The Identification of Novel Mechanisms to Regulate B cell Responses During Adaptive Immunity

Shannon Zenia Jones

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Approved by:

Barbara J. Vilen, Ph.D.

Stephen Clarke, Ph.D.

Ilona Jaspers, Ph.D.

David Diaz Sanchez, Ph.D

Robert Roubey, M.D.

ABSTRACT

SHANNON Z. JONES: Novel Mechanisms of Regulating B cell responses During Adaptive Immunity
(Under the direction of Barbara J. Vilen)

Initiation of the germinal center reaction during T-dependent adaptive immune responses gives rise to long-lived plasma cells (PCs) that produce high affinity, class switched antibodies. It also produces memory B cells to ensure a rapid, high affinity response to future pathogen exposure. Long-lived antibody and memory B cell responses underlie the success of vaccines and provide the host with durable, long-lasting protection from infectious disease. Although, the formation and maintenance of memory B cells and plasma cells are of critical importance, the mechanisms regulating these processes are poorly understood. Our lab has been interested in understanding the role of dendritic cells in regulating the germinal center reaction and adaptive immune response. We found that the formation of antigen/antibody immune complexes stimulate dendritic cells, through CD16 (FcgRIII), to secrete BAFF, a cytokine required initiation and maintenance of the germinal center as well as the formation of memory B cells. Specifically, our results indicate that DC-derived BAFF initially impacts the formation of T follicular helper cells, which are critical in seeding and initiating the germinal center response. Studies show that upon immunization with a T-

dependent antigen, mice that lack CD16 expression, as well as mice lacking BAFF production by hematopoeitic cells, display reduced numbers of T follicular helper cells, and as consequence, reduced germinal center number and size. Correlated with this deficit in germinal centers, these mice also display attenuated secondary immune responses, and fewer numbers of antigen-experienced memory B cells. This suggests that DCs and BAFF play a key role in germinal center dynamics and subsequent memory B cell formation and function. Collectively, our data highlight an additional role for BAFF in the initiation and maintenance of T-dependent adaptive immune responses.

Dedication

To all the wonderful people in my life, including my supportive family and amazing friends. Without you, this would have not been possible. To Aunt Vanessa, your strong spirit and perseverance continue to amaze me.

Thank you

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Ah receptor – Aryl hydrocarbon receptor

AID – activation induced deaminase

ADCC - antibody dependent cell cytoxicity

ASC – antibody secreting cell

Ag - antigen

APC – antigen presenting cell

BAFF – B cell activating Factor

BMCA – B cell maturation antigen

BCR - B cell Receptor

BR3 – BAFF Receptor

BMDC – bone marrow derived dendritic cells

BTK – Bruton's tyrosine kinase

CM – conditioned media

DC – Dendritic cell

FO – Follicular

FDC – follicular dendritic cell

GC – germinal center

IC – immune complex

Ig – immunoglobulin

IBDV – infectious bursal disease

IL – interleukin

i.p – intraperitoneal

i.v. - intravenous

ITAM – immunoreceptor tyrosine-based activation motif

ITIM – immunoreceptor tyrosine-based inhibition motif

LCMV - Lymphocytic choriomeningitis

MF – macrophage

MZ – marginal zone

NP – KLH – hydroxy-3-nitrophenylacetyl keyhole limpet hemocyanin

OVA – ovalbamin

PNA - peanut agglutinin

PFCs - perfluorinated compounds

PFOA - Perfluorooctanoic acid

PFOS - Perfluorooctanesulfonic acid

RSV – respiratory syncytial virus

s.c. - subcutaneous

SLE – systemic lupus erythematosus

TACI - transmembrane activator and calcium modulator and cyclophilin ligand interactor

TCDD – 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin

T_{FH}- T follicular helper

TCR – T cell receptor

TGF-b – transforming growth factor-beta

Tg – transgenic

TCE - trichloroethylene

TNF – tumor necrosis factor

CHAPTER 1

Introduction

1.1 Germinal Centers are the Hallmark of Adaptive Immunity

T cell dependent adaptive immune responses generate high affinity, antigen-specific antibodies that provide the host with long-lived protection against recurring infection. The ability of the immune system to generate such long-lasting responses is the basis for successful vaccination programs (1). One of the most critical events in initiating the adaptive immune response is the formation of germinal centers (GCs). Germinal centers are transient structures, located within B cell follicles of secondary lymphoid organs, where antigen-activated B cells, proliferate, undergo affinity maturation, and give rise to memory B cells, and long-lived plasma cells (2). Dysregulation of cell proliferation, mutation, and differentiation within the germinal center can lead to various pathologies, including tumor development, autoimmunity, and immunodeficiency. Therefore, understanding the mechanisms that regulate the survival, proliferation, and differentiation of B cells within the germinal center during T cell-dependent adaptive immune responses is of great importance.

A. Initiation of the germinal center response When naïve B cells bind antigen via the B cell receptor (BCR), they upregulate expression of the chemokine receptor, CCR7 and migrate to the outer T cell zones of secondary lymphoid organs, including the spleen and lymph nodes (3). Once B cells have migrated, they engage in cognate interactions with

helper T cells (3) and can now choose one of two cell fates. Specifically, these activated B cells can migrate into the extra-follicular space, under the influence of the G-protein coupled receptor, EBI-2 (4, 5), and differentiate into short-lived antibody secreting cells. Most plasma cells generated during extra-follicular responses survive for approximately 3 days before undergoing apoptosis (6, 7). Alternatively, activated B cells can move into the B cell follicle to establish germinal centers (8). The mechanisms responsible for this fate decision remain poorly understood, although several studies suggest that BCR affinity, BCR engagement, and co-stimulatory signals from helper T cells might all be involved (9-13). The initial germinal centers that are formed are oligoclonal, and are colonized on average, by one to three activated B cells within each follicle (14). Germinal center B cells differ from naïve B cells in many ways. These cells are much larger and can be identified by their expression of Fas, or CD95, GL-7, binding to peanut agglutinin (PNA), and loss of surface IgD expression (15-17). In the initial phase of the GC, there is extensive proliferation, or clonal expansion, but very little variation in the BCR. During this time, dark and light zones appear, which are thought to be sites of clonal expansion and selection, respectively (18). Intravital microscopy has shown that germinal center B cells can move between the dark and light zones (3). During clonal expansion, the chemokine receptor, CXCR4, is downregulated, and germinal center B cells are no longer retained within the dark zone, by the chemokine, CXCL12 (3). CXCL13 produced by follicular dendritic cell (FDCs) facilitates the trafficking of germinal center B cells to the light zone. Thus, germinal center B cell migration can be bidirectional, depending on the concentration gradient of CXCL13 and CXCL12 in each zone (19). Germinal centers are now characterized as open structures,

including naïve follicular B cells that can enter the light zone (3), where they are tested for their ability to bind antigen by competing for surface antigen on FDCs.

B. Clonal Expansion, somatic hypermutation, and affinity maturation During the germinal center response, stromal FDCs and T cells generate the signals required for isotype switching, somatic hypermutation, memory B cell formation, and terminal differentiation into plasma cells (20). The affinity of serum antibody for foreign antigen increases over time, due to somatic hypermutations in the B cell receptor. Most short-lived plasma cells in the extra-follicular foci are relatively unmutated. In contrast, a high frequency of somatic hypermutations is evident in GC B cells (14, 21). These B cells express the enzyme, activation-induced deaminase (AID), which deaminates cytidine residues in the VDJ and switch regions of the Ig gene, which leads to somatic hyper mutation and class switch recombination (22, 23). Clonal expansion of activated B cells followed by B cell receptor diversification, results in either retention of cells in the lymphoid organ and the formation of a second germinal center, or exit from the germinal center and entry into the long-lived memory B cell compartment (20). Over time, the extra-follicular response wanes, while the long-lived plasma cells and memory B cells begin to appear. Finally, during the contraction of a primary immune response, long-lived plasma cells migrate to the bone marrow, while memory cells may enter into the periphery, as non-secreting, class-switched B cells. However a significant portion of the memory B cell pool remains within the secondary lymphoid organs, in close proximity to the marginal zone (24). The antibody variable regions in most, but not all of long-lived PCs and memory cells display a high degree of mutations and show evidence of clonal selection, suggesting that these cells are derived from germinal centers (25, 26). The ultimate result of the germinal center is the generation of high-

affinity, antigen-experienced B cells that will enter into circulation until re-encounter with antigen.

C. Factors that govern germinal center formation and maintenance. Many factors govern the development and maintenance of the germinal center. Bcl-6 is highly expressed in germinal center B cells and is essential for their formation (27, 28). Bcl-6 functions by binding to the regulatory regions of genes in GC B cells, ultimately inhibiting the DNA damage response and promoting rapid cell proliferation (29). Another target of Bcl-6 is *Prdm1*, the gene encoding Blimp-1. Suppression of Blimp-1 expression by Bcl-6 prevents plasma cell differentiation within the germinal center (30). There is a large body of evidence indicating that signals from the BCR, from FDCs, and from T follicular helper (T_{FH}) cells all contribute to germinal center B cell survival. BCR signals support the survival of centrocytes (B cells undergoing selection within the light zone), since GC B cells that lack the BCR co-receptor, CD45, or the GTPase TC21, which links BCR signaling with PI3K activation, have increased rates of apoptosis (31). T_{FH} cells also provide proliferation, survival, and differentiation signals to GC B cells through CD40:CD40L interactions and the cytokines IL-4 and IL-21. Although it is clear that CD40L is essential in mediating GC B cell survival, the cytokines and signals that are required for GC B cell differentiation into either long-lived plasma cells or memory B cells remain undefined.

1.2 T follicular helper (T_{FH}) cells

T follicular helper (T_{FH}) cells are the specialized subset of $CD4^+$ T cells that are necessary for the initiation and GC maintenance, generation of memory B cells, and the development of plasma cells that arise from the GC reaction. T_{FH} cells are characterized by

their constitutive expression of the transcription factor Bcl-6, as well as the expression of CXCR5, PD-1 and ICOS (32, 33). The transcriptional repressor Bcl-6 is essential for T_{FH} cell development, but it does not act alone in controlling T_{FH} cell development. A network of transcription factors is involved, including c-Maf, STAT3, Batf, and Bcl-6 all have roles in T_{FH} cell differentiation and function (34, 35). It is postulated that Bcl-6 regulates T_{FH} cell differentiation by repressing alternative differentiation pathways, including those of the Th_1, Th_2, or Th_{17} lineages (32). Early Bcl-6 up-regulation occurs in the first division of activated $CD4^+$ T cells, and is strongly induced by antigen presenting dendritic cells. This first wave of Bcl-6 expression is observed within the first three days following antigen-priming at the B and T cell border and in the inter-follicular regions of lymph nodes in both mice and humans (36, 37). An additional wave of Bcl-6 expression coincides with CXCR5 induction and results in the development of a distinct $BCL\text{-}6^+CXCR5^+$ T_{FH} cell population, independent of cognate B cell interactions (38). In contrast to their induction, the maintenance of the T_{FH} cell population in the follicle is dependent on cognate B cell interactions (37, 38).

Once activated, T_{FH} cells secrete IL-21, which has been shown to be the most potent cytokine involved in regulating plasma cell differentiation in mice and humans (39, 40). IL-21 induction of plasma cell differentiation is STAT3 dependent (41), and involves the upregulation of Blimp-1, the master regulator of plasma cells (42, 43). IL-21 is also important for optimal germinal center B cell proliferation. The expression of Bcl-6 in GC B cells is somewhat reduced in the absence of IL-21 (44, 45). In addition, IL-21 has been shown to be important for isotype switching to several IgG isotypes and it is suggested that IL-21 is the master regulator of class switching to IgG_1 (46). In addition to IL-21, T_{FH} cells provide survival signals to GC B cells through additional pathways including CD40L, IL-4,

PD-1 and BAFF, which ultimately compete with death signals induced by Fas-FasL interactions (35).

T_{FH} cells are now defined as T cells that express the chemokine receptor CXCR5, localize to the follicles, and are specialized in providing help to germinal center B cells. Although T_{FH} cells are one of the first identified T cell subsets, the nature of their formation and function has only recently begun to be defined. Many of the cell surface molecules expressed by these specialized cells are critical for their interactions with B cells during the germinal center reaction. CXCR5 is a defining molecule for T cells, which enables them to home to the B cell follicle. T_{FH} cells migrate in response to CXCL13, produced by FDCs (35). The migration of T_{FH} cells into the follicle allows for the critical B cell-dependent phase of T_{FH} cell differentiation to occur. Activated B cells express ICOSL, and the expression of ICOSL by B cells is required for T_{FH} cells. ICOS-mediated PI3 kinase signaling is required for T_{FH} cell differentiation, and for the production of IL-21 by T_{FH} cells (47). Global analysis of the gene expression profile in T_{FH} cells has revealed that these cells are of a totally separate lineage from Th_1 and Th_2 cells (48). For example, Bcl-6 is preferentially expressed by T_{FH} cells, but not by Th_1 or Th_2 cells. IL-21 is also preferentially expressed by T_{FH} cells, and plays a critical role in regulation immunoglobulin production and germinal center formation (44, 49).

