REQUIREMENTS FOR MEMORY B CELL AND LONG LIVED PLASMA CELL DEVELOPMENT DURING ADAPTIVE IMMUNE RESPONSES AND *STAPHYLOCOCCUS AUREUS* INFECTION

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ABSTRACT

Amanda Beth Keener: Requirements for memory B cell and long lived plasma cell development during adaptive immune responses and *Staphylococcus aureus* infection
(Under the direction of Barbara Vilen)

Memory B cells and plasma cells (PCs) are terminally differentiated B cells that contribute to recall responses vital for protection against infections. Formation of high affinity memory B cells requires cognate T cell help, somatic hypermutation and affinity maturation within germinal centers (GCs); however, the signals that commit B cells to the GC and the memory pool remain unclear. This work identifies IgG immune complexes (ICs), FcγRs, and BAFF as vital players in the GC response and formation of memory B cells. Early secretion of IgG lead to IC-FcγR interactions that induced dendritic cells to secrete BAFF, which in turn promoted Bcl-6 expression in activated B cells. Loss of FcγRIII, hematopoietic cell-derived BAFF, or blocking Ig-Fc regions all diminished the expression of Bcl-6, the frequency of GC and memory B cells, and secondary antibody responses. This work highlights a key role for IC-FcγR interactions in B cell fate decisions.

ICs may be targeted by pathogens known to disrupt adaptive immune responses, like *Staphylococcus aureus*. Infection with *S. aureus* does not induce long-lived protective immunity. Although vaccine studies in mice and humans support the role of antibodies in protecting against recurring infections, it is not clear whether memory B cells or long-lived plasma cells are formed after *S. aureus* infection. The activity of B cells in response to *S. aureus*

is compromised by Protein A (SpA), a surface protein that interferes with IgG-FcγR interactions and opsonization of the bacteria, and induces polyclonal expansion of VhIII B cells. Using a SpA deletion mutant, we found that GCs, antibody responses, and memory B cell formation were unaffected by the presence of SpA. However, SpA drove an enhanced short-lived extrafollicular response and reduced the pool of long-lived bone marrow resident PCs that were derived from activated memory B cells. This led to a rapid decline in antigen-specific, class-switched antibody. Thus, failure to establish long-term protective antibody titers against *S. aureus* was not a consequence of diminished B cell memory formation, but a lack of long-term antibody maintenance.

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LIST OF ABBREVIATIONS

AID	Activation-induced (cytidine) deaminase
APRIL	A proliferation-inducing ligand
ASC	Antibody secreting cell
BAFF	B cell activating factor
BCMA	B cell maturation antigen
BCR	B cell receptor
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cell
BMMF	Bone marrow-derived macrophage
CM	Conditioned media
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
F(ab)	Antigen-binding fragment (of Ig)
Fc	Constant fragment (of Ig)
FcγR	Receptor for the constant fragment of Ig
FDC	Follicular dendritic cell
GC	Germinal center
i.p.	Intraperitoneal
IFN	Interferon
Ig	Immunoglobulin
IV	Intravenous
LLPC	Long-lived plasma cell

LPS	Lipoploysaccharide
MF	Macrophage
MRSA	Methicillin-resistant *Staphylococcus aureus*
NP-KLH	(4-hydroxy-3-nitrophenyl)-acetyl hapten conjugated to Keyhole limpet hemocyanin
OVA	Ovalbumin (egg protein)
PB	Plasmablast
PC	Plasma cell
pDC	Plasmacytoid dendritic cell
s.c.	Subcutaneous
SpA	Staphylococcus Protein A
TACI	TNFR homolog transmembrane activator and Ca^{2+} modulator and CAML interactor
Tfh	T follicular helper
Tg	Transgenic
TLR	Toll-like receptor
Vh	Heavy chain variable region (of Ig)
WT	Wild-type

CHAPTER1: INTRODUCTION

Adaptive immune responses

Successful activation of the adaptive immune response results in long-term immunological memory, which protects against re-infection. Memory B and T cells are long-lived, antigen-specific cells with the capacity to mount rapid and expanded responses upon secondary exposure to antigens or pathogens [1]. Memory responses form the basis of vaccination and protection from re-exposure to commonly encountered pathogens. Despite this, the adaptive immune mechanisms required for memory, particularly for memory to bacterial pathogens, are not completely understood. This understanding is needed in order to design vaccines that are best suited for their target pathogens and target populations.

Antibody production by B cells is a major arm of immune memory. The selection and survival of memory B cells during a primary adaptive immune response is a carefully regulated process. Initial activation of naïve B cells via B cell receptors (BCRs) causes them to migrate to T cell-rich areas of secondary lymphoid organs in search of cognate interaction and co-stimulation [2,3]. T cell help allows B cells to respond to antigen and proliferate. Proliferating cells differentiate into short-lived antibody-secreting cells (ASCs) or are recruited into structures called germinal centers (GCs) within the B cell follicle [4,5]. Both pathways can result in BCR isotype switching from immunoglobulin (Ig) M or D to IgG since activation-induce deaminase (AID) is activated during the initial T:B interaction [2].

Both ASC and GC pathways may also be T cell-dependent or independent [6,7]. High affinity or avidity BCR ligation and/or Toll-like receptor (TLR) signaling may initiate T-independent B cell responses [6,8]. In general, T-independent responses drive short-term proliferation of ASCs in extrafollicular foci, (though extrafollicular responses may also involve T cell help), and T-dependent responses promote GC recruitment.

The Germinal Center

Early in the immune response, follicular dendritic cells (FDCs) create a reticular network that presents Ag to B and T cells and secretes chemokines such as CXCL13 to bring cells together to form GC structures [5,9]. Within GCs, T follicular helper cells (Tfh) provide co-stimulation and survival signals to GC B cells, through interactions like CD40:CD40L and ICOS:ICOSL [10]. More recently, Tfh cells have been shown to produce local levels of BAFF within GCs to support affinity maturation of GC B cells in closest contact with Tfh cells [11].

Tfh cells and FDCs provide survival signals for GC B cells as they undergo clonal expansion, somatic hypermutation, and affinity maturation. Over time, B cell clones with BCRs of high affinity and specificity for antigen are selected for survival, while other clones die via apoptosis [9,12]. During this process, expression of the transcription factor, Bcl-6 directs B cells to maintain a GC phenotype and withstand the DNA mutations required for somatic hypermutation [9,13]. Signals from Tfh cells, in particular IL-21, contribute to the maintenance of Bcl-6 expression by GC B cell [13,14]. Although Bcl-6 is required for GC B cells, the factors required to initiate Bcl-6 expression in B cells are not well defined.

Memory B cells

The ultimate products of the GC reaction are memory B cells and affinity matured plasma cells (PCs). The mechanisms that drive one fate over the other are on an ongoing area of investigation. Expression of the transcriptional master regulator, Blimp-1, along with XBP-1 and IRF-4, direct plasmablast (PB) and PC differentiation [15–17]. The transcriptional control of memory B cell development is less clear. Blimp-1 and Bcl-6 are mutually repressive [13,18,19], but complete differentiation of memory B cells requires that Bcl-6 ultimately be downregulated [18]. Further, Bcl-6 and GCs are dispensable for generating low-affinity, under-mutated memory B cells [18,20], although secondary responses are impaired when the primary GC reaction is disrupted [1,10].

Memory B cells are long-lived, quiescent, antigen-specific cells, capable of mounting a rapid and robust antibody response upon re-exposure to antigen [21,22]. Classically, memory B cells have been defined as having high affinity class-switched BCRs; however, in recent years, it has become clear that memory B cells can be formed without class switching or even T cell help [23,24]. Both IgM^+ and IgG^+ memory B cells participate in rapid secondary responses; IgM^+ memory cells contribute to secondary GCs and IgG^+ memory cells produce PBs/ASCs that make high affinity IgG [25–27].

Memory B cells are uniquely poised to respond quickly to their cognate antigen. Memory B cells typically localize close to T cells and constitutively express co-stimulatory molecules, which allows them quickly obtain T cell help in a secondary response [22,26,28]. Both circulating IgG from the primary response and high BCR affinity improve antigen presentation

and T cell help [28]. Finally, genetic and epigenetic changes to anti-apoptotic and pro-cell cycle genes allow memory B cells to survive for long periods of time and divide rapidly when they re-engage antigen [22,29].

Plasma cells

Plasma cells are terminally-differentiated B cells that secrete antibody but do not divide. There are many paths leading to PC differentiation. First, primary T-dependent or T-independent responses can lead to short-term proliferation of antibody-secreting, dividing PBs in the extrafollicular spaces of secondary lymphoid organs [6,30]. These cells produce an early surge of low affinity antibody and may undergo somatic hypermutation outside the GC [31]. As PBs mature, they lose surface expression of B cell markers like B220, CD19 and the BCR and cease dividing and become PCs [2,30]. Second, PCs may come out of the GC reaction. Post-GC PCs typically secrete more highly mutated, higher affinity antibody [32]. Third, during a secondary immune response, activated memory B cell clones divide and differentiate into PBs, many of which mature into PCs [32–37].

Long-lived Plasma Cells

Most PBs and PCs die within two weeks of forming [8,38,39], but some, particularly those derived from GCs or memory B cells, migrate to survival niches in the spleen, bone marrow (BM), or at sites of inflammation, and become long-lived plasma cells (LLPCs) [32]. LLPCs constitutively secrete antibody for years and perhaps for the lifetime of an organism [36,40]. It is not yet clear whether subsequent infections produce PCs that oust previously established LLPCs from long-term niches. There is evidence that this may be the case during severe infections [34,41], but not after immunization with common vaccines like polio, tetanus,

or diphtheria [42]. Activation of memory B cells results in the largest proportion of LLPCs, but they can be formed after primary immune responses [12,43]. It has been speculated that intermittent activation of memory B cells is required to repopulate the LLPC niche over time, but most evidence indicates that LLPCs persist in the absence of antigen [43] and are regulated independently of memory B cells [40,44,45].

There are no defining markers of LLPCs or of ASCs that are destined to become LLPCs. Blimp-1 is highest in BM LLPCs, and Blimp-1 expression increases in splenic ASCs as they mature, but it's not clear if high Blimp-1 expression in the spleen is a pre-requisite for longevity in the BM [15]. Short and long-lived PCs express the glycoprotein CD138, which binds extracellular matrix, acts as a ligand for the PC survival factor, APRIL (A Proliferation-Inducing Ligand), and is expressed at higher levels as PCs mature [30,46,47]. T cell help and affinity maturation within the GC have both been implicated as prerequisites for LLPC differentiation [32,48]; however, it is possible for LLPCs to form without class switching or going through a GC reaction [49].

PBs and PCs express chemokine receptors that direct them to extrafollicular spaces, toward medullary cords of secondary lymphoid organs, and out of those organs into the blood [2]. These include CXCR4, which follows CXCL12 toward extrafollicular spaces and later in the response, toward the bone marrow [33,50,51]. $S1P_1$ is also required to exit secondary lymphoid organs into the blood [52,53].

Though it is not clear whether the fate of LLPCs is designated before or after entry into the BM niche, it is clear that both PCs and PBs reach the niche and must compete for limited survival factors. BM niche requirements are not completely defined, but they include stromal

cells, monocytes, eosinophils, and megakaryocytes [50,54]. Niche cells secrete APRIL, BCMA, CXCL12 and TNF-alpha [50,54–56]. These survival signals activate anti-apoptotic factors like Mcl-1 [57] and proteins like XBP-1 and ATG5 to regulate endoplasmic reticulum stress caused by constitutive Ig production[58–60]. Recently, the protein ZBTB20 has been described as a vital BM PC survival factor that is regulated as a result of adjuvant present during the immune response [61]. This supports the possibility that PC longevity is determined in secondary lymphoid organs before migration to the BM.

Long-term immunity and Staphylococcus aureus *infection*

LLPCs play an important role in immunity to viral, parasitic, and bacterial infections [35,44,49]. LLPCs constitutively secrete protective antibody that acts as an early line of defense and enhances secondary responses [35,49,62]. Therefore, it is vital to understand the mechanisms required for LLPC persistence. In the case of *Staphylocuccus aureus* infection, long-term maintenance of antigen-specific antibody is protective in humans [63–66]. However, *S. aureus* infections commonly recur and humoral immunity wanes unpredictably [67–71].

S. aureus is one of the most common causes of skin and soft-tissue infections and up to 65% of *S. aureus* infections are methicillin-resistant (MRSA), which is difficult to treat and life-threatening when it causes a systemic infection [72–74]. Therefore, there is great interest in generating a vaccine to protect vulnerable populations against *S. aureus* infection. However, it remains unknown whether memory B cells or LLPCs are formed in response to *S. aureus* infection.

S. aureus expresses many virulence factors that allow it to avoid or manipulate the immune response to infection and allow the bacteria to form abscesses in the skin or organs

[75,76]. The surface-expressed Staphylococcus protein A (SpA) is well known for its ability to impede B cell responses. SpA interferes with IgG-Fc receptor (FcγR) interactions by binding the Fc regions of IgG, interfering with opsonization and phagocytosis of *S. aureus* [77–79]. Work from our lab (Chapter 2) demonstrates that immune complex (IC) ligation of FcγRs induces B cell activating factor (BAFF) production by dendritic cells (DCs), which in turn directs B and T lymphocyte populations to generate GCs and memory. Therefore we hypothesized that SpA blocks BAFF production by interfering with IC- FcγR interactions thereby reducing DC BAFF production and memory B cell formation. We used an isogenic *Δspa* mutant to examine DC BAFF (Appendix 1), B cell memory, and GC formation (Chapter 3) in the presence of absence of SpA during *S. aureus* exposure or infection. We found that SpA did not interfere with any of these outputs, and characterization of the primary B cell response to *S. aureus* revealed that memory B cells and secondary amnestic antibody responses were not affected by SpA.

In addition to its interference with IgG- FcγR interactions, SpA can act as a super-antigen that binds naïve B cell receptors of the VhIII clan (up to 50% of naïve B cells in humans and 5-10% in mice) [80,81], and drives polyclonal B cell proliferation [75,82]. Injection of purified SpA can also induce to B cell deletion due to the absence of a second signal, like TLR stimulation or T cell help [83]. Immunization with a SpA variant that cannot bind IgG Fc or IgM induces production of neutralizing anti-SpA antibodies and mice that produce such antibodies also produce higher titers of more diverse anti-*S. aureus* antibody specificities upon challenge infection. These qualities have led to the hypothesis that SpA prevents B cell participation in the immune response to *S. aureus* [84]. We reasoned that SpA present during challenge infection may impede or alter memory B cell responses through its IgG-binding and/or super-antigen activity. We found that PB numbers were significantly increased by WT *S. aureus* compared to

challenge with *Δspa.* LLPCs, however, were only detectable after challenging mice with an *Δspa*, suggesting that SpA impedes either the migration or maturation of PC, or the retention and maturation of PC once they reach the BM. The physiological consequence of the lack of LLPC in WT-challenged mice was the waning of antigen-specific IgG production. In Chapter 3, the nature of the secondary PB and PC response is examined in detail in an effort to determine how SpA impedes LLPC accumulation and antibody maintenance.

CHAPTER 2: IgG immune complexes promote B cell memory by inducing BAFF[1]

Introduction

Adaptive immunity requires the commitment of activated B cells to either the memory or PC compartments, the differentiation of $CD4^+$ T cells to Tfh cells, and coordinated expression of chemoattractant receptors to position T and B cells within the follicle for cognate interactions [3,85,86]. The specialized microenvironment of the GC provides a site for rapid expansion and selection of B cell clones whose somatically mutating Ig V regions compete for a limiting amount of antigen displayed on follicular dendritic cells [9,87–90]. Although many steps in the cyclic process of somatic hypermutation and clonal selection are defined, the events required to induce memory cell responses are incompletely understood [91–94].

During the GC response, Tfh cells are critical effectors that provide help to B cells [3,95]. Tfh cells engage activated B cells at the T:B border, and their secreted cytokines promote Ig isotype switching and the selection of cells with high affinity B cell receptors in GCs [13,85,96,97]. The expression of CXCR5, ICOS, PD-1, and the secretion of IL-21 distinguish Tfh cells from other $CD4^+$ T cell subsets [98,99]. The formation of Tfh cells is dependent on the

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expression of Bcl-6, a process linked to ICOS expression on $CD4^+$ T cells [99,100] and influenced by IL-2 [101,102]. This commits primed T cells to the Tfh pool and inhibits their differentiation to other T cell subsets [103–105]. Bcl-6 is also required for GC B cell formation [103,106,107]. In activated B cells, Bcl-6 downregulates Blimp-1; thus, directing B cells away from PC differentiation and toward the memory pathway [17,108]. Cytokines such as IL-6 and IL-21 have been shown to affect Bcl-6 expression in B and T cells [14,109]; however detailed events upstream of Bcl-6 expression are of interest in understanding B and T cell differentiation in GC responses.

BAFF plays an essential role in controlling the development and survival of B2 and marginal zone B cells [110,111], enhancing PB survival [112], and the survival of affinity-matured B cells in the GC [11]. Early studies that neutralized BAFF suggested BAFF played a role in the GC response; however, interpretations of those results were complicated by the loss of B cells associated with BAFF depletion [113–116]. Others have shown that BAFF and anti-CD40 increase ICOSL expression on B cells [117], and that TACI serves to limit the expression of ICOSL and the expansion of Tfh cells and GC B cells [118]. These studies suggest an indirect role for BAFF family members and CD40L in the formation of Tfh cells through ICOS:ICOSL interactions. Thus, BAFF has been implicated in events that contribute to GC responses; however, how BAFF is induced and where it acts in the GC response remains unclear.

In this study we identify a previously unrecognized role for IgG-ICs, CD16, and BAFF in the formation of B cell memory. We found that early production of anti-NP-IgG promotes the formation of ICs that activate DCs through CD16. This induces the secretion of BAFF, which acts upstream of Bcl-6 to promote the formation of GC B cells and proper memory cell

formation. Although BAFF is not involved in the formation of Tfh cells, it plays a role in stabilizing the population at the peak of the GC response. Thus, IgG-ICs and FcγRs, through BAFF, act at or upstream of Bcl-6 expression in GC B cells and in the maintenance of Tfh cells to support the formation of B cell memory.

Results

BAFF$^{-/-}$ bone marrow chimeras exhibit reduced secondary responses

Previous studies have linked BAFF with an enhanced response to vaccination, suggesting that it plays a role in adaptive immune responses [119–121]. To assess this, we generated BAFF$^{-/-}$ bone marrow chimeras by engrafting irradiated B6 mice with B6 (BAFF$^{+/+}$) or BAFF$^{-/-}$ bone marrow. This approach limits BAFF deficiency to hematopoietic cells, allowing other sources of BAFF to maintain the peripheral B cell population [122] (data not shown). In BAFF$^{-/-}$ chimeras, we found that the primary IgG response to NP_{14}KLH (**Figure 1A**) was comparable to B6 control chimeras. However, 14 days after secondary immunization, the BAFF$^{-/-}$ bone marrow chimeras showed a 2-fold reduction in the levels of IgG compared to B6 chimeric control mice (1.4-fold lower on day 42) (**Figure 1B**). The reduced IgG during the secondary response could reflect diminished class switch since BAFF can induce AID expression [123,124]. However, AID mRNA levels in B cells from B6 control and BAFF$^{-/-}$ chimeras were not different (data not shown), suggesting that BAFF has a role other than in class switch.

BAFF$^{-/-}$ chimeras exhibit defects in the frequency of memory B, Tfh cells, and GC B cells

Rapid, high titer secondary immune responses require the activation of memory B cells [1]. Although IgG memory B cells do not require BAFF for maintenance [125], it is not known whether BAFF is important for their formation. To determine whether BAFF affects the frequency of memory B cells, we immunized BAFF$^{-/-}$ chimeras with NP_{14}KLH and measured the frequency of NP-specific memory B cells ($CD19^{+}IgG^{+}$Ac38 Id^{+}) on day 28 post-immunization. We found that in B6 mice, immunization significantly increased the frequency of memory B cells, but not in the BAFF$^{-/-}$ bone marrow chimeras (**Figure 1C**).

Tfh cells are critical to the early GC response and required for the differentiation of memory B cells [126–128]. It is possible that BAFF affected memory responses by influencing germinal center responses and/or Tfh cells. To assess whether BAFF affects formation and maintenance of Tfh cells, B6 and BAFF$^{-/-}$ chimeras were immunized and the frequencies of Tfh cells (CXCR5^{+}PD-1^{+}CD4^{+}) were quantitated on days 3 and 7 post-immunization (**Figure 1D and 1E**). On day 3, the frequency of Tfh cells in B6 chimeras increased by 1.5-fold while in BAFF$^{-/-}$ chimeras it increased 1.2-fold. This suggests that BAFF does not play a significant role in the formation of Tfh cells. However on day 7 post-immunization, B6 chimeras displayed an increased frequency (1.9-fold) of Tfh cells, whereas their frequency in BAFF$^{-/-}$ chimeras did not change. This suggests that BAFF may stabilize Tfh cells or support their expansion.

Germinal centers are necessary for the formation of high affinity, class-switched memory B cells [129,130]. To determine whether BAFF impacted the frequency of GC B cells, we enumerated CD19^{+}GL-7^{+}CD95^{+} GC B cells 7 days after immunization. In BAFF$^{-/-}$ chimeras, the frequency of total (**Figure 1F and 1G**) and Ag-specific (**Figure 1H**; CD19^{+}Ac38^{+}IgG^{+}GL-7^{+}CD95^{+}) GC B cells in BAFF$^{-/-}$ chimeras was lower than the response in B6 chimeras. Thus, BAFF significantly contributes to antigen-specific GC responses.

BAFF acts at or upstream of Bcl-6 expression in B cells

Bcl-6 plays a critical role in initiating GC responses and committing activated B cells to a memory cell phenotype [106,131,132]. Thus, one possibility is that BAFF affected Bcl-6 expression. To test this, we measured Bcl-6 levels in GC B cells after immunization. We found that on day 7, the levels of Bcl-6 in GC B cells from immunized BAFF$^{-/-}$ chimeras were lower than in B6 chimeras (**Figure 1I and 1J**). The data show that hematopoietic cell-derived BAFF

acts at, or upstream of, Bcl-6 expression in B cells. Collectively, our data indicate that BAFF impacts the expansion and stabilization of the Tfh cell pool, and the formation of GC and memory B cells.

