CELLULAR BASIS OF ANTIBODY MAINTENANCE:

HETEROGENEITY OF THE BONE MARROW PLASMA CELL POOL

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ABSTRACT

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David M. Allman

Plasma cells are the immune system cells responsible for producing antibodies, the key mediators of protective humoral immunity. Long-lived plasma cells (PC) are thought to be responsible for maintaining antibody titers and are believed to populate unique survival niches in the bone marrow (BM). Current models predict that bone marrow plasma cells (BM PC) consist chiefly of long-lived, slowly renewing cells. In **chapter 2**, we show the turnover rate of the BM PC pool to be much higher than predicted by these models; in fact, more than 50% of BM PC exhibit characteristics of recently formed PC. Intriguingly, these $B220^+$ PC do not appear to be cycling and are depleted upon ablation of peripheral B cell pools. In **chapter 3**, we extend our studies to antigen-induced responses. We find that very long-term maintenance of the antigen-specific BM PC pool is dependent on a CD40-independent B cell precursor. Despite the rapid turnover rate exhibited by $B220^+$ BM PC, antigen-induced antibody secreting cells are found within this population for more than 100 days post-immunization. These cells secrete exclusively low affinity, unswitched, κ type antibodies in sharp contrast to the high affinity, isotype switched cells found within the slowly renewing BM PC pool. Finally, we identify a population of rapidly renewing memory B cells that appear to be the precursors of the $B220^+$ BM PC. Together these data suggest that BM niches are continuously repopulated by newly generated plasma cells long after antigenic exposure and identify the memory B cell precursors of BM PC.

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CHAPTER 1: INTRODUCTION

I. The B cell in Adaptive Immunity

A. Adaptive Immunity: A Summary

The ability to avoid succumbing to disease prior to reproduction is critical to the evolutionary success of all organisms. While many lower order organisms rely on innate immune mechanisms for protection, most vertebrates have evolved adaptive immune systems to better protect themselves from an onslaught of viruses, bacteria, and parasites that are ubiquitous on our planet. Cells of the adaptive immune system consist of two major types: T cells and B cells. Both of these cell types share the four main attributes of adaptive immunity: inducibility, specificity, tolerance to self and memory. During their development, both B and T cells use rearrangement of DNA segments to generate an incredibly diverse array of cell surface receptors (B cell receptors (BCRs) and T cell receptors (TCRs)) capable of recognizing millions of antigenic determinants in a specific manner. Further testing of these receptors at tolerance checkpoints results in the elimination of cells with specificities against self. Mature, self-tolerant B and T cells then take up residence in secondary lymphoid organs where they are perfectly poised to encounter antigen.

Upon antigen encounter, B and T cells are induced to proliferate and to carry out specific effector functions. T cells expressing the molecule CD8 (CD8+ killer T cells) are capable of directly mediating killing of the pathogen. T cells expressing CD4 (CD4+ helper T cells) will instead aid B cells in becoming better effectors. Depending on the

nature of the antigen (see **section I.C**), B cells can assume one of a number of fates. Within days of antigen encounter, B cells whose BCR has a strong affinity for the antigen will differentiate into plasma cells (PC), antibody-secreting cells capable of secreting amounts of specific antibody that far exceed the weight of the cell (Conrad and Ingraham, 1974; HELMREICH et al., 1961; 1962; Hibi and Dosch, 1986). These cells provide the first wave of protective antibody early in the response and are thought to persist only on the order of days, though concrete evidence for this is scarce (**see section II.A**). For antigens that require T cell help (thymus-dependent (TD) antigens), B cells will migrate toward the T cell zone of the secondary lymphoid organ and form a structure called a germinal center (GC). Here the B cell receptor will be subjected to further processes in order to increase its affinity for antigen (**see section II.B**). B cells with the highest affinity BCRs will be selected into the memory components of humoral immunity: memory B cells (B_{mem}) and long-lived plasma cells (LLPC) (Radbruch et al., 2006; Weiss and Rajewsky, 1990). Memory B cells are antigen-experienced cells that can persist long-term and mediate rapid secondary responses. Upon a second encounter with the same antigen, memory B cells are ideally poised to respond with enhanced kinetics and differentiate into plasma cells producing high affinity antibodies (Toellner et al., 1996). Long-lived plasma cells lose their BCR (Manz et al., 1998) and thus their ability to respond to antigen; instead, they migrate to the unique survival niche of the bone marrow (BM) where they are capable of persisting for the lifetime of the animal (Manz et al., 1997; McMillan et al., 1972; Slifka et al., 1998). This dissertation will focus on further characterizing the longevity of the bone marrow plasma cell pool.

The ability to generate and maintain high titers of pathogen-specific antibodies is a major goal of the adaptive immune system. While non-humoral components of the

immune system can also be protective, antibody induction is the mechanism by which almost all human vaccines work (Amanna and Slifka, 2011). Moreover, many of the modern vaccines (such as polio, measles, diphtheria to name just a few) induce antibody protection that can last for decades (Amanna et al., 2007). Humans (and mice) that are unable to produce antibodies for whatever reason are immunocompromised and succumb to many bacterial and some viral infections, occasionally with fatal consequences (Boes et al., 1998b; Ehrenstein and Notley, 2010; Hoernes et al., 2011; Luther et al., 1997). For example, humans and mice that lack the kinase *btk*, a protein essential for B cell development (de Weers et al., 1993), are unable to generate antibodies and suffer from constant infections, requiring lifelong supportive care (de Weers et al., 1994; Khan et al., 1995). The nature of the antibody produced is also critical for appropriate protection. Antibodies of individuals who lack CD40L, a protein important for T cell – B cell interactions in the GC, are unable to undergo affinity maturation and class-switch recombination (Allen et al., 1993; Aruffo et al., 1993; DiSanto et al., 1993; Korthäuer et al., 1993). These individuals produce only low-affinity IgM antibodies and are consequently compromised in their ability to efficiently clear many pathogens (Allen et al., 1993; Aruffo et al., 1993; DiSanto et al., 1993; Korthäuer et al., 1993). On the other hand, people with a pathogenic overabundance of B cells (B cell malignancies such as lymphomas) are also unable to mount adequate antibody responses (Ballester et al., 1981; Shildt et al., 1983). While this seems paradoxical at first, the underlying mechanism is actually similar to people with B cell deficiencies. The expansion of the pathogenic B cell clones decreases the diversity of the B cell receptor repertoire, limiting the person's ability to respond to a wide variety of antigens (Ballester et al., 1981; Shildt et al., 1983). As all of these scenarios illustrate, generation and

maintenance of antibody titers are crucial components of effective immunity and are worth understanding in-depth.

B. B cell development and subsets

B cells are cells of the hematopoietic lineage and as such are derived from hematopoietic stem cells (HSCs) (Osawa et al., 1996a; 1996b). Hematopoiesis occurs in the liver during fetal life and in the bone marrow throughout adulthood (Christensen et al., 2004; Ema and Nakauchi, 2000; Ikuta and Weissman, 1992; Johnson and Moore, 1975; Moore and Metcalf, 1970; Weissman, 2000). Unlike T cells, B cells do not require an exogenous organ (such as the thymus) to complete their maturation and undergo the vast majority of their development in the bone marrow. On the road to becoming a B cell, HSCs gradually lose their pluripotency in favor of lineages with more restricted potential (Rieger et al., 2009). Studies have identified multipotent common lymphoid progenitors (CLPs) as an intermediate stage in this process (Allman et al., 1999; Li et al., 1996). Adoptive transfer studies have shown that CLPs give rise to B and T cells and have limited myeloid potential (Inlay et al., 2009; Kondo et al., 1997; Rumfelt et al., 2006). Expression of canonical B cell transcription factors such as early B cell factor 1 (EBF1) and paired box protein 5 (Pax5) then further defines the cells destined for B cell greatness (Inlay et al., 2009).

One of the major developmental milestones in the life of a B cell is the generation of its unique B cell receptor for antigen. To create the enormous diversity of BCRs found in the peripheral B cell pool, B cell precursors in the bone marrow undergo a DNA rearrangement process known as V(D)J recombination. In some ingenious experiments

over 30 years ago Tonegawa and Alt demonstrated that the antigen receptors of lymphocytes were assembled via random recombination events of hundreds of different gene segments: V, D, and J segments (Alt et al., 1984; Tonegawa, 1983). Recombinase activating genes (RAG1/2) that mediate this process were discovered a few years later (Oettinger et al., 1990; Schatz et al., 1989). Additional DNA diversification mechanisms increase the number of unique nucleotide sequences (and subsequent protein structures) to an even greater extent (Alt and Baltimore, 1982; Tonegawa, 1983). Upon pairing with a surrogate light chain, these newly formed, VDJ-rearranged heavy chains are able to form a signaling pre-BCR at the cell surface of the B cell precursor and signal to inhibit further recombination at the other IgH allele (Shaffer and Schlissel, 1997). Similar recombination events at either the kappa or lambda light chain loci yield functional light chains that can now pair with the rearranged IgH and form a functional BCR (Tonegawa, 1983). In addition to its role in antigen recognition, tonic BCR signaling has been shown to be indispensible for B cell survival (Lam et al., 1997); as such, all B cells which fail to make productive rearrangement or maintain BCR expression will be targeted for apoptosis (Lam et al., 1997). Finally, the random nature of this rearrangement mechanism inevitably results in BCRs with specificities against self-antigens (Wardemann et al., 2003). Through a number of mechanisms in the bone marrow and much-argued-over mechanisms at “tolerance checkpoints” in the periphery, these specificities are largely eliminated from the final mature B cell pool (Goodnow et al., 1988; Hartley et al., 1991; Nemazee and Weigert, 2000; Tiegs et al., 2011). These mechanisms appear to be mostly successful as only a minority of the population (<10%) suffers from autoimmune diseases (Goodnow et al., 2005). All of these finely tuned processes result in a population of IgM-bearing, non-self specific B cells ready to egress from the bone marrow and take up residency in peripheral lymphoid organs.

In peripheral lymphoid organs, B cells further specialize into a number of B cell subsets. While the life histories of these subsets are currently being debated (Martin and Kearney, 2001), differences in functionality are apparent. B1 B cells are an "innate-like" subset predominating in the peritoneal cavity and found in small numbers (<5%) in the spleen (Haas et al., 2005; Hardy and Hayakawa, 2001; Kantor, 1991; Tung et al., 2006). Marginal zone (MZ) B cells are defined by their anatomic location at the marginal sinus of the spleen or lymph node (Kraal, 1992; Martin et al., 2001). As blood first enters the organ at the marginal sinus, MZ B cells are *de facto* the first lymphocytes to encounter blood-borne antigens (Kraal, 1992; Martin et al., 2001). The majority (~95%) of the splenic B cell pool is composed of slowly-renewed follicular (FO) B cells (Allman and Pillai, 2008; Förster and Rajewsky, 1990). In comparison to their B1 and MZ counterparts, FO B cells respond to antigens (especially blood-borne antigens) with slower kinetics (Martin et al., 2001). BCR specificities of MZ B cells in particular are enriched for recognizing blood-borne bacterial pathogens (Carey et al., 2008; Gu et al., 1990). MZ B cells are also thought to be transcriptionally "poised" to differentiate into plasma cells with enhanced kinetics (Genestier et al., 2007; Oliver et al., 1997). While a spirited debate rages about the ability of B1 B cells to secrete antibody constitutively (without further differentiating into a plasma cell), transcriptional studies suggest that these cells are "poised" for activation similarly to MZ B cells (Fairfax et al., 2007; Savitsky and Calame, 2006; Tumang et al., 2005). In consequence, the early wave of IgM is believed to derive largely from these small MZ and B1 B cell populations (Martin et al., 2001). In contrast, FO B cells are the major players in subsequent stages of the immune response, including the production of class-switched antibodies (Förster and Rajewsky, 1987; Martin et al., 2001). While the precise details of ontogeny and transcriptional

regulation of these subsets remain to be elucidated, their specialized functions are undisputed and are critical to the successful resolution of many infections (Alugupalli et al., 2004; Martin et al., 2001).

C. B cell activation and the NP-system

While there is limited evidence that B cells can get activated in the bone marrow (Benner and Van Oudenaren, 1975; Cariappa et al., 2005), conventional models conceive of B cell activation as taking place in the secondary lymphoid organs (Koch et al., 1981). The nature of the antigen will dictate the specifics of the B cell response. Antigens can be grouped into two broad categories: thymus-dependent (TD) or thymus-independent (TI) antigens. TD antigens are often proteins capable of engaging the B cell receptor directly and activating T cells with antigen presented in the context of MHCII (MacLennan, 1994). Clonal expansion and differentiation of the antigen-responsive B cells results in a large pool of extrafollicular plasma cells, which are responsible for the early wave of low affinity, IgM antibody (Jacob et al., 1991; Smith et al., 1996). While the specific role of T cells during these early events is unclear, studies from our lab suggest that T cells are essential for a robust extrafollicular plasma cell response to TD antigens (Bortnick A, unpublished data). In parallel, B cells migrate to the T cell border following antigen encounter (Liu et al., 1991; Okada and Cyster, 2006; Reif et al., 2002; Schwickert et al., 2011), eventually forming a structure known as the germinal center (GC) (MacLennan, 1994). In the GC specialized T cells, known as follicular helper T cells (T_{FH}), provide help to B cells in the form of cytokines and cell surface interaction signaling (e.g. IL-21 and CD40-CD40L interactions, respectively) (Kawabe et al., 1994; Linterman et al., 2010; Xu et al., 1994; Zotos et al., 2010). These signals lead to the processes of

somatic hypermutation (SHM) and class-switch recombination (**see section II.B)** and ultimately result in the production of high affinity, class-switched B cells (MacLennan, 1994; Tarlinton, 2008). These B cells are subsequently selected into the memory B cell and long-lived plasma cell pools (Tarlinton, 2008). In the last ten years, a number of groups, including ours, have shown that the memory B cell and long-lived plasma cell fates are not restricted to GC-derived progeny and can occur in a GC-independent and even T-cell-independent manner (Bortnick et al., 2012; Hosokawa et al., 1984; Obukhanych and Nussenzweig, 2006).

Antigens that do not utilize T cells are subdivided into two groups: TI-I and TI-II antigens. TI-I antigens do not signal through the BCR and most often stimulate B cells through innate Toll-like receptors (TLRs) (Gronowicz et al., 1980). TI-II antigens, in contrast, are large repetitive structures, reminiscent of polysaccharide chains on the surface of bacteria, which are capable of eliciting B cell activation through extensive BCR crosslinking. Early characterization of responses to these antigens in athymic (nude) mice has firmly established their T cell independence (Feldmann et al., 1972). In contrast to their TD counterparts, the early response to TI antigens engages many of the innate-like B1 and MZ B cells, most likely due to the preponderance of BCR specificities recognizing common bacterial antigens amongst these B cell pools (Carey et al., 2008; Gu et al., 1990). Furthermore, B cells responding to TI antigens can only form a nascent germinal center that is aborted without ever being able to support the processes of somatic hypermutation or class switch recombination (de Vinuesa et al., 2000; Goodlad and Macartney, 1995; Toellner et al., 2002). This has been extended to mean that TI antigens are incapable of engendering immunological memory (memory B cells or long-lived plasma cells) (McHeyzer-Williams and McHeyzer-Williams, 2005; Mond et al.,

1995). Together with early studies suggesting that TI-antigen-specific antibody titers did not persist long-term, TI responses were long thought to be limited to short-term low affinity IgM production by extrafollicular plasma cells (Fidler, 1975; Mond et al., 1995). Recently, a number of papers have defined protective, persisting IgM responses to a variety of antigens including spirochetes, intracellular bacteria and encapsulated bacteria (Alugupalli et al., 2004; 2003; Racine et al., 2011; Taillardet et al., 2009). Shortly after, our lab defined the existence of IgM-secreting, T-cell-independent, long-lived bone marrow plasma cells in a model antigen system (Bortnick et al., 2012). Another lab has described memory B cells generated in response to a TI-II antigen (Hosokawa et al., 1984; Obukhanych and Nussenzweig, 2006); IgM-expressing memory B cells have recently come back into vogue, though it is unclear whether they are similarly generated in a GC-independent manner ((Dogan et al., 2009; Pape et al., 2011)**, see section II.C**) While it remains a rapidly evolving field, recent insights into the nature of TI B cell responses have significantly updated the model that has predominated for decades and raised new questions about the cellular and signaling requirements of generating immunological memory.

The success of many of the early studies on B cell activation relied heavily on utilizing well-defined, controllable model systems. In particular, tracking the response to haptenated antigens quickly became the standard that is still widely used today (including in this dissertation). Haptens are small molecules that are incapable of eliciting an immune response without being attached to a "carrier". Varying the nature of the carrier will determine whether a TI-I, TI-II or TD response will be induced: haptenated LPS elicits a TI-I response via TLR4 signaling (Persson and Möller, 1975; Poltorak et al., 1998), haptenated Ficoll, a synthetic sucrose polymer, crosslinks the BCR in a TI-II response

(Mosier et al., 1974), haptenated proteins engage T cell help in a TD response (Mitchell and MILLER, 1968). In addition to having the tools to track hapten-specific antibodies and antibody-secreting cells (Fidler, 1975), over the past 20 years a few groups have perfected the tools to track hapten-specific B cells using flow cytometry (Lalor et al., 1992). Importantly, these studies can be successfully performed in conventional C57BL/6 mice, without the need to resort to existing transgenic mouse models (Cascalho et al., 1996; Lalor et al., 1992; McHeyzer-Williams et al., 1993). In C57BL/6 mice, immunization with the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) conjugated to a carrier has the additional surprising property of eliciting a primary B cell response dominated by antibodies utilizing the λ1 light chain (Jack et al., 1977). The secondary response, in contrast, is said to contain many κ clones (Jack et al., 1977; Jacob et al., 1991). In addition to providing an alternative means for tracking the hapten-specific cells, this peculiar feature of the NP response has perplexed immunologists for decades and will be further addressed in later chapters of this dissertation (**chapter 3**). Finally, a big advantage of the NP system is the in-depth understanding we possess about the kinetics of the germinal center and plasma cell responses (Fidler, 1975; Jacob et al., 1991) as well as the molecular details of somatic hypermutation and subsequent affinity maturation of the NP-specific BCR (Takahashi et al., 1998). For example, the tryptophan to leucine mutation at codon 33 of CDR1 results in a 10-fold increase of the BCR affinity for NP and clones bearing this mutation come to dominate late primary and secondary NP responses (Allen et al., 1988; Weiss and Rajewsky, 1990). Altogether, these features of the NP-carrier system have been integral to its success as a model approach and made it ideally suited for the studies described in **Chapter 3.**

II. Long-term Humoral Immunity

A. Short-lived versus Long-lived Plasma Cells

Plasma cells are terminally differentiated cells of the B cell lineage that are uniquely adapted to produce large amount of antibodies without succumbing to ER-stress induced cell death (Oracki et al., 2010; Reimold et al., 2001). Upon antigen encounter in the periphery, naïve B cells whose BCRs have the highest affinity for the antigen selectively differentiate into an early wave of plasma cells (Chan et al., 2009; O'Connor et al., 2006; Paus et al., 2006; Phan et al., 2006). Antigen-specific B cells with a lower initial BCR affinity are recruited into GCs where they undergo multiple rounds of somatic hypermutation and selection, ultimately giving rise to progeny with BCR affinities several fold higher than those found in the naïve pool (Phan et al., 2006). Conventionally, the early wave of plasma cells was believed to have a half-life of 3-5 days (Ho et al., 1986; Smith et al., 1996), while the burden of immunological memory fell entirely upon the memory B cell progeny of the GC reaction. However, it had been noted that many antigens induced standing antibody titers after a single inoculation of antigen, without the apparent need for a *bona fide* memory response dependent upon reintroduction of antigen (Amanna and Slifka, 2010; Amanna et al., 2007). This gave rise to a model whereupon memory B cells underwent continuous rounds of division and differentiation into short-lived plasma cells, which secreted antibody for 3-5 days before succumbing to death by apoptosis (Schittek and Rajewsky, 1990; Smith et al., 1996). Whether this process was stochastic or driven by antigen persisting on follicular dendritic cells (FDCs) in the secondary lymphoid organs was a matter of much debate that has never been addressed to satisfaction (Amanna and Slifka, 2010; Bernasconi et al., 2002; Karrer et al., 2000; Liu et al., 1996; MacLennan, 1994; Tew and Mandel, 1979; Tew et al., 1990).

Alternatively, persistence of antibody titers was consistent with a model where long-lived plasma cells maintained antibody titers for the duration of the response. Cleverly using BrDU labeling and irradiation studies, two seminal papers changed the notion that antibody titer maintenance was dependent on constant waves of antigen-induced plasma cell differentiation and showed that instead it depended on bone marrow plasma cells that could persist for the lifetime of the animal (Manz et al., 1997; Slifka et al., 1998). Manz et al performed pulse-chase BrDU labeling studies on plasma cells generated in response to a TD antigen (ovalbumin) and demonstrated the persistence of antigen-specific plasma cells in the bone marrow for over 100 days (Manz et al., 1997). In addition to defining the concept of a long-lived plasma cell, this report also reiterated the importance of the bone marrow as a key site of antibody production (Manz et al., 1997; McMillan et al., 1972; Slifka et al., 1995). The following year, Slifka et al extended these studies by taking advantage of the well-known radioresistance property of plasma cells (Lowenthal and Harris, 1985; Slifka et al., 1998). Using irradiation to ablate all cells of the B lineage other than plasma cells, Slifka was able to show that the plasma cell response to LCMV persisted in the absence of a feeder B cell pool (Slifka et al., 1998). Moreover, that same year, the original group of Manz and Radbruch was able to demonstrate the antigen independence of bone marrow plasma cell maintenance by using *in vivo* cell transfer studies (Manz et al., 1998). I have personally evidenced the absence of a functional BCR on plasma cells by failing to detect the BCR signaling component Igβ flow cytometrically **(Fig. 2-2 C)**. Other studies from our lab have further expanded our conception of long-lived plasma cells by identifying a GC-independent pathway of long-lived plasma cell formation by using both irradiation and BrDU labeling approaches (Bortnick et al., 2012).

The proof that plasma cells can persist for very long periods of time has profoundly shaped our understanding of humoral immunity and has had significant medical implications. On the one hand, generating long-lived plasma cells capable of secreting high affinity, protective antibody is the major goal of vaccine design; on the other, the ability to deplete pathogenic plasma cells is much sought after. When B cell tolerance checkpoints are compromised and cells with self-reactive BCR specificities enter the effector pool, plasma cells (both short-lived and long-lived) secreting self-reactive antibodies become the culprit behind many manifestations of autoimmune disease (Hoyer et al., 2005; 2004; Martin and Chan, 2004). The drug rituximab, an anti-CD20 antibody capable of effectively eliminating B cells *in vivo*, is now widely used in the context of autoimmune disease and B cell malignancies (Edwards and Cambridge, 2006; Edwards et al., 2004). Importantly, while rituximab effectively depletes all major cells of the B cell lineage (including memory B cell effectors), CD20 is not expressed on long-lived plasma cells at sufficient levels to mediate depletion upon rituximab treatment (DiLillo et al., 2008). In fact, it is believed that rituximab is most effective in autoimmune diseases whose autoantibody production is largely due to short-lived plasma cells (Edwards and Cambridge, 2006). Another group took advantage of the rituximab resistance of long-lived plasma cells to further define the maintenance of the bone marrow long-lived plasma cell pool as being independent of memory B cells by ablating all B cells with anti-CD20 antibodies; however, their studies were limited as they examined only one timepoint post-ablation (Ahuja et al., 2008). Some studies in this dissertation will also take advantage of this discrepancy in CD20 expression between B cells and plasma cells to further examine the nature of the bone marrow plasma cell pool (**Fig. 2-6 B**). While the inability of anti-CD20 therapies to deplete plasma cells may be discouraging in the context of autoimmune disease, it has proven useful scientifically. Patients undergoing rituximab

treatment maintain their antibody titers to previously encountered antigens, providing the best evidence for the existence of long-lived plasma cells in humans (Cambridge et al., 2003).

