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# EARLY MEMORY B CELLS IN HUMANS ARE PRIMED FOR PLASMA CELL DIFFERENTIATION AND ARE REFRACTORY TO REACTIVATION

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# Abstract

A growing literature has identified the enrichment of germinal center experienced B cells that do not belong to the classical memory or long lived plasma cell compartments in individuals with chronic infection and autoimmunity. In this study, we sought to determine if any of those populations were present in healthy individuals and to characterize their transcriptional program and their phylogenetic relationships with other germinal center experienced populations. We report that CD19+CD27+CD21lo (CD21lo) B cells in humans isolated from peripheral blood are transiently enriched for antigen specificity 14-28 days post-immunization, at the time germinal centers (GCs) peak. While clonally related to memory B cells and plasmablasts, CD21lo cells form distinct clades within phylogenetic trees based on accumulated variable gene mutations, supporting a separate differentiation pathway. Accordingly, CD21lo cells express the Blimp-1 transcriptional program suggesting they are primed for plasma cell differentiation and refractory from GC differentiation, though they do not secrete antibody. Further, they down-regulate tissue and GC homing molecules, are inhibited from re-activation, and appear more susceptible to negative selection. Together, this data supports a model in which CD21lo cells are recent GC graduates that are refractory to GC reentry, preventing disruption of the affinity maturation process of lower affinity B cells.

# Chapter I: Introduction

## Overview

Immunological memory is the ability to have a more robust immune response to previously encountered pathogens. This ability has been documented since the ancient world. Thucydides, when writing about the Athens plague in 430BC, noted that no person who survived a primary infection had a fatal secondary infection (Littman 2009). Furthermore, people have attempted to harness this protective immunity via vaccination as early as the 10$^{th}$ century China, where they used inoculation and variolation against smallpox (Gross and Sepkowitz 1998). Later, Edward Jenner popularized the concept when he demonstrated that inoculating a healthy child with cowpox lesions protected against future smallpox infection (Riedel 2005). Today, vaccination has led to the control or eradication of 12 major diseases from most of the world: smallpox, diphtheria, tetanus, yellow fever, meningitis, pertussis, poliomyelitis, measles, mumps, rubella, typhoid and rabies, and immunological memory remains a critical component to infectious disease management.

Antibodies, also known as immunoglobulins, are specialized proteins secreted by B cells that can bind and facilitate the destruction of pathogens and they play a key role in immunological memory. Antibody based immunity is primarily mediated through high affinity memory B cells and long lived plasma cells generated during T-dependent immune responses. When memory B cells encounter their cognate antigen, they are rapidly reactivated and can differentiate into plasmablasts that secrete large amounts of protective antibodies into the bloodstream (L. J. McHeyzer-Williams and McHeyzer-Williams 2005; Kurosaki, Kometani, and Ise 2015). In contrast, long lived plasma cells continuously secrete

antibody over extended periods of time, providing constant serum-level protection. The secreted antibodies from either population can bind pathogens and protect by directly inhibiting receptor-ligand interactions or by facilitating the phagocytosis or lysis of the pathogen (Hart, Smith, and Dransfield 2004; Katz et al. 1980). Together, these two cellular components of humoral memory can provide potent, long lasting protection against previously encountered pathogens. However, the process of generating this immunological memory is complex and remains incompletely understood. The inability to generate effective immunity to common pathogens like influenza underscores the need to better understand the generation of immunological memory.

## Memory B Cell Development

### Generation of the Naïve B Cell Repertoire

The generation of B cell memory begins with B cell development in the bone marrow. At this site, hematopoietic precursor cells transition into lineage committed B cells. However, more importantly, the development of a diverse B cell repertoire capable of responding to a multitude of pathogens begins in the bone marrow as well. The large number of unique sequences, and consequently, the wide range of specificities of the naïve B cell compartment is a result of VDJ recombination. The heavy and light chains of antibodies are not encoded as single genes, but as a large number of gene segments that are randomly recombined into a functional gene and are then expressed on the cell surface of immature B cells as the B cell receptor (BCR). Each of these gene segments is flanked by non-coding DNA sequences called recombination signal sequences (RSS). During

recombination, the RSSs are brought together, creating a loop in the DNA. The RAG 1/2 complex then binds the RSSs and cleaves away the looped DNA, generating DNA hairpins at each broken end. Terminal deoxynucleotidyl transferase then modifies the ends by adding more nucleotides and DNA repair enzymes then rejoin them (Bassing, Swat, and Alt 2002; Schatz, Oettinger, and Schlissel 1992). The once separate gene segments now code for a complete gene that can be transcribed. This recombination and repair process introduces a high degree of diversity in the antibodies of immature B cells.

However, not all this diversity is useful. Immature B cells may recombine receptors that are self-reactive and potentially harmful. Single cell antibody cloning experiments have shown that up to 75% of immature B cells are autoreactive (Wardemann et al. 2003; Grandien et al. 1994). If self antigen in the bone marrow causes BCR crosslinking in an immature B cell, the cell may undergo one of four fates: clonal deletion, receptor editing, anergy, or immunological ignorance (Pelanda and Torres 2012). In some cases, the self reactive cells are simply removed from the repertoire by apoptosis. This mechanism of clonal selection was first proposed by Burnet and later proven in studies using transgenic mice (Burnet 1976). Immature B cells that bind their cognate antigen with high affinity in the bone marrow are eliminated via apoptosis (Hartley et al. 1991; Nemazee and Bürki 1989). While many self-reactive B cells are eliminated through clonal deletion, B cells with self-reactive receptors can avoid apoptosis if they can recombine a new non-self reactive receptor through receptor editing. In this process, the recombination machinery will generate a new light chain to generate a non-self-reactive receptor. If successful, receptor editing can rescue formerly self-reactive B cells, further expanding the diversity of the repertoire in the naïve B cell compartment (Radic et al. 1993; Gay et al. 1993; Tiegs,

Russell, and Nemazee 1993). Removal of autoreactive antibodies from the repertoire can also be achieved through anergy. If the avidity for the self-antigen is relatively low, the B cell may avoid deletion and may simply enter a state of unresponsiveness to antigen (Goodnow et al. 1988; Hippen et al. 2005). The anergic state allows for an expanded repertoire while also controlling for autoimmunity. Given sufficient stimulus, these anergic cells may later be activated in the periphery and participate in germinal center reactions where self-reactivity can be removed by somatic hypermutation of antibody-encoding genes and affinity for foreign antigen is increased (Sabouri et al. 2014). Self-reactive cells may also escape central tolerance as a result of immunological ignorance. Not all self antigens are expressed in the bone marrow and B cells with receptors reactive to those self antigens will not undergo negative selection and will graduate to the periphery. About 30-40% of B cells remain autoreactive after central tolerance, showing that many self-reactive cells are not removed from the repertoire (Grandien et al. 1994; Wardemann et al. 2003).

**B Cell Receptor Signaling**

When the B cell receptor binds antigen, it initiates a complex signaling cascade that results in changes in gene transcription that leads to the activation of the cell. The BCR complex consists of the membrane bound immunoglobulin, along with the Ig$\alpha$/Ig$\beta$ heterodimer, which contains the cytosolic ITAM motifs required for signaling (Flaswinkel and Reth 1994). When the BCR is crosslinked by antigen, src family kinases are activated and phosphorylate the ITAM motifs (Kurosaki 1999). This signaling cascade can be enhanced by the presence of the co-receptors CD19, CD21, and CD81 in the BCR complex. CD21 can bind complement (C3d) coated antigen, which leads to the phosphorylation of

CD19 (Fearon, Carroll, and Carroll 2000). When CD19 is phosphorylated, it recruits PI3K, which is critical for the propagation of the signaling cascade (Wang et al. 2002). This ability is partially redundant. BCR signaling can still occur in CD19 deficient mice, as BCAP, Cbl, Gab1 and Gab2 can all recruit PI3K as well (Dal Porto et al. 2004). PI3K is necessary for signaling because it generates PIP3 from PIP2 on the plasma membrane. The presence of PIP2 leads to the recruitment of PLCγ2 (Deane and Fruman 2004). PLCγ2 cleaves PIP2 into the second messengers IP3 and DAG, leading to calcium flux. DAG and elevated calcium levels lead to the activation of PKCb, which mobilizes the NF-kb signaling complex (Guo, Su, and Rawlings 2004). The NF-kb transcription factors can then translocate to the nucleus, and induce changes in transcription that result in B cell activation and proliferation(Ruland and Mak 2003).

This complex amplification cascade that mediates BCR signaling is negatively regulated by a number of inhibitory receptors. In general, these inhibitory receptors function by recruiting proteins that modulate signaling by dephosphorylating key members of the BCR cascade (Walker and Smith 2008). FcγRIIb is one of the best characterized signaling regulators. It inhibits BCR signaling by recruiting SHIP, which prevents PIP3 accumulation by hydrolyzing it to PIP2 (Scharenberg et al. 1998). The loss of PIP3 results in decreased recruitment of PLCγ, Btk, and Akt, which inhibits BCR signaling. FcγRIIb also negatively affects BCR signaling by dephosphorylating CD19, which will inhibit CD19's ability to recruit PI3K (Hippen et al. 2005). The SIGLEC family of receptors, which includes CD22, SIGLEC6, and SIGLEC10, can also negatively regulate BCR signaling. They act by recruiting SHP-1 and SHP-2 to docking sites formed after phosphorylation by Lyn. The SHP proteins dephosphorylate members of the BCR signaling cascade, including Igα, Igβ, Syk,

CD19 and SLP-65 (Walker and Smith 2008; Jellusova and Nitschke 2012). PD-1 and the FCRL family of receptors has a similar mechanism of inhibition to SIGLECs. PD-1, upon co-ligation with BCR, recruits SHP-2, while FCRL4 recruits SHP-1 and SHP-2, and FCRL5 recruits SHP-1 to their ITIM motifs (Ehrhardt et al. 2003; Haga et al. 2007; Okazaki et al. 2001).

The balance between the levels of positive and negative regulators of BCR signaling influences the signal intensity and quality generated by ligand engagement. Furthermore, the stage of BCR maturation, the availability of T cell help and the type of antigen may also affect the outcome of BCR signaling.

**Antigen Sampling and B Cell Activation**

Once B cells reach maturity, they position themselves in the follicles of the spleen and lymph nodes to maximize their ability to sample circulating antigens. This is critical because only a small proportion of naïve B cells will have BCRs that can effectively bind antigen and develop into effector or memory B cells. Once generated, memory B cells position themselves similarly so they too can sample antigen. The follicles are directly adjacent to the site of antigen entry in both organs: the subcapsular sinus in the lymph nodes and the marginal zone in the spleen. This positioning facilitates transport of antigen from the lymph and blood to the follicle with the waiting B cells (Batista and Harwood 2009).

Small soluble particles can localize to B cells in the follicle very rapidly. Fluorescently labeled protein antigen injected intradermally is found associated with antigen specific B cells in the lymph nodes within a few minutes (Pape et al. 2007).

Transport of larger particles requires specialized cells to capture them from the lymph and blood. In the lymph nodes, antigen can be delivered to the follicle by CD169+ macrophages in the subcapsular sinus (Buiting et al. 1996; Veninga et al. 2015). Vesicular stomatitis virus injected into the footpad can be found on CD169+ macrophages within 3 hours of injection and their role in antigen delivery was further confirmed in a PE immunization model (Junt et al. 2007; Phan et al. 2009). Additionally, CD11b+ SIGNR1+ dendritic cells (DCs) can also capture antigen in the lymph node medulla. The DCs then shuttle the antigen towards the follicular dendritic cell (FDC) region (Gonzalez et al. 2010). A similar mechanism for transporting antigen exists in the spleen. Marginal zone macrophages have been shown to pick up antigen from the blood flowing through the sinus and shuttle it into the follicle (Cinamon et al. 2007). Once antigen enters the follicle in either organ, it is displayed on the surface of follicular dendritic cells (FDCs) for extended periods of time (Nossal et al. 1968). B cells in the follicle can then scan and capture their cognate antigen from the FDC network (Suzuki et al. 2009). The B cells are activated after binding antigen, and migrate to the T cell zone to receive help from follicular T helper cells.

B cells that have found their cognate antigen on the FDC network still require T cell help before they can proceed to the germinal center or extrafollicular foci to develop into plasmablasts, memory cells, or long lived plasma cells. After BCR engagement, B cells migrate to the border between the T cell zone and B cell zone in a CCR7 dependent fashion (Y.-J. Liu et al. 1991; Okada et al. 2005). Here, they internalize antigen bound to the BCR and present processed antigen on MHCII molecules and attempt to find a T cell specific for the same antigen. When a B cell pairs with a T cell that can engage its peptide-MHC complex, the B cell proliferates briefly (Coffey, Alabyev, and Manser 2009). The B cell

clones can then enter either an extrafollicular focus and rapidly differentiate into antibody secreting plasmablasts or participate in a germinal center where their BCRs are mutated to increase their ability to bind antigen. The extrafollicular focus functions to generate early effector responses while the germinal center is the primary site for the development of high affinity antibodies that provide lasting protection against secondary challenges. The repertoires generated from the germinal center and the extrafollicular response are clearly distinct. The BCRs of cells exiting the germinal center are far more somatically mutated and generally bind with higher affinity than those exiting the extrafollicular response (Paus et al. 2006a). However, it is unclear whether the repertoires that seed the germinal center and extrafollicular response are at all distinct, though there is at least some overlap. Histological studies have found that some B cells in neighboring foci and germinal centers have a common clonal origin (Jacob 1992). It is likely that the progeny of some B cells that proliferate at the T-B border are able to enter both the follicle and focus.

**Extrafollicular Response**

While the germinal center reaction is responsible for generating high affinity antibodies, the process does not begin until about 7 days after the initial exposure to pathogen. However, the extrafollicular response occurs in the first days after the initiation of the immune response, providing an alternative source of antibodies and can generate some memory B cells as well. B cells activated in the follicle that acquire the necessary T cell help can migrate to the medullary cords in the lymph node or the bridging channels of the spleen. There, B cells aggregate in foci and can differentiate into antibody secreting plasmablasts. Each focus is estimated to be seeded by 1-3 cells (Jacob, Kassir, and Kelsoe

1991). When the germinal center is just forming, the cells participating in the extrafollicular response are of higher affinity than those in the germinal center (Paus et al. 2006b). This distribution may be the result of the preferential expansion of higher affinity cells within the extrafollicular foci. When mice with transgenic BCR receptors for HEL were challenged with intermediate and low affinity antigen, there was no significant difference in the number of B cell recruited to the extrafollicular response. However, by day 6, at the peak of the response, there were significantly fewer cells outside the follicle compared to the high affinity antigen response, though the germinal center was also affected. Alternatively, recruitment into the extrafollicular response may be affinity based. BCR signaling strength is reflected in the levels of IRF4 induction and cells with higher levels of IRF4 preferentially entered the extrafollicular response (Ochiai et al. 2013).

Early in the extrafollicular response, the plasmablasts primarily secrete unmutated IgM antibodies (M. G. McHeyzer-Williams 1993). However, as the response progresses, class switching can occur (Takahashi et al. 1998; Lee et al. 2011). Class switching is the process by which a B cell changes the isotype of antibody it produces. Histological studies have found that up to 40% of the foci will be completely isotype switched at the peak of the extrafollicular response (Jacob, Kassir, and Kelsoe 1991). Somatic hypermutation may also occur in the focus. In a model of lupus, autoreactive extrafollicular B cells were found to be mutated (William et al. 2002; Herlands et al. 2007). It is unclear whether this repertoire diversification in the focus occurs in a healthy host and whether there are any means of selection for beneficial mutations. The extrafollicular response resolves once the germinal centers ramp up. After about a week, in response to a still unknown signal, these early

plasmablasts undergo apoptosis and the foci disappear (Kenneth G. C. Smith et al. 1996; Jacob, Kassir, and Kelsoe 1991).

**Germinal Center Response and Affinity Maturation**

The germinal center is the site of a focused expansion of the B cell repertoire. A remarkable aspect of humoral immunity is the ability to increase the diversity of the B cell repertoire after development. When an individual mounts a humoral response to a pathogen or vaccine, antigen specific B cells can undergo further modifications of their BCR genes, thus increasing the diversity of the B cell repertoire. This process of further BCR refinement results in the increase in the affinity of the repertoire for that pathogen or vaccine (Eisen and Siskind 1964). The germinal center is responsible for this increase in diversity and specificity, through a process called affinity maturation. This expanded repertoire is utilized immediately in the plasmablast effector response and is also preserved in the memory compartment.

Entry of activated B cells into the germinal center is competitive. In studies done with high affinity and low affinity hapten specific BCR transgenic cells, the germinal centers in mice immunized with hapten were dominated by the high affinity clones (Shih et al. 2002). However, lower affinity B cells are capable of seeding and partaking in germinal center reactions in the absence of competition. Both high and low affinity cells can bind antigen and present peptide, but the lower affinity cells are unable to compete for T cell help at the T-B border (Schwickert et al. 2011). Thus, B cells that are antigen experienced, but bind with comparatively low affinity, are excluded from contributing progenitors to the somatically mutated antibody repertoire.

Affinity maturation occurs by somatic hypermutation, the process in which activation induced cytidine deaminase (AID) makes random point mutations in the BCR genes that may increase the BCR's ability to bind antigen (Teng and Papavasiliou 2007). To generate the most effective repertoire, the germinal center must then select the B cells with the highest affinities for survival and target the B cells with lower affinities or defunct receptors for apoptosis. This is achieved by creating a Darwinian competition. Germinal center B cells are all poised for apoptosis but can be rescued if they demonstrate the superior binding ability of their BCR by successfully competing for limited amounts of antigen and T cell help.

This competition occurs in two anatomically distinct zones: the dark zone where B cells are rapidly dividing and mutating, and the light zone, where B cells compete for survival. The light zone is established first, with clones seeding the germinal center as it is first forming and with the cells clustering around the FDC network (Y.-J. Liu et al. 1991; Jacob, Kassir, and Kelsoe 1991). While those earlier reports suggested that early GCs are pauciclonal, with 1-5 clones contributing progenitors, a recent study that sequenced the antibody genes of early GCs found that they were far more diverse, with an estimated 50-200 clones seeding each GC (Tas et al. 2016).