DC-derived BAFF regulates the frequency of GC B and Tfh cells

Myeloid cells are a major source of BAFF following infection or immunization [112,119,133]. To assess whether the source of BAFF that impacted the GC response was derived from DCs or macrophages (MFs), we adoptively transferred bone marrow-derived dendritic cells (BMDCs) and bone marrow-derived macrophages (BMMFs) from BAFF transgenic (Tg) mice into $BAFF^{-/-}$ chimeras by subcutaneous injection (s.c.) at the time of immunization. We reasoned that if BAFF was required for the maintenance of Tfh cells or the formation and/or maintenance of GC B cells, then enforced expression of BAFF by DCs and/or MFs might restore their frequencies. We previously established that 70% of s.c. injected BMDCs migrated to the inguinal lymph nodes, and that the magnitude of the subcutaneous anti-NP response was comparable to intraperitoneal (i.p.) immunization (data not shown). We found that constitutive expression of BAFF by Tg DCs, but not Tg MFs, restored the frequencies of GC B cells (**Figure 2A),** Tfh cells **(Figure 2B**), and the levels of Bcl-6 in GC B cells (**Figure 2C**). Conversely, transfer of $BAFF^{-/-}$ DCs did not increase the frequencies of GC B cells **(Figure 2A)**, indicating that the effects were not due to an increased number of DCs, or the secretion of other cytokines through activation of the transferred DCs. Thus, the previously reported role for DCs in the induction of GC and Tfh cells [134] might also involve BAFF.

The binding of immune complex to FcγRs induces BAFF secretion

Previous studies showed that exogenous ICs induce BMDCs to secrete a number of cytokines, including BAFF [135,136]. In another study, mice lacking the common gamma chain of the FcγRs ($Fc\gamma_c$) exhibited diminished secondary immune responses [62]. Because our data suggest that DCs may promote secondary responses via BAFF, we postulated that ICs formed by the early IgG antibody response might induce DCs to secrete BAFF. This model requires that the early IgG response occur concurrently, or precede BAFF secretion. To test this, we harvested spleens from B6 mice on days 2, 3, and 7 following NP_{14}KLH immunization and used ELISpot to measure the numbers of antibody (IgG) secreting cells (ASCs) and BAFF-secreting DCs. NP-specific (IgG) ASCs were present in the spleen by day 2 post-immunization and expanded over the course of 7 days (**Figure 2D**). Similarly, the number of splenic $CD11c^+$ DCs that secreted BAFF increased 6-fold between days 2 and 7 (**Figure 2E**). Thus, secretion of Ig by B cells, and production of BAFF by DCs, occurs simultaneously beginning approximately 2 days following immunization. This is consistent with the idea that IgG-ICs induce BAFF, which contributes to GC responses.

To further test the idea that IgG-ICs induce DCs to secrete BAFF, we blocked IgG-Fc:FcγRs interactions *in vitro* and assessed whether this impacted BAFF secretion by DCs. Stimulation of B6 BMDCs with pre-formed IgG-ICs (IgM bound by anti-μ) induced a1.8-fold increase in BAFF secretion (**Figure 2F**). This was not unique to IgM/IgG ICs because preformed anti-NP ICs (NP-OVA bound by anti-NP) stimulated a dose dependent 2.5-fold increase in BAFF secretion (data not shown). However, co-culture with a tetrameric tripeptide (TG19320) that blocks IgG Fc regions [137–139] reduced the number of BAFF-secreting DCs to levels indistinguishable from unstimulated cells.

To assess whether blocking Fc:FcγR interactions affected the adaptive immune response *in vivo*, we administered TG19320 at the time of immunization and measured the frequencies of GC B and Tfh cells. We found that co-administration of TG19320 with NP_{14}KLH blocked an increase in GC B and Tfh cells on day 7 (**Figure 2G and 2H**). This indicates that the interactions between IgG-ICs and FcγRs are necessary for optimal GC responses and for the stabilization of Tfh cells in response to immunization. Collectively, these data identify a mechanism wherein IgG-ICs formed early in the immune response ligate FcγRs on DCs to induce BAFF secretion, which in turn contributes to the GC response.

BAFF regulates the expression of Bcl-6 in activated B cells in vitro

To define whether the effects of BAFF on activated B cells was direct, we established an *in vitro* reconstitution system using the expression of Bcl-6 as a marker of memory B cell commitment.-In this model, B6 B cells were stimulated with anti-μ in combination with IL-4 and IL-5 to induce Ig secretion and a low level of Bcl-6 expression (**Figure 3A and B**). These activated B cells were then cultured in conditioned medium (CM) prepared from DCs treated with preformed IgG1-ICs (DC CM). Addition of DC CM increased Bcl-6 expression to approximately 3-fold over unstimulated B cells (**Figure 3A and 3B**). These levels were comparable to those achieved with recombinant BAFF. Bcl-6 expression was dependent on Ig-Fc regions as CM from DCs stimulated with $F(ab')_2$-containing ICs were ineffective at inducing Bcl-6 (**Figure 3C**). In this *in vitro* system, Bcl-6 expression was not induced in B cells cultured with DC CM where BAFF was neutralized with BR3-Fc, or where DC CM was made from $BAFF^{-/-}$ DCs (**Figure 3C**). As Bcl-6 levels become elevated, the plasma cell program is attenuated [17,140]. To further validate the *in vitro* model, we measured intracellular IRF-6 and

XBP-1 and found that DC CM diminished the levels of both transcription factors by 2.2-fold (**Figure 3D-G**), indicating that BAFF may act at or upstream of Bcl-6, directing B cell differentiation away from a plasma cell fate.

IgG-ICs bind CD16 during the anti-NP response

We reasoned that if FcγR stimulation promoted BAFF secretion, loss of the FcγR(s) that binds IgG1-anti-NP ICs would negatively affect the ability of DC CM to promote Bcl-6. To assess this, we tested whether CMs from BMDCs derived from B6 mice, or mice deficient in CD64 (FcγRI$^{-/-}$), CD32 (FcγRIIb $^{-/-}$), CD16 (FcγRIII $^{-/-}$), or CD16-2 (FcγRIV$^{-/-}$) induced Bcl-6 in the *in vitro* reconstitution system described above. DC CM from CD16$^{-/-}$ mice failed to induce Bcl-6 expression, while CMs from all other FcγR deficient mice induced Bcl-6 to levels comparable to, or above those induced by B6 DC CM (**Figure 3H**). This suggests that ligation of CD16 on DCs is required for the expression of Bcl-6 in B cells.

Impaired secondary responses in CD16$^{-/-}$ mice

Our data indicate that CD16 might be responsible for inducing DCs to secrete BAFF after NP_{14}KLH immunization. To test this *in vivo,* we quantitated the number of splenic BAFF secreting DCs in B6 and CD16$^{-/-}$ mice 7 days after immunization (**Figure 4A**). In the absence of CD16, we found that the number of BAFF secreting DCs was markedly diminished compared to wild type mice. This is consistent with the idea that CD16 is the FcγR responsible for initiating the IgG1-dominant immune response to NP-KLH [130].

To test whether loss of CD16 *in vivo* impaired adaptive immune responses, we measured primary and secondary antibody responses in B6 and $CD16^{-/-}$ mice following $NP_{14}KLH$ immunization. As in the $BAFF^{-/-}$ chimeric mice, the primary IgG response in $CD16^{-/-}$ mice was comparable to B6 mice (**Figures 4B**). In the secondary response, the levels of IgG in B6 mice increased 1.7-fold on day 42 (7 days after boost), and 4.5-fold on day 49 (14 days after boost), while IgG levels in the $CD16^{-/-}$ mice did not increase on days 42 or 49 (**Figure 4C)**. This was not an indirect consequence of altered cell populations due to CD16 deficiency since the frequencies of DCs, T, and B cells in $CD16^{-/-}$ mice were not different than B6 mice (**data not shown**). This indicates that CD16 plays a role in generating memory B cells and secondary immune responses to NP-KLH.

$CD16^{-/-}$ mice exhibit defects in forming Tfh and memory B cells

The loss of DC-derived BAFF in $CD16^{-/-}$ mice supports a role for CD16 in the memory response to $NP_{14}KLH$. To assess whether the diminished secondary response in $CD16^{-/-}$ mice reflects a reduction in memory B cells, we assessed the frequency of NP-specific memory B cells ($CD19^{+}Ac38^{+}IgG^{+}$) in B6 and $CD16^{-/-}$ mice 28 days following immunization. $CD16^{-/-}$ mice showed a 2.4-fold decrease in the Ac38 Id^{+} IgG memory B cell population compared to immunized B6 mice (**Figure 4D and 4E)**, indicating that CD16 regulates memory responses in part through BAFF production.

Our data with immunized $BAFF^{-/-}$ chimeras suggests that BAFF contributes to B cell memory by affecting the GC B and Tfh cell pool. If the source of BAFF was exclusive to IgG-ICs, then loss of CD16 would also diminish the GC B and Tfh pools. To assess this possibility,

we quantitated Tfh cells ($CD4^+CXCR5^+PD\text{-}1^+$) from immunized B6 and $CD16^{-/-}$ mice. After 3 days, $CD16^{-/-}$ mice had a comparable frequency of Tfh cells compared to immunized B6 mice (**Figure 4F**). However on day 7, $CD16^{-/-}$ mice had 3-fold fewer Tfh cells, similar to the defects observed in immunized $BAFF^{-/-}$ chimeras. Adoptive transfer of BAFF transgenic, but not $BAFF^{-/-}$ BMDCs into immunized $CD16^{-/-}$ mice, restored the frequency of Tfh cells on day 7 to levels seen in B6 mice (**Figure 4G**), suggesting that the defect was a consequence of reduced BAFF in $CD16^{-/-}$ mice. These findings demonstrate that CD16 ligation by IgG-ICs induces DCs to secrete BAFF and that CD16 is necessary for memory responses. Together with the findings from the $BAFF^{-/-}$ chimeras, our data suggest that IgG-IC binding to CD16 contributes to B cell memory through the effects of BAFF on Tfh cells and GC B cells.

Loss of CD16 diminishes germinal center responses

To assess whether loss of CD16 diminished GCs, we measured the frequency of splenic GC B cells ($CD19^+GL\text{-}7^+CD95^+$) and Ac38 Id^+ GC B cells 7 days after immunization. We found that both subsets of GC B cells were significantly reduced in $CD16^{-/-}$ mice (**Figure 5A-C**). Consistent with the diminished frequency of GC B cells, we found that the GCs in $CD16^{-/-}$ mice were 54% smaller on day 7 post-immunization, but as the size of the B6 GCs declined (by day 14 and 21) the sizes became similar (**Figure 5D**). The smaller size of the $CD16^{-/-}$ GC on day 7 was not the result of delayed kinetics because by day 28, the GCs in both B6 and $CD16^{-/-}$ mice were diminished, suggesting a comparable duration of the response (**data not shown**). We also found that immunized $CD16^{-/-}$ mice displayed a 2.3-fold reduction in the number of GCs on day 7; however, the differences were less apparent on day 14. By day 21, the $CD16^{-/-}$ mice had a similar number of GC structures (**Figure 5E**). Thus, although GCs form in the absence of CD16,

they are reduced in number and size early during the immune response. This suggests that stimulation of CD16 is an early event that impacts the GC response and subsequently, the memory B cell pool.

CD16 and BAFF are required for the expression of Bcl-6 and the formation of GC and memory B cells

Our data indicate that CD16 and ICs are required for DCs to make BAFF in response to NP-KLH **(Figure 4A**). To address whether DC-derived BAFF was sufficient to restore the GC and memory B cell pools in the $CD16^{-/-}$ mice, we adoptively transferred BMDCs from BAFF Tg mice at the time of immunization. We found that BAFF Tg DCs restored the frequency of GC B cells on day 7 (**Figure 6A**), and the frequency of Ac38 Id^{+} memory B cells on day 28 (**Figure 6B**). In contrast, BMDCs derived from $BAFF^{-/-}$ mice were unable to restore GC or memory B cells (**Figure 6A and B**), indicating that this effect was due to BAFF and not due to an increased number of DCs, or the secretion of other cytokines through activation of the transferred DCs. Since $BAFF^{-/-}$ DCs have intact CD16, the results also emphasizes that BAFF, induced by CD16 ligation, impacts the GC response and the frequency of memory B cells.

Our data suggest that DC-derived BAFF acts at or upstream of Bcl-6 (**Figure 1I, J, 2C, 3A-C**) and downstream of CD16 (**Figure 4A**). Thus, the absence of CD16 should also diminished Bcl-6 during NP immunization and restoring DC-derived BAFF by BMDC transfer should restore Bcl-6 levels. To test this, we adoptively transferred BAFF Tg BMDCs into $CD16^{-/-}$mice at the time of immunization. We found that compared to B6 controls $CD16^{-/-}$ GC B cells expressed low levels of Bcl-6 after immunization. Transfer of BAFF Tg BMDCs, but not $BAFF^{-/-}$ BMDCs, restored Bcl-6 levels in $CD16^{-/-}$ GC B cells (**Figure 6C**). Thus, reduced BAFF due to

loss of CD16 impacts Bcl-6 expression in GC B cells and the effects were not influenced by changes in DC numbers or activation of transferred BMDCs. Collectively, the data show that IgG-ICs and CD16 induce BAFF production by DCs. This suggests that BAFF acts upstream of Bcl-6 to induce the formation of GC B cells and to promote the maintenance of Tfh cells thereby promoting effective GC responses and B cell memory.

Discussion

Interactions between T cells, B cells, and DCs are necessary for the proper execution of the adaptive immune response [141–143]. This study identifies a previously unappreciated mechanism for DCs, FcγRs, and BAFF in the early events of the GC response. We show that ICs formed during the early IgG response to NP-KLH induced the production of BAFF by DCs. BAFF acted at, or upstream of, Bcl-6 to promote the optimal formation of GC B cells and in the stabilization of newly formed (day 7) Tfh cells. Loss of BAFF in hematopoietic cells, or loss of CD16, diminished the levels of Bcl-6, the frequencies of GC B cells, Tfh cells and memory cells, and the magnitude of the secondary antibody response. We found that DC-derived BAFF was sufficient to initiate these events. This series of events depends on the formation of IgG-ICs, suggesting that productive early antibody responses contribute to the optimal formation of B cell memory.

IgG-ICs and BAFF in the GC response

ICs have previously been implicated in promoting adaptive immune responses through enhanced Ag presentation [144] and stimulating cytokine production by DCs [135,136]. We observed that IC ligation of CD16 was required for BAFF production by DCs after NP-KLH immunization. Although the loss of $Fc\gamma_c$ reduces secondary antibody responses [62], it is not clear what role IC- FcγR interactions play in GC and memory B cell formation. Our data demonstrate that GCs and memory responses are disrupted in the absence of CD16 or when IC-FcγR interactions were blocked, indicating that FcγR signaling is important for these events. We also detected antigen-specific IgG by ELISpot within 2-3 days of immunization, making it possible for ICs to form and signal BAFF production before the initiation of GCs. This IgG was

likely secreted by class-switched extrafollicular PCs [140] that produce local levels of IgG early in the immune response [106,132,140].

Although at day 7 of the primary response to NP-KLH, GCs, GC B cells, memory B cells and Tfh cells were diminished in the absence of FcγR-IC signaling, they were not completely absent and the numbers of GCs was increased on days 14 and 21. This suggests that signals other than BAFF, and perhaps other receptors for ICs are required for GC responses. We also observed incomplete loss of these populations after immunization of $BAFF^{-/-}$ chimeric mice, suggesting DC-derived BAFF is only one of many signals required for optimal GC formation and function.

Early in the adaptive immune response, B cells interact with cognate T cells and co-stimulatory signals induce AID activation and class switch [145]. These signals include CD40L, which is also required for GC formation [146,147]. We found that $BAFF^{-/-}$ chimeras had normal AID expression and primary IgG production, suggesting that cognate interactions mediated by CD40L were intact. Thus, BAFF likely acts downstream of initial T:B interactions. Our *in vitro* data show that recombinant BAFF directly induced Bcl-6 in activated B cells, and DC CM (elicited under conditions that induce BAFF) diminished the expression of XBP-1 and IRF-4. BAFF, therefore, may act on B cells after cognate T interaction, extinguishing the PC phenotype and either committing cells to the memory pathway or supporting the survival of GC B cell precursors. This is consistent with studies showing that DC secretion of the PC-inducing cytokine, IL-12, is dampened by IC signaling [148].

Another signal required for a productive GC reaction is IL-21. It is possible that BAFF acts in concert with IL-21 to induce and/or sustain expression of Bcl-6 in GC B cells [14,149]. T cell-secreted IL-21 acts on B cells both during initial T:B interactions and after GCs are formed

to promote either Blimp-1 or Bcl-6 expression, depending on the context [18], BAFF may serve as a contextual signal early in the adaptive response to direct B cells toward a GC fate. BAFF may also maintain Bcl-6 in B cells destined for the GC, allowing IL-21 from Tfh cells to take over as the response progresses.

DCs as mediators of IC signaling

Although DCs, MFs, and neutrophils are among the major producers of BAFF, [122,150–154], our data support a role for DCs as the cells responsible for producing BAFF specifically in response to ICs after immunization. We found that transfer of BAFF-producing DCs, but not $BAFF^{-/-}$ DCs, restored the numbers of GC B cells in $BAFF^{-/-}$ chimeras and $CD16^{-/-}$ mice. This indicates that the defects in $CD16^{-/-}$ mice were mediated through the loss of BAFF. Further, BAFF-producing MFs were unable to restore the defects suggesting a unique role for DCs in this process consistent with tripartite interactions between DCs, T cells and B cells during adaptive immune responses. Other cells with Fc receptors may have roles similar to DCs that are worthy of further investigation. This current study indicates that DC-derived BAFF is sufficient to improve T-dependent GC responses and B cell memory. It also supports the use of DCs as a means to augment vaccine responses where better adjuvants are needed.

BAFF and Tfh cells

Previous studies showed that cells with a Tfh phenotype appear by day 3 after immunization [99,155,156]. These cells migrate towards follicles where they interact with B cells at the T-B border [156–159] to promote continuous expression of Bcl-6 [103,105,134,158,160], and entry of Tfh cells into the GC [105,160,161]. Our data suggest that

B cells and IgG-ICs are not involved in the formation of Tfh cells (day 3). This is consistent with previous studies showing that on days 1-3 post-immunization, the expression of Bcl-6 and CXCR5 in $CD4^+$ T cells is independent of B cells [99,134,155]. Instead, BAFF acted downstream of the formation of Tfh cells, possibly to stabilize the population or promote its expansion. Thus, previous studies showing a role for B cells in maintaining Tfh cells [109,155,158] might reflect the need for B cell-elicited Ig, ICs, and DC-derived BAFF. The role of BAFF in maintaining the Tfh population may be indirect. One possibility is that BAFF promotes the expression of ICOSL on B cells. Previous studies showed that signaling through the BAFF-R regulates the expression of ICOSL on B cells [117,162], thereby sustaining interaction between Tfh cells and B cells at the T:B border and within GCs [99,100]. This interaction could also stabilize the expression of Bcl-6 and the downstream molecules CXCR5 and PD-1[105,163,164] to maintain the "Tfh phenotype". This possibility is supported by studies showing that the absence of ICOSL on B cells reduces the frequency of $CXCR5^+$ $CD4^+$ cells after immunization [105], that ICOS:ICOSL interaction prolongs the engagement between B cells and Tfh cells [161], and that follicular bystander B cells support the formation and/or maintenance of Tfh cells by providing ICOSL in an antigen-independent manner [165].

The interaction between GC B cells and Tfh cells is bidirectional. Recent work described Tfh as a source of local BAFF within GC B cells that is required for affinity maturation [11]. Tfh-derived BAFF, however, is not required for GC initiation or maintenance [11], and its role in memory B cell development remains unknown. Our work indicates that early production of BAFF is upstream of GC formation and memory B cell development. Interestingly, we observed a more complete loss of antigen-specific GC B cells ($Ac38^+$) than total GC B cells in $BAFF^{-/-}$ chimeras and $CD16^{-/-}$ mice. This may reflect a loss of local BAFF within the GC due to lack of

Tfh maintenance. However, more work would be needed to determine whether affinity maturation is altered in these models.

Overall, our studies highlight a novel role for IgG-ICs and DC-derived BAFF in the GC response. Elucidating the events that initiate GC responses may impact our understanding of ICs and BAFF in autoimmunity. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by elevated levels of BAFF, autoantibody/autoantigen ICs, and multi-organ pathology. The formation of autoreactive memory is thought to be instrumental in driving long-lived PCs and sustaining autoantibody production [166,167]; however, the mechanisms that regulate memory formation to self-antigens are unclear. Our findings suggest that chronically high levels of ICs containing self-antigens could contribute to a break in B cell tolerance at the GC checkpoint. In SLE, elevated levels of circulating ICs may elevate BAFF and promote the GC response [137,166,168–171]. This suggests that neutralization of BAFF in patients with SLE may affect both B cell survival and GC responses that are necessary in the formation of autoreactive memory cells [110,150,152,172].

Materials and Methods

Animals

B6-Ly5.2 congenic mice were purchased from NCI, BAFF$^{-/-}$ mice (Schiemann et al., 2001), and CD16-2$^{-/-}$ (FcγRIV) mice (Nimmerjahn et al., 2010) were obtained from, Glenn Matsushima, and Charles Jennette at UNC-Chapel Hill. CD16$^{-/-}$ and CD64$^{-/-}$ mice (Hazenbos et al., 1996) were obtained from Dr. Anne Sperling at the University of Chicago, and BAFF Tg mice (Gavin et al., 2005) from Jeffrey Rathmell at Duke University. CD32$^{-/-}$ mice (Takai et al., 1996) were purchased from Jackson labs. Mice were used at 8-12 weeks of age and maintained in an accredited animal facility.

Reagents and Antibodies

Antibodies against mouse CD4, CD19, CD95, GL-7, ICOS, ICOSL, PD-1, and B220-647 were purchased from Biolegend, CXCR5, B220, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 antibodies from BD Biosciences, Bcl-6, XBP-1, and IRF-4 from Santa Cruz, and BAFF (1C9) from Enzo. Anti-μ $F(ab)_2$ was purchased from Jackson ImmunoResearch. Anti-μ (clone B7.6), anti-NP (clones H33L and B1-8), 2.4G2 (FcμRIIb/FcγRIII block) and Ac38 idiotype antibodies were purified from hybridoma supernatants. (Ac38 is an idiotype antibody that recognizes B-1-8 specificities generated during NP immunization). Recombinant mouse BR3-Fc and isotype control protein were generated using mammalian expression systems and standard purification protocols. H33L and B1-8 were gifts from Dr. Garnett Kelsoe (Duke University). IL-4 and IL-5 were purchased from Peprotech, recombinant BAFF (rBAFF) from R&D Systems, NP-OSu from Biosearch Technologies, KLH and PNA-biotin from Sigma Aldrich, alum from Thermo Scientific, and

streptavidin-Alexa 488 and Alexa 647 from Invitrogen. Streptavidin-alkaline phosphatase and anti-IgG alkaline phosphatase were purchased from Southern Biotech. The Fc-binding TG19320 peptide was synthesized as described (Fassina et al., 1998; Marino et al., 2000).

