CSR, this process occurs within the variable region, rather than at the switch

regions, and the DNA nicks are typically single-stranded, rather than double-stranded breaks[38]. The mechanisms of DNA repair also differs between the two processes. Mismatch-repair enzymes have been shown to play a role in both CSR and SHM, but error-prone polymerases, including Pol μ, Pol η, ζ or Pol ι, are necessary for SHM[38,44].

Though the process of SHM is error-prone, it is not entirely random. AID specifically targets cytosine residues, creating a G:U mismatch, and DNA repair mechanisms preferentially repair this site with thymine (T) in place of a cytosine (C), and an adenosine (A) in place of guanine (G), or vice versa. These C↔T and G↔A transitions are much more frequent than mutations involving a G, or any other change, commonly referred to as transversions. Transitions occur at least twice as often as transversions, and much more frequently than would be expected if the process of mutation were random[3,58]. This increased frequency of transitions is characteristic of AID induced mutations[3]. Position within the variable gene also effects the distribution of mutations, preferentially targeting WRC (W=A/T, R=A/G) sites, creating 'hotspots' and 'coldspots' independent from selection[3,59].

There is some evidence of mutations accumulating in B cells outside of the germinal centers, particularly in autoimmune hosts. Schlomchik and colleagues have demonstrated that autoreactive B cells bordering the T cell zone in the red pulp had undergone SHM through microdissection[60]. They additionally demonstrated that SHM could occur in plasmablasts in response to *Salmonella* infection, even without germinal center formation[61]. Similarly, our own lab has recently shown evidence of low-level mutations in long-lived IgM plasma cells

occurring even when germinal center formation is inhibited by anti-CD40L antibody treatment.

The accumulation of point mutations creates a pool of B cells clones of varying affinity that must then undergo clonal selection. Clonal selection, together with somatic hypermutation, leads to affinity maturation in germinal center B cells. This selection process is antigen-driven, and typically selects for high affinity B cell clones – those with an increased frequency of replacement mutations at antigen-binding sites. Replacement mutations, unlike silent mutations, alter the animo acid structure of the antibody. These are typically found in the complimentary-determining regions (CDRs), which bind antigen, and can often be deleterious when they occur in the framework regions (FRWs), which support the structure of the antibody. A high frequency of replacement mutations in the CDRs, and fewer in the frameworks, is commonly seen in memory B cells and bone marrow-resident IgG plasma cells, which are of high affinity (Figure 2)[3,62]. However, recent evidence has shown that clones of varying affinity may also be selected in response to complex antigens such as *Bacillus anthracis* and the hemagglutinin of influenza[63]. Evidence from our own lab additionally suggests that IgM plasma cells, though mutated, do not undergo antigen-driven selection for high affinity clones.

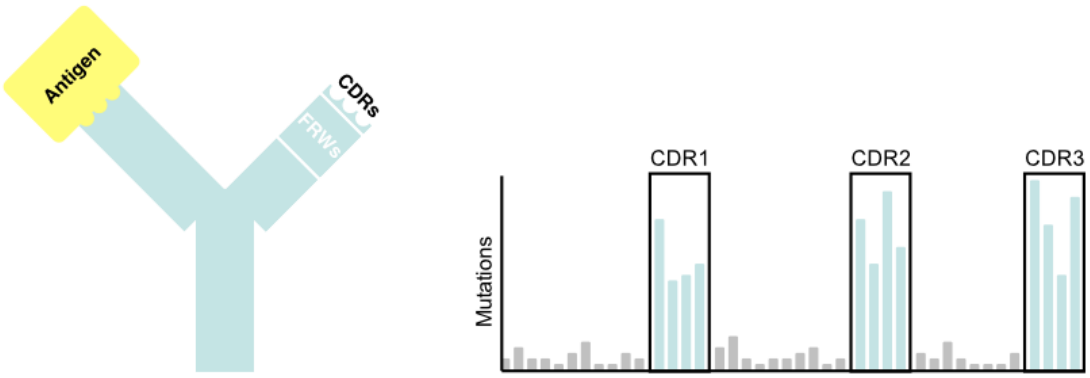


Figure 2. High frequency of mutations in CDRs following antigen-selection

B cell trafficking and survival

The chemokine CXCL12, formerly known as stromal-derived factor 1 (SDF-1), plays a critical role in the migration and retention of both B cells and plasma cells. Early B cell progenitors and developing B cells express the CXCL12 receptor, CXCR4[64]. This expression is critical to the retention of these cells in the bone marrow, where CXCL12 is constitutively produced by stromal cells, sometimes referred to as nurse cells[64,65].

Several adhesion molecules present on stromal cells are also critical to the homing and retention of both immature B cells and long-lived plasma cells within the bone marrow. Bone marrow epithelial cells expressing VCMA-1, LFA-1, and E-selectin bind to VLA-4, ICAM-1, and polysaccharide chains, respectively, on the surface of B cells – though VCAM-1 has been shown to be the key adhesion molecule. Loss of this adhesion in conditional VCAM-1 knock out mice results in

the depletion of both immature B cells and plasma cell populations in the bone marrow [6,66].

After exiting the bone marrow, naïve B cells initially home to secondary lymphoid tissues through an upregulation of CCR7 and CXCR5 chemokine receptors on their surface[64]. CCR7 expression drives activated B cells towards the T cell zone of the spleen and lymph nodes. This migration is in response to the chemokines CCL19 (ELC) and CCL21 (SLC), which are produced by stromal cells in the secondary lymphoid organs. Here, naïve B cells interact with helper T cells to initiate germinal center formation[67]. Expression of CXCR5 then drives B cells further into the B cell follicle in response to CXCL13 (BLC), produced by stromal cells as well as follicular dendritic cells[28,68,69].

CCR7 and CXCR5 are then downregulated during the differentiation into antibody-secreting cells (ASCs) and memory populations[64]. CXCL12 again becomes critical to localization, as activated B cells upregulate CXCR4 expression as they develop into plasmablasts[64,67,70]. It is suggested that CXCL12 may play a role in the egress of plasmablasts out of the B cell follicles and into the red pulp of the spleen, or into the medullary cords of the lymph nodes, where CXCL12 is found to be highly expressed as in the bone marrow. From there, plasmablasts can then migrate to CXCL12-expressing niches within the bone marrow. These CXCL12 microenvironments of both the bone marrow and secondary lymphoid organs are also sites of plasma cell retention, whether short or long-lived[64]. Similarly, memory B cells express CXCR4 and are also responsive to CXCL12, resulting in their localization within the red pulp of the spleen[71]. CXCR7, which

binds both CXCL12 and CXCL11 and is expressed at late stages of B cell maturation, may also help fine tune responses of CXCR4 to CXCL12[72].

Other chemokines and their cognate receptors also play an important role in the migration of plasmablasts specifically to the bone marrow. Two such receptors are CCR1 and CCR2, which respond to RANTES (CCL5) and MIP-1 (CCL4) or to MCP-1 (CCL2), respectively, which are expressed by bone marrow stromal cells. CCR2 is found to be upregulated on plasmblasts, while CCR1 is found on mature, bone-marrow resident plasma cells[73-75]. Our lab has additionally found CCR2 to be upregulated on IgG, but not IgM, long-lived plasma cells.

Antibody-secreting cells also have the capacity localize to sites of tissue inflammation. This is accomplished through expression of the CXCR3 receptor, induced by IFNγ, which responds to the inflammatory cytokines CXCL9, CXCL10, and CXCL11[70,76]. This tissue homing has been shown to be particularly critical for controlling persistent viral infections in the central nervous system[77-82]. It may also contribute to the pathology of autoimmune inflammation, such as in Rheumatoid Arthritis, where CXCR3-expressing antibody-secreting cells are recruited by CXCL9-expressing synovial fibroblasts, or in the inflamed kidneys of NZB/W mice[83,84]. Additionally, antibody-secreting cells have even been shown to differentiate into plasmablasts within the inflamed tissues, and can remain localized to these sites even after the inflammation is resolved[79].

Survival microenvironments

Once plasmablasts terminally differentiate into plasma cells within the survival microenvironment, they are thought to lose their migratory capacity[64,85]. However, CXCL12 still plays a key role in their differentiation and survival[6,70]. Long-lived plasma cells are primarily thought to localize to survival niches within the bone marrow, though our group and others have shown that long-lived plasma cells also persist within the splenic niche in both humans and mice[20,24,86]. CXCL12 is constitutively expressed by stromal cells in both organs, supporting long-term survival of these plasma cells[64,87].

IL-6R is also critical to the differentiation, survival, and IgG production of plasma cells. IL-6 is secreted by stromal cells, T cells, and macrophages in both the spleen and bone marrow, as well osteoblasts specifically within the bone marrow microenvironment[6,88-90]. There is evidence that plasma cells actually manipulate the stromal microenvironment to better support longevity, as stromal cells cultured with plasma cells are induced to express IL-6, though stromal cells do not express IL-6 in their absence[90]. IL-6 induces IL-21 secretion by T cells, driving B cell differentiation and antibody production through the STAT3 pathway[88,89]. This secreted IL-6 can also interact directly with IL-6R on the plasma cell surface to support plasma cell survival, though there is conflicting evidence whether IL-6 is required for plasma cell maintenace[6,90,91]. IL-6-/- deficient mice have delayed induction of plasma cells, but no reduction in long-term antigen-specific antibody titers[91]. There are many potentially redundant factors that contribute to the survival of long-lived plasma cells.

In addition to CXCL12 and IL-6, B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) also play a key role in the survival of class-switched plasma cells[92,93]. Both of these cytokines bind to the receptor B cell BCMA (B cell maturation antigen), which is highly expressed on plasma cell population, to sustain longevity. Bone marrow plasma cell survival is critically impaired in BCMA-/- deficient mice[94]. BAFF and APRIL do not seem to be required for memory B cell survival, however[92,95]. TNFα and CD44 signaling also contribute to sustaining long-lived plasma cells[6,91].

Some additional differences can be found between the splenic and bone marrow microenvironments. Osteoblasts and osteoclasts within the bone marrow may further shape the organization of the plasma cell survival niche and provide key survival signals such as CXCL12[64,96]. The splenic microenvironment additionally produces IL-5, as well as CD54 in humans, and consequently an upregulation of IL-5R and CD11a, respectively, has been found in splenic resident plasma cells[87,97]. Additionally, though plasma cells in the bone marrow are usually found in close proximity to stromal cells, in what often appears to be a one-on-one interaction, our *in situ* observations of the spleen reveal that plasma cells are clustered more closely with one another[6,98]. It is not currently known whether or to what extent adhesion molecules affect the retention of plasma cells within the spleen.

B1 B cells

There are several distinct lineages of B cells, each with a unique role within the immune response. This review has primarily focused on the development and

differentiation of follicular B2 cells, which are the largest B cell subset and the main effectors of the adaptive humoral response. B1 B cells, conversely, are a much smaller subset, and the key source of natural antibodies and innate-like B cell responses. B1 B cells, further subdivided into B1a and B1b cells based on their CD5 expression, are typically found within the peritoneum and pleural cavities, though they can also be found within the spleen and bone marrow at low frequencies. Unlike follicular B2 cells, they do not require T cell help, nor do the require B cell stimulation. Instead they respond to innate signals, even in the absence of antigen, in order to produce broadly specific, natural antibodies that are maintained in the steady state. These natural antibodies are essential to the early immune response, and are also key in maintaining tissue homeostasis[99]. Additional B cell subsets, including marginal zone B cells and regulatory B cells, have been reviewed elsewhere[100,101].

B1 cell development and selection

B1 B cells are the first B cells generated in both mice and humans, before B2 B cell development. The majority of these B1 cells arise prior to or shortly following birth, efficiently generated from the fetal liver tissue or the splanchopluera region in the case of B1a (CD5+) B cells[99]. These fetal B1 cells are primarily maintained through self-renewal in the adult organism[102]. However, both B1b (CD5-) and to a lesser extent B1a (CD5+), can also develop *de novo* from adult bone marrow precursors under certain conditions[103,104].

The antibody repertoire of fetal B1 cells is more limited than that of adult derived B2 B cells. Fetal B1 cells employ a restricted number of variable heavy

chains compared to B2 cells[105,106]. They also contain fewer P and N nucleotide substitutions, consistent with the fact that TdT is expressed only after birth, further limiting their junctional diversity[107,108]. However, adult-derived B1b cells and some splenic B1a cells are not restricted in their heavy chain usages, and do have non-templated nucleotide substitutions in the CDR3 junctions[109,110].

The poly-reactive antibody repertoire of B1 cells is generated in the absence of antigen, unlike B2 cells. It is also is enriched for low-affinity, self-reactive B cells[99]. These self-reactive B cells do not appear to undergo negative selection during the clonal selection process, and some evidence suggest they may in fact be positively selected for[111]. The resulting natural antibody repertoire is very different from the foreign antigen-specific, high-affinity antibodies of B2 cells.

Because of these limitations, the infant immune system is generally considered immature in comparison to that of adults. The infant B cell compartment is primarily composed of fetal-derived B1a cells, which secrete natural IgM. Any IgG is transferred by the mother, via the placenta or through breastfeeding, which can aid in protection but also may create a barrier to infant immune response. It is still heavily debated whether infants, and fetal-derived B1 cells, are able to generate long-lasting plasma cells in response to antigen[112,113].

B1 differentiation, function, and survival

Natural antibody secretion occurs spontaneously or through innate signaling pathways. Activation is BCR- and T cell-independent, and is instead stimulated through IL-5, IL-10, or TLR ligands such as LPS. Activated B cells upregulate

Blimp-1, like B2 cells, to differentiate into antibody secreting cells, though they do not undergo clonal expansion. These ASCs then migrate from the peritoneal and pleural cavities to the spleen, lymph nodes, and lamina propria, where they secrete high titers of natural antibody[99,114]. CXCL13 and IL-5, produced in the peritoneal cavity as well as the spleen, are critical to B1 localization, survival, and antibody production[97,114]. Unlike B2 cells, B1 B cells do not require BAFF or APRIL for their survival[95,115]. These natural antibodies are critical to clearing bacterial infections[51,116]. They have also been shown to be important for viral clearance[117].

B1 cells can undergo class-switching, preferentially switching to IgA secretion though they are able to switch to all immunoglobulin isotypes[99]. There is also some evidence that indicates that B1 cells may also undergo SHM, though at a lower frequency than in follicular B2 cells[51,118]. There is even some evidence that B1 cells, particularly B1b cells, can produce antigen-specific antibody in response to stimulation. This so-called immune IgM is distinct from natural IgM, and is a much smaller percentage of total circulating IgM. But studies of B1a and B1b responses to *S. pneumonia* have suggested that B1b cells contribute long-lasting, antigen-specific antibody to the adaptive immune response[119]. IgM from B1b cells in AID-deficient mice was also sufficient to protect against reinfection by *B. hermsii*[116]. Our own lab has further found similar evidence of long-lived IgM plasma cells, generated from B1b precursors, in response to viral influenza infection.

The evolution of humoral immunity

B cells were first discovered in the bursa of Fabricius of chickens by Max Cooper, in the lab of Robert Goode. They demonstrated that irradiated chickens, with no bursae, were unable to produce antibody, and identified B cells as the source of these immunologlobulins[120]. Antibodies, and with them the adaptive immune response, are found across all species of jawed vertebrates - though jawless vertebrates have recently been shown to possess a novel system of variable lymphocyte receptors (VLRs) which can be uniquely rearranged to target pathogens[121]. IgM appears as far back as in cartilaginous fish, and is found in all jawed vertebrates, with IgG evolving at a much later timepoint. Thus far, IgG has only been found present in mammals and is evidence of a second major event in FcR evolution – the first having occurred in bony fish and allowing for the process of class-switching. Distinct isotype classes with unique effector functions have added increasing complexity to the immune system as these animals evolved[122].

Somatic diversification processes similarly trace their roots back as far as cartilaginous fish. These primitive vertebrates have been shown to express RAG proteins and undergo a cluster-type of heavy chain rearrangement[123]. Similarly, AID and its homologs have co-evolved with their target immunoglobulins to add increasing diversity to the antibody repertoire[124]. In mice and humans, it does this through the process of somatic hypermutation, and there is evidence of SHM occurring to a limited degree in sharks and Xenopus (frogs) as well[3,125-127]. Sharks, however, have limited antibody specificity and lower affinity to many antigens[123]. In chickens, sheep, and rabbits, there is limited VDJ rearrangement

and much of the repertoire diversity is created through a process known as gene conversion. During this process, portions of the variable gene are replaced by homologous recombination, allowing for the creation of a diverse array of variable genes with less risk of disrupting their structure[128]. In chickens, there is only a single full-length variable gene, with many pseudogenes that can donate to the variable chain through homologous recombination[129].

B cells in clinical disease

In healthy hosts, B cells and antibody secretion are critical to neutralization and clearance of pathogens. However, aberrant regulation of B cell development, growth, or self-tolerance can result in immunodeficiencies, B cell cancers, or autoimmune disorders, respectively.

B cell immunodeficiencies

B cells are critical to host protection, and deficiencies at any stage can increase disease susceptibility. As previously discussed, deficiencies in RAG proteins, critical to early B cell development and to formation of antibodies, can result in the loss of both B and T cells and cause severe combined immunodeficiency (SCID)[9]. The loss of only B cells is referred to as Common Variable Immunodeficiency (CVID), and is the result of deleterious mutations in key B cell surface receptors such as CD19, 20, TACI, or BAFFR[130]. X-linked agammaglobulinemia is caused by a deficiency in Bruton's tyrosine kinase (Btk), critical to B cell maturation, and B cell development and antibody production are severely reduced in these patients[131]. Agammaglobulinemia can also result from

defects in the μ heavy chain (*IGHM*) gene, alongside additional complications[132]. Following B cell maturation, any impairment in the expression of CD40L, AID, or UNG1 can lead to hyper-IgM syndrome[36,46,133]. Certain immunoglobulin isotypes may also be absent in some patients, such as in Selective immunoglobulin M (IgM) deficiency or Selective immunoglobulin A (IgA) deficiency[134,135]. These are collectively referred to as primary immunodeficiencies, where an essentially function of the immune response is either missing or impaired. Recurrent infections are common in these patients, particularly bacterial infections. Treatments for these disorders depend on the particular deficiency, but often require transfer of immunoglobulin, or a bone marrow hematopoietic stem cell transplant into patients inflicted with severe immunodeficiencies[136].

B cell malignancies

Conversely, excessive or unregulated B cell proliferation can result in lymphoma, leukemia, or multiple myeloma. B cell lymphomas can include both Hodgkin and non-Hodgkin lymphomas (NHLs), with most NHLs being of B cell origin (80-85%). Diffuse large B-cell lymphoma (DLBCL) are the most common NHL, but this category also includes follicular lymphomas, mantle cell lymphomas, and Burkitt's lymphoma[137]. B cell leukemias are also common, and B-cell chronic lymphocytic leukemia (CLL) is the most common form of leukemia in adults. In CLL, B cells experience unregulated growth in the bone marrow, and crowd out healthy cells[138]. In Acute myelogenous leukemia (AML), B cell precursors are arrested at an early stage of development in the bone marrow, but continue to proliferate - causing bone marrow failure[139]. Multiple myeloma is specifically a

plasma cell malignancy. Like B cell leukemias, the malignant plasma cells can often crowd out healthy cells in the bone marrow through unregulated proliferation, making patients more susceptible to disease and bone failure[140].

Hypogammaglobulinemia and increased susceptibility to infection can result from both the disease and its treatment. Patients are typically treated with cytotoxic chemotherapy combined with B cell targeting monoclonal antibodies such as anti-CD20 (rituximab)[137,138,141]. Other therapies target Btk, P13 kinase,or Bcl-2, all directed against the BCR[142]. Multiple myeloma patients are commonly treated with bortezomib, a protease-inhibitor which targets the continued protein production of plasma cells, both healthy and malignant, or with other immunomodulatory drugs, such as thalidomide or lenalidomide[143]. These treatments are not always successful, nor are they well-tolerated, especially in pregnant women and in older patients, and there is a need to develop a better-tolerated, more highly targeted therapies in response to decreasing effectiveness.

B cell-linked autoimmunity

B cells also play a role in a number of autoimmune disorders, either through production of self-reactive antibodies, presentation of self-antigen to T cells, or through expression of inflammatory cytokines. Here we will focus on antibody-mediated autoimmunity. The buildup of autoimmune complexes and the resulting inflammation lead to such disorders as systemic lupus erythematosus (SLE), rheumatoid arthritis, sclerosis, and Sjögren's syndrome[144]. In SLE, multiple organ systems may be affected by autoantibodies and damaged by inflammation[145]. In arthritis, rheumatic factors against gamma globulins buildup

and cause an inflammatory response within the connective tissues, particularly within the joints[146]. Sclerosis also affects specific tissues, such as the skin in scleroderma or the arteries in atherosclerosis[144]. Sjögren's particularly affects the mucus membranes[147]. Autoantibodies may also target a specific receptor function, such as the thyroid stimulating hormone in Grave's disease or acetylcholine receptors in Myasthenia Gravis. Diabetes was formerly considered to be mostly T-cell mediated, but autoantibodies have also been shown to play a critical role in disease progression[144].

B1 cells in particular, along with polyreactive IgM, are often linked with autoimmunity. SLE, atherosclerosis, arthritis, and rheumatism have all been linked to expanded B1 populations[148-150]. However, depleting B1 cells does not appear to be a viable solution, as B1 cells are also involved with tissue homeostasis and regulatory functions, and depletion can actually worsen the progression of autoimmune disease[151,152]. More targeted approaches are critical to fighting autoimmunity without impairing healthy B cell functioning.

The importance of antibody responses in vaccine design

Neutralizing antibodies are the key correlate of long-term vaccine efficacy in the majority of currently licensed vaccines. Antibodies to smallpox have been observed in vaccinated individuals up to 75 years post-vaccination[23]. But the highly mutagenic nature of some viruses - including HIV and influenza - have made it difficult to induce long-lasting protection through vaccination. However, recent advances in our approach to vaccine design, including deep sequencing of the resulting B and T cell populations, have made it easier to predict the efficacy

of newly developed vaccines[153]. Rationally designed vaccines eliciting broadly neutralizing antibodies may succeed where previous attempts have failed[154,155].

Additional difficulties face the vaccination of infants as well as elderly patients. Infant B cell compartments are populated with fetal-derived B1 cells, which produce natural IgM rather than adaptive IgG responses, and generating long-lasting responses through vaccination has proved difficult. Infants typically respond poorly to vaccination, requiring multiple boots to maintain immunity. Maternal antibody can be passed on from mother to child, through the placenta or through breast-feeding, to provide protection within the first few months following birth, but this also poses a significant barrier to vaccination[113]. Elderly patients experience a similar lack of IgG due to the loss of long-lived IgG plasma cells and a reduced ability to generate new IgG plasma cells, limiting the effectiveness of vaccination[156]. Enhancing IgM production in these patients may help to overcome these barriers.

Summary

A better understanding of how to generate long-lasting, antigen-specific immune responses is essential to rational vaccine design and to targeting aberrant regulation, such as B cell malignancies and antibody-linked autoimmunity. Thus, this body of work focuses on characterizing a novel population of induced, long-lived IgM plasma cells in the spleen and their role in the humoral response.

Chapter 2 first identifies these cells and distinguishes them from other plasma cell subsets, and demonstrates the ability of long-lived IgM plasma cells to protect against viral infection *in vivo*. Here we show that these IgM plasma

cells are long-lived, comparable to IgG plasma cells, and that they preferentially populate the spleen. Via anti-CD40L depletion of the germinal centers, we demonstrate that IgM plasma cells, unlike IgG, do not require the germinal center to develop. Further, through high-throughput sequencing, we observed that these IgM antibodies are somatically mutated, even in the absence of germinal center formation. Finally, we demonstrate that long-lived IgM plasma cells alone are sufficient to protect against influenza challenge in immunized mice.

In Chapter 3, we further explore the development of these long-lived IgM plasma cells, focusing on their B1 lineage and the kinetics of the long-lived IgM response. Adoptive transfer of either B1b or B2 cells into B-cell deficient mice revealed that B1 rather than B2 cells were the source of long-lasting IgM responses, even in the absence of the germinal center. Further, we show that this long-lived IgM response develops prior to the IgG response, as early as day 4.

Altogether we have used this data to develop a model of B1-derived, long-lived IgM plasma cell development, which undergoes SHM but not CSR, and does not require germinal center formation. Several hypotheses for why this pathway may exist and how it may prove to be clinically significant are described in Chapter 4.

REFERENCES

1 Tonegawa, S. Somatic generation of antibody diversity. *Nature* **302**, 575-581 (1983).

2 Paige, C. J. & Wu, G. E. The B cell repertoire. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **3**, 1818-1824 (1989).

3 Di Noia, J. M. & Neuberger, M. S. Molecular mechanisms of antibody somatic hypermutation. *Annual review of biochemistry* **76**, 1-22, doi:10.1146/annurev.biochem.76.061705.090740 (2007).

4 Litman, G. W. *et al.* Phylogenetic diversification of immunoglobulin genes and the antibody repertoire. *Molecular biology and evolution* **10**, 60-72 (1993).

5 Guelpa-Fonlupt, V., Tonnelle, C., Blaise, D., Fougereau, M. & Fumoux, F. Discrete early pro-B and pre-B stages in normal human bone marrow as defined by surface pseudo-light chain expression. *European journal of immunology* **24**, 257-264, doi:10.1002/eji.1830240140 (1994).

6 Shapiro-Shelef, M. & Calame, K. Regulation of plasma-cell development. *Nature reviews. Immunology* **5**, 230-242, doi:10.1038/nri1572 (2005).

7 Kitamura, D., Roes, J., Kuhn, R. & Rajewsky, K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* **350**, 423-426, doi:10.1038/350423a0 (1991).

8 Ma, Y., Pannicke, U., Schwarz, K. & Lieber, M. R. Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell* **108**, 781-794 (2002).

9 Schwarz, K. *et al.* Severe combined immunodeficiency (SCID) in man: B cell-negative (B-) SCID patients exhibit an irregular recombination pattern at the JH locus. *The Journal of experimental medicine* **174**, 1039-1048 (1991).

10 Mombaerts, P. *et al.* RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, 869-877 (1992).

11 Lafaille, J. J., DeCloux, A., Bonneville, M., Takagaki, Y. & Tonegawa, S. Junctional sequences of T cell receptor gamma delta genes: implications for gamma delta T cell lineages and for a novel intermediate of V-(D)-J joining. *Cell* **59**, 859-870 (1989).

12 Alt, F. W. & Baltimore, D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 4118-4122 (1982).

13 Lewis, S. M. The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. *Advances in immunology* **56**, 27-150 (1994).

14 Candeias, S., Muegge, K. & Durum, S. K. Junctional diversity in signal joints from T cell receptor beta and delta loci via terminal deoxynucleotidyl transferase and exonucleolytic activity. *The Journal of experimental medicine* **184**, 1919-1926 (1996).

15 Iwasato, T. & Yamagishi, H. Novel excision products of T cell receptor gamma gene rearrangements and developmental stage specificity implied by the frequency of nucleotide insertions at signal joints. *European journal of immunology* **22**, 101-106, doi:10.1002/eji.1830220116 (1992).
16 Shimizu, T. & Yamagishi, H. Biased reading frames of pre-existing DH--JH coding joints and preferential nucleotide insertions at VH--DJH signal joints of excision products of immunoglobulin heavy chain gene rearrangements. *The EMBO journal* **11**, 4869-4875 (1992).
17 Sanz, I. Multiple mechanisms participate in the generation of diversity of human H chain CDR3 regions. *Journal of immunology* **147**, 1720-1729 (1991).
18 Hardy, R. R. & Hayakawa, K. B cell development pathways. *Annual review of immunology* **19**, 595-621, doi:10.1146/annurev.immunol.19.1.595 (2001).
19 Ohashi, P. S. & DeFranco, A. L. Making and breaking tolerance. *Current opinion in immunology* **14**, 744-759 (2002).
20 Slifka, M. K., Antia, R., Whitmire, J. K. & Ahmed, R. Humoral immunity due to long-lived plasma cells. *Immunity* **8**, 363-372 (1998).
21 Sciammas, R. *et al.* Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity* **25**, 225-236, doi:10.1016/j.immuni.2006.07.009 (2006).
22 Zotos, D. & Tarlinton, D. M. Determining germinal centre B cell fate. *Trends in immunology* **33**, 281-288, doi:10.1016/j.it.2012.04.003 (2012).
23 Hammarlund, E. *et al.* Duration of antiviral immunity after smallpox vaccination. *Nature medicine* **9**, 1131-1137, doi:10.1038/nm917 (2003).
24 Ho, F., Lortan, J. E., MacLennan, I. C. & Khan, M. Distinct short-lived and long-lived antibody-producing cell populations. *European journal of immunology* **16**, 1297-1301, doi:10.1002/eji.1830161018 (1986).
25 Nutt, S. L., Hodgkin, P. D., Tarlinton, D. M. & Corcoran, L. M. The generation of antibody-secreting plasma cells. *Nature reviews. Immunology* **15**, 160-171, doi:10.1038/nri3795 (2015).
26 DiLillo, D. J. *et al.* Maintenance of long-lived plasma cells and serological memory despite mature and memory B cell depletion during CD20 immunotherapy in mice. *Journal of immunology* **180**, 361-371 (2008).
27 Fukuda, T. *et al.* Disruption of the Bcl6 gene results in an impaired germinal center formation. *The Journal of experimental medicine* **186**, 439-448 (1997).
28 Allen, C. D. & Cyster, J. G. Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. *Seminars in immunology* **20**, 14-25, doi:10.1016/j.smim.2007.12.001 (2008).
29 Lee, C. H. *et al.* Regulation of the germinal center gene program by interferon (IFN) regulatory factor 8/IFN consensus sequence-binding protein. *The Journal of experimental medicine* **203**, 63-72, doi:10.1084/jem.20051450 (2006).
30 Reljic, R., Wagner, S. D., Peakman, L. J. & Fearon, D. T. Suppression of signal transducer and activator of transcription 3-dependent B lymphocyte

terminal differentiation by BCL-6. *The Journal of experimental medicine* **192**, 1841-1848 (2000).