T_{FH} cells provide germinal center B cells with survival and selection signals; therefore limiting the numbers of these cells is critical to prevent inappropriate B cell responses and the emergence of autoantibodies. Little is known about the regulatory mechanisms that control these T_{FH} cells. A population of $Foxp3^+$ follicular regulatory T cells have been identified that can exert suppressive effects on T_{FH} cells and the germinal center response

(50, 51). In response to immunization with T-dependent antigen, a potion of naïve regulatory T cells can express Bcl-6 (51). Expression of Bcl-6 allows them to express CXCR5 and home to the B cells follicle and localize to the germinal center. These T follicular regulatory cells control the germinal center reaction by limiting the number of T_{FH} cells that are formed and by inhibiting the selection of non-antigen specific B cells, including those with self-reactive B cell receptors (50).

Understanding mechanisms that regulate T_{FH} cell differentiation and function is of great important for improved vaccine design, since nearly all approved human vaccines function on the basis of protective T-dependent immune responses. In addition, T_{FH} cells play important roles in common autoimmune diseases, such as systemic lupus erythematosus (SLE), since mice with expanded T_{FH} populations and over-production of IL-21 develop lupus-like autoimmune disease (52).

1.3 Formation and Maintenance of B cell memory

The pool of quiescent, non-immunoglobulin secreting B cells that are produced during the germinal center reaction is largely composed of antigen specific, affinity-matured memory B cells. Some reported studies suggest that early memory B cell development can occur independent of a germinal center response (44, 53), although how well these germ-line BCR-expressing memory B cells compete with post-germinal center B cells during recall responses remains to be determined. Secondary responses resulting from re-challenge of memory B cells are faster, larger, and qualitatively different from primary responses (1). The ability to mount a recall response can be maintained for decades in humans and through the majority of a rodent's life (1).

Many factors, such as antigen and cytokine availability, can affect the differentiation and survival of memory B cells. The role of cognate antigen in memory B cell maintenance has been heavily debated. It was originally shown that recall responses were attenuated over time after sorted memory B cells were transferred to naive recipients in the absence of antigen (54). Based on this study, in addition to others, it was proposed that antigen containing immune complexes deposited on the surface of FDCs (via complement receptor and Fc receptors) was essential for memory cell maintenance (54, 55). However, more recent studies by Shlomchik and colleagues have demonstrated that memory B cell survival and function is not dependent upon immune complex deposition on FDCs (56). Rajewsky and colleagues have also demonstrated that there is no requirement for continued exposure to antigen to sustain memory B cells. Specifically, the BCR of memory B cells can be altered to recognize an antigen that has never been encountered by the host and memory B cells would continue to persist (57). The basis of the controversy and conflicting data surrounding the factors required for memory B cell maintenance could result from the fact that there are multiple layers of B cell memory, with each layer consisting of multiple effector function. Sophisticated studies by Dogan et. al have demonstrated that the memory B cell compartment can be composed of several layers, consisting of an antigen-independent layer located outside of the B cell follicles, in the spleen, blood, and secondary lymphoid organs. It also consists of an antigen-dependent layer of proliferating centroblasts in GC-like structures that depend on the presence of antigen (58). The authors have shown that after challenge, the IgG^+ memory cells have an immediate effector and protective response (the hallmark of B cell memory). This subset seems to have little capacity to reinitiate a GC response. In contrast, the IgM^+ subset of B cell memory ensures the replenishment of the memory compartment, by its rapid

mobilization and immediate isotype switching to IgG. In this model, the memory compartment is able to quickly neutralize the invading antigen, while continuing to replenish the memory pool. Other studies have also confirmed a hierarchy of maturity with the memory B cell compartment in mice (59). These studies demonstrated that there is a spectrum of memory B cells, consisting of a progression from more naïve-like to more memory-like properties.

Although we understand the basic features of the adaptive immune response, the signals that are required for memory B cell formation and reactivation are still relatively unknown. The mechanisms that regulate the quality and quantity of the memory population are also unclear. To date, no single factor has been identified irrefutably as being essential for memory formation. A recent study has hinted towards a role for IL-21 and BAFF in regulating memory B cell formation in humans (39). However, there are contrasting studies in mice demonstrating that memory B cell survival is independent of BAFF (60).

1.4 The role of BAFF in adaptive immunity

The TNF family member, BAFF and its homologue, APRIL, are homotrimeric type II transmembrane proteins that are necessary for B cell homeostasis. It was previously believed that only cells of a myeloid origin produced BAFF. It is now known that in addition, non-hematopoietic stromal cells can secrete BAFF and APRIL, which provide local niches to modulate the survival and function of B cells and plasma cells, especially in the bone marrow (61-63). BAFF producing cells include monocytes, macrophages, neutrophils, activated T and B cells, FDCs, stromal cells, astrocytes, osteoclasts, and epithelial cells (64, 65). The expression of BAFF is increased in the presence of type one interferons, IL-10, and G-CSF,

CD40L, as well as by the activation of Toll-like receptors (TLR4 and TLR9) (64, 65). Although it can be found in a membrane bound form, BAFF is usually produced in soluble form as a result of furin cleavage (61, 65). In myeloid cells, the binding of immune complexes also increases BAFF processing through an Fc receptor-dependent mechanism (66, 67). Processed, soluble BAFF adopts a trimeric form, as seen with other TNF family members; but it is the only member capable of further assembly as a 60-mer (65). BAFF has been shown to bind to three receptors, BCMA, BAFF-R (BR3), and TACI, all of which are expressed on B cells. BCMA is preferentially expressed on plasma cells, plasmablasts and tonsillar germinal center B cells (65). TACI is expressed by all peripheral B cells, particularly marginal zone B cells and B-1 B cells (65). BR3 is the dominant BAFF receptor expressed on all mature murine and human B cells. In mice, the expression of BAFF-R is initially low on newly formed immature B cells, but increases during B cell maturation. BAFF-R is the key receptor that is responsible for BAFF-mediated B cell survival, as mice deficient in BAFF-R display a phenotype similar to that of BAFF deficient mice (61). BCMA-deficient mice display no abnormalities in immune function, aside from impaired survival of long-lived plasma cells in the bone marrow (68). TACI, in contrast, has emerged as a negative regulator of B cell activation and expansion, since B cell numbers are increase in TACI deficient mice. In addition, TACI deficient mice also eventually develop SLE-like disease and lymphoid cancers (69).

It has been well documented that BAFF has an important role in B cell homeostasis. For reasons that are not fully understood, this homeostasis requires BAFF that is produced by radio-resistant stromal cells, instead of bone marrow-derived cells (70). BAFF and BR3 are known to play a fundamental role during the transition from immature T1 to T2 B cells and

therefore the generation of the mature B cell compartment in the spleen. This was demonstrated by an almost complete lack of follicular and marginal zone B cells and by a block at the T_1 cell stage in BAFF/BAFF-R deficient mice (71). In these mice, the B-1 compartment was unaffected, indicating that the development of this B cell subset is independent of BAFF and BAFF-R signaling. In contrast, transgenic mice over-expressing BAFF display an increase in all B cell subsets (72). This suggests that all mature B cells express BAFF receptor on their surface and are capable of responding to BAFF. The expanded B cell compartment in BAFF Tg mice also corresponded with the onset of autoimmunity and lupus-nephritis like disease (72). Recent studies have demonstrated a role for BAFF in plasma cell survival, however, memory B cell *survival* is independent of BAFF (60, 73). There is no current literature addressing whether BAFF plays a role in either plasma cell or memory cell *differentiation.*

The role of BAFF in germinal center formation and maintenance has been controversial. Given that $BAFF^{-/-}$ mice have a deficit in mature B cells, one would predict that the ability to form germinal centers would be compromised. Surprisingly, BAFF and BAFF-R deficient mice have the ability to form germinal centers subsequent to challenge with antigen. However, the kinetics of the germinal center reaction were altered in these mice (74). Studies have shown that immunized BAFF-R deficient and BAFF deficient mice displayed normal numbers of germinal centers early on in the immune response; however, the germinal centers dissipated more rapidly than wild type mice (74). There was also an increase in frequency of smaller germinal centers and decline in larger germinal centers, indicating the quickened dissipation of germinal center structures (64, 74). The lack of germinal center stability was attributed the failure to develop a competent FDC reticulum and

lack of antigen stimulation (74). These results suggest that BAFF receptor signaling is required for germinal center maintenance and that BAFF is also required for the formation of the FDC reticulum and efficient antigen stimulation of B cells.

A. An Emerging Role for BAFF in Modulating T cell responses Although BAFF and its cognate receptors have a dominant role in B cell biology, it is now clear that either directly, or indirectly, BAFF can also modulate T cell function *in vitro* and *in vivo*. Several *in vitro* studies have confirmed the role of BAFF as a co-stimulatory and survival factor for activated T cells (75). In addition to providing survival signals, BAFF also impacts T cell activation. In the presence of suboptimal T cell receptor stimulation, BAFF enhances T cell proliferation and cytokine production (76-78). Neutralization of BAFF *in vitro*, using both TACI-Ig and BAFF-R-Ig, resulted in decreased T cell activation. In addition, BAFF deficient mice show impaired T cell mediated graft rejection. These studies suggest that physiological levels of BAFF are necessary for the generation of sufficient T cell responses. BAFF may also play a role in stimulating T cell function during T cell mediated pathogenesis. Studies by Mackay et al have shown that the over-expression of BAFF in BAFF Tg mice exacerbates the severity of Th_1-mediated delayed-type hypersensitivity responses, by enhancing T cell proliferation and IFN-g production in lymph nodes (79). Although a role for BAFF has been demonstrated in modulating Th_1 responses, it remains to be determined whether BAFF is also involved in regulating other T cell subsets, including T follicular helper (T_{FH}) cells.

B. BAFF and autoimmunity. Elevated levels of soluble BAFF have been found in the sera and target organs of disease in mouse models of SLE, collagen induced arthritis, and chemically induced autoimmunity (80-82). Similarly, high levels of serum BAFF have been found in a subpopulation of human patients with varying autoimmune diseases (83). In many

studies, BAFF levels have been correlated with increased disease activity and titers of pathogenic autoantibodies (84). These studies provide a strong case for the pathogenic role of excess BAFF and APRIL in autoimmune disease.

The pathogenic role of BAFF in autoimmunity is further evidenced by the therapeutic benefits of BAFF neutralization. Studies have shown that lupus-prone mice treated with TACI-Ig and BAFF-R Ig displayed less proteinuria, and as a result, prolonged survival. *In vivo* BAFF antagonism has also provided protection in murine models of other autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, and Graves' disease (63, 85-87). Neutralization using TACI-Ig and BAFF-R Ig is thought to have therapeutic effects, due to B cell depletion, similar to what has been observed with anti-CD20 immunotherapy (Rituximab). Blocking BAFF may have some distinct advantages over B cell depletion using Rituximab. For example, BAFF receptor and CD20 expression overlap in many B cell subsets, but they also differ in other populations, including plasma cells, which express BCMA, but not CD20 (84). B cell depletion using anti-CD20 therapy also results in elevated serum BAFF levels (88). Therefore, newly generated immature B cells could be exposed to high levels of BAFF, which could cause another breakdown of immune tolerance and the resurgence of autoimmunity. Therefore the risk of another breach in immune tolerance could be avoided by targeting BAFF in the treatment of SLE and other autoimmune disease.

Treatment of lupus-prone mice with TACI-Ig and BAFF-R Ig resulted in the depletion of both follicular and marginal zone B cell subsets, but did not affect immature B cells or the B-1 B cell subset Interestingly, *in vivo* BAFF neutralization does not impact the survival of memory B cells in wild type mice (60). However, the effects of BAFF

neutralization on memory B cell formation have not yet been addressed. Elucidating the role of BAFF in maintaining memory B cell function will be especially important in regulating B cell memory formed to self-antigens, in the setting of autoimmune disease.

1.5 Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a complex, multi-organ autoimmune disease, affecting approximately 250,000 Americans, and is characterized by the production of autoantibodies to nuclear components (89-91). Alternating periods of flares and remissions are associated with an increased burden of apoptotic cells, the formation and deposition of immune complexes, and subsequent inflammation (92). Although the etiology of SLE remains unknown, multiple defects in immune regulation have been identified in lupus-prone mice and SLE patients. These include, complement deficiencies, T cell receptor (TCR) signaling abnormalities, and defective cytokine secretion (89, 92-94). These defects have been shown to contribute to the onset and pathogenesis of SLE (95). It is likely that environmental factors act on genetically prone individuals to induce a breakdown in tolerance mechanisms that regulate autoreactive lymphocytes. Some of these environmental factors include exposure to air pollutants and cigarette smoke, heavy metal poisoning (96), and previous viral infections, including Epstein Barr virus (97, 98). Hormonal influences have also been implicated, with SLE primarily affecting women (10:1 ratio). Genetic differences and environmental factors may interact in the pathogenic processes and also influence disease development and course. Identification of these factors and their interaction is vital to understanding the disease and may also contribute to the identification of new treatment targets, and possibly aid in disease prevention.