The dark zone and light zone have several phenotypic differences. The light zone is defined by the presence of FDCs and T cells. FDCs are vital to functioning germinal centers because they trap and display antigen. The antigen is retained for extended periods of time by trapping antibody coated antigen using CR1, CR2 and FcR gamma receptors expressed on their surface (Cyster et al. 2000; C. D. C. Allen and Cyster 2008). FDCs also define the character of the light zone by secreting the chemokine CXCL13. The presence of CXCL13

attracts Tfh cells, which express CXCR5. These Tfh cells provide signals to GC B cells that are indispensable for affinity maturation. Athymic mice lacking T cells are unable to develop germinal centers and humans that have mutated CD40 or CD40L, which are molecules required for T-B interactions, have a similar phenotype (Sverremark and Fernandez 1998; R. C. Allen et al. 1993).

A similar network of stromal cells supports the dark zone. While some FDC processes extend into the dark zone, the stromal cells in the dark zone consistent primarily of reticular cells that are characterized by their expression of CXCL12. The network of these cells is far less dense than that of the FDCs and they do not express CR1 or CR2 (Bannard et al. 2013; Rodda et al. 2015). The reticular cells' expression of a different chemokine from the light zone and their inability to bind immune complex generates an antigen and chemokine gradient that leads to the functional polarization of the germinal center.

GC B cells localize to the dark zone or the light zone based on their expression of CXCR4 and CXCR5, the receptors for CXCL12 and CXCL13 respectively. GC B cells with high levels of CXCR4 are drawn to the dark zone where reticular cells are secreting CXCL12. Dark zone GC B cells undergo somatic hypermutation, accumulating random point mutations in their antibody genes, and proliferate rapidly (C. D. C. Allen et al. 2004). The mutations may improve the binding abilities of the BCR or result in a lower affinity or nonfunctional receptor, but this is not tested in the dark zone. Eventually, in response to a still unknown signal that is independent of positioning, dark zone cells will decrease CXCR4 expression, and increase CXCR5, allowing them to migrate to the light zone (Bannard et al. 2013).

Once in the light zone, GC B cells compete for survival. GC B cells express high levels of Fas, which predisposes them to apoptosis, but they can be rescued from cell death if they receive signals from Tfh cells (Hao et al. 2008; Takahashi, Ohta, and Takemori 2001; Schwickert et al. 2011; Victora et al. 2010). To receive these survival signals, GC B cells compete to bind antigen displayed on the FDC network. The antigen is then processed into peptides and displayed on MHC molecules on the B cell surface for presentation to Tfh cells. B cells with the highest affinity receptors will be able to pick up the most antigen from the FDC network and will have more peptide-MHC to present to the Tfh cells. Increased T cell help is directly linked to GC B cell survival. The role of T cells as gatekeepers to B cell survival is supported from experiments where antigen was delivered to B cells in a BCR independent manner. GC B cells that received antigen in a BCR independent manner had decreased levels of affinity maturation despite higher levels of survival in the germinal center compared to GC B cells that picked up antigen from FDCs in a BCR-dependent manner (Victora et al. 2010).

GC B cells in the light zone that are unable to capture antigen from the FDC network or do not receive T cell help will undergo apoptosis, thus removing cells with low affinity BCRs or self reactive BCRs from the repertoire. GC B cells that do receive T cell help will either return to the dark zone to undergo further rounds of affinity maturation, or may receive a graduation signal. At any one time, the cells selected for survival are the cells with the highest affinity BCRs in a particular germinal center (C. D. C. Allen et al. 2007; Victora et al. 2010; Shih et al. 2002). There are many mechanisms that may contribute to the overall affinity of the germinal center increasing over time. Higher affinity may simply come from more mutation. The longer the germinal center reaction is in progress, the more mutations

a B cell can accumulate as they cycle repeatedly through the dark and light zones. Recently, a study showed that the amount of T cell help a GC B cell receives in the light zone is directly correlated to the amount of proliferation and mutation it will undergo when it returns to the dark zone (Gitlin, Shulman, and Nussenzweig 2014). This amplification of clones that can effectively bind antigen further accelerates the affinity maturation process. Additionally, increasingly limited amounts of antigen may also contribute to higher affinity. As the immune response progresses, antigen will become more scarce. At this point, only the B cells with the very highest affinity receptors would be able to compete for antigen and receive T cell help.

**Post-Germinal Center Differentiation**

The main role of the germinal center is to produce cells that can protect against future infections. Cells that graduate from the germinal center can differentiate into plasmablasts, memory B cells or long lived plasma cells, but the mechanism that drives this fate decision remains controversial. While early studies suggested that differentiation decision may be stochastic, the discovery that memory B cells and plasma cells have different affinities and specificities suggested otherwise (Hasbold et al. 2004). In mice given a primary immunization with NP, only a proportion of NP specific splenic memory B cells are high affinity, while nearly all of the NP specific bone marrow plasma cells are high affinity (K. G.C. Smith et al. 1997). Another study from this group showed that BCL-2 deficient mice, which have reduced apoptosis in the germinal center, accumulate large numbers of low affinity memory B cells after immunization, but the bone marrow plasma cell compartment contains only high affinity cells at a similar proportion to wild type mice

that received the same immunization (Kenneth G. C. Smith et al. 2000). Memory B cells and plasma cells were also shown to have different specificities when immunized with more complex antigens. In mice immunized with the West Nile Virus vaccine, memory B cells were able to bind an escape mutant, but plasma cells were not, showing that memory B cells may have a wider range of specificities (Purtha et al. 2011). Because the memory compartment contains cells with both high and low affinity BCRs for the immunizing strain, the low affinity cells may have a greater potential to cross react to an escape mutant. The differences in affinity in the two compartments may have evolved to maintain this functional flexibility.

There are multiple models that attempt to explain how this difference in affinity and specificity in the memory B cells and plasma cells arises as cells graduate from the germinal center. One model suggests that the affinity of the BCR determines GC B cell fate because the affinity and specificity of memory B cells and long lived plasma cells are distinct. In studies performed on BCR transgenic mice immunized with HEL, GC B cells with high affinity BCRs can differentiate into plasma cells with significantly greater frequency than GC B cells with low affinity BCRs, suggesting there is an affinity threshold cells must overcome to join the plasma cell compartment (Phan et al. 2006). Further evidence of an affinity threshold comes from studies of IRF4 signaling. The strength of BCR signaling a cell receives is correlated with the amount of IRF-4 expression, which is directly linked to plasma cell differentiation (R. Sciammas et al. 2014). Additionally, a recent study showed that higher levels of the Blimp-1 antagonist, Bach2, in light zone cells are correlated with lower affinity and less T cell help, and a greater propensity to differentiate into memory B cells (Shinnakasu et al. 2016). In sum, these studies suggest that Blimp-1 expression is

affected by BCR affinity in the germinal center, and is directly correlated with plasma cell differentiation. However, the molecular mechanism for translating BCR signaling strength or T cell help into changes in Blimp-1 expression remains unclear.

An alternate model of the germinal center fate decision suggests that differentiation decisions are made in a temporal manner. The affinity of germinal centers increases over time and BrdU pulse labeling experiments showed that plasma cells are exported from the germinal center later than memory B cells (Weisel et al. 2016). There may be an affinity independent temporal switch that changes the germinal center from producing memory B cells early in the response to producing plasma cells later in the response. The increasing affinity of germinal center B cells over time is therefore reflected in the difference in affinity of the two populations. However, it is unknown what the non-affinity based signal in this model would be.

## Differentiation and Function of B Cell Memory Populations

### Plasma Cell Differentiation and Function

Plasma cells are a highly specialized memory population that persists for years or decades in the bone marrow after they are generated. They secrete antibody into the serum to provide frontline protection against invading pathogens. This long term antibody secreting ability is largely controlled by Blimp-1, a transcription factor that inhibits the mature B cell program by repressing a large swath of genes and promoting the expression of plasma cell associated genes like XBP-1(Shaffer et al. 2002; Angelin-Duclos et al. 2000; Shapiro-Shelef et al. 2003). IRF4 also plays a critical role in differentiation, but in a dosage

dependent manner. At low levels, it promotes the germinal center phenotype, but at higher levels, it activates Blimp1 and represses BCL-6 (Roger Sciammas et al. 2006; Ochiai et al. 2013). This transcriptional transformation begins while the cell is still in the germinal center. Studies in transgenic mice with a fluorescent reporter for Blimp-1 show that a portion of GC B cells express Blimp-1 (Fooksman et al. 2010).

The Blimp-1 program is antagonized by a number of other transcription factors. Blimp-1 is inhibited by PAX5, a transcription factor that is expressed exclusively by B cells and regulates many aspects of B cell function, including BCR signaling, trafficking, metabolism and transcription (Fuxa and Busslinger 2007; Delogu et al. 2006). PAX5 downregulation is required for plasma cell development (Lin et al. 2002). Blimp-1 is also regulated by BCL-6. BCL-6 is expressed primarily in germinal center cells and promotes B cell activation, rapid proliferation, and inhibits the DNA damage response to allow for affinity maturation (Basso et al. 2010; Dent et al. 1997; Ye et al. 1997). In germinal center cells, it directly represses Blimp-1 to prevent the adoption of the plasma cell phenotype (Tunyaplin et al. 2004; Shaffer et al. 2000). However, in plasma cells, Blimp-1 directly represses BCL-6, creating a molecular axis of mutual antagonism. Repression of BCL6 is required to turn off the mature B cell program and transition to a plasma cell phenotype (Shaffer et al. 2002). Bach-2 is also a direct inhibitor of Blimp-1, and as mentioned previously, also plays a direct role in memory B cell differentiation (Ochiai et al. 2008; Shinnakasu et al. 2016).

The Blimp-1 program is also used by short lived plasmablasts that rapidly secrete large amounts of antibody before dying. While the developmental relationship between plasmablasts and plasma cells is still unclear, shorter lived plasma cells in mice express

lower levels of Blimp-1 and maintain more phenotypic similarities to mature B cells, while still secreting antibody (Kallies et al. 2004). Some groups have suggested that a portion of short lived plasma cells differentiate into long lived plasma cells, but that has not been definitively shown. However, it is clear that long lived plasma cells are germinal center derived; not only is there clear evidence of somatic hypermutation and class switching in the population, but also ablation of the germinal center leads to the abrogation of plasma cell generation (Good-Jacobson and Shlomchik 2010). Furthermore, lineage marking of germinal center cells show that a portion of those cells differentiate into bone marrow resident plasma cells (Weisel et al. 2016).

Once long lived plasma cells graduate from the germinal center, they migrate to the bone marrow by upregulating S1P to egress from the lymph node or spleen, and CXCR4 to enter the bone marrow. Once in the bone marrow, they are dependent on receiving survival signals from other cells in the environment, including IL-6, TNF, and APRIL. APRIL is required for the long-term survival of long lived plasma cells. When APRIL's receptor, BCMA, is knocked out in mice, plasma cells do not survive in the bone marrow (O'Connor et al. 2004). BCMA is also required to establish high levels of Mcl-1, an anti-apoptotic factor that is also required for survival (Peperzak et al. 2013).

**Memory B Cell Differentiation and Function**

Compared to plasma cell differentiation, memory B cell differentiation is still a poorly understood process. Bach2 has been recently shown to control memory B cell differentiation, but it is unclear whether it is a master transcriptional regulator that drives the memory B cell phenotype, as it's only known function in B cells is to repress Blimp-1

(Ochiai et al. 2008). Further, the memory B cell population is heterogeneous, and it is unclear whether the same rules govern differentiation into these phenotypically distinct subsets. However, it is well established that in T-dependent responses, the majority of high affinity memory B cells differentiate from the germinal center response. While this is intuitive from the observation that the BCRs are isotype switched and somatically mutated receptors, this was more formally shown in mice with disrupted germinal center formation due to deficiencies in CD40, SAP, or BCL-6. The mutant mice had memory compartments that were diminished or absent (Kawabe et al. 1994; Foy et al. 1994; Xu et al. 1994; Crotty et al. 2003; Toyama et al. 2002). More recently, fate-mapping experiments with labeled germinal center cells definitively showed that they differentiate into memory B cells (Dogan et al. 2009; Weisel et al. 2016). In addition to the germinal center, memory B cells can also be generated from the extrafollicular response (Blink et al. 2005; Weisel et al. 2016; Inamine et al. 2005; Toyama et al. 2002; Linterman et al. 2010). These cells tend to be less mutated and predominantly express IgM BCRs.

Memory B cells tend to be long lived. In a study of survivors of the 1918 Spanish flu pandemic, antigen specific B cells were identified 90 years after initial contact with the pathogen (Yu et al. 2008). The long-term survival of B cells varies by isotype. IgM B cells persist for longer than 500 days post immunization in mice, while IgG B cell compartment size was significantly diminished at that time point (Pape et al. 2011). The difference in life span may be a result of peripheral tolerance removing B cells that cross react with self, as IgG memory B cells, which undergo a greater degree of somatic hypermutation, also tend to be more polyreactive (Gitlin et al. 2016).

The hallmark of memory B cells is their ability to rapidly respond to a secondary challenge by differentiating into plasmablasts at a much more rapid rate than naïve B cells (Ahmed and Gray 1996; Dixon, Maurer, and Deichmiller 1954). While this can happen without T cell help, the number of plasma cells generated during a recall response is significantly greater with the presence of T cell help (Zuccarino-Catania et al. 2014). The mechanism that controls this ability is still unclear. Some groups have suggested a BCR intrinsic model and found quantitative and qualitative differences in the signaling abilities of IgM and class switched BCRs. All naïve B cells have IgM BCRs, whereas a large portion of memory B cells have class switched BCRs. IgG was shown to signal differently from IgM because of enhanced oligomerization after antigen binding (W. Liu et al. 2010; Davey and Pierce 2012). Furthermore, the cytoplasmic tails of IgG and IgE switched BCRs can also recruit additional adaptors, Grb2 and SAP97, which also leads to enhanced responses to antigen stimulus(Engels et al. 2009; W. Liu et al. 2012). However, there is likely also a role for BCR extrinsic differences that regulate the memory phenotype. Nuclear transplant experiments, where the nuclei of memory or naïve B cells were implanted in IgG expressing B cells, showed that only cells with memory nuclei differentiated into plasma cells upon stimulation (Kometani et al. 2013). This suggests there may be a distinct transcriptional program or other mechanism of gene regulation that controls the memory B cell phenotype.

During a secondary response, memory B cells also have the ability to re-enter the germinal center and remodel their receptors. However, it is unclear whether this ability is present across the memory compartment, or isolated to certain subsets. One study that separated cells by isotype found that IgM memory cells preferentially re-enter the germinal

center compared to IgG cells (Dogan et al. 2009). However, other groups have showed that IgG memory cells have an equal ability to participate in germinal center reactions, and one suggested that CD80 and PDL2 are better markers for determining germinal center re-entry (L. J. McHeyzer-Williams et al. 2015; Zuccarino-Catania et al. 2014). The differences in the studies may result from the confounding effects of differing immunization antigens and it is unclear whether these findings translate into human memory populations. Additionally, no molecular mechanism for determining germinal center re-entry in either of these models has been proposed.

**Non-Classical Memory B Cell Populations**

In addition to the well-characterized memory and plasma cell compartments, there is a growing literature describing the existence of non-classical memory B cells. These cells have distinct phenotypic markers, transcriptional profiles, and functional capabilities. Non-classical memory B cells have largely been identified in the context of chronic viral or parasitic infection, or in chronic autoimmunity, but also have been documented in healthy tonsils and peripheral blood.

**Chronic Infection Associated Non-Classical Memory B Cells**

Non-classical memory B cells were first identified in viremic HIV patients. While classical memory B cells in humans express CD27 and CD21 at the cell surface, a population of CD27-CD21lo memory-like B cells is expanded in viremic HIV patients. These patients also had other changes in surface expression, including elevated CD95 (Fas) and BCMA, and decreased BAFF-R (Moir et al. 2004). Further study of this population revealed that CD27-

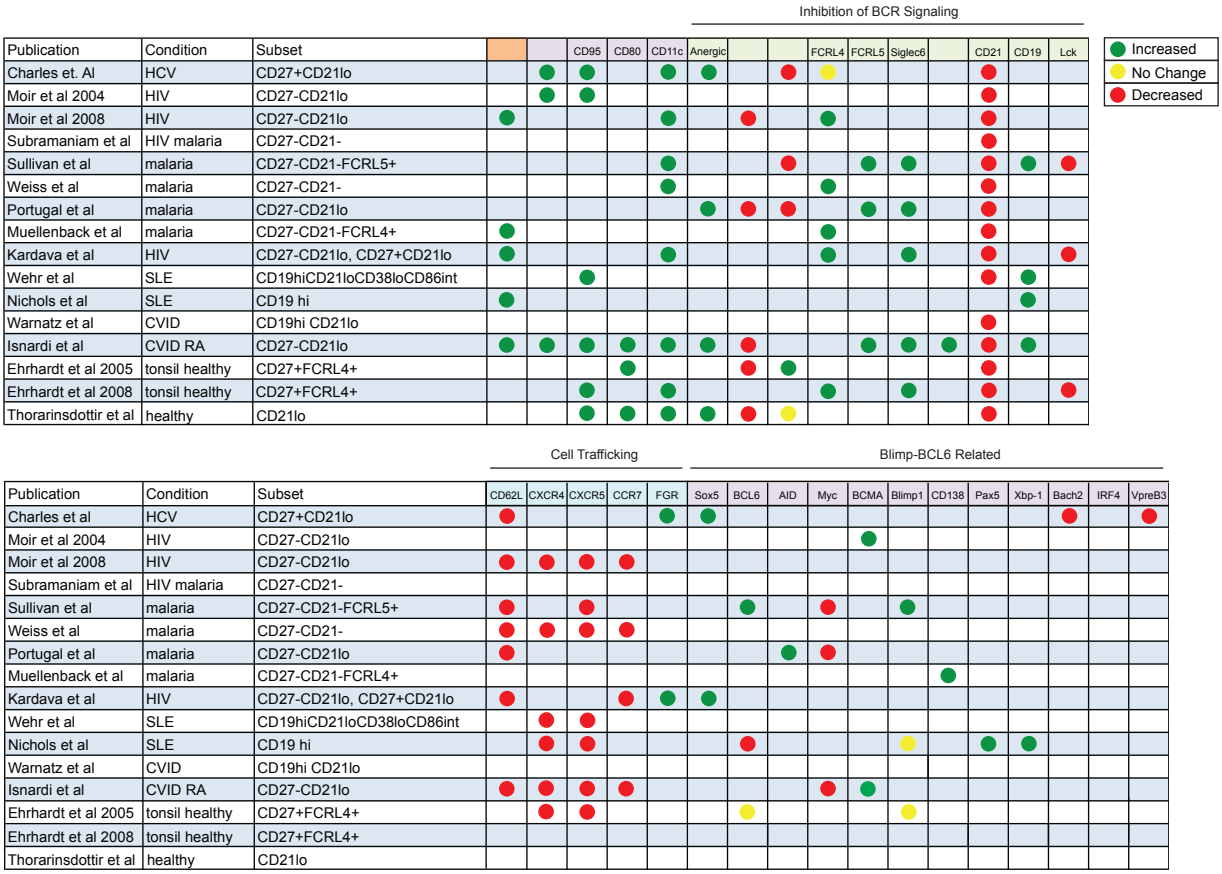


**Table 1: Summary table of characteristics of non-classical memory populations.**

Graphical summary of literature surrounding non-classical memory populations. The dot colors represent whether a particular gene or biological process was upregulated, downregulated or was unchanged. The absence of a dot indicates that no assay for that gene or pathway was performed.