31 Shaffer, A. L. *et al.* Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* **17**, 51-62 (2002).

32 Shaffer, A. L. *et al.* BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. *Immunity* **13**, 199-212 (2000).

33 Gray, D., Dullforce, P. & Jainandunsing, S. Memory B cell development but not germinal center formation is impaired by in vivo blockade of CD40-CD40 ligand interaction. *The Journal of experimental medicine* **180**, 141-155 (1994).

34 Takahashi, Y., Dutta, P. R., Cerasoli, D. M. & Kelsoe, G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *The Journal of experimental medicine* **187**, 885-895 (1998).

35 Taylor, J. J., Pape, K. A. & Jenkins, M. K. A germinal center-independent pathway generates unswitched memory B cells early in the primary response. *The Journal of experimental medicine* **209**, 597-606, doi:10.1084/jem.20111696 (2012).

36 Lougaris, V., Badolato, R., Ferrari, S. & Plebani, A. Hyper immunoglobulin M syndrome due to CD40 deficiency: clinical, molecular, and immunological features. *Immunological reviews* **203**, 48-66, doi:10.1111/j.0105-2896.2005.00229.x (2005).

37 Elgueta, R. *et al.* Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunological reviews* **229**, 152-172, doi:10.1111/j.1600-065X.2009.00782.x (2009).

38 Durandy, A. Activation-induced cytidine deaminase: a dual role in class-switch recombination and somatic hypermutation. *European journal of immunology* **33**, 2069-2073, doi:10.1002/eji.200324133 (2003).

39 Rajewsky, K. Clonal selection and learning in the antibody system. *Nature* **381**, 751-758, doi:10.1038/381751a0 (1996).

40 Muramatsu, M. *et al.* Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *The Journal of biological chemistry* **274**, 18470-18476 (1999).

41 Muramatsu, M. *et al.* Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**, 553-563 (2000).

42 Nussenzweig, M. C. & Alt, F. W. Antibody diversity: one enzyme to rule them all. *Nature medicine* **10**, 1304-1305, doi:10.1038/nm1204-1304 (2004).

43 Okazaki, I. M., Kinoshita, K., Muramatsu, M., Yoshikawa, K. & Honjo, T. The AID enzyme induces class switch recombination in fibroblasts. *Nature* **416**, 340-345, doi:10.1038/nature727 (2002).

44 Kinoshita, K. & Honjo, T. Linking class-switch recombination with somatic hypermutation. *Nature reviews. Molecular cell biology* **2**, 493-503, doi:10.1038/35080033 (2001).

45 Imai, K. *et al.* Human uracil-DNA glycosylase deficiency associated with profoundly impaired immunoglobulin class-switch recombination. *Nature immunology* **4**, 1023-1028, doi:10.1038/ni974 (2003).

46 Revy, P. *et al.* Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* **102**, 565-575 (2000).

47 Brekke, O. H. & Sandlie, I. Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nature reviews. Drug discovery* **2**, 52-62, doi:10.1038/nrd984 (2003).

48 Vieira, P. & Rajewsky, K. The half-lives of serum immunoglobulins in adult mice. *European journal of immunology* **18**, 313-316, doi:10.1002/eji.1830180221 (1988).

49 Janeway, C. *Immunobiology : the immune system in health and disease.* 6th edn, (Garland Science, 2005).

50 Racine, R. *et al.* IgM production by bone marrow plasmablasts contributes to long-term protection against intracellular bacterial infection. *Journal of immunology* **186**, 1011-1021, doi:10.4049/jimmunol.1002836 (2011).

51 Racine, R. & Winslow, G. M. IgM in microbial infections: taken for granted? *Immunology letters* **125**, 79-85, doi:10.1016/j.imlet.2009.06.003 (2009).

52 Jayasekera, J. P., Moseman, E. A. & Carroll, M. C. Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. *Journal of virology* **81**, 3487-3494, doi:10.1128/JVI.02128-06 (2007).

53 Diamond, M. S. *et al.* A critical role for induced IgM in the protection against West Nile virus infection. *The Journal of experimental medicine* **198**, 1853-1862, doi:10.1084/jem.20031223 (2003).

54 Boes, M. *et al.* Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *Journal of immunology* **160**, 4776-4787 (1998).

55 Jacob, J., Kelsoe, G., Rajewsky, K. & Weiss, U. Intraclonal generation of antibody mutants in germinal centres. *Nature* **354**, 389-392, doi:10.1038/354389a0 (1991).

56 Jacob, J., Przylepa, J., Miller, C. & Kelsoe, G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. III. The kinetics of V region mutation and selection in germinal center B cells. *The Journal of experimental medicine* **178**, 1293-1307 (1993).

57 Martin, A. *et al.* Activation-induced cytidine deaminase turns on somatic hypermutation in hybridomas. *Nature* **415**, 802-806, doi:10.1038/nature714 (2002).

58 Golding, G. B. & Glickman, B. W. Evidence for local DNA influences on patterns of substitutions in the human alpha-interferon gene family. *Canadian journal of genetics and cytology. Journal canadien de genetique et de cytologie* **28**, 483-496 (1986).

59 Cohen, R. M., Kleinstein, S. H. & Louzoun, Y. Somatic hypermutation targeting is influenced by location within the immunoglobulin V region. *Molecular immunology* **48**, 1477-1483, doi:10.1016/j.molimm.2011.04.002 (2011).

60 William, J., Euler, C., Christensen, S. & Shlomchik, M. J. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* **297**, 2066-2070, doi:10.1126/science.1073924 (2002).

61 Di Niro, R. *et al.* Salmonella Infection Drives Promiscuous B Cell Activation Followed by Extrafollicular Affinity Maturation. *Immunity* **43**, 120-131, doi:10.1016/j.immuni.2015.06.013 (2015).

62 Wagner, S. D., Milstein, C. & Neuberger, M. S. Codon bias targets mutation. *Nature* **376**, 732, doi:10.1038/376732a0 (1995).

63 Kuraoka, M. *et al.* Complex Antigens Drive Permissive Clonal Selection in Germinal Centers. *Immunity*, doi:10.1016/j.immuni.2016.02.010 (2016).

64 Hargreaves, D. C. *et al.* A coordinated change in chemokine responsiveness guides plasma cell movements. *The Journal of experimental medicine* **194**, 45-56 (2001).

65 Ma, Q., Jones, D. & Springer, T. A. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity* **10**, 463-471 (1999).

66 Koni, P. A. *et al.* Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. *The Journal of experimental medicine* **193**, 741-754 (2001).

67 Roth, K. *et al.* Tracking plasma cell differentiation and survival. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* **85**, 15-24, doi:10.1002/cyto.a.22355 (2014).

68 Pereira, J. P., Kelly, L. M. & Cyster, J. G. Finding the right niche: B-cell migration in the early phases of T-dependent antibody responses. *International immunology* **22**, 413-419, doi:10.1093/intimm/dxq047 (2010).

69 Cyster, J. G. *et al.* Follicular stromal cells and lymphocyte homing to follicles. *Immunological reviews* **176**, 181-193 (2000).

70 Hauser, A. E. *et al.* Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. *Journal of immunology* **169**, 1277-1282 (2002).

71 Roy, M. P., Kim, C. H. & Butcher, E. C. Cytokine control of memory B cell homing machinery. *Journal of immunology* **169**, 1676-1682 (2002).

72 Humpert, M. L., Pinto, D., Jarrossay, D. & Thelen, M. CXCR7 influences the migration of B cells during maturation. *European journal of immunology* **44**, 694-705, doi:10.1002/eji.201343907 (2014).

73 De Vos, J. *et al.* Microarray-based understanding of normal and malignant plasma cells. *Immunological reviews* **210**, 86-104, doi:10.1111/j.0105-2896.2006.00362.x (2006).

74 Vande Broek, I. *et al.* Chemokine receptor CCR2 is expressed by human multiple myeloma cells and mediates migration to bone marrow stromal

cell-produced monocyte chemotactic proteins MCP-1, -2 and -3. *British journal of cancer* **88**, 855-862, doi:10.1038/sj.bjc.6600833 (2003).

75 Moller, C., Stromberg, T., Juremalm, M., Nilsson, K. & Nilsson, G. Expression and function of chemokine receptors in human multiple myeloma. *Leukemia* **17**, 203-210, doi:10.1038/sj.leu.2402717 (2003).

76 Muehlinghaus, G. *et al.* Regulation of CXCR3 and CXCR4 expression during terminal differentiation of memory B cells into plasma cells. *Blood* **105**, 3965-3971, doi:10.1182/blood-2004-08-2992 (2005).

77 Marques, C. P. *et al.* CXCR3-dependent plasma blast migration to the central nervous system during viral encephalomyelitis. *Journal of virology* **85**, 6136-6147, doi:10.1128/JVI.00202-11 (2011).

78 Tschen, S. I. *et al.* Recruitment kinetics and composition of antibody-secreting cells within the central nervous system following viral encephalomyelitis. *Journal of immunology* **168**, 2922-2929 (2002).

79 Tschen, S. I. *et al.* CNS viral infection diverts homing of antibody-secreting cells from lymphoid organs to the CNS. *European journal of immunology* **36**, 603-612, doi:10.1002/eji.200535123 (2006).

80 Tyor, W. R. & Griffin, D. E. Virus specificity and isotype expression of intraparenchymal antibody-secreting cells during Sindbis virus encephalitis in mice. *Journal of neuroimmunology* **48**, 37-44 (1993).

81 Zuo, J. *et al.* Mouse hepatitis virus pathogenesis in the central nervous system is independent of IL-15 and natural killer cells. *Virology* **350**, 206-215, doi:10.1016/j.virol.2006.01.027 (2006).

82 Ramakrishna, C., Bergmann, C. C., Atkinson, R. & Stohlman, S. A. Control of central nervous system viral persistence by neutralizing antibody. *Journal of virology* **77**, 4670-4678 (2003).

83 Tsubaki, T. *et al.* Accumulation of plasma cells expressing CXCR3 in the synovial sublining regions of early rheumatoid arthritis in association with production of Mig/CXCL9 by synovial fibroblasts. *Clinical and experimental immunology* **141**, 363-371, doi:10.1111/j.1365-2249.2005.02850.x (2005).

84 Cassese, G. *et al.* Inflamed kidneys of NZB / W mice are a major site for the homeostasis of plasma cells. *European journal of immunology* **31**, 2726-2732 (2001).

85 Radbruch, A. *et al.* Competence and competition: the challenge of becoming a long-lived plasma cell. *Nature reviews. Immunology* **6**, 741-750, doi:10.1038/nri1886 (2006).

86 Ellyard, J. I. *et al.* Antigen-selected, immunoglobulin-secreting cells persist in human spleen and bone marrow. *Blood* **103**, 3805-3812, doi:10.1182/blood-2003-09-3109 (2004).

87 Ellyard, J. I., Avery, D. T., Mackay, C. R. & Tangye, S. G. Contribution of stromal cells to the migration, function and retention of plasma cells in human spleen: potential roles of CXCL12, IL-6 and CD54. *European journal of immunology* **35**, 699-708, doi:10.1002/eji.200425442 (2005).

88 Carbone, G. *et al.* Interleukin-6 receptor blockade selectively reduces IL-21 production by CD4 T cells and IgG4 autoantibodies in rheumatoid

arthritis. *International journal of biological sciences* **9**, 279-288, doi:10.7150/ijbs.5996 (2013).

89 Diehl, S. A., Schmidlin, H., Nagasawa, M., Blom, B. & Spits, H. IL-6 triggers IL-21 production by human CD4+ T cells to drive STAT3-dependent plasma cell differentiation in B cells. *Immunology and cell biology* **90**, 802-811, doi:10.1038/icb.2012.17 (2012).

90 Minges Wols, H. A., Underhill, G. H., Kansas, G. S. & Witte, P. L. The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. *Journal of immunology* **169**, 4213-4221 (2002).

91 Cassese, G. *et al.* Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *Journal of immunology* **171**, 1684-1690 (2003).

92 Benson, M. J. *et al.* Cutting edge: the dependence of plasma cells and independence of memory B cells on BAFF and APRIL. *Journal of immunology* **180**, 3655-3659 (2008).

93 Mackay, F. & Schneider, P. Cracking the BAFF code. *Nature reviews. Immunology* **9**, 491-502, doi:10.1038/nri2572 (2009).

94 O'Connor, B. P. *et al.* BCMA is essential for the survival of long-lived bone marrow plasma cells. *The Journal of experimental medicine* **199**, 91-98, doi:10.1084/jem.20031330 (2004).

95 Scholz, J. L. *et al.* BLyS inhibition eliminates primary B cells but leaves natural and acquired humoral immunity intact. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 15517-15522, doi:10.1073/pnas.0807841105 (2008).

96 Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B. I. & Nagasawa, T. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* **20**, 707-718, doi:10.1016/j.immuni.2004.05.001 (2004).

97 Moon, B. G., Takaki, S., Miyake, K. & Takatsu, K. The role of IL-5 for mature B-1 cells in homeostatic proliferation, cell survival, and Ig production. *Journal of immunology* **172**, 6020-6029 (2004).

98 Sze, D. M., Toellner, K. M., Garcia de Vinuesa, C., Taylor, D. R. & MacLennan, I. C. Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival. *The Journal of experimental medicine* **192**, 813-821 (2000).

99 Baumgarth, N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nature reviews. Immunology* **11**, 34-46, doi:10.1038/nri2901 (2011).

100 Cerutti, A., Cols, M. & Puga, I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nature reviews. Immunology* **13**, 118-132, doi:10.1038/nri3383 (2013).

101 Mauri, C. & Bosma, A. Immune regulatory function of B cells. *Annual review of immunology* **30**, 221-241, doi:10.1146/annurev-immunol-020711-074934 (2012).

102 Lalor, P. A., Herzenberg, L. A., Adams, S. & Stall, A. M. Feedback regulation of murine Ly-1 B cell development. *European journal of immunology* **19**, 507-513, doi:10.1002/eji.1830190315 (1989).

103 Duber, S. *et al.* Induction of B-cell development in adult mice reveals the ability of bone marrow to produce B-1a cells. *Blood* **114**, 4960-4967, doi:10.1182/blood-2009-04-218156 (2009).

104 Holodick, N. E., Repetny, K., Zhong, X. & Rothstein, T. L. Adult BM generates CD5+ B1 cells containing abundant N-region additions. *European journal of immunology* **39**, 2383-2394, doi:10.1002/eji.200838920 (2009).

105 Hardy, R. R., Carmack, C. E., Li, Y. S. & Hayakawa, K. Distinctive developmental origins and specificities of murine CD5+ B cells. *Immunological reviews* **137**, 91-118 (1994).

106 Tornberg, U. C. & Holmberg, D. B-1a, B-1b and B-2 B cells display unique VHDJH repertoires formed at different stages of ontogeny and under different selection pressures. *The EMBO journal* **14**, 1680-1689 (1995).

107 Gregoire, K. E., Goldschneider, I., Barton, R. W. & Bollum, F. J. Ontogeny of terminal deoxynucleotidyl transferase-positive cells in lymphohemopoietic tissues of rat and mouse. *Journal of immunology* **123**, 1347-1352 (1979).

108 Kantor, A. B., Merrill, C. E., Herzenberg, L. A. & Hillson, J. L. An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells. *Journal of immunology* **158**, 1175-1186 (1997).

109 Gu, H., Forster, I. & Rajewsky, K. Sequence homologies, N sequence insertion and JH gene utilization in VHDJH joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. *The EMBO journal* **9**, 2133-2140 (1990).

110 Kretschmer, K. *et al.* Antibody repertoire and gene expression profile: implications for different developmental and functional traits of splenic and peritoneal B-1 lymphocytes. *Journal of immunology* **171**, 1192-1201 (2003).

111 Hayakawa, K. *et al.* Positive selection of natural autoreactive B cells. *Science* **285**, 113-116 (1999).

112 Hayward, A. R. The human fetus and newborn: development of the immune response. *Birth defects original article series* **19**, 289-294 (1983).

113 Siegrist, C. A. Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants. *Vaccine* **21**, 3406-3412 (2003).

114 Ansel, K. M., Harris, R. B. & Cyster, J. G. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity* **16**, 67-76 (2002).

115 Crowley, M. T., Reilly, C. R. & Lo, D. Influence of lymphocytes on the presence and organization of dendritic cell subsets in the spleen. *Journal of immunology* **163**, 4894-4900 (1999).

116 Alugupalli, K. R. *et al.* The resolution of relapsing fever borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes. *Journal of immunology* **170**, 3819-3827 (2003).

117 Baumgarth, N. *et al.* B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to

influenza virus infection. *The Journal of experimental medicine* **192**, 271-280 (2000).
118 Roy, B. *et al.* Somatic hypermutation in peritoneal B1b cells. *Molecular immunology* **46**, 1613-1619, doi:10.1016/j.molimm.2009.02.026 (2009).
119 Haas, K. M., Poe, J. C., Steeber, D. A. & Tedder, T. F. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae. *Immunity* **23**, 7-18, doi:10.1016/j.immuni.2005.04.011 (2005).
120 Cooper, M. D., Peterson, R. D. & Good, R. A. Delineation of the Thymic and Bursal Lymphoid Systems in the Chicken. *Nature* **205**, 143-146 (1965).
121 Pancer, Z. *et al.* Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature* **430**, 174-180, doi:10.1038/nature02740 (2004).
122 Akula, S., Mohammadamin, S. & Hellman, L. Fc receptors for immunoglobulins and their appearance during vertebrate evolution. *PloS one* **9**, e96903, doi:10.1371/journal.pone.0096903 (2014).
123 Stavnezer, J. & Amemiya, C. T. Evolution of isotype switching. *Seminars in immunology* **16**, 257-275, doi:10.1016/j.smim.2004.08.005 (2004).
124 Conticello, S. G., Thomas, C. J., Petersen-Mahrt, S. K. & Neuberger, M. S. Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases. *Molecular biology and evolution* **22**, 367-377, doi:10.1093/molbev/msi026 (2005).
125 Hinds-Frey, K. R., Nishikata, H., Litman, R. T. & Litman, G. W. Somatic variation precedes extensive diversification of germline sequences and combinatorial joining in the evolution of immunoglobulin heavy chain diversity. *The Journal of experimental medicine* **178**, 815-824 (1993).
126 Wilson, M. *et al.* What limits affinity maturation of antibodies in Xenopus--the rate of somatic mutation or the ability to select mutants? *The EMBO journal* **11**, 4337-4347 (1992).
127 Diaz, M., Flajnik, M. F. & Klinman, N. Evolution and the molecular basis of somatic hypermutation of antigen receptor genes. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **356**, 67-72, doi:10.1098/rstb.2000.0750 (2001).
128 Chen, J. M., Cooper, D. N., Chuzhanova, N., Ferec, C. & Patrinos, G. P. Gene conversion: mechanisms, evolution and human disease. *Nature reviews. Genetics* **8**, 762-775, doi:10.1038/nrg2193 (2007).
129 Ota, T. & Nei, M. Evolution of immunoglobulin VH pseudogenes in chickens. *Molecular biology and evolution* **12**, 94-102 (1995).
130 Abbott, J. K. & Gelfand, E. W. Common Variable Immunodeficiency: Diagnosis, Management, and Treatment. *Immunology and allergy clinics of North America* **35**, 637-658, doi:10.1016/j.iac.2015.07.009 (2015).
131 Bruton, O. C. Agammaglobulinemia. *Pediatrics* **9**, 722-728 (1952).
132 Lopez Granados, E. *et al.* Clinical and molecular analysis of patients with defects in micro heavy chain gene. *The Journal of clinical investigation* **110**, 1029-1035, doi:10.1172/JCI15658 (2002).

133 Kroczek, R. A. *et al.* Defective expression of CD40 ligand on T cells causes "X-linked immunodeficiency with hyper-IgM (HIGM1)". *Immunological reviews* **138**, 39-59 (1994).
134 Hammarstrom, L., Vorechovsky, I. & Webster, D. Selective IgA deficiency (SIgAD) and common variable immunodeficiency (CVID). *Clinical and experimental immunology* **120**, 225-231 (2000).
135 Hong, R. & Gupta, S. Selective immunoglobulin M deficiency in an adult with Streptococcus pneumoniae sepsis and invasive aspergillosis. *Journal of investigational allergology & clinical immunology* **18**, 214-218 (2008).
136 Porta, F. *et al.* Stem cell transplantation for primary immunodeficiencies. *Bone marrow transplantation* **41 Suppl 2**, S83-86, doi:10.1038/bmt.2008.61 (2008).
137 Chu, Y. W. & Polson, A. Antibody-drug conjugates for the treatment of B-cell non-Hodgkin's lymphoma and leukemia. *Future oncology* **9**, 355-368, doi:10.2217/fon.12.189 (2013).
138 Lachance, S. *et al.* A Canadian perspective on the use of immunoglobulin therapy to reduce infectious complications in chronic lymphocytic leukemia. *Current oncology* **23**, 42-51, doi:10.3747/co.23.2810 (2016).
139 Saultz, J. N. & Garzon, R. Acute Myeloid Leukemia: A Concise Review. *Journal of clinical medicine* **5**, doi:10.3390/jcm5030033 (2016).
140 Kyle, R. A. & Rajkumar, S. V. Multiple myeloma. *The New England journal of medicine* **351**, 1860-1873, doi:10.1056/NEJMra041875 (2004).
141 Moreau, P. *et al.* Rituximab in CD20 positive multiple myeloma. *Leukemia* **21**, 835-836, doi:10.1038/sj.leu.2404558 (2007).
142 Jain, N. & O'Brien, S. Targeted therapies for CLL: Practical issues with the changing treatment paradigm. *Blood reviews*, doi:10.1016/j.blre.2015.12.002 (2015).
143 Lonial, S. & Boise, L. H. The future of drug development and therapy in myeloma. *Seminars in oncology* **40**, 652-658, doi:10.1053/j.seminoncol.2013.07.004 (2013).
144 Hampe, C. S. B Cell in Autoimmune Diseases. *Scientifica* **2012**, doi:10.6064/2012/215308 (2012).
145 Mahajan, A., Herrmann, M. & Munoz, L. E. Clearance Deficiency and Cell Death Pathways: A Model for the Pathogenesis of SLE. *Frontiers in immunology* **7**, 35, doi:10.3389/fimmu.2016.00035 (2016).
146 Renaudineau, Y., Jamin, C., Saraux, A. & Youinou, P. Rheumatoid factor on a daily basis. *Autoimmunity* **38**, 11-16, doi:10.1080/08916930400022574 (2005).
147 Theander, E. & Jacobsson, L. T. Relationship of Sjogren's syndrome to other connective tissue and autoimmune disorders. *Rheumatic diseases clinics of North America* **34**, 935-947, viii-ix, doi:10.1016/j.rdc.2008.08.009 (2008).
148 Hayakawa, K., Hardy, R. R., Parks, D. R. & Herzenberg, L. A. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *The Journal of experimental medicine* **157**, 202-218 (1983).

149 Binder, C. J. & Silverman, G. J. Natural antibodies and the autoimmunity of atherosclerosis. *Springer seminars in immunopathology* **26**, 385-404, doi:10.1007/s00281-004-0185-z (2005).
150 Duan, B. & Morel, L. Role of B-1a cells in autoimmunity. *Autoimmunity reviews* **5**, 403-408, doi:10.1016/j.autrev.2005.10.007 (2006).
151 Lampropoulou, V. *et al.* Suppressive functions of activated B cells in autoimmune diseases reveal the dual roles of Toll-like receptors in immunity. *Immunological reviews* **233**, 146-161, doi:10.1111/j.0105-2896.2009.00855.x (2010).
152 Boes, M. *et al.* Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 1184-1189 (2000).
153 De Gregorio, E. & Rappuoli, R. From empiricism to rational design: a personal perspective of the evolution of vaccine development. *Nature reviews. Immunology* **14**, 505-514, doi:10.1038/nri3694 (2014).
154 Gitlin, A. D. & Nussenzweig, M. C. Immunology: Fifty years of B lymphocytes. *Nature* **517**, 139-141, doi:10.1038/517139a (2015).
155 Kwong, P. D., Mascola, J. R. & Nabel, G. J. Rational design of vaccines to elicit broadly neutralizing antibodies to HIV-1. *Cold Spring Harbor perspectives in medicine* **1**, a007278, doi:10.1101/cshperspect.a007278 (2011).
156 Frasca, D., Van der Put, E., Riley, R. L. & Blomberg, B. B. Reduced Ig class switch in aged mice correlates with decreased E47 and activation-induced cytidine deaminase. *Journal of immunology* **172**, 2155-2162 (2004).

Chapter 2

Long-lived, antigen-induced IgM plasma cells demonstrate somatic mutations and contribute to long-term protection

In press at *Nature Communications, 2016.*

Caitlin Bohannon performed all of the experiments in this manuscript, except as follows:

Lakshmipriyadarshini Satyabhama preformed the influenza and LCMV time course ELISPOTs (Figure 1, c-h)

Ang Cui performed the hotspot analysis on the plasma cell heavy chain sequences (Figure 3, a-c)

Caitlin Bohannon and Joshy Jacob wrote the manuscript.

ABSTRACT

Long-lived plasma cells are critical to humoral immunity as a lifelong source of protective antibodies. Antigen-activated B cells - with T cell help - undergo affinity maturation within germinal centers and persist as long-lived IgG plasma cells in the bone marrow. Here we show that antigen-specific, induced IgM plasma cells also persist for a lifetime. Unlike long-lived IgG plasma cells, which develop in germinal centers and then home to the bone marrow, IgM plasma cells are primarily retained within the spleen and can develop even in the absence of germinal centers. Interestingly, their expressed IgV loci exhibited somatic mutations introduced by the activation-induced cytidine deaminase (AID)-dependent somatic hypermutation mechanism. However, these IgM plasma cells were likely not antigen-selected, as replacement mutations were spread through the variable segment and not enriched within the CDRs. Finally, antibodies from long-lived IgM plasma cells provided protective host immunity against a lethal virus challenge.

INTRODUCTION

Immune memory can last a lifetime, in no small part due to antigen-specific, long-lived plasma cells that continuously secrete antibodies and provide long-term protection[1-5]. These plasma cells develop from antigen-specific B cells through one of two pathways, resulting in either short-lived or long-lived plasma cells. Initially, following activation with either T-dependent or –independent antigens, antigen-specific B cells are able to progress immediately into short-lived plasma cells. These short-lived cells exist only transiently in the spleen and lymph nodes, and do not undergo affinity maturation[3,5]. In contrast, long-lived plasma cells, which are of higher affinity than their short-lived counterparts and can survive for many years, develop primarily in response to T-dependent antigens. T cell help initiates the germinal center reaction, where antigen-specific B cells undergo affinity maturation, and develop into either memory B cells, which remain in the spleen, or long-lived IgG plasma cells that home to the bone marrow and persist for a lifetime[1-3].

The development of both memory B cells and long-lived IgG plasma cells is significantly impaired in the absence of germinal center formation[6,7]. The interaction between CD40 on B cells and CD40L on activated T follicular helper cells (T_{FH}) is essential to germinal center function, and disrupting this interaction prevents germinal center formation and differentiation of activated B cells into long-term memory populations. It has been shown that blockade with αCD40 antibodies impairs memory B cell formation[6], and that αCD40L treatment blocks the development of long-lived IgG plasma cells[7], and both studies show significantly reduced antigen-specific IgG titers.

Following activation, B cells clonally expand and undergo the process of class-switching and somatic hypermutation as well[8,9]. During class-switching, B cells switch from surface IgM expression to secreted IgG isotypes, as well IgA and IgE. These cells, within the germinal centers, also undergo somatic hypermutation (SHM) and antigen-driven selection[4,8]. SHM is the process by which point mutations accumulate in the immunoglobulin (Ig) variable loci. This is initiated by activation-induced cytidine deaminase (AID, or AICDA) and point mutations are targeted to G:C pairs. This creates a pool of antigen-specific B cells clones with varying affinities and, out of these, high affinity clones are selected by antigen-driven selection. This process typically selects for replacement mutations (mutations that result in a different amino acid) at higher frequency within the antigen-binding, complementary determining regions (CDRs) than in framework (FRW) regions, as is commonly seen in both memory B cells and bone marrow resident, long-lived IgG plasma cells[4,10].