1.6 IgG Fc Receptors

IgG Fc receptors link innate and adaptive immune responses by their ability to mediate cellular interactions with antigen-antibody immune complexes. Two general classes of IgG FcRs are now recognized by the presence of an activating ITAM motif within the cytoplasm, and the inhibitory receptor, characterized by an ITIM sequence (99-102). These two classes of receptors function in concert and are usually co-expressed on the cell surface. The co-engagement of both the activating and inhibitory signaling pathways set the appropriate thresholds, which ultimately balances protective and pathogenic effector responses after IgG immune complex engagement. Imbalances between activating and inhibitory FcγR functions can contribute to autoimmunity and other pathologies in both mice and humans (103).

A. ITAM-containing, Activating Fc Receptors There are three different activating FcγRs expressed on murine effector cells: FcγRI (CD64), FcγRIII (CD16), and the recently described FcγRIV (also identified as CD16-2) (101). All three receptors generate signals through the ITAM sequences found within a shared common γ chain subunit (FcγR). Expression of the common γ chain is critical for the assembly of the activating Fc receptors (100). Common gamma chain knockout ($Fc\gamma^{-/-}$) mice were shown to have significant defects in antibody-dependent effector responses, including ADCC, phagocytosis of immune complexes, and some inflammatory responses (104). In contrast, deletion of the individual activating Fc receptors showed less pronounced phenotypes, especially in responses involving IgG_{2a} and IgG_{2b} antibody isotypes (105). These activating Fc receptors are found on most effector cells, including macrophages, monocytes, dendritic cells, NK cells, mast cells, eosinophils, neutrophils, and are absent from lymphoid cells (100, 102, 104, 106, 107).

The only high-affinity Fcγ receptor in both humans and mice, FcγRI, binds IgG_{2a} in mice, or IgG_1 and IgG_3 in humans, with an affinity of $10^8 – 10^9$ M^{-1}. All other receptors have a 100-1000 fold lower affinity, and show broader specificities (107). IgG_1 is the only isotype that is consistently assigned to an individual activating Fc receptor, FcγRIII. The deletion of FcγRIII abrogates IgG_1-mediated effector functions in many models of pathogenesis, including arthritis, glomerulonephritis, IgG-dependent anaphylaxis, IgG-mediated hemolytic anemia, and immunothrombocytopenia (108-112). FcγRIV binds with intermediate affinity to IgG_{2a} and IgG_{2b} *in vitro* (103). Studies have shown that even if several of activating Fc receptors with the same isotype specificity are present on the same cell surface, only those Fc receptors will be engaged that show the optimal affinity for the respective isotype. Therefore, IgG_1 immune complexes will trigger only FcγRIII, since it is the only activating Fc receptor that can bind IgG_1 (106).

B. FcγRIIb, the single ITIM-containing, inhibitory Fc Receptor In contrast to the activating FcγRs, FcγRIIb (or CD32) contains an ITIM sequence that inhibits effector cell responses (102). In addition to its expression on B cells, where it is the only IgG Fc receptor, FcγRIIb is also expressed on neutrophils, macrophages and mast cells, and is absent on T and NK cells (101). FcγRIIb expression on the surface of B cells is critical in setting BCR signaling thresholds and B cell effector functions. Signaling within the ITIM motif of FcγRIIb results in the recruitment of the phosphatases, SHIP and SHP-1, which prevents the recruitment of kinases such as BTK or PLCγ to the cell membrane, thereby diminishing events that are downstream of BCR activation such as intracellular calcium fluxes (107). The importance of FcγRIIb in modulating B cell activity and immune tolerance is supported by several studies in both mice and humans. Studies using $FcγRIIb^{-/-}$ mice showed enhanced

B cell responses, autoimmunity, and augmented IgG-mediated inflammation, demonstrating the inhibitory role of FcγRIIb in immune responses (113).

C. Fc receptors mediate important effector functions in immune cells Fcγ receptors play a significant role *in vivo* in maintaining peripheral tolerance, in augmenting T cells responses through antigen presentation, and in mediating antigen recognition and effector cell activation. Although Fc receptors have many roles in linking innate and adaptive immunity, their role in promoting secondary adaptive immune responses has only recently began to emerge. A recent study has shown that immune complex binding by Fc receptors enhances secondary antibody responses, although the precise mechanism by which this occurs is unknown (114). Immune complex binding by Fc receptors can induce many effector cell functions, including cytokine production and phagocytic functions. It remains unclear how these effector functions impact secondary immunity.

1.7 The role of TGF-β in maintaining peripheral immune tolerance

The immune system has evolved to initiate robust responses to invading pathogens while maintaining tolerance to self-antigens. Multiple mechanisms exist to ensure normal immunological function. The removal of self-reactive B and T cells during development creates a repertoire within the periphery that will preferentially recognize and eliminate non-self-antigens (115-117). Although central tolerance mechanisms exist within the bone marrow and thymus to eliminate autoreactivity, there is clear evidence that self-reactive immune cells can enter the periphery and secondary lymphoid organs. Therefore, peripheral tolerance mechanisms must also exist to limit the activation of autoreactive lymphocytes. Regulatory T and B cells, and their secreted cytokines have been recently recognized as

essential components of peripheral tolerance (118, 119). Transforming growth factor β (TGF-β) is one such regulatory cytokine with a critical role in regulating immune responses to self.

TGF-βs are regulatory proteins with pleiotropic effects on cell proliferation, differentiation, migration and survival, which affect many biological processes, including development, carcinogenesis, fibrosis, wound healing, and immune responses (120). The generation and analysis of TGF-β deficient mice established the role of the cytokine in inhibiting inflammation and autoimmunity, and has fostered an interest in this cytokine in immune regulation (121, 122). The immunoregulatory role of TGF-β was demonstrated in null mice. TGF-$\beta^{-/-}$ mice that are born, die shortly after weaning as a result of severe inflammatory disease, with lymphocyte infiltration into multiple organs and autoimmune disease (122, 123). The identification of the receptors for TGF-β s and the Smad proteins, as mediators of signaling, has also provided much needed information on the role of TGF-β s in regulating immune responses.

TGF-β is an important regulator of B cells activity, as demonstrated by the phenotype of mice with a B cell specific inhibition of TGF-β signaling (124). Secretion of TGF-β by regulatory cells inhibits B cell proliferation, induces apoptosis of immature or resting B cells, BCR activation, and isotype switching to most IgG isotypes. Consistent with TGF-β mediated inhibition of IgG class switching, conditional deletion of TGF-β signaling in B cells, *in vivo*, resulted in elevated serum Ig. These mice also developed enhanced IgG_3 responses to a normally weak antigen (124). These studies implicate TGF-β in attenuating B cell responses to low affinity antigens. Interestingly, titers of anti-DNA antibodies were also increased in mice lacking B cell derived TGF-β signaling, which suggests that TGF-β is an

important regulator of B cell tolerance to self-antigens *in vivo* (124). In contrast to its inhibitory roles in immune function, TGF-β has a unique role in promoting switching to IgA and IgG_{2b} in murine B cells and IgA in human B cells, *in vitro* (124-126). Upon optimal stimulation with antigen, and cytokine stimulation, the inhibitory activity of TGF-β subsides, and TGF-β can strongly promote IgA secretion. TGF-β mediated IgA class switching is associated with increased transcription of alpha heavy chain transcripts. The role of TGF-β in IgA production is further demonstrated in mice with a condition deletion of TGF-β signaling in B cells, where there is almost a complete absence of IgA within the serum (124). Thus, similar to T cells, TGF-β has both inhibitory and stimulatory effects on B cell function.

The adaptive immune response gives rise to long-lived humoral responses that protect the host from recurring infection. Although dendritic cells indirectly promote adaptive immunity through antigen presentation, it remains unclear whether DCs actively shape these responses. The goal of the work described herein is to identify mechanisms by which DC cells may impact the long-lived plasma cell response and memory B cell formation that occurs during T cell-dependent adaptive immune responses.

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CHAPTER 2

CD16-mediated BAFF production promotes Bcl-6 expression

2.1 Introduction

The success of adaptive immune responses requires appropriate interactions between antigen specific B and T cells leading to germinal center (GC) responses (1, 2). The specialized microenvironment provides sites for rapid expansion and mutation of antigen-specific B cell clones, antibody isotype switching, and finally commitment to the plasma cell fate or memory B cell pool (3-5). The formation of GCs and the emergence of memory B cells are subsequent to the production of antibody induced by T-independent formation of extrafollicular foci (2, 6), suggesting that early antibody responses may play a role in early GC events.

The affinity matured memory B cells that emerge from the GC require T cell help. Recent studies have identified $CD4^+$ follicular helper T cells (T_{FH}) as critical effectors that provide help to B cells during the GC response (7, 8). The formation of T_{FH} cells is dependent on the expression of Bcl-6 and the attenuation of Blimp-1, which inhibits the differentiation of other T_H subsets (7, 9). T_{FH} cells are distinguished from other $CD4^+$ subsets in that they express CXCR5, ICOS, PD-1, and IL-21. T_{FH} cells are important in forming and maintaining the GC. Together with follicular dendritic cells, T_{FH} cells provide

the proliferative and survival signals that are necessary to expand antigen-specific B cells and form the emerging germinal centers (10).

In addition to regulating T cell effector function, Bcl-6 is also highly expressed in germinal center B cells and is essential for their formation (11, 12). Bcl-6 binds to the regulatory regions of genes in GC B cells, ultimately inhibiting the DNA damage response and promoting rapid proliferation within the germinal center (13). Another target of Bcl-6 is *Prdm1*, the gene encoding Blimp-1. Suppression of Blimp-1 expression by Bcl-6 prevents plasma cell differentiation in the germinal center (14). Because Bcl-6 upregulation mediates several B and T cell effector functions that are critical for the germinal center reaction, the factors that promote Bcl-6 expression are of great interest. Mitogenic signals (including anti-mu, LPS, PMA and ionomycin and CD40L) that promote B and T cell proliferation are known to induce Bcl-6 expression using *in vitro* culture systems (15). Few studies have addressed the mechanisms that regulate Bcl-6 expression *in vivo*. IL-21 signaling has been linked to Bcl-6 expression in $CD4^+$ T cells and GC B cells, although the expression of Bcl-6 was only marginally reduced in the absence of IL-21 (16, 17). Thus, other factors exist to regulate Bcl-6 expression within B and T cells, germinal center formation, and other key events of the adaptive immune response.

Because of its essential role in B cell survival and homeostasis (18), BAFF may have additional roles in the adaptive immune response. Although BAFF and its cognate receptors have a dominant role in B cell biology, it is now clear that either directly, or indirectly, BAFF can also modulate T cell function *in vitro* and *in vivo* (19). In addition to providing survival signals, BAFF also impacts T cell activation. In the presence of suboptimal T cell receptor stimulation, BAFF enhances T cell proliferation and cytokine production (20-22). It is likely

that because of its effects on B and T cell function *in vivo*, BAFF can also impact the germinal center. Previous reports have shown that the role of BAFF in the dynamics of the germinal center is complex and controversial. Relatively little is known about the role of BAFF in GC formation, generation of the B cell memory compartment, and plasma cell development. A single study using TACI-Fc to reduce serum BAFF levels, reported a complete lack of germinal centers in treated mice (23). In complete contrast, a study using BCMA-Fc as a mechanism BAFF blockade, *in vivo*, show that germinal center formation is independent of BAFF (24). Therefore, the role of BAFF in germinal center dynamics remains unclear.

During adaptive immune responses, DCs play an important role in activating T cells through their ability to present peptides. In this study, we show that DCs actively shape early events in the adaptive immune response, by sensing a productive early antibody response and secreting BAFF. We find that BAFF induces the expression of Bcl-6 in activated B cells thereby extinguishing the plasma cell program and committing the cells to the memory pathway. Simultaneously, BAFF promotes the expression of Bcl-6 in activated $CD4^+$ T cells, promoting T_{FH} cell differentiation. Overall our studies define ICs sensed by DCs as critical initiators of the GC response and through their production of BAFF, they promote memory B cell differentiation.