B cell purification and bone marrow derivation of MFs and DCs

Splenic B cells were isolated from B6 mice by negative selection (StemCell Technologies) and were 95-99% pure, as determined by flow cytometry. Splenic DCs were purified by positive selection of $CD11c^+$ cells (Miltenyi) from enriched low-density cells (OptiPrep; Sigma). Purified cells were 80% $CD11c^+$.

Bone marrow-derived DCs (BMDCs) and bone marrow-derived MFs (BMMFs) were prepared from single cell suspensions from the tibias and femurs of B6, $CD64^{-/-}$, $CD32^{-/-}$, $CD16^{-/-}$, and $BAFF^{-/-}$mice. Following RBC lysis, cells were cultured 7 days in a 24 well low-cluster plate (Costar 3471) with 10 ng/ml GM-CSF and IL-4 to derive DCs and in 20 ng/ml M-CSF to derive MFs.

Cell Culture

We pre-formed immune complexes by stimulating B6 B cells (1.5×10^5) with an excess of anti-μ (B7.6; IgG_1; 30 μg/ml). The polyclonal IgM produced after seven days forms a complex with the excess anti-μ resulting in IgG1-IgM ICs in the supernatant. These B cell supernatants were used as a source of ICs in preparing DC conditioned medium (DC CM).

DC CM was prepared by culturing 2×10^4 BMDCs (day 7) in a 96 well plate in the presence of IC-containing supernatants (see above; 20% of volume), IL-4 (25 ng/ml), and IL-5 (25 ng/ml). After seven days, supernatants were harvested and frozen at -80°C.

For *in vitro* co-cultures, 1.5 x 10^5 purified B6 B cells were co-cultured with 1 x 10^4 BMDCs or *ex vivo* DCs in a 96 well plate stimulated with IL-4 (25 ng/ml), IL-5 (25 ng/ml) and 30 μg/ml anti-μ with or without recombinant murine BAFF (5 ng/ml) or DC CM (20% of total volume). Intracellular Bcl-6 was assessed by flow cytometry after 48 hours.

ELISAs

NP-specific IgG levels were quantitated from serum using microtiter plates coated with NP_{13}BSA and blocked with 0.5% BSA. Serially diluted serum samples were incubated overnight at 4^{o}C. Anti-NP was detected using an alkaline phosphatase conjugated rabbit anti-mouse IgG antibody (1/1000 dilution) followed by phosphatase substrate. Optical density (OD) values were converted to concentration based on standard curves using the H33L (anti-NP) hybridoma.

ELISpot

For the analysis of NP-specific B cells, multiscreen ELISpot plates (Millipore) were coated with NP_{13}BSA in PBS and blocked with 1% BSA. Single cell suspensions of spleen were prepared from immunized or naïve B6 mice. After RBC lysis, cells were plated in serial dilutions on washed ELISpot plates. Anti-NP IgG-secreting spots were detected with anti-IgG-biotin and streptavidin-HRP (BD Biosciences). Plates were developed with 3-amino 9-ethylcarbazole.

To enumerate BAFF-secreting DCs, $CD11c^+$ cells (1 x 10^6) were purified from spleens and cultured for 60 hours on BR3-Fc coated ELISpot plates. BAFF-secreting cells were detected using anti-BAFF (clone 1C9). To enumerate BAFF secreting cells from BMDCs, day 7 cells

(2.5×10^5) were plated on ELISpot plates as above and incubated 24 hours with preformed ICs (IgM + anti-μ) prior to addition of 1C9. Anti-μ ICs were made by combining the supernatant from stimulated B cells (20 ng of IgM) with anti-μ (5 μg). In some experiments, TG19320 was added at 50 μg/ml to inhibit IgG binding to Fc□Rs.

Bone Marrow Chimeras

B6-Ly5.2 congenic mice (6-8 weeks of age) were lethally irradiated (10.5 Gy; 1050 rads) and reconstituted with 8×10^6 bone marrow cells from either B6 (B6 control chimeras) or $BAFF^{-/-}$ ($BAFF^{-/-}$ chimeras) mice. After 8 weeks, we monitored reconstitution by assessing the frequency of $CD45.1^+$ and $CD45.2^+$ splenocytes by flow cytometry.

Immunization and Adoptive Transfers of BMMF/BMDCs

B6, $BAFF^{-/-}$ bone marrow chimeras, and $CD16^{-/-}$ mice (8-10 weeks of age) were immunized by intraperitoneal (i.p.) or subcutaneous (s.c.) injection with 100 μg of $NP_{14}KLH$ precipitated in an equal volume of alum (Imject® Thermoscientific). Mice were boosted by intravenous (i.v.) injection with the same dose of soluble $NP_{14}KLH$ at day 35. To assess the contribution of DCs or MFs in the secretion of BAFF, 8×10^6 BAFF Tg or $BAFF^{-/-}$ BMDCs or BMMFs were injected at the time of s.c. immunization. Draining lymph nodes were harvested on day 7 for flow cytometry analysis.

TG peptide injections

B6 mice were immunized with 100 μg $NP_{14}KLH$ in alum (1:1) via i.p. injection and administered three (i.p.) injections (15-30 mg/kg) of Fc blocking peptide (TG19320) or equal amount of unrelated control peptide over the course of seven days.

Flow Cytometry

GC B cells and Tfh were analyzed on day 7 post-immunization and were defined as $CD19^+$, $GL\text{-}7^+$, $CD95^+$ and $CD4^+$, $CXCR5^+$, $PD\text{-}1^+$. Ac38 was used to define NP-specific GC B cells. NP-specific memory B cells were defined as $Ac38^+$ IgG^+ double positive CD19+ lymphocytes. The lymphocyte gate was determined by forward and side scatter properties. To quantitate expression of intracellular IRF-4, Bcl-6 and XBP-1, splenocytes from immunized B6, $CD16^{-/-}$, B6 control chimeras and $BAFF^{-/-}$ chimeras mice were washed, fixed (4% paraformaldehyde), and permeabilized with methanol for a minimum of 24 hours at -20^oC. Fixed cells were washed and FcγRIIb/FcγRIII blocked with 2.4G2 before staining. Data are expressed as fold change in MFI/isotype control MFI.

Real Time PCR

Splenic B cells from B6 and $BAFF^{-/-}$ chimeras were purified after $NP_{14}KLH$ immunization. mRNA was isolated from 5-10 x 10^6 purified B cells and cDNA synthesized using Superscript VILO cDNA Synthesis Kit (Invitrogen). DNA was subsequently amplified using FastStart Universal SYBR Green Master mix (Roche). Relative values were compared using the $2^{-\Delta\Delta CT}$ method. 18s rRNA was used as an internal control in all experiments. Primers included: murine *Aicda* forward 5'GGGAAAGTGGCATTCACCTA3', murine *Aicda* reverse 5'GAACCCAATTCTGGCTGTGT3' murine 18s rRNA forward 5'TCAAGAACGAAAGTCGGAGGTT3', murine 18s rRNA revese 5'-GGACATCTAAGGGCATCACAG-3'.

Germinal Center Staining and Counting

Spleens were harvested from B6 or $CD16^{-/-}$ mice on days 7, 14, and 21 after immunization and flash frozen in OCT (Optimum Cutting Temperature; Fisher). Tissue sections (6 micron) were fixed in 1:1 MeOH/Acetone, blocked with 10% FBS in PBS containing 2.4G2, and stained with PNA-biotin and B220-Alexa647, and Streptavidin-Alexa488. Germinal centers were defined as PNA^+ cell clusters within $B220^+$ follicles (Han et al., 1997). The number of germinal centers per mm^2 of $B220^+$ area was determined by dividing the number of germinal centers counted in a field by the area of $B220^+$ follicles in the same field. This accounted for follicles that were only partially represented in a given field (Holl et al., 2011). This was done for 10-30 fields per mouse, totaling 30-100 follicles per mouse at each time point.

Microscopy

Macroscope images were obtained on a Leica MX16FA fluorescence stereo microscope/macroscope (0.63x objective; numerical aperature of 1.0). Other images were obtained using an Olympus Fluoview 500 (10x objective; numerical aperature of 0.45).

Statistics

The one-sided, or one-sample *t* test was used to compare changes in transcription factor levels and *in vitro* cytokine and antibody secretion to control values. Two-sample student's *t* tests were used to assess statistical differences between cell populations measured by flow cytometry and ELISpot, and for serum antibody secretion on days 42 and 49. Analyses were performed in GraphPad Prism.

Table 2. 1 Mice used for Chapter 2

Mouse line	Description
C57BL/6 (B6)	Wild-type
B6 Chimera	Lethally irradiated B6 reconstituted with congenic B6 bone marrow
BAFF$^{-/-}$	Lacks BAFF
BAFF$^{-/-}$ Chimera	Lethally irradiated B6 reconstituted with congenic BAFF$^{-/-}$ bone marrow
CD16$^{-/-}$	Lacks FcγRIII
BAFF Tg	Constitutively expresses BAFF in Myeloid cells

Chapter 2 Figures

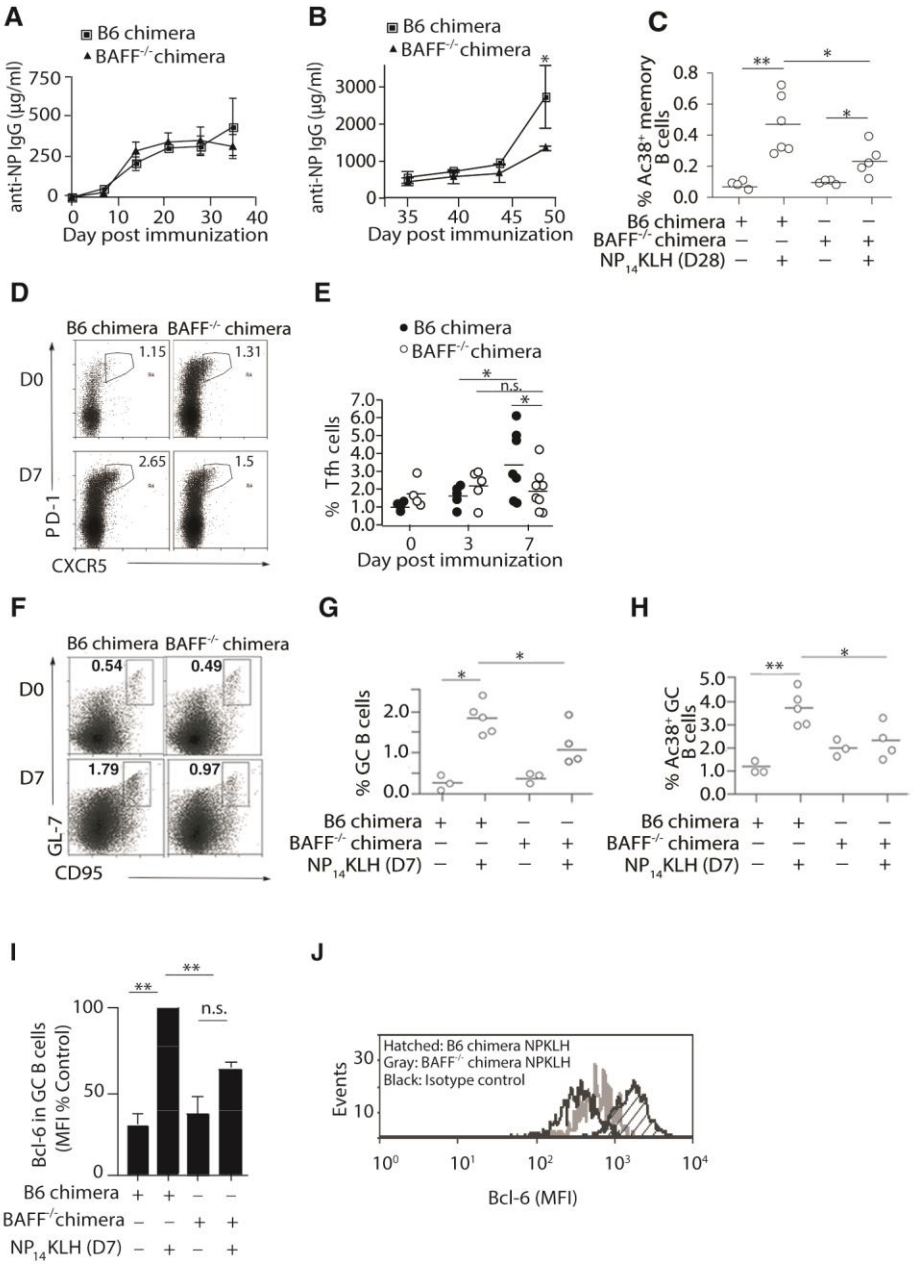

Figure 2. 1 BAFF$^{-/-}$ chimeras have defective memory and GC B cells, diminished Bcl-6 levels and decreased frequency of Tfh cells.

(A) B6 and BAFF$^{-/-}$ chimeric mice were immunized (i.p) with 100 µg NP_{14}KLH in alum. Serum IgG anti-NP responses in B6 and BAFF$^{-/-}$ chimeric mice were measured by ELISA on days 7, 14, 21, 28, and 35 post immunization. n=4-6 mice per group over 3 experiments. **(B)** Serum IgG anti-NP levels measured on days 39, 42, and 49 (4, 7, and 14 days) following boost

of 100 μg of soluble NP_{14}KLH on day 35. n = 3-6 mice over 3 experiments. **(C)** $CD19^+Ac38^+$ IgG^+ B cells enumerated by flow cytometry from the spleens of B6 and $BAFF^{-/-}$ chimeras immunized for 28 days. n = 4-6 mice over 4 experiments. **(D and E)** $CD4^+CXCR5^+PD\text{-}1^+$ T cells from B6 and $BAFF^{-/-}$ chimeric mice enumerated on days 0, 3 and 7 following immunization. n = 3-6 mice per time point over 2 experiments. **(F and G)** $CD19^+$, $GL\text{-}7^+$, $CD95^+$ GC B cells enumerated by flow cytometry on day 7 post-immunization. n = 3-5 mice over 3 experiments. **(H)** $CD19^+Ac38^+CD95^+GL\text{-}7^+$ B cells enumerated on day 7 post-immunization. n = 3-5 mice over 3 experiments. **(I and J)** Relative expression of Bcl-6 in GC B cells ($CD19^+CD95^+GL\text{-}7^+$) from B6 and $BAFF^{-/-}$ chimeras measured 7 days after NP_{14}KLH immunization (i.p.). n = 4-6 mice per group over 2 experiments. ** $p \leq 0.01$, *$p \leq 0.05$, n.s. = not significant

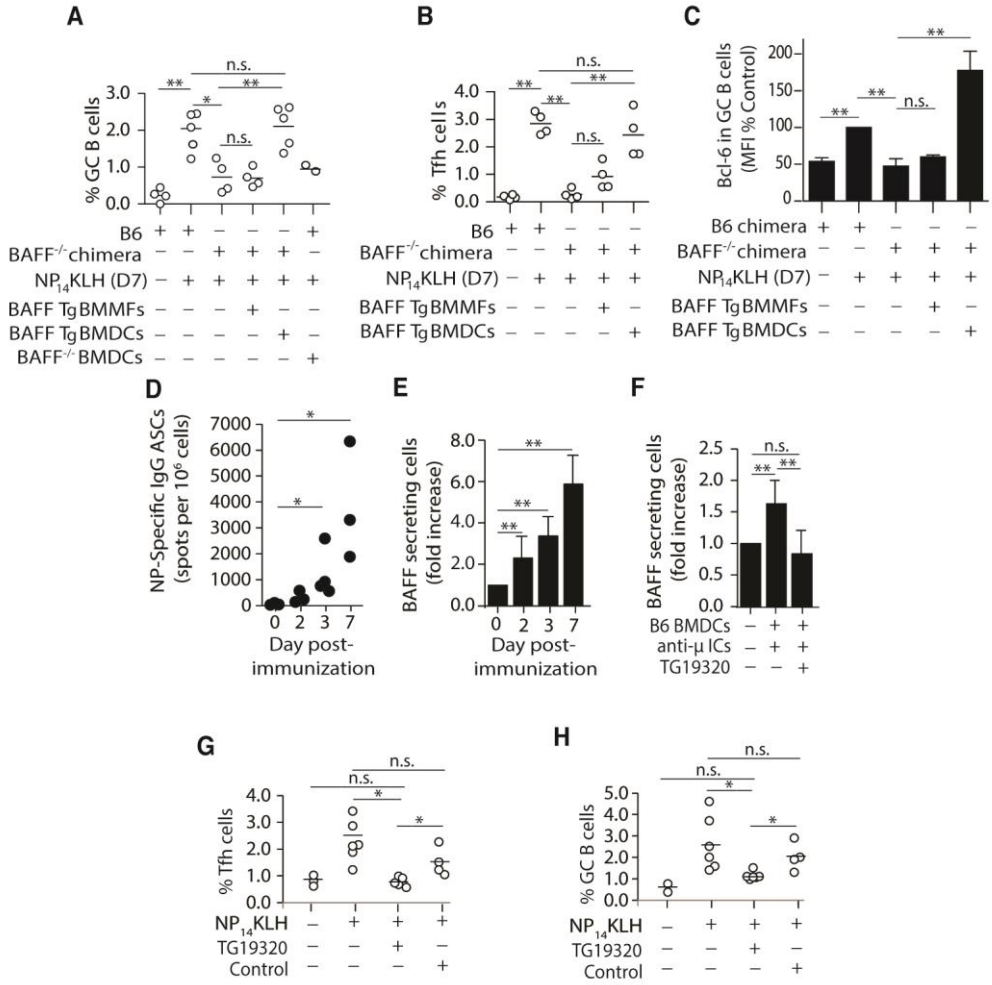

Figure 2. 2 Secretion of BAFF by DCs restores Bcl-6 levels and the frequency of GC B and Tfh cells**.**

(A-C) BAFF Tg BMDCs, BMMFs, or BAFF$^{-/-}$ BMDCs (8 x 10^6) were injected (s.c.) into B6 or BAFF$^{-/-}$ chimeric mice that were simultaneously immunized (s.c.) with $NP_{14}KLH$. On day 7, inguinal lymph nodes were harvested and the frequencies of **(A)** GC B cells (GL-7$^+$CD95$^+$ cells in CD19$^+$ B cells), **(B)** Tfh cell (CXCR5$^+$PD-1$^+$ cells in CD4$^+$ T cells), and **(C)** Bcl-6 expression in GC B cells from inguinal lymph nodes was measured by flow cytometry. n = 3-5 over 3 experiments. **(D)** On days 2, 3, and 7 following immunization, 1 x 10^6 splenocytes from B6 mice were plated on NP-BSA-coated ELISpot plates. After 18 hours, NP-specific ASCs were

quantitated. n = 3-4 mice per time point over 3 experiments. **(E)** 1 x 10^6 purified $CD11c^+$ cells from B6 mice immunized for 2, 3, and 7 days were plated on BR3-Fc coated ELISpot plates. After 60 hours, the number of BAFF secreting cells was enumerated. n = 3-6 mice per time point over 3 experiments. **(F)** BAFF secreting cells enumerated from B6 BMDCs (2.5 x 10^5) cultured for 24 hrs in the presence or absence of anti-μ ICs, with or without Fc blocking peptide (TG19320; 50 μg/ml). n = 3-10 over 6 experiments. **(G-H)** B6 mice were immunized with NP_{14}KLH and dosed with 15-30 mg/kg of Fc blocking peptide (TG19320) or an unrelated scrambled peptide (Control) via i.p. injection. On day 7, the frequency of Tfh cells **(**CD4+CXCR5+PD1+, **G)** and GC B cells **(**CD19+GL7+CD95+, **H)** was determined. n = 3-6 over 2 experiments. **$p \leq 0.01$, *$p \leq 0.05$, n.s. = not significant

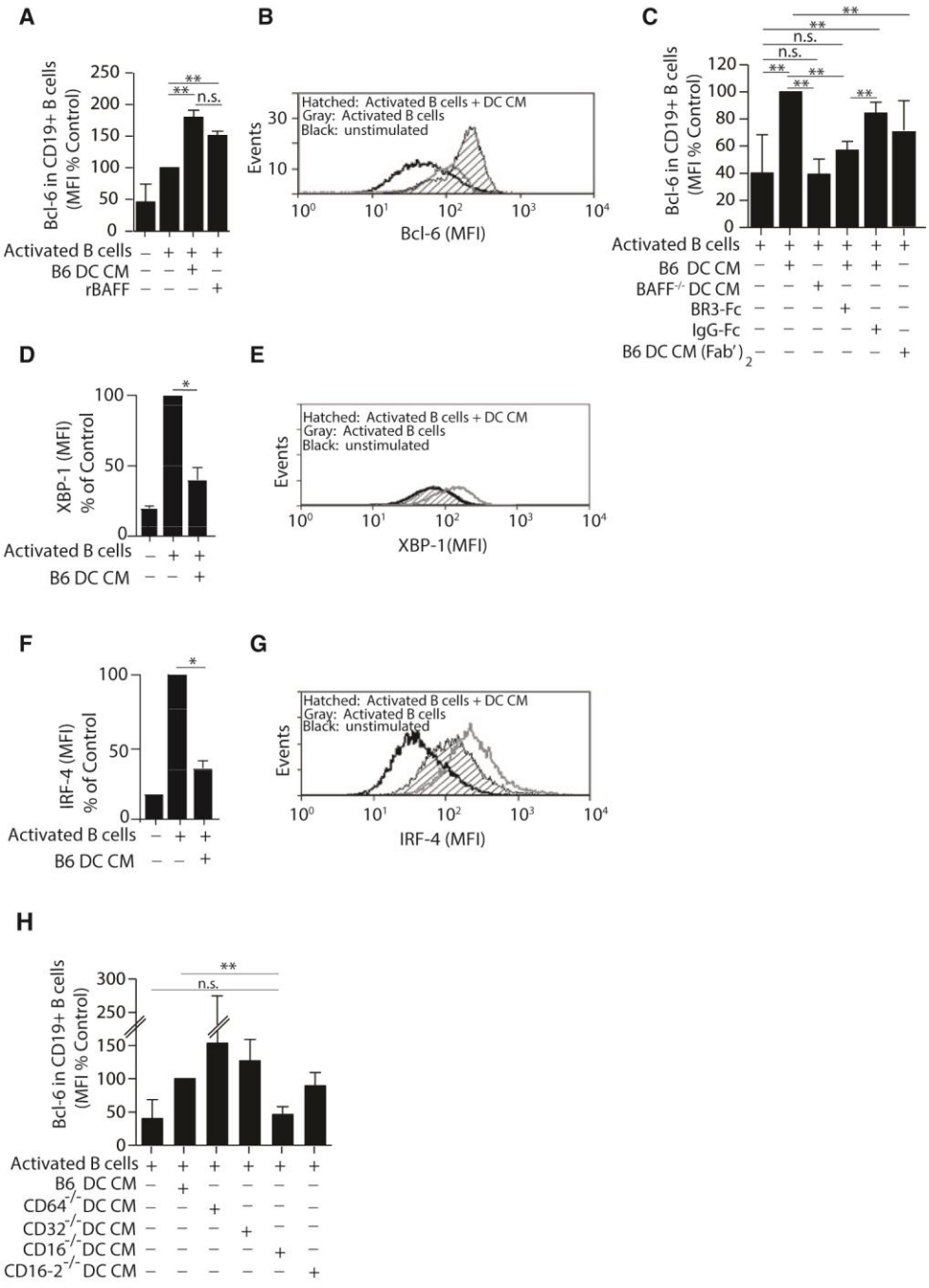

Figure 2. 3 DC-derived BAFF regulates the expression of Bcl-6 in cultured B cells. Purified B6 B cells (1 x 10^5) stimulated with anti-μ (30 μg/ml), IL-4 (25 ng/ml), and IL-5 (25 ng/ml) were co-cultured with DC CMs. Intracellular levels of key transcription factors (Bcl-6, XBP-1, IRF-4) were measured by flow cytometry on day 2. **(A)** Bcl-6 expression in B cells co-cultured with B6 DC CM or rBAFF (5 ng/ml). **(B)** Representative histogram of Bcl-6 expression

in B cells activated by DC CM as in (A). n = 3-4 mice over 4 experiments. **(C)** Purified B6 B cells were co-cultured with CM generated from B6 DCs treated with ICs containing intact Fc regions (B6 DC CM), $F(ab')_2$ (B6 DC CM $(Fab')_2$), $BAFF^{-/-}$ DC CM, or B6 DC CM neutralized with BR3-Fc (10 μg/ml) or control IgG-Fc (10 μg/ml). n = 4-7 over 4 experiments. **(D-G)** B cells were co-cultured with B6 DC CM. Intracellular levels of XBP-1 **(D, E)** and IRF-4 **(F, G)** were quantitated. n = 3 mice per group over 3 experiments. Histograms are representative of 3 experiments. **(H)** Bcl-6 expression in B cells cultured with DC CM from B6, $CD64^{-/-}$, $CD32^{-/-}$, $CD16^{-/-}$, or $CD16\text{-}2^{-/-}$ (FcγRIV) mice. n=6-7 mice per group over 4 experiments. $p \leq 0.01$, * $p \leq 0.05$, n.s. = not significant.