Long-lived IgG plasma cells, rather than memory B cells, are the key source of long-term IgG secretion[1,11]. This was demonstrated elegantly by Tedder and colleagues who showed that depletion of B cells, including memory B cells, via αCD20 antibody treatment did not affect antigen-specific IgG titers or long-lived IgG plasma cell survival[11]. Plasma cells persist long-term in the bone marrow microenvironment with the support of resident stromal cells, which provide critical survival signals. They also showed that disruption of this interaction, via blockade of the adhesion molecules LFA-1 and VLA-4, depleted bone-marrow IgG plasma cells and significantly decreased levels of serum IgG[11]. Three months later, however, antibody levels returned to previous levels - most likely due to differentiation of

memory B cells into long-lived plasma cells. Tedder and colleagues additionally showed that when both memory B cells and bone marrow-resident plasma cells were simultaneously depleted, serum IgG titers did not recover[11].

Here we report that a distinct population of antigen-specific, induced long-lived IgM plasma cells persists in the murine spleen, responding to vaccination or infection and secreting significant titers of antigen-specific IgM for the lifetime of the animal. These cells, unlike IgG plasma cells, are able to develop and somatically mutate even in the absence of germinal centers. These mutations were found in mutational hotspots typically induced by AID[4], but unlike IgG plasma cells, did not show evidence of antigen driven selection. IgM plasma cells that develop in the absence of germinal centers are also mutated, although at lower levels. Additionally, long-lived IgM plasma cells were found to protect against viral challenge *in vivo*.

RESULTS

Long-lived IgM plasma cells persist in the spleen

We initially sought to compare IgG plasma cell longevity in the bone marrow compartment over a time course of up to 2 years following protein immunization. As a control, we also quantitated IgM plasma cells in the spleen with the expectation that these would be short-lived. Age-matched naïve cohorts served as control for natural IgM cells. Natural IgM cells produce natural IgM antibodies, which are constantly present in the serum and provide an early, poly-reactive response against pathogens[12]. Recently, Kelsoe and colleagues have elegantly shown that these are long-lived plasma cells that develop from a fetal-lineage progenitor that is neither B1a nor B1b and persist in the bone marrow[13]. In order to assess longevity, we first

immunized mice with $NP_{22}CGG$ in alum and quantitated antigen-specific antibody-secreting cells (ASCs) by ELISPOT in both the bone marrow and spleen. We found comparable numbers of antigen-specific ASCs in both the bone marrow (Fig. 1a) and spleen (Fig. 1b) persisting at 2 months, 6 months, 1 year, and even 2 years post-immunization. Interestingly, when we compared IgM ASCs in the immunized cohorts to the naïve controls we found a significant number of long-lived, antigen-specific IgM ASCs in the spleen. These long-lived IgM ASCs were found in significantly higher numbers than both IgG and natural IgM cells seen in naïve controls, and persisted almost 2 years following immunization ($p<0.05$ for all time points up to 1 year). As with long-lived IgG plasma cells, we observed an initial spike in IgM ASCs in immunized mice, followed by a slow decay over the lifetime of the animal. By contrast, natural IgM ASCs accumulated in mice over time, up to around 6 months, and then remained relatively constant for the rest of their lifespan. To confirm that this observation on long-lived, induced IgM plasma cells was not merely an artifact of the hapten-carrier system, we also infected (Fig. 1c-d) or immunized mice (Fig. 1e-f) with live or inactivated A/Puerto Rico/8/1934 influenza virus (PR8), respectively. Again we found similarly long-lived responses in the spleen, with a significant population of IgM ASCs in infected or immunized mice compared to naïve controls. In particular, the infected mice showed significantly greater numbers of IgM ASCs in the spleen up to 11 months post-infection, compared with immunization. We additionally extended these studies to infection with the Armstrong strain of Lymphocytic Choriomeningitis Virus (LCMV) (Fig. 1g-h), and we again observed significantly higher numbers of antigen-specific, long-lived IgM plasma cells in the spleen when compared with a 6 month control naïve controls.

Antigen-specific IgM plasma cells persist in the spleen post-adoptive transfer

To determine whether IgM plasma cells are inherently long-lived, we sorted 10^4 splenic or bone marrow $CD138^+B220^-$ plasma cells, and adoptively transferred cells into B-cell deficient μMT mice, or lymphocyte-deficient *Rag*$^{-/-}$ mice. To demonstrate that IgM plasma cells are antigen-specific, and when transferred did not contain natural IgM plasma cells, we sorted 10^4 splenic and bone marrow $CD138^+B220^-$ plasma cells (Fig. 2a) from mice 2 months post-$NP_{22}CGG$ immunization or age-matched naïve controls, and assessed specificity via ELISPOT. Only plasma cells from immunized mice bound $NP_{22}CGG$ (Fig. 2b), and neither immunized nor naïve plasma cell populations reacted with irrelevant antigen - PR8 influenza virus (Fig. 2c). We hypothesized that if IgM plasma cells were inherently short-lived, we would observe a greater decay of IgM antibody titers in recipient μMT mice, which lack naïve B cell pools to draw on or antigen to stimulate cells. However, we found comparable $NP_{22}CGG$-specific IgG and IgM antibody titers post-adoptive transfer as measured by ELISA (Fig. 2d-e), indicating that IgM plasma cells are not inherently short-lived. These results are not unique to the μMT recipient mice, as we observed similar titers of IgM antibodies upon transfer of plasma cells into *Rag-/-* recipient mice (Supplementary Figure 1). To confirm IgM plasma cell survival and determine their post-transfer localization, we sacrificed the recipients after two months and assayed for the presence of IgG and IgM plasma cells in the spleen and bone marrow by ELISPOT assay. Our results demonstrate that antigen-specific IgM plasma cells survive 2 months post-transfer. Interestingly our results indicate a marked

preference for IgG plasma cells to localize to the bone marrow and IgM plasma cells to localize to the spleen (Fig. 2f-g).

To further characterize the longevity of splenic and IgM plasma cells, we determined their half-life via time-course ELISPOT (Fig. 2h). By transferring $CD138^{+}B220^{-}$ plasma cells from donor mice at 2 months post-immunization, we have not only excluded short-lived plasma cells, but also natural IgM cells (Fig. 2b,c) from our analysis. We then followed the decay of splenic IgG and IgM cells over the course of two months, and determined the half-life of each population from its linear regression. We were unable to determine using BrDU labeling whether these cells were still undergoing proliferation, due to the paucity of these plasma cells following transfer.. We did, however estimate the half-life of IgG and IgM plasma cells transferred from the spleen to be $t_{1/2}$ = 145 days and $t_{1/2}$ = 86 days, respectively. The half-life of these IgM plasma cells is relatively long compared to their short-lived counterparts, and the rate of decay is not significantly different than that of IgG plasma cells (p=0.82).

Long-lived IgM plasma cells develop in the absence of germinal centers

Long-lived, bone marrow-resident IgG plasma cells are dependent on germinal centers for their development. Disruption of germinal centers by blocking T cells help via αCD40L antibody treatment[7], depleting complement by cobra venom factor (CVF)[14], or the use of gene knockout animals[15] completely ablates the long-lived IgG plasma cell pool. To determine whether IgM long-lived plasma cells are germinal center-dependent, we administered αCD40L antibodies at days 6, 8, and 10 following immunization in order to block the formation of the germinal center.

αCD40L treatment has been previously shown to block germinal center formation and consequently the development of long-lived IgG plasma cells[7]. As expected, in mice where germinal center formation was disrupted, long-lived IgG plasma cells were depleted ($p<0.005$) in both the spleen and bone marrow (Fig. 3a). Surprisingly, depletion of germinal centers did not impair the formation of long-lived IgM plasma cells in either the bone marrow or the spleen (Fig. 3b). We also used a second approach to ablate germinal centers by treatment with CVF, and observed that the development of IgM plasma cells still occurs while long-lived IgG plasma cells are significantly impaired (Supplementary Figure 2). Taken together, this suggests that long-lived, induced IgM plasma cells, unlike their IgG counterparts, can develop even in the absence of germinal centers.

Long-lived IgM plasma cells are somatically mutated

Somatic mutation is a hallmark of long-lived IgG plasma cells in the bone marrow[4,8,16]. Thus we wanted to determine whether IgM plasma cells exhibited mutations in their Ig loci, analogous to IgG plasma cells. However, plasma cells lack surface Ig and hence they cannot be sorted based on antigen specificity. To overcome this limitation, we immunized mice with NP_{22}CGG, which induces a well-documented response in which NP-specificity is encoded by the IgHV186.2 gene segment in Igh^b mice[17,18]. Briefly, at 1 or 3 months post-NP_{22}CGG immunization, and with and without αCD40L treatment in C57/BL/6 cohorts, we have amplified and sequenced those cells expressing the canonical IgHV186.2 variable segment. We then analyzed these sequences using The International Immunogenetics Information System®'s (IMGT) HighV-QUEST[19] in order to identify the germline heavy chain

V(D)J gene segments and map mutations by comparison with a database of known sequences. Supplementary Table 1 shows the numbers of full-length sequences and unique clones found in each animal with a greater than 90% homology to IgHV186.2. As a control, we also sequenced the heavy chains of pre-B cells isolated from the bone marrow of these same mice. Pre-B cells only express rearranged heavy chains, but not light chains, and would have had no previous exposure to antigen[20]. A small number of IgHV186.2 sequences were found in the pre-B population, all identical to the published germline sequence. Though there were some rare instances of diverging base pairs seen in the total pre-B population, these did not result in amino acid changes. In contrast, IgHV186.2 IgG from bone marrow-resident plasma cells showed an abundance of mutations, with an average of 25.5 mutations per sequence. IgM plasma cells showed significantly fewer mutations, with an average of 3.3 mutations per sequence, and a third of sequences remained unmutated. In addition, with germinal center depletion there was a small but significant decrease in the number of mutations observed in IgM plasma cells – on average 2.1 – although they were still significantly mutated when compared to pre-B controls (average 0.4) ($p<0.001$) (Fig. 3c). As a control, we also sorted out $CD138^{+}B220^{-}$ plasma cells from unimmunized mice and tried to amplify IgHV186.2 V gene segments that encode NP-binding antibodies, but these rearrangements could not be found. Combined with the absence of NP-specific ASCs in the sorted $CD138^{+}B220^{-}$ plasma cells from unimmunized mice (Figure 2b), this data further illustrates that long-lived IgM plasma cells are induced rather the natural IgM cells.

During B cell development and assembly of the B cell receptor from Ig gene segments, imprecise joining of the V,D, and J segments creates a unique junction at

the CDR3 region[21]. These unique CDR3s can be used to identify individual clones. Figure 3d-e illustrates the clonality of representative plasma cell populations detailed in Supplementary Table 1. Each clone is identified by a unique CDR3 junction and may have multiple members based on diversification by somatic mutation. In a representative mouse, 79 individual IgG sequences from bone marrow plasma cells contained 7 clonal populations, with 3 clones accounting for 89% of the sequences (38, 36, and 15%, respectively), showing significant levels of clonal expansion (Fig. 3d). We see similar evidence of clonal expansion in IgM plasma cell sequences, with and without αCD40L treatment, though the effect is less pronounced in some samples (Fig. 3e-f). 56 IgM heavy chains from the bone marrow of a representative mouse can be grouped into 24 unique clones, with the 3 largest clones comprising 45% of all sequences (20,15, and 10%). 42 IgM heavy chains from the spleen include 11 unique clones, with the 3 largest comprising 74% of all sequences (51, 16, and 7%). 53 αCD40L treated bone marrow IgM sequences contained 17 clones, with the 3 largest comprising 50% of total sequences (34, 10, and 6%), while 26 αCD40L spleen IgM sequences contained 21 unique clones with the 3 largest comprising 17% of sequences (12, 8, and 8%). Within individual clones, the diversification via somatic mutation and the establishment of parent-daughter clones can be visualized by constructing lineage trees. We have constructed these trees for both IgG and IgM plasma cell clones, with and without germinal center depletion, using the IgTree software[22] (Fig. 3g-i). These lineage trees demonstrate that the mutational diversity generated within a common B cell clone persists in the periphery, for both IgG and IgM plasma cell subsets. This indicates that the mutants generated at the activated B cell stage were not lost but many were selected for and

can be found in the plasma cell compartments. These trees also further illustrate the clonal expansion that has taken place in both IgG and IgM plasma cell populations, though there are fewer mutations in IgM clones - and fewer still without germinal center help - mirroring the pattern we see in all IgHV186.2-encoded plasma cells.

Mutations in IgM plasma cells occur in motifs preferentially targeted by AID

We next sought to determine whether mutations in IgM sequences were introduced by the somatic hypermutation (SHM) mechanism. SHM is initiated by AID, which mutates DNA by deaminating the cytidine base, creating a uracil. This G:U mismatch leads to daughter DNA strands containing either the original G:C or an A:U at the site of mutation. Uracil-DNA glycosylase (UNG) and apurinic endonuclease (APE) are then involved in excising uracil out, and DNA repair mechanisms add back a complementary thymidine[23,24]. This process preferentially creates transition mutations (C<->T and G<->A) over transversions (all other changes)[4]. Here we compared the frequency of transitions vs. transversions in IgG and IgM plasma cells, with and without germinal center depletion. Transitions were much more common for all populations compared with transversions in IgHV186.2 sequences - characteristic of AID induced mutation[4,16,25] (Fig. 4a). To further testify the involvement of the AID-induced SHM mechanism, we analyzed whether mutations in IgG and IgM plasma cells occurred in SHM 'hotspot' motifs, where mutations are most likely to occur. The known hotspots are WR<u>C</u>/<u>G</u>YW and W<u>A</u>/<u>T</u>W, where the underlined nucleotide is mutated, W=weak (A,T), R=purine (A,G), and Y=pyrimidine (T,C). In contrast, SY<u>C</u>/<u>G</u>RS is considered a coldspot, where S=strong

(G,C), and other motifs are considered 'neutral'[26,27]. The data is depicted as 'hedgehog' plots that show individual IgG (Fig. 4b) and IgM (Fig. 4c) 5-mers, with sequences read 5' to 3', and the radiating bars indicating mutability at that motif. Mutations occur at the central base and are dependent on surrounding bases, with classical hotspots indicated by red or green bars, coldspots by blue, and neutral sites by grey. Figure 4c shows the cumulative hotspot and coldspot mutabilities as a percentage of total mutations. Both IgG and IgM plasma cell sequences predominantly exhibited mutations in hotspots (>60%) and, to a lesser degree, in coldspots (<7%), with no significant differences seen between IgG and IgM plasma cells. Taken together these data indicate that the pattern of mutation in IgM plasma cell is similar to that of IgG.

Mutations in IgM plasma cells are AID-dependent

Having demonstrated that mutations in IgM occur predominantly in AID-targeted hotspots, we wanted to confirm that AID was, in fact, the primary mechanism of these mutations. It has been previously shown that in AID knockout mice, class-switching does not occur, and long-lived IgG plasma cells do not develop[28]. We immunized cohorts of AID-/- mice with NP_{22}CGG, and observed that the formation of induced IgM plasma cells was not impaired in these mice. We then sequenced sorted out IgM plasma cells AID-/- cohorts as well as wild-type C57/BL/6 plasma cells, alongside pre-B controls and PCR amplified and sequenced their expressed IgV heavy chain gene segments. We found that mutations do not occur in $AID^{-/-}$ cohorts, equivalent to what we see in pre-B cell controls (Fig. 4e, 5d). This data demonstrates that mutations found in IgM plasma cells are indeed introduced by AID.

Mutations in IgM plasma cells are likely not antigen-selected

Having demonstrated that long-lived IgM plasma cells accumulate somatic mutations, we next analyzed the position of these mutations in order to compare the extent of antigen-driven selection in Ig heavy chains of IgG and IgM plasma cells. Antigen-selection typically enriches for replacement mutations within the CDRs, which bind the antigen, while mutations that disrupt the framework structure are not tolerated[4,10]. When we mapped out the replacement and silent mutations that occur in sample bone marrow IgG plasma cells, we observe a high frequency of replacement mutations within CDR1 and an even greater frequency in CDR2, as expected (Fig. 5a). Additionally, we observed a clear pattern of increased replacement to silent mutation (R/S) ratios in CDRs regions (R/S >3) compared to those in framework regions (R/S <2). No significant mutations are seen in AID-/- mice, also as expected (Fig. 5d). However, while a greater number of mutations are seen in IgM plasma cells of wild-type mice, we did not observe a high frequency of mutations, especially replacement mutations, in the CDRs of IgM. Furthermore, the R/S ratio shows a very different pattern of replacement mutations compared to IgG, with a low R/S ratio in the CDRs (<1) and a greater ratio in the framework regions 2 and 3 (<4) (Fig. 5b). A similar pattern is seen in IgM sequences from immunized mice in which germinal center function has been inhibited (Fig. 5c). Based upon the distribution of mutations and the R/S ratios, it is unlikely that IgM plasma cells are antigen-selected as in IgG plasma cells, and our results may indicate the necessity of the germinal center in antigen-driven selection.

Long-lived IgM plasma cells enhance protective immunity

We next sought to determine whether IgM antibodies from long-lived plasma cells could aid in host protection against a viral pathogen. In influenza infection, the antibody response has long been regarded as the key correlate of protection, and it is critical to the neutralization of the virus[29]. To assess protection, we first immunized cohorts of mice with 1400 HA A/PR/8/34 influenza virus. We then treated one cohort with αCD40L antibody in order to deplete germinal centers and long-lived IgG plasma cells, such that these cohorts would produce only IgM plasma cells. We verified that IgG plasma cells were ablated in αCD40L-treated cohorts (Fig. 6a) while PR8-specific IgM plasma cells persisted at greater numbers than in naïve controls (Fig. 6b). Sera taken at day 150 post-immunization confirmed the absence of IgG antibodies following αCD40L treatment (Fig. 6c). This sera was then assessed for neutralization of PR8 virus and compared to sera from mice with intact germinal centers, which had both IgG and IgM plasma cells, and naïve controls (Fig. 6d). Sera from αCD40L treated mice, which was predominantly IgM, effectively neutralized PR8 virus - particularly with addition of complement. IgM from naïve mice was significantly less effective, even with the addition of complement.

We additionally show that induced IgM from long-lived plasma cells is more effective than natural IgM in naïve mice in protecting against lethal viral challenge *in vivo*. At 1 year post-immunization, cohorts of immunized mice - with and without germinal center inhibition - and age-matched unimmunized controls were challenged intranasally with live mouse-adapted 10 x LD_{50} PR8 virus. Mice were weighed daily and those animals losing >20% of their initial body weights were euthanized (Fig. 6e). Following lethal virus challenge, all of the naive control mice

lost significant body weight (high morbidity) and had to be euthanized by day 7. Immunized mice with intact germinal centers and IgG plasma cells exhibited 100% survival with minimal morbidity. The αCD40L treated cohort, with IgM but not IgG plasma cells, showed an 80% survival rate - thus demonstrating effective protection (Fig. 6f). All surviving mice, with or without germinal center blockade, began to recover body mass by day 10 (Fig. 6e). Taken together these data demonstrate that mice lacking IgG plasma cells are still effectively protected against lethal influenza virus challenge up to 1 year post-immunization, and that IgM plasma cells enhance host protection in their absence, while natural IgM alone is not sufficient to protect naïve mice *in vivo*. However, a limitation of this experiment was the presence of memory CD4 and CD8 T cell populations, which would have contributed to survival and recovery in all immunized cohorts. Reactivated, germinal center-independent memory B cells may also have played a role in viral clearance[30].

To rule out the role of memory T cells, as well as memory B cells which require CD4 help for reactivation[31], we repeated the experiment but this time depleted both CD4 and CD8 T cells from all murine cohorts prior to challenging them with virus. In this way we could assess the role of antibody alone in neutralizing the virus – IgG and IgM combined, IgM from long-lived plasma cells alone, or natural IgM by itself. Briefly, mice were first infected intranasally with 0.1 x LD_{50} of live mouse-adapted PR8 virus, with one cohort again depleted of germinal centers and long-lived IgG plasma cells via αCD40L antibody treatment. Sera from these mice were collected at 1 month and assessed for the presence of αPR8 IgG and IgM as well as neutralization (Figure 7. a-b). Similar to our previous experiment (Fig. 6c,d), we do not see significant IgG in either GC-depleted mice or in age-matched unimmunized controls.

Higher titers of IgM in immunized/GC-depleted sera equated with significantly higher neutralization titers when compared to unimmunized mice, particularly when complement was added. We next depleted T cells from all cohorts and challenged mice with virus. At 1 and 3 days prior to viral challenge, mice were treated with 500 μg anti-CD4 and anti-CD8, effectively depleting the T cells as well as preventing memory B cell reactivation. Sample flow plots demonstrating depletion of CD4 and CD8 T cells in blood samples from each cohort are shown in Supplementary Figure 3. We then challenged mice with 2 x LD_{50} of live mouse-adapted PR8 virus – the lower dosage ensuring that immunized cohorts would survive long enough for post-challenge assessment. Once again all immunized/non-GC-depleted mice that bear virus-specific IgG and IgM antibodies survived the challenge, while all of the unimmunized controls succumbed to infection and 75% of the mice had to be euthanized (Fig. 7d). Not surprisingly, of the germinal center-depleted cohort that bears only virus-specific IgM antibodies, 75% survived (Figure 7c-d). Following PR8 challenge, titers of PR8-specific IgG were measured by serum ELISA to determine whether reactivation of memory B cells had occurred. No significant IgG titers were observed in the post-challenge sera of αCD40L-treated mice (Fig. 7e), indicating that their survival was due to IgM from long-lived plasma cells and not from reactivation of memory B or T cells, though they may have factored in the previous challenge.

DISCUSSION

In conclusion, our studies have characterized a distinct subset of long-lived, induced IgM plasma cells that persist within the spleen. Unlike bone marrow-resident IgG plasma cells, these cells develop either prior to or in the absence of the germinal

center. With the aid of high-throughput sequence analysis we have shown that these cells are somatically mutated, even in the absence of germinal center formation – though the lower number of mutations in the absence of germinal center may indicate it still plays some role in maturation. Mutations in plasma cell IgM antibodies were found in AID-induced 'hotspots', but did not show any evidence of antigen selection for replacement mutations within the CDRs. Further, we demonstrate that these IgM plasma cells are functionally relevant, and, even in the absence of IgG plasma cells, may enhance protection *in vivo* against lethal influenza challenge.

The longevity and distinct localization of IgM plasma cells highlight the spleen as a unique niche for predominantly IgM long-lived plasma cells. Previous studies have noted the presence of antigen-specific long-lived IgG plasma cells in the spleen - originally observed in response to LCMV infection in mice[1] - but they did not characterize IgM plasma cells. Here we demonstrate that IgM plasma cells in the spleen also have long half-lives, though not quite as long as IgG plasma cells from the spleen. MacLennan and colleagues have characterized the longevity and somatic mutation profile of IgG^+ and IgG^- plasma cells in response to NPCGG immunization in the spleen, but only up to day 21 post-immunization and not long-term[32]. These IgG^- plasma cells may be similar to the long-lived IgM populations we have found at much later time points post-immunization in our study. Additionally, our LCMV infection data demonstrates that virus-specific, IgM long-lived plasma cells are the predominant population in the spleen for up to 2 years post-infection, significantly higher than the IgG plasma cell population (Fig. 1h). This is also seen in immunization with $NP_{22}CGG$ and influenza, as well as in infection with influenza.

Interestingly, this preferential localization is recapitulated following adoptive transfer of long-lived plasma cells into B-cell deficient μMTs (Fig. 2f-g), wherein IgM plasma cells are retained primarily in the spleen and survive in the absence of antigen stimulation or naïve B cells in the adoptive transfer recipient.

These long-lived, induced IgM plasma cells that we have described are distinct from natural IgM plasma cells. While natural IgM populations remain steady over time, as we observe in our naïve controls, we see a sharp initial increase and then a gradual decay of induced IgM plasma cells in the spleen following infection and immunization, and in the bone marrow to a lesser degree (Fig. 1a-f). There is also evidence of the expansion of NP-specific VH186.2 clones following NP immunization (Fig. 3e-f, h-i), while natural IgM is thought to remain constant and does not expand in response to antigen stimulation[33]. That no NP-specific VH186.2 clones were found in the CD138+B220- plasma cell compartment of unimmunized mice further emphasizes that this population is antigen-induced. In addition, induced IgM plasma cells are somatically mutated (Fig. 3c), further distinguishing them from either short-lived plasma cells or natural IgM cells. Taken together, these data demonstrate that the long-lived, induced IgM plasma cell compartment of the spleen is distinct from not only bone marrow-resident IgG plasma cells but also natural IgM cells - also found primarily in the bone marrow of resting mice[12,13].

Additionally we have shown that long-lived IgM plasma cells were able to develop in the absence of germinal center formation, unlike IgG plasma cells (Fig. 3a-b, 6a-b, Supplementary Figure 2). This parallels the discovery of germinal center-independent IgM memory B cells, which develop outside of or in the absence of germinal centers[30]. Marc Jenkins and colleagues demonstrated that IgM memory B

cells are still able to develop in *Bcl6*$^{-/-}$ mice - which lack germinal centers – following immunization. They also observed in wild type mice that that IgM memory cells were somatically mutated, though less frequently than IgG memory B cells[34]. However, these studies were preformed separately and did not address whether somatically mutated memory cells could occur in the absence of germinal center formation.

The occurrence of somatic mutation outside of germinal centers has previously been observed in B cells in autoimmune hosts. Schlomchik and colleagues demonstrated in autoimmune MRL.Fas(lpr) mice that autoreactive B cells, microdissected from the border of the T cell zone and red pulp, had undergone somatic hypermutation outside of germinal centers, though germinal centers were present in this model[35]. They also recently demonstrated extrafollicular somatic hypermutation in plasmablasts in response to Salmonella infection, without significant germinal center formation[36]. In human patients suffering from hyper-IgM disorder due to a defect in CD40L, which inhibits germinal center function, low levels of somatic hypermutation are observed in memory B cells[37]. Here, we directly compare the frequency and position of germinal center-independent mutations from hundreds of sequences. We have additionally controlled for any potential error introduced by the sequencing method by running unmutated B220^{+}CD138^{-} CD19^{+}CD25^{+}IgM^{-}CD43^{-} pre-B cells (with rearranged heavy chain but not light chain) as a control alongside thousands of sample plasma cells. Compared to the pre-B sequences from the same animals, we found that IgM plasma cells exhibit low but significant levels of somatic mutation in the Ig variable loci, even in the absence of germinal center formation (Fig. 3c). However, we do find a significantly lower

frequency of IgM mutations in mice with impaired germinal centers. With or without the germinal center these mutations occurred primarily in AID-induced SHM hotspots and consisted frequently of transitions, typical of AID induction (Fig. 4a-d). We do not observe these mutations in $AID^{-/-}$ cohorts, confirming that they are indeed AID-dependent (Fig. 4e). Further, that these mutations do not show evidence of antigen selection indicates the necessity of the germinal center in antigen-driven selection (Fig. 5a-d). It is possible that these IgM plasma may still cells undergo some alternative form of antigen-selection, but it is unlike the mechanism we see in IgG plasma cells in which the CDRs are enriched for replacement mutations.

Functionally, IgM antibodies can confer protection to the host against viral and bacterial infections[38-41]. In mammalian hosts, which are also capable of producing IgG, IgM from memory cells has shown to be sufficient to protect against viral infection[40]. In another study, monoclonal IgM antibodies isolated from human patients were shown to protect mice against a lethal challenge (10 or 25 x LD_{50}) of H5N1 and H1N1 influenza viruses, respectively. Monoclonal IgM specific to H5N1 has also been shown to neutralize a diverse range of influenza subtypes including H1, H2, H5, H6, H8 and H9[40]. That IgM plasma cells also develop in response to T-independent antigens[42], or in the absence of the germinal center (Fig. 3a-b, 6a-b), may imply that this pathway continues to serve as an auxiliary pathway to protect the host even in the absence of T cell help. In this paper, we demonstrate the role of long-lived IgM plasma cells in protecting against influenza viral infection in the absence of IgG plasma cells (Fig. 6). We depleted the long-lived IgG response by blocking germinal centers via treatment with αCD40L antibody to demonstrate that serum containing only IgM can effectively neutralize virus, and that these animals

can also survive a lethal viral challenge (Fig. 6), even in the absence of T cell help and memory B cell activation (Fig. 7).

Another potential role for a long-lived IgM plasma cells is suggested by recent evidence showing that IgM antibodies may be necessary to generate an optimal IgG response[43]. In mice deficient for secretory IgM (sIgM$^{-/-}$) but still able to express surface IgM (as well as all other secreted isotypes), increased viremia and an impaired IgG response was observed following infection with West Nile Virus[43]. IgM titers were further able to accurately predict survival – low titers signaling poor prognosis – and wild-type immune sera were sufficient to rescue infected sIgM$^{-/-}$ cohorts. It has also been shown in several studies that IgM, when coupled with antigen, may also help to sustain long-term IgG titers following vaccination, even with suboptimal antigen doses[44]. This could be due to the ability of antigen-specific IgM to function as an effective adjuvant. It has been observed in murine models that IgM in conjunction with malarial antigens offered greater protection than conventional nonspecific adjuvants. Additionally, IgM antibodies may be important in overcoming maternal antibody's impairment of the infant immune response, as has been demonstrated with the same IgM/malarial Ag vaccine when administered to infant mice with immune mothers – those that were given the IgM-coupled vaccine all survived parasite challenge and developed high IgG titers, while all of the mice vaccinated with a non-specific adjuvant succumbed to the disease[45]. It is hypothesized that IgM's role in opsonization and complement recruitment may be responsible for enhancing both humoral and cell-mediated adaptive responses. It may also have the ability to stimulate innate cells by acting as a TLR ligand, though this is still an area of ongoing study[44]. Taken together, these studies imply that IgM

may serve a non-redundant role in humoral memory, providing a broadly neutralizing response as well as augmenting host IgG and overcoming impairment by maternal antibodies. Understanding the development of IgM plasma cells is critical not only to discerning the evolution of these pathways, but also to developing better vaccines and therapeutics[46].