With all the evidence for the importance of long-lived plasma cells in mice and humans, what is the role of short-lived plasma cells in immune responses? Current thinking holds that the secretion of low affinity IgM by the early wave of plasma cells is crucial for immediate protection against a pathogen, *de facto* buying time for a GC reaction to occur and generate high affinity effectors (Jacob et al., 1991; MacLennan, 1994; Nossal, 1992). While recent work by our lab and others has shown that some plasma cells from this early, extrafollicular wave are capable of entering the long-lived plasma cell pool and persisting long-term (Bortnick et al., 2012; Taillardet et al., 2009), the vast majority of these cells will apoptose within 3-5 days of their generation, as predicted by early models (Bortnick et al., 2012; Fidler, 1975; Liu et al., 1991; Smith et al., 1996). These cells are most often termed "plasmablasts" implying that these are antibody-secreting cells that are capable of cell division. The only direct evidence for the existence of these cells is one study that is over 40 years old (Claflin and Smithies, 1967); subsequent studies that have claimed the existence of plasmablasts have been less than rigorous in their interpretation of BrDU labeling data or focused on malignant plasma cells (Drewinko et al., 1981; Jego et al., 1999; Sze et al., 2000; Yaccoby and Epstein, 1999). More recent studies have shown that the plasma cell transcriptional program is necessarily incompatible with proliferation; in fact, the master regulator of the plasma cell lineage, Blimp-1, has been described as a director repressor of c-myc, a key player in cell cycle control (Lin et al., 1997). Similarly, expression of CDK inhibitor p18(INK4c) has been found to be essential for the proper generation of antibody-

secreting cells arrested in the G1 phase of the cell cycle (Bretz et al., 2011; Morse et al., 1997; Tourigny et al., 2002). While the term “plasmablast” persists in the literature it is now often used interchangeably with “short-lived plasma cell”, making no claims about the cell cycle status of the cell.

The question about the identity of the cell that will eventually colonize the plasma cell survival niches of the bone marrow (“plasmablast” or non-secreting precursor) is a hotly debated one (Jourdan et al., 2011; Medina et al., 2002; Moser et al., 2006; O'Connor et al., 2002; Tarte et al., 2003). One group has described a proliferating, non-antibody-secreting cell that leaves the GC as the relevant plasma cell precursor (O'Connor et al., 2002). Another lab has histologically demonstrated the presence of Blimp-1^{+} cells in GCs and, because many of these cells are Ki67^{+}, concluded that they are proliferating centroblasts (Angelin-Duclos et al., 2000). At least one paper finds antigen-specific plasma cells in the blood to parallel the maturity of BM PC, raising the possibility that these cells seed the bone marrow directly without further need for maturation (Blink et al., 2005). Despite these findings, most researchers seem to agree that phenotypically immature plasma cells are the population of cells that will exit secondary lymphoid organs and take up residence in the bone marrow (Medina et al., 2002). Using a Blimp1-GFP reporter system, Kallies et al (2004) describe the presence of low numbers of GFP^{int} (newly formed) plasma cells in the bone marrow and the classic Manz et al (1997) report suggests a degree of bone marrow plasma cell heterogeneity even whilst describing the longevity of the pool (Kallies et al., 2004; Manz et al., 1997). More recently, Racine et al have described a population of IgM plasmablasts in the bone marrow that facilitated long-term protection against intracellular bacterial infection (Racine et al., 2011). While these reports in the literature hint at the presence of newly formed

(short-lived) plasma cells in the bone marrow, to our knowledge a comprehensive study of the bone marrow plasma cell pool kinetics and dynamics has not been performed and will be the focus of **Chapter 2** of this dissertation.

B. Germinal Centers

According to the classic model of humoral immunity, humoral memory in the form of memory B cells and long-lived plasma cells is derived exclusively from germinal center (GC) structures. While recent studies have challenged this notion ((Bortnick et al., 2012; Racine et al., 2011; Taillardet et al., 2009; Toyama et al., 2002)**, section II.A, C**), the import of GCs to the production of high affinity, class-switched antibodies is undisputed (Tarlinton, 2008). GCs are microanatomical structures enriched with antigen-stimulated B cells undergoing class switch recombination (CSR), somatic hypermutation (SHM), and affinity-based selection before generating memory B cells and plasma cells (Tarlinton, 2008). Class-switch recombination is a permanent DNA recombination event whereby the constant region of the antibody will be replaced from IgM to one of the other isotypes (IgG, IgA, IgE) without a concomitant change in specificity (Martinez-Alonso and Coutinho, 1982). The nature and location of the antigen are key, albeit poorly understood, factors that determine which antibody class will be produced (Hasbold et al., 2004; He et al., 2007; Snapper and Mond, 1993). Somatic hypermutation is the mechanism responsible for affinity maturation, a process describing the increasing binding affinity antibodies acquire for the antigen during the course of the infection/immunization (EISEN and SISKIND, 1964). The variable chain of the antibody accumulates random point mutations, in some cases resulting in a structure with a higher than starting affinity for antigen (Berek et al., 1985). This process is known as somatic

hypermutation and is believed to occur almost exclusively in germinal centers (Berek et al., 1991; Jacob et al., 1993). While SHM outside the GC has only been described in transgenic models of autoimmune mice (William et al., 2005; 2002), CSR occurring outside a GC is a well-recognized phenomenon and has been documented both in T-independent and very early T-dependent responses (Mond et al., 1995; Pape et al., 2003; Snapper and Mond, 1993; Toellner et al., 1996). Both processes have been shown to be dependent upon activation-induced deaminase (AID), with AID knockouts producing only low affinity, unswitched antibodies (Muramatsu et al., 2000; 1999).

While T-independent antigens are capable of forming nascent GC structures (de Vinuesa et al., 2000; Goodlad and Macartney, 1995; Toellner et al., 2002), $CD4^+$ T cells are essential components of fully-fledged GC responses to T-dependent antigens (Thorbecke et al., 1994). Classically, access to both antigen and T cell help was believed to be limiting in the GC (Allen et al., 2007); however, recent studies tracking GC B cell migration have suggested that T cell help was the limiting factor (Victora et al., 2010). In fact, B cell migration within the histologically distinguishable light and dark zones of the GC is largely construed as a selection event determined by the ability to receive the requisite T cell help (Allen et al., 2007; Victora et al., 2010). T cells within the GC have been described as a unique helper T lineage defined by a transcriptional regulator: T follicular helper (T_{FH}) cells controlled by Bcl-6 (Fazilleau et al., 2009; Johnston et al., 2009; Nurieva et al., 2009). Recently, the steps of T_{FH} maturation have been elucidated, both in terms of location within a GC and the cell types involved (Baumjohann et al., 2013; Choi et al., 2011; Goenka et al., 2011; Kerfoot et al., 2011; Kitano et al., 2011). Both B-T cell interactions and cytokine secretion by T_{FH} have been identified as essential for a stable GC structure. First, CD40L-CD40 interactions have long been known to be

important for T-B interactions and indeed a lack of either of those components results in abrogated GCs and lack of high affinity, switched antibodies (Kawabe et al., 1994; Xu et al., 1994). In a human correlate, CD40L deficiency has long been known to be the cause of hyper-IgM syndrome, characterized by the inability of patients to undergo class-switch recombination (Allen et al., 1993; Aruffo et al., 1993; DiSanto et al., 1993; Korthäuer et al., 1993). Moreover, an exogenous source of CD40 antibodies can induce protective, isotype switched antibodies to T-independent, polysaccharide antigens (Dullforce et al., 1998), though too much of a CD40 agonist can short-circuit humoral immunity (Erickson et al., 2002). Secondly, lack of co-stimulator CD28 similarly resulted in a decrease of antibody titers (Shahinian et al., 1993), although those studies potentially warrant a reinterpretation in light of recent work suggesting that CD28 plays a direct role in controlling plasma cell survival and function (Nair et al., 2011; Njau et al., 2012; Rozanski et al., 2011). Additionally, molecules such as SAP and PD-1 on T cells and PD-L2 on B cells have been shown to regulate the stability of the GC and the number of T_{FH} and long-lived plasma cells (Cannons et al., 2010; Good-Jacobson et al., 2010; Qi et al., 2008). Finally, the cytokine IL-21 secreted by T_{FH} is critical for supporting GCs, in particular via maintaining Bcl-6 expression in GC B cells (Linterman et al., 2010; Zotos et al., 2010).

While many of the soluble and cell surface molecules controlling GC formation and maintenance are known, the regulation of GC output and dissolution is poorly understood. Recent work has suggested that differential distribution of IL21R during asymmetric division determines the fate of the GC B cell, with the IL21R-retaining daughter cell reentering the GC while the other daughter cell was free to leave the GC as a plasma cell (Barnett et al., 2012). Other work has suggested that plasma cells derived from a GC participate in a negative feedback loop, dampening T_{FH} responses and

resulting in GC dissolution (Pelletier et al., 2010). However these studies did not address the crucial role of affinity selection within the GC. Since GCs put out memory B cells and long-lived plasma cells, there must be mechanisms for selecting the highly mutated, high affinity cells into these pools. While B cells with a relatively low affinity for antigen are initially selected into the GC reaction (Chan et al., 2009; O'Connor et al., 2006; Paus et al., 2006), plasma cells exiting the GC have accumulated many mutations and are high affinity (Amanna and Slifka, 2010; Smith et al., 1997). In fact, it appears that the selection events for long-lived plasma cells are quite stringent: while low affinity clones can be found in the memory B cell compartment, GC-derived plasma cells are exclusively high affinity (Phan et al., 2006; Smith et al., 1997; 2000; 1994). Intriguingly, GC B cells appear to undergo a fixed mutational and proliferation program regardless of their initial BCR affinity; in other words, low affinity and high affinity cells undergo the same number of cell divisions and low affinity B cells accumulate more V_H mutations than their high affinity counterparts, making the two pools indistinguishable (Anderson et al., 2009; Shih et al., 2002). Instead, differential survival capacities appear to provide the selective force behind the memory B cell *versus* long-lived plasma cell fate decision. Stringent affinity selection was relaxed in *bcl-x*$_L$ transgenic mice with low affinity PC persisting in the bone marrow (Takahashi et al., 1999). *Bcl-2* transgenic mice evidenced higher numbers of plasma cells and memory B cells, though without a change in the fraction of high-affinity plasma cells, perhaps suggesting that different pro- and anti-apoptotic molecules have distinct roles in this process (Smith et al., 1994; 2000). In fact, both the pro-apoptotic molecule Bim and the anti-apoptotic Mcl-1 have been implicated in the process (Fischer et al., 2007; Peperzak et al., 2013; Vikstrom et al., 2010). Overall, while many studies have been carried out to elucidate these processes, it is clear that many aspects remain poorly understood.

C. Memory B cells

Memory B cells (B_{mem}) are antigen-specific components of humoral memory generated during primary immune responses and capable of rapidly differentiating into antibody-secreting cells upon antigen re-encounter. BrDU labeling studies have shown memory B cells to be long-lived (Schittek and Rajewsky, 1990); other work has demonstrated that memory B cells persist in the marginal zone of secondary lymphoid organs, perfectly positioned for antigen encounter soon after reinfection (Liu et al., 1988; Yates et al., 2013). While many groups have failed to find evidence for memory B cells in the BM (Shepherd and Noelle, 1991; Slifka et al., 1998), some data suggest that memory B cells can differentiate into plasma cells in the bone marrow in mice lacking secondary lymphoid organs (Koch et al., 1981; Ochsenbein et al., 2000; Paramithiotis and Cooper, 1997). T cell help appears dispensable for memory B cell survival; however, the role of T cells in memory B cell activation upon antigen re-encounter is less clear (Vieira and Rajewsky, 1990). Secondary immune responses did not occur when T cells were depleted using anti-CD4 antibodies (Ochsenbein et al., 2000; Vieira and Rajewsky, 1990); however, more recent studies using genetic knockout models found that memory B cell responses were unimpaired in the absence of cognate or bystander T cell help (Hebeis et al., 2004). Similarly, antigen presentation on FDCs was found to be dispensable for memory B cell maintenance in knockout models (Karrer et al., 2000). A number of historical cell transfer studies conflicted with this observation, concluding that antigen persistence was requisite for memory B cell maintenance (Askonas et al., 1972; Gray and Skarvall, 1988). The debate was elegantly ended by the lab of Klaus Rajewsky, who used a Cre recombinase based system to revise the BCR specificity away

from the immunizing antigen, demonstrating the antigen independence of memory B cells (Maruyama et al., 2000). The role of antigen in memory B cell activation and differentiation into plasma cells is less clear (Zinkernagel, 2002), with some workers arguing that it can be achieved via polyclonal, antigen-independent stimuli (Bernasconi et al., 2002).

One facet of memory B cell biology that has been extensively studied in the last few years is the provenance and heterogeneity of the memory B cell pool. Classically, memory B cells are thought to be high affinity emigrants from the GC (MacLennan, 1994; Weiss and Rajewsky, 1990); however, a number of recent papers have challenged this notion. In T-dependent, GC-forming responses low affinity cells have been detected in the memory B cell compartment, with up to 35% of the memory B cell pool being made up of low affinity clones (Smith et al., 1997; 2000). Another group found that memory B cells, unlike their long-lived plasma cell counterparts, represented a polyclonal cellular pool containing specificities for viral escape variants (Purtha et al., 2011). The heterogeneous nature of the memory B cell pool is further demonstrated by the presence of both isotype switched and IgM-bearing cells, with the provenance of the IgM^+ memory B cells being the matter of much debate. Studies in both mice and humans have detected evidence of somatic hypermutation in IgM-bearing memory B cells, highly suggestive of a GC origin for these cells (Dogan et al., 2009; Seifert and Küppers, 2009; Yates et al., 2013); however, other work has indicated that IgM^+ memory B cells were subject to a lesser degree of affinity maturation and differentiation than isotype switched memory B cells (Pape et al., 2011; Taylor et al., 2012). Unexpectedly, two independent studies in mice have found IgM^+ memory B cells to be longer-lived than IgG^+ memory B cells and both groups have proposed a model whereupon IgG^+ memory B cells

differentiate into plasma cells upon reinfection while IgM^+ memory B cells reinitiate GCs (Dogan et al., 2009; Pape et al., 2011); no further studies have been done to corroborate these surprising findings. It appears that IgM^+ and $IgG1^+$ memory B cells can also be generated in a GC-independent pathway from Bcl6-deficient B cells (Toyama et al., 2002). Generation of memory B cells to T-independent antigens and in a T cell-independent manner in humanized mice serves as further proof toward the existence of a GC-independent memory B cell generation pathway (Obukhanych and Nussenzweig, 2006; Scheeren et al., 2008). Characterization of memory B cell heterogeneity using phenotypic surface markers has identified at least 5 subsets of memory B cells (Tomayko et al., 2010); the relationship of these markers to the mutational status of the cells and subsequently their provenance is just beginning to be understood (Anderson et al., 2007). Overall, the multi-layered, intricate nature of humoral memory has been increasingly appreciated in recent years; hopefully future insights will result in an integrated, comprehensive model of memory B cells fairly soon.

D. Plasma cell transcriptional networks

Acquisition of a plasma cell fate involves a series of finely tuned steps including suppression of the B cell transcriptional program and expansion of protein secretion machinery in order to handle the prodigious amounts of antibodies being made. Blimp-1 has been described as a master regulator of the plasma cell fate with roles in both generation and maintenance of short- and long-lived plasma cells (Angelin-Duclos et al., 2000; Cattoretti et al., 2005; Messika et al., 1998; Sciammas and Davis, 2004; Shaffer et al., 2002; Shapiro-Shelef et al., 2003; 2005; Turner et al., 1994). A transcriptional repressor, Blimp-1 suppresses the B cell transcriptional program through direct

downregulation of Pax-5, the master regulator of the B cell lineage, and c-myc, an important cell cycle regulator often dysregulated in malignant plasma cells (Chesi et al., 2008; Delogu et al., 2006; Lin et al., 2002; 1997; Nera et al., 2006; Shou et al., 2000; Soro et al., 1999; Usui et al., 1997). Repression of c-myc was found to be necessary, but not sufficient for the assumption of the plasma cell fate (Lin et al., 2000). In contrast, Pax-5$^{-/-}$ B cells took on a secretory phenotype and evidenced increased expression of Blimp-1, establishing Pax-5 as a director repressor of Blimp-1 (Kallies et al., 2007; Nera et al., 2006; Usui et al., 1997). Similarly, Blimp-1 and Bcl-6 were found to be directly antagonistic, implying a reciprocal regulatory loop of plasma cell *versus* GC B cell fates (Alinikula et al., 2011; Chevrier et al., 2009; Corcoran et al., 2005; Emslie et al., 2008; Lin et al., 2004; Muto et al., 2010; 2004; Ozaki et al., 2004; Reljic et al., 2000; Sciammas and Davis, 2004; Shaffer et al., 2002; 2000; Tunyaplin et al., 2004). Largely as a consequence of Bcl-6 and Pax-5 repression, plasma cells downregulate many cell surface molecules associated with B cells, such as CD19, CD20, CIITA and BCR signaling components (Piskurich et al., 2000; Shaffer et al., 2002; Tai et al., 2012). Some of these conclusions will be re-examined in this dissertation, particularly in the finding that retention of the classical B cell marker B220 marks a functional difference among plasma cell subsets (**chapter 2**).

XBP-1 is one of the few critical plasma cell factors that are induced (rather than repressed) by Blimp-1 (Reimold et al., 2001; Shaffer et al., 2004). XBP-1 is critical for mediating efficient protein synthesis and secretion as well as the control of the unfolded protein response (UPR) in plasma cells and secreting B-1 cells (Goldfinger et al., 2011; Iwakoshi et al., 2003b; Savitsky and Calame, 2006; Shaffer et al., 2004); however, XBP-1 is not sufficient as it cannot rescue plasmacytic differentiation in the absence of Blimp-1

(Shapiro-Shelef et al., 2003; Taubenheim et al., 2012). It appears that, within the B cell lineage, XBP-1 function is restricted to plasma cell fates as other components of humoral memory are XBP-1-independent (Todd et al., 2009). Additionally, XBP-1 may have a role in plasma cell colonization of the bone marrow niche (Hu et al., 2009). IRF4 is the 3rd and final major player in plasma cell transcriptional networks whose early graded expression is thought to induce transcription of the *prdm1*, the Blimp-1 gene (Cattoretti et al., 2006; Hauser et al., 2009; Sciammas et al., 2006). Other work has indicated that IRF4 and Blimp-1 are regulated independently or even suggested that IRF4 may be downstream of Blimp-1 (Kallies et al., 2007; Klein et al., 2006). Additionally, IRF4-deficient B cells lack class-switch recombination, suggesting an independent role for IRF4 in this process (Klein et al., 2006; Sciammas et al., 2006). Over the years additional transcriptional regulators of the plasma cell fate have been identified: Oct2, CD93 and OBF-1 have been shown to be positive regulators while Bach2 and Mitf act as negative regulators by repressing *Prdm1* and *Irf4*, respectively (Alinikula et al., 2011; Chevrier et al., 2009; Corcoran et al., 2005; Emslie et al., 2008; Lin et al., 2004; Muto et al., 2004; 2010; Ochiai et al., 2006; 2008; Shen and Hendershot, 2007).

The intricacy of the plasma cell transcriptional networks has important medical implications. The majority of key transcriptional players are conserved between normal and malignant plasma cells; comparative microarray studies have identified ~250 genes that are differentially expressed, most of them not unique to malignant plasma cells (De Vos et al., 2002). For example, XBP-1 levels have been found to be highest in multiple myeloma cells (Carrasco et al., 2007; Reimold et al., 1996). A lot of work has focused on exploiting these differences in degree of expression. XBP-1 knockdown in myeloma lines has resulted in smaller cells and subsequent cell death (Shaffer et al., 2004). Although

IRF4 is not genetically altered in most myelomas, Shaffer et al showed that the malignant plasma cells were nevertheless “addicted” to the IRF4 regulatory network and suggested that a “therapeutic window” may exist where targeting IRF4 would kill myeloma cells while sparing healthy plasma cells (Shaffer et al., 2008). Blimp-1 target and classic phenotypic marker of plasma cells, CD138 (syndecan-1), has also been validated as a viable multiple myeloma target (Shaffer et al., 2002; Wu et al., 2012; Yang et al., 2007). Perhaps the most successful class of multiple myeloma drugs are the proteasome inhibitors. Antibody secretion via the ER pathway has been identified as a major component of the plasma cell death mechanism (Pelletier et al., 2006; Pengo et al., 2013). The accumulation of unfolded proteins due to extensive immunoglobulin production sensitizes normal plasma cells as well as myeloma cells to proteasome inhibition (Meister et al., 2007; Neubert et al., 2008). In addition, proteasome inhibition renders myeloma cells functionally XBP1-deficient, further exacerbating the effect (Lee et al., 2003). In combination with survival factor modulation described in the next section, the molecular toolkit for plasma cell manipulation is prodigious and constantly growing.

E. Plasma cell survival niches

The role of the bone marrow as a unique survival niche for long-lived plasma cells has been established since the longevity of the plasma cell population was first characterized (Manz et al., 1997; Slifka et al., 1995; 1998). When removed from this niche and placed in *ex vivo* cultures, bone marrow plasma cells remained viable for just a few hours, strongly suggesting that this plasma cell population depends on exogenous survival signals in the bone marrow (Minges Wols et al., 2002). Subsequently, a number of cellular, cell surface and soluble factors have been described as playing key roles in

the maintenance of this exclusive survival niche. The cytokine IL-6 (Bataille et al., 1989; Chauhan et al., 1997; Cheung and Van Ness, 2002; Hardin et al., 1994; Kawano et al., 1988; Kopf et al., 1994), the B Lymphocyte stimulator family cytokines BLyS and APRIL (Benson et al., 2008; O'Connor et al., 2004), bone marrow stromal cells (Cassese et al., 2003; Minges Wols et al., 2002), eosinophils (Chu and Berek, 2012; Chu et al., 2011) and megakaryocytes (Winter et al., 2010) and the CXCR4-CXCL12 chemokine axis (Hargreaves et al., 2001; Hauser et al., 2002; Tokoyoda et al., 2004) are some of the important factors that have been implicated in plasma cell and malignant (myeloma) plasma cell longevity. Furthermore, the factors that control the entry requirements as well as the size and composition of the bone marrow plasma cell pool are of considerable clinical interest (Moser et al., 2006; Radbruch et al., 2006). This section will review the current knowledge about all the various aspects of plasma cell survival in specialized niches.

It has been proposed that bone marrow stromal cells are central players in orchestrating bone marrow plasma cell survival, similar to their role in HSC survival niches (Shiozawa et al., 2008; Sugiyama et al., 2006). A number of groups have succeeded in culturing plasma cells *in vitro* in the presence of bone marrow stroma (Cassese et al., 2003; Minges Wols et al., 2002; Tokoyoda et al., 2010). These groups concluded that it was the synergistic effects of the soluble factors secreted by bone marrow stromal cells that resulted in optimal plasma cell survival, with no one factor being able to recapitulate the *in vivo* phenotype (Cassese et al., 2003; Minges Wols et al., 2002; 2007). While many soluble factors, including TNFa, IL-4, IL-5 and IL-10, were all identified as plasma cell survival factors, the role of two factors (IL-6 and CXCL12) has engendered the most scrutiny (Cassese et al., 2003). While a role in plasma cell survival

has been proposed for stromal cell-derived CXCL12, the majority of studies on CXCL12 have focused on its role in plasma cell mobilization and will be reviewed later in this section (Tokoyoda et al., 2010). On the other hand, the role of IL-6 in plasma cell differentiation and survival has been extensively studied (Bataille et al., 1989; Dedera et al., 1996; Kawano et al., 1988; Kishimoto, 1989; Kopf et al., 1994; Roldán and Brieva, 1991; Skibinski et al., 1998). Interestingly, IL-6 has been shown to induce XBP-1 in myeloma cell lines, suggesting one potential mechanism of action by which IL-6 mediates survival (Iwakoshi et al., 2003a; Wen et al., 1999). However, while IL-6 had profound effects on plasma cell survival *in vitro* (Minges Wols et al., 2002), $IL6^{-/-}$ mice had a more mild defect in antibody titers and recovered their plasma cell numbers over time (Kopf et al., 1994).

Two cytokines with roles in B cell homeostasis have key roles in plasma cell survival: BLyS (B lymphocyte stimulator; also known as BAFF) and APRIL (A proliferation inducing ligand) (Ingold et al., 2005; Mackay and Schneider, 2009; Moreaux et al., 2004; 2009; Novak et al., 2004; Scholz and Cancro, 2012). These cytokines have differing binding affinities for 3 distinct receptors expressed on B-lineage cells: BR3 (also known as BAFF-R), TACI and BCMA. Mature peripheral B cells are double positive for BR3 and TACI, while BCMA expression is thought to be restricted to memory B cells and long-lived PC (Darce et al., 2007; O'Connor et al., 2004; Rodig et al., 2005). While BLyS is capable of binding all three receptors with differing affinities (BR3>TACI>BCMA), APRIL cannot bind to BR3 and instead has the highest affinity for BCMA (Bossen and Schneider, 2006; Day et al., 2005). BLyS, through interactions with BR3, is thought to be the key molecule controlling the size of the naïve B cell pool (Scholz and Cancro, 2012; Woodland et al., 2008). Genetically lacking functional BR3 expression and *in vivo* depletion of BLyS in

adult mice result in the same phenotype: a profound peripheral B cell depletion (Lentz et al., 1996; Scholz et al., 2008). By contrast, excess BLyS is believed to result in less stringent selection of B cell clones, allowing autoreactive clones into the periphery and resulting in autoimmune disease (Cancro et al., 2009). The role of TACI in the regulation of the naïve B cell pool is less clear: unexpectedly, $TACI^{-/-}$ show evidence of increased B cell numbers, hinting at a negative regulator role for this molecule (Bülow et al., 2001; Yan et al., 2001). Interestingly, humoral immunity remains intact when BLyS is depleted *in vivo*, suggesting a functional redundancy of BLYS and APRIL in plasma cell survival (Scholz et al., 2008). This deduction is, in fact, borne out by experiments demonstrating a significant reduction in the number of bone marrow plasma cells when BLyS is neutralized in $APRIL^{-/-}$ mice (Benson et al., 2008). The same report demonstrates the complete independence of memory B cells on BLyS or APRIL, making memory B cells the only B2 lineage cells which do not rely on these two survival factors (Benson et al., 2008). Moreover, this group's results are internally consistent with their previous finding that bone marrow long-lived plasma cells depend on the promiscuous BLyS/APRIL receptor, BCMA (O'Connor et al., 2004).