CD21- cells had a decreased proliferative capacity in response to a variety of stimuli, but were enriched for HIV specific cells. This suggested that CD27-CD21lo cells consist of exhausted cells that have undergone repeated stimulation due to the constant presence of antigen in a chronic inflammatory environment. This exhaustion likely contributes to inability to control HIV in viremic individuals (Moir et al. 2008). The expanded population of non-classical memory cells also affects HIV patients' ability to respond to other pathogens. In patients with HIV and malaria co-infection, the portion of CD27-CD21lo cells

are increased and the breadth and magnitude of the malaria specific antibody response is reduced (Subramaniam et al. 2015).

Further studies focused on antigen specific cells in HIV patients and found that cells specific for the HIV protein gp140 were most enriched in the CD27+ CD21lo compartment, followed by the CD27+CD21+ classical memory compartment, and then the CD27-CD21lo population described above. There was an inverse correlation between the fraction of gp140 specific cells in the non-classical CD27+/- CD21lo compartment and the level of HIV plasma viremia (Kardava et al. 2014). This suggests that while these non-classical populations contain antibodies with protective capabilities, their presence is associated with negative outcomes in HIV patients due to their functional inhibition.

A similar CD21lo population has also been identified in HCV patients. In HCV+ patients with mixed cryoglobulinemia, there is an expanded memory-like B cell population that is CD27+, CD21lo, CD11c+, FCRL4hi, and IL-4Rlo. These cells have a distinct transcriptional profile, and differentially express genes including SOX5, CD11c, galectin-1 and FGR. Similar to the cells identified in HIV, they also have decreased calcium flux upon BCR triggering, and do not differentiate into plasmablasts as efficiently compared to classical memory B cells.

Non-classical memory B cells can also be identified in healthy individuals living in malaria endemic areas. In these individuals, CD27-CD21lo cells have elevated FCRL5 and CD11c, and lower CD62L, CXCR5, CCR7, and CXCR4 compared to classical memory B cells. These CD21lo cells also had reduced BCR signaling, proliferation and cytokine secretion. However, there is no difference in the distribution of isotypes or mutation rate, suggesting that despite their functional and phenotypic differences, classical and non-classical

memory B cells may share a common precursor (Weiss et al. 2009; Portugal et al. 2015). A similar population of non-classical memory cells, defined by elevated FCRL5 expression, was also identified in individuals living in malaria endemic areas. These FCRL5+ cells have a distinct transcriptional profile, and differential expression of surface markers including lower CD21 and elevated FCRL3, CD20, and CD19. Similar to the other previously described populations, FCRL5+ cells have a decreased ability to differentiate into antibody secreting cells, compared to the classical memory population (Sullivan et al. 2015).

While the previously described studies explicitly did not observe an expansion of an FCRL4+ non-classical population, another group showed that repeated malaria infection leads to the accumulation of FCRL4+ cells (Muellenbeck et al. 2013). Single cell antibody cloning of the FCRL4+ cells showed that they produce *Plasmodium* neutralizing antibodies, but sequencing of these antigen specific cells did not identify any that were clonally related. While there was no difference in the frequency of *Plasmodium* specific antibodies between FCRL4+ cells and classical memory cells, polyreactive antibodies are enriched in the FCRL4+ compartment. The enrichment of polyreactive antibodies in this compartment suggests there is either a selection defect in malaria infected individuals, or that this population is recently generated and has not yet had the time to be pruned by peripheral selection.

**Non-Classical Memory B cells in Autoimmunity**

Non-classical memory B cells have also been observed in individuals with chronic autoimmune disorders. Two studies have identified a population of expanded CD19 hi memory B cells in systemic lupus erythematosus (SLE) patients that is correlated with

worse clinical outcomes. The CD19hi compartment is enriched with autoreactive B cells, are responsive to BCR stimulation, but have decreased CXCR4 and CXCR5(Wehr et al. 2004; Nicholas et al. 2008). Another group identified an expansion of CD27- cells in SLE patients that was also correlated with more severe disease. Similar to the CD19hi cells, the CD27- population was enriched for autoantibodies (Wei et al. 2007).

Similar non-classical memory B cells populations were also found in patients with common variable immune deficiency (CVID) or rheumatoid arthritis (RA). A CD19hi CD21lo population is expanded in CVID patients with autoimmune cytopenia, and to a lesser extent, in SLE patients (Warnatz et al. 2002). Additionally, an expanded CD27- CD21lo population was also observed found in RA and CVID patients. Similar to the CD19hi population in SLE patients, the CD27-CD21lo cells are also enriched for autoreactive antibodies. However, unlike the CD19hi population, these cells have reduced calcium flux upon BCR stimulation. They also have a decreased proliferative capacity and a higher proportion of apoptotic cells. Gene expression profiling revealed the differential expression of many genes that are similar to that of non-classical genes identified in individuals with chronic viral infections (Isnardi et al. 2010).

**Non-Classical Memory B Cells in Healthy Individuals**

Non-classical memory B cells have also been identified in healthy individuals. The first observation of these populations in people without chronic infection or autoimmunity identified an FCRL4+ population in the tonsils. The cells have a cell surface phenotype that is distinct from classical memory B cell, but is similar to other non-classical populations, primarily in the upregulation of Fas and CD11c, and the decreased levels of CD21. FCRL4+

cells were tested for their expression of plasma cell related genes, but there was no detectable expression. However, microarray analysis revealed significant differences in the transcriptional programs between FCRL4+ and classical memory B cells, including and upregulation of inhibitory molecules like SIGLEC6 and transcriptional factors like SOX5 (Ehrhardt et al. 2005; Ehrhardt et al. 2008).

CD21lo cells have also been identified in healthy adults. They make up about 5% of the CD19+ population and similar to other non-classical populations, have elevated CD95 and decreased CD62L. Other similarities include a decreased proliferative capacity and reduced ability to differentiate into plasmablasts after non-specific stimulus (Thorarinsdottir et al. 2016, 21).

## Influenza as a Model of Acute B Cell Responses in Humans

The variety of memory B cell populations identified in humans suggests that further study is needed to better understand their role in the generating protective immunity. Influenza vaccination is one experimental system that is a useful model in interrogating acute B cell responses in humans. Influenza is an RNA virus that infects both humans and a number of animal species, including birds, swine, dogs, horses and cattle (Parrish, Murcia, and Holmes 2015). There are three types of influenza: A, B and C. Only A and B are associated with the seasonal flu in humans and are the only types people are vaccinated against. Within each type, there are many different strains of the virus that have evolved. Influenza's RNA polymerase lacks an error correction mechanism, and therefore many mutations are introduced during the life cycle of the virus. Mutations that provide a

selective advantage to the virus are preserved and amplified in the population. Additionally, new strains can develop when multiple influenza viruses infect the same host and exchange genetic material via recombination (Cox and Subbarao 2000).

The tremendous genetic diversity amongst influenza viruses makes generating lasting protective immunity challenging. Even if effective immunological memory is developed against an infecting strain, a person may still be susceptible new strains that are antigenically distinct. This lack of protective immunity is a major epidemiological problem because infection with influenza carries non-trivial health risks. It remains a significant source of mortality, which results from pneumonia, respiratory and cardiorespiratory conditions, as well as the exacerbation of underlying, non-respiratory disease. Influenza is responsible for over 41,000 deaths a year in the US, and the elderly and young are particularly susceptible (Simonsen et al. 2013; Beigel 2008).

The most effective tool against influenza infection remains the influenza vaccine. There are many versions of the vaccine, but the type given to the majority of adults is generated with a mixture of formaldehyde-inactivated strains of the virus and is delivered via intramuscular injection (Beigel 2008). The inactivated vaccine contains 3-4 strains of the virus, which are selected by the World Health Organization based on the prevalence of strains are currently in circulation. The vaccine is estimated to be 59% effective in adults and yearly vaccination is recommended for the majority of the population (Osterholm et al. 2012).

Due to the global health implications of influenza infection, there has been extensive research into the human B cell response to the influenza vaccine. In addition to providing knowledge on how to better target the vaccine to increase effectiveness, it has also become

an excellent experimental system to study acute B cell responses in humans. First off, it is relatively easy to find people who meet the qualifications to participate in the study. The vaccine is approved for yearly use for most healthy individuals (Beigel 2008). Additionally, the B cell response to the vaccine is relatively well characterized compared to other vaccines in humans. It has been well documented that the influenza protein, hemagglutinin (HA), is the target for most neutralizing antibodies and reagents to use HA in antigen baiting experiments are available (Wrammert et al. 2011; Whittle et al. 2014). Additionally, the kinetics of the B cell response are relatively consistent between people. Most people have a plasmablast burst 7 days post immunization and begin to generate flu specific memory after 14 days (K. Smith et al. 2009). This consistency reduces the number of time points that need to be collected in order to capture the vaccine responding B cells. Finally, B cell responses to influenza tend to be robust, and isolation of antigen specific cells is relatively simple (Wrammert et al. 2011). In comparison, vaccination with de novo antigens, like the anthrax vaccine, results in far fewer responding cells (unpublished data).

However, there are some complications to using influenza vaccination as an experimental model. First, due to the prevalence of flu, people have had repeated exposures to the virus, leading to varied immune histories. Immune history affects the vaccine response because memory B cells contribute to the pool of responding B cells and determine the specificity of the B cell response (Andrews, Huang, et al. 2015). Thus, the varying flu specific memory B cell repertoires in study participants can potentially confound results. Further, the vaccine changes from year to year, and the efficacy can vary as well (Beigel 2008). Thus, results from samples collected in multi-year studies may lack consistency due to varying responses to the different vaccines. Neither of these potential

confounding factors are easy to control, but influenza vaccination remains an attractive experimental model to study acute B cell responses in humans.

**Summary and Hypothesis**

The current model of post-germinal center B cell differentiation leaves much unexplained. Germinal center graduates have multiple possible fates, including differentiation into the plasmablast, memory B cell, or long lived plasma cell lineages. Much research has gone into the biology of these cells and has found they are distinctive in their function, kinetics of appearance and affinity of their BCRs. However, the mechanisms that determine the fate of GC graduates remains unknown. This is due, in large part, to the challenges of tracking recent germinal center emigrants that recirculate throughout the body upon leaving the germinal center. It is unclear whether germinal center graduates differentiate immediately into memory or plasma cells upon exiting the germinal center or if there is a transitional stage. Additionally, it is unknown what mechanism prevents recently graduated cells from being reactivated immediately if they encounter lingering antigen in the periphery. This would result in both the disruption of the ongoing germinal center response and the depletion of the long-term memory populations meant to protect against future immune challenges. Finally, it is unknown exactly how the immune system tolerizes B cells that acquire reactivity to peripheral self antigen during affinity maturation. To address these questions, we hypothesized that recent germinal center graduates enter a transitional state during which they are refractory to stimulus to prevent their immediate reactivation and thus protect the ongoing affinity maturation response and preserve these new additions to the memory compartment. Additionally, as they recirculate throughout

the body, these transitional cells would have the chance to test their receptors for reactivity with peripheral antigens they would not encounter in the germinal center.

The growing literature describing non-classical memory B cells in humans suggested that these cells might be good candidates for this transitional population. While their phenotypic descriptions vary to some degree, the majority of these populations downregulate CD21 and are functionally non-responsive, but contain antigen specific, germinal center experienced cells. These cells have been primarily observed in individuals with chronic infection or autoimmunity, but have also been identified in healthy individuals. The identification of non-classical memory B cells in healthy adults suggests that these cells are a part of the normal post-germinal center differentiation process rather than a reflection of a dysregulated or exhausted immune system, as has been postulated for the populations found in individuals with autoimmunity or chronic infection.

It is clear that the previously identified CD21lo and FCRL4/5+ populations are germinal center experienced. Not only do they contain large numbers of isotype switched cells and an equal or larger number of mutations compared to classical memory B cells, but they are also enriched for antigen specific cells. However, there is a large body of evidence showing they are functionally inhibited, compared to classical memory B cells. The CD21lo and FCRL4/5+ populations are more prone to apoptosis compared to classical memory B cells. Multiple studies have found elevated levels of CD95 (Fas) expression at the gene and protein level, and an increased propensity for apoptosis as measured by annexin staining both with and without stimulus. These non-classical populations also have a reduced capacity for BCR signaling compared to memory B cells. Not only do the non-classical populations have elevated levels of BCR inhibitory molecules like FCLR4, FCLR5, SIGLEC6,

and SIGLEC10, and decreased levels of the BCR co-receptor CD21, they also have decreased calcium flux after BCR stimulation, a decreased ability to proliferate after BCR specific and non-specific stimulation, and a diminished potential to differentiate into antibody secreting cells.

These varied populations have also downregulated receptors required to participate in germinal center reactions, including L-selectin, CCR7, CXCR4 and CXCR5. Furthermore, many of these populations have been found to upregulate FGR, a gene found to negatively regulate chemokine signaling. There is evidence at the transcriptional level of this blockade against re-assuming a germinal center phenotype as well. There is some limited evidence that the Blimp-1 program may be elevated in these non-classical memory populations. Blimp-1 is a well known antagonist to BCL-6, the driver of the GC phenotype. One study found an upregulation of Blimp-1 by QPCR in FCRL5+ cells in individuals exposed to malaria. However, there was no detectable expression in a similar assay performed on FCRL4+ cells in healthy tonsils. Bach2, a Blimp-1 inhibitor, was significantly decreased in CD21lo cells in HCV patients, and XBP-1, which is induced by Blimp-1, is upregulated in cells from SLE patients. The Blimp-1 pathway may be upregulated in non-classical cells as a mechanism to prevent the re-adoption of the BCL-6 driven germinal center phenotype.

The diverse contexts in which these non-classical populations have been observed suggest that understanding their role is important in delineating a complete picture of post-germinal center differentiation. Because the majority of previous studies have been performed on individuals with chronic infection or autoimmunity, it is unclear whether these cells play a role in an acute immune response in healthy humans. We set out to test our central hypothesis by identifying and comprehensively characterizing one of these

populations, the CD19+CD27+CD21lo cells, in a cohort of healthy individuals who received the seasonal influenza vaccine. We tested the antigen specificity of CD21lo cells, analyzed their phylogenetic relationships to other B cell populations, and compared their transcriptional profile to memory B cells, the cells they phenotypically resemble the most. There are substantial differences in all regards between CD21lo cells and classical memory B cells, leading us to conclude that the CD21lo population is a developmentally distinct stage of B cell development. In addition, we found evidence that CD21lo cells are recent germinal graduates that are inhibited from re-entering the germinal center and may be subject to peripheral tolerance. This has important implications in understanding the biology of the B cell vaccine response and post-germinal center differentiation.

# Chapter 2: Materials and Methods

PBMC Collection and Isolation: Peripheral blood samples were collected in accordance with the University of Chicago Institutional Review Board (#09-043-A). Peripheral blood mononuclear cells were isolated from 40 mL of venous blood from healthy adults 0, 14, 28, and/or 60 days after vaccination with the 2011-2012 or 2014-2015 quadrivalent seasonal influenza vaccine. B cells were first enriched using the RosetteSep Human B Cell Enrichment Cocktail (STEMCELL Technologies) and washed in PBS/0.2% BSA and then isolated using a Lymphoprep gradient. For frozen PBMC stocks, cells were frozen in FBS/10%DMSO before the B cell enrichment step and stored in liquid nitrogen. Frozen PBMCs were thawed in a 37C degree water bath and diluted in warm RPMI/10%FBS. The cells were washed in media and then PBS prior to staining for flow cytometry.

Flow Cytometry and Cell Sorting: Flow cytometry analysis was performed on fresh or frozen purified PBMCs from day 14 or 21 after vaccination using the BD Fortessa. Cells were sorted for RNASeq and for monoclonal antibody generation using a BD FACSAriaII machine. Plasmablasts were identified as CD19+ CD38++ CD27+, classical memory cells were CD19+CD38loCD27+CD21+, and CD21lo cells were CD19+, CD38lo, CD27+, CD21lo. The following antibodies (from BioLegend, unless noted) were used for cell sorting: anti-CD19 Pacific Blue, anti-CD38 PECy7, anti-CD27 BV605, anti-CD21 FITC. All flow cytometry data was analyzed using FlowJo. The following antibodies were used for assaying surface expression of a panel of proteins (from BioLegend, unless noted): anti-CD80 FITC (Caltag),

anti-CD95 BV421 (BD), anti-CD11c PE, anti-FCRL4 PE, anti-FCRL5 APC, anti-CXCR4 APC, anti-CXCR5 PE, and anti-CD73 PE-Dazzle.

Recombinant HA Production: Recombinant influenza HA was produced in 293T cells transfected with plasmids donated by the Mascola lab. Supernatant of the cells was collected and washed through a Ni-NTA column. Eluted protein was then further purified using size exclusion chromatography. The purified protein was then biotinylated using a BirA biotinylation kit (Avidity) and conjugated to SA-PE or SA-APC.

Monoclonal Antibody Cloning: Cells were single cell sorted into a catching solution of 2 uL of cell lysis buffer (0.2% Triton X-100, RNase Inhibitor), 1uL oligo-dT (10 uM), and 1uL dNTP (10mM) and cDNA was generated as per the SmartSeq2 protocol(Picelli et al. 2014). The cDNA was then used to produce monoclonal antibodies as previously described (K. Smith et al. 2009). In brief, 1 uL of the cDNA was further amplified for sequencing and cloning using nested PCRs. Sanger sequencing of the single cells was performed, and analyzed using IMGT V-Quest. Restriction enzyme sites were incorporated onto the amplicons by PCR and the genes were cloned into IgG1, IgK, or IgL expression vectors. Antibodies were expressed in HEK293T cells and purified using protein A beads (Pierce).

ELISA: Vaccine or IgG ELISAs were performed on starting concentrations of 1:20 dilution of the 2014-2015 seasonal influenza vaccine or 2ug/uL of anti-IgG antibody. Microtiter plates were coated with diluted vaccine or anti-IgG antibody. The well characterized Cr9114 antibody was used as a positive control for the vaccine ELISAs, recombinant human IgG

was used for the IgG ELISAs. Intermediate washes were performed with PBS/0.05% Tween. A horseradish peroxidase-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories) was used as a secondary and plates were developed with SuperAquaBlue (EBioscience) until the positive controls reached an OD405 of 3.0.