Evolutionarily, the emergence of IgM occurred long before that of IgG in mammals, appearing as far back as cartilaginous fish and occurring in all vertebrate species[47]. AID and its homologs similarly appeared at this early timepoint, coevolving with its target immunoglobulins as a means of repertoire diversification[48]. There is evidence for somatic hypermutation in the IgM heavy chain locus in sharks[49] as well as in *Xenopus* (frogs)[50]. These mutations are typically at low levels and are frequently found in mutation hotspots (targeting G:C pairs), but are less frequent in the CDR regions than would be expected for antigen-driven mutation. It has been suggested that the absence of germinal centers in these animals prevents antigen-mediated selection[50]. We observe a similar pattern of low-level mutations in the AID-induced hotspots of murine plasma cell IgM occurring in the absence of germinal centers, with no evidence of antigen-selection. It is possible that these mutated IgM plasma cells exist as a remnant of this pre-germinal center pathway, which protects cartilaginous fish and cold-blooded vertebrates. Further study of this pathway will expand our knowledge of how the function of adaptive humoral responses has evolved. Altogether, these data suggest a unique role in humoral memory for long-lived IgM plasma cells within the splenic niche, and adds further complexity to our knowledge of long-lived plasma cell development.

METHODS AND MATERIALS

Mice

C57BL/6 mice, BALB/c, B6.129S2-*Ighmtm1CGGn*/J (μMT), and B6.129P2-*Aicda*$^{tm1(cre)Mnz/J}$ (AID$^{-/-}$) mice were purchased from The Jackson Laboratory. All mice were maintained in a specific pathogen-free facility in accordance with the institutional guidelines of The Animal Care and Use Committee at Emory University. Mice were immunized or infected at 4-8 weeks of age and all controls were age- and sex-matched. Sample size was determined by the "resource equation" method, taking into account possible attrition during infection assays[51]. Animals were randomly assigned to treatment groups but were not blinded.

Immunizations and infections

Cohorts of female C57BL/6 or AID$^{-/-}$ mice were immunized intraperitoneally with 50μg hydroxy-3-nitrophenylacetyl chicken-γ-globulin ($NP_{22}CGG$) (Biosearch Technologies) with 50μl alum in PBS for a total volume of 200μl. Other cohorts were infected with 2×10^5 pfu of the Armstrong strain of Lymphocytic choriomeningitis (LCMV). Balb/c mice were immunized intramuscularly with 1400 HA, infected intranasally with 0.01 x LD_{50}, or rechallenged with 10 x LD_{50} of A/Puerto Rico/8/1934 (PR8) virus. Sera, bone marrow, and spleens were collected at given time points post-immunization.

Plasma cell ELISPOTs

96-well plates were coated with 20 μg/ml $NP_{22}CGG$ or OVA, 512 HA units per mL PR8 virus, or 10 μg/ml LCMV virus and blocked with complete RPMI (10% FBS, 1%

penicillin/ streptavidin, 1% HEPES, and 50μM 2-mercaptoethanol). Bone marrow and splenic cells isolated from immunized mice were serially diluted in complete RPMI and incubated 16 hrs at 37°C. The plates were then treated with either anti-IgG- or -IgM-biotin (Southern Biotechnology) followed by incubation with streptavidin-alkaline phosphatase (Sigma). Plates were then developed with 5-bromo-4-chloro-3-indolylphosphate (Sigma) until spots developed, and spots counted with CTL Immunospot® software.

Adoptive transfer of long-lived plasma cells

Bone marrow or splenocytes were enriched for B cells via EasySep B cell enrichment kit (Stemcell technologies) and $CD138^{+}B220^{-}$ plasma cells were FACS sorted from C57BL/6 mice 2 months post-immunization. 10^5 marrow or splenic plasma cells were then transferred via tail-vein injection into cohorts of sex-matched μMT mice, or measured for NP_{22}CGG-specificity via ELISPOT. Serum antibody titers were measured by ELISA at days 7, 14, 21, 30, and 60. Plates were coated with 20μg/ml NP_{22}CGG (or 2μg/ml anti-IgG or -IgM for standard wells) and blocked with 3% BSA. Sera from recipient μMT mice was serially diluted in PBS and incubated for 2 hours. Plates were then treated with either anti-IgG-HRP or –IgM-biotin, followed by incubation with streptavidin-HRP for IgM plates (Southern Biotechnology). TMB substrate (Pierce) was added for 30s and then the reaction stopped with 0.16M sulfuric acid. Plates were read at 450nm. Plasma cell numbers in the bone marrow and spleen were assayed by ELISPOT at days 7, 14, 30, and at 2 months post-adoptive transfer as previously described, or at 2 months for bone marrow transfer cohorts. Half-lives were determined by linear regression of transferred cells,

comparing the slopes for statistically significant differences (Graphpad Prism Version 5.0c).

High-throughput heavy chain sequencing

Total plasma cells ($CD138^+B220^-$) and bone marrow pre-B cells ($B220^+CD138^-CD19^+CD25^+IgM^-CD43^-$) were isolated via cell sorting from the marrow and spleens of C57/BL/6 or $AID^{-/-}$ mice 1 or 3 months post-immunization with $NP_{22}CGG$ (Supplementary Table 1), as well as from unimmunized controls. RNA from these cells was isolated with the RNeasy kit (Qiagen) and transcribed into cDNA with ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis (Invitrogen). cDNA was then amplified using the consensus heavy chain primers and PCR protocol as described by Tiller et al.[52], and the IgHV186.2-specific primer as described by Jacob et al.[17], with the 2nd PCR amplification primers adapted and barcoded for 454 pyrosequencing. Barcoded products were amplified on sequencing beads using the GS Junior Titanium Lib-A emPCR Kit (Roche), and sequenced on the GS Junior 454 sequencer using the matching kit (Roche). Sequences were then analyzed for heavy chain homology and mutations using The International Immunogenetics Information System® HighV-QUEST (IMGT.org)[19]. Sequences with >90% identity with IgHV186.2 are numbered in Supplementary Table 1. Partial or non-productive sequences were excluded from analysis.

Germinal center disruption

Cohorts of mice were treated with either 300 μg αCD40L (clone MR-1) antibody or control IgG at days 6, 8, and 10 post $NP_{22}CGG$ immunization. Additional cohorts

were treated with 25 μg Cobra Venom Factor (CVF) or control Ig 6 hours prior to immunization and again at 4 days post-immunization. At 45 days post-immunization, bone marrow cells and splenocytes were assayed by ELISPOT for the presence of IgG and IgM secreting plasma cells.

IgTree analysis

Ig lineage trees were made using IgTree software[22], information for which can be found at http://immsilico2.lnx.biu.ac.il/Software.html. Clones were identified by their unique CDR3 sequences and measured against the germline sequence to generate trees for the variable regions only. Boundaries were defined by the beginning and end of alignment with IgHV186.2 sequence.

SHM Hotspot analysis

IgHV186.2 sequences were partitioned into clonally-related groups based on shared CDR3 junctions. To avoid double-counting mutational events, a consensus mutated sequence was generated for each clone through majority vote at each mutated position. The positions differed from IMGT inferred germline segments in the V region up to the start CDR3 were considered mutations for substitution matrices and hotspot analysis. The 5-mer motif mutability values were generated by computing the mutation level of each motif adjusted for background frequency. The model and source code can be found at http://clip.med.yale.edu/SHM[26].

Virus neutralization

Cohorts of Balb/c mice were immunized intramuscularly with 1400 HA of PR8 virus, and treated with either αCD40L or control Ig as previously described. At 2 months post-immunization, 5 mice from each cohort were euthanized and PR8 specific plasma cells were measured by ELISPOT for IgG ablation in αCD40L blockade mice. At day 150 post-immunization, sera was collected from remaining 10 mice and measured by ELISA (plates coated with 512 HA PR8) to assess IgG clearance from the sera of the αCD40L cohort. RDE II-treated sera from either cohort or age-matched naïve controls, with or without complement added (1:1 ratio), was then assessed for neutralization of 2×10^3 $TCID_{50}$/ml of PR8 virus when incubated with MDCK cells overnight at 37°C. The presence of viral nucleoprotein in infected cells was determined by ELISA, as previously described, using anti-nucleoprotein biotinylated antibody (Chemicon International). The highest serum dilution where less than half of the cells were infected was determined to be the neutralization titer, where 50% specific signal = (OD_{450} virus control – OD_{450} cell control)/2 + OD_{450} cell control)[39].

Viral challenge

At 1 year post-immunization with 1400 HA of virus, cohorts of PR8-immunized mice, with or without αCD40L blockade, as well as age-matched unimmunized controls were infected intranasally with 10 x LD_{50} of PR8 virus. Similarly, at 2 months post-infection with 0.1 x LD50 of virus, cohorts were challenged intranasally with 2 x LD_{50} of virus following T cell depletion (described below). Mice were weighed each day for 14 days and those falling below 80% of their initial weight were euthanized.

T cell depletion

At 3 days and 1 day prior to viral challenge, all cohorts of mice were treated i.p. with 500 μg each of both anti-CD4 (clone GK1.5) and anti-CD8a (YTS 169.4) (BioXcell)[53,54]. Blood samples from each mouse were then assessed for the presence of CD4 and CD8 T cells by flow cytometry. Cells were fluorescently labeled with anti-CD4 (clone RM4-5, ebioscience) and anti-CD8 (clone 53-6.7, BD).

Statistical analysis

Statistical significance was verified by the Student's *t*-test (two-tailed), unless otherwise indicated.

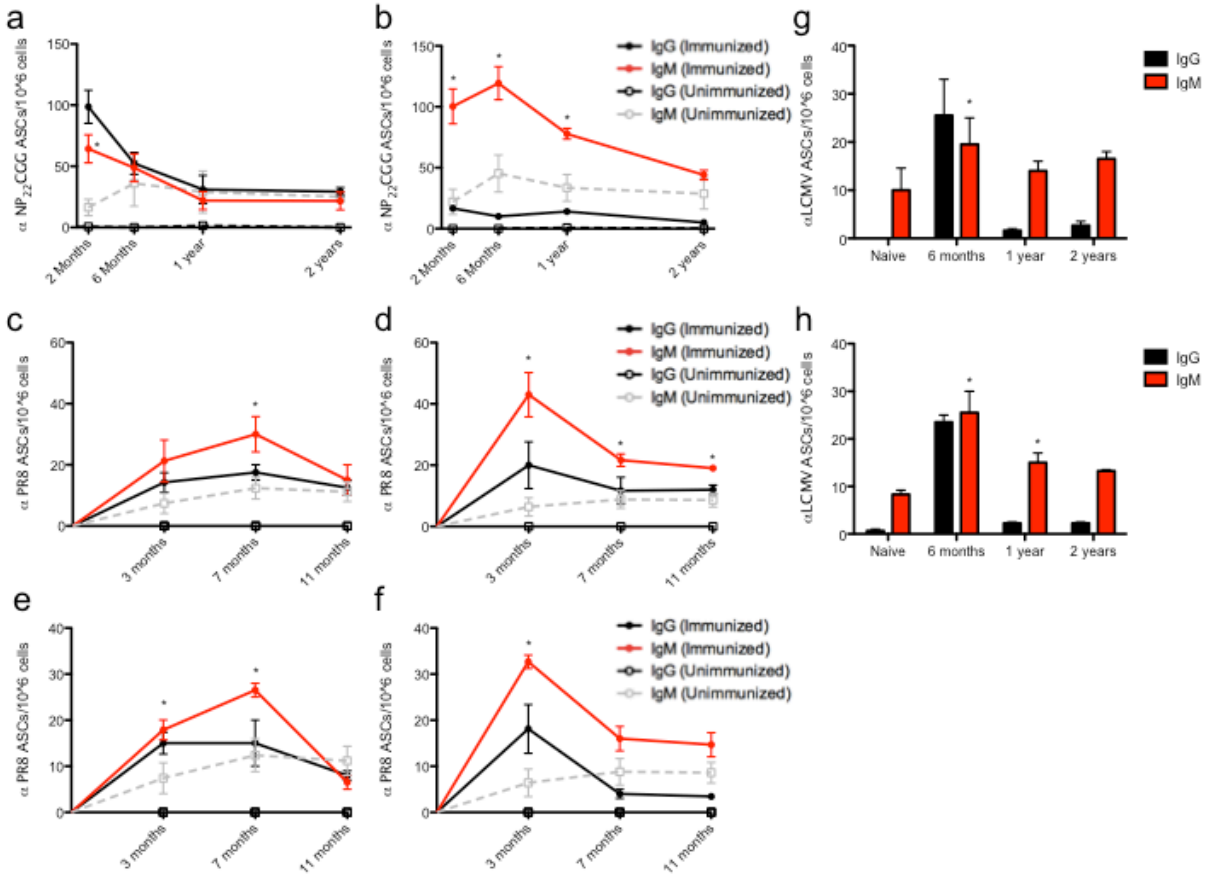


Figure 1: Expanded populations of IgM plasma cells persist in the spleen in response to diverse immunogens and pathogens. Cohorts of mice were immunized or infected with $NP_{22}CGG$, influenza, or LCMV virus and IgG (●) and IgM (●) ASCs from immunized and unimmunized (□ IgG, □ IgM) were measured via ELISPOT over time. **(a-b)** $NP_{22}CGG$-specific IgG and IgM plasma cells at 2 months, 6 months, 1 year, and 2 years post-immunization (n=6) or in naïve controls (n=3) were quantitated in the bone marrow and spleen, respectively. **(c-d)** A/PR/8/34 influenza virus-specific plasma cells at 3, 7, and 11 months post-infection with 0.01 x LD_{50} of virus (n=4) or in naïve controls (n=3) within the bone marrow and spleen, respectively. **(e-f)** A/PR/8/34-specific plasma cells at 3, 7, and 11

months post-immunization with 1400 HA of virus (n=4) or in naïve controls (n=3) within the bone marrow and spleen, respectively. **(g-h)** LCMV-specific IgG and IgM plasma cells in the bone marrow and spleen, respectively, at 6 months, 1 year, and 2 years post-infection with 2×10^5 pfu of LCMV Armstrong virus compared to a 6 month naïve control (n=3). The mean (±SEM) is shown for each timepoint and a * indicates time points where IgM ASCs are significantly higher ($p<0.05$) than in naïve animals.

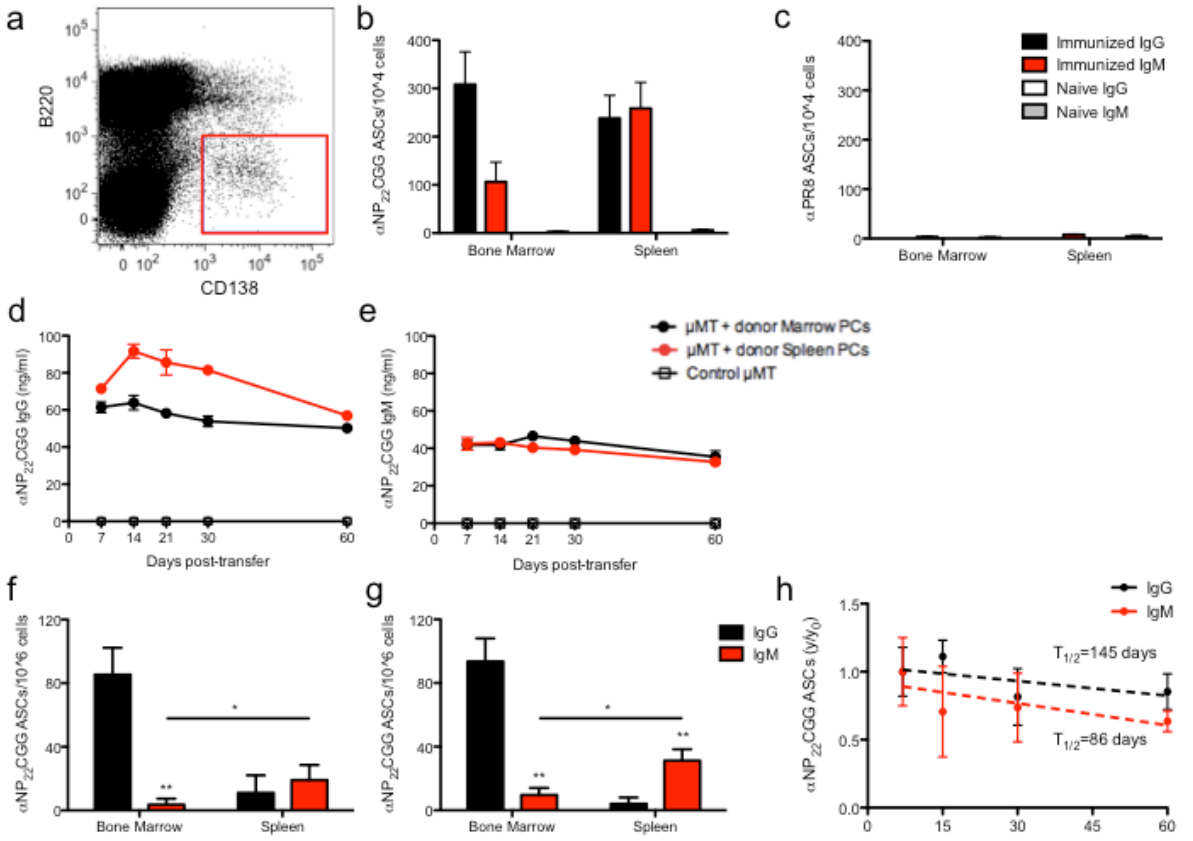


Figure 2: Antigen-specific, long-lived IgM plasma cells persist post-adoptive transfer and preferentially localize in the spleen, IgG plasma cells localize in the bone marrow. (a) Representative $CD138^{+}B220^{-}$ plasma cell gating strategy for FACS. **(b)** $NP_{22}CGG$-specific IgG (■) and IgM (■) ASCs in 10^4 sorted plasma cells, 2 months post-$NP_{22}CGG$ immunization (n=3) or in age-matched controls (□, n=3). **(c)** Non-specific (A/PR/8/34-binding) ASCs in 10^4 sorted plasma cells, 2 months post-$NP_{22}CGG$ immunization (n=3) or in age-matched controls (n=3). **(d)** Antigen-specific serum IgG titers found in recipient μMT mice that have been adoptively transferred with bone marrow plasma cells (●) or splenic plasma cells (●) as measured by ELISA at days 7 through 60 (n=5). **(e)** Antigen-specific

serum IgM titers found in recipient μMT mice that have been adoptively transferred with bone marrow plasma cells (●) or splenic plasma cells (●) as measured by ELISA at days 7 through 60 (n=5). **(f)** Localization of donor, NP_{22}CGG-specific IgG (■) and IgM (■) ASCs, adoptively in bone marrow plasma cell recipient mice. **(g)** Localization of donor, NP_{22}CGG-specific IgG (■) and IgM (■) ASCs, adoptively in splenic plasma cell recipient mice. The mean (±SEM) of 5 immunized mice or 3 naïve controls is shown, with ** indicating $p<0.005$. **(h)** The half-life of transferred cells was determined by fitting the time course data of either IgG or IgM plasma cells in splenic recipient mice (n=4) as a function of their ratio ($y/y_{initial}$) with its linear regression**.**

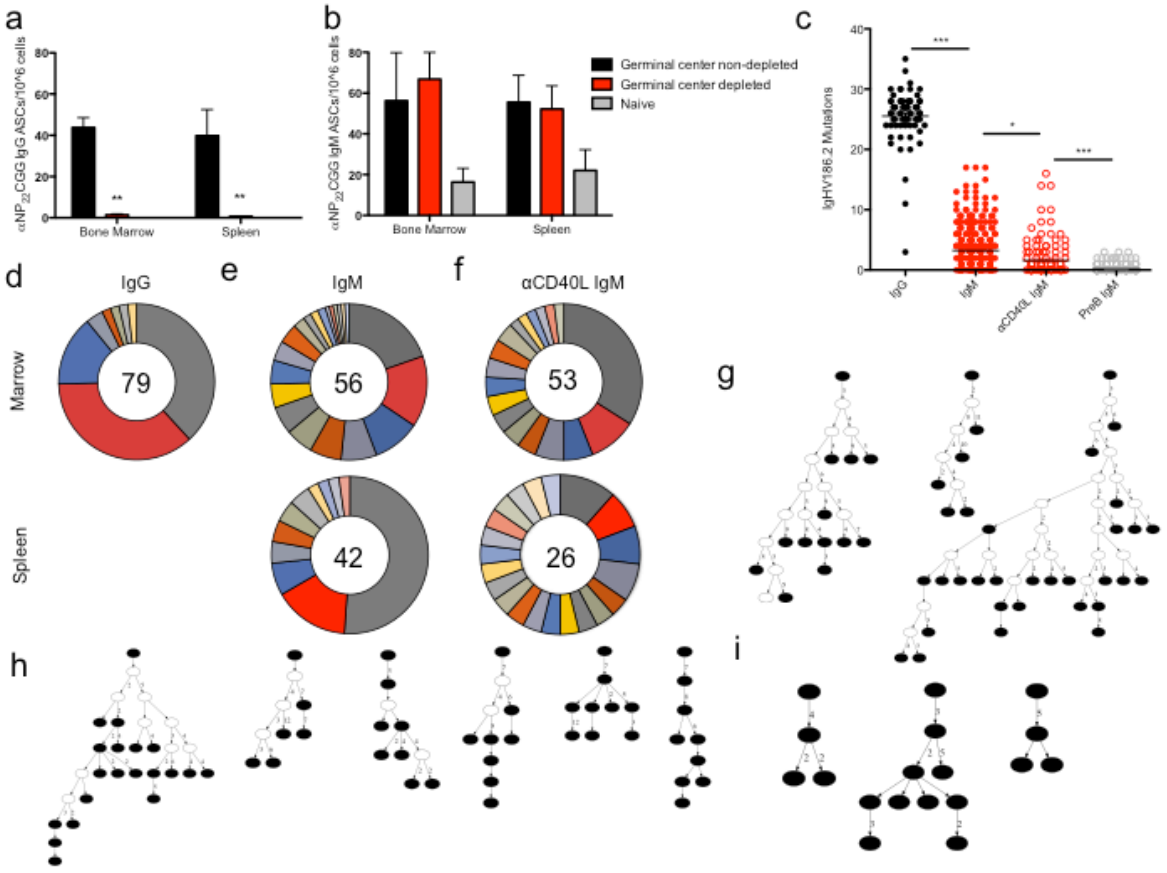


Figure 3: IgM plasma cells show evidence of somatic hypermutation, even when germinal centers are ablated. (a-b) NPCGG-specific IgG (■) and IgM (■) ASCs in mouse cohorts treated with either control IgG (n=5), αCD40L antibody to deplete germinal centers (n=7), or in age-matched naïve controls (■, n=3), as measured via ELISPOT at day 45 post-immunization. The mean (±SEM) is shown with ** indicating $p<0.005$. **(c)** IgG (●) and IgM IgHV186.2 sequence mutations in cohorts of C57/BL/6 mice at 1 and 3 months post-$NP_{22}CGG$ immunization (pooled), with (○) or without (●) αCD40L treatment, compared to total heavy chain sequences from pre-B cell unmutated controls (○) isolated from immunized C57/BL/6 cohorts (n=4). Spleen and bone marrow plasma cells are

pooled, and *= $p<0.05$ and ***= $p<0.001$. Refer to Supplementary Table 1 for sequence numbers. **(d)** Representative clonality of C57/BL/6 bone marrow IgG plasma cells post-immunization – each section represents a shared CDR3 junction as a percentage of total sequences as listed in the center. Representative clonality of C57/BL/6 IgM plasma cells in the spleen and bone marrow, treated with control Ig **(e)** or with αCD40L **(f)**. Ig lineage trees for IgG **(g)** and IgM plasma cells, treated with with control Ig **(h)** or with αCD40L **(i)**. Filled nodes represent germline or sample sequences, empty nodes indicate inferred precursors. Each line denotes either a single mutation between parent and daughter if no number is given.

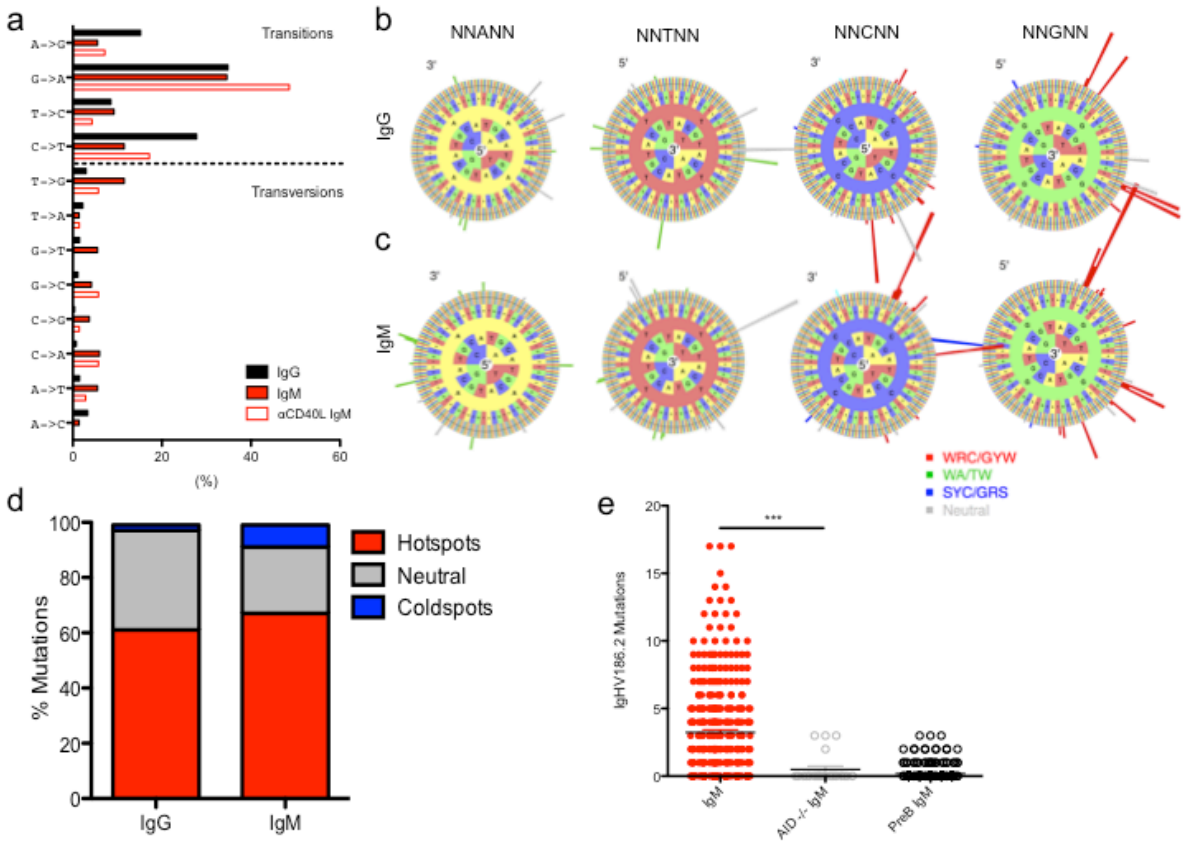


Figure 4: IgM heavy chain mutations are predominantly transitions and found in AID hotspot motifs, are AID-induced and AID-dependent. (a) Frequency of transition and transversion mutations for IgG (■) and IgM plasma cells, with (□) or without (■) αCD40L germinal center depletion. **(b-c)** Individual 5-mer mutabilities estimated from IgG and IgM sequences, respectively. Bars represent mutabilities of the central base as a function of the surrounding bases, read 5' to 3'. Green (WA/TW) and red (WRC/GYW) bars indicate hotspot motifs, grey the neutral sites, and blue the coldspots (SYC/GRS). Analysis was performed on total IgHV186.2 sequences for each population. **(d)** Percentage of total mutations found hotspot motifs (WRC/GYW or WA/TW) in red, neutral sites in grey, or coldspot motifs (SYC/GRS) in blue for IgG and IgM sequences. **(e)** IgM IgHV186.2

sequence mutations in cohorts of C57/BL/6 (●, n=4) or $AID^{-/-}$ (○, n=3) mice post-$NP_{22}CGG$ immunization, compared to total heavy chain sequences from pre-B cell unmutated controls (**O**) isolated from immunized C57/BL/6 cohorts. Spleen and bone marrow plasma cells are pooled, and *= $p<0.05$ and ***= $p<0.001$. Refer to Supplementary Table 1 for sequence numbers.