The cellular sources of pro-survival factors such as BLyS and APRIL constitute a further level of regulation in the formation of plasma cell survival niches. Radioresistant bone marrow stromal cells are significant sources of BLyS and APRIL in both mice and humans (Gorelik et al., 2003; Moreaux et al., 2005; Schaumann et al., 2007). Using a bone marrow chimera approach, one group established that other cellular sources of BLyS are insufficient to support normal B cell homeostasis (Gorelik et al., 2003). For plasma cell survival in particular, neutrophils, monocytes, osteoclasts, eosinophils and megakaryocytes have all been implicated as important sources of APRIL (Chu et al.,

2011; Huard et al., 2008; Moreaux et al., 2005; Winter et al., 2010). Moreover, both eosinophils and megakaryocytes have also been described to secrete IL-6, making these cell populations important sources of two key plasma cell survival factors: IL-6 and APRIL (Chu and Berek, 2012; Chu et al., 2011; Winter et al., 2010). The list of potential cellular sources of plasma cell survival factors is constantly expanding: recently, iNKT cells have been described as BLyS and APRIL secretors (Shah et al., 2013). Survival niches in other organs are less well established but are thought to rely on similar survival mechanisms (Cassese et al., 2001; Ellyard et al., 2005; Mahévas et al., 2013). Interactions between plasmablasts in secondary lymphoid organs and dendritic cells (as well as monocytes/macrophages) are thought to be associated with plasmablast survival, most likely via APRIL and IL-6 (García de Vinuesa et al., 1999; Mohr et al., 2009). Basophils in both spleen and bone marrow appear to affect plasma cell survival *in vitro* and *in vivo* (Rodriguez Gomez et al., 2010). B cell depletion studies in immune thrombocytopenia suggest that the spleen can provide a niche for long-lived plasma cells, not just plasmablasts; studies in an SLE mouse model establish inflamed kidneys as a site of plasma cell homeostasis (Cassese et al., 2001; Mahévas et al., 2013).

In addition to all the soluble factors described above, a whole slew of surface molecules are thought to play a role in plasma cell survival: CD28, CD44, CD54, FcγRIIb, integrin $\alpha 4\beta 1$, VLA-4, LFA-1, S1P1, CXCR3 and CXCR4 (Bahlis et al., 2007; Cassese et al., 2003; DiLillo et al., 2008; Ellyard et al., 2005; Hamilton et al., 1991; Kabashima et al., 2006; Nair et al., 2011; Reif et al., 2002; Tokoyoda et al., 2004; Underhill et al., 2003; Xiang et al., 2007). Here the question of plasma cell survival quickly becomes intertwined with the issue of plasma cell migration and adherence in new niches (Radbruch et al., 2006). It is hypothesized that the bone marrow plasma cell niche is of a

fixed size and thus subject to stringent entry requirements and competition (Radbruch et al., 2006). It is believed that a "coordinated change in chemokine responsiveness" is responsible for the migration of plasma cells out of secondary lymphoid organs and lodgment in the bone marrow (Hargreaves et al., 2001). This includes increased chemotactic activity toward CXCR4 ligand, CXCL12, and decreased responsiveness to B cell chemoattractants CXCL13, CCL19, and CCL21 (Hargreaves et al., 2001; Hauser et al., 2002).

CXCL12-secreting stromal cells are well-recognized as being essential to the formation of B lymphocyte niches in the bone marrow and some microscopy evidence exists for co-localization of bone marrow plasma cells with this subset of bone marrow stromal cells (Tokoyoda et al., 2004). It has even been hypothesized that the B cell lymphopenia noted in multiple myeloma patients may result from malignant plasma cells overwhelming B cell precursor survival niches (Alsayed et al., 2007; Pilarski et al., 1984; Tokoyoda et al., 2004). A similar competition mechanism has been proposed for new plasma cells entering the bone marrow, although it is based on a single human study (Odendahl et al., 2005; Radbruch et al., 2006). After a second vaccination with tetanus toxoid, researchers noted an increase in both tetanus-specific plasmablasts and plasma cells of other specificities in the blood (Odendahl et al., 2005). They concluded that newly formed plasma cells can only enter the long-lived pool by upregulating CXCR4, homing to the bone marrow and supplanting members of previously established cohorts (Hauser et al., 2002; Odendahl et al., 2005; Radbruch et al., 2006). Another group noted a decrease in bone marrow plasma cell numbers after immunization with a model antigen; neither group controlled for the nature of the antigen or traced the fate of the "displaced" cells to determine whether they re-colonized long-lived plasma cell niches

(Odendahl et al., 2005; Xiang et al., 2007). Based on the number of plasma cells necessary to maintain protective titers against a given antigen, Radbruch et al calculated that the competitive BM could support 1,000 PC specificities, arguably enough to preserve humoral memory against any encountered antigen for a lifetime (Radbruch et al., 2006). The limitations of this view of the bone marrow plasma cell population – largely long-lived, with some new immigrants – will be explored in this dissertation.

Summary

Plasma cells are B-lineage derived cells responsible for maintaining antibody titers (both protective and pathogenic) and are the source cell of the malignancy multiple myeloma. Plasma cells are believed to populate unique survival niches in the bone marrow. Current models predict that bone marrow plasma cells consist chiefly of long-lived, slowly renewing cells (**Fig. 1-1**). However, we find the turnover rate of the bone marrow plasma cell pool to be much higher than predicted by these models; in fact, more than 50% of bone marrow plasma cells exhibit characteristics of recently formed plasma cells. Studies in **chapter 2** of this dissertation will focus on characterizing the bone marrow plasma cell turnover kinetics as well as the phenotypic characteristics of the different bone marrow plasma cell subsets. **Chapter 3** will extend these studies by focusing on a response to a model antigen and will define the distinct precursor populations that give rise to the various bone marrow plasma cell pools.

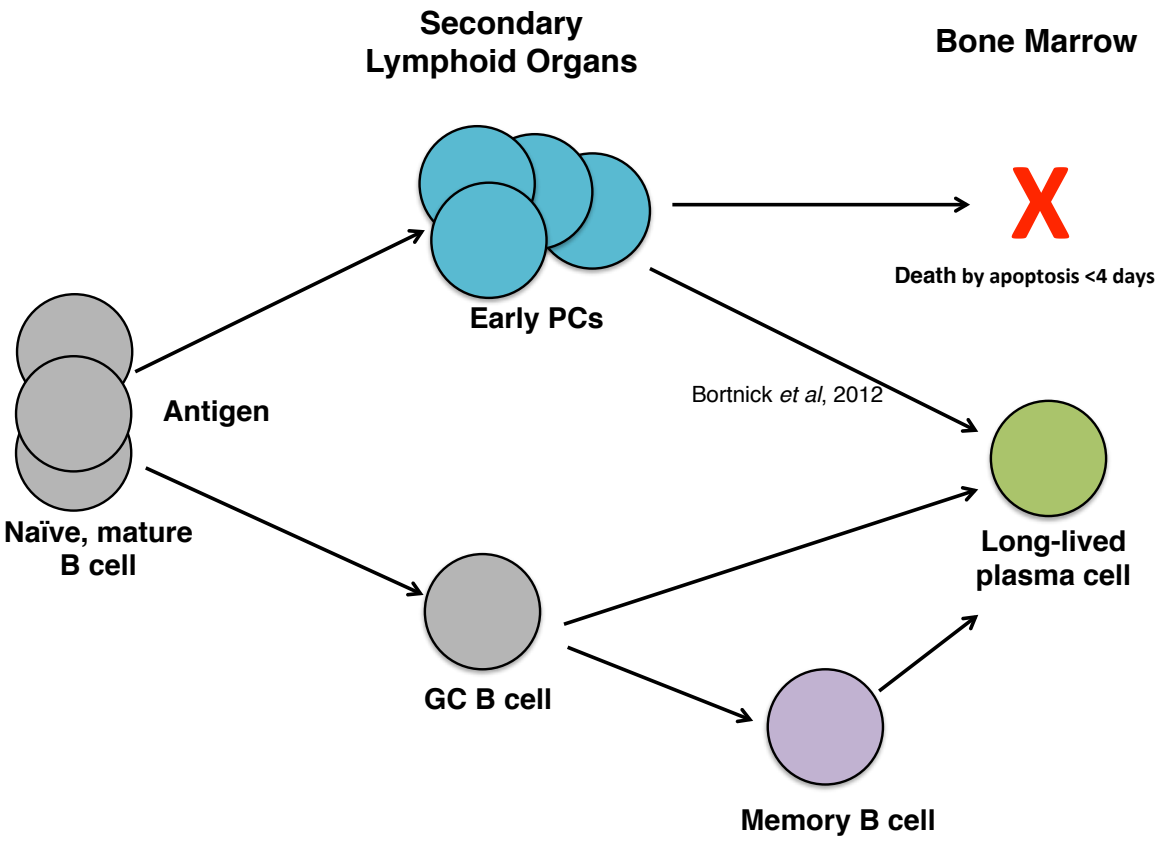

Figure 1-1. Conventional (1997-2012) model of plasma cell fates and precursors.

CHAPTER 2

THE BULK OF THE STEADY STATE BONE MARROW PLASMA CELL POOL IS MAINTAINED VIA CONTINUOUS GENERATION FROM A B CELL PRECURSOR

Abstract

Current models predict that bone marrow plasma cells (BM PC) consist chiefly of long-lived, slowly renewing cells. Here we find the turnover rate of the BM PC pool to be much higher than predicted by these models; in fact, more than 50% of BM PC exhibit characteristics of recently formed PC. These characteristics include surface expression of the canonical naïve B cell surface protein B220, and a 50% renewal rate of less than 3 days. Intriguingly, these PC do not appear to be cycling and are depleted upon ablation of peripheral B cell pools. Together these data show that a large fraction of the BM PC pool is continuously repopulated by plasma cells newly generated from B cell precursors.

The main findings are:

- **The bone marrow plasma cell pool is markedly heterogeneous: the bulk of bone marrow plasma cells are rapidly renewed.**
- **Rapidly renewing plasma cells do not appear to be in cell cycle.**
- **B cell precursors are needed to maintain the bone marrow plasma cell pool long-term.**

I. RESULTS

To explore the cellular dynamics with which cells enter and persist within the bone marrow plasma cell pool, we first employed continuous *in vivo* BrdU labeling in B6 mice that were not intentionally immunized. We find that within 30 days of labeling, >40% of bone marrow plasma cells have incorporated BrDU, indicative of a renewal rate much higher than predicted by other models (**Fig. 2-1 A**). To probe the bone marrow plasma cell heterogeneity suggested by this result, we further characterized the pool by examining the expression of the naïve B-cell signaling protein CD19 and the B-lineage marker B220 (CD45R) on the bone marrow of unimmunized adults. These cells were readily resolved into $B220^{+}CD138^{low}$ and $B220^{+/-}CD138^{high}$ populations. The $B220^{+}$ $CD138^{low}$ bone marrow fraction did not contain plasma cells, and likely corresponds to pre-B cells (Tung et al., 2006). By contrast, we resolved three subpopulations of $CD138^{high}$ subsets defined as $B220^{+}CD19^{+/-}$, $B220^{-}CD19^{+}$, and $B220^{-}CD19^{-}$. Notably, only a minority of all plasma cells corresponded to the classic $B220^{-}CD19^{-}$ plasma cell phenotype (**Fig. 2-1 B**). All three subsets contained high frequencies of antibody secreting cells when sorted from B6 adults (**Fig. 2-1 C**), clearly distinguishing these cells from a proposed, non-secreting plasma cell precursor (O'Connor et al., 2002). Microarray data (not shown) and real-time PCR showed that all $CD138^{high}$ subsets had high levels of Blimp-1 and low levels of B-lineage master regulator Pax5, consistent with previously published data for plasma cells (**Fig. 2-2 A, B**) (Horcher et al., 2001; Shaffer et al., 2002). Interestingly, it appears that bone marrow plasma cells retain surface expression of CD19 despite an absence of high levels of CD19 transcripts (**Fig. 2-1 B, Fig. 2-2 B**). Furthermore, cells within the $CD138^{high}$ $B220^{+}$ fraction exhibited signs of relative immaturity, as they expressed lower levels of Blimp-1 as revealed with Blimp1-GFP reporter mice (Kallies et al., 2004). In sharp contrast, $B220^{-}$ plasma cells, including

$CD138^{high}CD19^{+}$ cells, consisted of mature cells as judged by their relatively high Blimp-1 expression (**Fig. 2-1 D**). Consistent with their relative immaturity, $B220^{+}$ $CD138^{high}$ bone marrow cells labeled rapidly, indicating a 50% renewal rate of less than 3 days (**Fig. 2-1 E**). By contrast, bone marrow $B220^{-}$ plasma cells including $CD138^{high}$ $CD19^{+}$ cells exhibited protracted BrdU labeling kinetics indicative of pools of mature long-lived plasma cells. Indeed, the labeling kinetics for $CD19^{+}$ and $CD19^{-}$ plasma cells within the $CD138^{high}$ $B220^{-}$ fraction were indistinguishable (**Fig. 2-1 E, see Fig. 2-3 A** for representative gates). Finally, we performed pulse-chase BrDU studies which revealed no evidence of interconversion between the bone marrow plasma cell subsets with disparate BrDU labeling kinetics (**Fig. 2-3 B**). Altogether these data reveal a striking and previously unappreciated heterogeneity among bone marrow plasma cells.

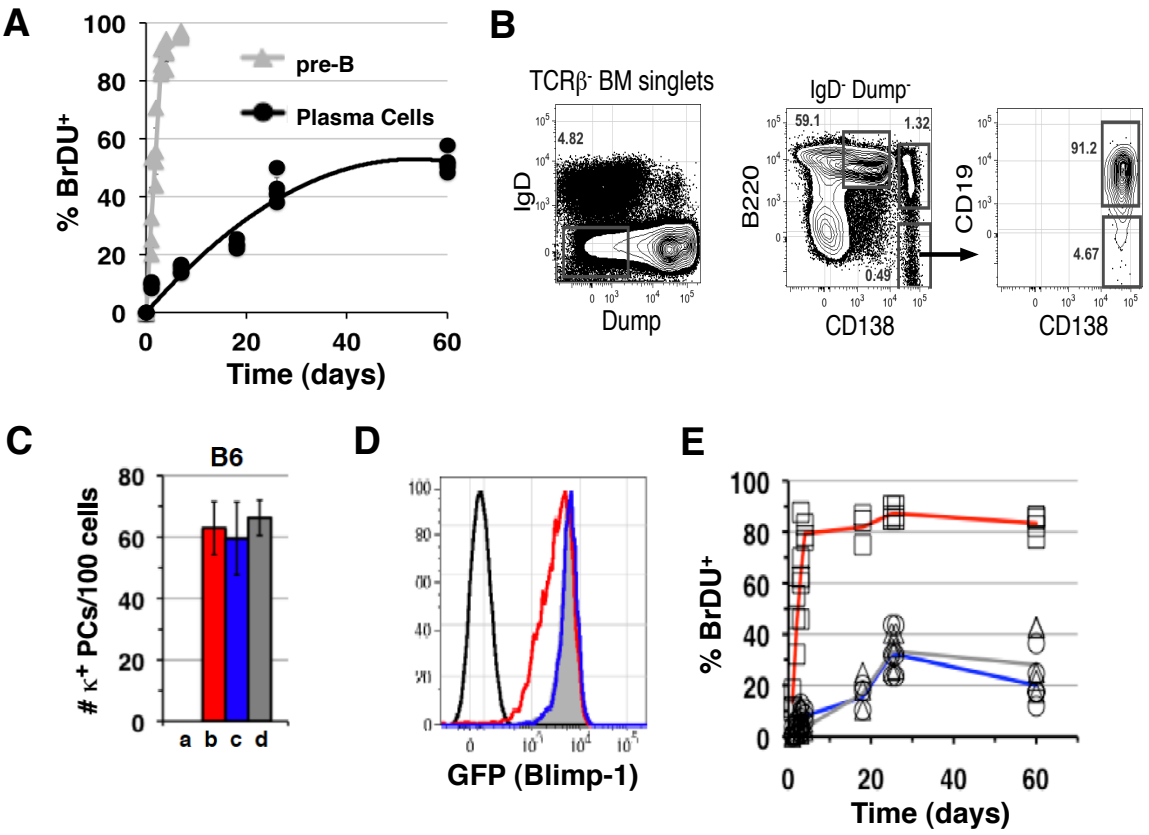

Figure 2-1. Adult bone marrow contains both rapidly and slowly renewing plasma cells. **(A)** B6 mice were fed BrdU for the indicated days before determination of the % of $Dump^{-}IgD^{-}CD138^{high}$ cells that are $BrdU^{+}$. Pre-B cells were used as a labeling control. Best-trend lines were drawn across the mean $BrdU^{+}$ cells for each subset using 3-4 mice per timepoint; error bars represent SEM. **(B)** BM cells from unimmunized B6 adults were analyzed by flow cytometry with the indicated antibodies (see **chapter 5**). **(C)** Cells within the indicated gates were sorted directly into ELISPOT plates to determine frequencies of total antibody secreting cells in each $CD138^{high}$ subset in naïve B6 mice. **(D)** Relative Blimp1/GFP expression among the subsets identified and labeled in 2-1B was determined using $B6.Blimp1^{+/Blimp1}$ mice. **(E)** Determination of % $BrDU^{+}$ cells within each gate, BrDU labeling as in 2-1A.

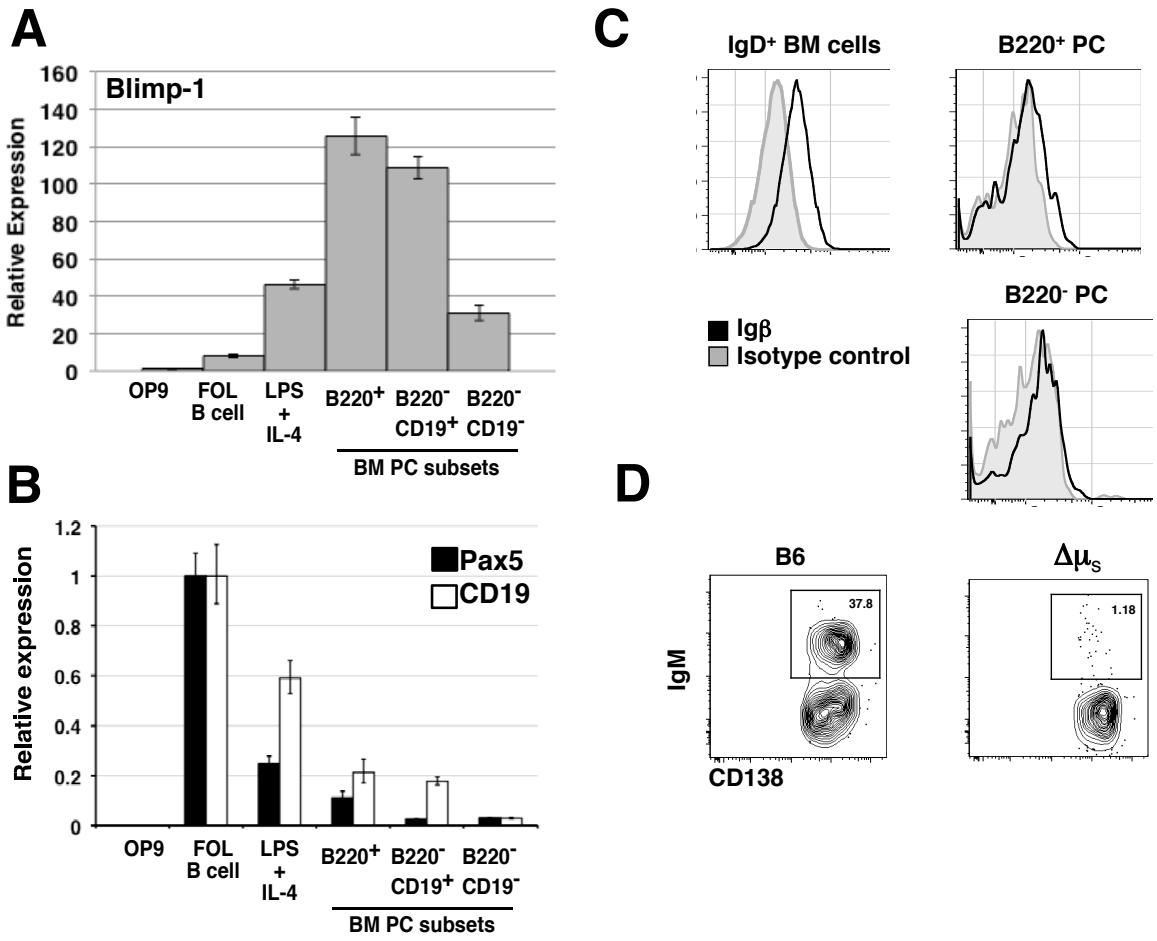

Figure 2-2. BM PC upregulate Blimp-1 and downregulate Pax5 and BCR components. (A) Relative Blimp-1 transcript expression in sorted BM plasma cell subsets was measured by qRT- PCR. Additional samples included cDNA prepared from the BM stromal cell line OP9, splenic FOL ($CD23^+$) B cells, and FOL B cells stimulated for 3 days with LPS and IL-4. Data are expressed relative to OP9 cells. Error bars indicate the relative quantity minimum and relative quantity maximum for each sample. **(B)** Relative CD19 and Pax5 transcript expression from samples prepared as in part A. Data are expressed relative to naïve FOL B cells. **(C)** BM PC subsets were stained for the presence of Igβ. Representative of n=3 mice. **(D)** BM PC were assayed for the presence of a membrane BCR in B6 mice and mice lacking secretory, but not membrane IgM ($\Delta\mu_s$) (Boes et al., 1998b; 1998a; Ehrenstein et al., 1998). Representative of n=3 mice.

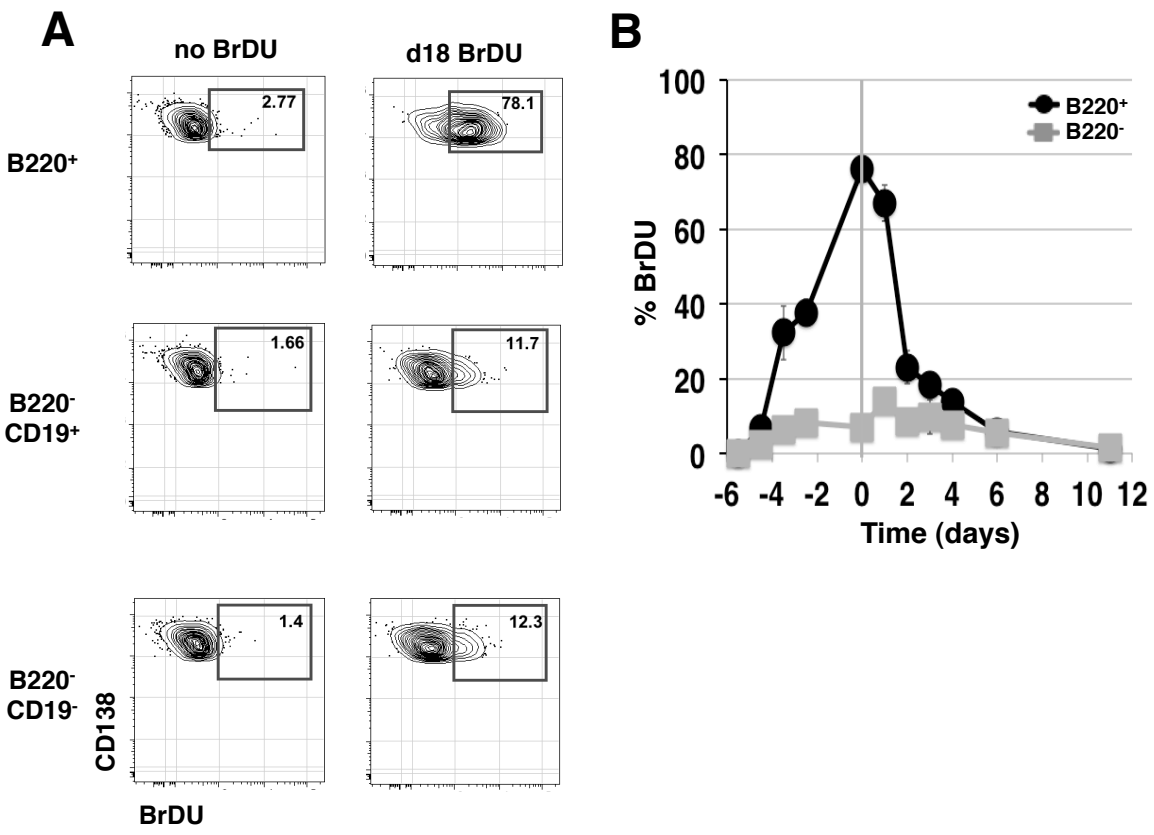

Figure 2-3. BrDU labeling kinetics of BM PC subsets. (A) Representative flow plots showing BrDU incorporation of the BM PC subsets defined in Fig. 2-1B and graphed in Fig. 2-1E at d18 post continuous BrDU labeling. **(B)** B6 adult mice were given BrDU in the drinking water and sacrificed after 1, 2, 3, 4, or 6 days of continuous BrDU (negative numbers on the x-axis) or were chased on regular water after 6 days of continuous BrDU administration (positive numbers on the x-axis). Average 3-4 mice per group; error bars represent SEM of individual animals.