ELISPOT: Vaccine and immunoglobulin ELISPOTs were performed as previously described (Wrammert et al. 2011). In short, ELISPOT filter plates were coated overnight with influenza vaccine, anti-IgG or anti-IgA at 4C. Plates were sorted and blocked with RPMI with 10% FCS for 2 hours at 37C. Sorted cells in RPMI with 10% FCS, 1% HEPES, 1% L-Glu, and 1% Pen/Strep were added to the plates in a 1:2 dilution series. Cells were incubated for 5 hours or overnight. Plates were then washed with PBS with .05% Tween and biotinylated anti-IgG or anti-IgA (Southern Biotech) was added at a 1:1000 dilution. Streptavidin-AP (Southern Biotech) was used as a secondary antibody and the ELISPOT was revealed using NBT/BCIP (Thermo Scientific).

Memory B Cell Stimulation: B cells were activated to differentiate into antibody secreting cells as previously described (Andrews, Kaur, et al. 2015; Crotty et al. 2004). In short, CD21lo and memory B cells were sorted from peripheral blood and cultured for 5 days with SAC (Sigma-Aldrich), CpG (InvivoGen) and PWM (gift from Shane Crotty). The cells were then placed on vaccine coated ELISPOT plates in a 1:2 dilution series and an ELISPOT was performed as described above.

Library Preparation and Sequencing: For RNASeq, RNA was extracted for sequencing using

TRIzol. Libraries were prepared using the SmartSeq2 protocol that was lightly modified to make low input bulk RNASeq libraries. 250 pg of input RNA was used to generate cDNA that was transcribed from mRNA primed with oligo-dTs and then purified with Ampure XP beads. The cDNA was then tagmented and adapters were added using the Illumina Nextera kit. 50 bp single end sequencing was performed on an Illumina HiSeq2000 at the University of Chicago Functional Genomics core. For repertoire sequencing, RNA was extracted using a Qiagen RNeasy Micro kit. cDNA was prepared by PCR amplifying the antibody transcripts using degenerate primers for the V region and specific primers for the constant region. The cDNA libraries were prepared and sequenced by iRepertoire.

RNASeq Analysis: Differential gene expression was performed on Galaxy (usegalaxy.org) using the Tuxedo suite as previously described (Trapnell et al. 2012). In short, sequences were aligned to the hg19 version of the human genome using Tophat and Bowtie2. Cuffmerge was used to combine transcript assemblies generated by Cufflinks and differential gene expression was determined using Cuffdiff. A differentially expressed gene is defined in our study as one with a q value less than 0.05 as calculated in Cuffdiff using the Benjamini-Hochberg correction. Additionally, the FPKM values for each sample were determined using Cuffquant and Cuffnorm on the transcript assemblies.

Quantitative PCR: QPCR was performed on cDNA made from using RNA extracted using Trizol as described above for RNASeq. The PRDM1, XBP1,TNFRSF17, and B2M Taqman Gene Expression Assays were used for QPCR and were run on an Applied Biosystems 7300 machine. The delta-delta Ct method was used for analysis.

Antibody Repertoire Analysis: Alignment and Gene Usage Analysis: Sequences were aligned to the reference IMGT database to using IMGT High V-Quest to identify the V, D, and J genes, the CDR3 nucleotide and amino acid sequence and the number of mutations in each region.

Gene-usage trees: IGHV gene frequency vectors and IGHV-IGHJ gene pair frequency vectors were generated after the alignment step. The number of occurrences of each gene was tabulated in the vectors, which were then scaled and used to generate a distance matrix. The similarity between the samples from the 3 subjects was then determined by generating a neighbor-joining tree using the APE package in R.

Clonal assignments: Sequences were segregated into clones, which we define as a group of sequences that have the same IGHV gene, IGHJ gene, and CDR3 length, and are at least 85% similar in the CDR3 region, similar to previous studies. Sequences were segregated into subgroups based on IGHV gene, IGHJ gene and CDR3 length and a hierarchical clustering tree was generated for each subgroup (code adapted from (Laserson et al. 2014)). The trees were then cut at the Levenshtein edit distance of 0.15*CDR3 length, rounded to the nearest integer. The clusters generated by this cut were then defined as B cell clones.

Maximum Likelihood Trees: We generated a multiple alignment for the sequences in each clone using MUSCLE and then estimated a maximum likelihood tree for each clone using RAxML with the GTR+G+I model. We analyzed the probability that the neighboring sequence would belong to each cell type by calculating the empirical probability. We

excluded small clones (less than 10 sequences) and clones with less than 3 sequences of each B cell subset from the analysis. Rooted trees were generated using the germline IGHV-IGHJ sequence. The rooted trees were visualized using the APE package in R.

Flu Positive Clones: Flu positive clones were identified using sequencing data from experimentally verified flu binding antibodies generated from Day 7 plasmablasts from the subjects in the study. Clones containing sequences with the identical CDR3 to any of the flu binding antibodies were considered flu positive clones for analysis.

# Chapter 3: Results

## CD21lo B cells are enriched for antigen specific cells after influenza immunization

Memory B cells, which are CD27+CD21+ and naïve B cells, which are CD27-CD21+ are the predominant components of the CD19+ B cell population in the peripheral blood. However, previous studies have observed that populations of CD27+ CD21lo and CD27- CD21lo B cells, which are poorly understood, are enriched for antigen specific cells during chronic infection (Moir et al. 2008; Muellenbeck et al. 2013; Kardava et al. 2014). To our knowledge, this enrichment has not been previously explored in healthy subjects undergoing an acute immune response. We used flow cytometry to measure the proportion of hemagglutinin (HA) specific cells present after vaccination with the seasonal influenza vaccine in four B cell subsets based on CD27 and CD21 expression. HA is a glycoprotein on the surface of influenza and is the predominant target of the antibody response (Kaur, Sullivan, and Wilson 2011). Fluorescently tagged HA from the H1N1 A/California/04/09 and H3N2 A/Perth/16/09 influenza vaccine strains were used to stain B cells to determine the proportion of cells that could bind HA within the different subsets. We verified the specificity of the HA staining by single cell sorting HA+B cells from two individuals and generating monoclonal antibodies. 92% of mAbs generated were specific for HA, thereby confirming the specificity of the approach (Figure 1A-B).

We found that the CD27+CD21lo non-classical memory population contained a significantly larger proportion of HA+ cells compared to the CD27+CD21+ classical

memory, the CD27-CD21+ naïve, and the CD27-CD21lo "tissue-like" memory populations (Fig 2A-C). Because of the predominance of antigen specific cells in the CD27+CD21lo compartment after immunization compared to the more frequently studied CD27-CD21lo cells, we decided to focus our study on this population. This enrichment was consistent across all six individual we sampled.

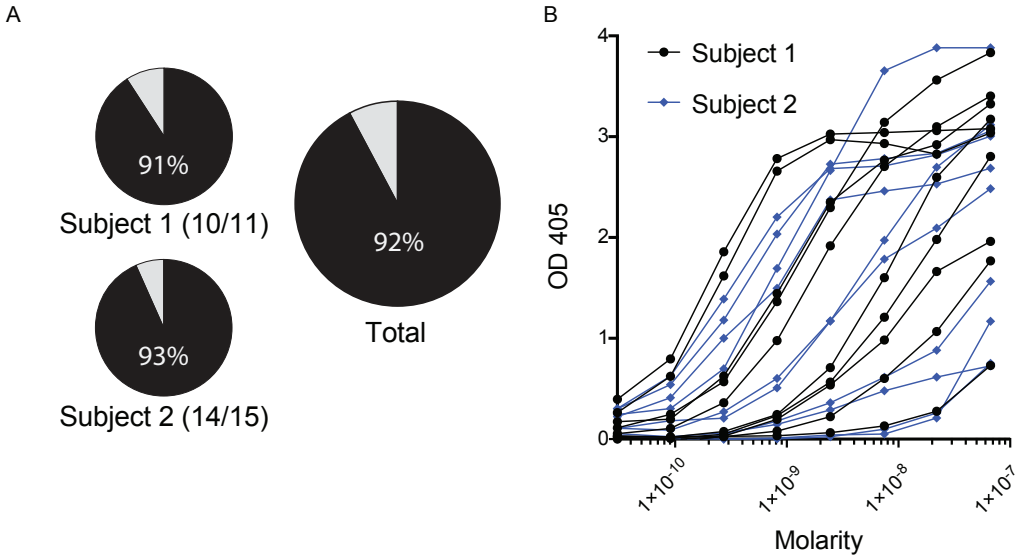


**Figure 1: Validation of the HA binding FACS assay.**

Monoclonal antibodies were generated from single cell sorted CD19+CD27+ HA binding B cells isolated from two individuals that received the seasonal influenza vaccine. ELISAs were performed to test the specificity of the monoclonal antibodies to the vaccine. (a) Percentage of monoclonal antibodies derived from HA+ memory B cells that bind vaccine by ELISA in each subject. (b) ELISAs binding curves of antibodies from (a).

We first established that CD27+CD21lo memory B cells make up approximately 5% of the CD27+ memory B cell population before vaccination and that proportion remains relatively constant during the vaccine response (Fig. 2B). In six subjects who received the seasonal 2014-2015 quadrivalent influenza vaccine, the CD21lo B cells consistently had more H1N1 HA and H3N2 HA specific B cells than did the predominant CD21+ memory B cells 14 days

post-immunization, as measured by flow cytometry. On average, 11.45% of CD21lo cells bound HA from the A strains in the vaccine, while only 0.85% of memory B cells were able to bind (Fig. 2D-E).

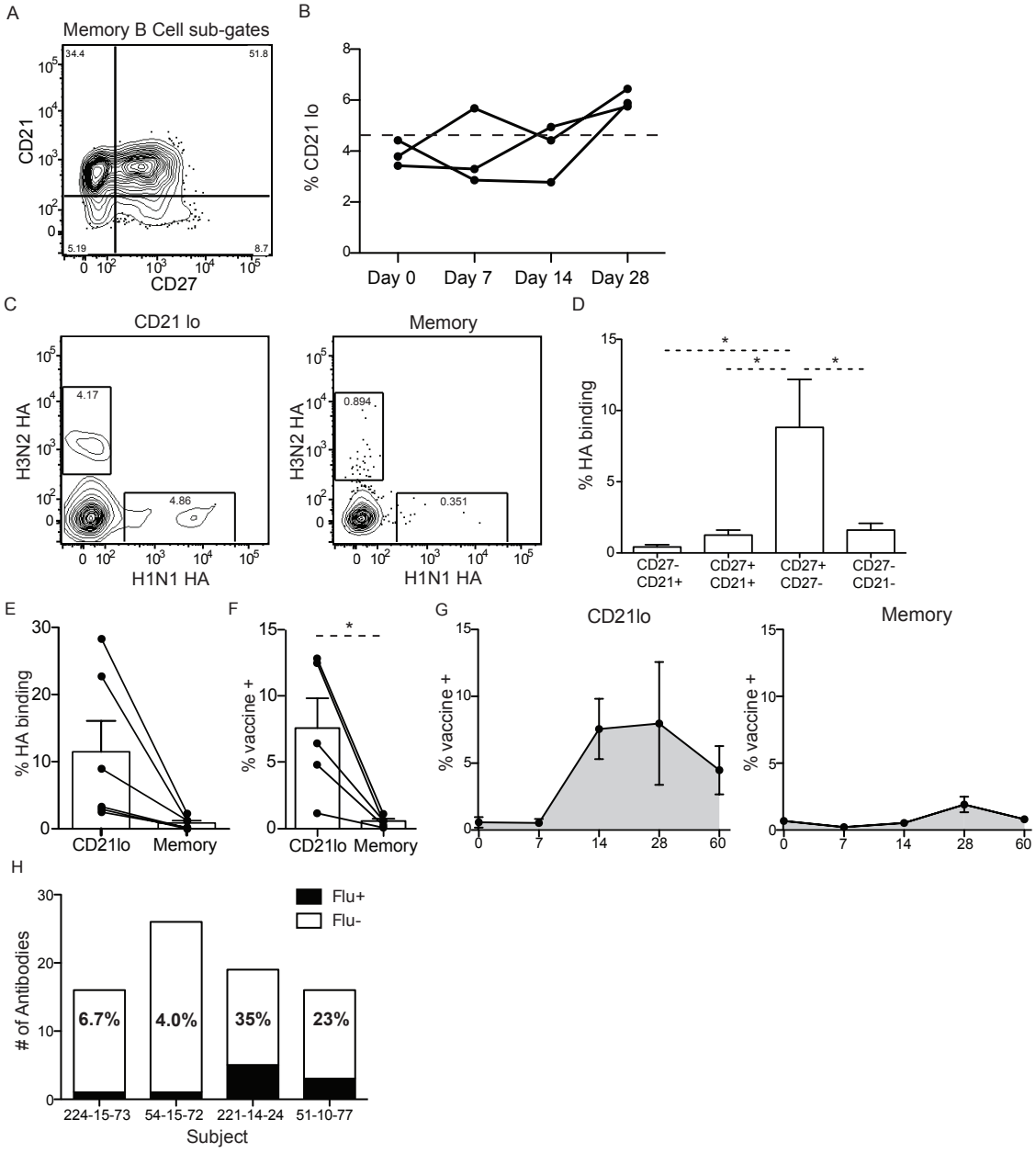


**Figure 2: CD21lo cells are enriched for influenza specific cells.**

The proportion of flu specific cell in the CD21lo compared to memory B cells was evaluated from cells isolated from the peripheral blood. (A) Representative FACS gating of CD19+ B cells by CD27 and CD21. (B) Frequency of CD21lo cells in the CD27+ peripheral blood

**Figure 2, continued.**

compartment at 0, 7, 14 and 28 days post-immunization (n=3) as measured by FACS. (C) Representative FACS gating of hemagglutinin (HA) staining for classical memory or CD21lo cells 14 days post-immunization. (D) Percentage of H1N1 and H3N2 HA binding cells in the CD27-CD21+, CD27+CD21+, CD27+CD21lo, and CD27-CD21lo subsets as determined by FACS analysis (n=6). (E) Percentage of H1N1 and H3N2 HA binding cells in the CD21lo or memory compartment as determined by FACS analysis (n=6). Lines link data points from the same individual. (F) Percentage of vaccine specific CD21lo of classical memory B cells as measured by ELISPOT 14 days post-immunization (n=5). (G) Percentage of vaccine specific CD21lo or classical memory B cells as measured by ELISPOT from 0 (n=4), 7 (n=3), 14 (n=5), 28 (n=5), and 60 (n=3) days post-immunization. Cells were sorted and stimulated for 5 days with CpG, PWM and SAC before being plated on vaccine coated ELISPOT plates. (H) Proportion of vaccine binding monoclonal antibodies generated from each individual. Numbers in each bar refer to the percentage of flu binding antibodies for that person.

The enrichment of vaccine specific B cells in the CD21lo compartment 14 days after immunization was further verified in 5 additional subjects using a polyclonal stimulation ELISPOT assay, as previously described (Crotty et al. 2004). On average, 7.5% of CD21lo cells were vaccine-specific, while only 0.52% of memory B cells were (Fig 2F). This assay was also performed 0, 28, and 60 days after vaccination. Notably, the percentage of vaccine specific cells peaks at different times for CD21lo and classical memory B (Fig 2G). While the peak of vaccine specific CD21lo B cells began at 14 days post-vaccination and was waning at day 28, the peak of vaccine specific classical memory B cells did not begin until 28 days post-vaccination. The kinetics of both these populations is distinct from the well-characterized day 7 peak of the plasmablast response (Wrammert et al. 2011). Further, unlike classical memory B cells, the CD21lo population contained virtually no influenza-specific cells before vaccination, demonstrating that antigen-specificity in this population is transient.

In a third assay, monoclonal antibodies (mAbs) were expressed recombinantly from the variable genes of single cell sorted CD21lo B cells from 4 individuals. Similar to the

findings from the flow cytometry and ELISPOT experiments, 15% (10/67) of CD21lo B cells isolated 14 days after influenza vaccination were specific to the vaccine, as measured by ELISA (Fig 2H). In addition to verifying our previous results, this experiment also demonstrates that the CD21lo subset is a ready source for high affinity mAbs, as antigen specific cells are present in this population for a 2-week period following immunization and can also be isolated from frozen samples (Fig 3A-B).

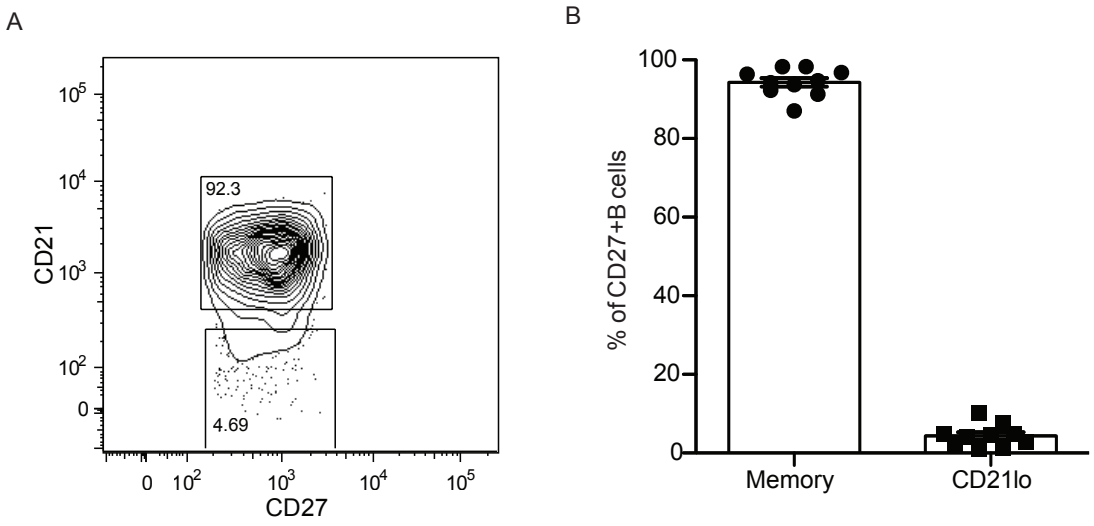


**Figure 3: CD21lo cells can be isolated from frozen PBMC stocks.**

Whole PBMCs were isolated from peripheral blood using a Ficoll gradient 14 days post immunization with the 2013-2014 or 2014-2015 seasonal influenza vaccine. PBMCs were stored in liquid nitrogen in FCS with 10% DMSO until they were thawed and analyzed. (A) Representative gating of memory and CD21lo cells in the CD27+ B cell compartment from thawed PBMCs. (B) Percentage of memory and CD21lo cells in the CD27+ B cell compartment after freeze-thaw.