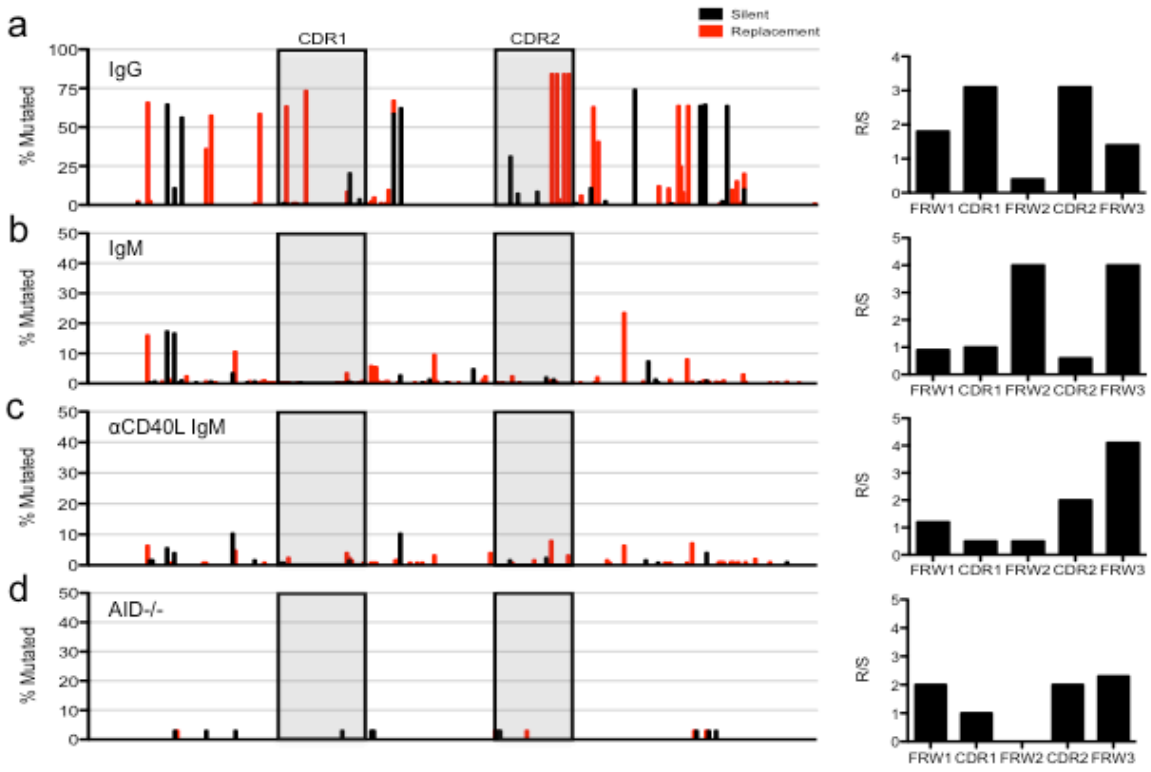


Figure 5: IgM heavy chain mutations are not enriched in CDR regions, unlike IgG, and show no evidence of antigen-selection. Mutational frequency or replacement (in red) and silent mutations are plotted for IgHV186.2 regions of IgG **(a)** and IgM **(b)** plasma cells, as well IgM plasma cells with αCD40L blockade **(c)**, and AID$^{-/-}$ IgM plasma cells **(d)**, with replacement to silent amino acid changes (R/S ratios) given to the right**.**

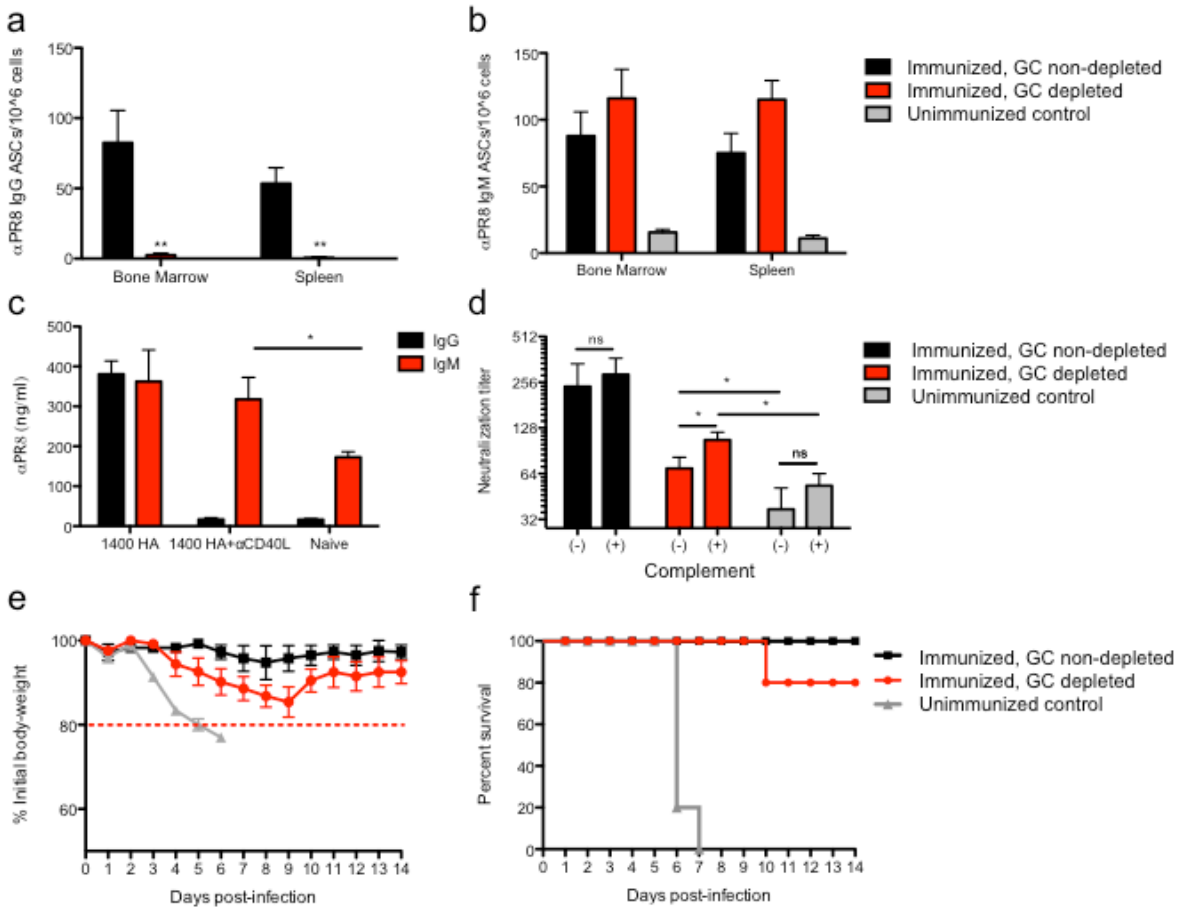


Figure 6: Germinal center independent IgM plasma cells are capable of neutralizing influenza virus *in vitro* and protecting the animal against infection *in vivo*. (a-b) PR8-specific IgG (■) and IgM (■) ASCs, respectively, as measured by ELISPOT from cohorts two months post-immunization with 1400 HA PR8 (n=5) - treated with control Ig or αCD40L at days 6, 8, and 10 to inhibit germinal centers – or age-matched naïve control mice (■, n=3). **(c)** Antigen-specific IgG (■) and IgM (■) titers as measured by serum ELISA at 150 days post-immunization, with or without αCD40L treatment. **(d)** Neutralization of PR8 virus by day 150 sera from PR8-immunized cohorts (n=5), with (■) or without (■)

αCD40L treatment, or age-matched naïve controls (■, n=3), with or without complement, and ** indicates $p<0.005$. **(e-f)** Mice immunized with A/PR/8/34 virus, with or without αCD40L treatment, were challenged with 10 x LD_{50} of virus 1 year post-immunization, with morbidity (body weight loss) and survival rate measured, respectively. The mean (±SEM) of 5 mice in each cohort is shown.

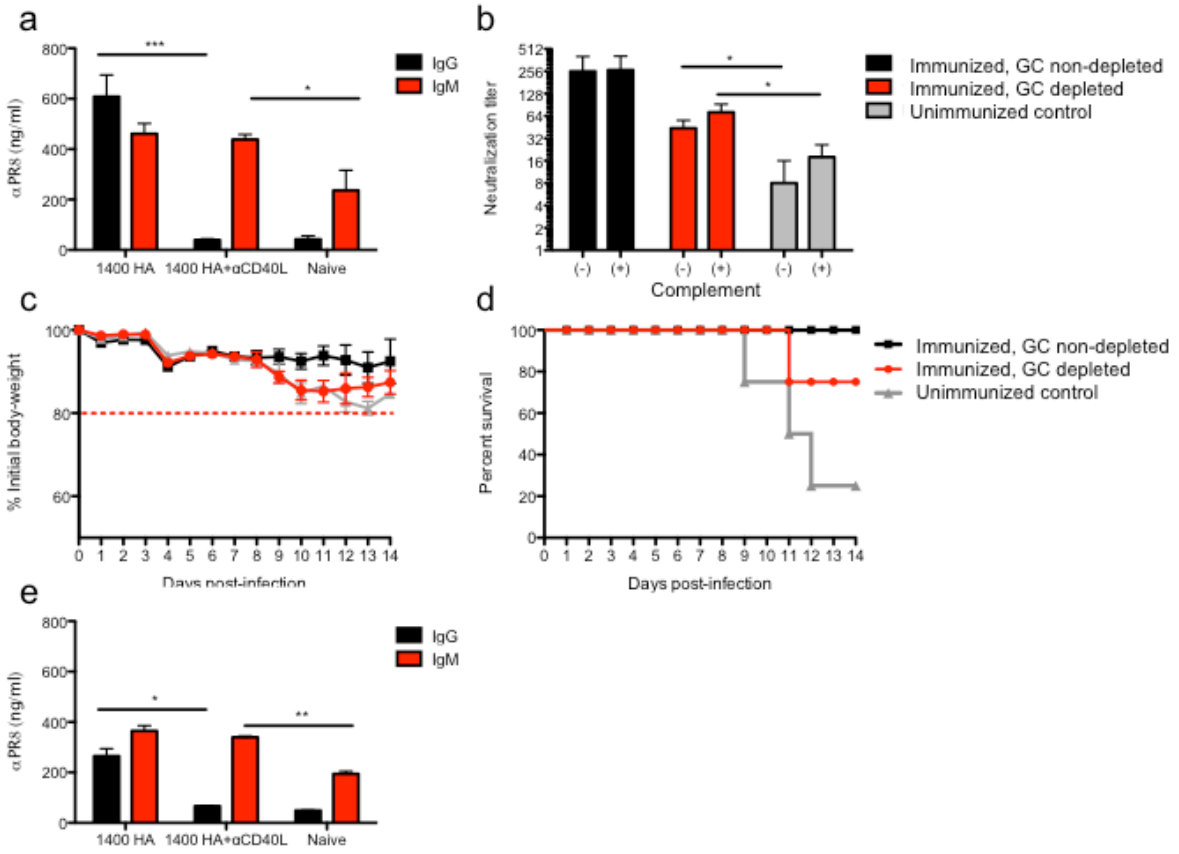
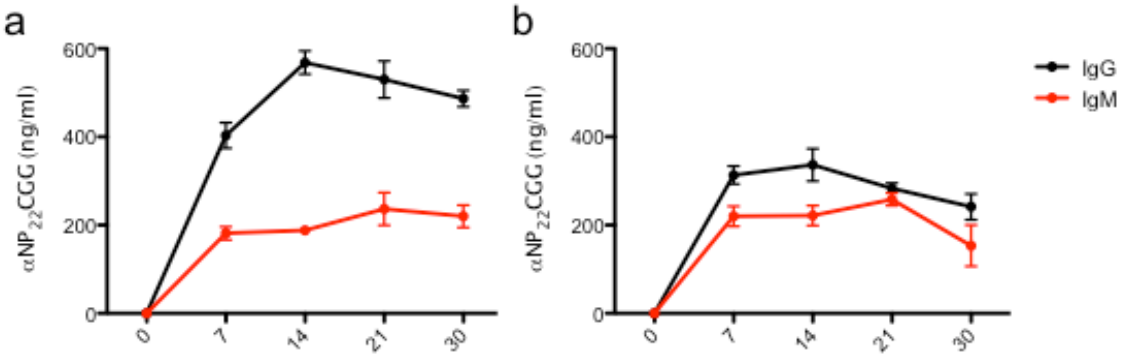


Figure 7: IgM plasma cells are capable of protecting the animal against infection *in vivo* in the absence of IgG plasma cells, memory B cells, and T cell help. (a) PR8-specific IgG (■) and IgM (■) titers, respectively, as measured via serum ELISA from cohorts at 1 month post-infection with 0.1 x LD_{50} PR8 virus - treated with control Ig or αCD40L at days 6, 8, and 10 to inhibit germinal centers – or age-matched naïve control mice (■, n=4). **(b)** Neutralization of PR8 virus by 1 months sera from PR8-immunized cohorts, with (■) or without (■) αCD40L treatment, or age-matched naïve controls (■, n=4), with or without complement. Mice immunized with A/PR/8/34 virus, with or without αCD40L treatment, were then treated with anti-CD4/CD8 at 1 and 3 days prior to infection and challenged

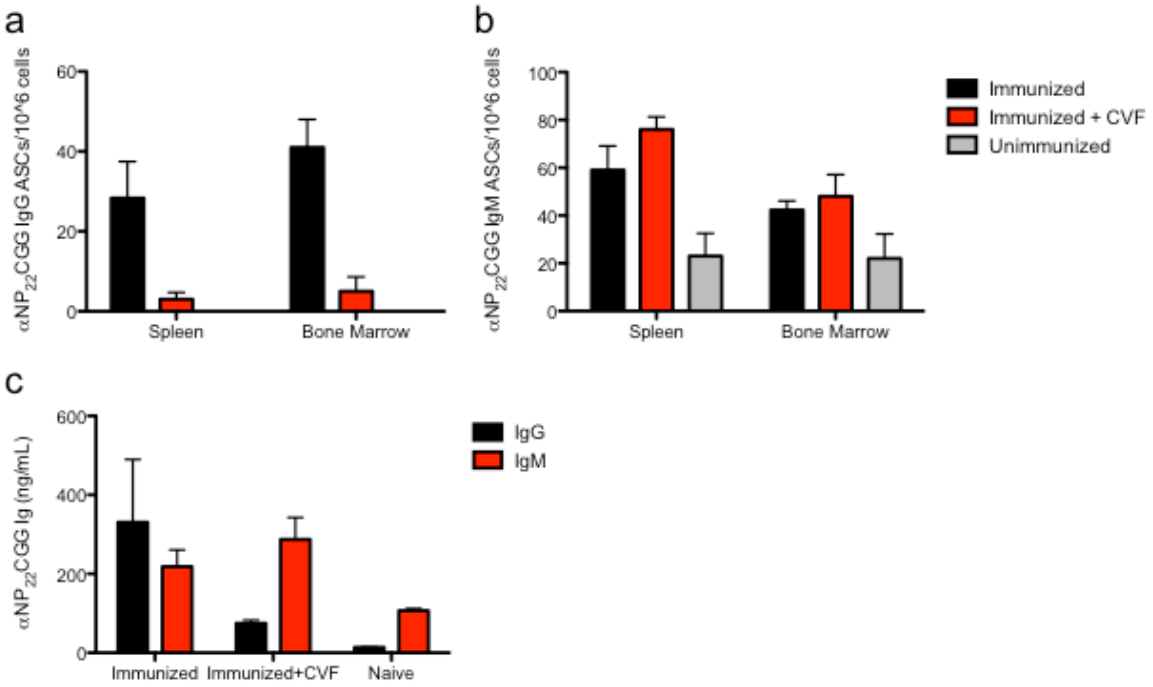
with 2 x LD_{50} of virus at 2 months post-immunization. **(c-d)**, Morbidity (body weight loss) and survival rate measured, respectively, post-PR8 challenge. The mean (±SEM) of 4 mice in each cohort is shown. (e) PR8-specific IgG (■) and IgM (■) titers, respectively, as measured via ELISA 14 days post-PR8 challenge.

			IgM		IgG	
			# of sequences	# of unique CDR3s	# of sequences	# of unique CDR3s
Wild type	Mouse 1	Spleen	18	8	N/A	N/A
	(1 month)	Marrow	161	14	66	6
	Mouse 2	Spleen	42	11	N/A	N/A
	(1 month)	Marrow	56	21	10	2
	Mouse 3	Spleen	32	14	N/A	N/A
	(3 months)	Marrow	15	5	9	2
	Mouse 4	Spleen	25	23	N/A	N/A
	(3 months)	Marrow	12	7	79	8
αCD40L depleted	Mouse 1	Spleen	26	21		
	(1 month)	Marrow	53	18		
	Mouse 2	Spleen	17	13		
	(1 month)	Marrow	34	19		
	Mouse 3	Spleen	27	17		
	(3 months)	Marrow	N/A	N/A		
	Mouse 4	Spleen	15	12		
	(3 months)	Marrow	2	2		
AID-/-	Mouse 1	Spleen	10	8		
	(1 month)	Marrow	3	3		
	Mouse 2	Spleen	15	10		
	(1 month)	Marrow	7	3		
	Mouse 3	Spleen	8	5		
	(1 month)	Marrow	3	1		

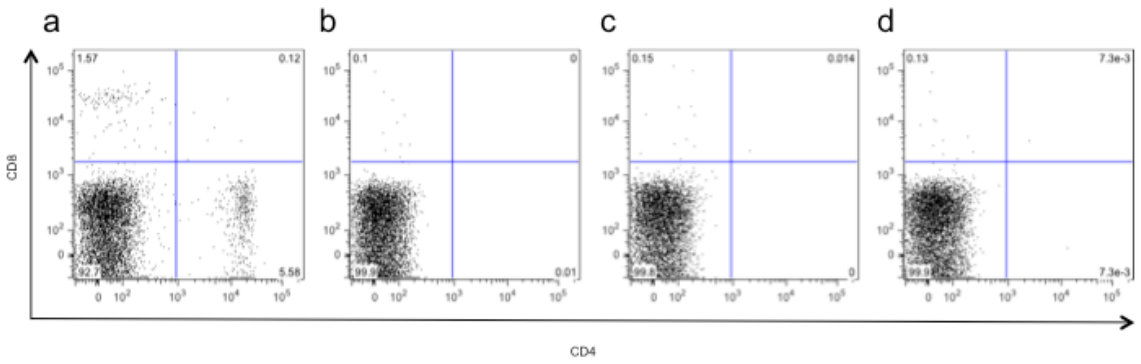
Table 1: Total heavy chain sequences with >90% homology to IgHV186.2. Total number of IgHV186.2 sequences and unique complementary determining region 3 (CDR3) sequences are shown from individual mice. Sequences were amplified from pooled $CD138^{+}B220^{-}$ plasma cells using a VH186.2-specific primer.



Supplementary Figure 1: Antigen-specific IgM and IgG titers persist, post-adoptive transfer of plasma cells into recipient Rag-/- mice. C57/BL mice were immunized with $NP_{22}CGG$, and then 2 months later 10,000 bone marrow or splenic plasma cells were sorted and adoptively transferred into Rag-/- mice. The recipients were then bled on days 7, 14, 21, and 30 and serum levels of anti-NP antibodies were measured by ELISA. **(a-b)** Antigen-specific serum IgG (●) and IgM (●) titers found in bone marrow or splenic plasma cell recipient mice (n=3), respectively, as measured by ELISA at days 7 through 30.



Supplementary Figure 2: Long-lived IgM, but not IgG, plasma cells are generated when germinal center formation is blocked by Cobra Venom Factor (CVF) treatment. Cohorts of mice were treated with CVF at 6 hours prior to and 7 days following $NP_{22}CGG$ immunization, to deplete complement and prevent germinal center formation. Untreated, immunized mice have intact germinal centers. **(a-b)** $NP_{22}CGG$-specific IgG and IgM ASCs, respectively, in immunized mouse cohorts (n=5), with (■) or without (■) CVF treatment, or in age-matched naïve controls (■, n=3), as measured via ELISPOT at day 45 post-immunization. **(c)** $NP_{22}CGG$-specific IgG (■) and IgM (■) antibodies in the sera as measured by ELISA. The mean (±SEM) is shown with * indicating $p<0.05$ and ** indicating $p<0.005$.



Supplementary Figure 3: Treatment with anti CD4 and CD8 antibodies leads to efficient depletion of CD4 and CD8 T cell. Representative flow plots of PBMC in control mice (n=3) and those treated with anti-CD4/CD8 Abs at 1 and 3 days prior to infection (n=4). **(a)** Untreated controls with healthy T cell populations. **(b-d)** Treated mice: PR8-immunized mice **(c)** with and **(b)** without αCD40L germinal center depletion, and **(d)** age-matched unimmunized controls.

LITERATURE CITED

1 Slifka, M. K., Antia, R., Whitmire, J. K. & Ahmed, R. Humoral immunity due to long-lived plasma cells. *Immunity* **8**, 363-372 (1998).

2 Hammarlund, E. *et al.* Duration of antiviral immunity after smallpox vaccination. *Nature medicine* **9**, 1131-1137, doi:10.1038/nm917 (2003).

3 Ho, F., Lortan, J. E., MacLennan, I. C. & Khan, M. Distinct short-lived and long-lived antibody-producing cell populations. *European journal of immunology* **16**, 1297-1301, doi:10.1002/eji.1830161018 (1986).

4 Di Noia, J. M. & Neuberger, M. S. Molecular mechanisms of antibody somatic hypermutation. *Annual review of biochemistry* **76**, 1-22, doi:10.1146/annurev.biochem.76.061705.090740 (2007).

5 Nutt, S. L., Hodgkin, P. D., Tarlinton, D. M. & Corcoran, L. M. The generation of antibody-secreting plasma cells. *Nature reviews. Immunology* **15**, 160-171, doi:10.1038/nri3795 (2015).

6 Gray, D., Dullforce, P. & Jainandunsing, S. Memory B cell development but not germinal center formation is impaired by in vivo blockade of CD40-CD40 ligand interaction. *The Journal of experimental medicine* **180**, 141-155 (1994).

7 Takahashi, Y., Dutta, P. R., Cerasoli, D. M. & Kelsoe, G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *The Journal of experimental medicine* **187**, 885-895 (1998).

8 Jacob, J., Miller, C. & Kelsoe, G. In situ studies of the antigen-driven somatic hypermutation of immunoglobulin genes. *Immunol Cell Biol* **70 (Pt 2)**, 145-152, doi:10.1038/icb.1992.19 (1992).

9 Victora, G. D. & Nussenzweig, M. C. Germinal centers. *Annual review of immunology* **30**, 429-457, doi:10.1146/annurev-immunol-020711-075032 (2012).

10 Wagner, S. D., Milstein, C. & Neuberger, M. S. Codon bias targets mutation. *Nature* **376**, 732, doi:10.1038/376732a0 (1995).

11 DiLillo, D. J. *et al.* Maintenance of long-lived plasma cells and serological memory despite mature and memory B cell depletion during CD20 immunotherapy in mice. *Journal of immunology* **180**, 361-371 (2008).

12 Choi, Y. S., Dieter, J. A., Rothaeusler, K., Luo, Z. & Baumgarth, N. B-1 cells in the bone marrow are a significant source of natural IgM. *European journal of immunology* **42**, 120-129, doi:10.1002/eji.201141890 (2012).

13 Reynolds, A. E., Kuraoka, M. & Kelsoe, G. Natural IgM is produced by CD5- plasma cells that occupy a distinct survival niche in bone marrow. *Journal of immunology* **194**, 231-242, doi:10.4049/jimmunol.1401203 (2015).

14 Fehr, T. *et al.* Antiviral protection and germinal center formation, but impaired B cell memory in the absence of CD19. *The Journal of experimental medicine* **188**, 145-155 (1998).

15 Fukuda, T. *et al.* Disruption of the Bcl6 gene results in an impaired germinal center formation. *The Journal of experimental medicine* **186**, 439-448 (1997).

16 Teng, G. & Papavasiliou, F. N. Immunoglobulin somatic hypermutation. *Annu Rev Genet* **41**, 107-120, doi:10.1146/annurev.genet.41.110306.130340 (2007).

17 Jacob, J., Przylepa, J., Miller, C. & Kelsoe, G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. III. The kinetics of V region mutation and selection in germinal center B cells. *The Journal of experimental medicine* **178**, 1293-1307 (1993).

18 Toellner, K. M. *et al.* Low-level hypermutation in T cell-independent germinal centers compared with high mutation rates associated with T cell-dependent germinal centers. *The Journal of experimental medicine* **195**, 383-389 (2002).

19 Alamyar, E., Duroux, P., Lefranc, M. P. & Giudicelli, V. IMGT((R)) tools for the nucleotide analysis of immunoglobulin (IG) and T cell receptor (TR) V-(D)-J repertoires, polymorphisms, and IG mutations: IMGT/V-QUEST and IMGT/HighV-QUEST for NGS. *Methods in molecular biology* **882**, 569-604, doi:10.1007/978-1-61779-842-9_32 (2012).

20 Zhang, M., Srivastava, G. & Lu, L. The pre-B cell receptor and its function during B cell development. *Cellular & molecular immunology* **1**, 89-94 (2004).

21 Sanz, I. Multiple mechanisms participate in the generation of diversity of human H chain CDR3 regions. *Journal of immunology* **147**, 1720-1729 (1991).

22 Barak, M., Zuckerman, N. S., Edelman, H., Unger, R. & Mehr, R. IgTree: creating Immunoglobulin variable region gene lineage trees. *Journal of immunological methods* **338**, 67-74, doi:10.1016/j.jim.2008.06.006 (2008).

23 Bransteitter, R., Pham, P., Scharff, M. D. & Goodman, M. F. Activation-induced cytidine deaminase deaminates deoxycytidine on single-stranded DNA but requires the action of RNase. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 4102-4107, doi:10.1073/pnas.0730835100 (2003).

24 Chaudhuri, J. *et al.* Transcription-targeted DNA deamination by the AID antibody diversification enzyme. *Nature* **422**, 726-730, doi:10.1038/nature01574 (2003).

25 Golding, G. B., Gearhart, P. J. & Glickman, B. W. Patterns of somatic mutations in immunoglobulin variable genes. *Genetics* **115**, 169-176 (1987).

26 Yaari, G. *et al.* Models of somatic hypermutation targeting and substitution based on synonymous mutations from high-throughput immunoglobulin sequencing data. *Frontiers in immunology* **4**, 358, doi:10.3389/fimmu.2013.00358 (2013).

27 Cohen, R. M., Kleinstein, S. H. & Louzoun, Y. Somatic hypermutation targeting is influenced by location within the immunoglobulin V region. *Molecular immunology* **48**, 1477-1483, doi:10.1016/j.molimm.2011.04.002 (2011).

28 Honjo, T., Kinoshita, K. & Muramatsu, M. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. *Annual review of immunology* **20**, 165-196, doi:10.1146/annurev.immunol.20.090501.112049 (2002).

29 Potter, C. W. & Oxford, J. S. Determinants of immunity to influenza infection in man. *British medical bulletin* **35**, 69-75 (1979).
30 Taylor, J. J., Pape, K. A. & Jenkins, M. K. A germinal center-independent pathway generates unswitched memory B cells early in the primary response. *The Journal of experimental medicine* **209**, 597-606, doi:10.1084/jem.20111696 (2012).
31 Kurosaki, T., Kometani, K. & Ise, W. Memory B cells. *Nature reviews. Immunology* **15**, 149-159, doi:10.1038/nri3802 (2015).
32 Sze, D. M., Toellner, K. M., Garcia de Vinuesa, C., Taylor, D. R. & MacLennan, I. C. Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival. *The Journal of experimental medicine* **192**, 813-821 (2000).
33 Choi, Y. S. & Baumgarth, N. Dual role for B-1a cells in immunity to influenza virus infection. *The Journal of experimental medicine* **205**, 3053-3064, doi:10.1084/jem.20080979 (2008).
34 Pape, K. A., Taylor, J. J., Maul, R. W., Gearhart, P. J. & Jenkins, M. K. Different B cell populations mediate early and late memory during an endogenous immune response. *Science* **331**, 1203-1207, doi:10.1126/science.1201730 (2011).
35 William, J., Euler, C., Christensen, S. & Shlomchik, M. J. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* **297**, 2066-2070, doi:10.1126/science.1073924 (2002).
36 Di Niro, R. *et al.* Salmonella Infection Drives Promiscuous B Cell Activation Followed by Extrafollicular Affinity Maturation. *Immunity* **43**, 120-131, doi:10.1016/j.immuni.2015.06.013 (2015).
37 Weller, S. *et al.* CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 1166-1170, doi:10.1073/pnas.98.3.1166 (2001).
38 Harada, Y., Muramatsu, M., Shibata, T., Honjo, T. & Kuroda, K. Unmutated immunoglobulin M can protect mice from death by influenza virus infection. *J Exp Med* **197**, 1779-1785, doi:10.1084/jem.20021457 (2003).
39 Skountzou, I. *et al.* Influenza virus-specific neutralizing IgM antibodies persist for a lifetime. *Clinical and vaccine immunology : CVI* **21**, 1481-1489, doi:10.1128/CVI.00374-14 (2014).
40 Throsby, M. *et al.* Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. *PLoS One* **3**, e3942, doi:10.1371/journal.pone.0003942 (2008).
41 Racine, R. *et al.* IgM production by bone marrow plasmablasts contributes to long-term protection against intracellular bacterial infection. *Journal of immunology* **186**, 1011-1021, doi:10.4049/jimmunol.1002836 (2011).
42 Bortnick, A. *et al.* Long-lived bone marrow plasma cells are induced early in response to T cell-independent or T cell-dependent antigens. *Journal of immunology* **188**, 5389-5396, doi:10.4049/jimmunol.1102808 (2012).
43 Diamond, M. S. *et al.* A critical role for induced IgM in the protection against West Nile virus infection. *The Journal of experimental medicine* **198**, 1853-1862, doi:10.1084/jem.20031223 (2003).