While bone marrow plasma cells are classically believed to be post-mitotic (Radbruch et al., 2006), recent reports have described the presence of a proliferative plasmablast in the bone marrow (Racine et al., 2011), leading us to investigate whether the rapidly renewing $B220^{+}$ fraction corresponded to such a cell. Employing two different methods for assessing cellular proliferation (Schittek et al., 1991; Wilson et al., 2008), we were unable to find evidence of a dividing plasma cell in the bone marrow (**Fig. 2-4 A, B**). Moreover, we found no evidence of high *c-myc* expression, a trans-membrane BCR or associated signaling components (Igβ), which are often used to define a "plasmablast" (Racine et al., 2011) (**Fig. 2-4 C, Fig. 2-2 C, D**). The post-mitotic status of $B220^{+}$ plasma cells coupled with their rapid BrDU turnover kinetics suggested constant replenishment from a proliferating B cell precursor pool.

To probe this, we utilized two different approaches to deplete B cells and assess the effect on bone marrow plasma cell population frequency. First, I lethally irradiated C57BL/6 hosts and reconstituted them with $RAG^{-/-}$ *versus* control bone marrow. Since B cell development in $RAG^{-/-}$ arrests at the pro-B stage (Ceredig, 2002), there will be no mature B cell precursor available to continuously replenish the bone marrow plasma cell compartment in $RAG^{-/-}$ reconstituted mice. At eleven weeks post-reconstitution, we see ~40% of the bone marrow plasma cell pool persisting (**Fig. 2-5 A, B**). This is consistent with our BrDU turnover kinetics data demonstrating that ~60% of the total bone marrow plasma cell pool is replaced every two months (**Fig. 2-1 A**).

While a useful tool, irradiation has some serious downsides as an experimental approach including a potentially disruptive effect on bone marrow plasma cell survival niches (Moser et al., 2006; Zhang et al., 2010). To more rigorously address our question, we took advantage of novel transgenic mice that allow us to inducibly delete B cells and

assess the effect on bone marrow plasma cell population frequency. Specifically, we mated hCD20-TAMCre.C57BL/6 mice, where a B-cell specific Cre recombinase is activated upon tamoxifen treatment, to Gt(ROSA)26Sortm1(DTA)Jpmb/J mice, in which a floxed stop codon is placed before a diphtheria toxin cassette (from here on referred to as CD20Cre-DTA mice) (Ivanova et al., 2005; Khalil et al., 2012) (**Fig. 2-6 A**). While the majority of peripheral B cells are $hCD20^+$ (95%), only a minority of bone marrow plasma cells express low levels of hCD20, with no differences in expression detected between $B220^+$ *versus* $B220^-$ plasma cells (**Fig. 2-6 B**). We treated CD20Cre-DTA mice, as well as single transgene controls (CD20Cre and Rosa26-DTA), with tamoxifen for 3 days to induce B cell deletion and subsequently at 2 week intervals to deplete any newly maturing B cells. While $CD19^+$ B cell numbers were reduced 5-10 fold at all timepoints examined, the kinetics of decline in bone marrow plasma cells were more protracted (**Fig. 2-6 C, Fig. 2-7 A, B**). Early on (d17 post-tamoxifen induction) $B220^+$ and $B220^-$ plasma cell frequencies in CD20Cre-DTa mice did not differ significantly from the frequencies in control CD20Cre animals (**Fig. 2-7 A, B**). Consistent with their rapid turnover kinetics, $B220^+$ plasma cells were significantly reduced at days 40 and 160 post- B cell ablation, while the slowly renewing $B220^-$ plasma cells were only affected months post- B cell ablation (**Fig. 2-7 A, B**). These findings support our BrDU labeling studies and establish a role for a B cell feeder pool in the maintenance of polyclonal bone marrow plasma cell populations.

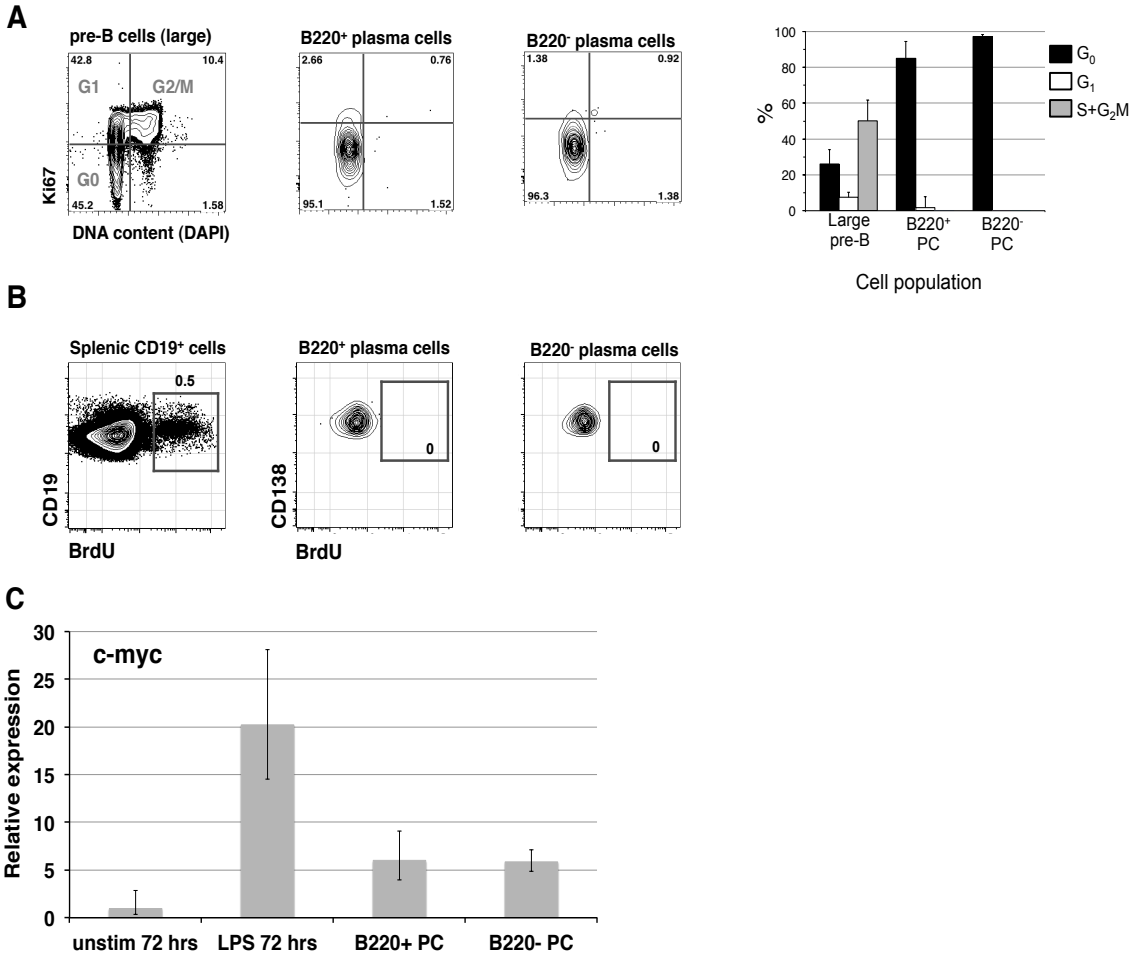

Figure 2-4. Bone marrow plasma cell subsets are not actively cycling. (A) Intracellular DAPI staining was used to quantitate DNA content in the BM of adult B6 mice (Wilson et al., 2008). Cycling (large) pre-B cells were used as a positive control. Representative of n≥3 individual mice. **(B)** B6 mice were injected with BrDU i.p. for 1 hour and analyzed by flow cytometry for BrDU incorporation . Labeling with BrDU is indicative of proliferation (Schittek et al., 1991). Representative of n≥3 individual mice. (**C**) Relative c-myc transcript expression in sorted BM plasma cell subsets was measured by qRT- PCR. Additional samples included cDNA prepared from unstimulated splenic FOL ($CD23^+$) B cells and FOL B cells stimulated for 3 days with LPS. Data are expressed relative to unstimulated FOL B cells. Error bars indicate the relative quantity minimum and relative quantity maximum for each sample.

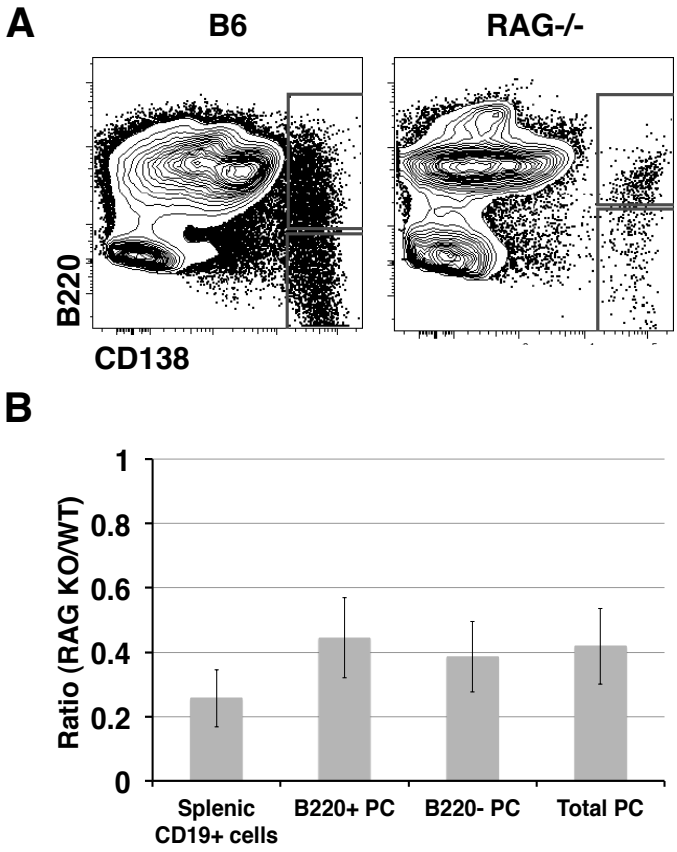

Figure 2-5. The BM PC pool is continuously replenished by a lymphoid precursor. (A) Representative BM PC flow plots from C57BL/6 and RAG$^{-/-}$ reconstituted mice. **(B)** The decay rate of B220^{+} and B220^{-} PCs was illustrated by dividing the number of cells in each subset in RAG$^{-/-}$ reconstituted mice by the average number of cells in each subset in a C57BL/6 control as previously described by Slifka et al (Slifka et al., 1998).

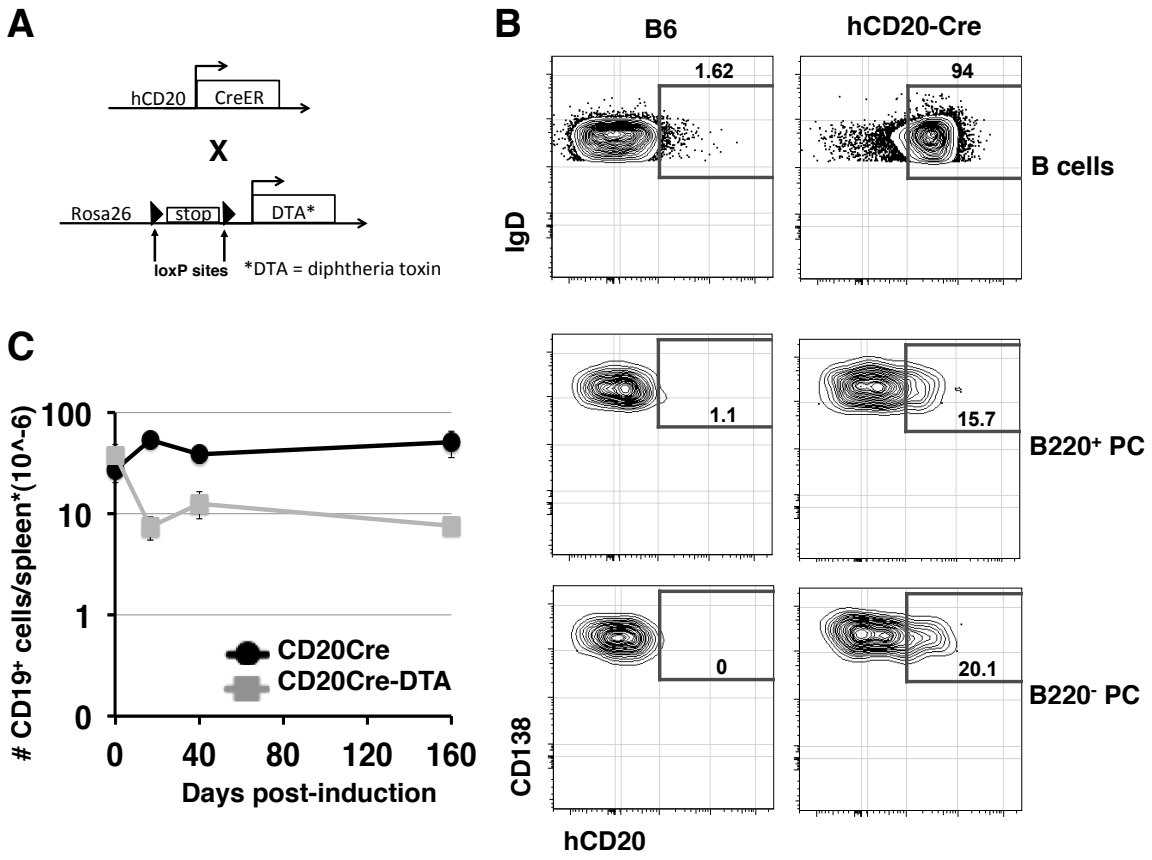

Figure 2-6. Design and characteristics of hCD20Cre-DTA mice. (A) Mating strategy used to generate CD20Cre-DTA mice (see text for details) **(B)** Representative splenic B cell and BM PC flow plots from C57BL/6 and hCD20Cre mice assessing for expression of surface hCD20 on each population. **(C)** hCD20-TAMCre.C57BL/6 mice (CD20Cre) and hCD20-TAMCre.C57BL/6 x Gt(ROSA)26Sortm1(DTA)Jpmb/J mice (CD20Cre-DTA) were treated with three induction doses and subsequent fortnightly doses of tamoxifen and sacrificed on days 0, 17, 40 and 160 post-induction (Ivanova et al., 2005; Khalil et al., 2012). Numbers of $CD19^+$ B cells were quantified using flow cytometry and total spleen counts. Average 3-4 mice per group; error bars represent SEM of individual animals.

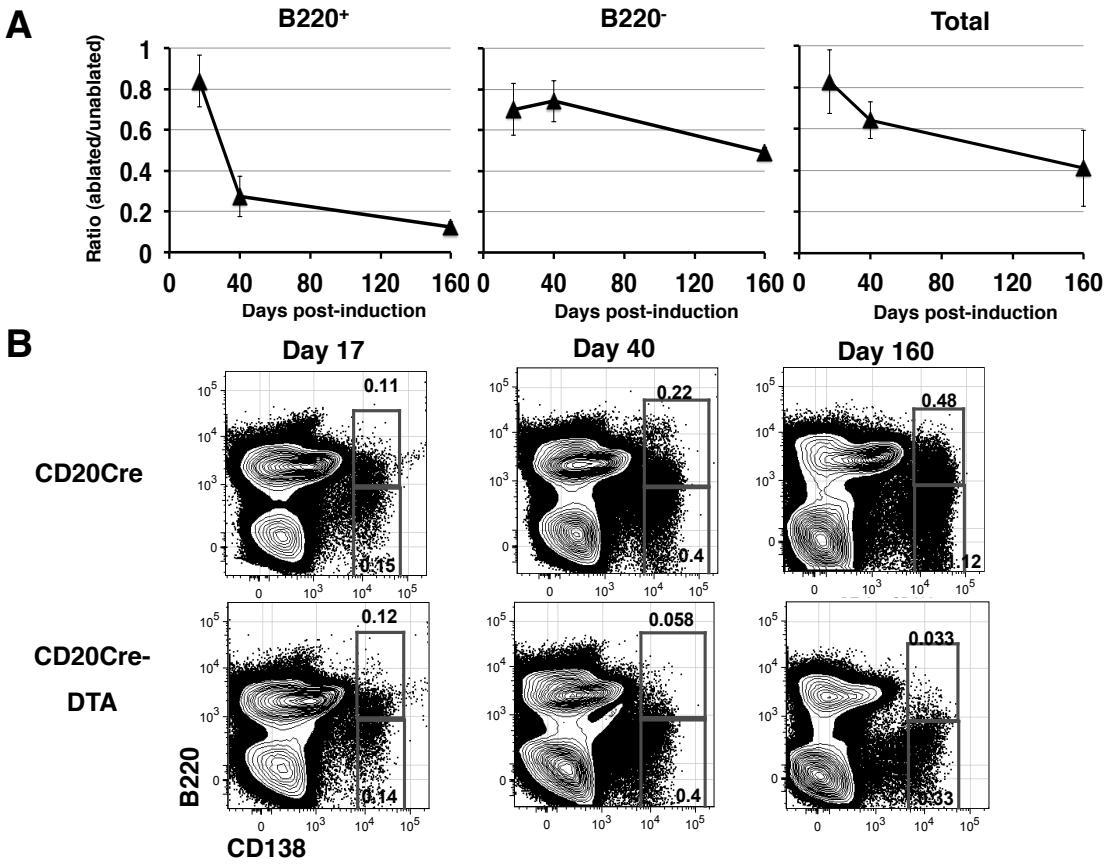

Figure 2-7. The BM PC pool is continuously replenished by a B cell precursor. (A) The decay rate of $B220^+$ and $B220^-$ PCs was illustrated by dividing the number of cells in each subset in CD20Cre-DTA (ablated) mice by the average number of cells in each subset in 2-4 CD20Cre controls (unablated) for each indicated timepoint as previously described by Slifka et al (Slifka et al., 1998). **(B)** Representative BM PC flow plots from CD20Cre and CD20Cre-DTA mice at three different timepoints.

II. DISCUSSION

Since the description of long-lived plasma cells by Manz et al in 1997 (Manz et al., 1997), the literature has focused on characterizing the bone marrow as a site of almost exclusively long-lived, high-affinity plasma cells. A closer examination reveals hints of bone marrow plasma cell heterogeneity: newly formed GFP^{int} plasma cells in the bone marrow of Blimp-1 reporter mice, kinetics of BrDU labeling in Manz et al, the presence of IgM-secreting plasma cells in the bone marrow (Bortnick et al., 2012; Kallies et al., 2004; Manz et al., 1997). However, most of the published works suggest that newly formed plasma cells constitute a minority (~10% as per Kallies et al) of the bone marrow plasma cell pool. Our results define the degree of turnover heterogeneity of bone marrow plasma cells and find that, surprisingly, roughly 50% of the bone marrow plasma cell pool is replaced every 60 days; a much higher number than predicted by previous models (**Fig. 2-1 A**). Moreover, we find that this unexpected extent of turnover represents the summation of two separate bone marrow plasma cell pools: a rapidly and a slowly renewing population. Although it is tempting to posit a dividing bone marorw "plasmablast" as the mechanism of BrDU incorporation in the rapidly renewing bone marrow plasma cell pool (Racine et al., 2011), our data support a role for continuous recruitment of peripheral B cells into the bone marrow plasma cell pool (**Fig. 2-4 and 2-7**). Whereas the mechanisms of bone marrow colonization by new plasma cells are not fully understood, our findings suggest a continuous entry of new plasma cells that coexist with more established, long-lived plasma cells. The issues surrounding the potential replacement of old specificities by newcomer plasma cells are of the utmost interest and will be further discussed in **Chapter 4**.

One of the areas where understanding the nature of the bone marrow plasma cell

pool is of particular importance is in the field of multiple myeloma. While it is clear that acquisition of malignant potential involves a number of gene expression changes between normal plasma cells and myeloma cells (for example, c-myc upregulation), comparing the kinetics of healthy *versus* malignant bone marrow plasma cell pools should prove informative (Bergsagel and Kuehl, 2001; De Vos et al., 2002; Shou et al., 2000). Models of the disease have long followed the classical cancer paradigm and postulated the existence of a rare myeloma stem cell. The exact nature of this cell has been a matter of much debate. For instance, Yaccoby et al reported that plasma cell-depleted bone marrow could not transfer disease to a SCID-hu host in a humanized mouse system (Yaccoby and Epstein, 1999). In contrast, later papers by Matsui et al utilizing NOD/SCID mice concluded that the myeloma stem cell was a $CD138^-$ B cell precursor (Matsui et al., 2004; 2008). Our data demonstrating the dependence of bone marrow plasma cells on a B cell precursor seem to parallel the findings of Matsui et al (Matsui et al., 2004), though they do not definitively rule out the acquisition of malignant potential by a terminally differentiated plasma cell.

The multi-component nature of the bone marrow plasma cell compartment broaches many questions about its regulation and survival requirements. While many factors have been identified as being important for the maintenance of bone marrow plasma cells (Belnoue et al., 2008; Benson et al., 2008; Chu et al., 2011; Kopf et al., 1994; Radbruch et al., 2006; Winter et al., 2010), our new appreciation of the extent of bone marrow plasma cell heterogeneity raises the strong possibility that these factors have differential effects on the different bone marrow plasma cell subsets. For instance, it has been proposed that plasma cells are located in proximity to CXCL12-producing stromal cells in the bone marrow (Tokoyoda et al., 2004). It would be informative to

determine whether access to these survival niches differed between rapidly *versus* slowly renewing bone marrow plasma cells. Alternatively, a re-examination of cell-intrinsic survival factors such as Mcl-1 could lead to an understanding of the observed survival differences (Peperzak et al., 2013). Overall, we submit that in light of the new data, a thorough reexamination of the survival requirements of long-lived plasma cells is warranted.

In sum, we propose that the bone marrow plasma cell compartment is uniquely heterogeneous, both in regards to phenotypic markers and functional turnover rates. The continuous replenishment of the bone marrow plasma cell pool from a B cell precursor and the resultant questions about plasma cell entry requirements and the niche size are some of the more interesting aspects of the work. To that end, the mechanisms involved in the formation, migration and maintenance of the various bone marrow plasma cell subsets all merit further investigation.

CHAPTER 3

SLOWLY *VERSUS* RAPIDLY RENEWING BONE MARROW PLASMA CELL SUBSETS ARE DERIVED FROM DISTINCT CELLULAR PRECURSORS

Abstract

T-dependent antigens are notorious for their ability to induce high affinity, long-lived PC. However, we find that very long-term maintenance of the antigen-specific BM PC pool is dependent on a CD40-independent B cell precursor. Specifically we find that, despite the rapid turnover rate exhibited by $B220^+$ BM PC (described in **chapter 2**), antigen-induced antibody secreting cells are found within this population for more than 100 days post-immunization. These cells secreted exclusively low affinity, unswitched, κ type antibodies, consistent with a germinal center (GC)-independent origin in sharp contrast to the GC-derived (high affinity, isotype switched) cells found within the slowly renewing BM PC pool. Finally, we are able to identify κ *versus* λ-expressing antigen-specific B cell populations we believe represent the cellular precursors of the different BM PC pools. Together these data suggest that BM niches are continuously repopulated by newly generated plasma cells long after antigenic exposure and offer intriguing insights into the identity of the cellular precursors of BM PC.