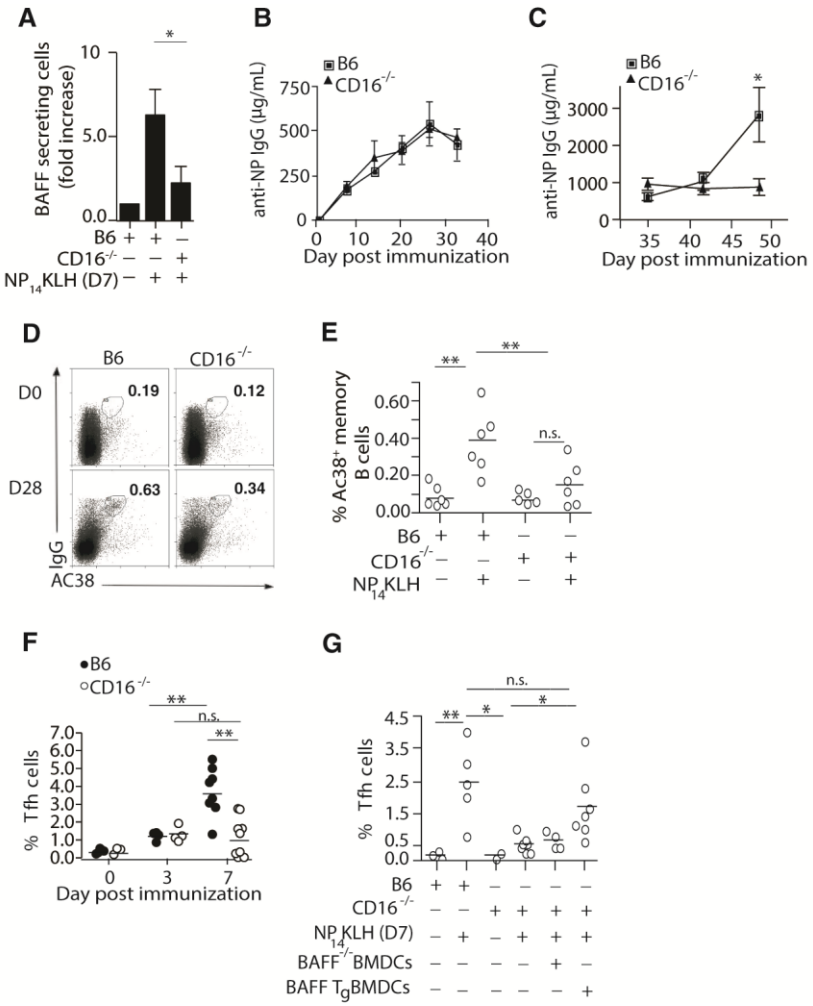

Figure 2. 4 CD16$^{-/-}$ mice have defective BAFF secretion, secondary antibody responses, frequency of memory B cell and Tfh cells**.**

(A) BAFF secreting cells enumerated from purified CD11c^{+} cells isolated from B6 and CD16$^{-/-}$ mice on day 7. n = 3 over 3 experiments. **(B and C)** B6 and CD16$^{-/-}$ mice were immunized (i.p) with 100 µg NP_{14}KLH in alum. Serum IgG anti-NP **(B)** primary responses measured by ELISA on days 0, 7, 14, 21, 28, and 35; serum IgG **(C)** secondary antibody levels measured on days 39, 42, and 49 (days 4, 7, and 14) following boost of 100 µg soluble NP_{14}KLH on day 35. n = 4-8 mice over 3 experiments. **(D and E)** CD19^{+}Ac38^{+}IgG^{+} memory B cells enumerated by flow cytometry from the spleens of B6 and CD16$^{-/-}$ mice immunized for 28 days. n = 5-6 mice over 3 experiments. Data are expressed as the percent of CD19^{+} cells that are IgG^{+} and Ac38^{+}. **(F)** The

frequency of $CD4^+CXCR5^+PD\text{-}1^+$ T cells from immunized B6 and $CD16^{-/-}$ mice on days 0, 3 or 7. n = 3-8 mice per time point over 4 experiments. **(G)** B6, BAFF Tg, and $BAFF^{-/-}$ BMDCs (8 x 10^6) were injected into $CD16^{-/-}$ mice that were simultaneously immunized with $NP_{14}KLH$. On day 7, the frequencies of $CD4^+CXCR5^+PD\text{-}1^+$ T cells from inguinal lymph nodes were enumerated by flow cytometry. n = 3-7 mice over 3 experiments. $p \leq 0.01$, $*p \leq 0.05$, n.s. = not significant

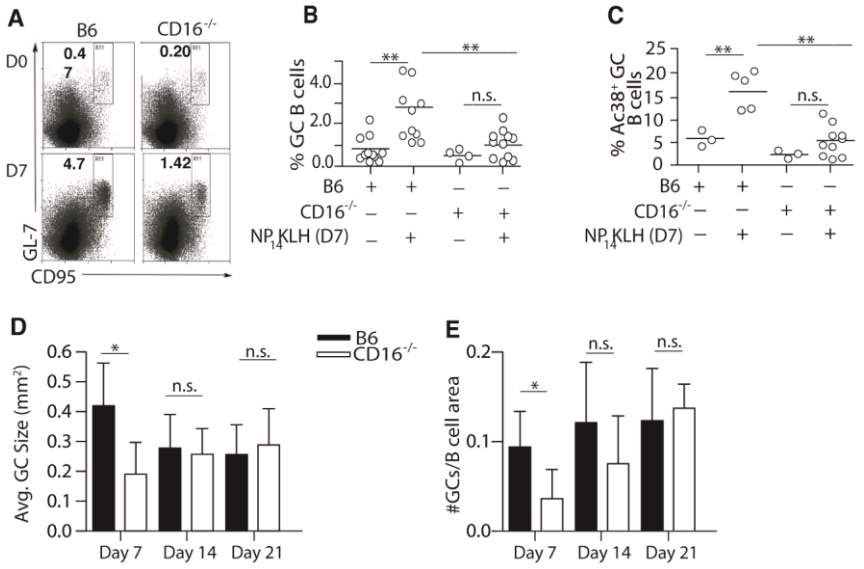

Figure 2. 5 CD16$^{-/-}$ mice have diminished germinal center responses.

(A and B) The frequency of splenic CD19^{+}GL-7^{+}CD95^{+} GC B cells was measured by flow cytometry from B6 and CD16$^{-/-}$ mice 7 days after immunization. n = 4-11 mice per group over 3 experiments. **(C)** The frequency of NP-specific GC B cells (CD19^{+}Ac38^{+}GL-7^{+}CD95^{+}) measured in B6 and CD16$^{-/-}$ mice 7 days after immunization. n = 3-10 mice over 3 experiments. **(D)** The size of GCs and **(E)** the number of GCs were enumerated from B6 and CD16$^{-/-}$ mice 7, 14, and 21 days after immunization using confocal microscopy. n = 3-6 mice per time point over 4 experiments.

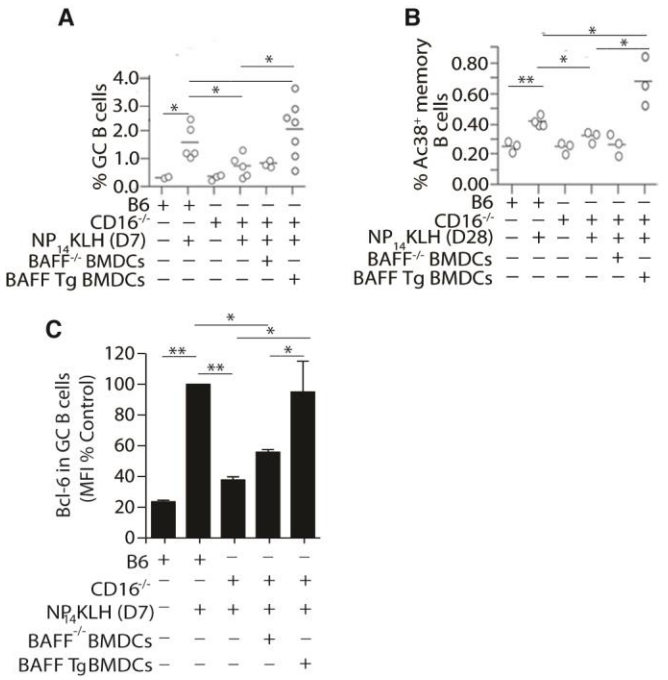

Figure 2. 6 Adoptive transfer of BAFF expressing DCs rescues GC and memory B cell populations and restores Bcl-6 levels in $CD16^{-/-}$ mice B cells.

(A) BMDCs (8×10^6) from BAFF Tg or $BAFF^{-/-}$ mice were injected (s.c.) into $CD16^{-/-}$ mice and simultaneously immunized (s.c.) with $NP_{14}KLH$. On day 7, the percentage of GC B cells ($CD19^+GL\text{-}7^+CD95^+$) were measured from inguinal lymph nodes. n = 3-7 mice per group over 3 experiments. **(B)** Same as (A) but on day 28, $CD19^+Ac38^+IgG^+$ memory B cells were enumerated. n = 3-4 over 2 experiments. **(C)** BAFF Tg, and $BAFF^{-/-}$ BMDCs (8×10^6) were injected into $CD16^{-/-}$ mice and simultaneously immunized with $NP_{14}KLH$. n = 4 mice over 3 experiments. On day 7, Bcl-6 expression in GC B cells from inguinal lymph nodes was measured by flow cytometry. $^{**}p \leq 0.01$, $^{*}p \leq 0.05$, n.s. = not significant.

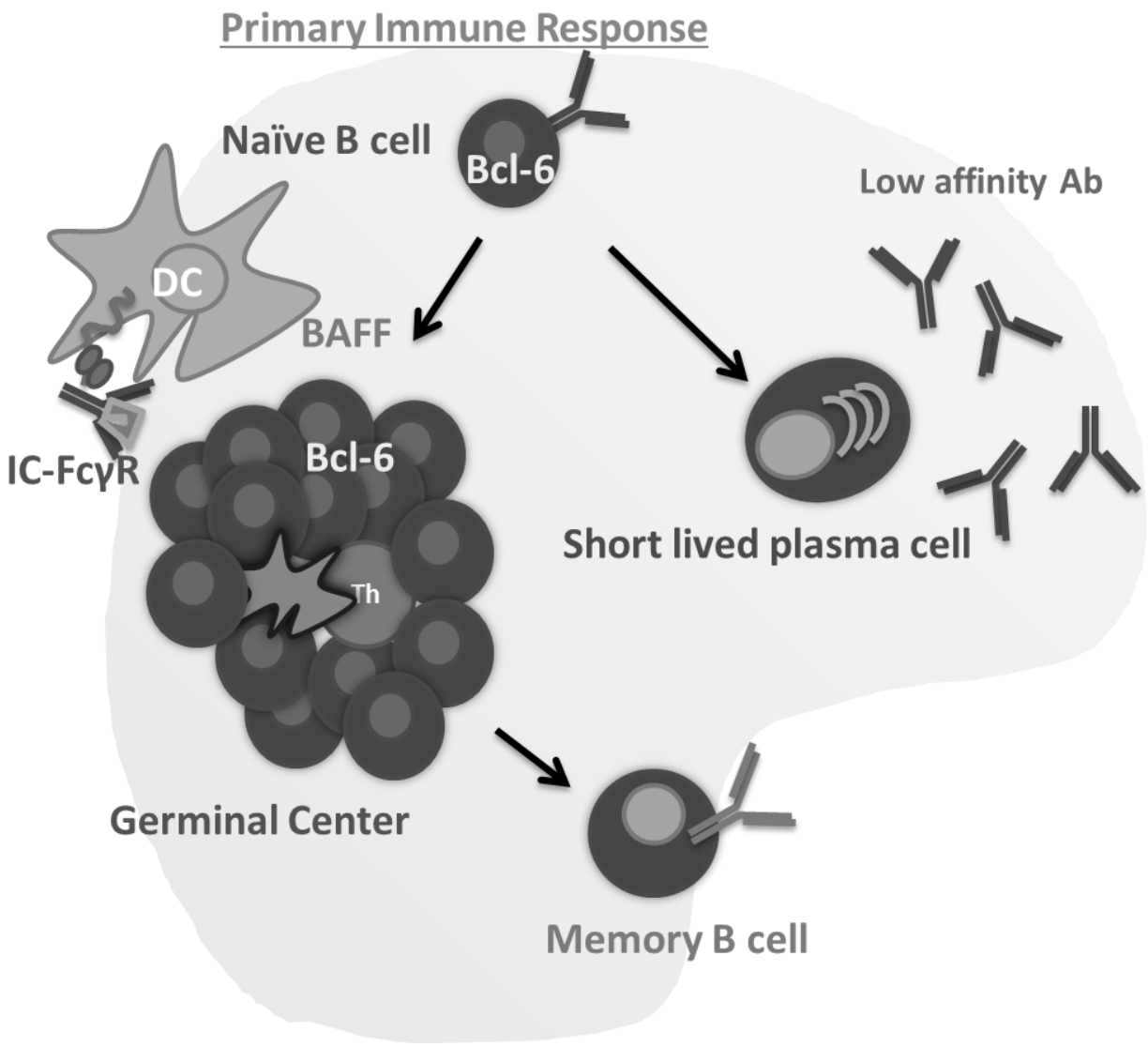

Figure 2. 7 Chapter Two Model.

During an adaptive immune response (in this example, to NP-KLH), B cells respond to BCR ligands and co-stimulation by differentiating into short-lived plasma cells that secrete IgG. IgG binds antigen and forms IC's that signal DCs to make BAFF by binding FcγR (in the case of NP-KLH, FcγRIII). Before and/or during the germinal center reaction, BAFF signals B cells directly or indirectly to upregulate Bcl-6, a transcription factor required to maintain germinal center B cells and form memory B cells.

CHAPTER 3: *Staphylococcus aureus* Protein A Disrupts Immunity Mediated by Long-Lived Plasma Cells[2]

Introduction

Staphylococcus aureus is a major cause of hospital and community acquired infections and has become more difficult to treat as antibiotic-resistant strains spread [72–74]. *S. aureus* infections commonly recur without inducing long-term immunity [67,68]. Attempts to design a human vaccine have failed despite the ability of some vaccine approaches to induce short-term protection in mouse models [173–180]**.** Sustained serum antibody is key for conferring long-term protection against infection. Antibodies against *S. aureus* enhance phagocytosis of the bacteria, block adhesion, and prevent abscess formation in mice [179,181,182]. In humans, high levels of pre-existing antibody are associated with reduced incidence of infection [64–66]. However antibody levels wane unpredictably, leading to the loss of vaccine efficacy [69–71]. This suggests that *S. aureus* disrupts the longevity of the humoral immune response.

Memory B cells and LLPCs are two cell types that confer long-term humoral immunity. Serum antibody levels are sustained by LLPCs that are derived mainly from memory B cells activated during secondary immune responses [33–37]. Memory B cells have been identified

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after infection with viral, parasitic, and bacterial pathogens [183–186], but whether these cells are formed after *S. aureus* infection has not been addressed. Memory B cells reside in an inactive state in the secondary lymphoid organs, and upon re-exposure to antigen, rapidly divide into daughter cells or differentiate into immature ASCs called PBs ($CD138^{+}$, $B220^{lo/neg}$, Ig^{+}) [32,187].

The majority of PBs become short-lived PCs ($CD138^{+}$, $B220^{lo/neg}$, Ig^{-}); however, some migrate to survival niches in the BM and mature into LLPCs [33,37,188]. LLPCs and their constitutively secreted antibody are now recognized as major contributors of protection against bacterial infection [35,44,49]. LLPCs can survive at least 100 days [43] and up to the lifetime of the organism [36,40]. In humans, LLPC survive for months to decades and are the main source of convalescent serum IgG [36,40,189,190]. Recent work has revealed a relationship between long-term antibody maintenance and resistance to recurring *S. aureus* infections [63], yet it remains unknown whether LLPCs are formed after *S. aureus* infection.

The factors that deter long-lasting immunity to *S. aureus* are not completely understood. The complex array of *S. aureus* virulence factors and surface proteins promote evasion from the adaptive immune response [76,191,192]. SpA is a virulence factor that directly modulates the B cell response [75,82]. SpA has five Fc-binding domains that sequester IgG and interfere with immune complex (IC)-mediated antigen presentation and phagocytosis [77–79]. SpA also activates B cells upon binding the F(ab) portion of B cell receptors of the VhIII clan (up to 50% of naïve B cells in humans and 5-10% in mice) [80,81]. In the context of infection, this binding enhances secretion of SpA-specific IgM [193]. Immunization with a mutant SpA that cannot bind either the Fc or Fab regions of Ig induces neutralizing anti-SpA antibodies that protect mice from sepsis and lead to an enhanced and more diverse antibody response [84,194]. This suggests

that the presence of SpA on *S. aureus* alters the B cell response to infection. However, whether SpA influences long-term B cell memory, or LLPCs has not been addressed.

In this study, we show that SpA disrupts BM accumulation of LLPC by enhancing the short-lived ASC response. Previously inoculated mice that were challenged with a SpA-deficient *S. aureus* mutant (Δ*spa*), but not with WT *S. aureus*, formed LLPCs and maintained serum antibody for at least 12 weeks after challenge. The lack of long-term humoral immunity to WT *S. aureus* was not due to defects in memory B cell formation or activation because primary infection with either WT or Δ*spa* induced the formation of germinal centers and functional memory B cells. Rather, SpA altered the differentiation of ASCs during the secondary response by expanding IgM^+ extrafollicular PBs, disrupting the LLPC compartment, and preventing long-term antibody maintenance.

Results

The primary and secondary antibody responses to S. aureus are unaffected by Protein A.

A successful adaptive immune response results in immunological memory that confers long-term protection during subsequent exposure. To assess whether *S. aureus* induces the formation of memory B cells, and whether this was impacted by Protein A (SpA) we subcutaneously inoculated mice with strain RN4220 expressing SpA (WT), or an isogenic *spa* mutant (Δ*spa*). We chose subcutaneous inoculation for the primary response because clearance of *S. aureus* could be readily monitored at the injection site. We found that infection was cleared within 3 weeks and the mild virulence of RN4220 allowed inoculated mice to be analyzed over a long period of time without significant weight loss, morbidity, or depletion of B cells **(Supplemental Figure 1A)**. To characterize the primary immune response, we measured the total *S. aureus*-specific IgG and IgG2 over time (**Figure 1A).** A modest level of IgG2 **(Figure 1B)** and total IgG **(Supplemental Figure 1B)** was detectable between 2 and 5 weeks after primary inoculation regardless of whether mice were inoculated with *S. aureus* Δ*spa* (1.5-fold) or WT (2-fold).

The increase of *S. aureus*-specific IgG between the first and second weeks after primary inoculation suggested that a T-dependent immune response had induced class switch. GCs are the sites of class switching and affinity maturation, and are required for the formation of high-affinity memory B cells [9,130,195]. To define whether *S. aureus* induced GCs, we quantitated GC B cells over the course of the primary response. Subcutaneous inoculation with *S. aureus* RN4220 Δ*spa* or WT induced GC B cells in the draining lymph nodes and spleen with similar kinetics, peaking between days 7 or 14 and resolving by day 35 **(Figures 1C-D).** Tfh cells,

which are required for GC development [3,95] were also induced in similar frequencies after primary inoculation with *S. aureus* Δ*spa* or WT **(Supplemental Figure 1C).**

One possible mechanism underlying the lack of long-term immunity to *S. aureus* is failure to promote the formation of memory B cells. We used ELISpot to indirectly detect the numbers of *S. aureus* antigen-specific memory B cells after *in vitro* polyclonal stimulation as no flow cytometry methods are available. IsdB was chosen as the target antigen since anti-IsdB IgG has been detected in infected mice, and has recently been studied in vaccination trials [84,173,196]. On day 35 post-inoculation, *ex vivo* lymphocytes from the spleens and lymph nodes were polyclonally activated with pokeweed mitogen. The 6-day culture period is too short to induce class-switch, thus all antigen-specific IgG-secreting cells detected by ELISpot were derived from memory B cells [183,184,189]. We detected significantly greater numbers of class-switched IsdB-specific ASCs in both Δ*spa* and WT-inoculated mice compared to naïve mice **(Figure 1E)**, suggesting that memory B cells were formed as a result of infection in the presence or absence of SpA. Together these data suggest that the lack of long-term protection against *S. aureus* is not due to failure to form memory B cells.