2.2 Materials and Methods

Animals

All animals were maintained in an accredited animal facility at University of North Carolina. C57BL/6 (B6) and $CD16^{-/-}$ mice were bred in house and used at 8-10 weeks of age. 5-7 week old B6-Ly5.2 congenic mice were purchased from NCI-Frederick. OTII TCR transgenic mice were obtained from Jenny Ting at the University of North Carolina (UNC). $BAFF^{-/-}$ mice were obtained from Glenn Matsushima, also from UNC. BAFF Tg mice were obtained from Dr. Jeffrey Rathmell of Duke University.

Reagents and Antibodies

Antibodies against mouse CD4, CD19, CD95, GL-7, ICOS, and PD-1 were purchased from Biolegend. Anti-CXCR5, anti-B220, anti-IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 antibodies were purchased from BD Biosciences. Anti-mouse Bcl-6 was purchased from Santa Cruz. Recombinant cytokines, IL-4 and IL-5 were purchased from Peprotech. NP-OSu was purchased from Biosearch Technologies. KLH and ovalbumin proteins were purchased from Sigma Aldrich. The adjuvant, alum, was purchased from Thermo Scientific. Anti-mu $F(ab)_2$ antibody was purchased from Jackson Immunoresearch. Anti-mu (B7.6), anti-NP (H33L and B1-8), and AC-38 anti-idiotype antibodies were purified from hybridoma supernatants. H33L and B1-8 were kind gifts from Dr. Garnett Kelsoe (Duke University). BR3-Fc and IgG-Fc were kind gives from Dr. Robert Benschop (Eli Lilly). B and T cell negative selection purification kits were purchased from StemCell Technologies. Splenic DC purification kit was purchased from Miltenyi Biotec. Streptavidin-alkaline phosphatase was purchased from Southern Biotech.

B cell purification

Splenic B cells were isolated from B6 mice by negative selection (StemCell Technologies). B cells were 95-99% pure, as determined by flow cytometry (with fewer than 5% DCs and MFs).

Bone marrow-derived DC (BMDC) cultures

Bone marrow single-cell suspensions were prepared from the tibias and femurs of B6, $CD64^{-/-}$, $CD32^{-/-}$, $CD16^{-/-}$, and $BAFF^{-/-}$mice. Following RBC lysis, cells were cultured in GM-CSF and IL-4 for 7 days in a 24 well low cluster dish.

Splenic DC isolation

$CD11c^{+}$ cells were purified from the spleens of B6 mice at 8-10 weeks of age. $CD11c^{+}$ DCs were approximately 80% pure, as determined by flow cytometry.

B cell and DC co-culture

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 for 7 days. B cells were stimulated with IL-4, IL-5 and anti-mu. Supernatants were harvested and IgM secretion was measured by ELISA.

B6 B cell and OTII T cell co-culture

2 X 10^5 purified B6 B cells were co-culture with 2 X 10^5 T cells purified from OTII TCR transgenic mice. Cells were cultured or 7 days in the presence of 100 μg ovalbumin (with or without 1 X10^4 B6 BMDCs). Supernatants were harvested on day 7 and IgM was measured by ELISA.

Preparation of DC Conditioned Media (CM)

2 X 10^4 BMDCs were cultured in a 96 well plate, in the presence of anti-mu (B7.6), conditioned media from antigen-stimulated B cells, IL-4 and IL-5. After seven days, supernatants from stimulated DCs were harvested. C67BL/6 B cells were purified, and cultured with or without DC CM (20% of total volume), in a 96 well plate, with additional anti-mu (B7.6), IL-4 and IL-5.

Total IgM ELISAs

IgM levels from were detected using anti-mouse IgM (33-60) and biotin-labeled anti-mouse IgM (B7.6).

CFSE-based Proliferation Assay

B cell proliferation was assessed by the dilution of 5-carboxyfluorescein diacetate succinimidyl ester (CFSE) loaded cells as previously described (25).

Bone Marrow Chimeras

B6-Ly5.2 congenic mice (6-8 weeks of age) were lethally irradiated (1050 rads), and reconstituted with 8 X 10^6 bone marrow stem cells from either wildtype (B6) or $BAFF^{-/-}$ mice. Reconstitution of the B cell compartment was assessed after 8 weeks, by flow cytometry.

NP-KLH Immunization

8-10 week old B6, $BAFF^{-/-}$ bone marrow chimeras, and $CD16^{-/-}$ mice were immunized i.p. with 100 μg NP-$_{11-14}$-KLH precipitated in alum for induction of primary responses. For

secondary immunization, mice were boosted (i.v. injection) with the same dose of soluble NP_{11-14}-KLH at least 30 days post- primary immunization. Mice were bled weekly for serum anti-NP levels. Antibody levels were measured 4, 7, 14 days after boost to quantitate memory recall (secondary) responses. Additional cohorts of B6 and $CD16^{-/-}$ mice were immunized s.c. with 100 μg NP-KLH precipitated in alum. 8 X 10^6 BAFF Tg or B6 BMDCs were adoptively transferred into immunized mice. Inguinal lymph nodes were harvested at seven days post-immunization and assessed by flow cytometry.

Memory B cell Enumeration

B6 and $CD16^{-/-}$ mice (8-10 weeks of age), and B6 and $BAFF^{-/-}$ chimeras were immunized as described. At 28 days post-immunization spleens were harvested from B6, $CD16^{-/-}$, b6 chimeras, and $BAFF^{-/-}$ chimeras. Single-cell suspensions were prepared, and antigen specific memory B cells were enumerated by flow cytometry. Antigen-specific, IgG^+ memory B cells were detected using the AC-38 hybridoma and biotinylated anti-IgG cocktail (containing anti-IgG_1, anti-IgG_{2a}, anti-IgG_{2b}, and anti-IgG_3).

T Follicular Helper Cell Enumeration

B6 and $CD16^{-/-}$ mice (8-10 weeks of age), and B6 and $BAFF^{-/-}$ chimeras were immunized as described. At day 7 post-immunization, the mice were sacrificed and spleens were harvested. Single cell suspensions were prepared, and $CD4^+$, $CXCR5^+$, $PD\text{-}1^+$, and $ICOS^+$ T cells were enumerated by flow cytometry.

NP ELISA

Sera were tested for IgG anti-NP Abs (ELISA). Microtiter plates were coated with NP_{13}-conjugated BSA (Biosearch Technologies). Serially diluted serum samples were incubated overnight at 4^oC. Anti-NP IgG levels were detected using an alkaline phosphatase conjugated goat anti-mouse IgG antibody (1/1000 dilution) followed by phosphatase substrate (Sigma-Aldrich). Optical density (OD) values were converted to concentration based on standard curves (generated using the H33L anti-NP hybridoma), using a four-parameter logistic equation (Softmax Pro 3.1 software; Molecular Devices).

AID Real Time PCR

Spleens were harvested from B6 and $BAFF^{-/-}$ chimeras seven days after NP-KLH immunization, and splenic B cells were isolated according to the Stem Cell Easy Sep protocol. mRNA was isolated from 5-10 X 10^6 purified B cells using TRIzol (Invitrogen) and chloroform. cDNA from 500 ng of mRNA per sample was synthesized using Superscript VILO cDNA Synthesis Kit (Invitrogen). DNA amplification was performed using FastStart Universal SYBR Green Master mix (Roche). Primers for qRT-PCR were synthesized by IDT. The PCR protocol was 95° for 10 minutes, and 40 cycles at 95° for 15 seconds and 60° for 1 minute. Relative values were compared using the $2^{-\Delta\Delta CT}$ method. 18s rRNA was used as an internal control in all experiments.

Germinal Center Staining and Counting

Spleens were harvested from B6 or $CD16^{-/-}$ mice on days 7, 14, and 21 after NP-KLH immunization and flash frozen in OCT (Fisher). Sections 6mm thick were fixed in 1:1 MeOH/Acetone, blocked with 10% FBS in PBS plus 1:100 2.4G2, and stained with PNA-

biotin (Sigma) and B220-APC (BD Pharmingen), and Streptavidin-Alexa488 (Invitrogen). Germinal centers were recognized as aggregates of PNA^+ B cells. The percent of total splenic area covered by germinal centers, as well as average number and average size of germinal centers were compared between B6 and $CD16^{-/-}$ mice.

Architecture Staining for B6 and $BAFF^{-/-}$ Chimeras

Spleens were prepared as above from naïve B6, B6 chimera, or $BAFF^{-/-}$ chimera mice. Sections of 8mm thickness were cut, fixed in MeOH/Acetone, blocked in Superblock (Pierce) plus 1:100 2.4G2, and stained with anti-B220-APC, anti-CD11b-PE, anti-CD11c-PE, and Thy1.2-biotin (all BD Pharmingen), followed by Strepdavidin Alexa 488 (Invitrogen).

Statistics

The one sample t test was used to compare levels of secreted IgM levels in anti-mu stimulated controls, and anti-mu stimulated B cells under various treatment conditions. The one sample *t* test was also used to compare expression levels of Bcl-6 in B6 and $BAFF^{-/-}$ bone marrow chimeras. The student's *t* test was used to compare levels of secreted IgM and IgG in immunized B6 mice, $CD16^{-/-}$ mice, and B6 and $BAFF^{-/-}$ bone marrow chimeras. The unpaired *t* test was used to compare percentages of memory B cells, GC B cells, and T_{FH} cells. Analyses were performed in GraphPad Prism.

2.3 Results

2.3.1 Dendritic cells regulate Ig secretion from antigen stimulated B cells

During adaptive immune responses, the presentation of antigen to primed T cells promotes the differentiation of activated B cells into memory and plasma cells suggesting that dendritic cells (DCs) play an indirect role in humoral immune responses. To address whether DCs play a more direct role in shaping the B cell response to foreign antigen, we co-cultured bone marrow derived DCs (BMDCs) with ovalbumin-stimulated B cells from B6 mice and antigen-pulsed OTII T cells (Figure 2.1 A). We found that BMDCs inhibited 98% of Ig secretion induced by ovalbumin stimulation. Inhibition of Ig secretion required OTII T cells and was ovalbumin-specific as BSA did not promote Ig secretion. Furthermore, B cells cultured with ovalbumin alone did not secrete a detectable amount of Ig. Repression of Ig secretion was not unique to ovalbumin in that BMDCs also repressed 85% of anti-mu induced Ig where T cell help was provided in the form of recombinant IL-4 and IL-5 (Figure 2.1 B). Similar to BMDCs, splenic $CD11c^+$ *ex vivo* cells repressed 70% of anti-mu induced immunoglobulin (Figure 2.1 B). B cell proliferation and viability were comparable to that of B6 B cells stimulated in the absence of DCs (data not shown).

The binding of immune complexes (ICs) to Fc receptors on DCs can induce cytokine production (26). In our *in vitro* model, IgG-ICs could form when secreted IgM binds to the anti-mu antibody (B7.6) used to stimulate the B cells in culture. Since B7.6 is an IgG_1 antibody, the IgM/anti-mu containing ICs could ligate FcγRs on the DCs. To assess whether DCs secrete cytokines in response to Fc receptor ligation, we prepared DC conditioned medium (CM) by stimulating BMDCs for 7 days with the culture supernatant from anti-mu

(B7.6) or anti-mu $(Fab)_2$ -stimulated B cells (Figure 2.1 C). We then tested whether these DC CMs repressed antigen-stimulated B cell Ig secretion. We found that DC CM containing the intact Fc portion significantly inhibited Ig secretion, indicating that DCs secrete a soluble factor(s) that represses B cell Ig secretion. This is consistent with transwell data showing that DCs regulate Ig secretion in a contact-independent manner (data not shown). In contrast, DC CM prepared using the anti-mu $F(ab)_2$ was unable to inhibit IgM secretion in stimulated B cells (Figure 2.1 C). This suggests that the binding of ICs to FcγRs on DCs inhibits IgM secretion. To determine which Fc receptors were responsible for the observed DC-mediated repression, we prepared a series of conditioned media, using BMDCs from B6, CD64 ($Fc\gamma RI^{-/-}$), CD32 $(Fc\gamma RIIb)^{-/-}$, and CD16 $(Fc\gamma RIII)^{-/-}$ mice. The data show that CM from $CD16^{-/-}$ mice was unable to repress B cell Ig secretion, while CMs from B6, $CD64^{-/-}$, and $CD32^{-/-}$ mice remained repressive (Figure 2.1 D). Thus, the binding of anti-mu/IgM containing ICs to CD16 induces the secretion of a soluble repressive factor(s) that limits the differentiation of B cells into antibody secreting cells.

To discern whether DCs decreased Ig secretion by inhibiting the Ig transcriptional program, we measured the intracellular levels of XBP-1, IRF-4, and Bcl-6. Coincident with reduced Ig secretion, we found that the levels of XBP-1 and IRF-4 were diminished two-fold, while Bcl-6 was increased 1.5-fold (Figure 2.1 E-G). This suggests that DCs block activated B cells from differentiating into plasma cells and may affect the PC/memory fate decision. Thus, the binding of IgG-ICs to CD16 induces the secretion of a soluble factor(s) that upregulates Bcl-6 and limits the differential of B cells into plasma cells.