44 Ilag, L. L. Immunoglobulin M as a vaccine adjuvant. *Medical hypotheses* **77**, 473-478, doi:10.1016/j.mehy.2011.06.013 (2011).

45 Harte, P. G., Cooke, A. & Playfair, J. H. Specific monoclonal IgM is a potent adjuvant in murine malaria vaccination. *Nature* **302**, 256-258 (1983).

46 Yoshida, T. *et al.* Memory B and memory plasma cells. *Immunological reviews* **237**, 117-139, doi:10.1111/j.1600-065X.2010.00938.x (2010).

47 Akula, S., Mohammadamin, S. & Hellman, L. Fc receptors for immunoglobulins and their appearance during vertebrate evolution. *PloS one* **9**, e96903, doi:10.1371/journal.pone.0096903 (2014).

48 Conticello, S. G., Thomas, C. J., Petersen-Mahrt, S. K. & Neuberger, M. S. Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases. *Molecular biology and evolution* **22**, 367-377, doi:10.1093/molbev/msi026 (2005).

49 Hinds-Frey, K. R., Nishikata, H., Litman, R. T. & Litman, G. W. Somatic variation precedes extensive diversification of germline sequences and combinatorial joining in the evolution of immunoglobulin heavy chain diversity. *The Journal of experimental medicine* **178**, 815-824 (1993).

50 Wilson, M. *et al.* What limits affinity maturation of antibodies in Xenopus--the rate of somatic mutation or the ability to select mutants? *The EMBO journal* **11**, 4337-4347 (1992).

51 Charan, J. & Kantharia, N. D. How to calculate sample size in animal studies? *Journal of pharmacology & pharmacotherapeutics* **4**, 303-306, doi:10.4103/0976-500X.119726 (2013).

52 Tiller, T., Busse, C. E. & Wardemann, H. Cloning and expression of murine Ig genes from single B cells. *Journal of immunological methods* **350**, 183-193, doi:10.1016/j.jim.2009.08.009 (2009).

53 Skountzou, I. *et al.* Immunity to pre-1950 H1N1 influenza viruses confers cross-protection against the pandemic swine-origin 2009 A (H1N1) influenza virus. *Journal of immunology* **185**, 1642-1649, doi:10.4049/jimmunol.1000091 (2010).

54 Penaloza-MacMaster, P., Provine, N. M., Blass, E. & Barouch, D. H. CD4 T Cell Depletion Substantially Augments the Rescue Potential of PD-L1 Blockade for Deeply Exhausted CD8 T Cells. *Journal of immunology* **195**, 1054-1063, doi:10.4049/jimmunol.1403237 (2015).

Chapter 3

B1b B cells give rise to long-lived IgM plasma cells early in immune response

Manuscript currently in submission.

Caitlin Bohannon performed all of the experiments in this manuscript.

Caitlin Bohannon and Joshy Jacob wrote the manuscript.

ABSTRACT

Long-lived plasma cells are critical to long-term host protection as a source of antigen-specific neutralizing antibodies. B1 B cells also play an essential role in the neutralization and clearance of pathogens, spontaneously secreting low-affinity natural IgM. Previously, our lab has characterized a novel population of induced, antigen-specific, long-lived IgM plasma cells, which are distinct from natural IgM cells. Here we demonstrate that these cells are derived from the B1 precursors, rather than follicular B2 cells, that they localize to the spleen *in vivo*, and that they arise early in the immune response following antigen stimulation.

INTRODUCTION

B cells play a critical role in the immune system as producers of neutralizing antibodies. Two distinct lineages comprise the B cell compartment in humans and in mice. The largest population, B2 B cells, includes bone-marrow derived follicular B cells, sometimes referred to as conventional B cells, as well as marginal zone B cells[1]. B1 B cells, divided into B1a (CD5+) and B1b (CD5-) classifications, are predominantly thought of as fetal in origin. Follicular B cells are key to the adaptive immune response, while other subsets produce more innate-like responses. B1 cells in particular are responsible for the spontaneous secretion of broadly reactive, natural antibodies, and are maintained in the steady state even in the absence of microorganisms[2,3]. These cells do not require T cell help nor do they require BCR stimulation, and are the predominant B cell population in the pleural and peritoneal cavities – though they may also be found in spleen and a low frequencies within the bone marrow. During adulthood, their numbers are largely maintained by self-renewal, and they do not expand in response to BCR stimulation. B2 cells, by contrast, outnumber B1 cells within the spleen and bone marrow, and can be reconstituted from bone marrow precursors[4]. Follicular B cells clonally expand and produce antigen-specific responses following BCR stimulation and can differentiate into high-affinity memory populations[1].

Humoral memory is key to long-term protection against pathogens, and plasma cells are the key source of long-lived antibody production[5]. Plasma cells develop from the antigen-specific B cell response through one of two pathways, differentiating into either short-lived or long-lived plasma cells[5,6]. Without the aid of T cells, specific B cells can immediately differentiate into short-lived plasma cells

that exist transiently in these spleen and lymph nodes. Long-lived plasma cells, which can survive as long as the lifetime of the host, primarily develop with the aid of T cells. T cells within the secondary lymphoid organs initiate germinal center formation shortly following exposure to antigen. The germinal center is fully established by day 7 and during this process B cells undergo affinity maturation and class-switching. From this, high-affinity plasmablasts emerge, homing to survival niches within the bone marrow where they terminally differentiate into long-lived plasma cells[5-7].

The initial homing of mature B cells to the secondary lymphoid organs occurs through upregulation of the CXCR5 and CCL7 receptors on the surface of B cells, which home to, respectively, CXCL13 (BLC) and CCL19 and CCL21 (SLC) in the spleen and lymph nodes. These receptors are then downregulated in the transition to plasma cells as they migrate to survival niches[8]. CXCR4, the major determinant of plasma cell localization and also critical to plasma cell survival, is found to be upregulated on plasmablasts and plasma cells[9]. CXCR4 responds to CXCL12, or SDF-1, which is constitutively expressed by stromal cells in the bone marrow, medullary cords, and the red pulp of the spleen – all sites of plasma cell retention, whether short- or long-lived[8]. Stromal cells in the bone marrow also produce MCP-1, and the cognate receptor, CCR2, is found on bone marrow resident plasma cells[9,10]. Once localized within the survival microenvironment, plasma cells are thought to lose their migratory capacity, though CXCR4 still plays a key role[11]. IL-6R is also critical to the differentiation, survival, and IgG production of plasma cells, responding to IL-6 secreted by T cells and macrophages, as well osteoblasts within

the bone marrow microenvironment[12,13]. Within the spleen, IL-5 is highly expressed, which promotes survival and antibody production in B1 populations[9,14].

Much of what we know about long-lived plasma cell populations comes from the study of those populations within the bone marrow, but significant numbers of long-lived plasma cells do remain in the secondary lymphoid organs such, including the spleen[5]. Our initial studies have shown a significant population of antigen-specific, long-lived plasma cells in murine spleens, and these cells predominantly express IgM rather than IgG (in press). Here we demonstrate that long-lived IgM plasma cells arise early in the immune response from B1b, rather than B2 cells. When naive B1b cells and B2 are adoptively transferred into B cell deficient uMT mice and then immunized, only B1b cells give rise to long-lived IgM plasma cells – even in the absence of germinal center formation. These cells are distinct from long-lived IgG plasma cell populations, expressing the Il-5R marker of B1 lineage as well as splenic survival, rather than bone marrow homing receptor CCR2, and they are commonly found *in situ* in the red pulp of the spleen. They also appear to develop prior to IgG plasma cells, possibly as early as day 4 post-stimulation, as seen when early plasmablasts are adoptively transferred into uMT mice. This early development suggests a significant, non-redundant role for long-lived IgM plasma cells in the humoral response.

RESULTS

Long-lived IgM plasma cells require T cell help

We first sought to determine whether T cell were required for the development of long-lived IgM plasma cells as they are for IgG plasma cells. We achieved this using two different methods. The first was accomplished through blockade of B and T cells interactions via αCD40L antibody treatment, which has been shown to disrupt formation of long-lived IgG plasma cells[15]. Our lab has shown previously that αCD40L blockade around day 7, which prevents germinal center formation but not early T cell help, does not affect the formation of long-lived IgM plasma cells (in press). In this experiment, 300 μg of αCD40L were given at days 2, 4, and 6 post-immunization with $NP_{22}CGG$ – blocking early T cell help as well. At day 45 post-immunization we measured the number of IgG and IgM antibody-secreting cells (ASCs) in the spleen and bone marrow of treated and control animals. In untreated, immunized animals we see both long-lived IgG and IgM plasma cells, significantly above the numbers seen in unimmunized cohorts (Fig. 1a,b). However, with αCD40L treatment at this early timepoint we do observe depletion of both IgG and IgM ASCs – with numbers similar to what we see in unimmunized controls. This distinguishes long-lived, induced IgM plasma cells from short-lived plasma cells and natural IgM cells, which do not require T cell help.

Long-lived IgM plasma cells are found in the red pulp of the spleen

Next we looked at the *in situ* localization of these plasma cells. At several months post-immunization, we have previously demonstrated that long-lived IgM plasma cells are most commonly found in the spleen (in press). Here we observe them *in situ*

via immunohistochemistry of frozen splenic sections. Overall we found that $CD138^{+}B220^{-}$ plasma cells, whether IgM-expressing or not, localized in the red pulp (as defined by Ter-119 staining) of murine spleens at the memory time point (Fig. 2a). IgM-expressing plasma cells were further shown to be NPCGG-binding in 3 months post-immunized mice but not in naïve controls, further distinguishing them from spontaneous natural IgM cells (Fig 2b). Taken together, our data shows that persistent and antigen-specific IgM plasma cells primarily localize in the red pulp of the spleen in immunized animals.

Long-lived IgM plasma cells express IL5R, not CCR2

Over the course of plasma cell development, the regulation of chemokine reception expression is critical to advancing the cell along its path and homing to the survival niche. In order to understand these differences in plasma cell subset homing, we compared the expression of some of the key chemokine receptors on these subsets by flow cytometry 3 months post $NP_{22}CGG$ immunization. Gating for $CD138^{+}B220^{-}$ plasma cells, and further for those NPCGG-specific cells and either IgG or IgM-expression, we measured the level of expression of CXCR4, CCR2, IL-6R, and IL-5R on the surface (Fig. 3), as well CCR7 and CXCR5 (data not shown). CXCR4 expression, linked toboth bone marrow and splenic homing as well as continued survival in both locations, is consistent across both niches and isotypes (Fig. 3a,b). CCR2, a marker of terminally differentiated plasma cells and bone marrow homing, is noticeably upregulated in IgG plasma cells in both the spleen and bone marrow (Fig. 3c,d). Conversely, CCR7, typically downregulated in plasma cells and a marker of secondary lymphoid homing B cells, appears to be more highly expressed by IgM+

cells in both the spleen and bone marrow. CCR7 and CXCR5, as anticipated, were found to be downregulated on all plasma cell subsets and no significant differences were found (data not shown). These data correlate with the preferentially homing of IgG plasma cells to the bone marrow, and the persistence of IgM plasma cells in the spleen. Similarly, both IgG and IgM express IL-6R, a key survival receptor, though we observe slightly less on the surface of IgM plasma cells (Fig. 3e,f). Instead, we found high levels of IL-5R expression on long-lived IgM plasma cells, significantly above expression on IgG plasma cells (Fig. 3g,h). This may contribute to their persistence in the spleen, and is it also suggestive that these cells could potentially be of B1 rather than B2 lineage. Overall, this differential expression of homing and survival receptors points to a potentially unique role for long-lived, IgM plasma cells.

Long-lived IgM plasma cells are B1b lineage

Next we sought to determine whether long-lived IgM plasma cells originated, by adoptively transferring naïve B1 and B2 cells into B cell deficient animals to follow their response to immunization. $B220^{+}CD138^{-}IgM^{lo}CD43^{-}CD23^{+}CD5^{-}$ B2 cells or $B220^{+}CD138^{-}IgM^{hi}CD43^{+}CD23^{-}CD5^{-}$ B1b cells were sorted from C57BL/6 murine spleens and transferred into sublethally irradiated uMT mice. Insufficient B1a cells were collected from the spleen, making them a less likely candidate for populating the sizeable long-lived IgM plasma cell compartment. B1b and B2 recipient mice were then immunized with PR8 virus, and half were treated with αCD40L antibody at days 6, 8, and 10 to prevent germinal center formation. PR8-specific IgG and IgM in the sera was measured at 1 and 2 months post-transfer, and PR8-specific IgG and IgM ASCs were measured in the spleens and bone marrow of recipient mice at the 2

month timepoint. Following immunization, and without αCD40L treatment, we found that B2 recipients predominantly had IgG ASCs in the bone marrow, indicating that they are not the source of long-lived IgM plasma cells (Fig. 4a,b). B1b recipients also had IgG ASCs in the bone marrow, but additionally had IgM ASCs in both the spleen and bone marrow. Thus, long-lived IgM plasma cells are derived from B1, rather than B2, populations, and are very likely from B1b cells specifically. This is further supported by our ELISA data, which indicates that B2 recipients lacked significant IgM, and B1b recipients have both PR8-specific IgG and IgM (Fig. 3c,d). Following αCD40L, IgG in the serum and IgG ASCs are depleted (Fig. 3e-h). But B2 populations did not give rise to a significant number of IgM ASCs in the absence of germinal center formation; they were still predominantly found in B1b recipient mice (Fig. e-f). This is again confirmed by ELISA results (Fig. g,h). Thus B1b cells, rather than B2, are the source of long-lived IgM plasma cells, even in the absence of germinal center formation.

Long-lived IgM plasma cells develop prior to IgG plasma cells

Finally, we wished to determine whether long-lived IgM plasma cells developed prior to or following germinal center formation, after which class-switching could take place. To do this, we followed the development of pre- (day 4) and post- (day 7) germinal center plasmablasts into plasma cells. Following NP_{22}CGG immunization, $5x10^5$ day 4 or day 7 $CD138^+B200^+$plasmablasts were sorted from C57BL/6 mice and transferred into uMT recipients (Fig. 5a). Plasmablasts from unimmunized animals were transferred to another cohort as a control. When measured by ELISPOT, plasmablasts from unimmunized animals do not show any evidence of NP_{22}CGG-

specific ASCs, compared to the significant number of IgM ASCs from day 4 sorted plasmablasts. This ensures that natural IgM cells are excluded from the transfer. NP22CGG-specific IgG is only found in day 7 recipients, and not in control plasmablasts or day 4 cohorts (Fig. 5c). Though there is a small amount of IgM found in control plasmablast recipients, significantly more is found in day 4 recipients, and furthermore is seen in day 7 recipients (Fig. 5d). IgM from both day 4 and day 7 transfers persists 45 days post-transfer, suggesting that these plasma cells are long-lived. This also suggests that long-lived IgM plasma cells may arise as early as day 4 post-immunization, prior to germinal center formation and the development of long-lived IgG plasma cells.

DISCUSSION

Here we have determined that long-lived IgM plasma cells are phenotypically and functionally distinct from IgG plasma cells. They persist within the red pulp of the spleen and express markers of splenic survival and B1 lineage (IL-5R), rather than bone marrow homing (CCR2). They are derived from B1 rather than B2 populations, and develop even in the absence of germinal center formation, though they do require T cell help. Additionally, our data suggests that long-lived IgM plasma cells develop prior to germinal center formation, and before the IgG plasma cell response. Overall the B1-derived long, lived IgM plasma cell response ensures early and continuous antibody secretion, making this population a significant and non-redundant aspect of humoral immunity.

This further is supported by similar findings of antigen-specific IgM induced in B1 populations. B1 cells have long been characterized as part of the innate arm of the immune system, and they were not thought to respond or proliferate in the presence of antigen stimulation, and instead secreting natural IgM spontaneously, or through innate, BCR-independent signals such as LPS stimulation[1,16,17]. Baumgarth and colleagues have reported that B1a cells can selectively accumulate in response to influenza infection, but do not find evidence of clonal expansion[18]. However, modest expansion of B1a populations has been seen in response to *F. tularensis*, likely through innate (LPS) rather than BCR signaling[19]. Similarly, B1b cells have also been shown to expand in response to *B. hermsii* infection[20]. Our studies have additionally found evidence of B1b cells expanding in response to stimulation with anti-IgM, rather than LPS (Supplementary Figure 1).

Furthermore, studies of B1a and B1b responses to *S. pneumoniae* suggest that B1b cells may play a role in adaptive immunity, producing long-lasting, antigen-specific antibody in response to stimulation[21]. As we observe in our own adoptive transfer experiments, they discovered that antigen-specific IgM was present in deficient mice reconstituted with peritoneal B1b, but not B2 cells. In addition, they were able to demonstrate the presence of natural antibodies in mice transferred with B1a B cells. However, they did not find that splenic B cells provided a significant IgM response, likely because the majority of B cells in the spleen are B2 rather the B1 lineage, nor did they follow recipients past day 14. Consistent with this finding, other groups have reported long-lasting IgM titers in response to both *F. tularensis* and NP-Ficoll, suggesting the possibility of long-lived IgM plasma cells[19,22,23]. Our experiments followed B1b and B2 recipient mice for 2 months post-immunization, and additionally show that virus-specific IgM ASCs were present at 2 months post-immunization in B1b recipients, confirming that these IgM-secreting cells are indeed long-lived.

IgM is critical for host protection early in infection. It is the primary component of the T-independent response, and is also expressed early in response to T-dependent antigens. Natural IgM from B1a cells is present at high levels in the steady state and makes majority of circulating IgM, but immune IgM can also be generated early in response to antigen stimulation. Natural IgM is an essential part of the innate defenses against pathogens, essential to both neutralization and clearance[17,23]. Because of this, natural IgM antibodies are often polyreactive. Despite their low affinity, the polymeric nature of IgM means it binds a diverse array of antigens with high avidity. Conversely, immune IgM is antigen-specific. It is secreted by B2 cells

very early in the immune response, prior to class-switching, but recent evidence, including our own research, has also demonstrated that B1 cells may be a significant source of long-lasting immune IgM[23]. In addition, secreted IgM is critical to promoting a healthy IgG response in B2 cells, and thus is necessary at a very early stage in the immune response[24,25]. In the absence of secreted IgM, IgG antibody responses are significantly impaired[25]. This is potentially due the lack of IgM antibody-antigen complexes that mediated presentation follicular B cells[26,27]. Thus we are not surprised to find that even long-lived IgM plasma cells are induced early in the immune response to a T-dependent antigen, NP-CGG, prior to germinal center formation and before IgG plasma cells. In this way IgM contributes both directly and indirectly to long-lasting humoral memory from the earliest timepoints.

Because the B1 cells, and not B2, are generated prior to and in the first few weeks following birth, an increased understanding of these cells is also critical to overcoming the obstacles that face infant immunization. The immune system of newborns is often considered immature in comparison to the adult immune system[28]. B1a cells, in particular, make up the majority of the B cell compartment in infants. These cells are able to secrete natural IgM in response to innate stimulation[1], but whether infants are able to generate adaptive, long-lasting responses to pathogens is still a subject of debate[28,29]. This may be one potential reason that infants often respond poorly to vaccination, usually requiring multiple boosts; in addition to the significant barrier that maternal antibody poses[29]. Vaccination of aging patients and maintaining long-lasting immunity in the elderly is similarly difficult because of the loss of IgG plasma cells[30]. Enhancing early IgM production by B1 cells may prove key to rational vaccine design.

B1 cells and polyreactive IgM are also often linked with autoimmunity. B1 cells have been shown to be enriched for poly- and self-reactive antibodies of low affinity[1]. Expanded populations of B1 cells are also linked to several B cell mediated autoimmune disorders, including lupus, atherosclerosis, arthritis, and rheumatism[31-33]. IgM itself is linked to a number of autoimmune diseases, including those previously listed as well as hyperIgM syndrome, caused by a defect in CD40L expression[34]. But B1 cells and IgM are also involved with tissue homeostasis and regulatory functions, and in some cases depletion of the B cell compartment or secreted IgM has been shown to worsen the progression of autoimmune disease[35,36]. A better understanding of B1 development and differentiation into long-lived IgM plasma cells can further help us to enhance vaccine design and target autoimmunity in patients.

METHODS AND MATERIALS

Mice

C57BL/6 and NOD.129S2(B6)-*Cd28tm1Mak*/JbsJ (CD28$^{-/-}$) mice were purchased from The Jackson Laboratory. All mice were maintained in a specific pathogen-free facility in accordance with the institutional guidelines of The Animal Care and Use Committee at Emory University. All controls were age- and sex-matched.

Immunizations and infections

Cohorts of female C57BL/6 or CD28-/- mice were immunized intraperitoneally with 50μg hydroxy-3-nitrophenylacetyl chicken-γ-globulin ($NP_{22}CGG$) (Biosearch Technologies) with 50μl alum in PBS, or intramuscularly with 1400 HA of A/Puerto Rico/8/1934 (PR8) virus. Sera, bone marrow, and spleens were collected and analyzed at given time points post-immunization.

Disruption of early T cell help

Cohorts of mice were treated with either 300 μg αCD40L (clone MR-1) antibody or control IgG at days 2, 4, and 6 post-$NP_{22}CGG$ immunization to prevent early T help. At 45 days post-immunization, cells isolated from the spleen and bone marrow were assayed for the presence of IgG and IgM secreting cells by ELISPOT as described below.

Plasma cell ELISPOTs

Plates were coated with 20 μg/ml $NP_{22}CGG$ or 512 HA PR8 and blocked with complete RPMI (10% FBS, 1% penicillin/ streptavidin, 1% HEPES, and 50μM 2-

mercaptoethanol). Cells isolated from spleens and bone marrow of immunized mice were serially diluted and incubated overnight at 37°C. The plates were then treated with either anti-IgG- or -IgM-biotin (Southern Biotechnology) followed by incubation with streptavidin-alkaline phosphatase (Sigma). Plates were then developed with 5-bromo-4-chloro-3-indolylphosphate (Sigma) until spots developed, and spots counted with CTL Immunospot® software.

ELISA

Plates were coated with 20 μg/ml NP_{22}CGG or 512 HA PR8 and blocked with BSA. Sera were serially diluted alongside standard controls. The plates were then treated with either anti-IgG-HRP or IgM-biotin followed by incubation with streptavidin-HRP (Southern Biotechnology). Plates were developed with 1x TMB substrate solution (ebioscience), and absorbance measured by Gen5 by BioTek software.

Immunostaining and cell sorting

Immunostaining of murine splenocytes and bone marrow cells was preformed as previously described[37]. Briefly, cells or frozen tissue sections were stained by incubating with CD138 (BioLegend), B220 (BioLegend), IgM (Abcam), NPCGG (Biosearch Technologies), Ter-119 (ebioscience), CD23 (BD), CD43 (ebioscience), CD5 (BioLegend), CCR2 (R&D Systems), CXCR4 (BD), CCR7 (ebioscience), CXCR5 (BD), IL5R (ebioscience), and/or IL6R (ebioscience) for 30 minutes prior to flow analysis or sorting. Intracellular staining for flow cytometry was preformed following fixation and permeabilization (BD kit), and cells were then incubated with NPCGG, IgM, and/or IgG (Abcam).

Adoptive transfer of B lineages

5x10^5 B220$^+$CD138$^-$IgM^{lo}CD43$^-$CD23$^+$CD5$^-$ B2 cells and B220$^+$CD138$^-$IgM^{hi}CD43$^+$CD23$^-$CD5$^-$ B1b cells were sorted from naïve C57BL/6 mice and transferred in sub-lethally irradiated (500 rads) μMT mice. The following day they were immunized with 1400 HA PR8 virus. Half of each cohort were treated with 300 μg αCD40L antibody at days 6, 8, and 10 post-NP_{22}CGG immunization to prevent germinal center formation. Serum antibody titers were measured by ELISA at 1 and 2 months post-transfer, and Ag-specific ASCs measured by ELISPOT at 2 months.

Adoptive transfer of plasmablasts

CD138$^+$B220$^+$ plasma cells were FACS sorted from C57BL/6 mice at 4 or 7 days post-immunization, or from unimmunized controls. 5 x10^5 marrow or splenic plasma cells were then transferred via tail-vein injection into cohorts of sex-matched μMT mice. Serum antibody titers were measured by ELISA (as previously described) at days 15, 30, and 45.

B cell stimulation

10^5 B220$^+$CD138$^-$IgM^{lo}CD43$^-$CD23$^+$CD5$^-$ B2 cells or B220$^+$CD138$^-$IgM^{hi}CD43$^+$CD23$^-$CD5$^-$ B1b cells were sorted from C57BL/6 spleens, and cultured in a 96 well plate with 2 μg/ml anti-IgM, 100 ng/ml anti-CD40, 10 ng/ml IL-2 and IL-4, and 5 ng/ml IFNγ in cRPMI. Cell numbers were determined after 6 days using the Countess Automated Cell Counter (Invitrogen).

Statistical analysis

Statistical significance was verified by the Student's *t*-test.

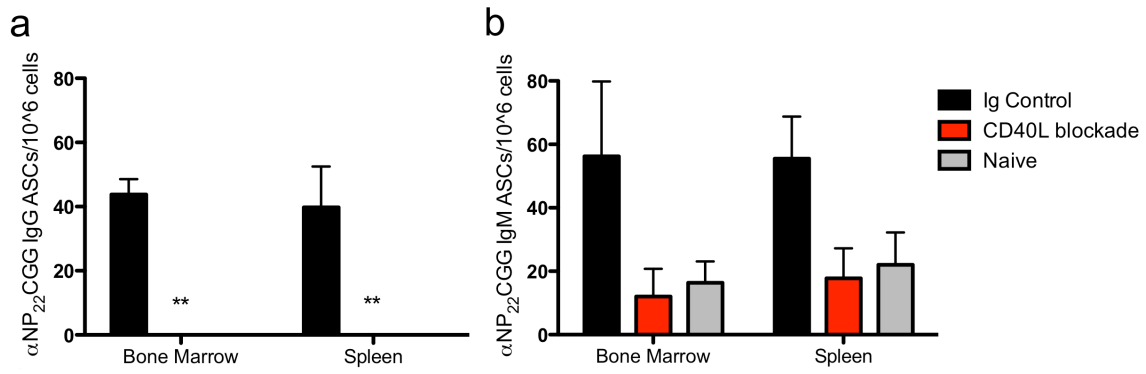


Figure 1: Long-lived IgM plasma cells require early T cell help but not germinal center formation, arise early in immune response. Cohorts of C57BL/6 mice immunized with $NP_{22}CGG$ were treated with either control IgG (■, n=5) or αCD40L antibody at days 2, 4, and 6 (■, n=4), compared to unimmunized/untreated controls (■, n=4). At day 45, mice were sacrificed and Ag-specific ASCS in the spleen and bone marrow were measured by ELISPOT. **(a)** $NP_{22}CGG$-specific IgG ASCs from the spleen and bone marrow. **(b)** $NP_{22}CGG$-specific IgM ASCs from the spleen and bone marrow.