Another possibility is that memory B cells develop normally, but fail to be reactivated upon re-infection. Memory B cell responses are more rapid and robust than primary responses to the same stimuli [1,22,197]. To test whether memory B cells are activated upon secondary exposure to *S. aureus*, we inoculated mice a second time (herein referred to as challenge) and measured antibody responses. We challenged mice intravenously (IV) with *S. aureus* RN4420 WT or Δ*spa* to mimic sepsis conditions, and measured antibody responses over two weeks **(Figure 1A).** Challenge of previously inoculated mice resulted in amnestic (2-6 fold) IgG and

IgG2 responses whether the primary inoculation was WT or Δ*spa* **(Figure 1F and Supplemental 1D).**

To ensure that the enhanced and rapid IgG response induced by IV challenge was dependent on previous infection rather than initiating a new primary response, we compared IgG and IgG2 responses on day 49 (14 post-challenge) to those produced 14 days after a primary IV infection. Consistent with memory cell activation IV challenge of previously inoculated mice induced significantly higher levels of IgG2 and IgG compared to naïve mice **(Figure 1G and Supplemental 1E)**. In addition, we found that challenge with either WT or Δ*spa S. aureus* induced amnestic IgG responses to IsdB **(Figure 1H),** that were also dependent on previous infection **(Figure 1I).** To ensure that the secondary response was not unique to RN4220, we subcutaneously inoculated and IV challenged mice with the clinical strains, Newman and USA300. We observed enhanced secondary responses similar to those induced by RN4220 **(Supplemental Figure 1F).**

A hallmark of memory B cells is their longevity [21]. It is possible that the memory B cells formed after *S. aureus* infection are not long-lived, thus diminishing long-term immunity. To assess this, we challenged mice 100 days after primary inoculation and found that challenge with WT or Δ*spa* induced an amnestic *S. aureus*-specific antibody response (2-3 fold) **(Figure 1J).** It is also possible is that failure of memory B cells to self-renew after repeated challenge contributes to the lack of long-term protection against *S. aureus.* To examine this, we challenged mice a second time (third infection) 6 weeks after the first IV challenge. The second challenge boosted antibody production (**Figure 1K)**, indicating memory B cells were not depleted or rendered unresponsive. Together these data indicate that regardless of the presence or absence of

SpA, long-lived memory B cells are formed and activated after *S. aureus* inoculation and challenge.

The presence of Protein A during challenge preferentially expands plasmablasts

When memory B cells are activated, they rapidly divide, forming antibody-secreting PBs ($CD138^{+}$ $B220^{lo/neg}$ surface Ig^{+}) that mature into plasma cells (PCs) ($CD138^{+}$ $B220^{neg}$ surface Ig^{-}) [22,24,32,33,198]. To assess whether SpA impacted the PC response, we enumerated PBs and PCs (collectively referred to herein as ASCs) [199]. Mice challenged (IV) with either WT or Δ*spa S. aureus* made more total ASCs compared to naïve mice given a primary IV infection (**Figure 2A-C and Supplemental Figure 2A).** However, the frequencies and numbers of total splenic ASCs were significantly higher after WT challenge. This expansion was induced by WT challenge regardless of whether mice were initially inoculated with WT or Δ*spa* **(Figure 2B-C and Supplemental Figure 2A),** and was unrelated to changes in total $CD19^{+}$/$B220^{+}$ B cells **(Supplemental Figure 2B).**

SpA has previously been identified as a B cell superantigen that binds the VhIII class of BCRs and drives polyclonal B cell expansion and apoptosis [75,82]. SpA is released from *S. aureus* during normal cell division, making it possible for the protein to reach B cells in the spleen in soluble form and through DC-mediated presentation during the course of an infection [200–202]. IgM binds SpA with a higher affinity than IgG [203], so we reasoned that if SpA drove ASC expansion by binding F(ab)s, the ASC population would predominately be PBs that retained surface IgM [6]. We found that 54% of the ASCs formed after WT challenge retained surface IgM whereas 30% of ASCs formed after Δ*spa* challenge retained surface IgM **(Figure**

2D). The frequency of ASCs retaining IgG was not significantly different between the two groups (20.5% of Δ*spa* and 17.8% of WT) **(Figure 2E)**. These data indicate that most of the expanded ASCs were derived from IgM^+ cells suggesting they were induced through the F(ab)-binding activity of SpA**.**

To assess whether expansion of IgM^+ cells impacted the antigen-specific IgG response, we quantitated the class-switched IsdB-specific ASC response by ELISpot on day 40 (day 5 post-challenge) **(Figure 2A).** Consistent with a memory response (Figure 1E), we found that mice challenge with *S. aureus* RN4220 Δ*spa* or WT, produced 2-6 times more IsdB-specific IgG ASCs than naive mice that were inoculated IV on the same day; however, the numbers of IsdB-specific ASCs did not differ between mice challenged with WT versus Δ*spa* **(Figure 2F)**. Collectively, these data suggest that long-lived, memory cells are formed regardless of whether mice are inoculated with WT or Δ*spa S. aureus*. Upon reactivation, the newly formed PCs produce comparable amounts of antigen-specific IgG antibody; however, challenge with WT *S. aureus* promotes a robust expansion of IgM^+ PBs.

SpA is a known virulence factor of *S. aureus* and kidney bacterial load is reduced after infection with *S. aureus* Δ*spa* compared to WT [204–206]. Thus, it is possible that the reduced virulence of *Δspa* limits the expansion or IgM^+ PBs [207,208]. To test this, we challenged mice with *S. aureus* Newman WT, Δ*spa*, or Δ*srrAB*, a mutant that expresses SpA but displays a virulence defect similar to that of Δ*spa* **(Supplemental Figure 3A).** Challenge with *S. aureus* Δ*srrAB* induced expansion of IgM^+ PBs similar to that seen after WT challenge **(Supplemental Figure 3B-C).** Thus, the lack of expansion of IgM^+ PBs was not caused by reduced virulence, but was specifically related to the absence of SpA.

The presence of Protein A during challenge enhances the short-lived extrafollicular response

IgM$^+$ ASC responses are typically short-lived [8,38,39]. To determine whether the expanded ASCs induced by SpA were short-lived, we monitored CD138$^+$ B220$^{lo/neg}$ cells by flow cytometry over the course of two weeks. We found that the frequencies and number of ASCs peaked in the spleens of both WT and Δ*spa*-challenged mice on day 40 (day 5 post-challenge) and declined by day 42 (day 7 post-challenge) **(Figure 3A)**. This is consistent with the expanded PB population consisting mainly of short-lived cells.

Short-lived responses are typically extrafollicular [30,209,210], so to examine whether the ASC expansion induced by WT challenge was extrafollicular, we first examined splenic DCs, which are key mediators of extrafollicular responses that carry intact antigen into lymphoid organs and produce cytokines conducive to PB differentiation [201,202,211–213]. We analyzed the frequencies of classical and plasmacytoid DC subsets and their expression of surface markers associated with activation (CD80, CD86, MHCII, CD11c and CD40). We found no significant differences in the DC populations induced by WT or Δ*spa* challenge **(data not shown).** TLR2 ligands expressed during *S. aureus* infection induce DCs to make IL-12, a cytokine that promotes ASC differentiation [197,214–217]. IL-12$^+$ DCs are expanded in immunized Fcγ$^{-/-}$ mice, indicating a role for immune complexes (ICs) in regulating DC IL-12 [148]. Because IC binding to FcγRs is impeded by SpA's Fc-binding function, we quantitated IL-12-producing CD11c$^+$ DCs on day 40 (day 5 post-challenge). We found that WT and Δ*srrAB* challenge induced 1.7-fold more IL-12$^+$ DCs than Δ*spa* challenge **(Figure 3B and Supplemental Figure 3D)**. This suggests that SpA binding to the Fc region of Ig could play a role in PB expansion by promoting DC IL-12.

Expansion of IL-12^{+} DCs is associated with enhanced short-lived extrafollicular responses [148]. To determine whether the expanded IgM^{+} PBs represented an extrafollicular response, we analyzed spleen sections on day 40 (day 5 post-challenge). WT challenge resulted in larger and more frequent extrafollicular foci composed of IgM$^{+/bright}$ cells compared to Δ*spa* challenge **(Figure 3C)**. These foci were located mainly in the red pulp of the spleen **(3C arrows)** and could be identified in bridging channels next to B cell follicles (**Figure 3C asterisks).**

ASCs fail to populate the bone marrow following challenge with WT S. aureus

Activation of memory B cells promotes the formation of ASCs that migrate to sites of inflammation, or to survival niches in the bone marrow (BM) where they constitutively produce protective IgG [33,43]. Expansion of splenic ASCs is observed in models of autoimmunity or in chronic inflammation where migration to, or retention in, the BM is disrupted by chronic production of short-lived PCs [207,218–220], defective migration to the BM [34], disruption of the BM PC niche [34,41] or a combination of these effects. Since challenge with WT *S. aureus* expanded extrafollicular short-lived PBs, we examined whether this expansion disrupted the establishment of long-lived PCs (LLPCs) in the BM.

The majority of antigen-specific PCs migrate to the BM within 2 weeks of forming [35–37]. Therefore, we measured IsdB-specific IgG ASCs from the spleen and BM by ELISpot on day 49 (day 14 post-challenge). We observed significantly more IsdB-specific IgG ASCs in the BM of mice challenged with Δ*spa* compared to mice challenged with WT *S. aureus* (**Figure 4A and B).** These BM ASCs likely derived from activated memory cells since the number of ASCs

present on day 49 (day 14 post-challenge) were significantly higher than the numbers present in the BM 14 days after primary IV Δ*spa* infection. This indicates that SpA interferes with the accumulation of BM ASCs derived from memory B cells during challenge.

The activation of memory B cells during challenge initiates GC reactions [23,25,185]. In many cases, expansion of short-lived extrafollicular ASCs occurs at the expense of GC formation; a critical event in the formation of LLPCs [10,48,190,221,222]. To assess this possibility, we quantitated the frequency of GC B cells during the course of the secondary response and found that GC B cells expanded after challenge with either Δ*spa* or WT **(Figure 4C).** Although the frequency of B cells that were positive for GL7 and Fas was higher after WT challenge on day 40 (day 5 post-challenge), the total numbers of GC B cells were not significantly different at this time point **(Supplemental Figure 4A).** By microscopy, GC structures of similar sizes were detected after Δ*spa* and WT challenge at similar frequencies and T-B architecture was not disrupted **(Supplemental Figure 4B).** Therefore, defective accumulation of BM ASCs after WT challenge was not caused by the inability to form secondary GCs.

Bone marrow ASCs do not accumulate after WT challenge despite normal migration and adequate niche factors

To determine whether SpA affected the kinetics of the ASC migration to the BM, we quantitated $CD138^{+}B220^{lo/neg}$ ASCs in the BM during the first 3 weeks after challenge. We found no increase in BM ASC numbers 35 days after primary subcutaneous inoculation with *S. aureus* RN4220 Δ*spa* or WT **(Figure 5A)**, consistent with reports that higher numbers of LLPCs

are formed during secondary responses than after primary responses [32,36]. ASCs appeared in the BM by day 40 (day 5 post-challenge) regardless of whether mice were challenged with WT or Δ*spa S. aureus* **(Figure 5A)**; however, the number of BM ASCs declined in the WT-challenged mice after day 45 (day 10 post-challenge). Conversely, ASC numbers continued to rise in the Δ*spa*-challenged mice. By day 49 (day 14 post-challenge) the Δ*spa*-challenged mice displayed significantly higher frequencies and numbers of BM ASCs than mice challenged with WT or Δ*srrAB* **(Figure 5A, Supplemental Figure 5A)**.

Surface expression of CXCR4 guides ASCs toward CXCL12 emanating from the BM [51]. Since SpA induces internalization of CXCR4 *in vitro* [223], alterations in CXCR4 expression or responsiveness after WT challenge could disrupt homing of ASCs to the BM. To assess this, we quantitated surface CXCR4 on ASCs on day 40 (peak response; day 5 post-challenge), when ASC start to migrate from the spleen. We found that the levels were not different regardless of whether mice were challenged with *S. aureus* WT or Δ*spa* **(Figure 5B)**. Consistent with comparable expression, ASCs from WT and Δ*spa* challenge mice showed comparable migration toward CXCL12 (SDF-1) through a trans well membrane **(Figure 5C)**. The presence of SpA in the challenge infection, therefore, did not appear to inhibit initial migration of ASCs to the BM, but rather impacted their accumulation 2 weeks after challenge.

Systemic inflammation can make the BM microenvironment inhospitable to LLPCs by depleting niche factors including eosinophils and APRIL [34,50,56]. It is possible that SpA disrupted these niche factors during WT infection, preventing LLPCs from establishing in the BM. We analyzed these factors on day 40 (day 5 post-challenge) at which point, eosinophils peak in the BM [55]. The frequencies and numbers of eosinophils and APRIL^{+} BM cells were similar between WT and Δ*spa*-challenged mice **(Figure 5D-E and Supplemental Figure 5B)**.

This suggests that the defect in PC accumulation after WT challenge was not caused by the loss of niche factors known to be disrupted by inflammation [188].

To confirm that the BM microenvironment was competent to support LLPCs, we assessed whether ASCs of a different specificity could populate the BM during WT or Δ*spa S. aureus* infection. We immunized mice with (4-hydroxy-3-nitrophenyl)-acetyl hapten conjugated to Keyhole limpet hemocyanin (NP-KLH), and 35 days later boosted mice with NP-KLH alone or co-infected IV with *S. aureus* RN4220 WT or Δ*spa*. We detected NP-specific IgG ASCs in the BM on day 49 (day 14 post-boost) at similar frequencies regardless of whether mice were co-infected with WT or Δ*spa* **(Figure 5F)**. This indicates that the BM niche was intact after IV infection with *S. aureus.* Collectively, these data indicate that the lack of BM PC accumulation after WT challenge was not caused by a disruption in the BM niche environment, but by an alteration in the ASC population itself. Consistent with this, ASCs present in the BM of WT-challenged mice displayed significantly reduced levels of APRIL receptor and maturation marker, CD138 **(Supplemental Figure 5C)**, indicating a less mature phenotype than ASCs induced in the absence of SpA [46,47].

Protein A prevents long-term maintenance of antigen-specific IgG by bone marrow plasma cells

Although the BM is the main site of LLPC survival, the spleen also harbors PCs for a significant period of time [220,224]. We examined whether expanded ASCs induced by WT challenge would persist in the spleen over time. First, to confirm that ASCs formed after WT challenge were not simply delayed in arriving to the BM, we measured IsdB-specific ASCs 8 weeks after challenge. BM ASCs induced by Δ*spa* challenge were maintained for 8 weeks;

however, the low numbers of ASCs present after WT challenge remained unchanged (**Figure 6A).** The numbers of ASCs present in the spleen declined in both WT and Δ*spa*-challenged mice by week 8, to levels that were not different from those in naïve mice (**Figure 6B)**. This indicates that WT challenge did not establish LLPCs in the spleen.

The main function of LLPCs is the long-term maintenance of antigen-specific Ig as an early defense against re-exposure to pathogens [35,44]. We observed similar *S. aureus*-specific IgG responses in the first 2 weeks after challenge with WT or Δ*spa* **(Figures 1 and 2).** Since we could not detect significant numbers of antigen-specific BM ASCs in WT challenged mice, we hypothesized that this serum IgG response would not be maintained over time. To test this, we measured serum IgG in groups of WT or Δ*spa*-challenged mice bi-weekly for 12 weeks after challenge. We found that by 2 weeks post-challenge the levels of IsdB-specific IgG diverged; mice challenged with Δ*spa* S. aureus maintained relatively high serum antibody for the 12 week period. In contrast, the serum antibody levels in mice challenged with WT *S. aureus* dropped to those detected in uninfected mice (**Figure 6C**). This is consistent with the lack of BM PC accumulation after WT challenge. Therefore, the presence of SpA during a challenge infection did not disrupt the memory B cell and secondary ASC responses, but impaired the accumulation of BM ASCs, thereby preventing long-term maintenance of antigen-specific serum IgG.

Discussion

S. aureus infections commonly recur without inducing protective immunity [68,225]. Effective vaccine design is hindered by waning antibody responses and it is unclear whether this is due to the inability to form long-lived memory B cells or PCs. We found that SpA expressed on the surface of *S. aureus* disrupted the long-term maintenance of serum antibody by preventing accumulation of LLPCs. The lack of LLPCs in the BM was not due to defects in memory B cell or GC formation and SpA did not impair secondary ASC responses. Rather, SpA induced an expanded short-lived IgM^+ PB response that did not contribute to the LLPC pool.

Several studies have described an inverse relationship between ASC expansion and the LLPC compartment. First, lowering the B cell activation threshold after secondary Ag exposure through the loss of the transcription factor Aiolos augments proliferation of short-lived splenic ASCs and relaxes the selection of high affinity PCs. This leads to a lack of LLPCs in the BM, despite normal GCs and chemokine homing [226]. Second, infection with *P. chabaudi* drives an expanded T-independent ASC response while inhibiting long-term maintenance of the T-dependent antibody response [207]. Third, chronic antigen exposure also favors splenic short-lived ASC proliferation over LLPCs by altering chemokine production and responsiveness [34]. In a model of systemic lupus erythematosus where autoantigen is chronically available, *ex vivo* splenic PCs are unresponsive to CXCL12 and diseased mice are unable to generate LLPCs [148,220,224]. In our model, *ex vivo* ASCs from WT challenged mice migrated toward CXCL12 and similar numbers of ASCs reached the BM within a week after WT or Δ*spa* challenge. This indicates that SpA did not affect chemokine/migration pathways. Despite normal migration to the BM, only Δ*spa* challenge resulted in a continued increase in BM ASC numbers. This was

not due to an inhospitable BM microenvironment because NP-specific ASCs were able to accumulate in the BM even when formed during WT *S. aureus* infection.

Our study shows an inability to maintain long-term antibody titers and an impaired capacity of ASCs to accumulate in the BM after WT challenge. This may be due to lack of survival and/or proliferation. The present study does not distinguish between these possibilities; however, these are ongoing areas of investigation. Although many requirements for LLPC survival have been defined, it is not clear what distinguishes short-lived PCs from LLPCs. There is evidence that short- and long-lived ASCs are derived from different precursor populations [227] and that their fate is influenced by the environment in which they originate [48,61,228,229]. Nonetheless, PBs must proliferate and compete for limited survival factors in BM niches in order to become LLPC [227]. Therefore, it is likely that some ASC have the potential to thrive in the niches better than others. How such potential is determined remains unclear. Some have suggested that T cell help is required, but LLPCs can be detected after T-Independent responses [48,49]. We observed increased levels of CD138 on the surface of ASCs in the BM after Δ*spa* challenge compared to WT challenge. Since CD138 is a receptor for APRIL, this phenotype may contribute to the ability of the Δspa-derived BMPC population to persist.

Our data rule out a role for defects in BM niches, suggesting that the context of ASC differentiation in the spleen was important for determining their fate. We speculate that the excess of short-lived PBs driven by SpA in spleen diluted the pool of ASCs that had the potential to survive or proliferate in the BM. Expansion of splenic short-lived extrafollicular ASCs has been described following high affinity/avidity BCR stimulation [222,230]. Such interactions are also characteristic of SpA binding the F(ab) regions of VhIII BCRs [82]. The excess of short-

lived ASCs induced by SpA during the secondary response may have occupied vital BM niches long enough for the long-lived precursors to miss a window of opportunity to become established. There is evidence that such a window is controlled by transient expansion and activation of chemokine-producing BM niche cells [188]. This might explain why the LLPC compartment in WT-challenged mice failed to catch up to that observed in Δ*spa*-challenged mice several weeks after the splenic ASC response resolved.

Changes in gene expression allow memory B cells to more rapidly divide compared to naïve B cells [22,29]. Memory B cells also express higher levels of co-stimulatory molecules and TLRs [231,232]. SpA-BCR binding increases TLR2 sensitivity, resulting in PB proliferation [233]. SpA also mediates BCR uptake of *S. aureus,* leading to intracellular TLR activation [234]. Thus, the effects of SpA may preferentially target the memory population. Because the expanded PBs in WT-challenged mice were IgM^+, memory B cells involved in the response would have been IgM memory cells [23,25]. IgM memory B cells may be more susceptible to SpA-induced activation because SpA binds IgM with higher affinity than IgG [203] and because IgM memory B cells are more likely to be positioned within the marginal zone than IgG memory [22,26,235] where exposure to soluble SpA may be enhanced such that short-lived extrafollicular responses are preferentially driven [236]. In our study, there was significantly greater expansion of splenic IgM^+ PBs after WT challenge compared to primary infection, suggesting a significant contribution of IgM memory B cells in addition to naïve cells. Nonetheless, the secondary antigen-specific IgG^+ ASC response, which derives from both IgG^+ and IgM^+ memory B cells [27] was similar after WT and Δ*spa* challenge. This indicates that the PB expansion driven by SpA did not diminish the ability of memory cells to respond to *S. aureus* antigens. It is possible that the two responses represent unique responses of the recently

described memory B cell subsets [27] to SpA superantigen binding. Development of a flow-based method to detect *S. aureus*-specific memory B cells would aid future studies to discern the respective roles of different memory B cell subsets in *S. aureus* infection.

Our work identifies SpA as a modulator of long-term humoral immunity and compliments previous work demonstrating its role in modulating B cells during infection [84,194,237]. In these studies, immunization with a purified SpA mutant that does not bind Fc or F(ab) regions of Ig (SpAkkaa) or infection with *S. aureus* expressing the SpAkkaa mutant induced anti-SpA neutralizing antibodies that disabled SpA during challenge with *S. aureus* USA300. As a result, *S. aureus*-specific antibody production was significantly increased and bacterial burden in the kidneys of immunized mice was reduced. These studies highlight the role of SpA in impeding short-term primary and secondary B cell response, but do not address long-term antibody maintenance. Our model is different in that SpA is deleted rather than neutralized and secondary *S. aureus*-specific ASC responses were similar in the first 2 weeks after either WT or Δ*spa* challenge. This may be because our choice of strain and inoculum did not induce B cell deletion; therefore memory B cells activated in the presence of SpA could still participate in the secondary response. Another possibility that is not mutually exclusive is that our model does not generate SpA-specific antibodies which may form ICs with SpA that may enhance B cell activation. Nonetheless, after 4 weeks antigen-specific antibody was not replenished in WT-challenged mice due to the lack of LLPC. Together, these data suggest that eliminating the effects of SpA during infection would promote both robust memory and long-term antibody maintenance, which are both key for preventing recurring infections [63].