2.3.2 Immunized CD16$^{-/-}$ mice have impaired secondary immune responses

Our transcription factor analysis suggests that the stimulation of DCs through CD16 directs the differentiation of activated B cells away from plasma cell towards the memory B cell program, or germinal center. To address these possibilities, we measured primary and secondary antibody responses over time in B6 and CD16$^{-/-}$ mice following immunization and boost with NP-KLH. We found that immunized CD16$^{-/-}$ mice mounted an IgM and IgG primary antibody response that was comparable to B6 mice (Figures 2.2 A and C). In contrast, IgM levels during the secondary response of immunized CD16$^{-/-}$ mice were reduced 2-fold, while the IgG antibody levels were 6-fold decreased (Figures 2.2 B and D). Collectively, these data suggest that immune complex binding and CD16 are critical for IgG and IgM production during secondary immune responses.

2.3.3 CD16$^{-/-}$ mice have diminished numbers of memory B cells

Rapid, high titer secondary immune responses require the activation of memory B cells. We reasoned that the defects observed in the recall response of CD16$^{-/-}$ mice are the result of defects in memory B cell formation. To address whether CD16 plays a role in forming B cell memory, we enumerated CD19^{+}, IgG^{+}, AC-38-binding (idiotype positive) B cells 28 days following immunization. As predicted, immunized B6 mice showed a 4-fold expansion of CD19^{+}, IgG^{+} antigen specific memory B cells, as compared to unimmunized controls (Figure 2.3). In contrast, CD16$^{-/-}$ mice showed very little expansion (1.5-fold) and compared to immunized B6 mice, the numbers of memory B cells were reduced by 59% (Figure 2.3). These results show that CD16 influences the secondary immune response prior to the formation of memory B cells.

2.3.4 Immunized CD16$^{-/-}$ mice exhibit defects in forming GCs

Memory B cells are a hallmark of a successful germinal center response. Thus, the diminished numbers of memory cells could reflect the failure to form or maintain germinal centers (GCs). To assess whether loss of CD16 diminishes the entry of B cells into GCs, we enumerated GL-7^{+}, CD95^{+} splenic B cells 7 days following immunization. As shown in Figure 2.4, immunized B6 mice showed a three-fold increase in the numbers of GC B cells, compared to unimmunized B6 mice. However, CD16$^{-/-}$ mice showed only a two-fold increase in GC B cells, compared to unimmunized CD16$^{-/-}$ mice (Figure 2.4 A and B). CD16$^{-/-}$ had 50% fewer GL7^{+}, CD95^{+}, CD19^{+} B cells, when compared to immunized B6 mice. Furthermore, the immunized CD16$^{-/-}$ showed a reduction in the number of AC-38 binding, antigen-specific B cells within the germinal center (Figure 2.5). Taken together, our data indicate that the CD16$^{-/-}$ mice harbor defects in germinal center formation.

To determine the size and frequency of germinal centers, spleen tissue sections were stained with peanut agglutinin (PNA) and the B cell marker, B220. The numbers of GCs per follicle were counted and the size of GC was measured in B6 compared to CD16$^{-/-}$ mice. We found that 7 days after immunization, GCs in the spleens of CD16$^{-/-}$ mice were 40% smaller than those in B6 mice and over the course of the next 2 weeks (day 14, 21) the GC size never reached that achieved in B6 mice (Figure 2.6 A and C). On day 28, the GCs were significantly smaller in both B6 and CD16$^{-/-}$ mice indicating the small size observed in CD16$^{-/-}$ spleens was not due to delayed maturation (data not shown). Immunized CD16$^{-/-}$ mice also displayed fewer GCs within the B cell follicle at days 7 and 14, although this difference was not statistically significant. Figure 2.6 B). These data suggest that CD16 is

also necessary to initiate the germinal center. Our data implicate the importance of events early in germinal center formation in defining the memory B cell pool.

2.3.5 Dendritic cells secrete BAFF in response to CD16 ligation

Fc receptor activation induces DCs to secrete a number of cytokines (27, 28). To define the underlying mechanism by which ICs, DCs, and CD16 regulate memory cell formation and GCs, we neutralized CM from DCs, using a panel of antibodies, whose cognate receptors are expressed on B cells. Neutralization with BR3-Fc, a decoy receptor for BAFF, showed a significant loss of repression, restoring antibody secretion to levels comparable to the control (Figure 2.7). Furthermore, DCs from $BAFF^{-/-}$ deficient mice were unable to repress anti-mu induced Ig secretion. Finally, treatment of anti-mu stimulated B cells with rBAFF significantly decreased IgM secretion (Figure 2.7). This suggests that DC-derived BAFF, induced by ICs, regulates B cell responses *in vitro*.

2.3.6 $BAFF^{-/-}$ bone marrow chimeras also have reduced secondary responses

Our data reported thus far suggest that activated B cells may rely on DCs and their ability to sense ICs and secrete BAFF to direct B cells towards the germinal center and into the memory B cell pool. Although BAFF has been implicated in the survival of mature, naïve B cells, and plasma cells, it has not been implicated in regulating the formation of B cell memory. To assess whether BAFF produced by hematopoietic cells plays a role in adaptive immune responses, we examined primary and secondary immune responses of reconstituted B6 and $BAFF^{-/-}$ chimeras. $BAFF^{-/-}$ mice lack mature peripheral B cells, while reconstituted $BAFF^{-/-}$ bone marrow chimeric mice have mature B cells (29), with all hematopoietic cells lacking BAFF production. Although immunized $BAFF^{-/-}$ chimeras have

a comparable primary anti-NP response to that of B6 mice (29), their response to secondary immunization with NP-KLH has not been assessed. Similar to the $CD16^{-/-}$ mice, $BAFF^{-/-}$ chimeras displayed comparable levels of anti-NP IgG and IgM antibody levels to B6 during the primary response (2.8 A and C). However, the $BAFF^{-/-}$ bone marrow chimeras showed an impaired secondary response to NP-KLH immunization, with approximately 2-fold less IgM and IgG production (Figures 2.8 B and D). BAFF can induce class switching in activated B cells (30-32), hence the loss of IgG in the secondary response could reflect diminished AID expression. Comparison of AID mRNA levels in B6 and $BAFF^{-/-}$ chimeras showed comparable levels of AID expression (Figure 2.9) Overall, the data indicate that BAFF derived from hematopoeitic cells is essential for secondary immune responses. Because we saw only a 50% decrease in antibody responses by the $BAFF^{-/-}$ chimeras, other factors may be involved in regulating secondary immune responses, and could contribute to the 6-fold decrease in the anti-NP response of the $CD16^{-/-}$ mice.

2.3.7 Enforced expression of BAFF in DCs restores secondary responses in $CD16^{-/-}$ mice.

The data suggest that activated B cells may rely on DCs to direct memory cell formation through their ability to sense ICs and secrete BAFF. To directly assess DC-derived BAFF was necessary for GC formation, we adoptively transferred (s.c.) BMDCs from BAFF transgenic mice into immunized $CD16^{-/-}$ mice to determine whether GC B cell ($CD19^{+}$, GL-7^{+}, $CD95^{+}$) numbers were restored. We previously established that 70% of subcutaneously injected BMDCs migrate to the inguinal lymph and that the magnitude of the subcutaneous anti-NP response was comparable to intraperitoneal immunization. Using this model, we found that immunized B6 mice showed a robust increase (4.8 fold) in GC B cells, as compared to the unimmunized control mice (Figure 2.10). Consistent with the

intraperitoneal immunization model, CD16$^{-/-}$ mice displayed fewer GC B cells (2.3 fold), as compared to immunized B6 mice. The transfer of BMDCs chronically expressing BAFF restored this defect. This effect was not due to increased numbers of DCs as BMDCs derived from B6 mice did not restore GC B cell numbers, as compared to immunized B6 mice (Figure 2.10). Overall, these data indicate that BAFF from DCs restores GC B cell numbers in immunized CD16$^{-/-}$ mice.

2.3.8 BAFF$^{-/-}$ chimeras have decreased formation of memory B cells and a delayed germinal center response

To assess whether memory B cells formed in the absence of hematopoietic cell-derived BAFF, we enumerated antigen-specific CD19^{+}, IgG^{+}, and AC-38^{+} B cells on day 28, post-immunization. We found that there was a 4-fold increase in memory B cells in immunized B6 chimeras. In contrast, BAFF$^{-/-}$ showed a significantly diminished increase in memory B cells and this level was reduced by 45% fewer memory B cells compared to immunized B6 chimeras (Figure 2.11 A). To determine whether BAFF also affects GC formation, we enumerated CD19^{+}, GL-7^{+}, and CD95^{+} B cells, seven days post-immunization. Compared to B6 chimeras, those lacking BAFF displayed 30% fewer germinal center B cells (Figures 2.11 B and C). Thus, early in the primary response, the numbers of memory and GC B cells is impaired in BAFF$^{-/-}$ chimeras, much like the phenotype observed in the CD16$^{-/-}$ mice. This implicates BAFF secretion by DCs in the entry of activated B cells into the GC.

2.3.9 CD16$^{-/-}$ mice and BAFF$^{-/-}$ chimeras have reduced T_{FH} cells

The development of T_{FH} cells is an important step in promoting B cell responses during adaptive immunity. T_{FH} cells co-express CXCR5, ICOS, and PD-1 allowing them to

properly home to GCs, to influence selection and survival (33). Defects in the differentiation of this T cell subset could account for the reduced germinal size and memory B cell numbers in $CD16^{-/-}$ mice and $BAFF^{-/-}$ chimeras. Further, our data indicate that *in vitro*, BAFF regulates Bcl-6. To define whether BAFF affects T_{FH} cells, B6 and $CD16^{-/-}$ mice were immunized with NP-KLH, sacrificed at day 7, and T_{FH} cells were enumerated. $CD16^{-/-}$ mice had 54% fewer $CD4^+$, $CXCR5^+$, PD-1^+ T cells, as compared to immunized B6 mice (Figure 2.12 A and B). This was not a consequence of secondary effects due to CD16 deficiency since $BAFF^{-/-}$ chimeras also exhibited a 40% decrease in numbers of T_{FH} cells (Figure 2.12 C and D).

We predicted that if BAFF regulated the formation or maintenance of T_{FH} cells, forced expression of BAFF in the DC population would restore the T_{FH} cell pool. Indeed, the adoptive transfer of BAFF transgenic BMDCs into immunized $CD16^{-/-}$ mice restored the number of T_{FH} cells at seven days post-immunization to levels that were not statistically different from immunized B6 mice (Figure 2.13). This effect was not observed in immunized $CD16^{-/-}$ mice that received BMDCs from B6 mice, indicating that increased numbers of DCs alone does not enhance T_{FH} cell numbers. Collectively, this suggests that BAFF, induced by ICs activating CD16 promotes the formation or maintenance of T_{FH} cells.

2.3.10 BAFF regulates the expression of Bcl-6

The upregulation of Bcl-6 by $CD4^+$ T cells promotes expression of CXCR5 and ICOS on T_{FH} cells, while expression in activated B cells extinguishes the PC program and directs activated B cells toward memory B cell pathway. ICOS has been shown to regulate Bcl-6 in T_{FH} cells, but whether other factors impact Bcl-6 remains unclear. We reasoned that since

BAFF increased Bcl-6 expression in our *in vitro* culture model, it might affect Bcl-6 expression in the GC response. We found that at day 7 post-immunization, Bcl-6 levels in $CD4^+$ T cells of $BAFF^{-/-}$ chimeras were reduced by 43% (Figure 2.14 A). BCl-6 expression in GC B cells in $BAFF^{-/-}$ chimeras was also reduced by a similar magnitude (Figure 2.14 B). Thus, the diminished numbers of T_{FH} cells and GC B cells is coincident with reduced Bcl-6 expression.

To define whether constitutive expression of BAFF by DCs restores Bcl-6 levels, we adoptively transferred BAFF Tg BMDCs into immunized $CD16^{-/-}$ mice by subcutaneous expression. We found that at day 7 post-immunization, enforced expression of BAFF by DCs Bcl-6 expression was restored Bcl-6 comparable to that of immunized B6 mice (Figure 2.15 A). Constitutive expression of BAFF by DCs also restored the Bcl-6 levels in GC B cells (Figure 2.15 B). The data show that in the absence of FcR stimulation and BAFF expression, Bcl-6 expression is reduced. As a consequence GC size and memory B cell numbers are reduced. This defect ultimately results in diminished recall responses.