Based on the three assays described above, we estimate that approximately 10% of CD21lo cells are influenza specific 14 days after vaccination. The rapid induction of antigen specific cells in the CD21lo population beginning 7 days after immunization and the presence of the CD21lo population at steady state suggests that these cells are a persistent

but overturning pool of B cells that reflect the most recent B cell responses. The peak of influenza-specific CD21lo cells 14-21 days after immunization coincides with the apogee of the GC reaction, suggesting that this population is the earliest stage of B cell differentiation after affinity maturation in the GC.

## CD21lo B cells are clonally related to but phylogenetically distinct from plasmablasts and classical memory B cells

The differential kinetics of the flu specific CD21lo cells from plasmablasts and memory B cells suggest they are developmentally distinct. To further investigate this hypothesis, we performed high throughput sequencing of PCR amplified heavy chain genes from the plasmablasts (CD19+ CD38+ CD27+), memory B cells (CD19+ CD38lo CD27+ CD21+), and CD21lo cells (CD19+ CD38lo CD27+ CD21lo). The plasmablasts were isolated from peripheral blood 7 days post-immunization and the other populations were isolated at 14 and 90 days post-immunization.

The majority of VH genes used across the compartments belonged to the IGVH1, IGVH3, and IGHV4 families, as previously observed (Fig 4A). However, there was great diversity in which VH gene and VH-JH gene pairings were used as measured by Simpson's Index (Fig 4B), indicating very low bias in the VH and JH usage between the different B cell subpopulations. Neighbor joining trees were generated using a distance matrix generated from the number of sequences that used each VH gene or VH-JH combination. The samples in the tree clustered by individual, showing that each person utilizes a diverse set of immunoglobulin genes in responding to influenza vaccination and has a unique signature of gene usage (Fig 4C-D).

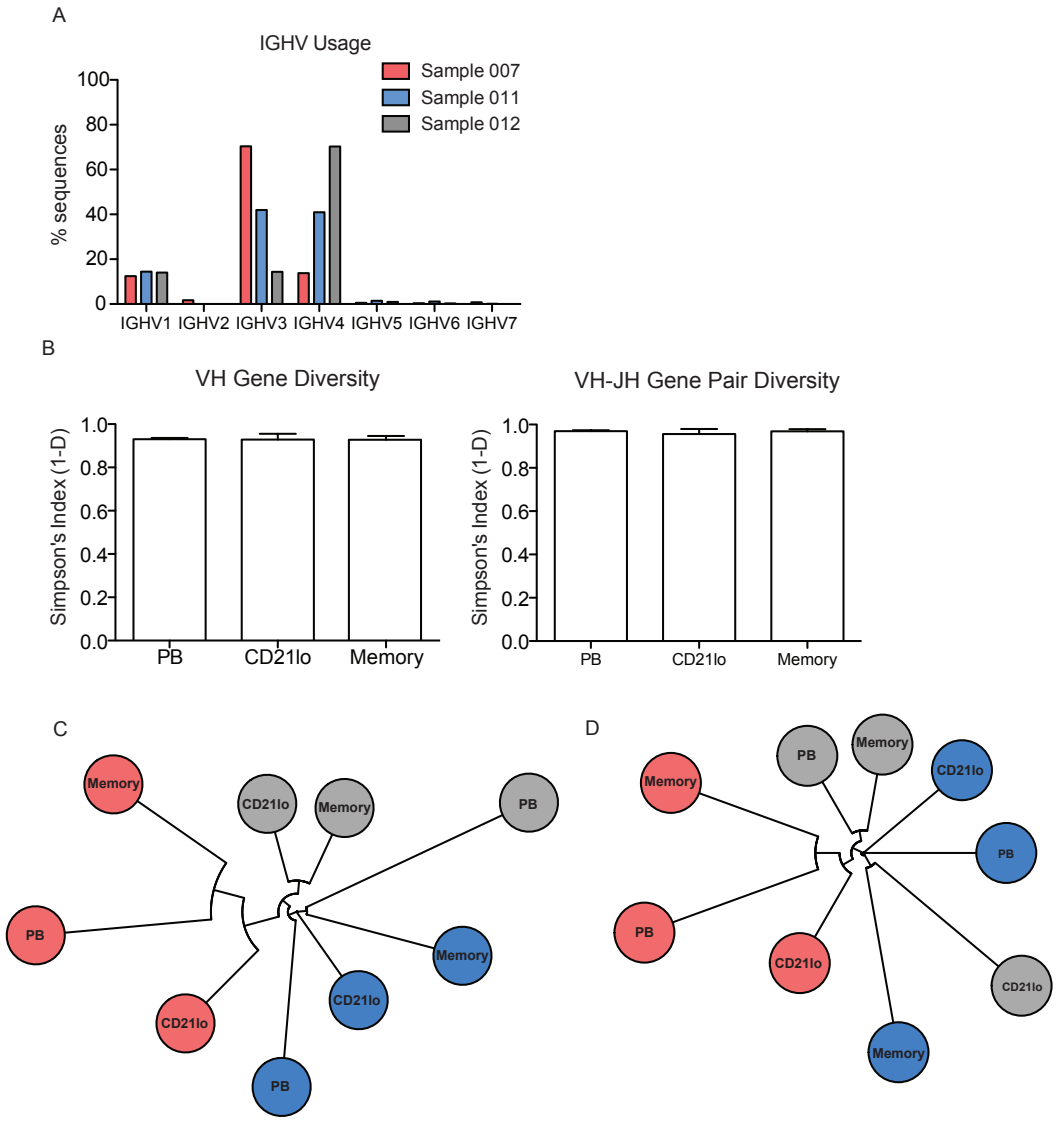


**Figure 4: PB, CD21lo and memory cells are diverse in their immunoglobulin gene usage.**

Variable gene usage was analyzed from high throughput immunoglobulin gene sequencing. Variable gene alignment was performed with IMGT High VQUEST. (A) Frequency of usage of genes from each IGHV family. (B) Diversity in IGHV gene usage and IGHV-IGHJ gene pairings as measured by Simpson's Index. Index represents the probability that two randomly drawn sequences will no be the same gene (or gene pair). (C) Neighbor joining trees built from frequency vectors of IGHV gene usage or (D) IGHV-IGHJ gene pairings.

We then analyzed the patterns of somatic mutation within expanded clones to determine the phylogenetic relationships between the plasmablast, memory, and CD21lo

sequences. Similar to previous studies, we grouped the sequences into clonotypes based on VH and JH gene usage, CDR3 length, and 85% nucleotide similarity in the CDR3 (Laserson et al. 2014; Tipton et al. 2015). The majority of clones (average 80%) were present at a low frequency with less than 5 unique sequences (Fig 5A). The clone size was normalized across subjects for the number of sequences in each sample by averaging the number of clones in each clone size bucket from 100 bootstrap samples that were equal in size to the sample with the fewest number of sequences. Despite the predominance of low-prevalence clones, there were numerous large clonal expansions in each sample. We found that there were CD21lo cells are clonally related to both plasmablasts and memory B cells in all 3 individuals. On average, about 20% of clones containing CD21lo sequences also contained sequences from plasmablasts or memory B cells, or both (Fig 5B). This high degree of clonal relatedness suggests that these cells share a common ancestor that underwent affinity maturation and generated multiple daughter lineages that had varied differentiation fates.

Due to the frequency of exposure to influenza, the common ancestor is likely from the reactivated memory B cell pool that dominates the immune response to the vaccine in most individuals (Andrews, Huang, et al. 2015). Reactivated memory B cells with high affinity for the immunizing antigen tend to differentiate directly into plasmablasts, while lower affinity cells re-enter the germinal center to undergo further affinity maturation (Phan et al. 2006). The extensive germinal center experience of these two populations was evident when we analyzed 13 clones that contained experimentally verified flu binding antibodies (Table 2). Monoclonal antibodies specific for the vaccine generated from plasmablasts were used as references to identify clones that contain sequences with the

same CDR3 sequence. While there were wide ranges of mutational load within each flu specific clone, both populations averaged 14 V region nucleotide mutations (Fig 5C,D Fig 6). There were an insufficient number of classical memory B cells in the verified flu specific clones to be included in this analysis. The small numbers of memory B cells in these flu binding clones is consistent with the kinetics identified by assays measuring antigen specificity (Fig 2G).

| Sample | Ab name | H1N1 | H3N2 | B | CDR3 |
|---|---|---|---|---|---|
| 007-10051 | 2C06 | | | ● | gcgagaatgttttcctcgtacttttttgacttc |
| 007-10051 | 2D02 | | | ● | gcgagaatgttttcttcgtactattttgactac |
| 011-10069 | 2A01 | ● | ● | ● | gcgagaggcggaaatgggcgacccttgacaac |
| 011-10069 | 2F01 | ● | ● | | gcccgcgggggtgactcgggcggtgggctcgactac |
| 011-10069 | 3B01 | ● | ● | ● | gcgagggcctattttgactcc |
| 011-10069 | 3B03 | ● | ● | | gcgagaaggtactttgactac |
| 011-10069 | 3D03 | | ● | | gcgcgagatgggagtgatactacgctgcctgtatattattatgatagtagtggccttgactac |
| 012-10081 | 4D03 | | ● | | gcgagagatctgtatagcaccagttggtatgagcccagatttaactactactacggtatggacgtc |
| 012-10081 | 4G05 | ● | | | gcgagagggctcggcattcttaacgttgacacacctatcatggactactttgactac |

**Table 2: Specificity of Flu Binding Antibodies.**

Monoclonal antibodies from all three subjects were generated from plasmablasts isolated 7 days post-vaccination. The specificity for each antibody was tested using ELISAs against live virus. The table shows the strain specificity and CDR3 sequences of monoclonal antibodies used to identify flu specific clones in the immunoglobulin repertoire sequencing.

The *de novo* affinity maturation of the CD21lo cells was evident when we modeled the germinal center evolution of each clone using maximum likelihood trees that were rooted on the germline VH-JH sequence. The presence of distinct CD21lo clades within each clone was obvious upon visual inspection (Fig 5E-G). Using those trees, we empirically determined the probability that the nearest neighbor in the phylogenetic tree for each

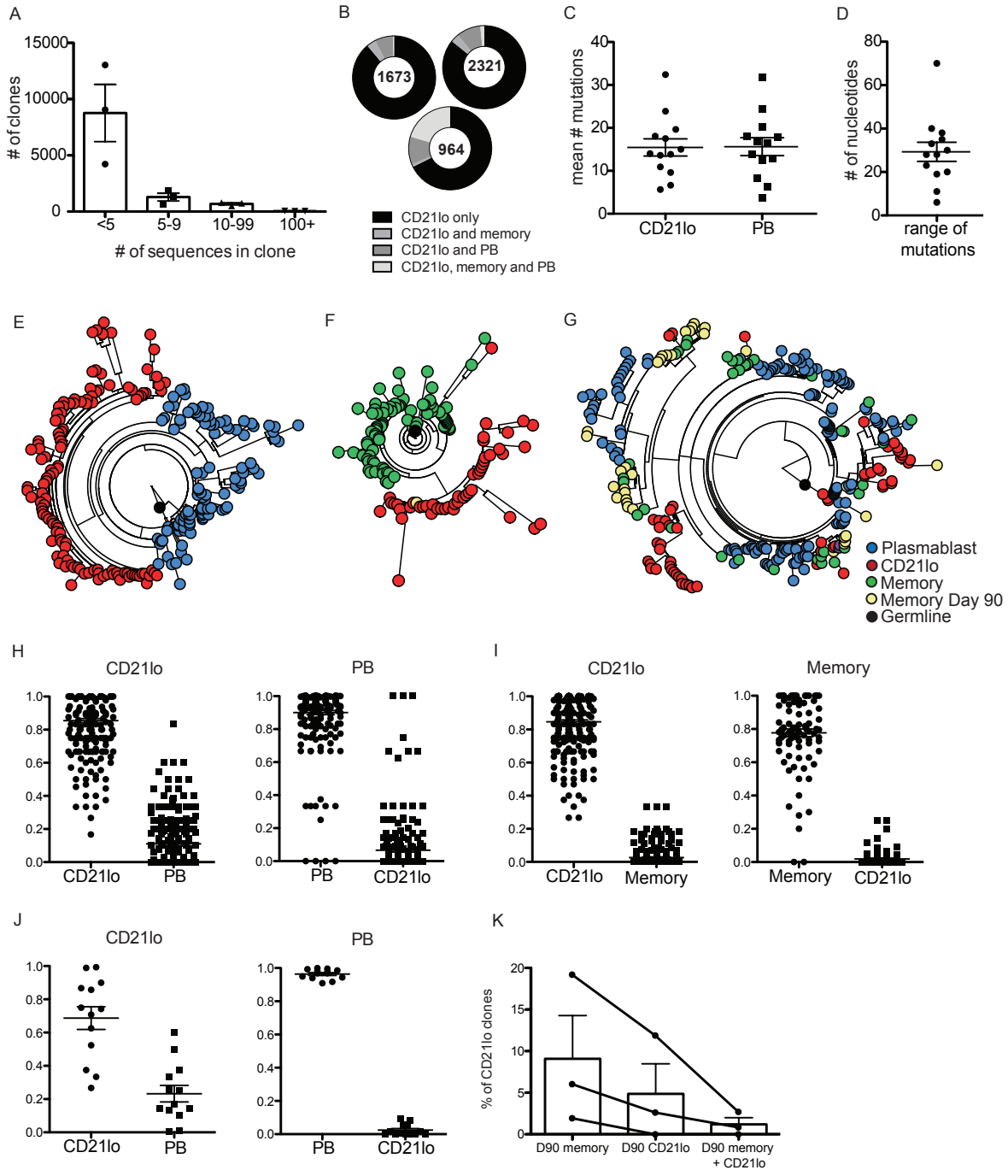


**Figure 5: CD21lo cells are clonally related to plasmablasts and memory B cells but form distinct clades.**

454 sequencing of cDNA libraries generated from PCR amplified antibody genes from plasmablasts, memory B cells, and CD21lo cells isolated from 3 subjects that received the 2010-2011 seasonal influenza vaccine. (A) Number of clonal families of each size (n=3). Clone numbers were normalized for sequencing sample size by rarefaction. (B) Pie charts

**Figure 5, continued.**

showing the proportion of Day 14 CD21lo containing clones that also contain PB or Day 14 memory sequences. (C) Mean number of mutations in the V region for Day 14 CD21lo and plasmablast sequences for each flu specific clone (n=13). (D) Difference in V region nucleotide mutation number between most and least mutated sequences within each flu specific clone (n=13). (E-G) Representative maximum likelihood trees. Trees are rooted on the germline VH-JH sequence of the clone. Empirical probability of a Day 14 CD21lo sequence will have another Day 14 CD21lo sequence as its nearest neighbor in the phylogenetic tree or vice versa for plasmablast (n=219) (H) or Day 14 memory B cell (n=87) (I) or sequences in experimentally verified flu specific clones (n=12) (J). (K) Percentage of clones that contain Day 14 CD21lo sequences that also contain sequences from Day 90 CD21lo, Day 90 memory, or both.

determined the probability that the nearest neighbor in the phylogenetic tree for each CD21lo sequence was another CD21lo sequence, a plasmablast sequence (Fig 5H) or a memory B cell sequence (Fig 5I). CD21lo sequences had a significantly higher probability of being neighbors with other CD21lo sequences than plasmablast or memory B cell sequences. The same analysis was performed for plasmablast and memory B cell sequences and revealed there is a greater probability that the nearest neighbor is a sequence from the same B cell subset than from a CD21lo sequence (Fig 5H-I). The same segregation of CD21lo cells was observed in the verified flu-binding clones mentioned above (Fig 5J, Fig 7). The distinct CD21lo clades in these trees shows that while this population may share a common germinal center ancestor with plasmablasts and memory B cells, they evolve separately during affinity maturation. This unique pattern of somatic mutations in CD21lo cells supports an origin of these cells from ongoing GC reactions.

Interestingly, a substantial number of classical memory B cells isolated 90 days post-immunization were clonally related to CD21lo cells found 14 days post-immunization, while very few CD21lo cells found at day 90 were (Fig 5G, 5K). In one individual, there were none. There were also no Day 14 CD21lo clones from any of our subjects that

contained sequences from both Day 90 CD21lo cells and Day 90 memory cells. This data, coupled with the ELISPOT experiments showing that the enrichment of antigen specific cells in the CD21lo compartment decays over time, supports the notion that CD21lo cells are in a post-GC transitional stage.