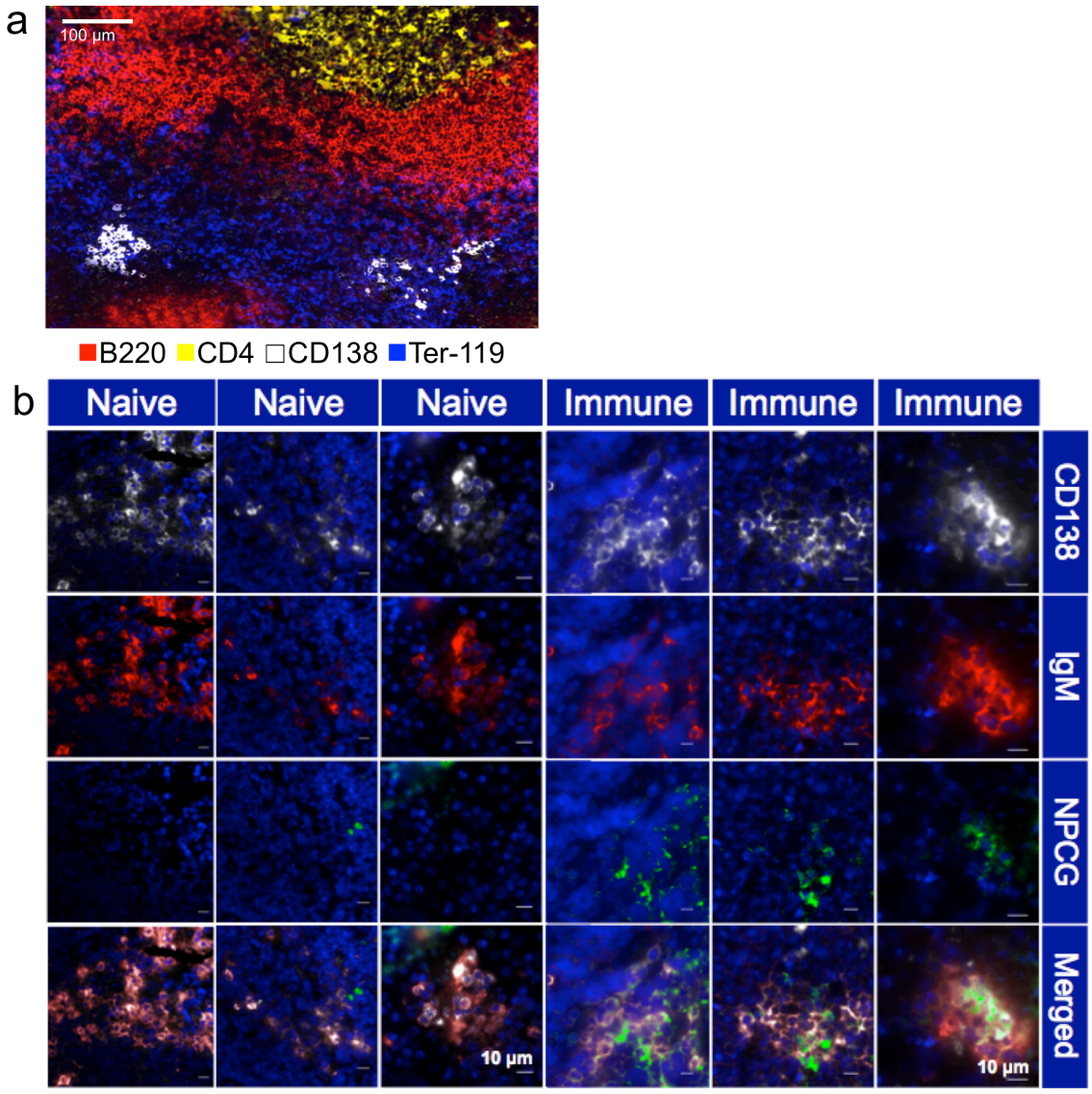


Figure 2: Plasma cells localize to the red pulp of the spleen, and only bind $NP_{22}CGG$ in immunized mice. (a) Representative *in situ* staining of plasma cells in the spleen at 1 month post-immunization with CD138 (white), Ter119 (blue), B220 (red), and CD4 (yellow) at 5x and 10x magnification, respectively. **(b)** Representative co-staining of CD138 (white), $NP_{22}CGG$ (green), and IgM (red) with

DAPI (blue) in mice 2 months post immunization with $NP_{22}CGG$ or in age-matched naïve controls (n=4).

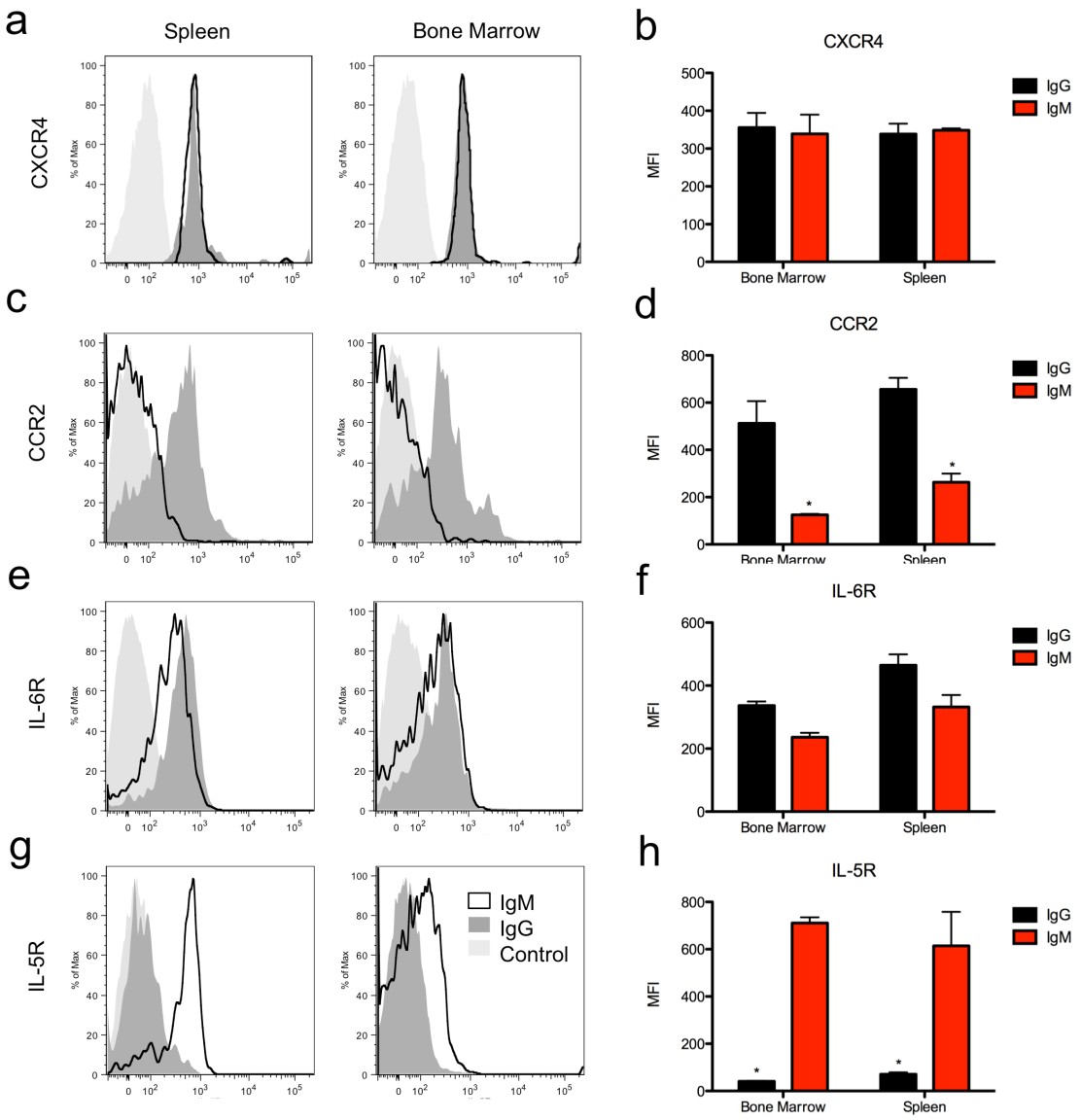


Figure 3: Differential expression of chemokine and survival receptors on the surface of IgG and IgM long-lived plasma cells. Splenocytes and bone marrow cells were gated on CD138+B220- plasma cells, and further on intracellular NPCG and either IgG or IgM to determine expression levels of surface receptors (n=3). **(a,b)** CXCR4 expression of IgG (■) and IgM (□) plasma cells, compared to a non-B cell control population (■) in the spleen and bone marrow, and MFI,

respectively. **(c,d)** CCR2 expression of IgG (■) and IgM (□) plasma cells, compared to a non-B cell control population (■) in the spleen and bone marrow, and MFI, respectively. **(e,f)** IL-6R expression of IgG (■) and IgM (□) plasma cells, compared to a non-B cell control population (■) in the spleen and bone marrow, and MFI, respectively. **(g,h)** IL-5R expression of IgG (■) and IgM (□) plasma cells, compared to a non-B cell control population (■) in the spleen and bone marrow, and MFI, respectively.

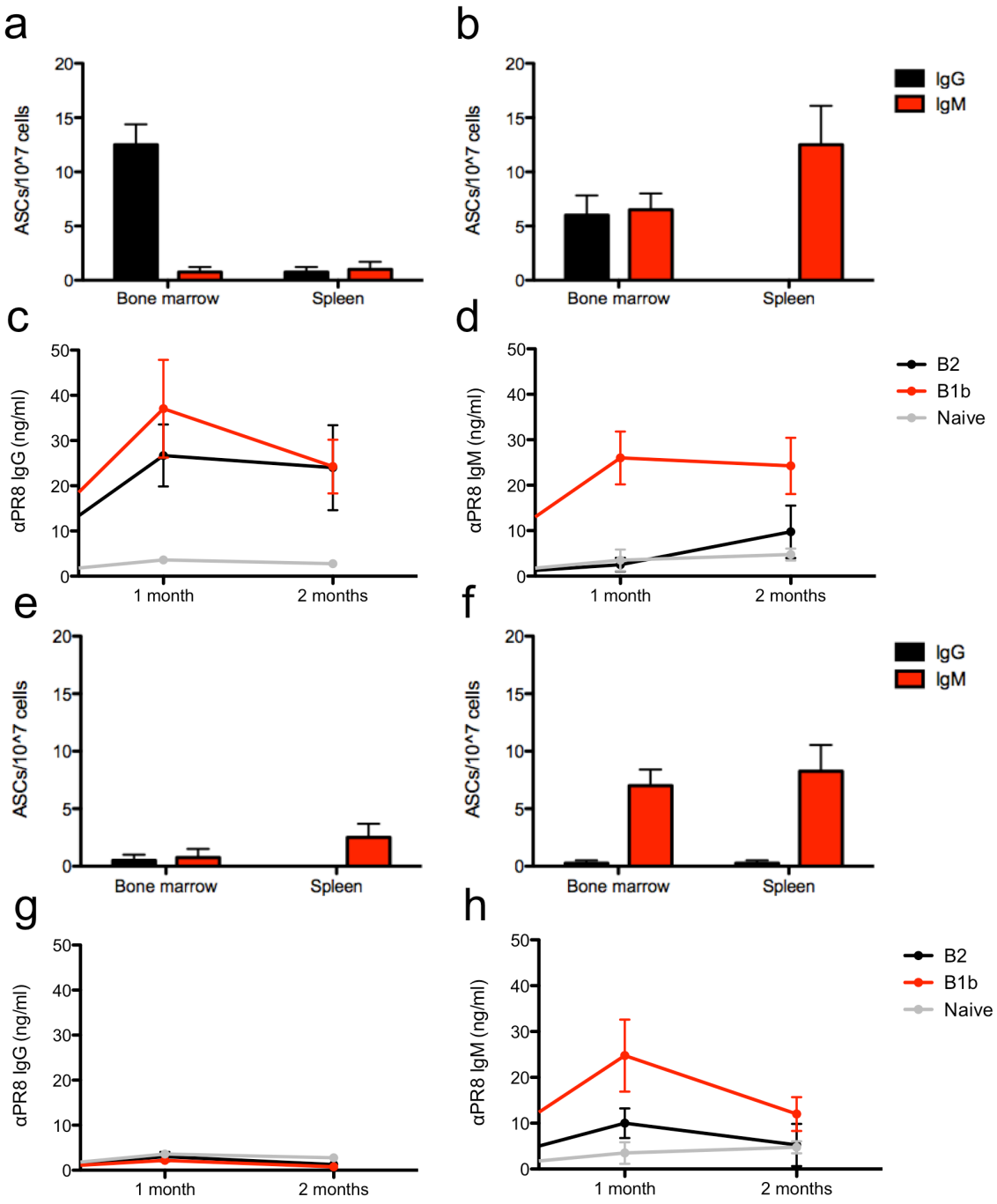


Figure 4: B1b, not B2, cells give rise to long-lived IgM plasma cells, even in the absence of germinal centers. 2.5 x 10^5 sorted B1b or B2 cells were adoptively transferred into sub-lethally irradiated uMT mice (n=4), which were then

immunized with PR8 virus. Half the cohorts were additionally treated with αCD40L at days 6, 8, and 10 to deplete germinal centers. **(a)** PR8-specific IgG (■) and IgM (■) ASCs from B2 recipient mice. **(b)** PR8-specific IgG (■) and IgM (■) ASCs from B1b recipient mice. **(c)** PR8-specific IgM in the sera of mice transferred with B1b (•) or B2 (•) cells, or from untransferred (•) controls. **(d)** PR8-specific IgG in the sera of mice transferred with B1b (•) or B2 (•) cells, or from untransferred (•) controls. **(e)** PR8-specific IgG (■) and IgM (■) ASCs from B2 recipient mice treated with αCD40L. **(f)** PR8-specific IgG (■) and IgM (■) ASCs from B1b recipient mice treated with αCD40L. **(g)** PR8-specific IgM in the sera of mice transferred with B1b (•) or B2 (•) cells and treated with αCD40L, or from untransferred (•) controls. **(h)** PR8-specific IgG in the sera of mice transferred with B1b (•) or B2 (•) cells and treated with treated with αCD40L, or from untransferred (•) controls.

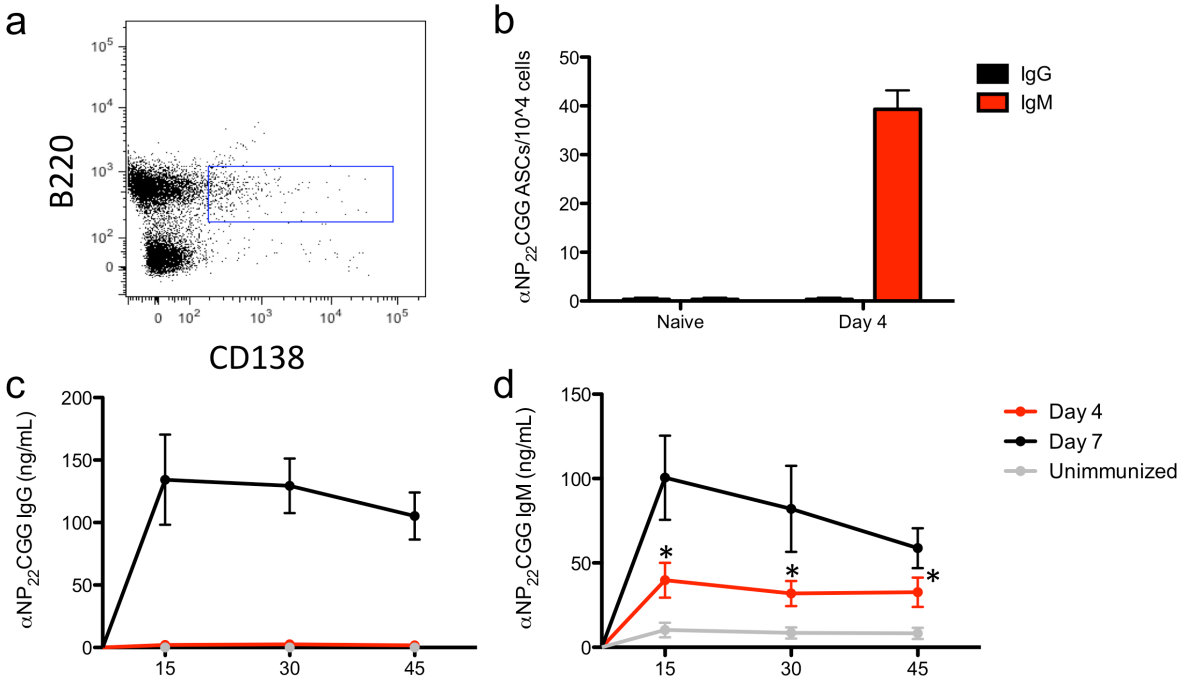
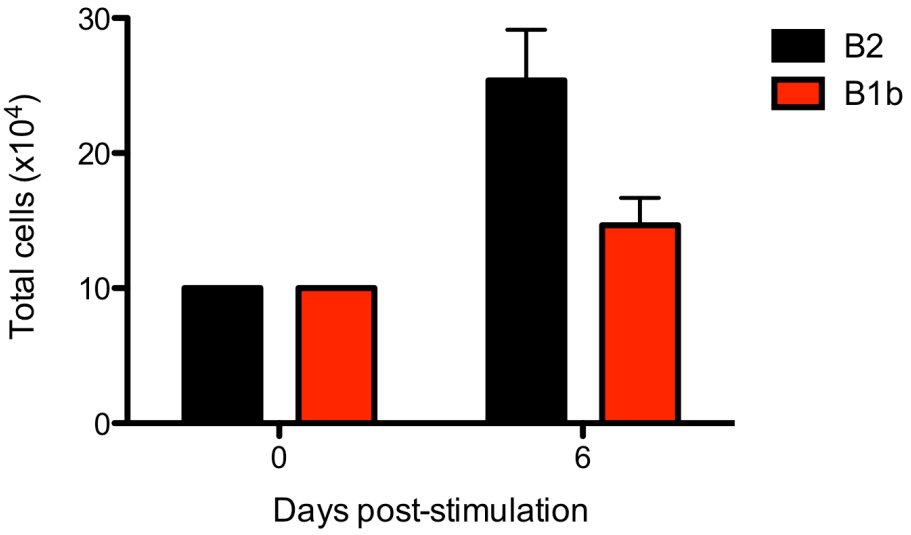


Figure 5: Long-lived IgM arises early in immune response. (a) Sample gating for plasmablasts sorted from C57BL/6 mice immunized either 4 or 7 days prior with $NP_{22}CGG$, or from unimmunized controls. **(b)** Ag-specific IgG (■) and IgM (■) ASCs from unimmunized and day 4 post-immunization sorted plasmablast populations. 10^5 day 4, day 7, or unimmunized plasmablasts were then adoptively transferred into uMT cohorts. Sera was collected and measured for Ag-specific IgG and IgM titers by ELISA at days 15, 30, and 45 post-transfer. **(c)** Antigen-specific IgG from mice transferred with day 4 (•), 7 (•), or unimmunized (•) plasmablasts. **(d)** Antigen-specific IgM from mice transferred with day 4 (•), 7 (•), or unimmunized (•) plasmablasts. * indicates $p < 0.05$.



Supplementary Figure 1: B1b B cells proliferate only modestly in response to stimulation when compared to B2 cells. 10^5 $B220^+CD138^-IgM^{lo}CD43^-CD23^+CD5^-$ B2 cells or $B220^+CD138^-IgM^{hi}CD43^+CD23^-CD5^-$ B1b cells were sorted from C57BL/6 spleens, and cultured for 6 days with anti-IgM, anti-CD40, IL-2, IL-4, and IFNγ.

LITERATURE CITED

1 Baumgarth, N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nature reviews. Immunology* **11**, 34-46, doi:10.1038/nri2901 (2011).

2 Haury, M. *et al.* The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. *European journal of immunology* **27**, 1557-1563, doi:10.1002/eji.1830270635 (1997).

3 Bos, N. A. *et al.* Serum immunoglobulin levels and naturally occurring antibodies against carbohydrate antigens in germ-free BALB/c mice fed chemically defined ultrafiltered diet. *European journal of immunology* **19**, 2335-2339, doi:10.1002/eji.1830191223 (1989).

4 Lalor, P. A., Herzenberg, L. A., Adams, S. & Stall, A. M. Feedback regulation of murine Ly-1 B cell development. *European journal of immunology* **19**, 507-513, doi:10.1002/eji.1830190315 (1989).

5 Slifka, M. K., Antia, R., Whitmire, J. K. & Ahmed, R. Humoral immunity due to long-lived plasma cells. *Immunity* **8**, 363-372 (1998).

6 Ho, F., Lortan, J. E., MacLennan, I. C. & Khan, M. Distinct short-lived and long-lived antibody-producing cell populations. *European journal of immunology* **16**, 1297-1301, doi:10.1002/eji.1830161018 (1986).

7 Nutt, S. L., Hodgkin, P. D., Tarlinton, D. M. & Corcoran, L. M. The generation of antibody-secreting plasma cells. *Nature reviews. Immunology* **15**, 160-171, doi:10.1038/nri3795 (2015).

8 Hargreaves, D. C. *et al.* A coordinated change in chemokine responsiveness guides plasma cell movements. *The Journal of experimental medicine* **194**, 45-56 (2001).

9 De Vos, J. *et al.* Microarray-based understanding of normal and malignant plasma cells. *Immunological reviews* **210**, 86-104, doi:10.1111/j.0105-2896.2006.00362.x (2006).

10 Vande Broek, I. *et al.* Chemokine receptor CCR2 is expressed by human multiple myeloma cells and mediates migration to bone marrow stromal cell-produced monocyte chemotactic proteins MCP-1, -2 and -3. *British journal of cancer* **88**, 855-862, doi:10.1038/sj.bjc.6600833 (2003).

11 Hauser, A. E. *et al.* Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. *Journal of immunology* **169**, 1277-1282 (2002).

12 Carbone, G. *et al.* Interleukin-6 receptor blockade selectively reduces IL-21 production by CD4 T cells and IgG4 autoantibodies in rheumatoid arthritis. *International journal of biological sciences* **9**, 279-288, doi:10.7150/ijbs.5996 (2013).

13 Diehl, S. A., Schmidlin, H., Nagasawa, M., Blom, B. & Spits, H. IL-6 triggers IL-21 production by human CD4+ T cells to drive STAT3-dependent plasma cell differentiation in B cells. *Immunology and cell biology* **90**, 802-811, doi:10.1038/icb.2012.17 (2012).

14 Moon, B. G., Takaki, S., Miyake, K. & Takatsu, K. The role of IL-5 for mature B-1 cells in homeostatic proliferation, cell survival, and Ig production. *Journal of immunology* **172**, 6020-6029 (2004).

15 Takahashi, Y., Dutta, P. R., Cerasoli, D. M. & Kelsoe, G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *The Journal of experimental medicine* **187**, 885-895 (1998).

16 Morris, D. L. & Rothstein, T. L. Abnormal transcription factor induction through the surface immunoglobulin M receptor of B-1 lymphocytes. *The Journal of experimental medicine* **177**, 857-861 (1993).

17 Baumgarth, N. *et al.* Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 2250-2255 (1999).

18 Choi, Y. S. & Baumgarth, N. Dual role for B-1a cells in immunity to influenza virus infection. *The Journal of experimental medicine* **205**, 3053-3064, doi:10.1084/jem.20080979 (2008).

19 Cole, L. E. *et al.* Antigen-specific B-1a antibodies induced by Francisella tularensis LPS provide long-term protection against F. tularensis LVS challenge. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 4343-4348, doi:10.1073/pnas.0813411106 (2009).

20 Alugupalli, K. R. *et al.* The resolution of relapsing fever borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes. *Journal of immunology* **170**, 3819-3827 (2003).

21 Haas, K. M., Poe, J. C., Steeber, D. A. & Tedder, T. F. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae. *Immunity* **23**, 7-18, doi:10.1016/j.immuni.2005.04.011 (2005).

22 Hsu, E. Mutation, selection, and memory in B lymphocytes of exothermic vertebrates. *Immunological reviews* **162**, 25-36 (1998).

23 Racine, R. & Winslow, G. M. IgM in microbial infections: taken for granted? *Immunology letters* **125**, 79-85, doi:10.1016/j.imlet.2009.06.003 (2009).

24 Baumgarth, N. *et al.* B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *The Journal of experimental medicine* **192**, 271-280 (2000).

25 Boes, M. *et al.* Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *Journal of immunology* **160**, 4776-4787 (1998).

26 Baumgarth, N. A two-phase model of B-cell activation. *Immunological reviews* **176**, 171-180 (2000).

27 Jayasekera, J. P., Moseman, E. A. & Carroll, M. C. Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. *Journal of virology* **81**, 3487-3494, doi:10.1128/JVI.02128-06 (2007).

28 Hayward, A. R. The human fetus and newborn: development of the immune response. *Birth defects original article series* **19**, 289-294 (1983).

29 Siegrist, C. A. Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants. *Vaccine* **21**, 3406-3412 (2003).

30 Frasca, D., Van der Put, E., Riley, R. L. & Blomberg, B. B. Reduced Ig class switch in aged mice correlates with decreased E47 and activation-induced cytidine deaminase. *Journal of immunology* **172**, 2155-2162 (2004).

31 Hayakawa, K., Hardy, R. R., Parks, D. R. & Herzenberg, L. A. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *The Journal of experimental medicine* **157**, 202-218 (1983).

32 Binder, C. J. & Silverman, G. J. Natural antibodies and the autoimmunity of atherosclerosis. *Springer seminars in immunopathology* **26**, 385-404, doi:10.1007/s00281-004-0185-z (2005).

33 Duan, B. & Morel, L. Role of B-1a cells in autoimmunity. *Autoimmunity reviews* **5**, 403-408, doi:10.1016/j.autrev.2005.10.007 (2006).

34 Kroczek, R. A. *et al.* Defective expression of CD40 ligand on T cells causes "X-linked immunodeficiency with hyper-IgM (HIGM1)". *Immunological reviews* **138**, 39-59 (1994).

35 Lampropoulou, V. *et al.* Suppressive functions of activated B cells in autoimmune diseases reveal the dual roles of Toll-like receptors in immunity. *Immunological reviews* **233**, 146-161, doi:10.1111/j.0105-2896.2009.00855.x (2010).

36 Boes, M. *et al.* Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 1184-1189 (2000).

37 Maris, C. H., Miller, J. D., Altman, J. D. & Jacob, J. A transgenic mouse model genetically tags all activated CD8 T cells. *Journal of immunology* **171**, 2393-2401 (2003).

Chapter 4: Discussion

A new model of plasma cell development

In chapter 2 we characterized a novel population of long-lived IgM plasma cells that persist in the spleen. These IgM plasma cells are antigen-induced and antigen-specific, and are distinct from natural IgM cells, and persist beyond short-lived IgM plasma cells. They are similar in longevity to long-lived IgG plasma cells, with a $t_{1/2}$ of 84 days, and are observed at significant levels over natural IgM antibody secreting cells (ASCs) for greater than a year post-immunization in mice. But unlike IgG, they preferentially localize to the spleen rather than the bone marrow both in wild-type mice and following adoptive transfer into B cell deficient μMT mice. Further, they continue to develop when germinal center formation is blocked by anti-CD40L treatment, which inhibits IgG plasma cell development[1]. They are not class-switched, but they do undergo somatic hypermutation, even in the absence of germinal centers. These mutations occur less frequently than in IgG, and are more often found in the framework regions than the antigen-binding CDRs. This suggests a lack of clonal selection, as is observed in IgG plasma cells and memory B cells[2,3]. However, these mutations are AID-induced, as confirmed by a lack of mutations observed in AID-/- mice, and they are found in typical AID hotspots like we see in IgG plasma cells. Additionally, we demonstrate that these long-lived IgM plasma cells provide long-term protection against viral influenza infection – even in the absence of IgG and memory B and T cells.

In chapter 3 we have described the early development of these long-lived plasma cells, demonstrating their B1 cell origin, T cell dependence, and quick response. We had previously shown that germinal center formation was not required for long-lived IgM plasma cell development, but blocking CD40:CD40L interactions prior to germinal center formation does inhibit IgM plasma cell development, demonstrating the need for early T cell help in generating this response. This finding was confirmed in CD28-/- mice, which also lack T cell help. This further distinguishes long-lived IgM plasma cells from natural IgM, which is produced without T cell help and to T-independent antigens[4]. These long-lived IgM plasma cells are found clustered *in situ* within the red pulp of the spleen, as observed in immunofluorescence staining of the murine spleen. Flow cytometry analysis revealed that these cells express high levels of CXCR4 and IL-6R, which promote localization and survival within either the spleen or bone marrow, as well as IL-5R, which is upregulated in splenic B cells, particularly of B1 origin[5-9]. By contrast, IgG cells, though also expressing high levels of CXCR4 and IL-6R, did not express significant levels of IL-5R on their surface. Instead they expressed high levels of CCR2, which promotes bone marrow homing[5,10]. These differences in receptor profiles may contribute to the differing localization of these plasma cell subsets, but it also suggests that long-lived IgM plasma cells may be of B1 origin. We confirmed that all IgM ASCs were B1 derived, even in the absence of the germinal center, via adoptive transfer of B1 and B2 naïve B cells into B cell deficient μMT mice. No significant IgM ASCs persisted in B2 recipient mice long-term. Finally, we demonstrated that long-lived IgM plasma cells develop prior to IgG plasma cells, as soon as day 4 post-immunization.

Taken together, these data demonstrate a novel pathway of long-lived plasma cell development. One that begins in the B1 compartment, but responds to T-dependent antigen stimulation to produce antigen-specific antibody, rather than natural IgM. This pathway does require T cell help, but also has the potential to bypass the germinal center. Even in the absence of germinal center formation, these cells still develop somatic mutations and can differentiate into long-lived IgM plasma cells, which preferentially inhabit the spleen. These somatic mutations are AID-induced, and occur in AID hotspots, but they do not appear to be clonally selected. Many of the replacement mutations are found within the framework regions, suggesting that the resulting plasma cells are of low or mixed affinity, compared to the high affinity IgG plasma cell populations.

In this model, B1 cells are recruited from the spleen and peritoneal cavity, and stimulated with antigen via T cell help. These cells undergo somatic hypermutation and clonal expansion, before differentiating into long-lived plasma cells as early as day four. Complete germinal center formation is not required for mutation or for development of these IgM plasma cells, though the absence of germinal centers does significantly reduce the number of mutations observed. The resulting plasma cells express high levels of CD138 and no B220, further distinguishing them from the B220-expressing short-lived plasma cells and natural IgM cells[11,12]. They also express CXCR4, IL-6R, and IL-5R, which allows them to populate and survive within the splenic niche. They express only low levels of CCR2 and are not as frequently observed in the bone marrow. These splenic plasma cells can survive for several years post-immunization, with a half-life of 84 days, comparable to IgG plasma cells (Figure 1).