2.4 Discussion

Proper execution of an adaptive immune response requires T cells, antigen presenting cells, such as DCs, and B cells. The current dogma is that DCs prime T cells to promote B cell Ig secretion. In this paper, we highlight a novel role for DCs in sensing a productive immune response through Fc receptors, followed by secreting BAFF to direct B cells towards the seeding of a germinal center. Our data show that BAFF is a critical factor in the formation of T_{FH} cells and diminished numbers of T_{FH} cells delay the number and size of newly forming germinal centers. As a consequence, the number of memory B cells is reduced, leading to a significant reduction in antibody levels during recall responses. These findings place an important role on DCs in defining the fate of activated B cells and identify BAFF as an important mediator that initiates the B cell memory response. Our studies indicate that DCs exert these regulatory effects through the sensing of a productive immune response via immune complex binding through Fc receptors. Upon Fc receptor activation, DCs secrete BAFF, which plays a critical role in initiating T follicular cell formation and subsequent germinal center formation. We also demonstrate the requirement for Fc receptor ligation and subsequent BAFF production in memory B cell differentiation.

Receptors for the Fc portion of IgG play a significant role *in vivo* in maintaining tolerance, augmenting T cells responses via antigen presentation, and in mediating effector cell activation during immune responses. We've now identified an additional role for the Fc receptor, CD16, in regulating germinal center formation and subsequent secondary immune responses through the production of BAFF by myeloid cells. Recent studies by Goins et al. support our findings, and also implicate Fc receptors in enhancing secondary immune responses. Although these studies linked Fc receptors and the enhancement of memory B

responses, the exact mechanism by which this occurs is still undefined. Furthermore, these studies did not define the specific Fc receptor involved in this regulatory process, since $Fc\gamma^{-/-}$ mice lack CD64, CD16, and CD16-2. From our studies, we have concluded that expression of a single Fc receptor, CD16, and its activation by immune complexes is required for DCs to regulate B cell responses *in vitro* and *in vivo*. Specifically, ligation of CD16 by immune complexes allows for the production of BAFF, which is critical for germinal center formation, T_{FH} cell differentiation, and downstream B cell effector function.

Although multiple Fc receptors are expressed on the surface of DCs, with varying affinities for IgG, our *in vitro* data suggest that only CD16 is critical in this mechanism. The implication of only CD16 can most likely be attributed to the fact that it preferentially binds the immune complexes generated in our *in vitro* cultures, which were composed of IgG_1. Several studies corroborate our findings and have reported the preferential binding of CD16 to IgG_1-containing immune complexes in both *in vitro* and *in vivo* systems (34-36). Because NP immunization results primarily in an IgG_1 response, it is likely that CD16 predominantly binds the ICs formed *in vivo* to induce regulatory immune functions. Although our work suggests that CD16 is indispensable for T_{FH} cell generation and germinal center responses, it is possible that immune responses that generate antibodies of another isotype will implicate an additional Fc receptor.

During T-dependent adaptive immune responses, B cells interact with T_{FH} cells within the follicle and these cellular interactions are indispensable for germinal center formation and the production of class-switched, high affinity antibodies. Consistent with their location in the B cell follicle, T_{FH} cells upregulate the chemokine receptor, CXCR5, allowing for migration toward the ligand CXCL13, which expressed exclusively in the

follicle. ICOS is also upregulated on T_{FH} cells and is required for their development and expansion. In addition, IL-21 has been reported to play a critical role in T_{FH} cell formation and in B cell growth, survival, and class switching (17). Although, T_{FH} cell differentiation is dependent on the availability of IL-21 (37), other cytokines could also be important in regulating T follicular helper T_{FH} cell fate. We herein report that myeloid cell-derived BAFF also regulates T follicular helper differentiation. We have found that mice that lack CD16 expression and BAFF production by hematopoietic cells have reduced numbers of T_{FH} cells, following immunization. As a consequence, we also saw a temporal defect in germinal formation, and an additional defect in memory B cell differentiation. This delay in germinal center formation and defect in secondary B cell responses were similar to defects seen in IL-21R deficient mice immunized with T-dependent antigens (38), wherein there was a delay at post-immunization day 7 in germinal center formation and a significant reduction in total antigen specific memory B cells at day 35 post-immunization. Thus, CD16 and BAFF appear to be as equally important as IL-21 in regulating T_{FH} cell function and adaptive immune responses. Most recently, Ettinger and colleagues have shown that BAFF and IL-21 synergize to enhance the formation of memory B cells in humans (39). Thus, it is possible that BAFF and IL-21 signaling integrate to contribute the adaptive immune responses and the generation of B cell memory

Our studies provide evidence of a direct molecular mechanism by which BAFF signaling impacts T_{FH} cell formation. Upregulation of the transcription factor Bcl-6 is also a key requirement for T_{FH} cell differentiation and germinal center initiation (40). We propose that BAFF plays a role in promoting the formation of T_{FH} cells and the seeding of the germinal center reaction, by impacting Bcl-6 expression in both B and T cells. It is possible

that BAFF/BAFF-R interactions lead to additional molecular changes that promote T_{FH} cell differentiation. A previous study has shown that BAFF-R signaling can induce NFkB activation and subsequent ICOSL expression in B cells, which is required for T_{FH} cell formation (41). Thus, signaling through the BAFF receptor may be necessary for the upregulation of cell surface receptors and molecules, such as CXCR5 and ICOS, that are required for the differentiation and function of T_{FH} cells. Overall, our results ultimately demonstrate that BAFF regulates T-dependent adaptive immune responses by initially impacting the differentiation of T_{FH} cells immediately following antigen encounter.

BAFF has been established as a key cytokine in primary B cell survival and homeostasis. However, recent studies have revealed a complex role for this cytokine in the germinal center reaction. A few conflicting studies have been published describing the role of BAFF in germinal center formation and maintenance. In studies using BR3-deficient mice, Kalled and colleagues have shown BAFF is not required for the initiation of the GC response, however, it is necessary for the maintenance of established GCs (42). The authors also report an increase in small germinal centers and a decline in large germinal centers, indicating an overall dissipation (42, 43). The authors attribute the lack of GC stability in BR3-deficient mice to failure of the mice to develop a mature FDC reticulum. However, other factors may contribute to the effects of BAFF on germinal center maintenance. In stark contrast, Yan et. al showed that the BAFF-TACI interaction is critical for germinal center formation. Specifically, they found that spleens from immunized, TACI-Fc-treated mice were devoid of germinal centers (23). These conflicting results could be due to the fact that different immunizing antigens were used (sRBCs vs. NP-CGG), in addition to the fact that germinal center reactions were assessed at varying time points. The observed differences

could also be attributed to the BAFF-depleting reagent that was used (BCMA-Fc vs. TACI-Fc). The complete absence of germinal centers in immunized, TACI-Fc treated mice could implicate a role for APRIL, since both BAFF and APRIL can bind TACI *in vivo*. The lack of GCs in immunized TACI-Fc mice might also implicate an additional, unidentified TACI ligand that regulates germinal center formation. Similar to studies by Yan et al, our experiments reveal a role for both Fc receptors and BAFF production in initiating the germinal reaction. We found that both $CD16^{-/-}$ and $BAFF^{-/-}$ chimeras had a delay in germinal center formation. Germinal centers in immunized $CD16^{-/-}$ mice were also significantly smaller than those in immunized control. Our data suggest that early events in the germinal center define are essential for formation of the memory B cell pool. Because B cells in $CD16^{-/-}$ have a shorter duration in the germinal center, it is also likely that the length of the germinal center response is critical in forming high affinity, antigen-experienced memory B cells.

Although survival and maintenance of the mature B cell compartment is dependent upon BAFF production by radiation resistant stromal cells, our data suggest that certain aspects of the adaptive immunity rely on BAFF produced by hematopoietic cells. Specifically, T_{FH} cell differentiation, germinal center initiation, and memory B cell formation all rely on hematopoietic stem cell-derived BAFF. Our data suggest that although serum BAFF levels in $BAFF^{-/-}$ chimeras are comparable to B6 chimeras, high local concentrations of BAFF within the spleen are required for efficient germinal center formation and memory B cell formation.

Overall, our studies highlight a role for dendritic cell-derived BAFF in regulating multiple events during the adaptive immune response. Activation of dendritic cells through

a CD16-mediated mechanism leads to BAFF production, which promotes Bcl-6 expression with B cells and germinal center formation. BAFF also actively shapes the development of T_{FH} cells by modulating Bcl-6 expression in $CD4^+$ T cells. In addition to its role early in the germinal center response, BAFF also impacts memory B cell formation and subsequent recall responses.

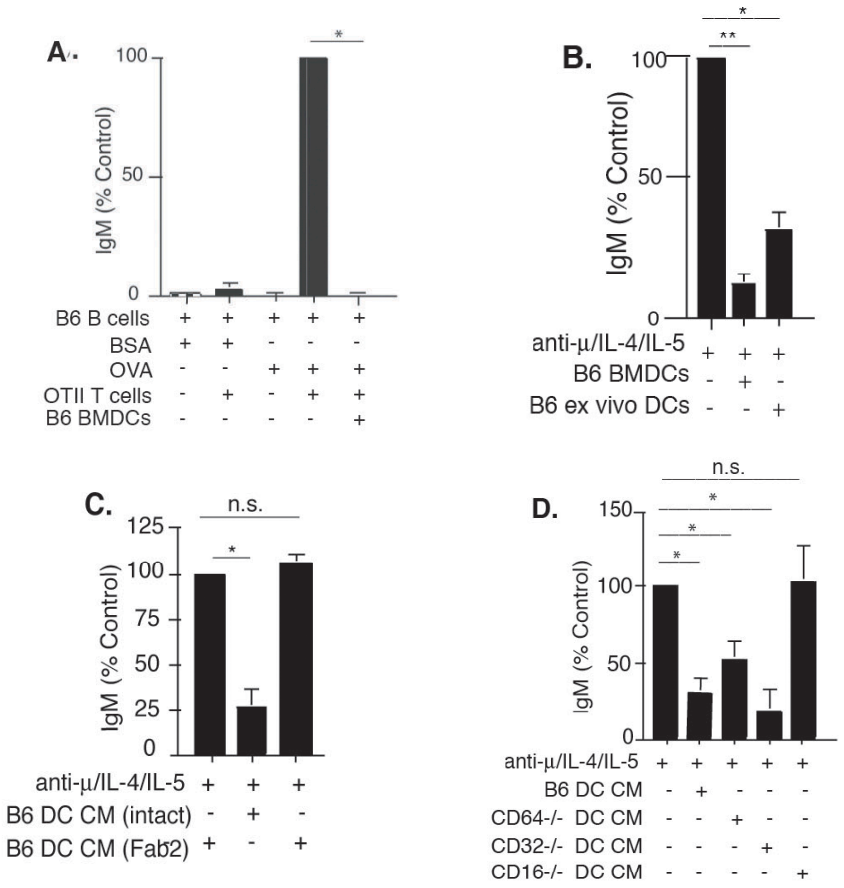


Fig. 2.1 DCs secrete soluble factors that inhibit Ig secretion. (**A.)** Purified B6 B cells were co-cultured with OTII TCR Tg T cells, and either ovalbumin or BSA (± B6 BMDCs). Ovalbumin stimulated B cells secreted 1.2-2 μg/mL IgM. n = 3 (**B.)** Purified B6 B cells were co-cultured with either B6 DMDCs or B6 *ex vivo* DCs in the presence of anti-mu, rIL-4, and rIL-5. Anti-mu stimulated B cells secreted 15-31 μg/mL IgM. n=5 (**C.**) B6 B cells were cultured with DC CM (containing either anti-mu with intact Fc or anti-mu $F(ab)_2$) in the presence of additional anti-mu, rIL-4 and rIL-5. n=5 (**D.).** Purified B6 B cells were cultured with DC CM from B6, $CD64^{-/-}$, $CD32^{-/-}$, or $CD16^{-/-}$ mice. n=5. Culture supernatants were harvested after seven days. Total IgM levels were quantitated by ELISA. * $p \leq 0.05$, one sample *t* test

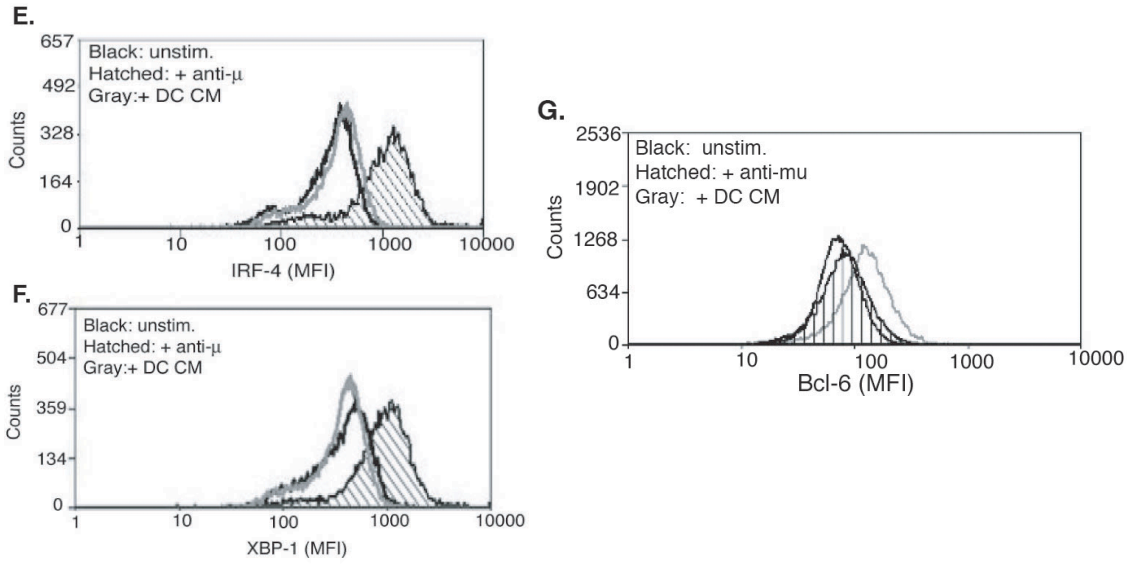


Fig. 2. 1 DCs secrete soluble factors that inhibit Ig secretion. Purified B6 B cells were stimulated with anti-mu, rIL-4, and rIL-5 for 48 hrs (± B6 DC DC). Cells were harvested permeabilized, and intracellular expression levels of **(E.)** IRF-4, **(F.)** XBP-1, and **(G.)** Bcl-6 were assessed by flow cytometry. Data are representative of 3 individual experiments.