The main findings are:

- **High affinity, isotype switched, λ-secreting antigen-specific cells are restricted to the slowly renewing bone marrow plasma cell pool.**
- **The long-term maintenance of the antigen-specific BM PC pool requires a CD40-independent, antigen-specific B cell precursor.**

I. RESULTS

To further understand the import of the various bone marrow plasma cell populations, we decided to probe the relevance of these subpopulations to long-term induced immunity. To determine the kinetics with which plasma cells induced by the T-dependent antigen NP-CGG enter and persist in each subpopulation, we quantified NP-specific plasma cells in each subset at several times after a single inoculation with NP-CGG (**Fig. 3-1 A**). Remarkably, we could detect antigen-specific cells in both pools at all timepoints examined, with a greater proportion of NP-specific plasma cells localizing to the $B220^-$ bone marrow fraction later in the response (**Fig. 3-1 A and Fig. 3-2 A**). Furthermore, since recent reports (Bortnick et al., 2012; Racine et al., 2011) have indicated that plasma cells induced with a T-independent antigen are capable of forming a long-standing pool in the bone marrow (Bortnick et al., 2012), we explored the role of the bone marrow plasma cell subsets in a primary NP-LPS response. While the kinetics differed from those seen with a T-dependent antigen, we were able to detect NP-specific cells in all subsets at different points of the response (**Fig. 3-2 C**). Additionally, we confirmed the presence of all three subsets in B6.TCR$\beta^{-/-}\delta^{-/-}$ mice, which have been genetically modified to lack T cells (**Fig. 3-2 B**). Together these data suggest that bone marrow plasma cell subsets have important roles in both T-dependent and T-independent humoral immunity.

Our studies in naïve mice indicated that the $B220^+$ plasma cell pool is rapidly renewing and largely dependent on continuous replenishment by B cells (**Fig. 2-1 and Fig. 2-7**); we wondered whether the fraction of the $B220^+$ plasma cell pool specifically induced by a T-dependent antigen was similarly regulated. The presence of antigen-specific cells in the rapidly renewing $B220^+$ bone marrow plasma cell subset for months

post-immunization suggested that some fraction of the long-standing bone marrow plasma cell pool is continuously replenished by antigen-specific, proliferative cells. To probe this directly, we continuously administered BrDU for 7 days to mice that had been immunized with NP-CGG months earlier (127 days) and found evidence of $NP^{+}BrDU^{+}$ cells (**Fig. 3-1 B**). Notably, we failed to observe robust evidence of cell division in the antigen-specific plasma cell pool (**Fig. 3-1 C**). These analyses demonstrated directly that antigen-specific plasma cells are found in the functionally rapidly renewing pool after the primary response has waned (**Fig. 3-1 B**) (Takahashi et al., 1998).

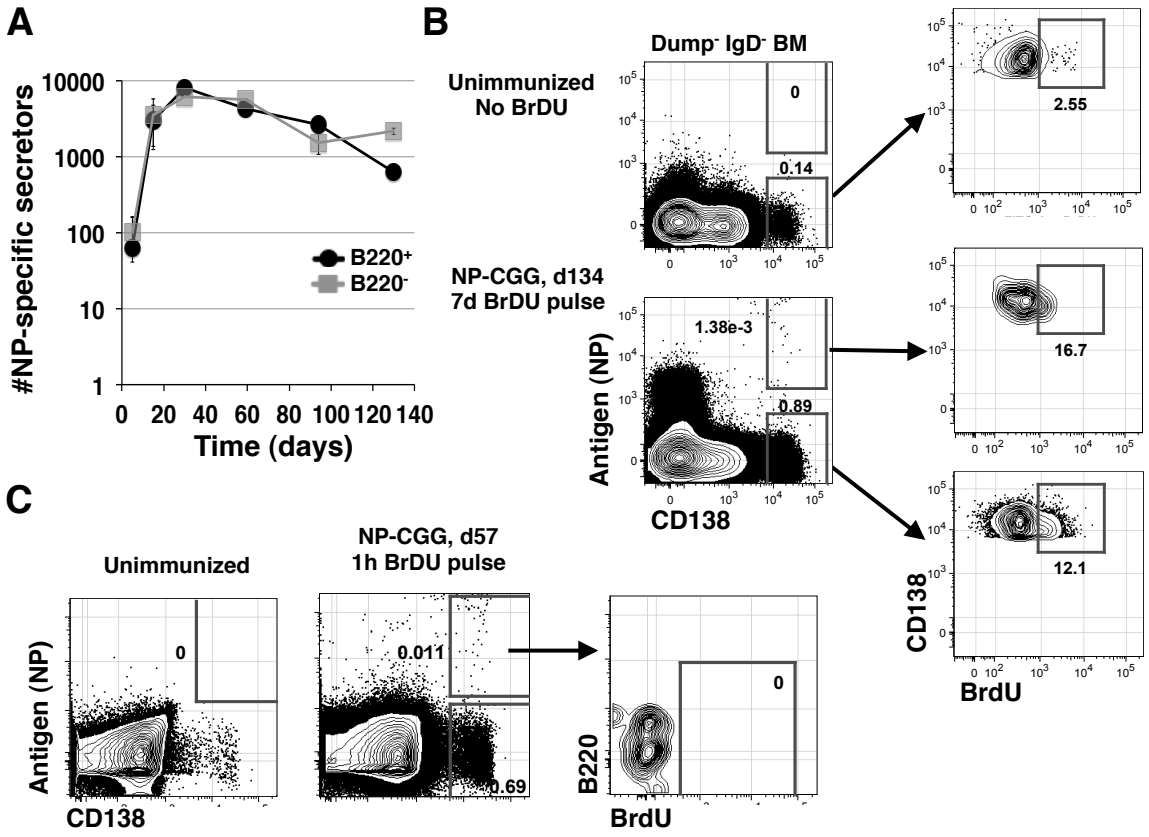

Figure 3-1. Antigen-specific plasma cells localize to the rapidly renewing BM PC pool for months post-immunization. **(A)** B6 mice were immunized with NP-CGG/alum, and cells within each PC pool were sorted into NP26-BSA coated ELISPOT plates to determine frequencies of NP-specific PC within each population at the indicated time points post-immunization. Data from 3 pooled adult B6 mice; error bars represent SEM of triplicate ELISPOT wells. Background has been subtracted out. $p<0.05$ at d130, N.S. at all other timepoints. **(B)** B6 mice were immunized with NP-CGG/alum. At d127 post-immunization the mice were fed BrDU for 7 days and analyzed by flow cytometry for the presence of intracellular antigen (NP) and BrDU incorporation. Data from 5 pooled mice are shown. **(C)** B6 mice were immunized with NP-CGG/alum. At d57 post-immunization the mice were injected with BrDU i.p. for 1 hour and analyzed by flow cytometry for the presence of intracellular antigen (NP) and BrDU incorporation . Labeling with BrDU is indicative of proliferation (Schittek et al., 1991). Representative of $n \geq 3$ individual mice.

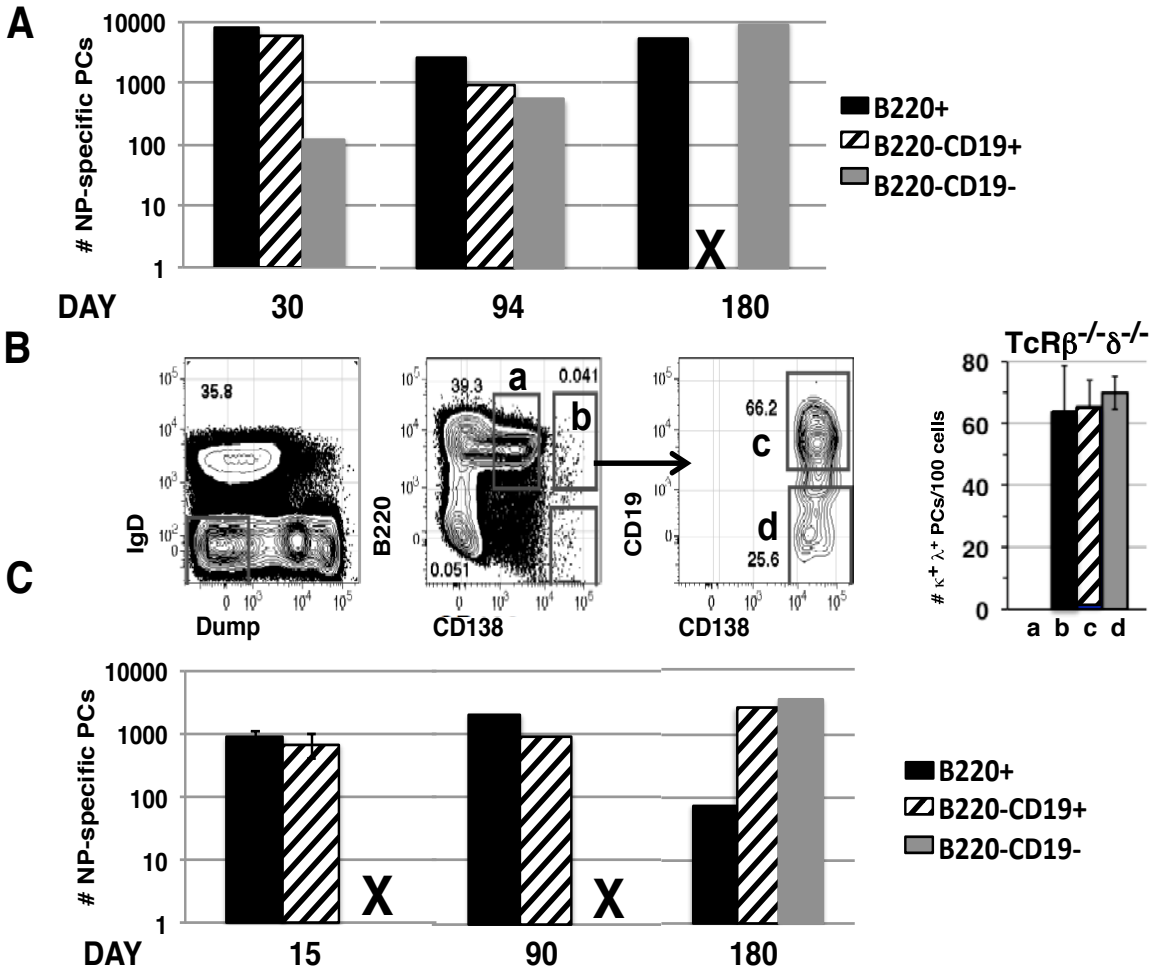

Figure 3-2. Localization of antigen-specific cells into BM PC subsets defined by CD19 in T-dependent and T-independent responses. (A) B6 mice were immunized with NP-CGG/alum and cells within each PC pool were sorted into NP26-BSA coated ELISPOT plates to determine frequencies of $\kappa^+ \lambda^+$ NP-specific PC within each population at the indicated time points post-immunization. Data show BM from 3 pooled mice. **(B)** Bone marrow cells from unimmunized TcR$\beta^{-/-}\delta^{-/-}$ mice were analyzed by flow cytometry. Cells were pre-gated on live singlets, IgD^- $Dump^-$ (CD4, CD8, F4/80, Ter119, Gr-1) populations and further subdivided by the plasma cell marker CD138, B220 and CD19. **(C)** B6 mice were immunized with NP-LPS/PBS and cells within each PC pool were sorted into NP26-BSA coated ELISPOT plates to determine frequencies of $\kappa^+ \lambda^+$ NP-specific PCs within each population at the indicated time points post-immunization. Data for day 15 represent means of 3 individual mice +SEM. Data from days 90 and 180 show BM from 3 pooled mice.

The persistent extrafollicular phenotype of the antigen-induced $B220^+$ bone marrow plasma cells (Hsu et al., 2006), combined with our data in **Fig. 2-7** and **Fig. 3-1**, strongly suggested that the maintenance of long-standing Ag-induced bone marrow plasma cells depended, in part, on continuous replenishment by Ag-specific B cell precursors. While NP^+ memory B cells in the spleen incorporated BrDU at rates comparable to the NP^+ bone marrow plasma cell pool (**Fig. 3-3 A**), I could not find evidence of robust cell division within this cell population (**Fig. 3-3 B**). To more rigorously examine the contribution of splenic NP^+ memory B cells to the NP^+ bone marrow plasma cells, we decided to ablate the memory B cell population. Other labs have attempted to address this question in the past, albeit without having insight into the turnover rates of different bone marrow plasma cell populations. In Slifka et al, plasma cells were shown to persist for months after antigen-specific B cells were eliminated using radiation (Slifka et al., 1998). However, radiation is a systemic treatment whose impact on stromal and lymphoid cell populations can obfuscate effects on the bone marrow plasma cells (Ochsenbein et al., 2000). Studies with anti-CD20 antibody as a method to deplete B cells circumvented the problem of systemic effects but examined only one timepoint, precluding the ability to accurately determine the decay rates of the bone marrow plasma cell pool (Ahuja et al., 2008).

We decided to genetically ablate B cells using CD20Cre-DTA mice previously described in **Fig. 2-6**. CD20Cre-DTA mice and single transgene controls were immunized with NP-CGG/alum and treated with tamoxifen on days 29-31 post-immunization as well as every 2 weeks subsequently in order to preclude any possible contribution from newly emerging B cells. NP^+CD19^+ cells were effectively depleted using this strategy and remained undetectable for the duration of the experiment (**Fig. 3-4**

A). Antigen-specific bone marrow plasma cells were quantified using NP26- or NP4-BSA coated ELISPOT plates to detect total or high affinity NP secretors, respectively. While we were unable to detect a decline in total or high-affinity antigen-specific plasma cells at earlier timepoints, by 140 days of continuous B cell ablation there was a significant decrease in the numbers of antigen-specific plasma cells (**Fig. 3-4 B, C**). Even when the antigen-specific pool was allowed a longer time to become established (tamoxifen treatment commenced at day 65 post-immunization as opposed to day 29), long-term B cell ablation resulted in a decline of the NP-specific secretors (**Fig. 3-5**). This result is largely consistent with previous published work on the long-lived nature of bone marrow plasma cells (Slifka et al., 1998), (Ahuja et al., 2008) and we believe that our data support the existence of *bona fide* long-lived bone marrow plasma cells (**Fig. 2-1 E, Fig. 3-1 B, C**). However, we opine that B cell ablation experiments performed by us and others obscure the true heterogeneity of the bone marrow plasma cell pool, as the expected two-fold decrease resultant from the ablation of the $B220^{+}$ bone marrow plasma cell fraction would be hardly detectable on an ELISPOT assay. We propose that it is not until the slowly renewing pool begins to be depleted that a detectable drop in numbers is observed. Overall, these data definitively show the existence of long-lived bone marrow plasma cells, yet highlight the limitations of this longevity and the dependence, albeit limited, on a B cell precursor.

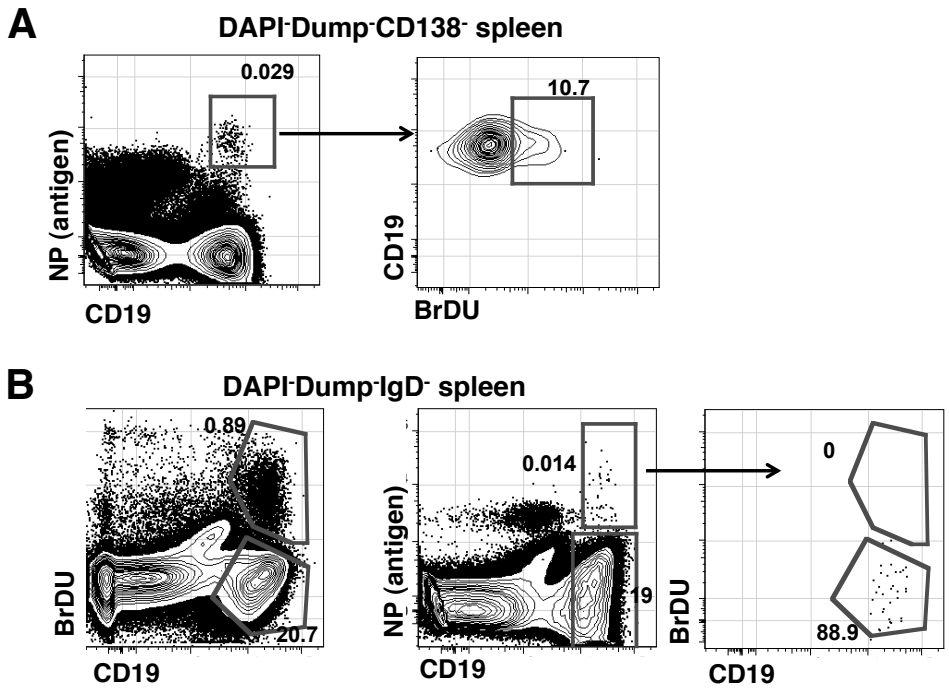

Figure 3-3. Antigen-specific memory B cells incorporate BrDU during a week-long pulse, but do not show evidence of robust cell division. **(A)** B6 mice were immunized with NP-CGG/alum. At d127 post-immunization the mice were fed BrDU for 7 days and analyzed by flow cytometry for the presence of surface antigen (NP) and BrDU incorporation. Data from 5 pooled mice are shown. **(B)** B6 mice were immunized with NP-CGG/alum. At d131 post-immunization the mice were injected with BrDU i.p. for 1 hour and analyzed by flow cytometry for the presence of surface antigen (NP) and BrDU incorporation . Labeling with BrDU is indicative of proliferation (Schittek et al., 1991). NP^+CD19^+ cells had phenotypic markers indicative of the memory B cell lineage (data not shown). Representative of $n \geq 3$ individual mice.

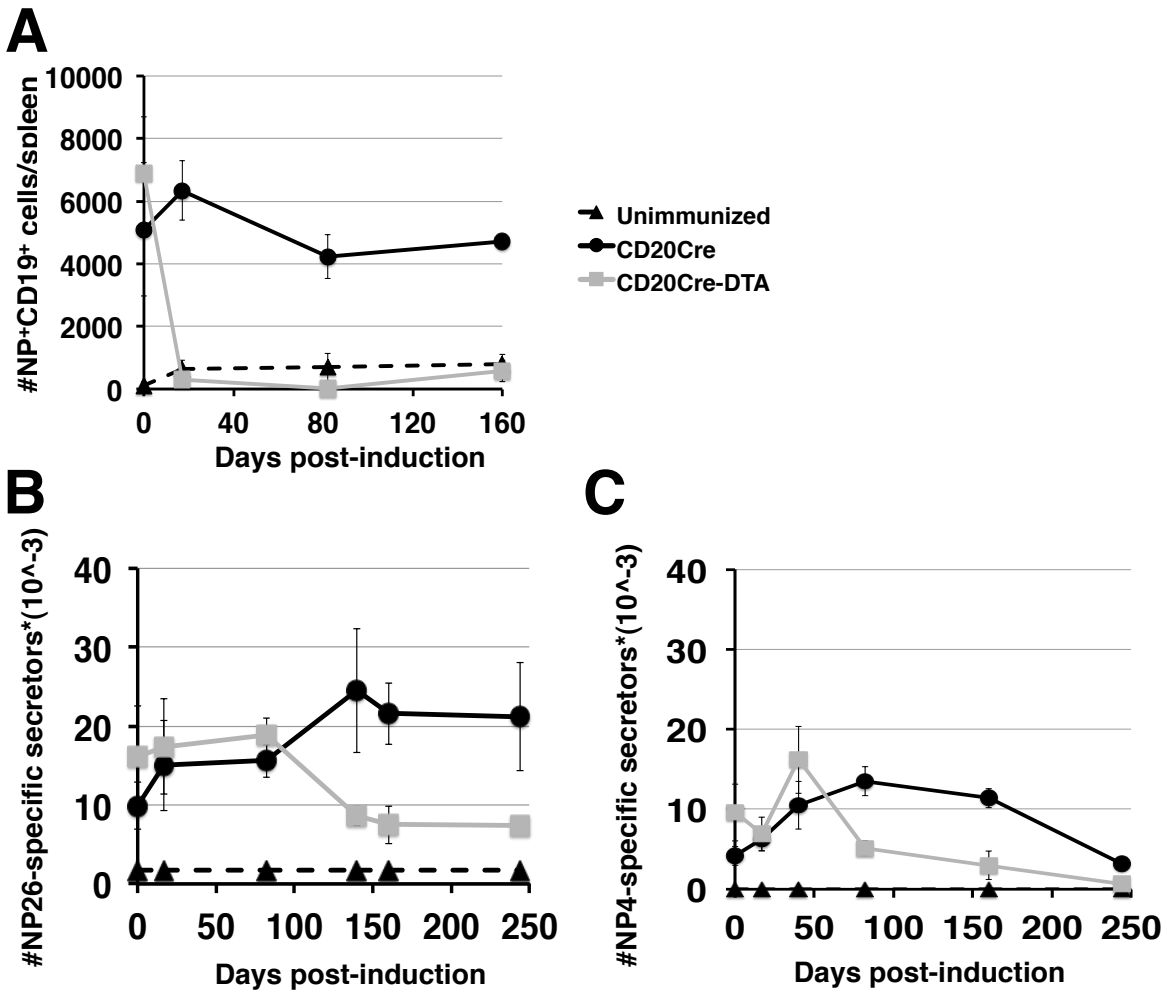

Figure 3-4. Antigen-specific BM PCs are affected by long-term B cell ablation at very late timepoints. (A) CD20Cre and CD20Cre-DTA mice were immunized with NP-CGG/alum, treated with three induction doses starting at d29 post-immunization and subsequent fortnightly doses of tamoxifen and sacrificed at multiple timepoints post-induction. Numbers of NP^+CD19^+ B cells were quantified using flow cytometry and total spleen counts. Average 3-4 mice per group; error bars represent SEM of individual animals. $p<0.05$ at all timepoints after d0. **(B)** NP-specific BM PC from mice described in Fig. 3-4 A were quantitated using NP26-BSA ELISPOTs. $p<0.05$ by d160 post-induction. **(C)** High affinity NP-specific BM PC from mice described in Fig. 3-4 A were quantitated using NP4-BSA ELISPOTs.

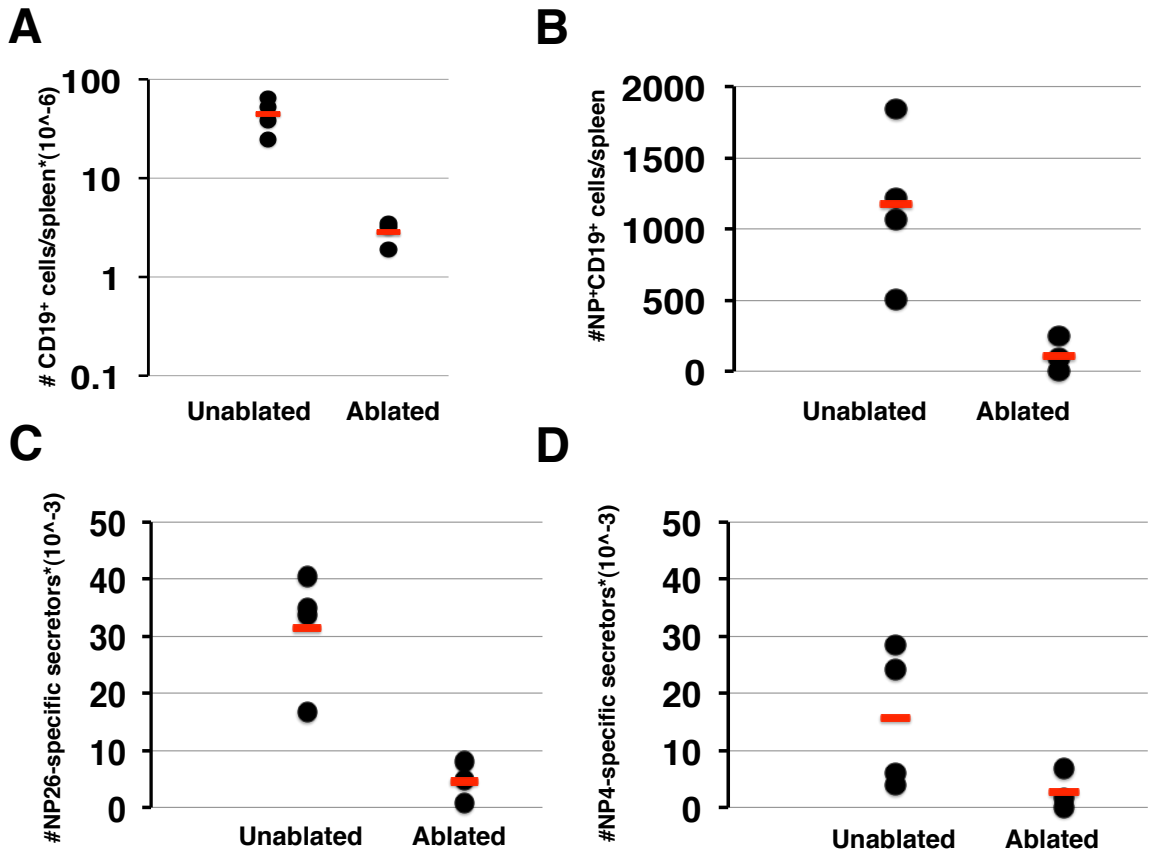

Figure 3-5. Antigen-specific BM PCs are affected by long-term B cell ablation at very late timepoints. **(A)** CD20Cre and CD20Cre-DTA mice were immunized with NP-CGG/alum, treated with three induction doses starting at d65 post-immunization and subsequent fortnightly doses of tamoxifen and sacrificed 160 days post-induction. Numbers of $CD19^+$ B cells were quantified using flow cytometry and total spleen counts. Average 3-4 mice per group; red dashes represent averages. **(B)** Numbers of NP^+CD19^+ B cells were quantified using flow cytometry and total spleen counts. **(C)** NP-specific BM PC from mice described in Fig. 3-5 A were quantitated using NP26-BSA ELISPOTs. **(D)** High affinity NP-specific BM PC from mice described in Fig. 3-5 A were quantitated using NP4-BSA ELISPOTs.