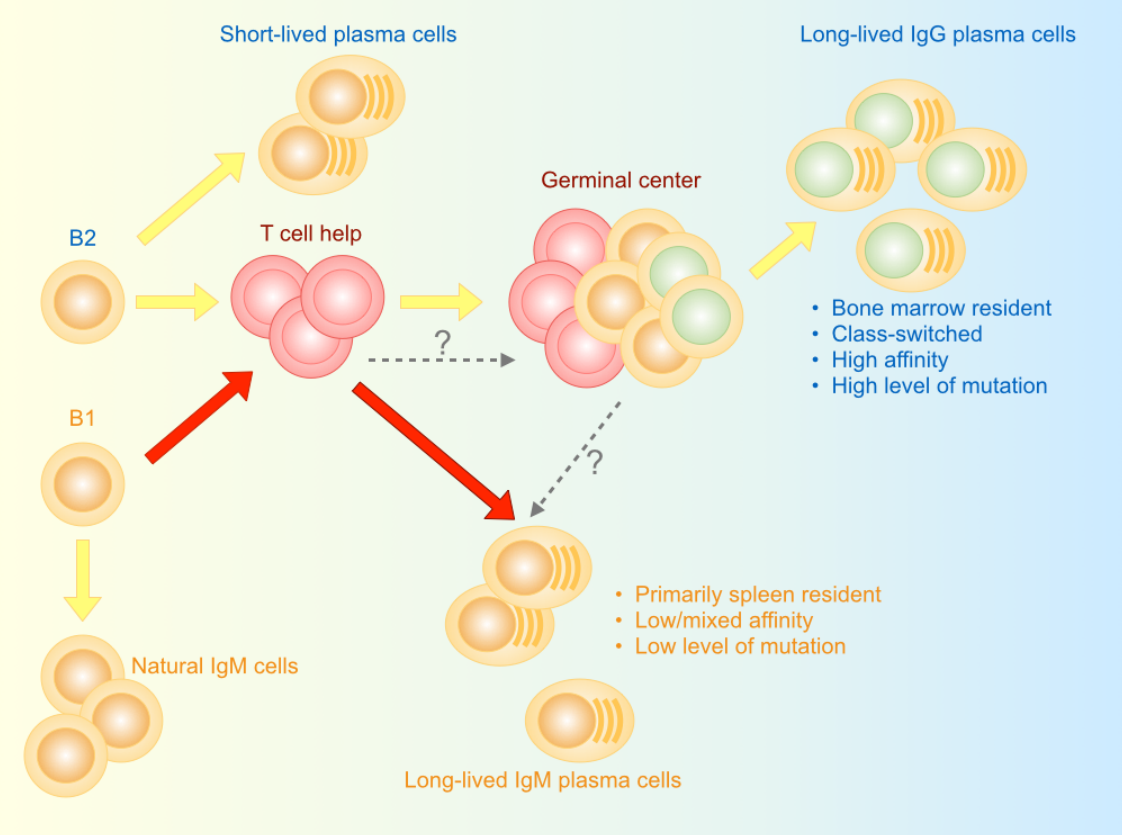


Figure 1. Model of B1- and B2-derived plasma cell differentiation pathways

This pathway was predicted, but never definitively characterized, by several previous studies. Engle and colleagues have demonstrated a role for induced IgM in combating West Nile Virus[13]. Studies of *Streptococcus pneumoniae* infection by Tedder and colleagues additionally suggest that B1b cells may produce antigen-specific IgM in response to infection, and showed that this antigen-specific IgM persisted for as long as 10 weeks post-infection[14]. This suggests the possibility of B1-derived memory populations or long-lived IgM plasmablasts as a potential source of induced IgM response[15].

The discovery of germinal center-independent, unswitched memory B cells in particular implies that the germinal center may not be essential to memory formation[16]. Low levels of somatic hypermutation have been observed in germinal-center independent memory B cells, in patients suffering from hyper-IgM disorder due to a defect in CD40L[17]. There is also evidence of somatic hypermutation occurring in extrafollicular plasmablasts, particularly in autoimmune cells, as well as evidence of mutations in B1 cells. Schlomchik and colleagues have demonstrated that autoreactive B cells, microdissected from the extrafollicular border, are somatically mutated[18]. They additionally show evidence of somatic hypermutation occurring in extrafollicular plasmablasts in *Salmonella* infection, in the absence of germinal center formation[19]. B1 cells have also been reported to undergo some degree of somatic mutation, though at a significantly lower frequency than follicular B2 cells[15,20]. Here we are the first to characterize the development of B1-derived, somatically mutated, long-lived IgM plasma cells in full, distinguishing them from natural IgM secreting B1 cells and other memory populations.

The role of long-lived IgM plasma cells

We have shown that these long-lived IgM plasma cells are functional, and that they are sufficient to protect against viral challenge, and to a greater degree than natural IgM alone. Natural IgM has previously been shown to protect against several bacterial infections, including *Borrelia hermsii, Streptococcus pneumoniae, Ehrlichia muris,* and vesicular stomatitis[15,21-26]. When sIgM is depleted, it significantly increases host susceptibility to systemic bacterial

infection, even with IgG present[25]. Natural IgM has also been shown to be necessary for both neutralization and clearance of influenza infection[27]. But here we have demonstrated that natural IgM alone was not enough to protect against >2 x LD_{50} influenza challenge, while 75-80% mice with long-lived IgM plasma cells survived, without IgG and even without T cells or memory B cells. Though we have demonstrated that these plasma cells play a role in host protection, it is not yet fully understood how or why this plasma cell pathway developed. Several possibilities exist, which are not mutually exclusive.

Evolutionary remnant?

IgM antibodies evolved long before IgG, and can be seen in animals as far back as sharks and other cartilaginous fish. IgG arose much later, and was first observed in mammals. IgG itself originated from a duplication of the IgM gene[28]. Class-switching itself did not come about until the evolution of jawed vertebrates - along with an increase in immunoglobulin isotypes. In cartilaginous fish, the variable gene segments are clustered with a single constant gene, predominantly IgM. AID and its homologues appeared prior to the advent of class switching, and even longer before the evolution of the IgG, IgA, or IgE isotypes[29].

Further evidence of the ancient origins of this adaptive IgM pathway stems from reports of somatic hypermutation observed in the IgM heavy chains of sharks and frogs. In *Xenopus* (frogs), these mutations are found at low levels and within AID induced hotspots – targeting cytosines in WRC motifs – but there is no enrichment for replacement mutations in the CDRs. This suggests that these mutations are not antigen-driven[19]. This G:C bias is also seen in sharks, which

also exhibit no evidence of affinity selection[20]. The absence of germinal centers in these animals, and in all cold-blooded species, may account for the lack of antigen-driven selection. This mirrors the IgM mutations we observe in mice – infrequent mutations, AID-targeted/WRC-biased, and no evidence of clonal selection. The same pattern is observed in mice with depleted germinal centers, demonstrating that this pathway is not dependent on their formation. The mutations in murine IgM more closely resembles that of frogs and sharks than mutations in murine IgG, which do require germinal centers, are far more frequent, and are enriched in the CDR regions by antigen-selection. An adaptive IgM response might simply be a remnant of pre-germinal center species. However, we find it unlikely that this pathway is merely an evolutionary artifact as IgM continues to play a critical role in the humoral response.

Long-lived IgM plasma cells in early response

Both natural and induced IgM are essential to early host defense. Natural IgM is spontaneously produced by B1 cells even in the absence of antigen, and is the primary component of circulating IgM. Natural IgM from B1 cells is one of the first lines of defense against pathogens, and is critical to the neutralization and clearance of both bacterial and viral infections[22-27]. In *Borellia hermsii* infection, B1 cells mediate bacterial clearance from the bloodstream within the first 2-3 days post-infection. Mice deficient for secreted IgM (sIgM-/-) were unable to clear bacteremia[22]. Similarly, when faced with acute peritonitis, 70% of sIgM-/- mice died within the first 32 hours, compared to only 20% of wildtype mice.

Reconstitution with polyclonal IgM restored bacterial resistance, while monoclonal IgM had a less significant effect[25].

Activated B cells also express IgM for several days post-infection, until class switching is initiated around day 7. Without requiring T cell help, activated B cells can differentiate into short-lived plasma cells, secreting protective IgM antibody until they are replaced by long-lived plasma cell populations. We additionally show in B-cell deficient mice adoptively transferred with day 4 plasmablasts, that induced long-lived IgM plasma cells are generated early in the immune response, prior to the germinal center. This induced IgM is critical to early defense, and occurs in response to both T-dependent and –independent antigen, unlike long-lived IgG plasma cells[30,31]. Long-lasting induced IgM, however, has been observed in response to T-independent antigen stimulation[32]. In the absence of T cell help and long-lived IgG plasma cells, the induced IgM response is necessary to both the early and continuing defense against infection.

IgM and B1 cells are also essential to infant immunity, prior to the development of follicular B2 cells and long-lived IgG plasma cells. These cells spontaneously secrete natural IgM and aid in the protection of infants for the first weeks (in mice) or months (in humans) following birth. It is currently debated whether infants are able to develop long-lived plasma cells in response to antigen, which may contribute to the difficulty of infant vaccination programs[4].

Tissue homeostasis

B1 cells and IgM also play a critical role in tissue homeostasis[4]. In particular, they are responsible for maintenance of a healthy mucosal microbiota[33]. B1 cells are

also involved in the anti-inflammatory response and the clearance of apoptotic cells[34]. Natural IgM can bind to lipoproteins to reduce atherosclerosis[35]. The absence of these populations can increase the incidence of autoimmunity and worsen disease progression[36,37]. These roles have been demonstrated for natural IgM, and it is currently unknown whether induced IgM may play a role in homeostasis and suppression of autoimmunity. And, as discussed in more detail below, B1 cells and IgM also may also provide feedback that support IgG expression.

Long-lived IgM promotes healthy IgG response

It has been previously demonstrated that IgM production is critical to a healthy IgG response. In mice that express surface IgM, but are unable to secrete IgM, the IgG2a response to limiting doses of T-dependent antigen was critically impaired. This study by Chen and colleagues demonstrated a potential feedback role for secreted natural IgM in IgG production[38]. This same group also demonstrated an increase in self-reactive IgG in mice deficient in secreted IgM (sIgM-/-) [36]. Engle and colleagues similarly demonstrated lowered IgG titers in sIgM-/- mice in response to West Nile Virus infection, as well as showing that induced IgM in particular played a role in protection against the virus[13]. It has also been demonstrated that IgM when used as adjuvant and coupled to malarial antigens stimulated high titer of protective anti-malarial IgG in mice[39]. It is currently unknown whether long-lasting IgM responses continue to enhance IgG expression long-term, and the mechanism by which IgM regulates IgG expression still remains to be elucidated.

Potential for broadly neutralizing antibodies

IgM molecules tend to be lower affinity, but due to the high valency of their pentameric structure, they are able to bind a wide range of antigens with high avidity. This enables natural IgM to broadly neutralize despite the low affinity of the individual molecule. The majority of IgM antibodies are polyreactive, and are especially effective at binding antigen with repeating epitopes – including viral particles, nucleotide sequences, and carbohydrates and phospholipids on the surface of pathogens[15]. In sIgM-/- mice, which are highly susceptible to bacterial infections, polyclonal but not monoclonal IgM (specific to the conserved cell membrane component phosphatidylcholine) restored resistance to bacterial infection[25]. The polyreactivity of IgM also makes it critical to both the clearance and neutralization of pathogens such as influenza, which can often escape specific antibodies[27]. IgM has additionally demonstrated cross-protectivity across multiple strains[40].

To date, there have been no studies that quantitate the affinity of induced IgM. It is typically assumed that like natural IgM, these induced IgM antibodies are lower affinity. Our own data show low levels of somatic hypermutation in induced IgM plasma cells, and these mutations rarely affect the amino acid sequence of the antigen-binding sites. Following SHM, it is unlikely that the affinity of these cells is significantly impacted. There is also no evidence of clonal selection, which typically selects for high affinity mutants. However, recently it has been shown that in the case of complex antigen – such as whole bacteria or influenza hemagglutinin – that antibodies of mixed affinity may actually be selected for[41]. This study may also provide insight into the purpose of generating

long-lived IgM plasma cells of low to mixed affinity – potentially as a means to target complex pathogens with a broader response.

Additionally, the relatively high frequency of replacement mutations in the framework regions of the IgM antibody may additionally increase the flexibility of the antibody and improve antigen-binding. The sequencing of broadly neutralizing antibodies to HIV by Nussenzweig and colleagues has demonstrated that framework mutations may actually be required for a broad response[42]. This sort of flexibility and broad response is essential to neutralizing highly evasive pathogens such as HIV and influenza.

Potential for clinical applications

Rational vaccine design

Neutralizing antibodies are the key correlate of long-term vaccine efficacy[43]. As previously discussed, the generation of broadly neutralizing antibodies is critical to overcoming the barriers to long-lasting HIV and influenza vaccination, as well as other pathogens that continue to evade effective vaccination strategies[25,27,40-42]. The induction of long-lived IgM plasma cells in particular may prove essential to designing a broadly protective vaccine, due to the somatic diversity of the induced IgM response. We have no only demonstrated the ability of long-lasting IgM responses to protect against influenza challenge, even in the absence of IgG, we have also demonstrated that long-lived IgM plasma cells undergo somatic hypermutation to further diversify the response. A better understanding of how somatic mutations in IgM affect the affinity, flexibility, and cross-protectivity of these antibodies will also aid in an understand of how production of these

broadly-neutralizing antibodies might be enhanced – whether through vaccine antigen, adjuvant, or delivery system.

IgM expression has also been shown to be critical for a healthy IgG response, particularly in response to limiting doses of antigen[13,38]. Thus a poor induction of IgG through vaccination may be attributable to a lack of IgM. Induced IgM, as well as natural IgM, plays a role in enhancing the IgG response, and stimulating either or both may improve IgG titers and long-lasting protection.

Induction of B1-derived long-lived IgM responses in infants may also prove to be a more effective strategy than current vaccination programs. There are numerous barriers to generating a long-lived IgG response in infants – a lack of B2 cells and the presence of maternal IgG suppression chief among them[4,44] – but it may be possible to induce long-lived IgM in these patients. Polyclonal IgM has also been additionally been shown to work as adjuvant in malarial vaccines given to infants with immune mothers, overcoming the obstacle of maternal antibody[39].

Similarly, the elderly suffer from decreasing antigen-specific antibody titers and increased susceptibility to disease[45-47]. The response to vaccination in aging patients is significantly reduced compared to younger individuals, as demonstrated in seasonal influenza vaccinations[48]. Both the number of antibody-secreting cells and the antibody production of individual cells also decrease with age[49]. The B cell repertoire may also decrease, limiting the ability to response to diverse antigens, and AID expression and CSR/SHM are also reduced in aging populations[45,50,51]. This is linked with a more limited germinal center response –

it has been shown in mice and in humans that the number of germinal center B cells is reduced and that the germinal centers themselves are smaller[52,53]. The response may also be delayed, and with reduced levels of class switching and SHM[54]. Because long-lived IgM plasma cells are able to develop in the absence of germinal centers, and we have shown that they can provide sufficient protection against viral pathogen, a focus on this stimulating this germinal center-independent pathway may aid in developing more effective vaccines strategies for elderly patients. A broadly neutralizing IgM response may also help to overcome limitations of the gaining B cell repertoire.

Targeting B cell disorders

Induced IgM plasma cells make up a smaller portion of total circulating IgM and total long-lived plasma cells, and it is currently unknown whether these cell factor into B cell disease. However, an increased understanding of how these cells differentiate may still aid in better targeting clinical approaches to B cell disorders. In particular, B1 cells and IgM have be linked to a number of autoimmune disorders, and B1 expansion is observed in lupus, atherosclerosis, arthritis, and rheumatism[55-57]. However, depletion of B1 cells and secreted IgM does not always reduce autoimmunity, and can actually worsen disease progression[36,37]. Targeting autoimmune cells, without negatively affecting healthy B1 and IgM responses, may require distinguishing between the natural and induced IgM compartments. A better understanding of the markers of a healthy B cell response may also aid in better targeting cancerous B cells.

Future directions of this study

Currently, we are in the process of preforming a microarray analysis on long-lived IgM cells in comparison to IgG plasma cells. The mRNA expression profiles will further be compared to naïve B1 and B2 cells. With this, we will better understand how B1 cells differentiate in long-lived IgM plasma cells, and how these cells differ from IgG plasma cells. In particular, we will focus on the expression of AID and the machinery of class switching and somatic hypermutation. We hypothesize that lowered expression of AID may result in both the low mutations and lack of class switching observed in long-lived IgM. This hypothesis is based in part on a study of aging mice by Riley, Blomberg, and colleagues, wherein lowered AID expression was observed with declining IgG production and infrequent somatic hypermutations[45]. Low AID expression may be inherent to B1 cells, as mutations are often observed at low levels in B1 variable genes, or it may be related to the timing of differentiation – it is possible that early plasma cells have differentiated before they were able to accumulate many mutations or class-switch. Our findings that long-lived IgM plasma cells can develop prior to germinal center formation also suggests that timing may play a role in mutational frequency and class-switching. A more detailed study of the kinetics of this response will further clarify how the long-lived IgM and IgG plasma cell pathways diverge.

Long-term, we also hope to understand more about the function of these long-lived IgM plasma cells. Determining what effect, if any, that somatic mutation has on the ability of induced IgM to bind antigen will provide further insight into the function of these cells. Using influenza as a model, we will further

be able to measure the cross-protective potential of IgM against diverse strains of the virus. The ability to generate broadly neutralizing antibodies is of great clinical significance, and will aid not only in our increasing our knowledge of the humoral response but it may also prove critical to designing better vaccines.

LITERATURE CITED

1 Takahashi, Y., Dutta, P. R., Cerasoli, D. M. & Kelsoe, G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *The Journal of experimental medicine* **187**, 885-895 (1998).

2 Di Noia, J. M. & Neuberger, M. S. Molecular mechanisms of antibody somatic hypermutation. *Annual review of biochemistry* **76**, 1-22, doi:10.1146/annurev.biochem.76.061705.090740 (2007).

3 Wagner, S. D., Milstein, C. & Neuberger, M. S. Codon bias targets mutation. *Nature* **376**, 732, doi:10.1038/376732a0 (1995).

4 Baumgarth, N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nature reviews. Immunology* **11**, 34-46, doi:10.1038/nri2901 (2011).

5 De Vos, J. *et al.* Microarray-based understanding of normal and malignant plasma cells. *Immunological reviews* **210**, 86-104, doi:10.1111/j.0105-2896.2006.00362.x (2006).

6 Moon, B. G., Takaki, S., Miyake, K. & Takatsu, K. The role of IL-5 for mature B-1 cells in homeostatic proliferation, cell survival, and Ig production. *Journal of immunology* **172**, 6020-6029 (2004).

7 Hargreaves, D. C. *et al.* A coordinated change in chemokine responsiveness guides plasma cell movements. *The Journal of experimental medicine* **194**, 45-56 (2001).

8 Carbone, G. *et al.* Interleukin-6 receptor blockade selectively reduces IL-21 production by CD4 T cells and IgG4 autoantibodies in rheumatoid arthritis. *International journal of biological sciences* **9**, 279-288, doi:10.7150/ijbs.5996 (2013).

9 Diehl, S. A., Schmidlin, H., Nagasawa, M., Blom, B. & Spits, H. IL-6 triggers IL-21 production by human CD4+ T cells to drive STAT3-dependent plasma cell differentiation in B cells. *Immunology and cell biology* **90**, 802-811, doi:10.1038/icb.2012.17 (2012).

10 Vande Broek, I. *et al.* Chemokine receptor CCR2 is expressed by human multiple myeloma cells and mediates migration to bone marrow stromal cell-produced monocyte chemotactic proteins MCP-1, -2 and -3. *British journal of cancer* **88**, 855-862, doi:10.1038/sj.bjc.6600833 (2003).

11 Foote, J. B., Mahmoud, T. I., Vale, A. M. & Kearney, J. F. Long-term maintenance of polysaccharide-specific antibodies by IgM-secreting cells. *Journal of immunology* **188**, 57-67, doi:10.4049/jimmunol.1100783 (2012).

12 Oh, J. Z. *et al.* TLR5-mediated sensing of gut microbiota is necessary for antibody responses to seasonal influenza vaccination. *Immunity* **41**, 478-492, doi:10.1016/j.immuni.2014.08.009 (2014).

13 Diamond, M. S. *et al.* A critical role for induced IgM in the protection against West Nile virus infection. *The Journal of experimental medicine* **198**, 1853-1862, doi:10.1084/jem.20031223 (2003).

14 Haas, K. M., Poe, J. C., Steeber, D. A. & Tedder, T. F. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in

innate and adaptive immunity to S. pneumoniae. *Immunity* **23**, 7-18, doi:10.1016/j.immuni.2005.04.011 (2005).

15 Racine, R. & Winslow, G. M. IgM in microbial infections: taken for granted? *Immunology letters* **125**, 79-85, doi:10.1016/j.imlet.2009.06.003 (2009).

16 Taylor, J. J., Pape, K. A. & Jenkins, M. K. A germinal center-independent pathway generates unswitched memory B cells early in the primary response. *The Journal of experimental medicine* **209**, 597-606, doi:10.1084/jem.20111696 (2012).

17 Weller, S. *et al.* CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 1166-1170, doi:10.1073/pnas.98.3.1166 (2001).

18 William, J., Euler, C., Christensen, S. & Shlomchik, M. J. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* **297**, 2066-2070, doi:10.1126/science.1073924 (2002).

19 Di Niro, R. *et al.* Salmonella Infection Drives Promiscuous B Cell Activation Followed by Extrafollicular Affinity Maturation. *Immunity* **43**, 120-131, doi:10.1016/j.immuni.2015.06.013 (2015).

20 Roy, B. *et al.* Somatic hypermutation in peritoneal B1b cells. *Molecular immunology* **46**, 1613-1619, doi:10.1016/j.molimm.2009.02.026 (2009).

21 Racine, R. *et al.* IgM production by bone marrow plasmablasts contributes to long-term protection against intracellular bacterial infection. *Journal of immunology* **186**, 1011-1021, doi:10.4049/jimmunol.1002836 (2011).

22 Alugupalli, K. R. *et al.* The resolution of relapsing fever borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes. *Journal of immunology* **170**, 3819-3827 (2003).

23 Briles, D. E. *et al.* Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 streptococcus pneumoniae. *The Journal of experimental medicine* **153**, 694-705 (1981).

24 Gobet, R., Cerny, A., Ruedi, E., Hengartner, H. & Zinkernagel, R. M. The role of antibodies in natural and acquired resistance of mice to vesicular stomatitis virus. *Experimental cell biology* **56**, 175-180 (1988).

25 Boes, M., Prodeus, A. P., Schmidt, T., Carroll, M. C. & Chen, J. A critical role of natural immunoglobulin M in immediate defense against systemic bacterial infection. *The Journal of experimental medicine* **188**, 2381-2386 (1998).

26 Jayasekera, J. P., Moseman, E. A. & Carroll, M. C. Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity. *Journal of virology* **81**, 3487-3494, doi:10.1128/JVI.02128-06 (2007).

27 Baumgarth, N. *et al.* B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *The Journal of experimental medicine* **192**, 271-280 (2000).

28 Akula, S., Mohammadamin, S. & Hellman, L. Fc receptors for immunoglobulins and their appearance during vertebrate evolution. *PloS one* **9**, e96903, doi:10.1371/journal.pone.0096903 (2014).

29 Stavnezer, J. & Amemiya, C. T. Evolution of isotype switching. *Seminars in immunology* **16**, 257-275, doi:10.1016/j.smim.2004.08.005 (2004).

30 Ho, F., Lortan, J. E., MacLennan, I. C. & Khan, M. Distinct short-lived and long-lived antibody-producing cell populations. *European journal of immunology* **16**, 1297-1301, doi:10.1002/eji.1830161018 (1986).

31 Zotos, D. & Tarlinton, D. M. Determining germinal centre B cell fate. *Trends in immunology* **33**, 281-288, doi:10.1016/j.it.2012.04.003 (2012).

32 Hsu, M. C., Toellner, K. M., Vinuesa, C. G. & Maclennan, I. C. B cell clones that sustain long-term plasmablast growth in T-independent extrafollicular antibody responses. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 5905-5910, doi:10.1073/pnas.0601502103 (2006).

33 Kroese, F. G. *et al.* Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. *International immunology* **1**, 75-84 (1989).

34 Chen, Y., Park, Y. B., Patel, E. & Silverman, G. J. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. *Journal of immunology* **182**, 6031-6043, doi:10.4049/jimmunol.0804191 (2009).

35 Shaw, P. X. *et al.* Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *The Journal of clinical investigation* **105**, 1731-1740, doi:10.1172/JCI8472 (2000).

36 Boes, M. *et al.* Accelerated development of IgG autoantibodies and autoimmune disease in the absence of secreted IgM. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 1184-1189 (2000).

37 Lampropoulou, V. *et al.* Suppressive functions of activated B cells in autoimmune diseases reveal the dual roles of Toll-like receptors in immunity. *Immunological reviews* **233**, 146-161, doi:10.1111/j.0105-2896.2009.00855.x (2010).

38 Boes, M. *et al.* Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *Journal of immunology* **160**, 4776-4787 (1998).

39 Harte, P. G., Cooke, A. & Playfair, J. H. Specific monoclonal IgM is a potent adjuvant in murine malaria vaccination. *Nature* **302**, 256-258 (1983).

40 Throsby, M. *et al.* Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. *PloS one* **3**, e3942, doi:10.1371/journal.pone.0003942 (2008).

41 Kuraoka, M. *et al.* Complex Antigens Drive Permissive Clonal Selection in Germinal Centers. *Immunity*, doi:10.1016/j.immuni.2016.02.010 (2016).

42 Klein, F. *et al.* Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. *Cell* **153**, 126-138, doi:10.1016/j.cell.2013.03.018 (2013).

43 De Gregorio, E. & Rappuoli, R. From empiricism to rational design: a personal perspective of the evolution of vaccine development. *Nature reviews. Immunology* **14**, 505-514, doi:10.1038/nri3694 (2014).

44 Siegrist, C. A. Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants. *Vaccine* **21**, 3406-3412 (2003).

45 Frasca, D., Van der Put, E., Riley, R. L. & Blomberg, B. B. Reduced Ig class switch in aged mice correlates with decreased E47 and activation-induced cytidine deaminase. *Journal of immunology* **172**, 2155-2162 (2004).

46 Dorshkind, K., Montecino-Rodriguez, E. & Signer, R. A. The ageing immune system: is it ever too old to become young again? *Nature reviews. Immunology* **9**, 57-62, doi:10.1038/nri2471 (2009).

47 Linterman, M. A. How T follicular helper cells and the germinal centre response change with age. *Immunology and cell biology* **92**, 72-79, doi:10.1038/icb.2013.77 (2014).

48 Govaert, T. M. *et al.* The efficacy of influenza vaccination in elderly individuals. A randomized double-blind placebo-controlled trial. *Jama* **272**, 1661-1665 (1994).

49 Burns, E. A., Lum, L. G., L'Hommedieu, G. & Goodwin, J. S. Specific humoral immunity in the elderly: in vivo and in vitro response to vaccination. *Journal of gerontology* **48**, B231-236 (1993).

50 Gibson, K. L. *et al.* B-cell diversity decreases in old age and is correlated with poor health status. *Aging cell* **8**, 18-25, doi:10.1111/j.1474-9726.2008.00443.x (2009).

51 Weksler, M. E. & Szabo, P. The effect of age on the B-cell repertoire. *Journal of clinical immunology* **20**, 240-249 (2000).

52 Kosco, M. H., Burton, G. F., Kapasi, Z. F., Szakal, A. K. & Tew, J. G. Antibody-forming cell induction during an early phase of germinal centre development and its delay with ageing. *Immunology* **68**, 312-318 (1989).

53 Kolar, G. R., Mehta, D., Wilson, P. C. & Capra, J. D. Diversity of the Ig repertoire is maintained with age in spite of reduced germinal centre cells in human tonsil lymphoid tissue. *Scandinavian journal of immunology* **64**, 314-324, doi:10.1111/j.1365-3083.2006.01817.x (2006).

54 Miller, C. & Kelsoe, G. Ig VH hypermutation is absent in the germinal centers of aged mice. *Journal of immunology* **155**, 3377-3384 (1995).

55 Hayakawa, K., Hardy, R. R., Parks, D. R. & Herzenberg, L. A. The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. *The Journal of experimental medicine* **157**, 202-218 (1983).

56 Binder, C. J. & Silverman, G. J. Natural antibodies and the autoimmunity of atherosclerosis. *Springer seminars in immunopathology* **26**, 385-404, doi:10.1007/s00281-004-0185-z (2005).

57 Duan, B. & Morel, L. Role of B-1a cells in autoimmunity. *Autoimmunity reviews* **5**, 403-408, doi:10.1016/j.autrev.2005.10.007 (2006).