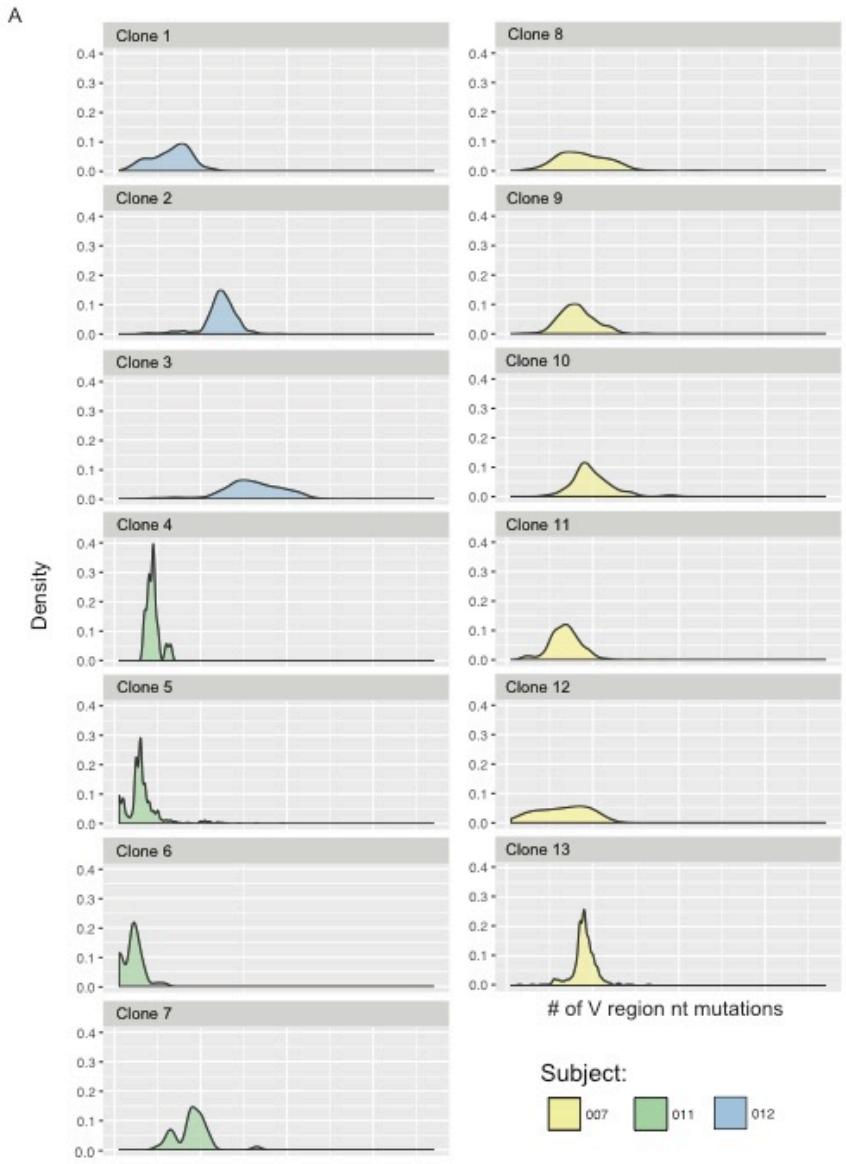


**Figure 6: Mutational load in flu specific clones.**

The number of mutations of each sequnece in the flu specific clones was determined using IMGT High VQUEST. (A) Distribution of number of V region nucleotide mutations within clones containing experimentally validated flu binding antibodies

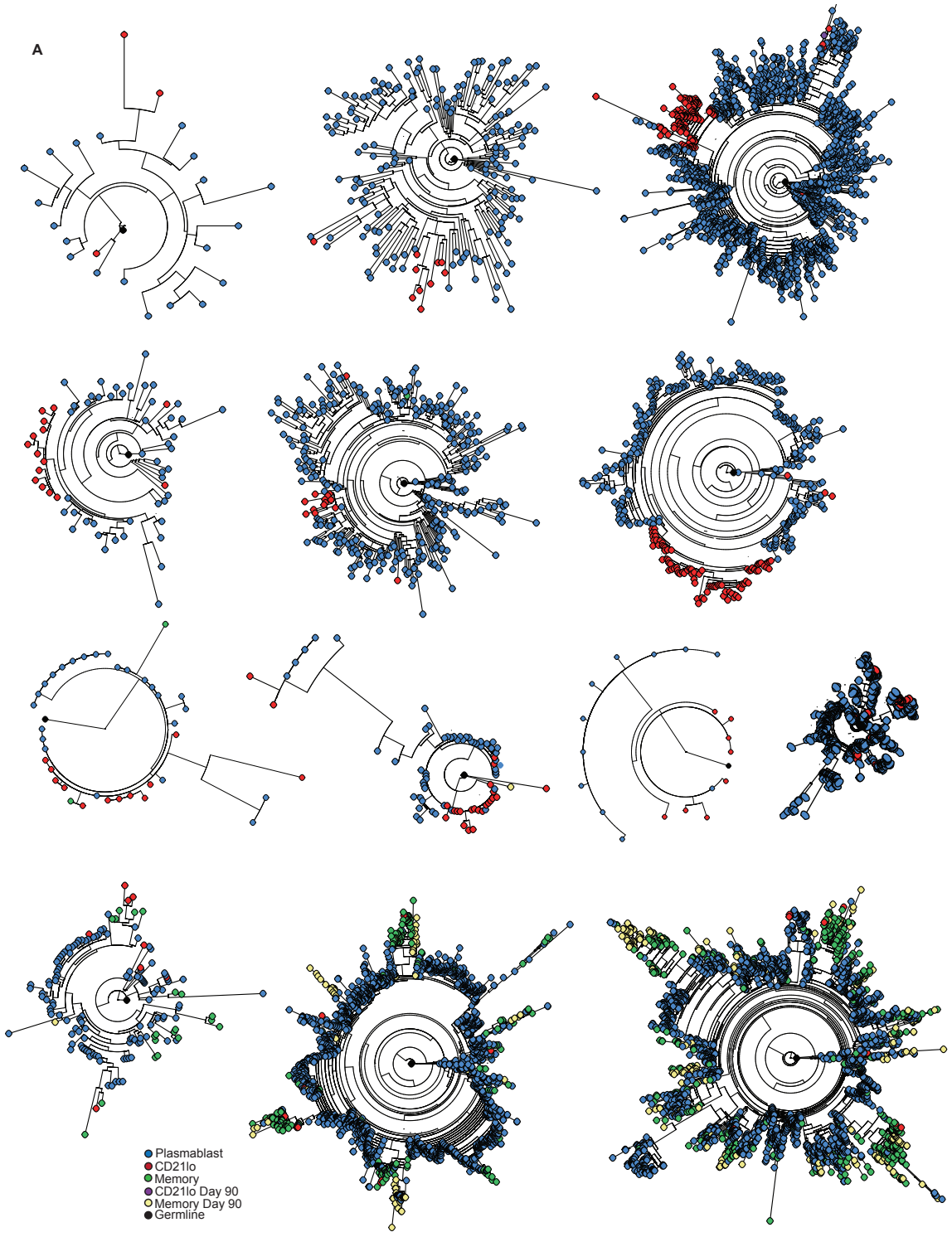


**Figure 7: Phylogenetic trees of experimentally validated flu binding clones.**

Repertoire sequencing data was filtered to identify clones that contained the same CDR3 sequence as experimentally confirmed flu binding antibodies. (A) Maximum likelihood

**Figure 7, continued.**

trees were generated using RaxML to determine the phylogenetic relationships between the different B cell subsets.

## Analysis of the transcriptional program of CD21lo cells

The phylogenetic segregation of CD21lo cells from classical memory B cells within the same clone suggests that the two populations may be functionally distinct, despite having a similar cell surface phenotype. To further explore the functional capacities of these cells and better understand the transcriptional programs of the different post-germinal center lineages, we performed RNASeq on CD21lo and memory B cells. The two populations were sorted from the peripheral blood of four healthy subjects 14 days after receiving the seasonal influenza vaccination. RNA was extracted using TRIzol and libraries were prepared using the SmartSeq2 protocol that we modified slightly for bulk RNASeq (Picelli et al. 2014). Single end 50 base pair sequencing was performed on an Illumina HiSeq2000 machine by the University of Chicago Functional Genomics core and sequence analysis was done as previously described (Trapnell et al. 2012). Differential expression analysis was performed using Cuffdiff and genes with a q value > 0.05 were considered to have significantly different expression between the two groups. We identified 260 genes that were differentially expressed between CD21lo and classical memory B cells (Fig 8A-C), many of which correlate with functional differences found in CD27+CD21lo B cell subsets previously characterized during chronic infections. As detailed below, the differentially expressed genes fell into various functional categories, including the promotion of recirculation, inhibition of activation or GC differentiation, increased susceptibility to peripheral tolerance, and differentiation into a plasma cell phenotype.

## CD21lo cells have decreased expression of tissue homing molecules and recirculate in the peripheral blood

CD21lo cells are a recirculating population isolated from the peripheral blood. However, compared to recirculating classical memory B cells, CD21lo cells differentially express many trafficking related molecules. The chemokine receptors, CCR7, CXCR4, and CXCR5 were all downregulated on CD21lo cells (Fig 8C). These receptors control trafficking to and within the GC and their decreased expression would inhibit entry of these cells to GCs and lymphoid tissues. FGR, which inhibits chemokine signaling, was upregulated in CD21lo cells. Decreased trafficking is further suggested by the decreased expression of L-selectin (CD62L), and CD73 in CD21lo cells (Fig 8C). L-selectin interacts with ligands in the high endothelial venules and is required for migration of B cells into peripheral lymph nodes, while CD73 regulates adhesion and transmigration of lymphocytes to the endothelium (Tang et al. 1998; Salmi and Jalkanen 2005). Downregulation of L-selectin, CD73, CXCR4 and CXCR5 was further verified at the protein level by flow cytometry (Fig 9A-B). In addition, CD21lo cells also express elevated levels of CD11c at both the transcript and protein level (Fig 8C, 9A-B). Increased CD11c has been identified in almost every non-classical memory B cell population in humans (Charles et al. 2011; Moir et al. 2008; Sullivan et al. 2015; Weiss et al. 2009; Kardava et al. 2014; Isnardi et al. 2010; Ehrhardt et al. 2008; Thorarinsdottir et al. 2016). CD11c is an integrin glycoprotein that can bind various ligands, including iC3b, fibrinogen, ICAM-1, and LPS, and assists cells in adhesion to the endothelium (Bilsland, Diamond, and Springer 1994; López-Rodríguez et al. 1995). Overall, the expression profile of CD21lo cells suggests they may traffick to different

anatomical locations compared to memory B cells and are more prone to continued recirculate throughout the body.

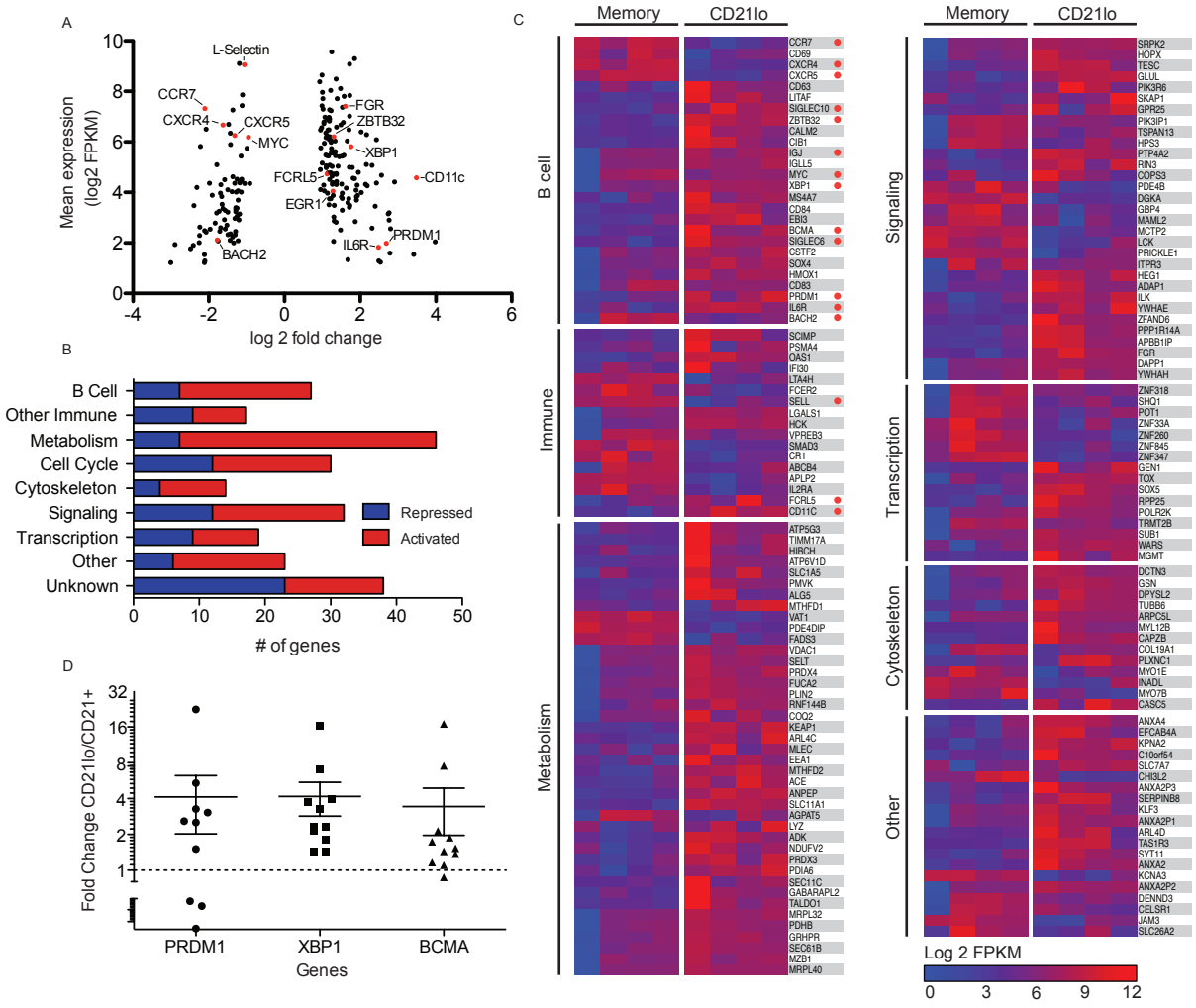


**Figure 8: CD21lo cells are transcriptionally distinct from memory B cells.**

RNASeq was performed on CD21lo and memory B cells isolated 14 days post-immunization. Sequence alignment and differential gene expression was analyzed using the Tuxedo suite on Galaxy. (A) Scatter plot showing the mean expression (log2 FPKM) plotted against log fold change of the 260 genes that are significantly differentially expressed between CD21lo and memory B cells (FDR < 0.05). Red dots represent genes highlighted in the text. (B) Functional pathway analysis of differentially expressed genes. (C) Heatmap representing the expression (log2 FPKM) of selected differentially expressed genes. Genes highlighted in the text are annotated with a red dot. (D) Expression fold change of CD21lo over memory from QPCR validation of key plasma cell associated genes (n=11).

## CD21lo cells express a program that suggests increased T-B interactions and susceptibility to Fas mediated apoptosis

We also found that proteins related to T-B cell interactions were differentially expressed. As mentioned earlier, CD11c levels were significantly higher in CD21lo cells compared to classical memory (Fig 8C, 9A-B). CD11c+ B cells in mice localize to the T-B border in the spleen and form more stable interactions with T cells than other B cells (Rubtsov et al. 2015). CD80, which binds CD28 and CTLA4, and is required for T cell interaction, is also upregulated on CD21lo cells, as well as on the CD11c+ B cells in mice (Fig 8C, 9A-B). Interestingly, CD21lo cells also have significantly higher levels of Fas compared to classical memory B cells, as seen in many other non-classical populations (Charles et al. 2011; Moir et al. 2004; Wehr et al. 2004; Isnardi et al. 2010; Ehrhardt et al. 2008; Thorarinsdottir et al. 2016). Higher levels of Fas are associated with a greater susceptibility to Fas mediated apoptosis (K. G. Smith, Nossal, and Tarlinton 1995). We speculate that the increase in T-B interactions that CD21lo cells likely experience suggests that they are further testing their newly generated BCRs for self-reactivity and are subject to Fas mediated apoptosis if they are activated by self antigen and are unable to find T cell help.

A conundrum of B cell biology is the site and stage of negative selection to avoid autoreactivity introduced by somatic mutations in GC reactions. Although purely self reactive cells would be counter-selected in GCs, cross reactive cells are not removed. Having a phenotype refractory to re-activation would be a likely stage for post-GC tolerance to avoid antibody secretion against auto-antigens. Further, the transient, recirculating phenotype of CD21lo cells would allow recent germinal center graduates to

test their new BCRs against body-wide self-antigens.

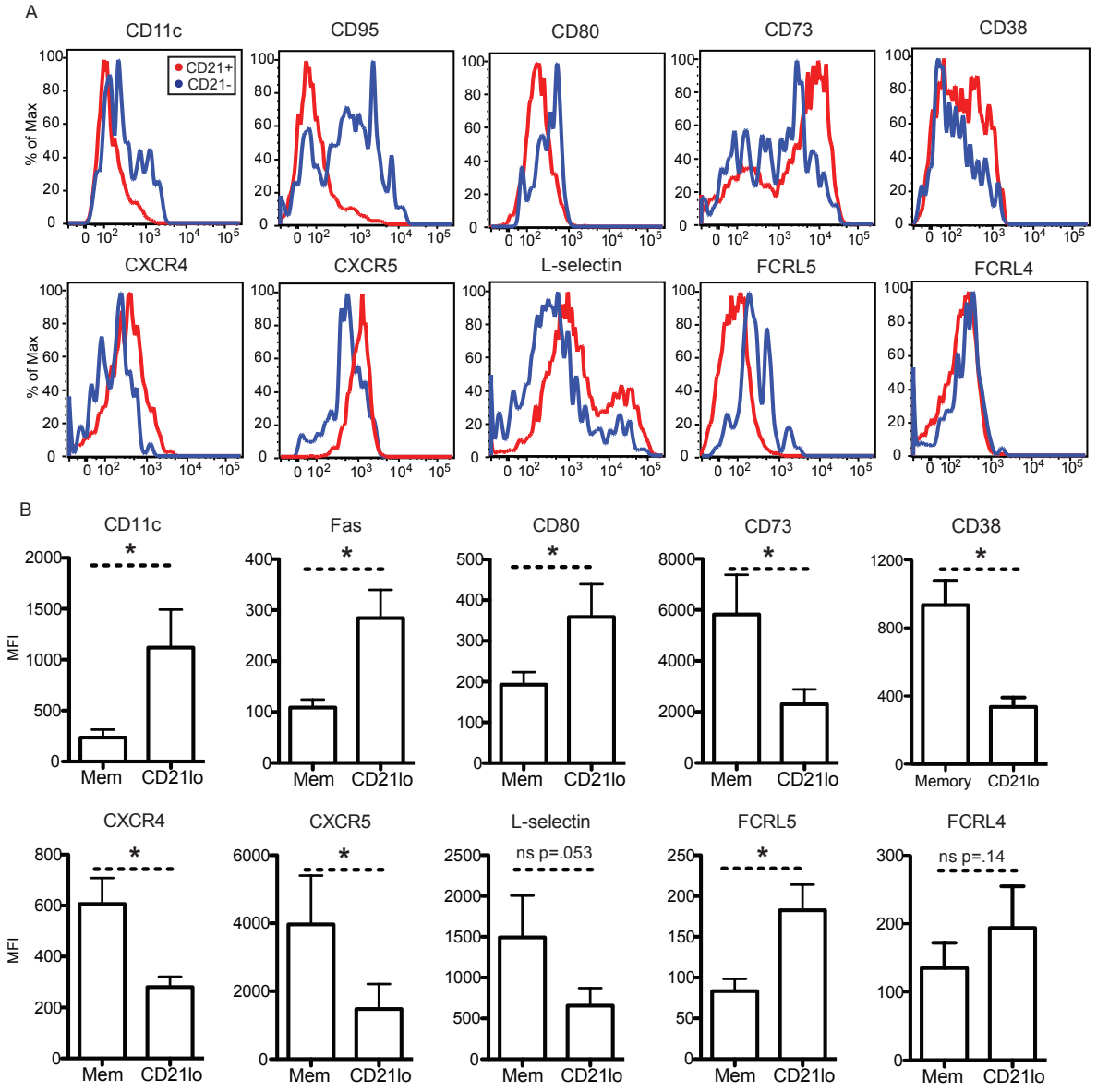


**Figure 9: Differential expression of surface markers by CD21lo cells compared to memory B cells.**

Flow cytometry analysis was performed on bulk PBMCs isolated 14 days after immunization with the 2013-2014 or 2014-2015 seasonal influenza vaccine. PBMCs were frozen after isolation and thawed prior to antibody staining. (A) Representative histograms from FACS analysis of CD21lo and memory B cells for a panel of markers. (B) Surface expression as represented by the MFI (median fluorescence intensity) for a panel markers (n>= 6).