In our quest to better understand the nature of the B cell precursor seeding the bone marrow plasma cell pool, we first wondered whether CD40-dependent T-B interactions were critical for bone marrow plasma cell maintenance. As it is known that $CD40L^{-/-}$ mice have extremely low numbers of plasma cells (perhaps implicating CD40-CD40L interactions in early plasma cell differentiation events), we sought to disrupt the interaction, thereby ablating the GC, during an ongoing immune response (Kawabe et al., 1994; Noelle et al., 1992; Renshaw et al., 1994). While it is widely believed that GC reactions wane over time and are not detectable by 35 days post introduction of antigen (Liu et al., 1991; MacLennan, 1994), many groups have described GC persisting for much longer time periods (Gatto et al., 2007). As our chief interest lies in the long-term maintenance and not the formation of the antigen-specific bone marrow plasma cell pool, we chose to mirror the design of our B cell ablation experiment (**Fig. 3-4**) and treat C57BL/6 mice with anti-CD154 (MR-1) antibodies starting at d29 post-immunization with NP-CGG/alum (Noelle et al., 1992; Takahashi et al., 1998). MR-1 treatment effectively disrupted GC reactions early on (**Fig. 3-6 A**); only partial recovery of GC was seen 6 weeks post-immunization (**Fig. 3-6 B**). As previously shown by our lab, NP^+ GC did not reemerge at any point post MR-1 treatment ((Bortnick et al., 2012) and data not shown); NP^+CD19^+ cells, most likely representative of memory B cells, were unaffected (**Fig. 3-6 C, D**).

To examine the effect of anti-CD154 treatment on bone marrow plasma cell populations, I first confirmed the absence of CD40 expression on both $B220^+$ and $B220^-$ plasma cell subsets (**Fig. 3-7 A**). Neither the total (polyclonal) bone marrow plasma cell subsets (**Fig. 3-7 B, C**) nor the antigen-specific bone marrow plasma cell pool (**Fig. 3-7 D**) were affected by anti-CD154 treatment. As expected, anti-CD154 treatment resulted

in a decrease in high-affinity antigen-specific IgG secretors (**Fig. 3-7 E**). The moderate effect size seen in **Fig. 3-7 E** was not unexpected; as suggested by Takahashi et al, prolonging the GC reaction by delaying MR-1 administration "allowed proportionate recovery" of high affinity NP-specific secretors (Takahashi et al., 1998). While it is well-established that early disruption of the GC will result in significant effects on the bone marrow plasma cell pool, GC output into the bone marrow plasma cell or memory B cell pools is considered negligible after day 40 post-immunization (Gatto et al., 2007; Takahashi et al., 1998). Our results support a limited (if any) role of CD40-CD40L dependent processes in the seeding of the bone marrow plasma cell pool later than d30 post-immunization. Thus, although our data clearly show a role for an antigen-specific precursor in the long-term maintenance of bone marrow plasma cells (**Fig. 3-4**), this precursor appears to CD40-independent (**Fig. 3-7**).

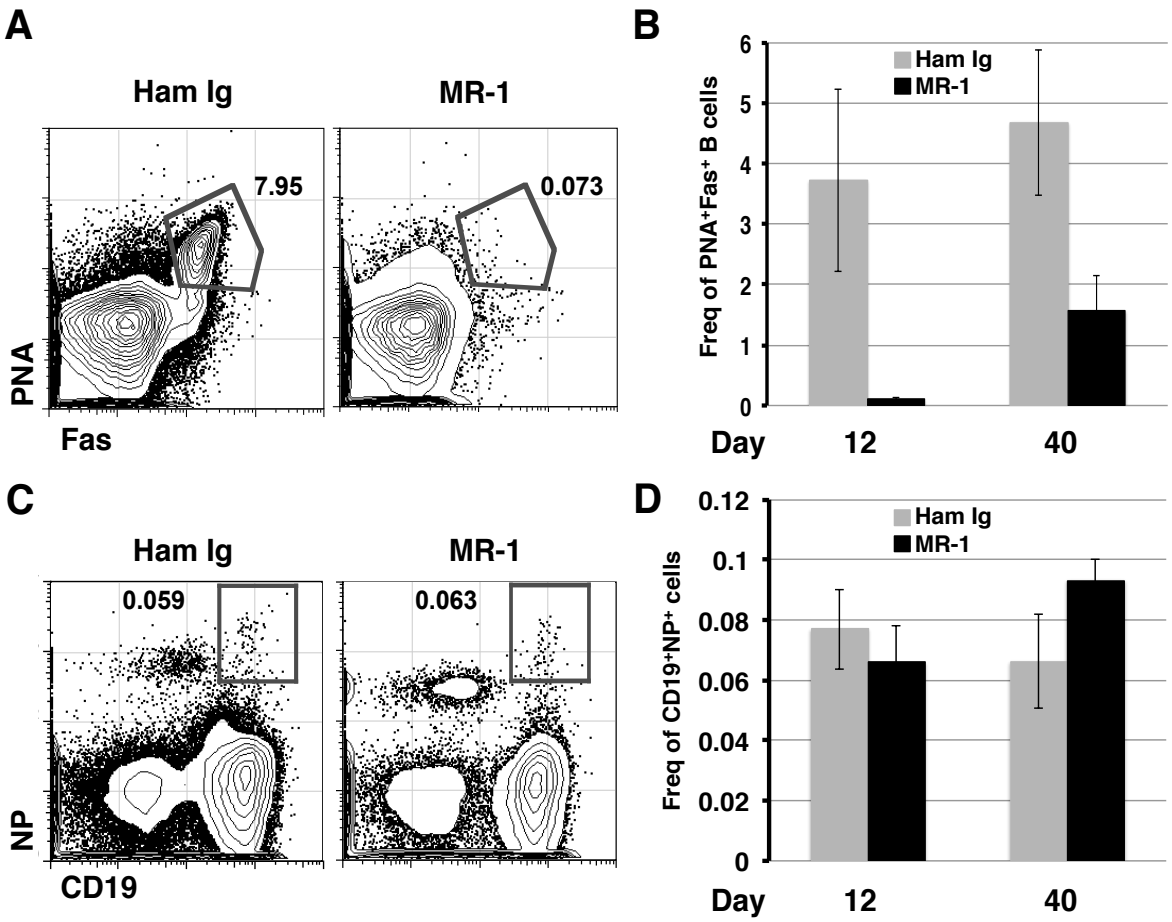

Figure 3-6. Effect of disrupting CD40-CD40L interactions on peripheral B cell populations. C57BL/6 were immunized with NP-CGG/alum and treated i.p. with anti-CD154 antibodies (MR-1) or control (Ham Ig) on days 29, 31, 33 post-immunization. Average 3-4 mice/group were analyzed using flow cytometry. **(A)** Representative flow plots of GC ablation at d12 post MR-1/Ham Ig administration. $DAPI^{-}CD19^{+}B220^{+}$ spleen cells are shown. **(B)** Fraction of $DAPI^{-}CD19^{+}B220^{+}$ spleen cells that are $PNA^{+}Fas^{+}$ is shown at d12 and d40 post MR-1/Ham Ig I administration. Average 3-4 mice/group; error bars represent SEM. **(C)** Representative flow plots of the effect of GC ablation on $NP^{+}CD19^{+}$ memory B cells at d40 post MR-1/Ham Ig administration. $NP^{+}CD19^{-}$ population likely represents antigen-laden macrophages as that population is $CD11b^{+}$ (data not shown). **(D)** Fraction of $DAPI^{-}$ splenocytes that are $NP^{+}CD19^{+}$ at d12 and d40 post MR-1/Ham Ig administration. Average 3-4 mice/group; error bars represent SEM.

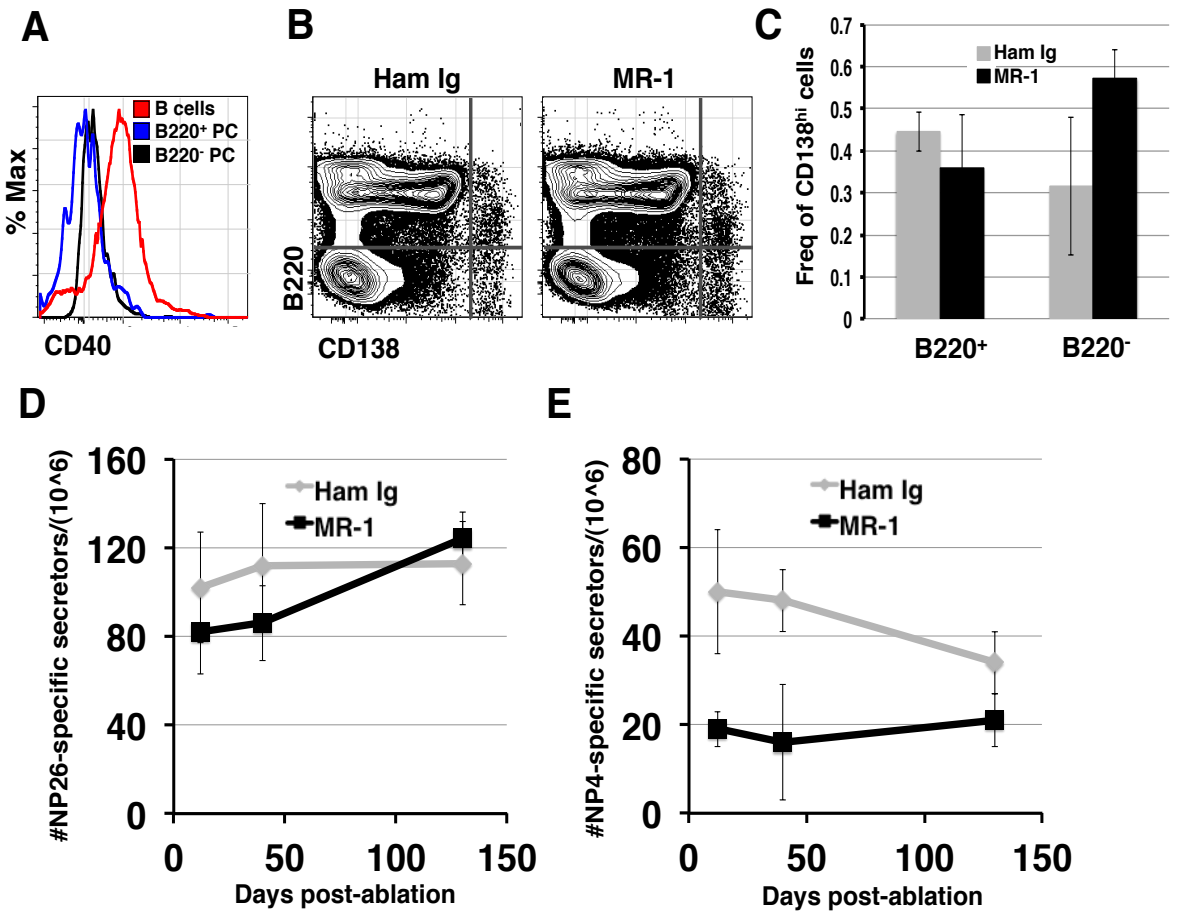

Figure 3-7. CD40-CD40L interactions are dispensable for long-term antigen-specific BM PC maintenance. (A) Expression of CD40 on splenic B cells and BM PC subsets by flow cytometry. Representative of n=3 mice. **(B-E)** C57BL/6 were immunized with NP-CGG/alum and treated i.p. with anti-CD154 antibodies (MR-1) or control (Ham Ig) on days 29, 31, 33 post-immunization. Average 4 mice/group; error bars represent SEM. **(B)** Representative flow plots of BM PC subsets on d40 post MR-1/Ham Ig administration. Gated on $DAPI^{-}Dump^{-}IgD^{-}$ cells. **(C)** Fraction of $DAPI^{-}Dump^{-}IgD^{-}$ cells that are $CD138^{high}B220^{+/-}$ on d40 post MR-1/Ham Ig administration. **(D)** NP-specific BM PC from mice treated as described above (B-E) were quantitated using NP26-BSA ELISPOTs. **(E)** High affinity NP-specific BM PC from mice treated as described above (B-E) were quantitated using NP4-BSA ELISPOTs.

The continuous replenishment of the bone marrow plasma cell pool by a CD40-independent precursor led us to wonder about the affinity and isotype profile of newly differentiated bone marrow plasma cell immigrants (largely represented by the $B220^+$ plasma cell fraction). Since CD40-CD40L interactions are crucial for affinity maturation of B cells (Takahashi et al., 1998), we hypothesized that the rapidly renewing $B220^+$ bone marrow plasma cells would contain mostly low affinity clones. This idea was further supported by multiple precedents in the literature stating that phenotypically immature plasma cells are often characterized by production of low affinity antibodies (Paus et al., 2006; Phan et al., 2006). We used the NP4- *versus* NP26-BSA ELISPOT system to examine the affinity of the different bone marrow plasma cell subsets at multiple times post-immunization with NP-CGG/alum. We find that at all timepoints examined high affinity cells localize exclusively to the slowly renewing $B220^-$ plasma cell pool (**Fig. 3-8 A, B**). Consistent with an extrafollicular phenotype, the $B220^+$ fraction consists entirely of unswitched IgM-secreting plasma cells, while all high affinity NP-specific IgG secretors are found in the $B220^-$ pool (**Fig. 3-8 B, C**). Surprisingly, a large fraction of the slowly renewing $B220^-$ pool also consists of IgM secretors (**Fig. 3-8 B, C**).

To confirm these surprising isotype findings with an independent approach, I took advantage of an observation recently made in our lab that plasma cells retain some surface expression of the Ig isotype secreted by those cells. While it is unclear whether this expression represents low levels of *bona fide* transmembrane BCR or passive acquisition of secreted antibodies (**Fig. 2-2 C, D**), our lab has confirmed that sorted surface IgM^+ plasma cells secrete only IgM antibodies in an ELISPOT assay, IgA^+ plasma cells secrete IgA, while IgM^-IgA^- plasma cells secrete IgG isotype antibodies (Joel

Wilmore, unpublished data). On day 133 post-immunization with NP-CGG/alum, I sorted bone marrow plasma cell subsets as indicated in **Fig. 3-9** ($B220^+IgM^+$, $B220^+IgM^-IgA^-$, $B220^-IgM^+$, $B220^-IgM^-IgA^-$) and assayed for NP-specific antibody secretion via the NP26-BSA ELISPOT assay. Consistent with our results in **Fig. 3-8**, NP secretors within the $B220^+$ bone marrow plasma cell pool were almost entirely of the IgM isotype, while the $B220^-$ plasma cell fraction contained both IgM and IgG secretors (**Fig. 3-9**). Together with the data in **Fig. 2-3 B**, which demonstrate a lack of detectable conversion of $B220^+$ into $B220^-$ plasma cells, the results in **Fig. 3-8** and **Fig. 3-9** suggest that the two bone marrow plasma cell lineages are distinct both functionally and with regard to their provenance. Finally, the preponderance of IgM secretors is unexpected given the T-dependent nature of this response and challenges our preconception of T-dependent bone marrow plasma cells as being a largely high-affinity, switched population.

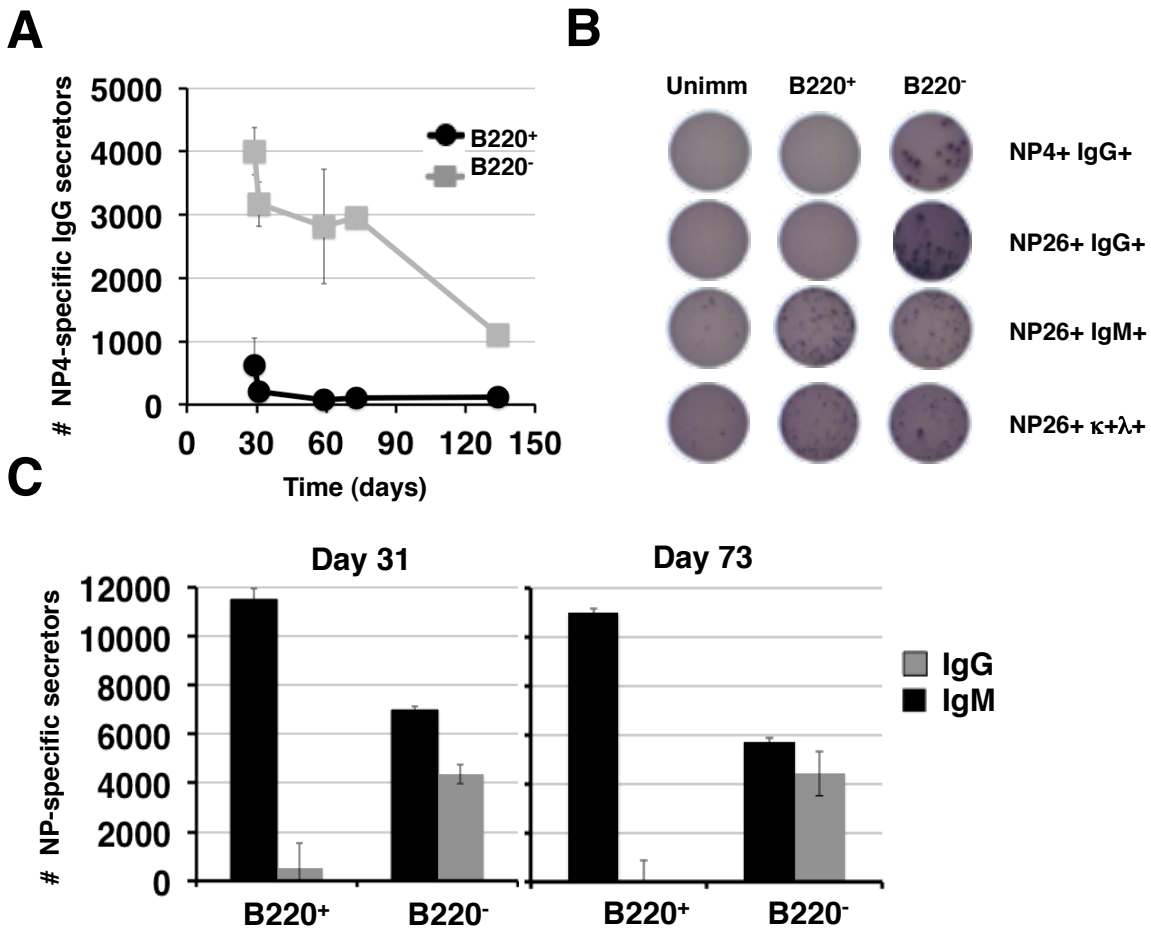

Figure 3-8. High affinity antigen-specific plasma cells are restricted to the slowly renewing BM PC pool. **(A**) BM PC subsets were assayed as described in Fig. 3-1 A, using NP4-BSA to detect exclusively high affinity cells. $p<0.01$ at all timepoints. **(B)** Representative ELISPOT wells from d73 post-immunization**. (C)** BM PC subsets were assayed as described in Fig. 3-1 A, using anti-IgM and anti-IgG to detect secretors of the respective isotype. Background has been subtracted out. $p<0.01$ for all groups (IgM *vs*. IgG) except d73 $B220^-$ where the difference is N.S.

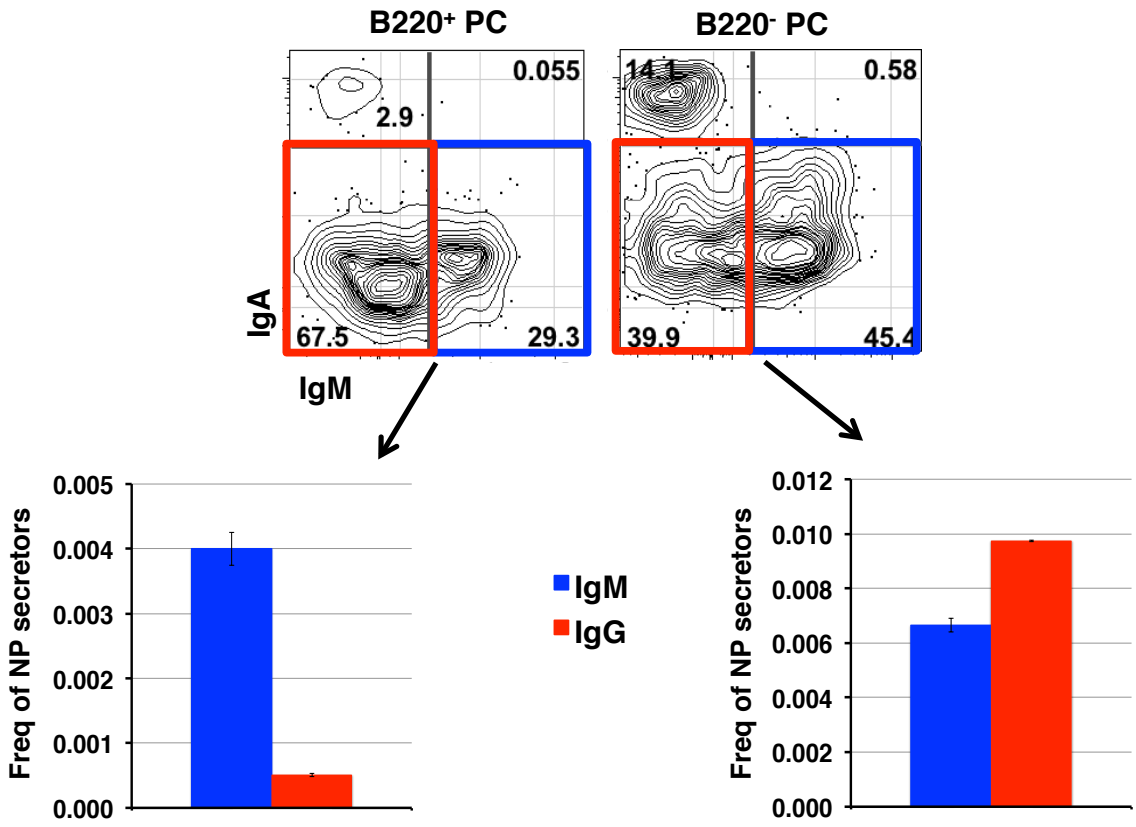

Figure 3-9. NP-specific IgG secretors are found almost exclusively within the B220$^-$ BM PC subset. B6 mice were immunized with NP-CGG/alum, and cells within each PC pool were sorted into NP26-BSA coated ELISPOT plates to determine frequencies of NP-specific PC within each population at day 133 post-immunization. Data from 3 pooled adult B6 mice; error bars represent SEM of triplicate ELISPOT wells. Background has been subtracted out.

The differences in affinity and isotype usage among the bone marrow plasma cell subsets suggested that the two pools were derived from distinct precursors. While the response to the NP hapten is classically thought to be dominated by cells bearing the λ1 light chain, the memory response is believed to be more heterogeneous, containing many κ clones (Jack et al., 1977). As our data suggest that the bone marrow plasma cell pool is replenished from a memory B cell precursor (**Fig. 3-4, 3-6, 3-7**), we hypothesized that the NP-specific rapidly renewing $B220^+$ plasma cell pool would consist mostly of κ secretors late after immunization. As a corollary, plasma cells formed during the primary, λ-dominated response would be found within the long-lived $B220^-$ pool.

Original studies suggesting preponderance of κ clones in secondary responses had been done decades earlier and had induced "memory" using a hyper-immunization strategy no longer considered standard (Jack et al., 1977). Later studies have failed to find evidence of NP-specific κ antibodies during late responses, but these researchers restricted their analysis to IgG antibodies (Takahashi et al., 1998). Thus, we first wanted to examine the light chain usage among the putative NP^+ B cell precursors of the bone marrow plasma cell pool. We find that, consistent with conventional wisdom, the early (day 20 post-immunization) response to NP-CGG is comprised largely of λ-bearing B cells (**Fig. 3-10 A, B**). In contrast, NP^+CD19^+ cells late in the response are substantially more heterogeneous with roughly half the pool consisting of κ-bearing cells (**Fig. 3-10 A, B**). Among the bone marrow plasma cell subsets, we find that early on NP-specific λ secretors can be found in both pools, while at late timepoints the rapidly renewing $B220^+$ plasma cell pool consists solely of κ secretors (**Fig. 3-11**). Importantly, we are able to detect a subset of antigen-specific B cells that evidences rapid BrDU turnover kinetics

and consists of IgM- and κ-expressing cells, strongly suggesting that it serves as the precursor for the $B220^+$ plasma cells (**Fig. 3-10 C**). The selection events underlying the entry of κ *versus* λ clones into the different bone marrow plasma cell pools remain poorly understood and will be discussed further in **chapter 4**. In sum, we believe that the differential light chain usage among the bone marrow plasma cell subsets further supports the notion that $B220^+$ and $B220^-$ plasma cells represent two unique lineages derived from distinct precursors.