In summary, the effects of SpA did not impede the formation of GCs or memory B cells, were independent of virulence, and did not disrupt the BM microenvironment. Instead, we found

that SpA altered the immune response during challenge infection. The presence of SpA directed the secondary ASC response to favor short-lived, extrafollicular PBs that impeded the accumulation of LLPCs and attenuated maintenance of long-term antibody. Thus, it is possible that previous vaccine attempts have generated B cell memory, but that reinfection in the presence of SpA drives an excess of short-lived PBs that contribute to clearance of the infection at hand, but not to protection from subsequent infections. It would be of interest to test the effect of a SpA vaccine on the accumulation of LLPC and long-term antibody levels. Further studies to understand the mechanisms by which SpA disrupts BM PC accumulation will inform vaccine design for *S. aureus* and other pathogens.

Materials and Methods

Mice

C57BL/6 mice were bred and maintained in a specific pathogen-free facility at UNC Chapel Hill according to the Institutional Animal Care and Use Committee (IACUC) standards. All experiments were performed according to the IACUC guidelines. At 7-8 weeks of age, mice were inoculated subcutaneously (s.c.) with 1 x 10^8 CFU of *S. aureus* RN4220 (Δ*spa* or WT), Newman, or USA300 in 20 μl of 1x PBS. For challenge experiments, 5 weeks after primary inoculation, 1 x 10^6 CFU were delivered intravenously (IV) via tail vein injection in 100 μl of 1x PBS. For NP-KLH experiments, 100 μg of NP_{20}KLH conjugated to alum (Imject) was delivered by intraperitoneal injection. Immunized mice were boosted (IV) with 100 ug of soluble NP_{20}KLH.

Bacteria

S. aureus RN4220 (Δ*spa* and WT), Newman and USA300 were grown in brain heart infusion (BHI) broth at 37°C overnight and washed and diluted in 1x PBS for infections. Colony forming units (CFUs) were confirmed by plating serial dilutions on BHI plates. Isogenic *S. aureus* Δ*spa* mutants in Newman and RN4220 were constructed by amplifying ≈ 1b 5' and 3' homology fragments flanking *spa* with primers Spa5'.1A (CGACTCTAGAGGAAAAACACACTTC) and Spa5'.1B (TAAGGATCGGGGACAATACCTACACC) for amplification of the 5' homology fragment and primers Spa3'1A (TACCGAGCTCGAATATCATTTTATCC) and Spa3'1B (CAGTGCAGCGGAATTGCTTGGAGTCC) for amplification of the 3' homology fragment.

The resulting fragments were cloned on either side of a Spec^r cassette in the *E. coli/S. aureus* shuttle vector pBT2ts. Allelic exchange was performed as previously described [238] *S. aureus* Δ*srrAB* mutants in RN4220 and Newman were generated as previously described [238].

Kidney burden analysis

To assess bacterial load in kidneys, 7-8 week-old C57BL/6 mice were infected IV with 1 x 10^6 CFU of *S. aureus* Newman or RN4220 WT, Δ*spa*, or Δ*srrAB*. Kidneys were harvested 5, 7 or 14 days later. Kidneys were homogenized in 1x PBS and serial dilutions of homogenate were plated on BHI. CFUs were counted after overnight incubation at 37°C.

ELISA

For *S. aureus* ELISAs, vinyl plates were coated with *S. aureus* RN4220 Δ*spa* grown to stationary phase, washed, and diluted 1:10 in 1x PBS. Plates were coated overnight at 4°C and blocked with 0.5% bovine serum albumin (BSA) overnight at 4°C. Samples were loaded in 0.5% BSA and incubated overnight at 4°C, incubated with anti-IgG, anti-IgG2, or anti-IgM conjugated to alkaline phosphatase for 1 hour at 37°C and developed with p-nitrophenyl phosphate. For IsdB ELISAs, polystyrene plates were coated with 3 μg/ml IsdB (AA40-614) in carbonate buffer overnight at 4° and blocked in 0.5% BSA for 1 hour at 37°C. Plates were loaded and developed as for whole *S. aureus* ELISAs.

Flow cytometry

For flow cytometry analysis, spleens and lymph nodes were crushed into single cell suspensions in cold 1x PBS. Bone marrow was harvested from femurs by flushing femur shafts with 1x PBS. Cells were washed and red blood cells (RBCs) lysed with 0.165M NH_4Cl. Cells

per sample were labeled with Pacific Orange Live/Dead dye and Fc receptors blocked with 2.4G2 before antibody staining. Lymphocytes were identified by forward and side-scatter profiles. GC B cells were identified as $CD19^{+}$ $GL7^{+}$ FAS^{+}; Tfh cells as $CD4^{+}$ $PD\text{-}1^{+}$ $CXCR5^{+}$; ASCs as $CD138^{+}$ $B220^{lo/neg}$; DCs as $CD11c^{hi}$ $CD19^{-}$ $CD3^{-}$ $CD49b^{-}$ $Ter119^{-}$ and $CD8\alpha^{+}$ or $CD8\alpha^{-}$; pDCs as $CD11c^{+}$ $CD19^{-}$ $CD3^{-}$ $CD49b^{-}$ $Ter119^{-}$ $CD45RA^{+}$; eosinophils as Siglec-F^{+} $CD11b^{int}$. For intracellular cytokine analysis, cells were fixed and permeabilized (BD Biosciences) overnight at 4°C before staining. For intracellular IL-12, cells were incubated for 3 hours at 37°C with 3ug/ml Brefeldin A before fixing. For chemokine receptor analysis, cells were labeled in azide-free buffer at 37°C, then washed in normal buffer on ice to halt recycling. Data for DC analyses were collected on a Becton Dickinson LSR II and analyzed with FlowJo. All other samples were run on a Beckman Coulter CyAn ADP and analyzed with Dako Summit.

Antibodies and reagents

Antibodies specific for CD19-PacBlue, CD3e-PE, CD4-PacBlue, PD-1-PE, APRIL-PE, CD11c-Alexa488, IL-12p40-Alexa647, Ter119-FITC, CD11c-PE-Cy7, MHCII IA/IE-APC were purchased from Biolegend; GL7-FITC, FAS-PE, CXCR5-Biotin, CD138-PE, B220-FITC, IgG-Biotin, Siglec-F-PacBlue, CD19-FITC, CD8-BrilliantViolet711, CD45RA-Biotin, Streptavidin-BrilliantViolet421, and Streptavidin-HRP from BD Biosciences; GR-1-Biotin, CXCR4-APC, CD3e-FITC, CD49b-FITC, CD40-PE, CD86-PE, CD80-APC from eBiosciences. IgM (B7.6) and anti-Fc (2.4G2) were purified from hybridomas. Live/Dead Pacific Orange and Fixable Blue dyes were purchased from Invitrogen/Life Technologies and SDF-1 from Shennendoah Biotechnology. Anti-IgG-Alkaline Phosphatase, Anti-IgM- Alkaline Phosphatase, Streptavidin-Alexa647, and Streptavidin-Alexa488 were purchased from Invitrogen; Anti-IgG2- Alkaline Phosphatase from Southern Biotech; p-Nitrophenyl Phosphate tablets, 3-amino-9-ethylcarbazole

tablets, PNA-biotin, and Lipopolysaccharide from Sigma; Anti-IgGFc-biotin from Jackson immunoresearch; Brefeldin A from LC laboratories. IsdB peptide (AA 40-613) was a generous gift from Dr. Benfang Lei (Montana State University). Pokeweed mitogen was a generous gift from Dr. Shane Crotty (La Jolla Institute for Allergy & Immunology).

ELISpot for ex vivo ASC

Multiscreen ELISpot plates were coated with 1.5 μg/ml IsdB (AA40-613) or NP_{13}BSA in 1x PBS overnight at 4°C and blocked in 1% BSA overnight at 4°C. Single cell suspensions of RBC-lysed splenocytes or BM cells were resuspended in RPMI supplemented with Sodium pyruvate, Gentamicin, Pen/Strep, L-Glutamine, β-ME, and 10% FBS (R10) and plated at 0.5-2 x 10^6 cells/well. Plates were incubated at 37°C for 18 hours, then washed and incubated with anti-IgG or anti-IgM conjugated to biotin overnight at 4°C. Plates were washed and incubated in streptavidin-HRP for 3 hours RT and developed with 3-amino-9-ethylcarbazole in Dimethylformamide.

ELISpot for memory B cells

Spleens and lymph nodes were harvested and single cell suspensions were washed and RBC-lysed. Cells were plated in RPMI supplemented with sodium pyruvate, gentamicin, pen/strep, L-glutamine, β-ME, and 10% FBS at 0.5 x 10^6 cells/ml 1:1 with irradiated (1200 rad) feeder cells and cultured with 5 μg/ml LPS, 1:10,000, and 1:1000 fixed *S. aureus* for 5 days at 37°C. After 5 days, cells were washed and plated in serial densities (0.25-2 x 10^6 cells/well) on IsdB-coated ELISpot plates prepared and developed as above.

Tissue immunofluorescence and microscopy

Spleens from challenged mice were harvested and fixed in 4% paraformaldehyde for 5hr RT. They were washed and hardened in 30% sucrose overnight, then washed and flash frozen in OCT (Tissue-Tek). Sections (6 microns) were fixed in 1:1 MeOH/Acetone and blocked in 0.5% Rat serum and 2.4G2 before staining for B220-Alexa647 and IgM-biotin and streptavidin-488 to detect extrafollicular foci. To detect GCs, fixed sections were blocked in 10% FBS and 2.4G2 and stained with B220-Alexa647 and PNA-biotin and streptavidin-488. Sections were imaged with an Olympus Fluoview 500 (10x objective; numerical aperture of 0.45) and analyzed using ImageJ.

Migration assays

Splenic B cells were purified by negative selection (Stem Cell Technologies), and 5 x 10^6 cells were plated in the top chamber in a 5 μm trans well insert (Corning). For control wells, cells were plated in the bottom chamber with no insert. 100 ng/ml of SDF-1 was added to the bottom chambers and plates were incubated for 3hr at 37°C. Samples from the bottom and control wells were harvested and labelled with CD138 and B220 and enumerated by flow cytometry for 120 seconds per sample. The number of migrated ASCs enumerated from the bottom of the trans well was divided by the number enumerated from the corresponding control well and expressed as a percent of ASCs in the control well.

Statistics

The 1-way ANOVA and Bonferroni-Holm post hoc tests were used to compare multiple groups. The two-sided, unpaired Student's *t* test was used for experiments comparing two

groups. In cases where the data was not normally distributed, the Wilcoxon rank sum test was used.

Ethics Statement

The University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (IACUC) approved animal care and use protocols performed for this work (12.276 and 14.018) in accordance with the National Institutes of Health/Public Health Services Animal Welfare Assurance Policy (A3410-01) and the United States Department of Agriculture Animal Research Facility Regulations (registration number 55-R-044).

Table 3. 1 *S. aureus* strains and mutants

Strain	Mutant	Description
RN4220	*Δspa*	Lacks gene for Protein A
RN4220	*Δsrrab*	Lacks SrrAB, a regulator of virulence factors
Newman	*Δspa*	Lacks gene for Protein A
Newman	*Δsrrab*	Lacks SrrAB, a regulator of virulence factors
USA300	*Δspa*	Lacks gene for Protein A

Chapter 3 Figures

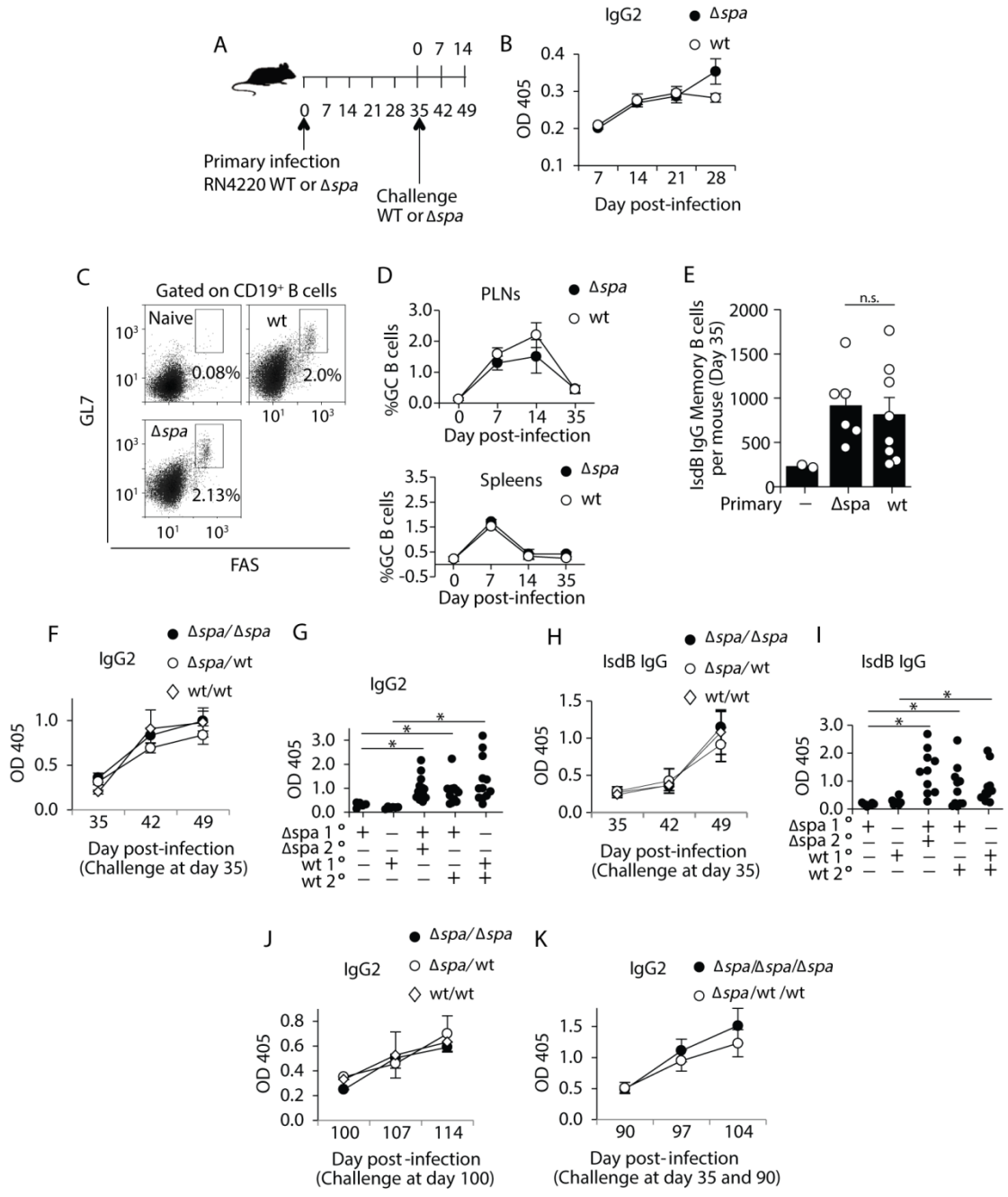


Figure 3. 1 Memory B cells are formed and activated in response to *S. aureus.*

(**A**) Schematic of inoculation and challenge course. (**B-K**) Mice were inoculated s.c. with *S. aureus* RN4220 WT or Δ*spa*. (**C-K**) Primed or naïve mice were challenged IV with *S. aureus* RN4220 WT or Δ*spa* on day 35 (**F-I**), 100 (**J**), or 35 and 90 (**K**). (**B**) *S. aureus*-specific IgG2

measured by ELISA from sera collected on indicated days. n=4-9 mice per group in 3 experiments. (**C**) Representative histograms of GC B cell analysis by flow cytometry on day 14 post-inoculation. (**C-D**) Expression of GC markers GL7 and Fas on B cells from (**C**) draining PLNs or (**D**) spleens. n=3-6 mice per group per time point in 4 experiments. (**E**) Memory B cells detected by ELISpot 35 days post-inoculation. n=6-8 mice per group in 3 experiments. (**F-K**) (**F,G, J, K**) *S. aureus*-specific IgG2 or (**H-I**) IsdB-specific IgG measured by ELISA from sera collected on the indicated days. Sera for G and I were collected on day 14 post-challenge (day 49 for primed mice). (**F**) n=9-21 mice per group in 7 experiments; (**G**) n=5-18 mice per group in 3 experiments; (**H**) n=5-16 mice per group in 10 experiments; (**I**) n=9-12 mice per group in 3 experiments; (**J**) n=3-5 mice per group in 2 experiments; (**K**) n-5-8 mice per group in 4 experiments. O.D.=optical density. *$p<0.05$. n.s. not significant. Error bars=standard error.

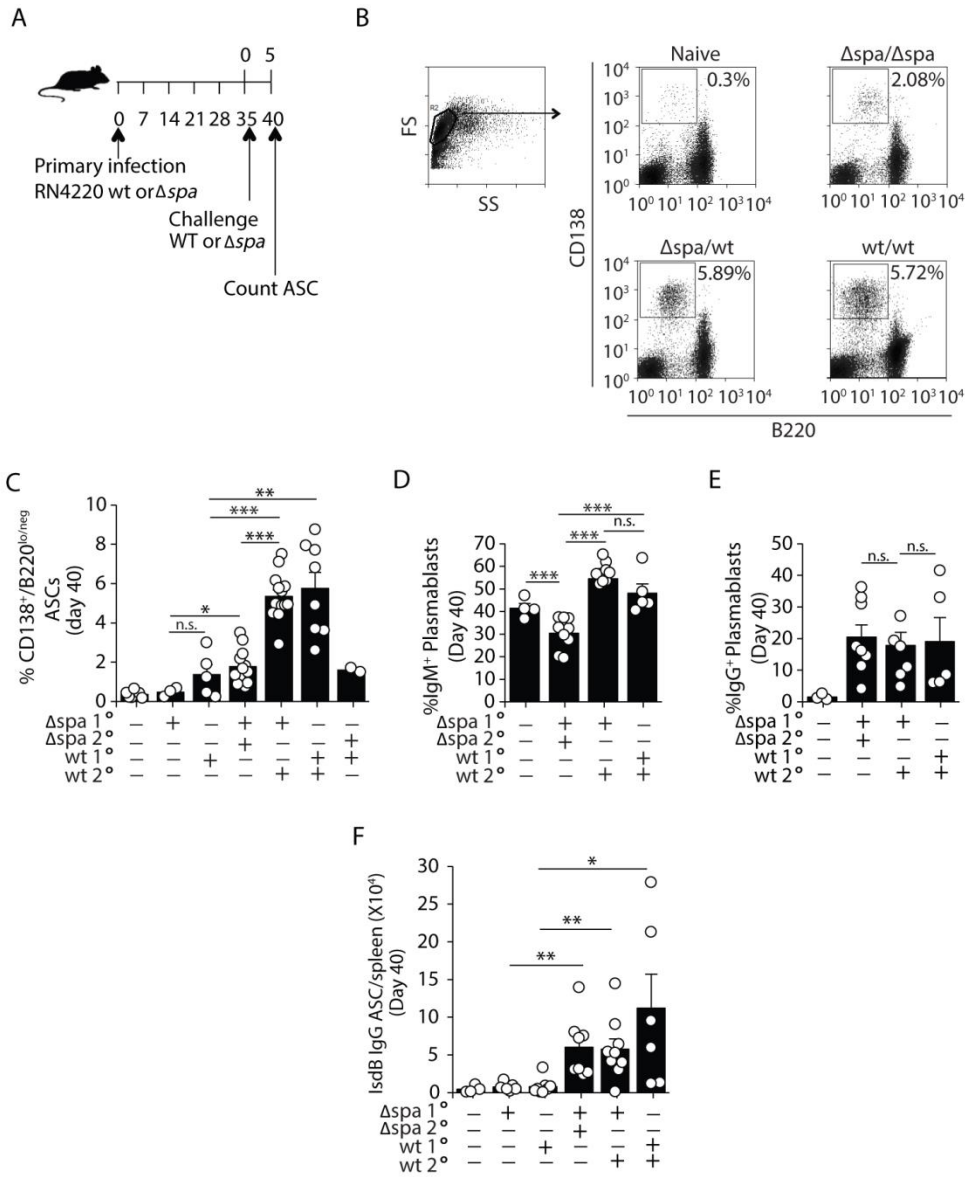


Figure 3. 2 Protein A induces the expansion of IgM^+ plasmablasts

(**A**) Schematic of inoculation and challenge course. Mice were inoculated s.c. with *S. aureus* RN4220 WT or Δ*spa*. On day 35 primed mice and naïve mice were challenged IV with *S. aureus* RN4220 WT or Δ*spa*. Spleens were harvested for analysis 5 days later (day 40). (**B**) Representative histograms of $CD138^+$ $B220^{lo/neg}$ ASC analysis in (C). (**C**) Compilation of the frequency of $CD138^+$ $B220^{lo/neg}$ ASCs from (B). n=2-13 mice per group in 7 experiments. (**D-E**)

Frequency of surface (**D**) IgM^+ and (**E**) IgG^+ ASCs. n=3-9 mice per group in 4 experiments. (**F**) Anti-IsdB IgG-producing ASCs quantitated by ELISpot. n=4-9 mice per group in 4 experiments. FS=forward scatter; SS=side scatter; *p<0.05, **p<0.01, ***p<0.001, n.s. not significant. Error bars=standard error.

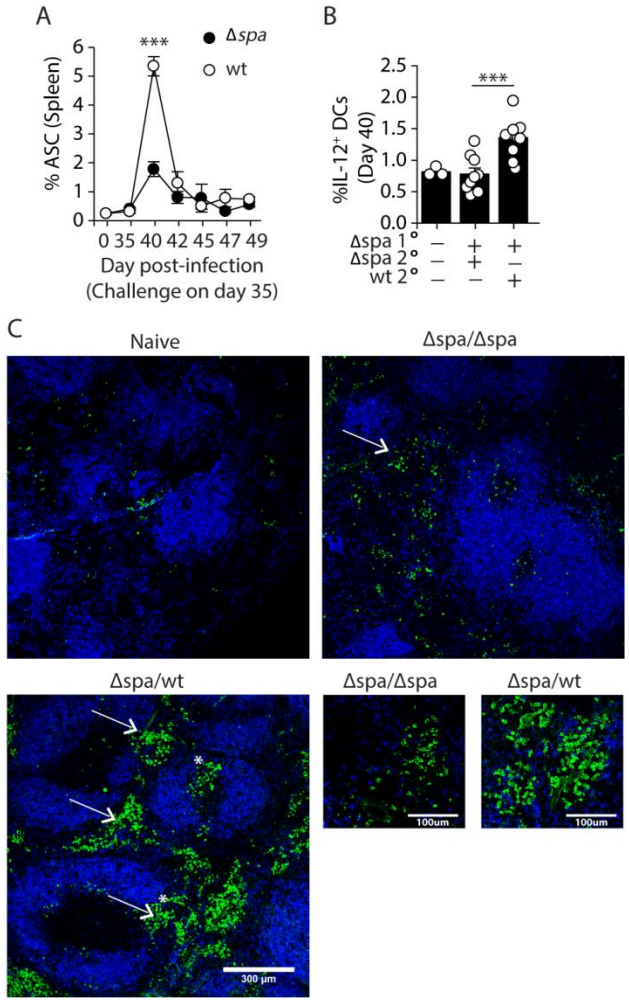


Figure 3. 3 Protein A drives an expanded short-lived extrafollicular response (A-C)
Mice were inoculated s.c. with *S. aureus* RN4220 Δ*spa* and challenged IV 35 days later with *S. aureus* RN4220 Δ*spa* or WT. (**A**) The frequency splenic ASCs at indicated time points. n=3-13 mice per group per time point over 2-7 experiments. (**B**) The frequency of IL-12-producing splenic DCs on day 40 (day 5 post-challenge). n=3-12 mice per group in 3 experiments.