## The transcriptional profile of CD21lo B cells suggests they are more difficult to reactivate and are blocked GC reactions

The upregulation of Blimp-1 also inhibits GC differentiation for CD21lo cells. BCL6 is the master transcriptional regulator driving GC B cell differentiation (Dent et al. 1997; Ye et al. 1997). The reciprocal antagonism between BCL6 and Blimp-1 in B cell differentiation is well documented and Blimp-1 directly represses BCL6 expression (Tunyaplin et al. 2004; Shaffer et al. 2002; Crotty, Johnston, and Schoenberger 2010). The upregulation of the Blimp-1 program in CD21lo cells shows that these recent GC graduates are transcriptionally inhibited from differentiating back into GC cells.

CD21lo B cells also have elevated levels of negative regulators of BCR signaling compared to classical memory B cells. BCR signaling and amplification involves a cascade of phosphorylation events that results in an influx of calcium ions that control transcriptional regulators like NFAT and NF-kb (Scharenberg, Humphries, and Rawlings 2007). This process can be modulated by various co-receptors that have ITIM or ITAM motifs. FCRL5, which is upregulated on CD21lo cells (Fig 8C, Fig 9A-B), facilitates the recruitment of SHP-1 after the phosphorylation of its ITIM motifs, which leads to an inhibition of BCR signaling (Haga et al. 2007). Additionally, SIGLEC10, which negatively regulates BCR signaling by modulating that calcium flux, was also upregulated in CD21lo cells, as was SIGLEC6, a related, but less characterized molecule (Fig 8C) (Müller and Nitschke 2014; Hoffmann et al. 2007). The increased expression of these inhibitory molecules, combined with the decreased levels of CD21, results in cells that are more difficult to activate. Accordingly, when CD27+CD21lo cells in HIV patients were treated with siRNA that silenced FCRL4 and SIGLEC6, the cells had an increased proliferation capacity after BCR stimulation, showing

that there are functional consequences to the inhibition of BCR signaling (Kardava et al. 2011).

We verified the hypoactive state of CD21lo cells by comparing their capacity to be activated and differentiate into antibody secreting cells to that of classical memory B cells. CD21lo and memory B cells were isolated 0, 14, 28, and 60 days post-immunization and were activated by polyclonal stimuli. The relative capacity of CD21lo cells to be activated and to differentiate into antibody-secreting cells was significantly reduced compared to memory B cells (Fig 10C).

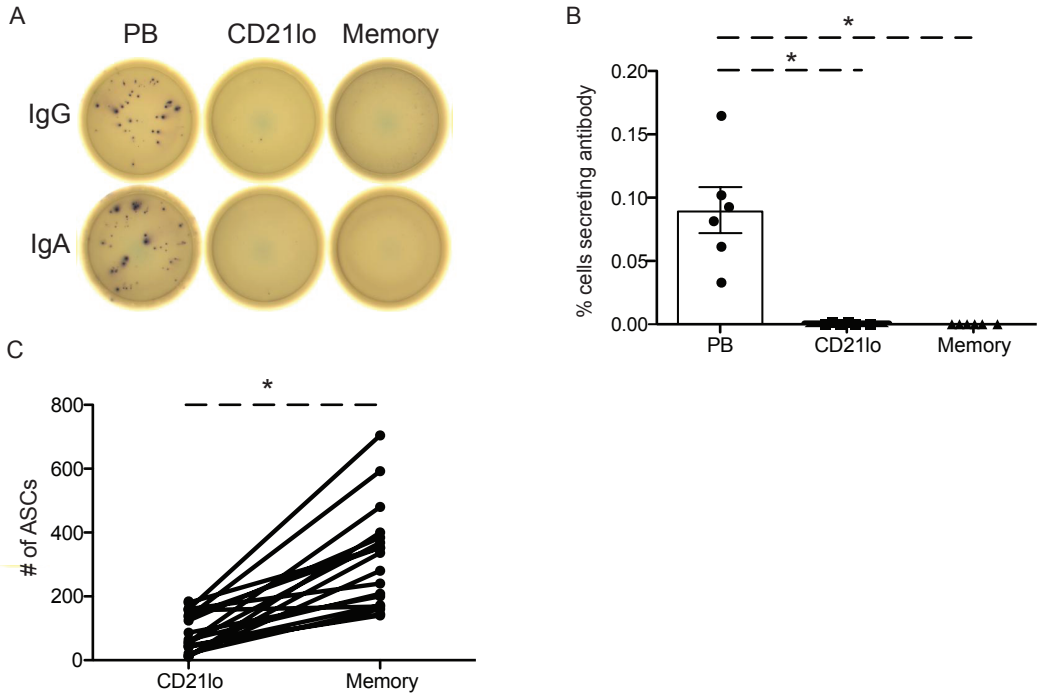


**Figure 10: CD21lo cells are functionally distinct from memory and plasma cells.**

(A) Representative ELISPOT image showing plasmablasts producing both IgG and IgA antibodies while memory and CD21lo cells produce neither. (B) Summary of the frequency of IgG (n=3) or IgA (n=3) antibody secreting cells in the plasmablast, CD21lo and memory populations. (C) Fraction of CD21lo or memory B cells that differentiate into antibody secreting cells, as measured by ELISPOT, after stimulating with CpG, SAC, and PWM (n=19).

## CD21lo cells upregulate the plasma cell transcriptional program but do not secrete antibody

CD21lo cells are transcriptionally primed for differentiation into a long lived plasma cell fate. In classical memory B cells, there was no or minimal expression of Blimp-1, the transcriptional regulator of the plasma cell program, and high expression of Bach-2, a Blimp-1 repressor, as expected (Fig 8C) (Shaffer et al. 2002; Ochiai et al. 2006). However, CD21lo cells significantly upregulated Blimp-1 and downregulated Bach-2 (Fig 8C), as previously observed in other non-classical populations (Charles et al. 2011; Sullivan et al. 2015).

In addition to Blimp-1 and Bach-2, several direct targets of Blimp-1 were also differentially regulated in CD21lo cells (Fig 8C). These genes include XBP-1, which controls the unfolded protein response in plasma cells, c-Myc, a GC associated proliferation marker, the J-chain, which links secretory multimeric immunoglobulins, and MZB1, an ER protein required for heavy chain synthesis (Johansen, Braathen, and Brandtzaeg 2000; Rosenbaum et al. 2014). Other plasma cell associated genes not regulated by Blimp-1 were also differentially expressed. Class II antigen presentation capabilities are decreased during plasma cell differentiation (Piskurich et al. 2000, 1; Silacci et al. 1994) and ZBTB32, an early repressor of CIITA and MHCII in long lived plasma cells, was upregulated in CD21lo cells (Yoon et al. 2012). BCMA and IL-6R, genes associated with the plasma cell niche in the bone marrow, were also significantly upregulated in CD21lo cells compared to classical memory. BCMA and IL-6R bind APRIL and IL-6 respectively, which are secreted by bone marrow eosinophils and stromal cells (Nutt et al. 2015). The shift in functional capabilities of these cells suggests that these cells are a pre-cursor stage to the long lived plasma cell

population. The differential expression of key plasma cell related genes including PRDM1, XBP1, and BCMA was verified using QPCR (Fig 8D).

However, despite the upregulation of plasma cell associated genes, CD21lo cells are not plasma cells. There is decreased surface expression of CD38, a plasma cell marker, on CD21lo cells than on memory B cells (Fig 9A-B). Furthermore, the CD21lo population does not secrete antibody. CD21lo cells, along with plasmablasts as a positive control and memory B cells as a negative control, were sorted from peripheral blood and then immediately plated on anti-IgG or anti-IgA coated ELISPOT plates. Despite the upregulation of the Blimp-1 gene network, the CD21lo cells do not actively secrete antibodies of either isotype (Fig 10A, B). This finding is consistent with past studies showing no influenza specific antibody secreting cells in the peripheral blood at day 14 post-vaccination (Wrammert et al. 2011; Brokstad et al. 1995). Because CD21lo cells peak after all detectable antigen-specific plasmablasts have declined, we do not believe these cells are pre-plasmablasts. Rather, we suggest these cells are primed to differentiate into bone marrow resident, long lived plasma cells.

## CD21lo cells are also present in the tissue

We also assayed for the presence of the CD21lo population in the tissue. We tested CD21lo and memory B cells isolated from healthy tonsils for the expression of the same panel of markers used on our peripheral blood samples. Similar to the peripheral blood, we found that CD21lo cells make up approximately 5% of the CD27+ compartment in the tonsil (Fig 11A-B). The tissue resident CD21lo cells shared many characteristics with the CD21lo cells in the periphery (Fig 11C-D). The tissue resident CD21lo cells also downregulated

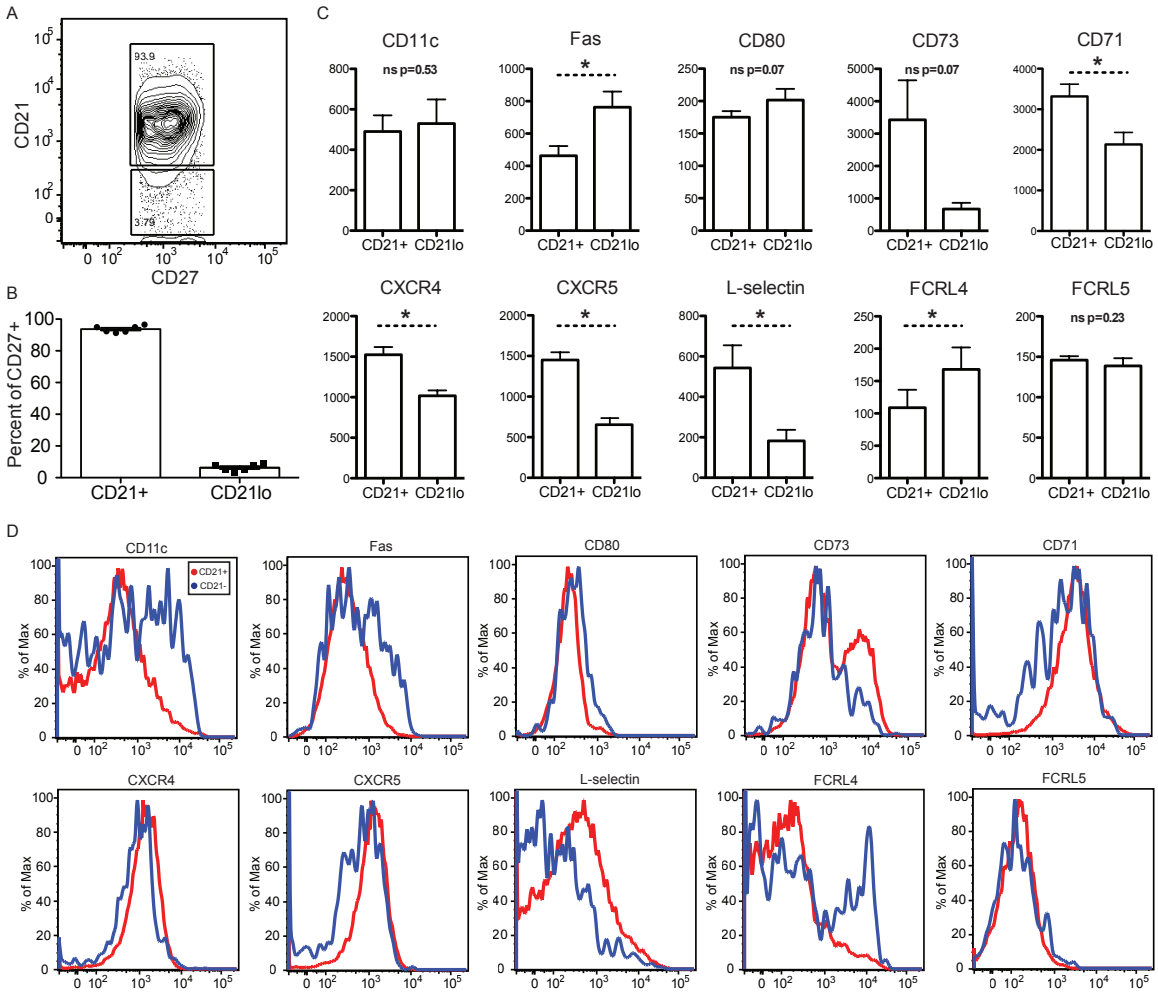


**Figure 11: CD21lo B cells are present in the tonsil and are phenotypically similar to those found in peripheral blood.**

Surface protein expression of tonsil resident PBMCs were analyzed by flow cytometry. Frozen samples were rapidly thawed in a 37C water bath prior to antibody staining on ice and analysis on the flow cytometer. (A) Representative gating of CD21 staining for classical memory or CD21lo cells in healthy tonsils. (B) Proportion of CD21lo cells in the CD27+ B cell compartment. (C) Representative histograms from FACS analysis of CD21lo and memory B cells for a panel of markers. (D) Surface expression as represented by the MFI (median fluorescence intensity) for the following markers (n=6).

CXCR4, CXCR5, L-selectin, and CD73, suggesting they in the process of leaving the germinal

center. They also have elevated levels of Fas, meaning they are also more prone to Fas

mediated apoptosis. Similar to previous studies in the tonsil, but unlike CD21lo cells in the

periphery, tissue resident CD21lo cells have upregulated FCRL4, but not FCRL5 (Ehrhardt et al. 2005; Ehrhardt et al. 2008). Both FCRL4 and FCRL5 inhibit BCR signaling and it is unclear why there are different patterns of expression in the tissue compared to the periphery. Overall, the tissue resident CD21lo cells are phenotypically similar to CD21lo cells found in the periphery. We suggest they are similar to peripheral CD21lo cells, and are potentially in an earlier stage of differentiation, captured as they are extravasating from the tissue to enter circulation.

# Chapter 4: Discussion

## Overview

The generation of memory and long lived plasma cells is critical to developing effective immunological memory. However, this complex biological process remains incompletely understood. Following affinity maturation, the germinal center cells that receive sufficient survival signals graduate to the periphery, where they differentiate into plasmablasts, memory B cells or long lived plasma cells. Much about this transition remains unknown, including whether transitional developmental stages exist, how recent germinal center graduates are prevented from being reactivated from encounters with lingering antigen, and whether these newly generated cells undergo negative selection. We hypothesized that non-classical memory B cell populations previously identified in individuals with chronic infection or autoimmunity actually represent a normal, post-germinal center, transitional stage that is primed to differentiate into long lived plasma cells. This population would be refractory to stimulus in order to protect the ongoing affinity maturation process and preserve the memory compartment for future immune responses. Further, we suggest that these transitional cells are subject to peripheral tolerance so that cells with BCRs that acquired reactivity for self in the germinal center can be removed before they join a long lived memory compartment.

In this work, we tested that hypothesis by identifying and characterizing a population of recent germinal center graduates isolated from healthy individuals that received the seasonal influenza vaccine. The findings presented herein led us to propose a

novel transitional stage of post-germinal center development. We report that in normal, healthy immune responses in humans, CD19+CD27+CD21lo cells are a potential early plasma cell precursor population that represents the earliest immigrants to the peripheral blood from GC reactions. A variety of phenotypic and functional characteristics support a model in which new germinal center graduates first enter circulation as CD21lo cells after affinity maturation, and will transiently recirculate while being inhibited from further stimulation. This phenotype will make CD21lo cells both ideally suited as the stage for post-GC tolerance induction and would avoid invasion of other GC reactions to maximize memory cell diversity. Upregulation of the Blimp1 program by CD21lo cells would also prime them to differentiate into long-lived plasma cells, though they phenotypically most resemble memory B cells. In total, our data suggests a new and critical transitional stage of post-germinal center differentiation.

**CD21lo cells are a novel transitional post-germinal center population**

We found substantial evidence that suggests that CD21lo cells are recent germinal center graduates that are in a transitional developmental stage. Flow cytometry, ELISPOT and monoclonal antibody experiments all showed that CD21lo cells are enriched for antigen specific cells 14 days after immunization, but that this enrichment decays over time. The elevated levels of antigen specific cells compared to the rest of the CD27+ B cell compartment suggests that the CD21lo cells are recent germinal center emigrants that underwent affinity maturation towards the vaccine antigen. Additionally, the kinetics of antigen enrichment parallels that of the germinal center reaction. The proportion of antigen specific cells peaks 14-28 days after vaccination, and is waning by 60 days after

vaccination. The changing nature of the antigen specific cell enrichment suggests that the CD21lo population is a transitional state that is constantly overturning and reflects the most recent germinal center response.

However, we did not definitively prove that CD21lo cells are germinal center derived, which is impossible in a human study. It is formally possible that these cells are derived from the extrafollicular response, as there is one study that shows that some somatic hypermutation can occur in extrafollicular foci. However, the extensive level of mutation of these cells, on par with that seen in the memory and plasmablast compartments, suggests that is not the case. Additionally, it is possible that these cells are differentiating from the flu specific plasmablast compartment. However, it is unlikely because plasmablasts are considered to be a terminally differentiated state. Further, while CD21lo cells upregulate Blimp-1, they do no secrete antibody lack the cell surface marker, CD38, which helps characterize plasmablasts.

The identification of the CD21lo population in healthy individuals and the observation of their enrichment for antigen specific cells has significant implications in the understanding of the role of CD21lo cells. The large majority of studies regarding these cells have utilized samples from individuals with chronic infection and autoimmunity, leading many to propose that these cells are the product of immune dysfunction or dysregulation. However, we find that these cells are a normal component of the vaccine response. We suggest that the previously identified CD21lo populations may simply reflect the massive ongoing immune responses in people with chronic infection or autoimmune conditions.

Additionally, the relative abundance of antigen specific cells in the CD21lo population compared to classical memory B cells marks them as potential targets for monoclonal antibody discovery. As we have shown, CD21lo cells can be used as an enriched source of antigen specific antibodies from peripheral blood isolated at time points outside of the narrow plasmablast burst that is now commonly used for mAb production. While the proportion of antigen specific cells in the CD21lo compartment is less than in the plasmablast compartment, antigen baiting can be used during single cell sorting to enrich for antigen specific CD21lo cells for mAb production.