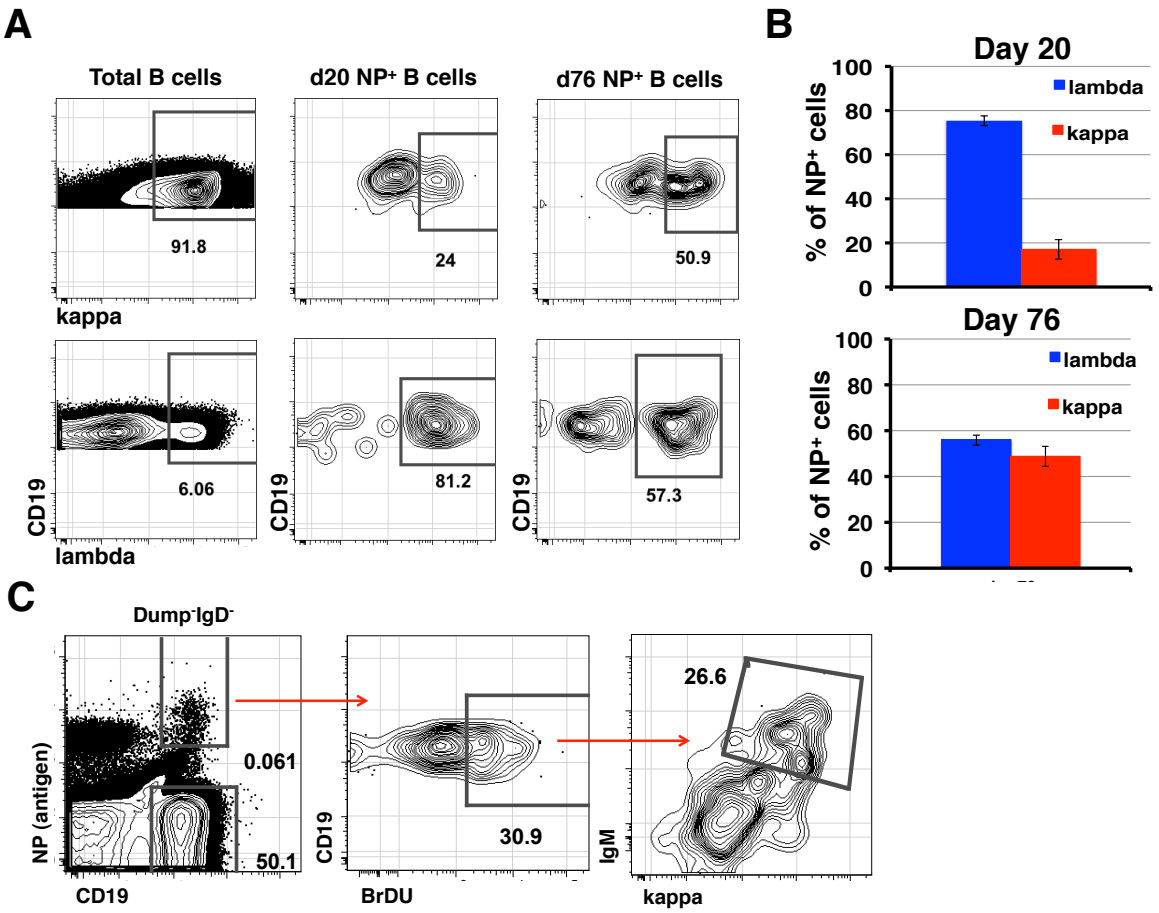

Figure 3-10. Light chain usage within the NP$^+$ B cell pool varies over the course of the NP-CGG response. Spleens from B6 mice were analyzed using flow cytometry on days 20 and 76 post immunization with NP-CGG/alum. (**A**) Representative flow plots of DAPI$^-$Dump$^-$IgD$^-$CD19$^+$NP$^{+/-}$ splenic cells. (**B**) Fraction of DAPI$^-$Dump$^-$IgD$^-$CD19$^+$NP$^+$ cells that are surface κ- *versus* surface λ-bearing on day 20 or day 76 post-immunization. Average 4 mice/group; error bars represent SEM. (**C**) B6 mice were immunized with NP-CGG/alum and administered BrDU in the drinking water between days 52-59 post-immunization. Spleens were analyzed using flow cytometry; representative plot of n=3 mice are shown.

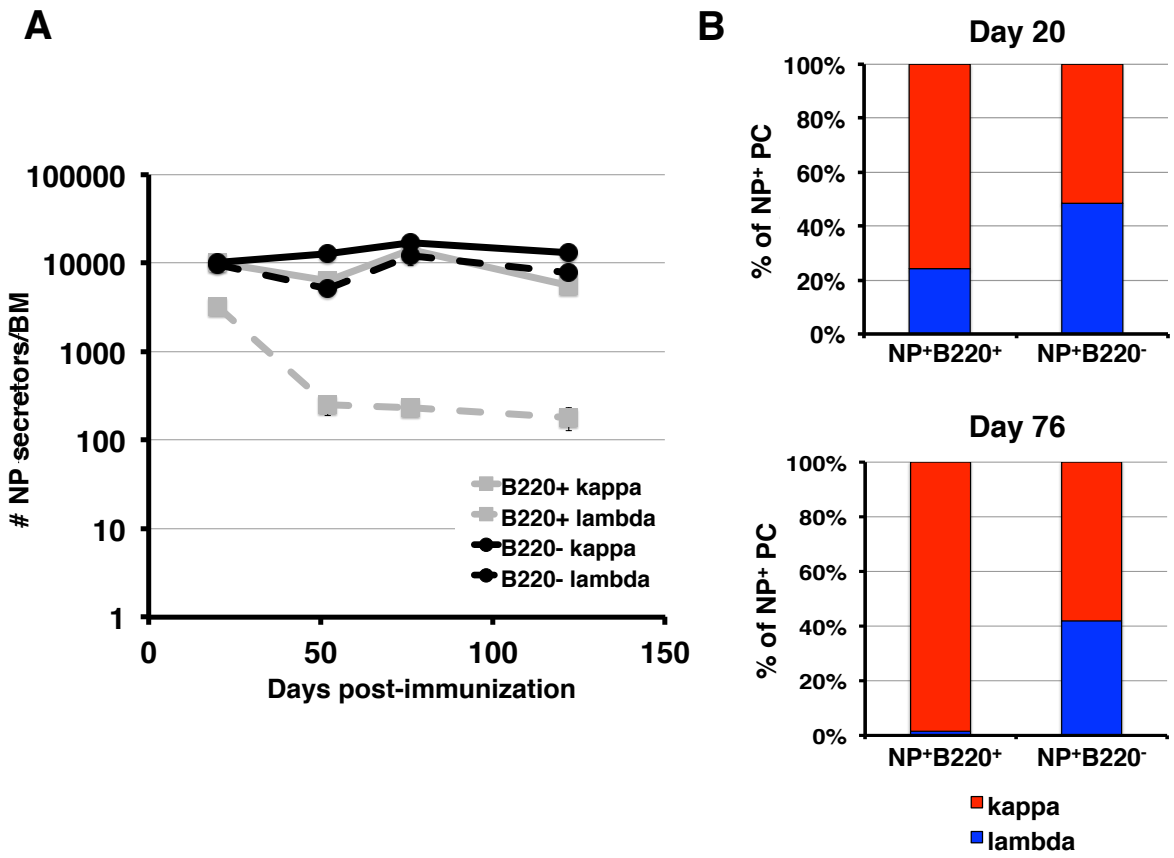

Figure 3-11. NP-specific λ-secreting PC are restricted to the slowly renewing B220$^-$ BM PC subset late in an NP-CGG response. (A) B6 mice were immunized with NP-CGG/alum, and cells within each PC pool were sorted into NP26-BSA coated ELISPOT plates and detected with κ *versus* λ antibodies at multiple timepoints post-immunization. Data from 3 pooled adult B6 mice; error bars represent SEM of triplicate ELISPOT wells. Background has been subtracted out. Differences between B220$^+$, κ$^+$ and B220$^+$, λ$^+$ are statistically significant at all timepoints with $p<0.01$. (**B**) BM from mice described in Fig. 3-11 A (d20 and d76 post-NPCGG) was analyzed by flow cytometry for the presence of intracellular antigen (NP) and intracellular κ and λ. Fraction of κ *versus* λ-bearing DAPI$^-$ Dump$^-$IgD$^-$NP$^+$CD138^{high}B220$^{+/-}$ cells is shown; average 4 mice/group.

II. DISCUSSION

While studies in unimmunized animals (**chapter 2**) allowed us to define some crucial aspects of the kinetics and dynamics of the bone marrow plasma cells, we turned to a more controlled immunization system to fully characterize the roles of the bone marrow plasma cell subsets in an antigen-induced T-dependent response. Upon immunization with the T-dependent antigen NP-CGG/alum, we find that antigen-specific plasma cells continue to be generated and localize to the rapidly renewing plasma cell pool for months after the introduction of antigen (**Fig. 3-1, 3-2**). Interestingly, we find these pools to be qualitatively distinct, with high-affinity cells localizing solely to the slowly renewing subset (**Fig. 3-8**). These data are in marked contrast with the concept of bone marrow plasma cell replacement, where newly formed plasma cells replace the long-lived veterans in the bone marrow (Radbruch et al., 2006, Odendahl et al., 2005). As data for the replacement theory are limited and based largely on difficult-to-interpret human experiments, our findings indicate that a reexamination of the question may be warranted (see **chapter 4** for further discussion).

By using genetically manipulated mice and monoclonal antibody approaches, we were able to disrupt CD40-CD40L interactions and to ablate all B cells to demonstrate that the maintenance of the antigen-specific bone marrow plasma cell pool relies on a CD40-independent B cell precursor. Previous reports in the literature have contradicted the notion that a CD20-expressing precursor is required for bone marrow plasma cell maintenance; however, one of the reports only examines one timepoint (Ahuja et al., 2008) while another does not look later than 70 days B cell ablation (DiLillo et al., 2008). In fact these reports do not necessarily contradict the conclusion that plasma cells induced in response to T-dependent antigens have a half-life on the order of 80-100 days

(Gatto et al., 2007; Slifka et al., 1998); our data correspond well to this notion for only by examining timepoints well exceeding the projected half-life do we see evidence of CD20-dependent replenishment of the plasma cell pool (**Fig. 3-4**). The CD40-independent replenishment of the bone marrow plasma cell pool is further consistent with a putative memory B cell precursor as this population is known to not rely on T cell signals for maintenance (Vieira and Rajewsky, 1990).

Our characterization of the bone marrow plasma cell response to NP-CGG/alum led to a further surprise: the preponderance of IgM secretors, even at late stages of this T-dependent response (**Fig. 3-8, 3-9**). A number of researchers utilizing this very well-characterized model antigen have reported a continuous increase in the affinity of the bone marrow plasma cell pool, culminating in 100% of the bone marrow NP-responders being high affinity by 28 days post-immunization (Smith et al., 1997) (Takahashi et al., 1998). However these researchers examined only the IgG1 part of the response, leading them to the conclusion that the majority of the NP-CGG response trended toward high affinity with time. Indeed our data confirm that the vast majority of NP-specific IgG secretors are high affinity (**Fig. 3-8 B**). It is only recently that work by a number of groups has identified IgM-secreting plasma cells in the bone marrow (Bortnick et al., 2012; Racine et al., 2011). Work from our lab in particular has suggested that IgM-secreting plasma cells induced in a T-dependent response may be able to localize to the bone marrow (Bortnick et al., 2012); prior to these studies there was no precedent for examining the bone marrow for IgM responses. Additionally, we have discovered profound differences in light chain usage among the antigen-specific secretors within the $B220^{+}$ *versus* the $B220^{-}$ subsets (**Fig. 3-11**). The NP-specific rapidly renewing subset contains exclusively IgMκ secretors (**Fig. 3-8, 3-11**) and we see evidence of renewing

NP-specific B cells that express markers consistent with a putative precursor for the rapidly renewing plasma cell pool (**Fig. 3-10 C**). Consequently, we propose that the rapidly and slowly renewing bone marrow plasma cell pools derive from distinct cellular precursors at different points of an induced response.

In sum, we suggest that the heterogeneity of the bone marrow plasma cell pool plays a key role in humoral immune responses to both T-dependent and T-independent antigens and should be further evaluated in systems involving disease antigens. We demonstrate that the maintenance of the antigen-specific bone marrow plasma cell pool is dependent on a CD40-independent B cell precursor and identify a putative B lineage precursor that shares certain key features with the plasma cells within the rapidly renewing $B220^{+}$ bone marrow pool. Together our data suggest a multi-layered organization of the key players in humoral immunity and raise interesting questions about the interrelationships among the participants of this structural framework.

CHAPTER 4: DISCUSSION

Plasma cells are essential components of humoral immunity, responsible for secreting large amounts of protective antibodies. The composition and longevity of the plasma cell pool has long been a matter of debate, with early models positing that all plasma cells were short-lived and dependent on constant replenishment from memory B cells and more current studies suggesting that the bone marrow provides a survival niche exclusively for high affinity, long-lived plasma cells. Our data suggest that the true nature of the bone marrow plasma cell pool is a conglomeration of the two models (**Fig. 4-1).** First, we identify two populations of bone marrow plasma cells that differ with regard to their turnover kinetics, providing evidence that both short-lived and long-lived plasma cells coexist in the bone marrow. Second, we can demonstrate that the long-standing bone marrow plasma cell pool appears to be non-dividing and relies on replenishment by a CD20-expressing cellular precursor. We further show that the different bone marrow plasma cell subsets contain functionally distinct secretors throughout the course of a T-dependent response. Secretors within the precursor-dependent $B220^{+}$ pool produce exclusively low affinity, IgM, κ antibodies. The classic purveyors of humoral immunity – high affinity, IgG secretors – are only found within the slowly renewing $B220^{-}$ bone marrow plasma cell pool, although, surprisingly, this pool also contains many IgM secretors. Finally, we present evidence for the putative cellular precursors of the different plasma cell subsets: rapidly renewing, IgM- and κ-bearing antigen-specific B cells which also express surface markers associated with memory B cells. These findings characterize the multi-faceted nature of the bone marrow plasma cell pool and highlight many unanswered questions about the roles of B cell precursors and persisting antigen

on the maintenance of standing antibody titers. We propose that our work has important implications for therapies aimed at eliminating cellular sources of pathogenic antibodies as well as for rational vaccine design.

I. Nature of the Bone Marrow Plasma Cell

Since the discovery of the plasma cell as the cellular source of antibody production, researchers have been trying to define the lifespan and survival requirements of these cells. Early work demonstrated that plasma cells were terminally differentiated, non-dividing cells whose numbers contracted many fold shortly after their induction (SCHOOLEY, 1961). This led to the notion that plasma cells were intrinsically short-lived and dependent on constant replenishment from a precursor (SCHOOLEY, 1961). Later experiments identified the GC B cells as the precursors of the high affinity, IgG-secreting plasma cells and postulated memory B cells as the feeder pool for this plasma cell population later in the response (Schittek and Rajewsky, 1990; Tarlinton, 2008). This model, whereby memory B cells turn over slowly and constantly differentiate into short-lived plasma cells either stochastically or in response to persisting antigen/polyclonal stimulators, persisted for decades and, in my experience, still holds sway over the minds of many practicing physicians (Schittek and Rajewsky, 1990; Smith et al., 1996). Two seminal papers in the late 1990s revolutionized our understanding of plasma cell longevity by demonstrating that these cells could persist for months in the absence of radioresistant precursors and even proposed that plasma cells can survive for the lifetime of the animal (Manz et al., 1997; Slifka et al., 1998). While the evidence in these papers did not exclude (and even supported) the possibility that short-lived plasma cells coexisted with their long-lived counterparts in the bone marrow, the notion that the bone

marrow was a unique survival niche reserved for the “crème de la crème” of antibody-secreting cells began to be perpetuated in the literature.

The models of the bone marrow plasma cell pool put forth over the last 15 years have had to reconcile the notion of the bone marrow as the repository of long-lived plasma cells with the manifest need of the bone marrow to accept new plasma cell specificities as the host encounters and battles new antigens. A number of studies have focused on characterizing the plasma cell or plasma cell precursor that colonizes the bone marrow; most have put forth evidence of “graded stages of increasing maturity” as the (pre) plasma cell transits from peripheral lymphoid organs to the bone marrow (Jourdan et al., 2011; Medina et al., 2002; O'Connor et al., 2002). In a 2004 report, Kallies et al demonstrate the presence of bone marrow plasma cells expressing intermediate levels of Blimp-1, a phenotype consistent with the splenic “plasmablast” stage, further supporting the notion that plasma cells undergo their final maturation stages in the bone marrow (Kallies et al., 2004). As our data clearly indicate the presence of two populations of bone marrow plasma cells, we wondered whether these subsets comprised the same lineage, with one pool representing an intermediate stage in plasma cell development. The immature phenotype of the $B220^{+}$ plasma cell subset casts this pool as a likely candidate for the precursor to the $B220^{-}$ pool. However, we fail to track the BrDU label from the $B220^{+}$ into the $B220^{-}$ pool during BrDU pulse-chase experiments and preliminary mathematical modeling work suggests that there is no inter-conversion between the two bone marrow plasma cell pools (**Fig. 2-3 and data not shown**).

Experiments in mice immunized with NP-CGG lend further support to this model, as the cells within the two pools differ with regard to their affinity as well as light chain and isotype usage (**Fig. 3-8, 3-9, 3-11**). While it has been suggested that plasma cells undergo further affinity maturation in the bone marrow (Takahashi et al., 1998), we do not believe that those data can be explained by a maturation of $B220^+$ PC into $B220^-$ plasma cells. Those studies examined solely IgG-secreting plasma cells, while our data indicate that the NP-specific $B220^+$ plasma cell pool is comprised entirely of IgM secretors (**Fig. 3-8**). As plasma cells lack the machinery to support class-switch recombination, it is extremely unlikely that $B220^+$ IgM secretors are maturing into the high affinity, IgG secretors characteristic of the long-lived $B220^-$ bone marrow plasma cell pool (Marshall et al., 2011; Muto et al., 2010). However, as both bone marrow plasma cell pools contain NP-specific IgM secretors we cannot rule out that $B220^+$ plasma cells enter the IgM fraction of the $B220^-$ pool. A mathematical analysis limited to the IgM-secreting bone marrow plasma cell fraction may help elucidate the interrelationships among these populations. Nonetheless, the high affinity IgG-secreting phenotype of a portion of the $B220^-$ pool strongly suggests that these cells are derived from a B cell precursor distinct from the one postulated for the $B220^+$ pool (**Fig. 3-10**). One group has reported that the maturation status of plasma cells found in the blood is indistinguishable from that of bone marrow plasma cells (Blink et al., 2005); a human study found two plasma cell subsets (low and high affinity) released into the circulation simultaneously following a tetanus booster (González-García et al., 2008). It is thus possible that the $B220^-$ plasma cell (or some fraction of this pool) complete their maturation in the periphery and then migrate to the bone marrow. It is important to note that there is no direct evidence of plasma cell maturation in the bone marrow and thus short-lived and long-lived plasma cells may exist

in parallel within this niche. If so, what could be the advantages/consequences of maintaining two different pools of antibody secretors targeting the same antigen?

II. The Role of the Precursor

Given the functionally heterogeneous nature of the bone marrow plasma cell response to NP-CGG/alum, it is tempting to speculate as to the potential advantage of such a multi-component system. It has been proposed that memory B cells are less mutated than bone marrow plasma cells and, as a result, are better able to respond to alternative epitope structures such as may be found in viral escape mutants (Purtha et al., 2011; Smith et al., 1997). As new antigen-specific plasma cells enter the rapidly renewing bone marrow plasma cell pool even months post-immunization (**Fig. 3-1 B**) and appear to be dependent on a memory B cell precursor (**Fig. 3-4, 3-10**), it is possible that these cells represent the arm of the humoral immune response designed to deal with the alternative structures of viral escape variants (Purtha et al., 2011). Two groups have reported functional heterogeneity of the long-lived memory B cell compartment (Dogan et al., 2009; Pape et al., 2011), making the existence of a parallel structure in the bone marrow plasma cell compartment an appealing one. In particular, these groups have suggested that IgM^+ memory B cells persist longer than their isotype switched counterparts (Dogan et al., 2009; Pape et al., 2011), raising the possibility that IgM secretors within the $B220^-$ slowly renewing bone marrow compartment share a similar fate. In fact, the effects of B cell ablation appear to manifest earlier and to a greater extent in the high affinity IgG-secreting plasma cell pool when compared to the effects on the total antigen-specific plasma cell pool (**Fig. 3-4 C *versus* Fig. 3-4 B**). These data could indicate that the IgM secretors within the $B220^-$ pool are constantly replenished

while the IgG responders wane over time, particularly if that pool is partially dependent on the shorter-lived IgG-bearing memory B cells. Alternatively, the data could be indicative of the exceptionally long-lived nature of the IgM secretors within the $B220^-$ pool. BrDU studies to better define the dynamics of the putative memory B cell precursors and IgM-secreting plasma cells specifically, as well as BrDU studies performed following B cell ablation protocols, should help distinguish between these possibilities in the near future.

By suggesting that the maintenance of the bone marrow plasma cell pool is (partially) precursor-dependent, our data raise many intriguing questions about the regulation of this process. What are the signals driving memory B cells to differentiate into plasma cells? Are certain memory B cell subsets more likely than others to gain entry into the bone marrow plasma cell pool? There is limited evidence in the literature suggesting that the entry into the bone marrow plasma cell compartment is subject to "previously unappreciated selective regulation" (Scheid et al., 2011). By sequencing IgG-bearing memory B cells and IgG-producing bone marrow plasma cells from humans, Hedda Wardemann's group was able to show a much higher frequency of self-reactive and polyreactive clones within the memory B cell pool as compared to the bone marrow plasma cell population. As our data also note clonal disparities between the memory B cell and bone marrow plasma cell pools, it is tempting to speculate as to the nature of the regulatory mechanisms involved. Specifically, while we can clearly identify antigen-specific, λ-bearing memory B cells at late timepoints during the NPCGG/alum response, we see no evidence of NP-specific λ secretors within the rapidly renewing $B220^+$ bone marrow plasma cell pool (**Fig. 3-10, 3-11**). BrDU studies reveal that the renewing ($BrDU^+$) portion of the NP^+CD19^+ splenic pool is not exclusively $IgM^+\kappa^+$, suggesting that

the turnover kinetics of λ- *versus* κ-bearing memory B cells are not the cause of the discrepancy (**Fig. 3-10**).

One potential mechanism of regulation could be related to the affinity of the population, whereby the λ-bearing memory B cells represent the high affinity fraction of the pool. Many models posit that affinity sensing is a key mechanism for egress from the GC (MacLennan, 1994); one report suggests that high affinity antibodies are capable of dampening ongoing GC responses via a negative feedback loop (Pelletier et al., 2010). Furthermore, Mark Shlomchik's group has reported a memory B cell subset that is enriched for the presence of high affinity-conferring mutations (Anderson et al., 2007). Thus affinity-based regulation for entry into the bone marrow plasma cell pool by memory B cells is a notion not without precedent. Examining the sequences of many of these memory B cell and bone marrow plasma cell populations should yield some insights and determine whether the proposed model has merit. Unfortunately, we have had trouble performing such analyses, largely because all protocols for sequencing NP-specific clones are restricted for use on λ-expressing cells (Lalor et al., 1992; Tomayko et al., 2010). As NP-specific $B220^+$ plasma cell clones are entirely κ late in NP-CGG/alum responses, standardizing sequencing protocols not restricted to λ-bearing populations would be of great interest in the future. In addition to resolving GC/memory B cell-bone marrow plasma cell interrelationships, such reagents would allow us to further define the affinities of the IgM-expressing populations of interest. While ELISPOT reagents for the NP system allow us to distinguish antibodies that bind the antigen with high *versus* low affinity, the system is limited to recognizing high affinity antibodies of the IgG isotype, as the pentameric nature of the IgM isotype interferes with the integrity of the assay. As the mechanisms for class-switch recombination and isotype switching are not always

coordinated (Toyama et al., 2002), and as hypermutated, GC-derived IgM clones have been previously described (Dogan et al., 2009; Seifert and Küppers, 2009; Yates et al., 2013), it is of great interest to us to determine whether $B220^+$ and/or $B220^-$ IgM secretors correspond to such a population. Assaying for the presence of the canonical high affinity mutations combined with more functional biochemical binding studies would greatly enhance our understanding of the provenance and affinity properties of these populations.