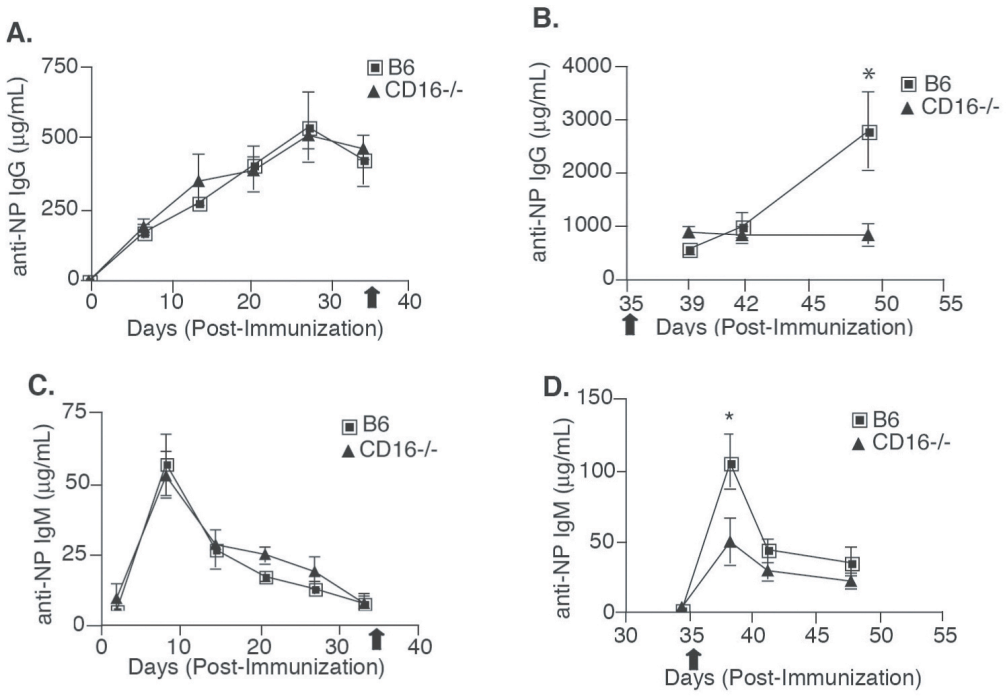


Fig. 2. 2 CD16$^{-/-}$ mice have defective secondary immune responses. B6 and CD16$^{-/-}$ mice were immunized with 100 µg NP-$_{11}$-KLH in alum (i.p). Mice were bled weekly and primary antibody responses **(A and C)** were measured by ELISA. At 35 days post-immunization, the mice were boosted with 100 µg soluble NP-KLH, and antibody secretion during the secondary immune response was assessed at days 4, 7, and 14 post-boost **(B and D)**. n = 9 for each group. *$p \leq 0.05$, Student's *t* test

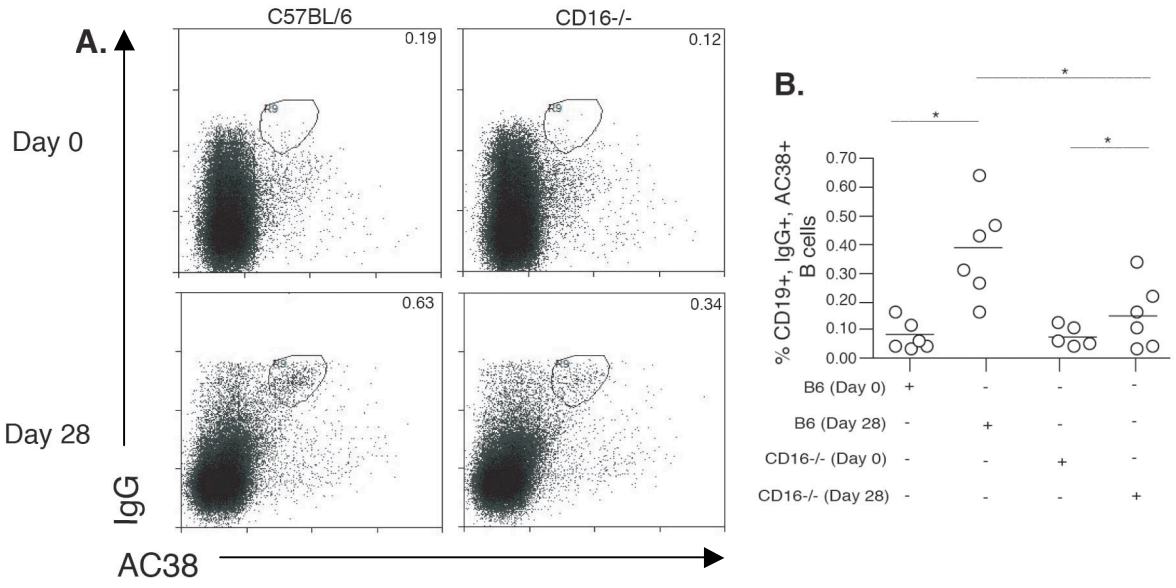


Fig. 2.3 Immunized CD16$^{-/-}$ mice have fewer Ag-specific memory B cells. (A. and B.) 8-10 week old B6 and CD16$^{-/-}$ mice were immunized with NP-$_{14}$-KLH. At 28 days post-immunization, spleens were harvested and CD19^{+}, IgG^{+}, AC-38-binding B cells were enumerated by flow cytometry. *p $\leq$ 0.05, unpaired *t* test

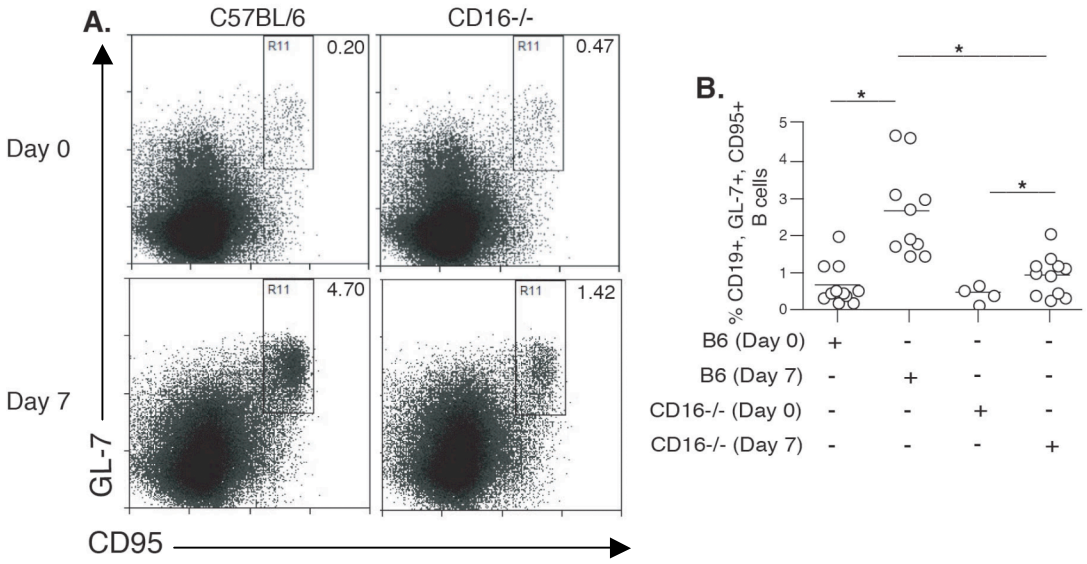


Fig. 2.4 Immunized CD16$^{-/-}$ mice have fewer GC B cells. (A. and B.) 8-10 week old B6 and CD16$^{-/-}$ mice were immunized with NP-$_{14}$-KLH precipitated in alum. At 7 days post-immunization, spleens were harvested and CD19^{+}, GL-7^{+}, CD95^{+} B cells were enumerated by flow cytometry. * $p \leq 0.05$, unpaired *t* test

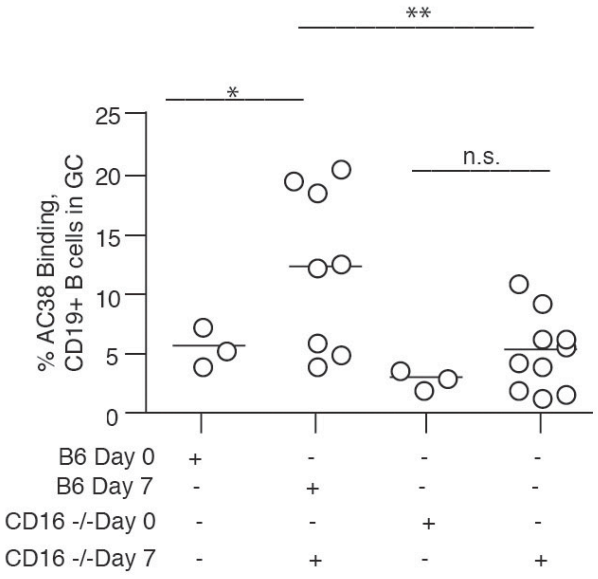


Fig. 2.5 Immunized CD16$^{-/-}$ mice have fewer Ag-specific B cells within the GC. 8-10 week old B6 and CD16$^{-/-}$ mice were immunized with NP-$_{14}$-KLH precipitated in alum. At 7 days post-immunization, spleens were harvested and AC-38-binding, CD19^{+}, GL-7^{+}, CD95^{+} B cells were enumerated by flow cytometry. * $p \leq 0.05$, **$p \leq 0.01$, n.s: not significant, unpaired *t* test