A comprehensive comparison of the specificity and affinity of plasmablasts and CD21lo mAbs would be of significant interest. CD21lo cells appear to arise as the product of ongoing GC reactions compared to plasmablasts that are likely from the direct activation of memory B cells. This suggests that CD21lo cells have undergone affinity maturation during the current immune response while plasmablasts are a reflection of the pre-existing memory B cell repertoire. Thus, there may be important qualitative differences in mAbs from CD21lo cells, such as adaptation to *de novo* epitopes or improved binding affinity. This is particularly important in isolating specific antibodies towards new strains of rapidly evolving pathogens like influenza and provide a new tool in predicting the level of immune protection that will be generated by the current germinal center reaction.

**CD21lo cells are phylogentically distinct from PB and memory B cells**

We observed that CD21lo cells are clonally related to both plasmablasts and memory B cells, but form distinct clades within phylogenetic trees. Previous work has shown that plasmablasts responding to influenza vaccine primarily derive from recalled

memory B cells (Andrews, Huang, et al. 2015). However, memory B cells are capable of differentiating directly into plasmablasts or re-entering the germinal center to undergo additional affinity maturation (Dogan et al. 2009). Because CD21lo cells are clonally related to plasmablasts, we suggest that CD21lo cells are recent germinal center emigrants that derive from a memory B cell ancestor that was recalled in the current vaccine response. Furthermore, the presence of clones containing large clades of both plasmablasts and CD21lo cells suggests that a recalled memory B cell can contribute progenitors to both populations. The alternative explanation for such tree patterns would be that two or more clonally related memory B cells were recalled and some differentiated into plasmablasts while others contributed to the germinal center.

It is also possible that CD21lo cells are generated from germinal center cells that had a naïve B cell pre-cursor. It is likely that germinal center graduates have similar differentiation pathways regardless of whether they had a naïve or memory B cell as a pre-cursor. We suggest that the clones that containing only CD21lo sequences, particularly the clones with lower mutational loads, are derived from the a naïve B cell precursor that expanded and mutated in the germinal center.

The observation of defined clades shows that the CD21lo population represents a distinct stage of B cell maturation. Because the CD21lo cells phenotypically resemble classical memory B cells in many ways, we wondered if they had just temporarily and/or stochastically downregulated CD21 expression, but fundamentally remained classical memory B cells. However, there is little evidence of the interleaving of CD21lo derived sequences with memory derived sequences, which would be the case if the CD21lo state was only a temporary state that memory cells transitioned in and out of. This is further

supported by our RNASeq data that showed that the CD21lo population was transcriptionally distinct from the rest of the CD27+ B cell compartment.

Our finding that CD27+CD21lo cells are a distinct post-germinal center subset has important implications in the study design of future research utilizing B cells isolated in the peripheral blood. The use of the classification system where all CD19+CD27+ are considered memory B cells should be reconsidered. Our studies show that there is greater heterogeneity in the CD27+ compartment than previously appreciated and that there are multiple distinct developmental stages contained within that population of cells.

**The CD21lo stage is a potential mechanism to protect the ongoing germinal center response and preserve the newly generated memory B cell pool**

When we characterized the CD21lo cells generated from the vaccine response in healthy individuals, we found they shared many characteristics with previously identified non-classical memory B cells. They are refractory to stimulus and reactivation, and appear to be blocked from re-entering the germinal center. This observation provides further evidence that the CD21lo cells we find in healthy people are the same group of cells as those found in individuals with chronic infection or autoimmunity. However, we disagree with the conclusion that their relative anergy compared to classical memory B cells is a result of exhaustion from repeated antigen stimulation. While that may be a reasonable explanation when they were first observed in situations with continuous immune stimulation, it is an unlikely reason for this phenotype in an acute vaccine response.

Instead, we suggest that their non-functional phenotype is protective to the generation of humoral memory in two ways. First, it prevents the disruption of ongoing

germinal centers. If there is an active germinal center response, there is likely to be lingering amounts of the immunizing antigen in the periphery. If recently generated cells could be reactivated upon binding that antigen and re-enter the germinal center, they would have a selective advantage in affinity maturation compared to the still evolving cells in the germinal center. The recent graduates would outcompete the still evolving germinal center cells for survival, leading to a severe loss of BCR diversity and a less protective humoral response. A mechanism to prevent this would be critical for effective protection from pathogens such as influenza that undergo antigenic drift mainly for key protective epitopes, resulting in low affinity B cell responses that would need to competitively mature for protection. This unresponsiveness to activation is also beneficial because it preserves recently generated memory B cells or plasma cell precursors for future immune responses. If these cells were reactivated rapidly after generation, the stores of memory cells would be immediately depleted.

**The CD21lo stage is ideal for peripheral tolerance**

We also observed that the recirculating CD21lo cells had an expression profile that suggested they were more susceptible to Fas mediated apoptosis and had increased T-B interactions. This implied that this transitional stage could be an opportunity for peripheral tolerance. During affinity maturation, random mutations are introduced into the variable region of the BCR. While purely auto-reactive B cells in the germinal center will not receive T cell help and will undergo apoptosis, there is no known mechanism to remove cells that acquire cross reactivity to peripheral self antigens not found in the germinal center. This transitional stage, where CD21lo cells are recirculating through the body, is an ideal time to

remove cross reactive receptors from the repertoire before final differentiation. CD21lo cells would be able to sample antigen from the periphery and if activated, search for T cell help. If those cells receive T cell help, they would be rescued. However, if they were activated by self antigen, they would likely not receive T cell help and would be deleted. A mechanism for peripheral tolerance like this is particularly important for long lived plasma cell precursors. LLPCs continuously secrete large loads of antibody to the bloodstream for years or decades and there is no known negative selection mechanism once they lodge in the bone marrow.

**CD21lo cells are potential LLPC precursors**

CD21lo cells appear to be a transitional population. Given the upregulation of the Blimp-1 program and other plasma cell associated genes, we suggest that they are primed to differentiate into long lived plasma cells. Though it has been long appreciated that the serum level protection provided by LLPCs is a potent defense against future infection, the developmental pathway of this population has been unclear. Various models have been proposed, including differentiating from plasmablasts or from plasma cell committed precursors. Our work suggests that CD21lo cells, or some proportion of them, may be a plasma cell committed precursor population that will eventually transition into bone marrow resident LLPCs. The upregulation of molecules like BCMA and ZBTB32, suggest they are differentiating to adapt to a bone marrow niche. Our identification of this population has important implications in plasma cell research. Little is known about the generation of LLPCs because they are challenging to obtain in humans. The ability to

sample LLPC precursors from the blood would allow for further characterization of the BCR specificity and the general biology of the population.

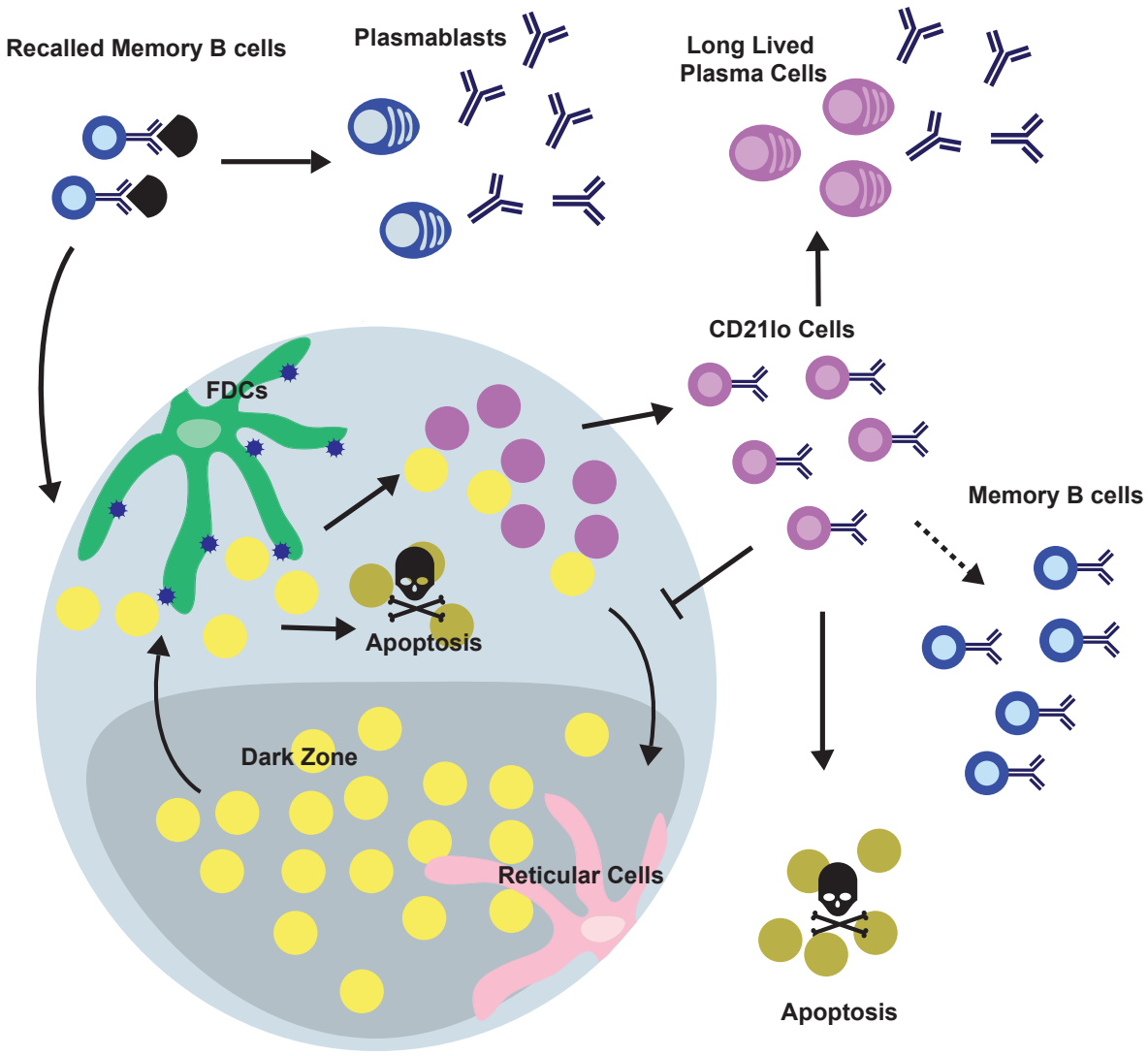


**Figure 12: Model of post-germinal center maturation.**

(A) Recalled memory cells can differentiate into plasmablasts or re-enter the germinal center to undergo additional affinity maturation. Cells destined for the long lived plasma cell compartment first enter circulation as CD21lo cells. They are refractory to activation and prone to apoptosis if they encounter their cognate antigen without receiving T cell help as a mechanism for peripheral tolerance. Eventually, they migrate to the bone marrow and become long lived plasma cells and provide long term serum level protection.

However, an alternative, but not exclusive, model to evaluate would be that the Blimp-1 program is upregulated as part of the mechanism to block germinal center re-entry that was discussed earlier. Blimp-1 directly antagonizes BCL-6, and recent germinal center emigrants may slightly upregulate Blimp-1 in order to prevent the re-adoption of the germinal center phenotype. If true, this would reinforce the role of the Blimp-1-BCL6 axis in regulating B cell differentiation and as a key genetic switch in the immune system.

**Limitations to the experimental model**

Studying the humoral immune system in humans is a critical component of developing a better understanding of B cell biology that can then be applied to improving disease treatment and prevention. However, there are limitations to experimental systems designed around human subjects, including our influenza vaccine model. First, experiments with human samples are limited in the types of samples that can be ethically acquired. In our study, we were only able to obtain a limited amount of peripheral blood at time points that had to be spaced at least 7 days apart. It is unknown whether cell in circulation are representative of the cells being generated in the vaccine response, which occurs in secondary lymphoid organs. It is possible that there are some cells that do not re-enter circulation and we do not sample. Additionally, because we are unable to obtain bone marrow samples, we cannot test whether CD21lo cells eventually differentiate into bone marrow resident long lived plasma cells. Finally, it is unclear how representative influenza immunization is of acute B cell responses and B cell responses in general. We did not perform any parallel studies with subjects receiving other vaccinations, or experiencing acute infections like the common cold. However, despite the challenges of working with an

experimental model that depends on human samples, further study of B cell populations in humans of clear importance in helping translate basic science to clinical use.

## Future Directions

### Identification and fate mapping of CD21lo-like cells in mice

In this work, we propose that the CD21lo compartment consists of cells transitioning from a germinal center phenotype into long lived plasma cells. However, this hypothesis is difficult to prove using human samples. Further work in dissecting the post-germinal center B cell transition necessitates the identification of a parallel population in mice and the development of a fate mapping mouse model.

It is likely a population similar to the CD21lo cells exists in mice. Previous work has identified a CD11c+ spleen resident B cell population that shares some characteristics with the CD21lo cells we have studied (Rubtsov et al. 2015). Because mice lack a marker like CD27 to identify memory B cells, an inducible Cre system, where AID, or some other germinal center specific gene used to drive Cre expression, can mark recent germinal center graduates by activating expression of a fluorophore. Flow cytometry analysis and ELISPOT stimulation experiments on fluorescently marked cells after immunization could be used to identify subpopulations that are enriched for antigen specific cells and phenotypically resemble the CD21lo compartment in humans. RNASeq could then be performed on these subsets to identify those that have a similar transcriptional profile to CD21lo cells. In particular, the upregulation of the plasma cell program and the downregulation of germinal center associated trafficking receptors would be key pathways to identify.

An alternative approach to identify a CD21lo-like population that is less biased, but more costly, would be perform single cell RNASeq on a large number of fluorescently marked cells that are not plasmablasts. These cells could then be grouped into subsets based on their transcriptional profile and a subset similar to the CD21lo cells may be identified.

With either approach, the identification of a gene that is upregulated upon differentiating into the CD21lo-like subset would be necessary to develop a mouse model for any fate mapping experiments. That marker gene could be used to drive inducible Cre expression so that the fate of the subset could be tracked. The mice would be immunized, cre expression would be induced, and then, at multiple time points after immunization, the location, phenotype, and transcriptome of the fluorescently marked cells would be assessed. If the fluorescently marked cells are found to differentiate into bone marrow resident plasma cells, then there would be compelling evidence that the CD21lo population is indeed a plasma cell precursor.

**Characterization of the heterogeneity of the human B cell compartment**

Our work on CD21lo cells shows that the CD27+ B cell compartment in healthy individuals is more heterogeneous than previously appreciated. However, our flow cytometry analysis also revealed that some key markers, like Fas and CD73, have a biphasic expression profile, suggesting there may be further heterogeneity within the CD21lo compartment. In addition, the literature surrounding non-classical memory B cell subsets has used a variety of panels of markers to identify the various populations of study. This

has led to uncertainty over whether there is overlap between subsets and if findings for one subset apply to a different, but similar subset.

Single cell RNASeq of recent germinal center graduates could potentially provide clarity in this regard. These cells could be identified in a non-biased way using antigen baiting, like we did with HA in this project. Analysis of these single cell transcriptomes could reveal whether there is underlying heterogeneity in previously studied subsets. Once the data has been correctly normalized, the transcriptomes can be sorted into subsets based on gene expression patterns using various clustering techniques.

**Mapping Developmental Trajectories**

Another way to analyze single cell RNASeq data from humans or mice would be to organize the transcriptomes in chronological order of differentiation. Assuming that most genes change their expression gradually, the ordering would be determined by patterns of change in gene expression. There are multiple algorithms with varying approaches that have been developed to solve this problem (Trapnell et al. 2014; Bendall et al. 2014; Setty et al. 2016). In general, they attempt to identify the shortest path through a graph structure, with the path representing the developmental progression and the nodes of the graph representing each cell. This analysis would provide insight to the relationships between previously identified subsets, as well as potentially reveal new developmentally distinct populations.

**Functional studies of memory B cell subsets in humans and mice**

The RNASeq and flow cytometry profiling showed that negative regulators of BCR signaling, like FCRL5, SIGLEC6, and SIGLEC10 were significantly upregulated on CD21lo cells. However, further studies of memory B cell subsets identified in humans and in mice should include functional studies to better understand the biological role of these cells and determine whether they are truly refractory to BCR stimulation. First, more detailed stimulation studies, including calcium flux and p-Erk FACS experiments, would lead to a greater understanding of the signaling capabilities of these cells. Preliminary studies in our lab suggest that CD21lo cells in humans are capable of fluxing calcium in response to strong BCR stimulus, but it is unclear whether they do so in more physiological conditions. Additionally, apoptosis assays would be critical in determining whether particular memory B cells are more susceptible to Fas induced apoptosis and peripheral tolerance.

**Develop paired heavy-light chain library preparation and analysis tools**

Immunoglobulin repertoire analysis was a key component in developing our understanding of the relationship between CD21lo cells and other established cell types. However, our analysis, and most NGS repertoire studies in the literature, has been constrained to the heavy chain alone. While the heavy chain sequences can provide significant information about the B cell repertoire, the most accurate analysis of B cell clones requires knowledge of the light chain. Likely, some of the clones we identified actually consist of multiple clones that would segregate separately if we had information about the light chain.

There have been recent advances in library preparation techniques that can pair the heavy and light chains together for sequencing. However, if we adapt those techniques in future studies to interrogate the phylogenetic relationships between B cell subsets, we would need to refine our analysis tools to accommodate information about the light chain. For instance, we would need to change our definition of a clone to factor in the presence of a light chain. Additionally, the generation of maximum likelihood trees to determine evolutionary lineage would be affected. Because we already assume that somatic hypermutation is a Markov process for these trees, one solution would be to merely append the heavy chain sequence with the light chain and proceed with the maximum likelihood estimation as before.

**Conclusions**

In this work, we present a critical advance in the understanding of the biological role of CD21lo cells in healthy immune systems and propose that they represent a critical transitional stage after germinal center graduation. For the first time, we show that this population is enriched for antigen specific cells after an acute immune response in healthy individuals, but are refractory to stimulus, blocked from germinal center re-entry, and are susceptible to negative selection. Additionally, we found that CD21lo cells are phylogenetically distinct from established B cell subsets and that they are primed to differentiate into plasma cells. In sum, we propose that CD21lo cells are a post-germinal center transitional population that is a long lived plasma cell pre-cursor. We hope these findings will help shape the current understanding of the role of non-classical memory B cells in the immune response and the developmental pathway of long lived plasma cells.

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