The question of the signals needed by memory B cells in order to differentiate into plasma cells remains a matter of much debate. While one high profile report has concluded that memory B cells differentiate into plasma cells in response to polyclonal stimuli such as bystander T cell help and CpG DNA (Bernasconi et al., 2002), most reports have focused on the role of persisting antigen as the driving force behind continuous differentiation of memory B cells (Karrer et al., 2000; Ochsenbein et al., 2000). It is now well-accepted that memory B cells are maintained in the absence of antigen (Maruyama et al., 2000), yet a reintroduction of antigen elicits potent proliferative and plasma cell-differentiating responses. Using cell transfer studies and mice deprived of secondary lymphoid organs, one group concluded that antigen stimulation of memory B cells was essential for the maintenance of antibody titers (Ochsenbein et al., 2000). As antigen is known to persist on follicular dendritic cells (FDCs), another group used $TNFR1^{-/-}$ mice, known to lack mature FDCs, to study the effects of persisting antigen on maintaining antibody titers. They found that, although memory B cells remained intact, antibody titers and bone marrow antibody-secreting cells declined 90-95% within 300 days (Karrer et al., 2000). These workers concluded that persisting antigen was essential for the maintenance of antibody titers and that only a minority of the bone marrow plasma

cell pool was long-lived; it is worth noting, however, that these studies were done in global knockout mice incapable of forming germinal centers and (much like the report by Ochsenbein et al) relied heavily on cell transfer studies, an infamously flawed technique in the field of antigen persistence. It is notoriously difficult to perform cell transfer studies without accidentally transferring antigen into the host, especially as the dose of antigen required for BCR stimulation is not well-known and may be quite small. Indeed, prior to the elegant studies by the group of Klaus Rajewsky who revised the B cell receptor specificity in order to circumvent the problem of antigen transfer (Maruyama et al., 2000), it was widely held that memory B cells were dependent on antigen for persistence (Gray and Skarvall, 1988). Interestingly, our only evidence that plasma cell survival is independent of antigen also comes from work utilizing cell transfer studies (Manz et al., 1998); while we do not believe that plasma cells have the requisite BCR signaling machinery to respond to antigen (**Fig. 2-2 C**), there may be merit to revisiting this question. Overall, the role played by persisting antigen in the maintenance of humoral immunity has proven to be extremely difficult to elucidate. To avoid issues inherent in cell transfer approaches, we are currently re-deriving the mice utilized by the Rajewsky group in their seminal paper (Maruyama et al., 2000). By enabling us to revise the specificity of the BCR directly, this system will allow us to assay the effects on antibody titers directly within the immunized animal and (hopefully) provide definitive answers about the role of persisting antigen in the maintenance of humoral responses.

III. Displacement and the Bone Marrow Niche

As seen above, responses to one model antigen can be immensely complex when considering the regulation of and competition within the bone marrow plasma cell

pool. When considering that the bone marrow has to accommodate large numbers of specificities generated throughout the lifetime of the individual, the problem intensifies. First, the nature of the bone marrow niche is scrutinized: can the niche support a fixed/limited number of plasma cell specificities, making it subject to competition between plasma cell clones, or is it expandable? While direct evidence is scarce, it is widely held that the bone marrow plasma cell niche is of a limited size (Radbruch et al., 2006). Workers from Ian MacLennan's group quantified numbers of plasma cells in the spleen during the first three weeks of the response to an experimental antigen and concluded that the spleen has "a finite capacity to sustain plasma cells produced" (Sze et al., 2000). Although studies in this work focused on extrafollicular splenic responses, the concept of a finite plasma cell niche has heavily influenced our thinking about the bone marrow's ability to provide suitable survival niches for an unlimited number of plasma cells.

Human studies have tended to support the notion of a non-expandable bone marrow plasma cell niche. A tetanus toxoid booster was capable of expanding both specific and bystander T cell memory; however, a transient increase of tetanus-specific antibody titers was not accompanied by expansion of other pre-existing antibody titers (Di Genova et al., 2006). In addition to highlighting the importance of antigen availability for memory B cell responses, these results suggest that inflammation alone is insufficient to expand pre-existing pools. Calculations extrapolated from the number of plasma cells the bone marrow is believed to support (based on cell counts of bone marrow sections in both mice and humans), new plasma cells generated in the course of a response and the amount of antibody manufactured per cell led to an estimate of 1,000 distinct specificities being supported by the bone marrow (Haaijman et al., 1977; Radbruch et al., 2006; Schauer et al., 2003; Simonsen et al., 1984; 1986; Trepel, 1974). The concept of displacement states that a fraction of each pool (as defined by a given specificity) will be

lost to allow space for new plasma cell immigrants during every immune response. It has been estimated that humoral immunity for an established antigen will wane by ~0.1% for every new specificity to be accommodated (Bernasconi et al., 2002; Radbruch et al., 2006). At that rate, it would take roughly 700 immune challenges (~23 years in humans) to affect a 50% drop in the number of plasma cells of any given specificity (Radbruch et al., 2006). Though the magnitude of the antibody response differs between antigens, for agents such as tetanus it has been estimated that less than 20% of plasma cells originally colonizing the bone marrow would be sufficient to maintain protective antibody titers (Radbruch et al., 2006; Schauer et al., 2003; Simonsen et al., 1986). It is thus conceivable that, despite the competitive loss of plasma cells from the bone marrow pool, established humoral memory against a given pathogen could last a lifetime.

While it may be comforting to think of humoral immunity as being long-lasting despite brutal competition for resources, the occurrence of displacement is far from certain. Such indirect observations as human adolescents having two-fold higher titers of antibody against tetanus toxoid have been interpreted as being due to a larger bone marrow capacity for plasma cells resultant from lack of competition (Schauer et al., 2003). It is worth noting that studying humoral immune responses in subjects of different ages is fraught with complications as T cell helper quality and subsequently GC responses are known to wane with age (Eaton et al., 2004; Haynes et al., 2003). Perhaps the best evidence comes from one human study which characterized the plasma cells found in the circulation following a tetanus booster (Odendahl et al., 2005). This group described two antibody-secreting cell populations in the blood: a tetanus toxoid-specific population with the phenotype and chemotaxis properties consistent with plasmablasts and a phenotypically long-lived plasma cell population of unknown specificities, believed to correspond to the displaced residents of the bone marrow (Odendahl et al., 2005). It has

been further proposed that newly generated “plasmablasts” physically compete for the requisite survival niches in the bone marrow, most likely by displacing bone marrow plasma cells from CXCL12-expressing bone marrow stromal cells (Radbruch et al., 2006). This conjecture is derived from data which indicates that bone marrow plasma cells co-localize with CXCL12-expressing cells and utilize CXCL12 as a survival factor, while CXCR4-expressing plasmablasts are capable of migrating toward a CXCL12 gradient (Cyster, 2003; Hauser et al., 2002; Odendahl et al., 2005; Tokoyoda et al., 2004; Wehrli et al., 2001). It is worth remembering that this work was unable to show directly that long-lived plasma cells were displaced from the bone marrow nor whether these cells returned to the bone marrow or took up residence in some other site capable of fostering plasma cell survival (Odendahl et al., 2005); as such the conclusions from this work remain tenuous.

How does the characterization of $B220^+$ *versus* $B220^-$ plasma cells fit into these complex paradigms? Due to the immature phenotype of the $B220^+$ plasma cells, it is tempting to characterize this pool as the plasmablasts that will displace the established, long-lived pool. However, the extremely rapid turnover rate of this population suggests that these cells are not colonizing the desirable survival niches (such as proximity to CXCL12-expressing stromal cells). On the other hand, the decline in antigen-specific plasma cells over time (**Fig. 3-1 A**) may be indicative of displacement by new plasma cell specificities (even in the specific pathogen-free conditions of the university mouse facility). Exogenous survival factors are crucial to plasma cell longevity, so perhaps displacement from survival niches is a major mechanism that limits the duration of this famed longevity. We hypothesize that $B220^+$ and $B220^-$ plasma cells localize to different locations within the bone marrow and are planning microscopy imaging studies to further investigate the issue. Finally, we remain intrigued by the concept of displacement

(independent of the $B220^{+/-}$ subsets discussed) and are interested in pursuing a sequential immunization strategy with various trackable antigens to more rigorously examine the displacement model.

IV. Implications of Work

The work presented herein has extended our understanding of the bone marrow plasma cell pool, allowing us to modify the conventional model of plasma cell differentiation and maintenance in several important ways (summarized in **Fig. 4-1**). Although this dissertation has focused on characterizing plasma cell responses to a model antigen, we fully believe the work has broad implications for many other fields. Inducing enduring, high affinity antibody responses is a major goal of vaccine design. Understanding the multi-parameter nature of the plasma cell response should help with designing vaccine antigens capable of driving differentiation of high affinity, IgG-secreting $B220^{-}$ plasma cells at the expense of their $B220^{+}$ counterparts. Moreover, these data call for a re-examination of the role of antigen persistence in the maintenance of antibody titers.

In the fields of autoimmunity and cancer, where plasma cells take on a pathogenic role, a deeper understanding of the different populations can provide new targets for drug development. Though both short-lived and long-lived plasma cells have been implicated in the pathogenesis of autoimmune disease (O'Connor et al., 2002), a more in-depth understanding of which autoimmune disorders are predominantly short-lived plasma cell *versus* long-lived plasma cell driven will help determine the best therapies to utilize in each case. Personally, I believe that the differential effects of rituximab in various autoimmune disorders may be explained by the relative preponderance of short-lived

versus long-lived plasma cells in any given disorder. Finally, my data may provide further insights into the long-debated question of the myeloma stem cell. Perhaps due to their less "mature" nature $B220^{+}$ plasma cells are more susceptible to accumulating the mutations essential for malignant transformation. Studies in mouse models of multiple myeloma are currently being planned in our lab to help address these questions. I, thus, remain extremely excited about the future directions and implications of this work.

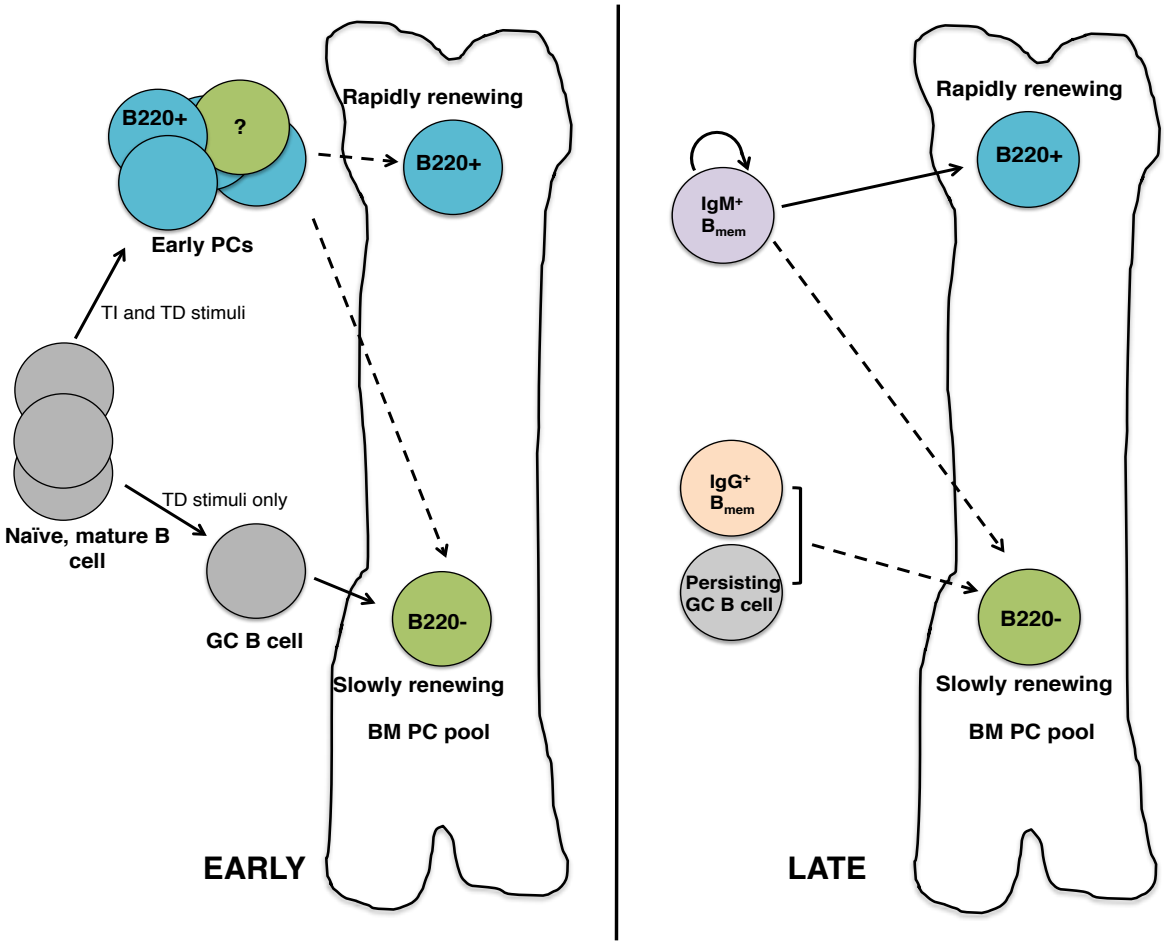

Figure 4-1. Amended model of the bone marrow plasma cell pool at early and late timepoints of a T-dependent immune response. Solid arrows indicate pathways strongly supported by the data, while dashed arrows represent conjectured, though formally unproven interrelationships.

CHAPTER 5: MATERIALS AND METHODS

Mice. C57BL/6 (B6) and B6.TcR$\beta^{-/-}\delta^{-/-}$ females (age 8-10 weeks) were obtained from Jackson Laboratories. B6.Blimp1$^{+/GFP}$ mice (Kallies et al., 2004) were kindly provided by the late Dr. Mark Pescovitz (Indiana University) with permission from Dr. Stephen Nutt (WEHI). Gt(ROSA)26Sortm1(DTA)Jpmb/J mice (Ivanova et al., 2005) were obtained from Dr. Taku Kambayashi (University of Pennsylvania) and maintained in our facility (JAX order # 00-6331). hCD20-TAMCre.C57BL/6 (Khalil et al., 2012) were provided by Dr. Terri Laufer (University of Pennsylvania) with permission from Dr. Mark Shlomchik (Yale University). All animal procedures were approved by the University of Pennsylvania Office of Regulatory Affairs.

Mouse genotyping. The progeny of Gt(ROSA)26Sortm1(DTA)Jpmb/J and hCD20-TAMCre.C57BL/6 mice were screened for the presence of GFP signal (indicative of the DTA transgene) and hCD20 expression (using APC-anti-hCD20, clone 2H7 from eBioscience) via flow cytometry. The presence of wild-type and Blimp^{GFP} alleles in B6. Blimp1$^{+/GFP}$ mice was determined by PCR purification of tail DNA with primer sequences provided by Dr. Stephen Nutt (bl-1: GGCAAGATCAAGTATGAGTGC, bl-2: TGAGTAGTCACAGAGTACCCA, bl-3: GCGGAATTCATTTAATCACCCA). PCR products were resolved on a 2% agarose gel (expected band sizes: 611bp wild-type, 531bp Blimp^{GFP}).

Cell preparation and staining. Spleen and BM cells were harvested and stained with

optimal dilutions of the indicated antibodies as described (Lindsley et al., 2007). Specifically, BM cells were flushed from tibias, femurs and iliacs and splenocytes prepared through perfusion of spleens with FACS buffer (PBS with 0.1% BSA and 1mM EDTA). Following lysis of red blood cells with 0.165M NH_4Cl, cells were washed and incubated with optimized dilutions of antibodies in 100μL final volume of FACS buffer. After 30 minutes of staining at 4°C, cells were washed and incubated with the relevant streptavidin antibodies as necessary (10-20 minutes at 4°C). To determine frequencies of κ+ and λ+ cells, BM or spleen cells were first stained with the anti-κ or anti-λ alone. Cells were subsequently washed twice and stained with the remaining antibodies in the presence of 1% mouse and rat serum. Total number of BM cells was estimated using the approach of Opstelten and Osmond (Opstelten and Osmond, 1983). For cell sorting of plasma cell populations, BM preparations were depleted of granulocytes using biotin-anti-Gr1, spleens were depleted of T cells using biotin-anti-CD3 and both organs were depleted of red blood cell lineage cells using biotin-anti-Ter119. Depletions were carried out on LS depletion columns (Miltenyi Biotec) and using streptavidin microbeads (Miltenyi Biotec).

Antibodies. Unless noted otherwise all of the following reagents were purchased from eBiosciences: FITC-anti-IgM (R26-46, BD Biosciences), and PNA (Sigma); phycoerythrin (PE)-anti-CD138 (281-2, BD Biosciences); PE- TexasRed-anti-B220 (RA3-6B2); Dump channel was comprised of the following antibodies: PE-Cy7-anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-Gr-1 (RB6- 8C5), anti-F4/80 (BM8), and anti-TER119; allophycocyanin (APC)-Cy5.5-anti-CD19 (1D3); Alexa405 anti-IgD (11-26), FITC-anti-κ (BD Biosciences),

PE-anti-κ (Southern Biotechnology), FITC-anti-λ (BD Bioscinces), PE-anti-λ (Southern Biotechnology) and Biotin-anti-CD138 (281-2, BD Biosciences). Biotinylated antibodies were revealed with Streptavidin-APC-Cy7 (BD Biosciences), Streptavidin-PE-TexasRed or Streptavidin-BrilliantViolet421 (BioLegend). APC-NP was conjugated in our laboratory using standard methods. Nonviable cells were eliminated from all analyses with the UV-excited DNA dye DAPI (Molecular Probes), and doublets were excluded from all analyses using the combined width parameter of the forward and side scatter parameters.

Intracellular Flow Cytometry Staining. For intracellular stains, live cells were identified by pre-incubation with AquaLIVE/DEAD fixable live/dead stain (Invitrogen): 1μL of AquaLIVE/DEAD/1mL PBS, 500μL per organ, 20 min at RT. All washes after the fixing step were carried out at 1800rpm for 7 minutes. After surface antibody staining as previously described, cells were fixed in 100μL of solution A (Caltag) for 15 min at RT, in the dark. Cells were then washed and permebealized with 100μL solution B (Caltag) containing NP-APC, anti-κ-PE or anti-λ-PE for 30 min at RT, in the dark. For staining with anti-BrDU-FITC, cells were further incubated with 1mL freshly prepared DNAse solution (17mL of dH_2O, 2mL 1.5M NaCl, 40μL 1M MgCl2, 0.9mL of 5mg/mL DNAse prepared in dH_2O (Sigma, DN25)). After spinning and decanting the supernatant, cells were incubated with 50μL diluted BrDU-FITC (1:6.25 – 8μL BrDU-FITC into 42μL FACS buffer) and NP-APC (as needed) for 35 minutes at RT, in the dark. After a final wash, cells were resuspended in 400μL FACS buffered and analyzed via flow cytometry.

Flow Cytometry and Cell Sorting. Analyses were carried out on one of two 4-laser LSR II flow cytometers, or a 3-laser FACAria cell sorter (all from Becton Dickinson, San Jose, CA). All flow cytometry data were analyzed by uploading file into FlowJo 8.8 (TreeStar, San Carlos, CA). Data collected on the LSR II or Aria were subjected to the data transformation algorithm in FlowJo that allows negative cell populations to be viewed as symmetrical clusters (Herzenberg et al., 2006). Multiple files per sample were often concatenated before analysis, allowing for the visualization of $>10^7$ cells per file. For cell sorting, stained cells were applied to the FACAria at a sheath pressure of 70 psi and a drop delay frequency of approximately 98000 drops/s. This resulted in sort trigger rates of 28000 to 30000 cells/s.

cDNA preparation and RT-PCR. BM or spleen cells were sorted directly into 200 μL RNeasy RLT buffer from the RNeasy kit (Qiagen) and RNA processed according to the manufacturer's instructions. cDNA was prepared using the First Strand cDNA Synthesis Kit (Roche). Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out using 5ng cDNA, Taqman reaction mix (Taqman) and inventoried Taqman probes for CD19, Pax5, Blimp1 and c-myc. GAPDH rRNA served as an endogenous control for all samples. Analyses were performed on an ABI Prism 7300 system (Applied Biosystems). Relative transcript abundance was determined with the ΔΔCT method.

***In vivo* BrdU labelling.** Adult B6 mice were fed drinking water containing 0.5mg/ml BrdU (Sigma) and 1mg/ml sucrose. For 1 hour BrDU pulses, mice were injected with 200μL

intraperitoneally with 0.5mg of BrDU in sterile PBS (2.5mg/mL BrDU). Flow cytometric analysis of BrdU incorporation was accomplished as described above.

BM chimeras. Donor cells from $RAG^{-/-}$ were purified as described above; C57BL/6 hosts were lethally irradiated (900R) on the day of cell injection. $1x10^6$ BM cells were retro-orbitally injected into anesthetized hosts (anaesthesia: 200μL intraperitoneal injection of 100mg/mL ketamine and 20mg/mL xylazine in PBS). Hosts were maintained on water containing a Bactrim suspension (400 mg sulfamethoxazole and 80 mg trimethoprim/500 mL water) for 4 weeks following lethal irradiation.

Immunizations. 8-12 week old mice were immunized intraperitoneally (i.p.) with 50μg NP16-CGG in alum or 50μg NP0.6-LPS in PBS.

Preparation of NP-CGG/alum. Add 400μL, 2.5mg/ml NP-CGG (Biosearch Technologies N-5055-5, conjugation ratio ~16, diluted in 2mL sterile PBS) to 2mL sterile PBS (for final concentration of 0.5mg/ml). Note: you may scale this protocol linearly to make larger amounts of the immunogen. Next, add 4mL Aluminum Potassium Sulfate (Sigma A-7167) 10% solution in sterile dH_2O (Dilute Aluminum Potassium Sulfate in water and warm in 42°C water bath if it does not go into solution). Adjust pH to 6.5 using 1M KOH dropwise (about 80 drops). Use pH strips to confirm pH in a sterile manner. Color will be yellowish white and cloudy like skim milk. Once pH is adjusted, keep in dark at 4°C overnight. Next day, spin at 2500rpm for 15 min. Pour off supernatant and precipitate

with sterile PBS (it will be the consistency of Crisco). Repeat steps 5 and 6 for a total of 3 rinses. Resuspend precipitant in 2mL sterile PBS for a total volume of 4mL. This is enough for about 15 injections.

CD40-CD154 blockade. On days 29, 31, and 33 post-immunization with NP-CGG/alum, mice were given intraperitoneal inoculations (300mg/injection) of anti-CD154 (MR-1) or control hamster IgG (both from BioXcell) as described by Takahashi et al (Takahashi et al., 1998).

Tamoxifen preparation and administration. Tamoxifen (Sigma, T5648-1g) was reconstituted in corn oil (Sigma, C8267-500 mL) to a concentration of 20mg/ml. Tamoxifen was first reconstituted in 1mL 100% ethanol and vortexed extensively. Subsequently, 25 mL of corn oil was added and incubated at 37°C with frequent vortexing until solution was brought up to 50 mL of corn oil. Note: it takes 2-8 hours for the tamoxifen to go into solution, depending on how often you vortex. 10 mL aliquots were stored at -20°C. 200mg/kg tamoxifen was administered via oral gavage on days 29-31 post-NPCGG immunization and every 2 weeks subsequently.

ELISPOT. Multiscreen HTS plates (Millipore) were coated with 10µg/well of either Goat anti-Mouse Ig(H+L) (Southern Biotech), or NP33-BSA, or NP4-BSA (BioSearch) in sodium bicarbonate buffer. Cells were serially diluted across the plate, and then

incubated for 8-16 hr at 37°C. Biotin-Goat anti-IgG, Goat-anti-IgM, Goat-anti-κ or Goat-anti-λ (Southern Biotech) diluted in block buffer was added, followed by three washes with 0.1% Tween-20 detergent, and a secondary incubation with ExtrAvidin-alkaline phosphatase (Sigma). Spots were detected using BCIP/NBT (Sigma) and scanned and counted with an ImmunoSpot Analyzer (Cellular Technology Ltd.). Number of antigen-specific plasma cells per BM was calculated based on the estimate of $3x10^8$ cells/mouse BM (Opstelten and Osmond, 1983; Osmond, 1986). In NP-CGG/alum immunization studies, background quantified in unimmunized controls was subtracted from the immunized experimental samples; this was particularly important for studies done with anti-κ, anti-λ and anti-IgM antibodies which have a high level of background and was not necessary for NP4-BSA, anti-IgG studies.

Statistical Analysis. Significances in differences in plasma cell frequencies between two experimental groups were evaluated with the unpaired two-tailed t-test using Excel software.

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