(**C**) Day 40 spleen sections labeled with B220 (blue) and IgM (green) to image extrafollicular foci. n=5-6 mice per group in 3 experiments. Arrows indicate foci and * indicates bridging channels. Full image scale bar=300 microns, inset scale bars=100 microns. ***p<0.001. Error bars=standard error.

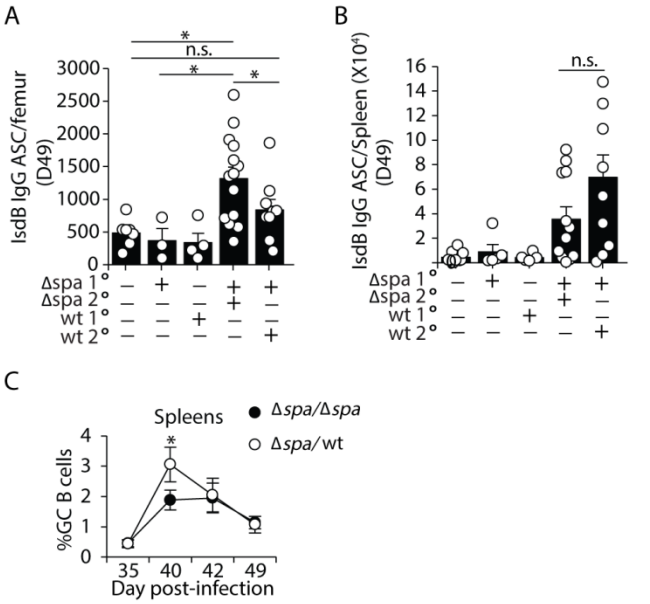


Figure 3. 4 *S. aureus*-specific ASCs formed after WT challenge fail to populate the bone marrow

(**A-C**) Mice were inoculated s.c. with *S. aureus* RN4220 Δ*spa* and challenged 35 days later with *S. aureus* RN4220 Δ*spa* or WT. (**A-B**) Anti-IsdB IgG-producing ASCs quantitated from (A) bone marrow and (B) spleens on day 49 (day 14 post-challenge). (**A**) n=3-14 mice per group in 7 experiments; (**B**) n=5-12 mice per group in 10 experiments. (**C**) $CD19^{+}$ B cells analyzed for expression of GC markers GL7 and Fas on the indicated days. n=3-13 mice per group in 9 experiments. *$p<0.05$, n.s. not significant. Error bars=standard error.

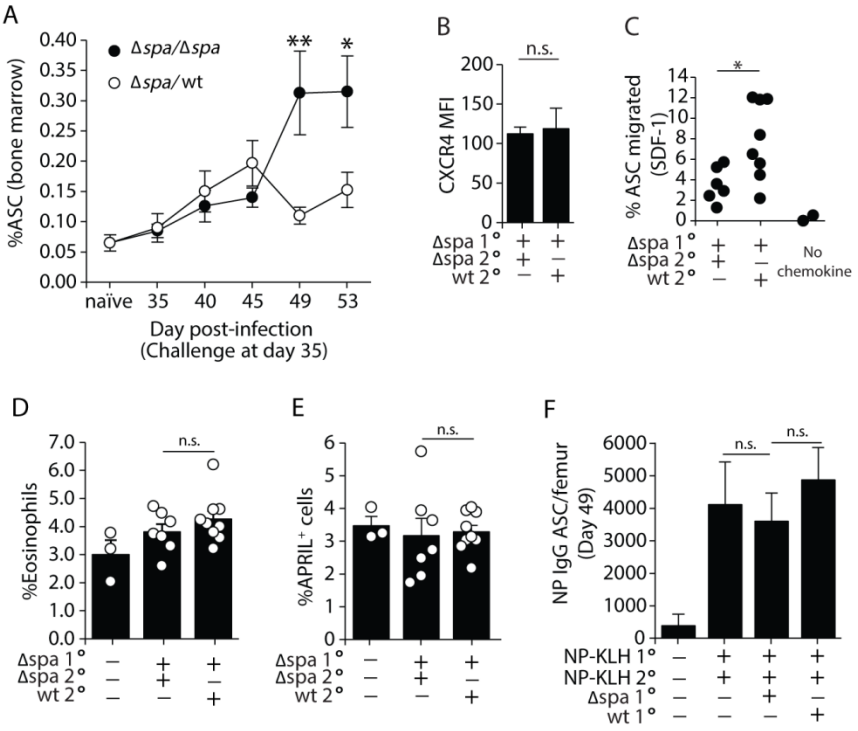


Figure 3. 5 Protein A does not disrupt BM niche factors or the migration of ASCs to the BM (**A-E**) Mice were inoculated s.c. with *S. aureus* RN4220 Δ*spa* and challenged 35 days later with *S. aureus* RN4220 Δ*spa* or WT. (**A**) BM CD138+ $B220^{lo/neg}$ ASCs quantitated by flow cytometry on the indicated days. n=3-12 mice per group per time point in 2-5 experiments. (**B**) Surface expression of CXCR4 on splenic ASC on day 40 (day 5 post-challenge). n=5-6 mice per group in 3 experiments. (**C**) Frequency of ASCs that migrated through a trans well membrane toward SDF-1(CXCL12). n=2-8 mice per group in 3 experiments. (**D-E**) The frequency of (D) eosinophils and (E) APRIL-producing cells present in BM on day 40 (day 5 post-challenge). n=3-6 mice per group in 3 experiments. (**F**) Mice were immunized with NP_{20}KLH by i.p. injection and 35 days later boosted with soluble NP_{20}KLH alone or co-infected IV with *S. aureus* RN4220 Δ*spa* or WT. On day 49 (day 14 post-boost/infection), NP-specific IgG-producing ASC were quantitated by ELISpot. n=2-6 mice per group in 2 experiments. *p<0.05, n.s.=not significant. Error bars=standard error.

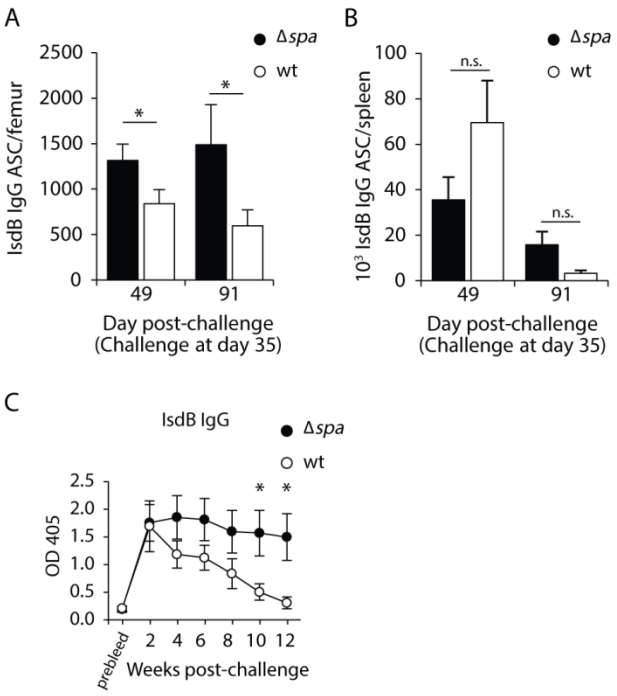
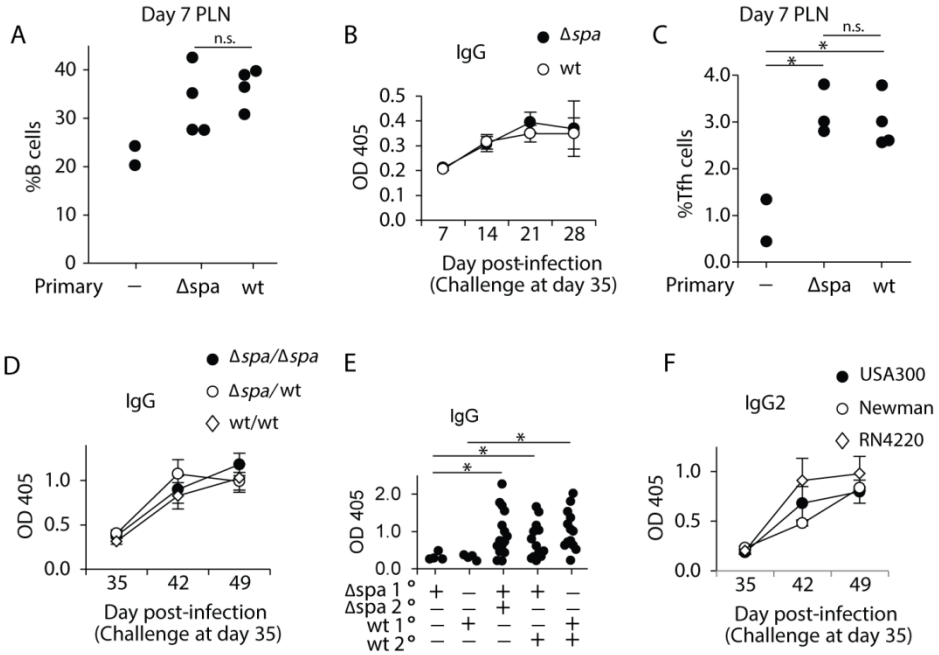
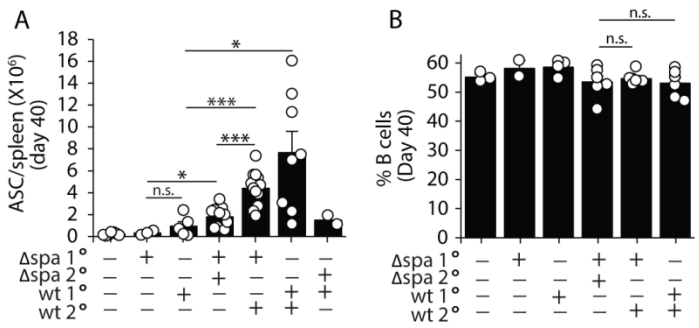


Figure 3. 6 Protein A prevents long-term maintenance of BM LLPCs and serum antibody (**A-C**) Mice were inoculated s.c. with *S. aureus* RN4220 Δ*spa* and challenged 35 days later with *S. aureus* RN4220 Δ*spa* or WT. (**A-B**) Anti-IsdB IgG-producing ASCs quantitated from the (A) BM and (B) spleens of challenged mice on days 49 and 91 (day 14 and 56 post-challenge). n=3-6 mice per group in 3 experiments. (**C**) IsdB-specific IgG measured by ELISA from sera collected on indicated days. n=4-10 mice per group in 5 experiments. OD=optical density, *$p<0.05$, n.s.=not significant. Error bars=standard error.



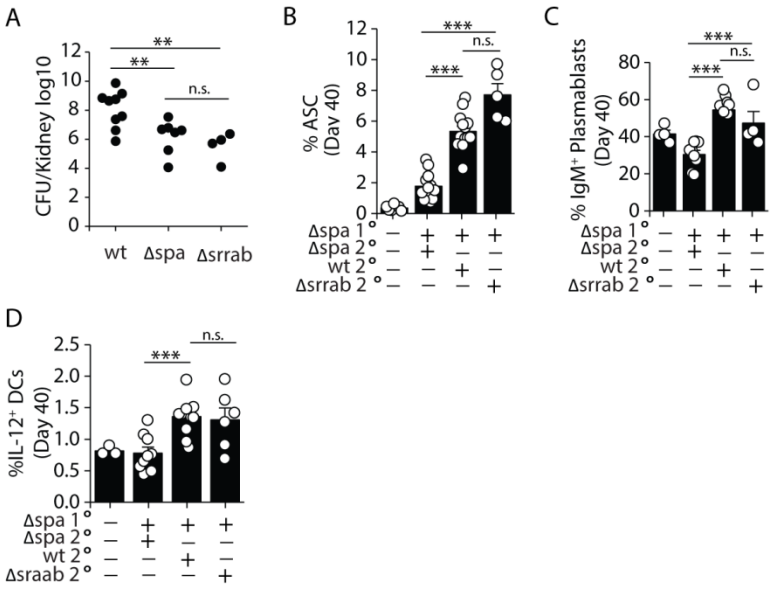
Supplemental Figure 3. 1

(**A-D**) Mice were inoculated s.c. with *S. aureus* RN4220 WT or Δ*spa*. (**A**) Frequency of B cells from PLNs. N=2-4 mice per group in 2 experiments. (**B**) *S. aureus*-specific IgG measured by ELISA from sera collected on indicated days. n=3-4 mice per group in 2 experiments. (**C**) Frequency of Tfh cells among CD4+ T cells in PLNs. N=2-4 mice per group in 2 experiments. (**D-F**) Primed or naïve mice were infected intravenously (IV) with *S. aureus* RN4220 WT or Δ*spa* and *S. aureus*-specific IgG was measured by ELISA. (**E**) was measured on day 14 post-challenge (day 49 for primed mice). (**D**) n=7-16 mice per group per time point in 8 experiments; (**E**) n=4-17 mice per group in 5 experiments. (**F**) *S. aureus*-specific IgG measured by ELISA in sera from mice inoculated s.c. with *S. aureus* RN4220, Newman, or USA300 and challenged with the same strain 35 days later. n=3-8 mice per group in 3 experiments. OD=optical density, *p<0.05.



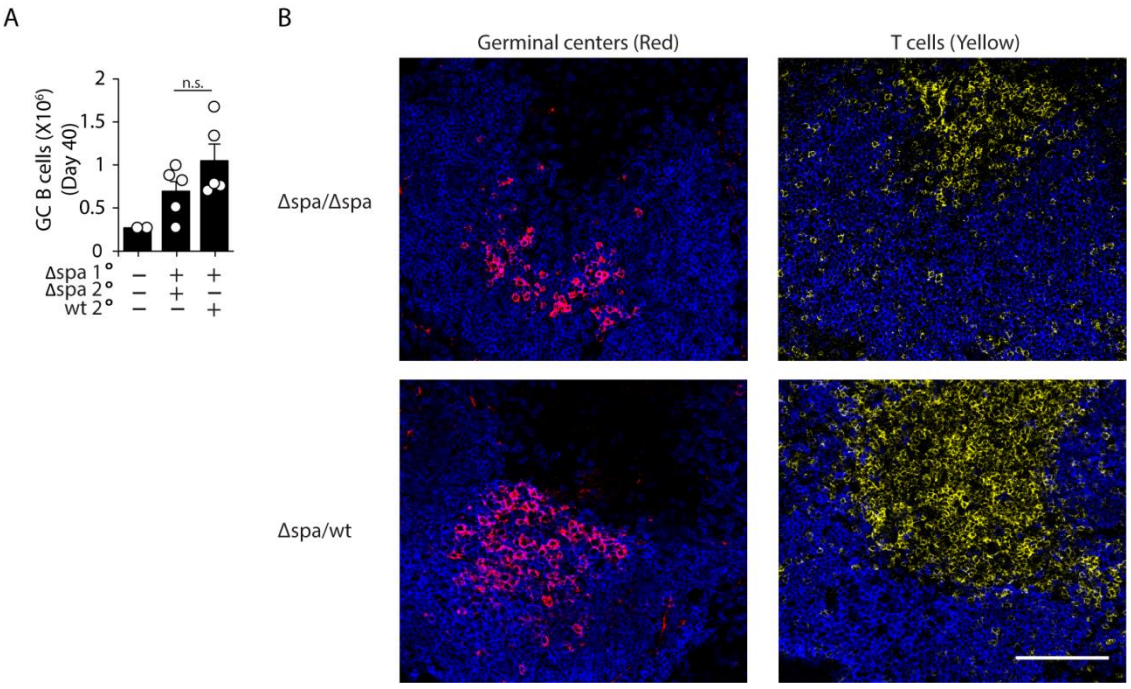
Supplemental Figure 3. 2

(**A**-**B**) Mice were inoculated s.c. with *S. aureus* RN4220 WT or Δ*spa* and challenged 35 days later with *S. aureus* RN4220 Δ*spa* or WT. (**A**) The numbers of $CD138^{+}$ $B220^{lo/neg}$ ASCs and (B) the frequency of $CD19^{+}$ B cells in spleens harvested 5 days later (day 40). (A) n=3-13 mice per group in 7 experiments; (B) 2-7 mice per group in 3 experiments. *p<0.05, ***p<0.001, n.s.=not significant. Error bars=standard error.



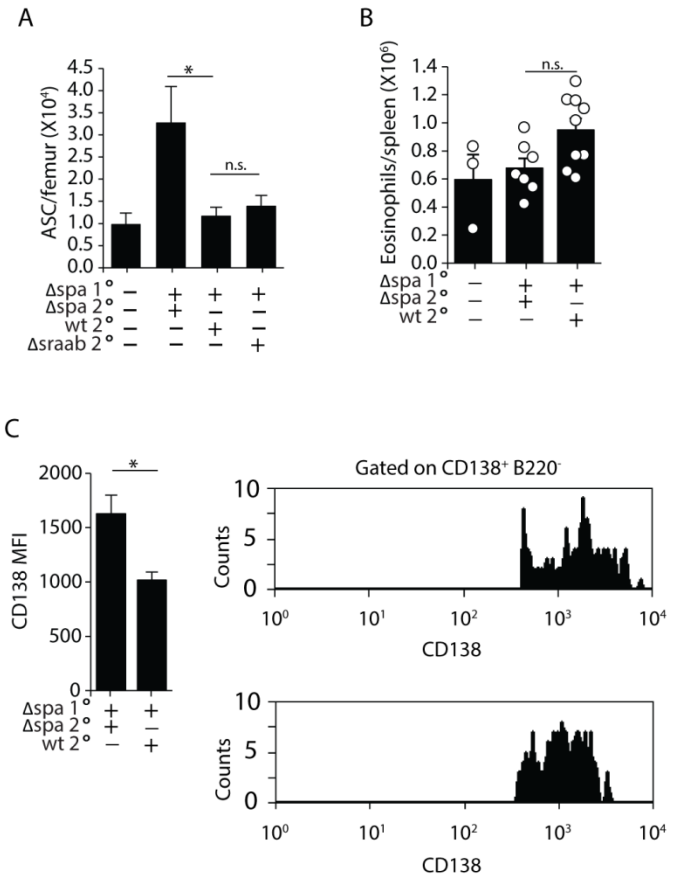
Supplemental Figure 3. 3

(**A**) Bacterial load in the kidneys of mice infected IV with *S. aureus* Newman WT or Newman Δ*spa* or Newman Δ*srrAB* 7 days earlier. n=4-9 mice per group in 2 experiments. (**B-D**) Mice were inoculated s.c. with *S. aureus* RN4220 Δ*spa* then challenged 35 days later with *S. aureus* RN4220 WT, Δ*spa*, or Δ*srrAB*. Spleens were analyzed 5 days later (day 40). (**B**) The frequency of splenic $CD138^{+}$ $B220^{lo/neg}$ ASCs. n=3-13 mice per group in 7 experiments. (**C**) Frequency of IgM+ PBs. n=3-9 mice per group in 4 cohorts. (**D**) Frequency of IL12+ DCs. n= 3-12 mice per group in 3 experiments. Error bars=standard error.



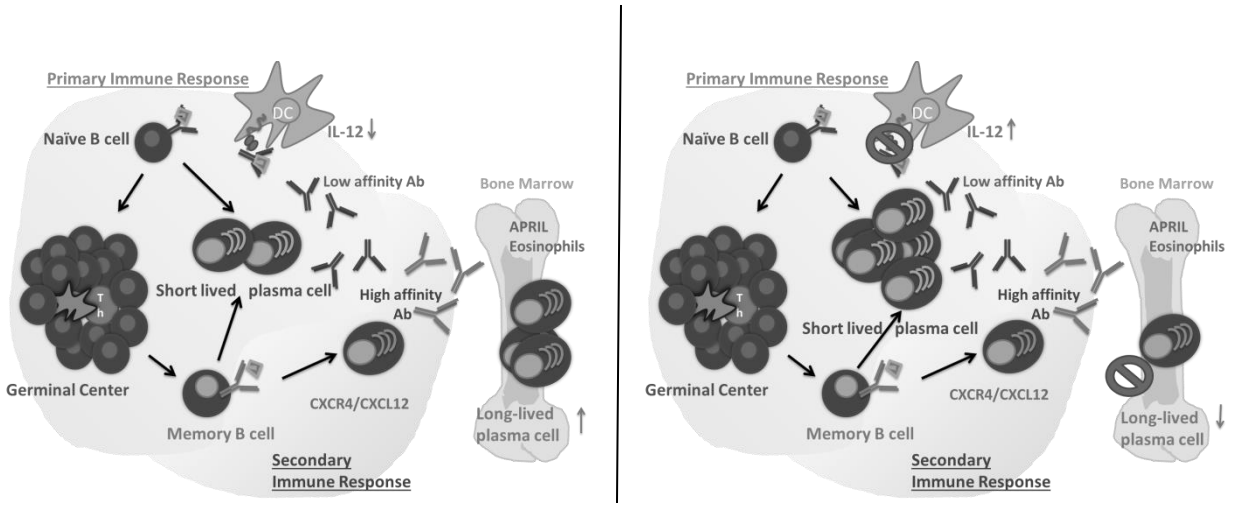
Supplemental Figure 3. 4

(**A-B**) Mice were inoculated s.c. with *S. aureus* RN4220 Δ*spa* and challenged 35 days later with *S. aureus* RN4220 Δ*spa* or WT. Spleens were harvested 5 days later (day 40). (**A**) The number of GC B cells measured. n=2-5 mice per group in 2 experiments. (**B**) Day 40 spleen sections labeled with B220 (Blue), PNA (Magenta), and Thy1.2 (Yellow). n=4-6 mice per group in 2 experiments. Scale Bar=100 microns. n.s.=not significant. Error bars=standard error.



Supplemental Figure 3. 5

(**A**) Mice were subcutaneously inoculated with *S. aureus* RN4220 Δ*spa* then challenged 35 days later with *S. aureus* RN4220 WT, Δ*spa*, or Δ*srrAB.* (**A**) Frequency of BM CD138^{+} B220$^{lo/neg}$ ASCs on day 49 (day 14 post-challenge). n=6-12 mice per group in 7 experiments. (**B**) Number of BM eosinophils. n=3-9 mice per group in 3 experiments. (**C**) Expression of CD138 on BM ASCs on days 45 and 47 (days 10 and 12 post-challenge). n=6-10 mice per group in 3 experiments. Error bars=standard error.



Supplemental Figure 3. 6 Chapter Three Model.

In the primary adaptive immune response to *S. aureus*, B cells differentiate into short-lived plasma cells, germinal center B cells, and ultimately memory B cells in the absence (A) or presence (B) of SpA. (**A**) Memory B cells respond to *Δspa S. aureus* (sepsis) during a secondary infection by differentiating into short-lived plasmablasts and long-lived plasma cells, mainly in the spleen. Long-lived plasma cells then follow the chemotactic signal, CXCL12 (via CXCR4) to survival niches in the bone marrow where they constitutively secrete IgG. (**B**) In response to a secondary wild-type infection, however, significantly more short-lived plasmablasts are produced, likely as a result of the superantigen activity of SpA. An increase in the frequency IL-12^{+} DCs due to SpA's interference of FcγR-IC interactions also potentially contributes to the rise in plasmablasts. Plasma cells and plasmablasts formed in the presence of SpA during the secondary response are able to respond to chemotactic signals to the bone marrow and some arrive within a week of secondary infection. However, the number of plasma cells in the bone marrow of wild-type infected mice is not sustained and antigen-specific IgG drops in the serum within 4 weeks. This occurs despite the presence of niche factors, APRIL and eosinophils, and

the ability of the bone marrow niche to support plasma cells. It is hypothesized that plasma cells "imprinted" to become long-lived during the splenic response are crowded out by cells that are not. During a window characterized by high expression of CXCL12 from the bone marrow, both types of plasma cells migrate to the bone marrow niches, but only the "imprinted" ones survive. Because the ratio of "imprinted" to "non-imprinted" is lower after wild-type infection due to short-lived plasmablast expansion (B), fewer long-lived cells are sustained compared to the number that survive after *Δspa* infection (A).

CHAPTER 4: CONCLUSION

Long-term humoral immunity in protection against disease

Long-term humoral immunity mediated by memory B cells and LLPCs is a key arm of protective immunity against many pathogens. Generating either cell type involves a series of check points that are still incompletely characterized, particularly in the context of infection. The work presented in this thesis identifies previously unappreciated regulatory mechanisms of memory B cell formation (Chapter 2) and an immune evasion strategy that disrupts LLPC function (Chapter 3) after *Staphylococcus aureus* infection.

Chapter 2 describes a role for ICs and BAFF in promoting GCs, Bcl-6, and B cell memory. Although it is known that GCs and Bcl-6 expression of GC B cells are pre-requisites for class-switched high affinity memory, the molecular mechanisms that decide GC B cell fate are not well defined. The work in Chapter 2 identifies BAFF as a key mediator of that decision that promotes the transcription factor Bcl-6 and downregulates signals required for PC differentiation. Another key feature of this work is the identification of ICs as regulators of the earlier primary adaptive immune response. The requirement of IC-FcγR interactions for BAFF in the primary response serves as a both a positive feedback mechanism and a regulatory checkpoint.

Chapter 3 represents the first known characterization of memory B cell and PC responses to the common pathogen, *Staphylococcus aureus* and the role of the bacterial protein SpA in

modulating long-term humoral immunity. This work provides evidence that B cells are capable of forming memory after *S. aureus* infection, but have not been appreciated as participants in protective immunity in part due to the expression of SpA during re-infection. It also identifies LLPCs as targets of immune modulation by *S. aureus* and provides insight that could improve vaccine design.

The Role of DCs and ICs in B cell responses

The work presented in Chapters 2 and 3 shed light on two ways that DCs participate in B cell fate decisions in response to IC ligation. In Chapter 2, DCs were found to be a sufficient source of BAFF required for GC and memory B cell responses. In Chapter 3, DC IL-12 production is implicated as a driver of PB differentiation. In both cases, DCs integrate input, or lack of input, from ICs into signals that are relevant to B cell differentiation.

Though they are most well-known for presenting antigen to and activating T cells, DCs also directly modulate B cells during immune responses. They induce T-independent class switch and B cell survival through secretion of BAFF and APRIL and expression of CD40L [239]. Other cells make cytokines that impact B cells during an immune response, but DCs are uniquely positioned in close proximity to B cells during both T-dependent and T-independent responses. DCs carry large intact antigen into spleen [201] and can be found in the FO and MZ, in the red pulp of the spleen, and in GCs [112,142,240,241]. B cells also survey incoming DCs for Ag that can be carried into the spleen in both MHC-processed and native forms [202,213], which places DCs at an ideal position to regulate B cells during their initial exposure to antigen.

DCs are also unique in their ability to integrate IC signaling into their responses to antigen. DCs co-express activating (FcγRI, III, and IV) and inhibitory (FcγRIIb) receptors for IgG, which provides an opportunity to set up thresholds for positive or negative regulation by ICs [242]. These signals can enhance antigen presentation [144] and promote secretion of cytokines like interferon (IFN) and BAFF [135,136,243], and downregulate other signals, like IL-12 [136,148].

In Chapter 2, we found that DCs lacking FcγRIII ($CD16^{-/-}$) make less BAFF, resulting in reduced B cell Bcl-6 and memory B cell numbers. An understanding of how DC BAFF is regulated has important implications for vaccine design. DC BAFF was recently identified as a positive regulator of malaria-specific memory B cells, but was defective during malaria infection [244]. It would be interesting to determine the role of ICs in BAFF dysregulation during malaria infection. Systemic overexpression of BAFF also successfully enhances immunity generated against a bacterial vaccine [121]. BAFF's role in B cell survival, however, makes it a potent driver of autoimmunity [245]. The work in Chapter 2 suggests that confining over-expression of BAFF to DCs may be a way to achieve specific regulation of antigen-specific B cells while limiting the potential for autoimmunity.

In *S. aureus* infection, DCs are the main producers of IL-12, which contributes to protection [214–216] and enhances T-independent B cell responses [217]. $Fc\gamma R^{-/-}$ DCs make more IL-12 than wild-type DCs after immunization [148]. IC-FcγR interactions, therefore, are needed to downregulate DC IL-12 made in response to innate signals. In Chapter 3, we observed an increase in $IL\text{-}12^{+}$ DCs and expansion of short-lived extrafollicular PBs after WT but not *Δspa* challenge, suggesting IC-mediated regulation of IL-12 is disrupted by SpA. More work should be done to determine if this observation was indeed a direct consequence of SpA

interfering with IC-FcγR interactions. This could be accomplished using the recently described SpAaa (non-Fab binding) and SpAkk (non-Fc-binding) *S. aureus* mutants [84].

Other cells with Fc receptors may have roles similar to DCs that are worth investigating. FcγR ligation on MFs and monocytes, for example, induces pro- and anti-inflammatory cytokine secretion [246]. In the NP-KLH model, MFs were not able to restore BAFF-dependent GCs or memory (Chapter 2), but they may play similar roles in other contexts. Another example is plasmacytoid DCs (pDCs), which make IFN-α as a result of IC-mediated internalization of intracellular TLR ligands [243]. Other cells with FcγRs that have been demonstrated to influence B cell differentiation include neutrophils, basophils, and mast cells [247]. These were not examined in Chapter 2, but it would be worthwhile to determine the respective roles of each $Fc\gamma R^+$ cell type in IC-mediated regulation of B cell responses.

Translating IC-mediated regulation of memory to S. aureus *infection*

Model antigens like NP-KLH have provided invaluable details about the requirements for GCs, memory B cells, and PCs. However, details learned in a model system are not always applicable to every type of immunogen or infection. Model antigens allow us to isolate the roles of individual factors in B cell responses that can then be tested in more biologically relevant situations. Using NP-KLH, we determined that BAFF made by IC-bound DCs plays a key role in successful memory and GC formation through secretion of BAFF (Chapter 2). Disruption of IC-FcγR interactions with either an Fc-blocking peptide that mimics SpA (Chapter 2) or native SpA on *S. aureus* (Appendix 1) also blocked BAFF secretion *in vitro*. Despite these findings, *in vivo* infection with *S. aureus* induced DC BAFF secretion, GC reactions, and B cell memory in the

presence or absence of SpA (Chapter 3). This may have occurred because, unlike single-antigen T-dependent immunization with NP-KLH, bacterial infection provides many ligands that could stimulate BAFF production by DCs or other myeloid cells. TLR stimuli and IFNs can induce myeloid DCs to make and/or secrete BAFF [153,248,249].

Another reason SpA may not block IC-driven BAFF production *in vivo* is because of its low affinity for IgG3 [250]. IgG3 is typically made during T-independent extrafollicular responses [8,207,251]. Therefore, the requirement for early IC-driven BAFF may be filled by IgG3 during *S. aureus* infection and perhaps in other contexts where T-independent responses are present.

B cells in Staphylococcus aureus *infection*

Some studies have concluded that B cells are not required for protection against *S. aureus* since B cell knockout mice fare no worse than wild-type mice in regards to bacterial organ burden after septic infection [196,252,253]. However, these studies focus on short-term responses (7 or 14 days after primary infection or immunization) and lack characterization of the B cell response. Further, in one study [253], the loss of B cells resulted in a significant reduction in systemic IFN-γ, indicating that B cells promote immunity to *S. aureus* even if they do not directly contribute to bacterial clearance.

Despite these findings, passive immunization with monoclonal antibodies is protective against *S. aureus* and pre-challenge antibody titers correlate with reduced bacterial organ burden in infected mice [179–182]. Clinical studies in humans have also shown correlations between pre-existing antigen-specific serum antibody levels and improved clinical outcomes [64,65], and

a recent study reported that maintenance of antigen-specific Ab during convalescence correlated with reduced recurrence [63]. Mutations that cause defects in antibody production also increase the risk of acquiring *S. aureus* infections [174,254]. Human antibody responses to *S. aureus*, however, are variable and there are no clear predictors indicating which patients will retain high titers for long periods of time [69,255].

Recent evidence suggests that these observations may be related SpA's ability to drive BCR-mediated activation and sequester IgG from FcγRs. Injection of SpA drives polyclonal B cell expansion or deletion of B cells, depending on the inoculum and the presence of co-stimulation [75,82]. A mutant form of SpA that cannot bind Fab or Fc (SpAkkaa) can be injected into mice as a purified protein without causing any B cell deletion, allowing it to be used as a vaccine [237]. Vaccination with SpAkkaa or passive immunization with mAbs raised against SpAkkaa result in antibody-mediated protection against *S. aureus* abscess formation [194,237]. Inoculation with live *S. aureus* that express SpAkkaa similarly protects against subsequent infection [84]. Serum IgG is also more effective at neutralizing *S. aureus* expressing the mutant SpA compared to wild-type *S. aureus* [84]. These data point to SpA's role in negating the effects of B cells and antibody on the immune responses to *S. aureus* and may explain why the absence of B cells does not appear to influence short-term clearance of the pathogen.

Chapter 3 adds to these observations by identifying SpA as a modulator of the long-term B cell response. Despite allowing memory B cells to form and function, SpA interfered with LLPCs, which are the ultimate products of B cell memory responses. So although affinity maturation may be able to proceed and provide protective antibody, the presence of SpA prevents that antibody from being maintained.

Negative regulation of long-lived plasma cells by pathogens

LLPCs have been recently recognized as important participants in protection against infection and as potential targets of immune modulation by invading pathogens. Disruption of long-term humoral immunity may result from defects in the GC response, as is the case for *Plasmodium chabaudi* infection [207]. Another pathogen, *Salmonella enterica,* disrupts LLPC-mediated immunity by altering the BM PC niche [34].

Sepsis and *P. chabaudi* infection deplete previously generated antigen-specific BM PCs, presumably by inducing high levels of IgG that negatively regulate LLPCs through FcγRIIb [41,256]; however FcγRIIb$^{-/-}$ mice were defective in initially generating BM PCs, making these results difficult to interpret [256]. It is possible that in the context of *S. aureus* infection, SpA prevents negative regulation of splenic PCs via FcγRIIb, which could make competition for LLPC establishment in the BM more difficult (discussed in Chapter 3). FcγRIIb-mediated regulation of BM PCs, however, is not intrinsic to BM PCs themselves, because FcγRIIb$^{-/-}$ PCs transferred into wild type mice persisted at similar frequencies for the same duration at wild type PCs transferred into wild type mice [257]. Therefore, FcγRIIb may not directly regulate BM PCs, but may indirectly affect their longevity by regulating their precursors during differentiation within secondary lymphoid organs.

This seems to be the case for PC regulation through the IgM-IC receptor, FcμR. FcμR negatively regulates T-independent B cell responses in the spleen [258,259] and loss of FcμR reduces PC trafficking to the BM after T-dependent immunization [258]. Interestingly, an IgM-binding protein expressed by *Plasmodium spp.* is hypothesized to block the interaction between FcμR and IgM [260]. This suggests that it is possible that pathogen disruption of FcμR -mediated

regulation is involved in the ultimate disruption of BM PCs by unregulated short-lived splenic ASC responses.

Determining the capacity to become a LLPC

In Chapter 3 we hypothesize that the mechanism that links short-lived ASC expansion to the lack of LLPC involves competition between PBs after migration to the BM. This is difficult to test because there is no clear way to distinguish PCs that are destined to be long-lived from those which are not. Blimp-1 expression is the most well characterized marker of maturity for splenic ASCs, though it is not clear whether Blimp-1 can be used to discern PC longevity because BM PCs are uniformly Blimp-1^{high} [15]. The surface B cell markers, CD19 and B220 decrease as Blimp-1 expression increases in splenic ASCs [15]. ASCs found in the BM after WT *S. aureus* challenge expressed higher levels of CD19 and lower levels of CD138 than BM PCs from *Δspa*-challenged mice at time points before the numbers of BM PC were equal (Chapter 3 and Appendix 2). CD138 is a marker of maturity and a receptor for the PC survival factor, APRIL [46,47]. Therefore this phenotype ($CD19^{+/hi}$ $CD138^{lo}$) may be associated with an inability to compete for survival in the BM niche.

This is consistent with a model of "imprinted" longevity for LLPCs which has previously been proposed [48]. In the "imprinted" model, some PBs and PCs differentiating in the spleen receive signals from T cells or via the BCR that induce expression of genes required for survival in the BM [32,48,61]. These genes may include the receptors for survival factors like BAFF or APRIL. The BAFF and APRIL receptor, transmembrane activator and CAML interactor (TACI) is required for PC differentiation and/or survival [118,261]. $TACI^{hi}$ cells, for example, are least likely to express cell-cycle or pro-apoptotic genes [262]. Expression of TACI is regulated by the

BCR, TLRs, and CD40L [263]. B cell maturation antigen (BCMA) is another receptor for APRIL found highly expressed on PCs. Although the control of BCMA is less well characterized, its expression is vital for LLPC survival and maintenance, and activation of the pro-survival factor, Mcl-1 [57,264].

It is possible that ASCs activated solely by SpA do not receive T cell help and consequently lack the signals required to activate receptors like TACI, CD138, or BCMA. This is consistent with the observation that $SAP^{-/-}$ mice cannot generate LLPCs [183]and that GC-derived PCs are formed late in the GC reaction, and thus have experienced multiple rounds of T cell help and affinity maturation [10,130]. It is also possible that ASCs derived in the presence of SpA lack the appropriate BCR signaling required to upregulate BAFF and APRIL receptors. Although a signaling pathway linking SpA to downmodulation of these receptors is not clear, it may involve Bruton's tyrosine kinase (Btk), which is downstream of the BCR and TLRs and is required for TACI expression [263,265]. Interestingly, the loss of Btk is associated with an increase in extrafollicular responses [2]. The fact that most of the expanded PB population induced by SpA was surface IgM^+ may also be important if the differences in IgG and IgM BCR-proximal signaling [266,267] are related to expression of genes required for BM LLPC survival. The frequency of IgM^+ ASCs in the BM as well as the expression of TACI and BCMA on ASCs from WT versus *Δspa*-challenged mice is currently under study.

The mechanism that prevents ASCs formed after WT *S. aureus* challenge from populating the BM between days 5 and 14 post-challenge remains to be defined. If the "imprinting" model is correct, both imprinted and non-imprinted ASCs would be competent to migrate to the BM during a window of high expression of chemotactic and survival signals in the BM [188]. After that window is closed, however, the imprinted cells would outcompete those

without high expression of survival signals. In the case of WT-challenge mice, the expansion of "non-imprinted" cells may limit the number of "imprinted" cells that arrive in the BM. For reasons that are not yet clear, the imprinted cells do not rebound in the WT-challenged mice since by eight weeks post-challenge, WT-challenged mice have a similar number of IsdB-specific BM PCs as naïve mice (Chapter 3). To understand whether "imprinting" provides a survival advantage, a proliferative advantage, or both, future work should be done to analyze the frequency of apoptotic (Annexin V^+) and dividing ($Ki67^+$ or $BrdU^+$) ASCs in the BM of WT and *Δspa*-challenged mice over time.

Relevance for human vaccine design

Both Chapters 2 and 3 have important implications for vaccine design. The results of Chapter 2 suggest that BAFF could be used to augment vaccine responses and that local, DC-derived BAFF is sufficient to improve T-dependent GC responses and B cell memory. Long-term, genetic manipulation or IC stimulation of DCs could be used to enhance vaccines for which better adjuvants are needed, or for DC-based cancer vaccine therapies.

Chapter 3 reveals an important mechanism of immune modulation that could be exploited to improve the efficacy of vaccines against *S. aureus*. Vaccines comprised of whole bacteria deficient for SpA do generate humoral memory, but are not protective against WT (SpA^+) infection ([268] and Appendix 3). Induction of SpA-neutralizing antibody is a better strategy that has been proven effective at reducing abscess formation and increasing short-term antibody responses in mice [84,194]. The data in Chapter 3 indicate that long-term antibody titers will be an important measure of the effectiveness of any vaccine strategy that neutralizes SpA. Two

important variables to consider in pursuing this strategy are the expression levels of SpA by an infecting strain and the frequency of VhIII host B cells. It would be useful to discern whether these factors are related to the observed variability in long-term antibody levels among individuals recovering from *S. aureus* infections.

APPENDIX 1

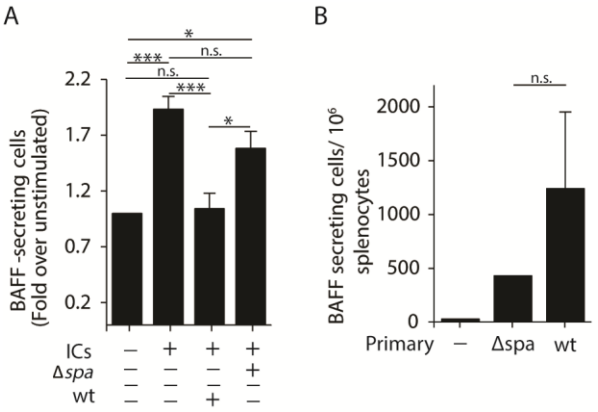


Figure 4. 1 Staphylococcus Protein A interferes with IC-induced DC BAFF *in vitro* but not *in vivo*

Previous work by our lab has shown that immune complex (IC) ligation of Fc receptors (FcγR) induces BAFF production by DCs which in turn directs B and T lymphocyte populations to generate memory. We hypothesized that SpA on *S. aureus* would sequester ICs and prevent FcγR ligation on DCs and BAFF production. To test this, DCs derived from the bone marrow were cultured with ICs and secreted BAFF was measured by ELISpot (see Chapter 2 Methods) in the presence or absence of *S. aureus* RN4220 WT or *Δspa* (1 x 10^6 CFU) (**A**). n=5. To determine whether SpA could inhibit IC-FyR interactions *in vivo*, we infected mice IV with 1 x 10^6 CFU *S. aureus* RN4220 WT or *Δspa.* After 7 days, $CD11c^+$ DCs were isolated from the spleen by positive selection and plated onto a BAFF ELISpot (**B**). n=2 mice per group. *p<0.05, **p<0.01, ***p<0.001, n.s.=not significant.

APPENDIX 2

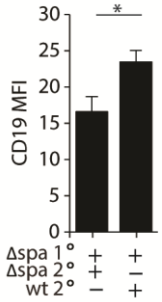


Figure 4. 2 BM ASCs formed in the presence of SpA express more CD19 than on those formed after *Δspa* challenge.

CD19 expression is reduced as ASCs mature into PCs. To compare the maturity of ASCs found in the BM of mice challenged with *S. aureus* RN4220 WT or *Δspa*, $CD138^{+}$ $B220^{lo/neg}$ BM ASCs were analyzed by flow cytometry 10 and 12 days after challenge to measure the levels of CD19 (see Chapter 3 Methods). n=6-10 mice per group in 4 experiments. *$p<0.05$

APPENDIX 3

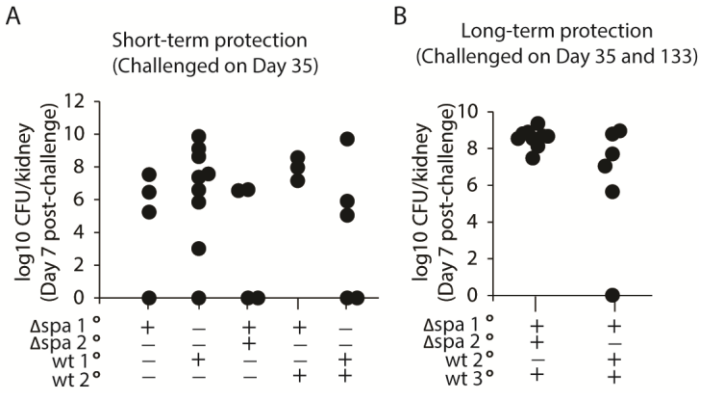


Figure 4. 3 Inoculation with *Δspa S. aureus* does not protect mice from septic infection after WT challenge

(A) To determine whether SpA interferes with the production of a protective immune memory response, we infected mice S.C. with *S. aureus* Newman *Δspa* (1 x 10^8 CFU). After 35 days, naïve or pre-infected mice were challenged I.V. with *S. aureus* Newman *Δspa* or WT (1 x 10^6). After 7 days, the numbers of CFUs in the kidneys were measured (see Chapter 3 Methods). n=4-9 mice per group in 2 experiments. (**B)** To test whether the long-term maintenance of BM PCs and circulating IgG was protective against septic infection, we infected mice S.C. and challenged them 35 days later as in (A). After 14 weeks (Day 133), mice were challenged I.V. a second time with *S. aureus* Newman WT and 7 days later, kidneys were harvested and analyzed for bacterial CFUs as in (A). n=6-8 mice per group in 3 experiments. Data are not statistically significant.

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