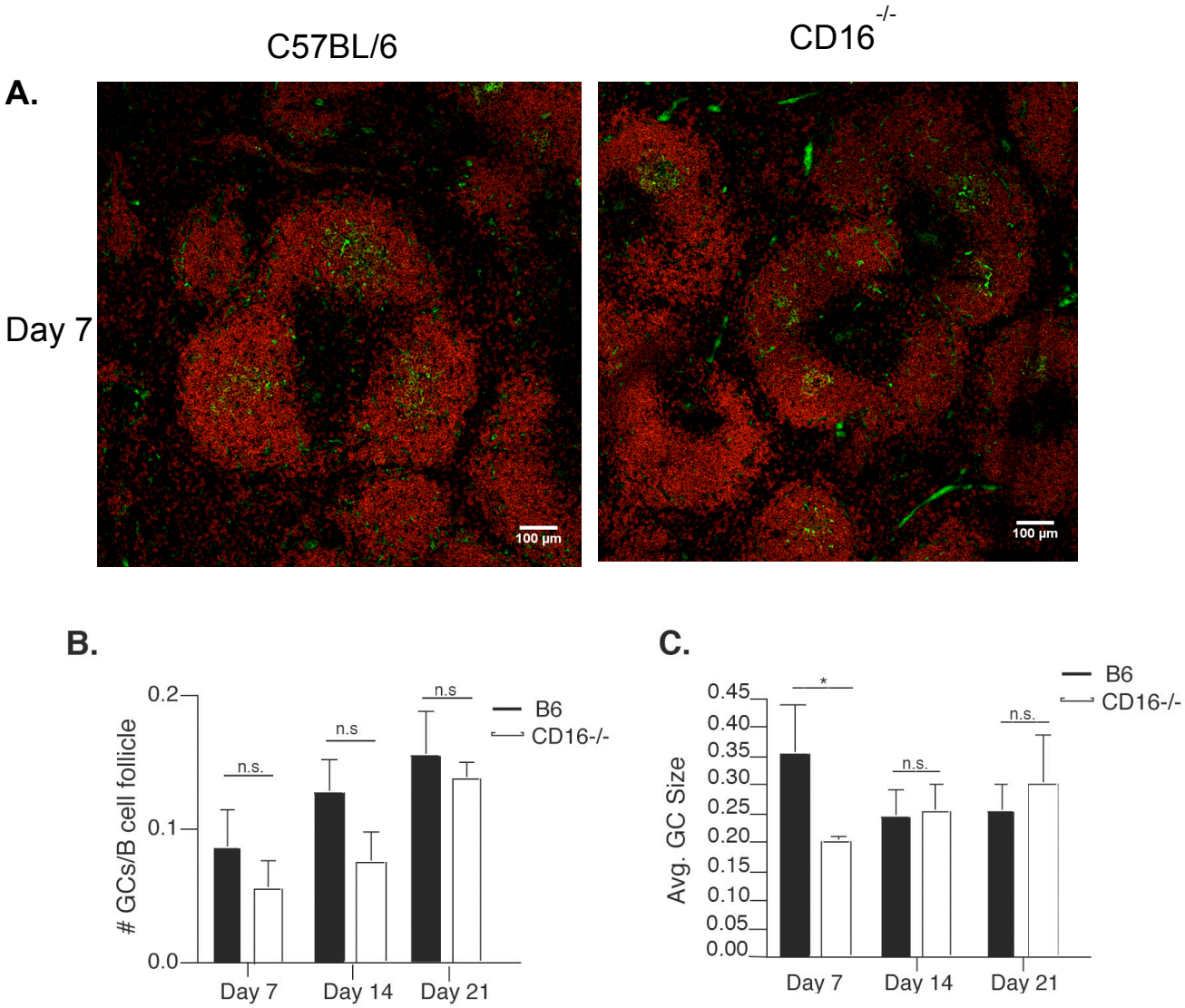


Fig. 2.6 Immunized CD16$^{-/-}$ mice have smaller GCs. 8-10 week old B6 and CD16$^{-/-}$ mice were immunized with NP-$_{14}$-KLH. At 7 days post-immunization, spleens were harvested, sectioned, immunofluorescently stained with anti-B220 (red) and anti-PNA (green) to assess germinal center number **(B.)** and size **(C.)** n= 7 * $p \leq 0.05$., n.s: not significant, unpaired *t* test

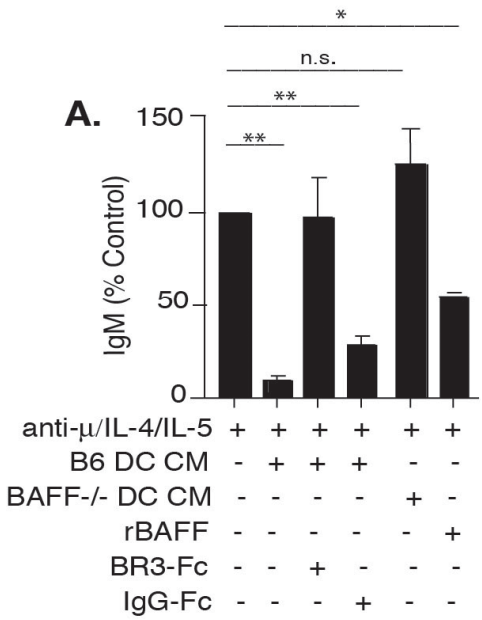


Fig. 2.7 Dendritic cells secrete BAFF to inhibit Ig secretion. (A.) Anti-mu stimulated B6 B cells were cultured with B6 DC CM neutralized with BR3-Fc or control antibody, IgG-Fc. Purified B6 B cells were also cultured with from either B6 or $BAFF^{-/-}$ mice in the presence of additional anti-mu, rIL-4 and rIL-5. n=4. Finally, anti-mu stimulated B cells were cultured with 10 ng/mL rBAFF. Culture supernatants were harvested after seven days. Total IgM levels were quantitated by ELISA. * $p \leq 0.05$, one sample *t* test

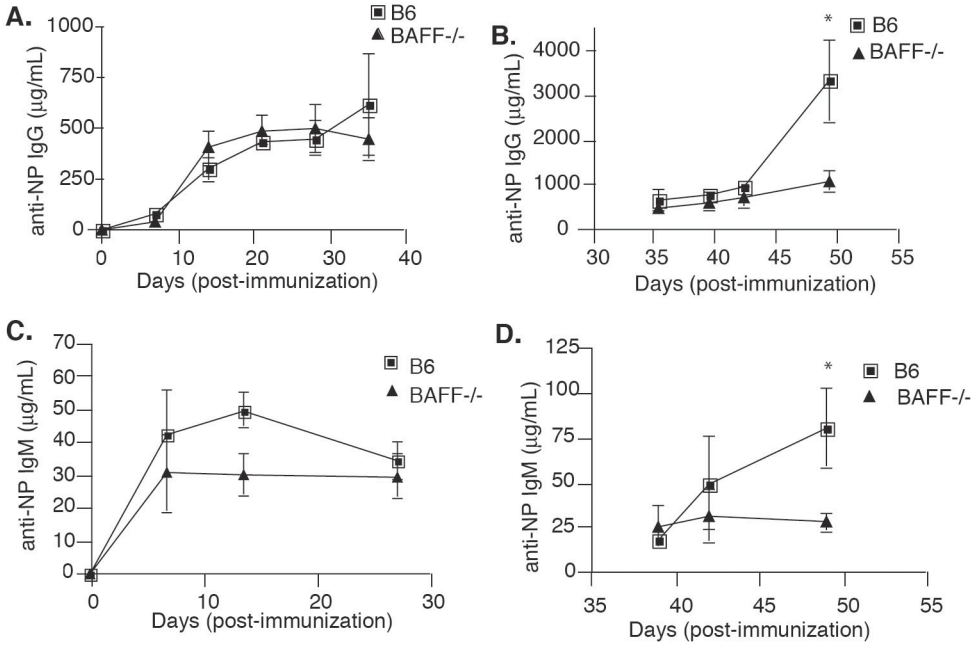


Fig. 2.8 BAFF$^{-/-}$ chimeras have defective secondary immune responses. B6 and BAFF$^{-/-}$ chimeras were immunized with 100 μg NP-$_{11}$-KLH in alum (i.p). The mice were bled weekly and primary antibody responses **(A and C)** were measured by ELISA. At 35 days post-immunization, the mice were boosted with 100 μg soluble NP-KLH, and antibody secretion during the secondary immune response was assessed at days 4, 7, and 14 post-boost **(B and D)**. *$p \leq 0.05$, Student's *t* test

Table 2.1 Real Time RT-PCR primer sequences

murine *Aicda*	5'-GGGAAAGTGGCATTCACCTA-3'
murine *Aicda*	5'-GAACCCAATTCTGGCTGTGT-3'
murine 18s rRNA forward	5'-TCAAGAACGAAAGTCGGAGGTT-3'
Murine 18s rRA	5'-GGACATCTAAGGGCATCACAG-3'

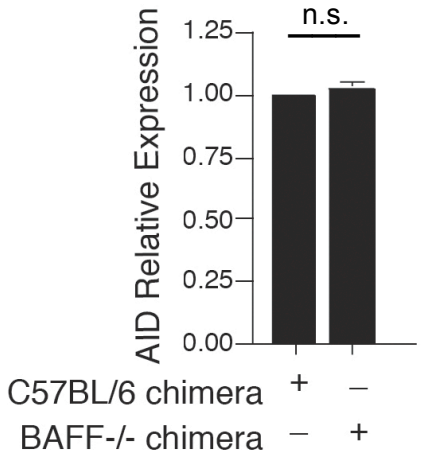


Fig. 2.9 BAFF$^{-/-}$ chimeras express comparable levels of AID. B6 and BAFF$^{-/-}$ chimeras were immunized with 100 μg NP-$_{11}$-KLH in alum (i.p). At seven days post-immunization, B cells were purified from the spleen and RNA was isolated. Expression of AID were quantitated by real time RT-PCR. n=3. n.s: not significant, one sample *t* test

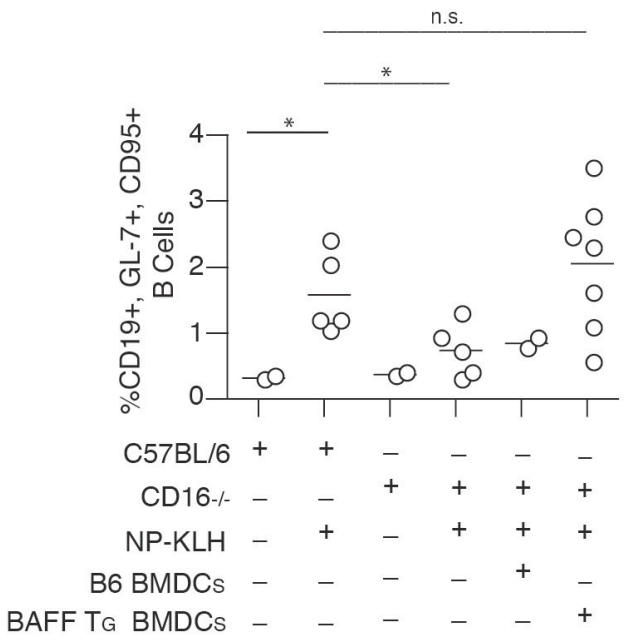
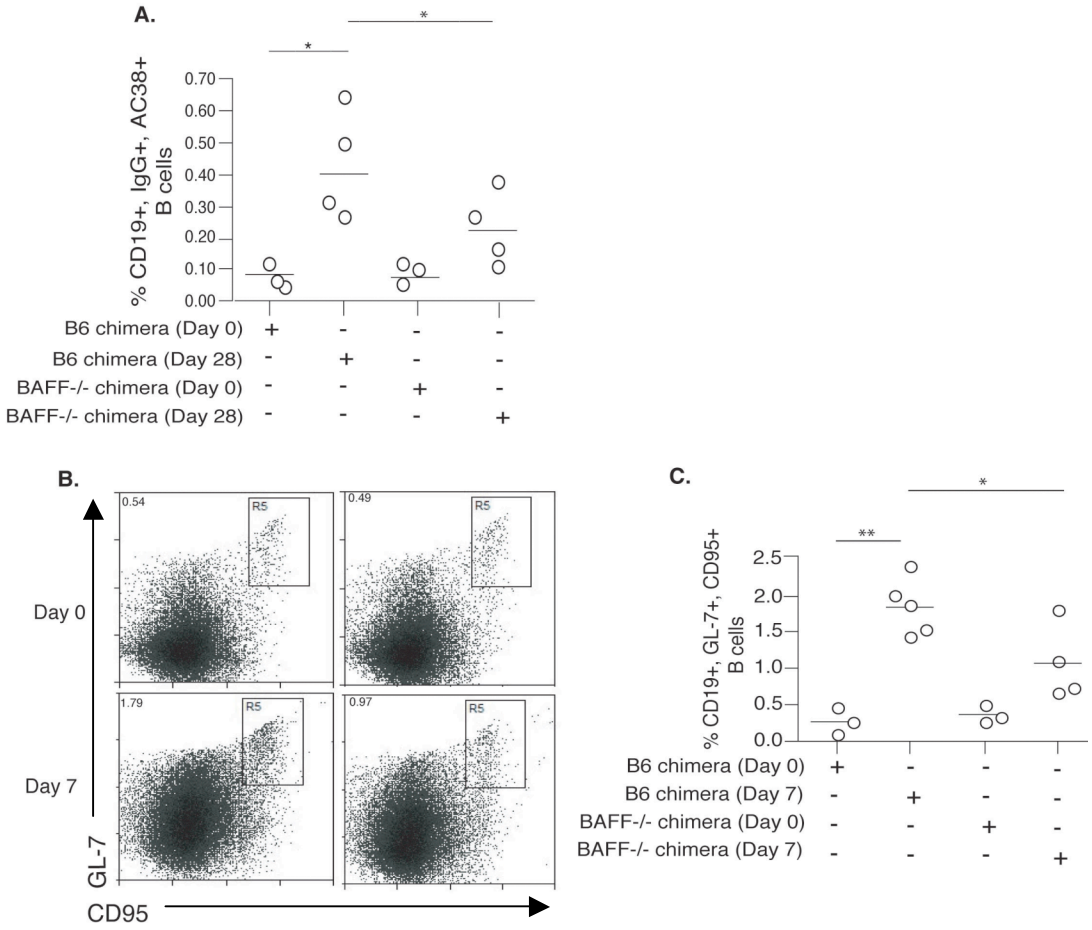
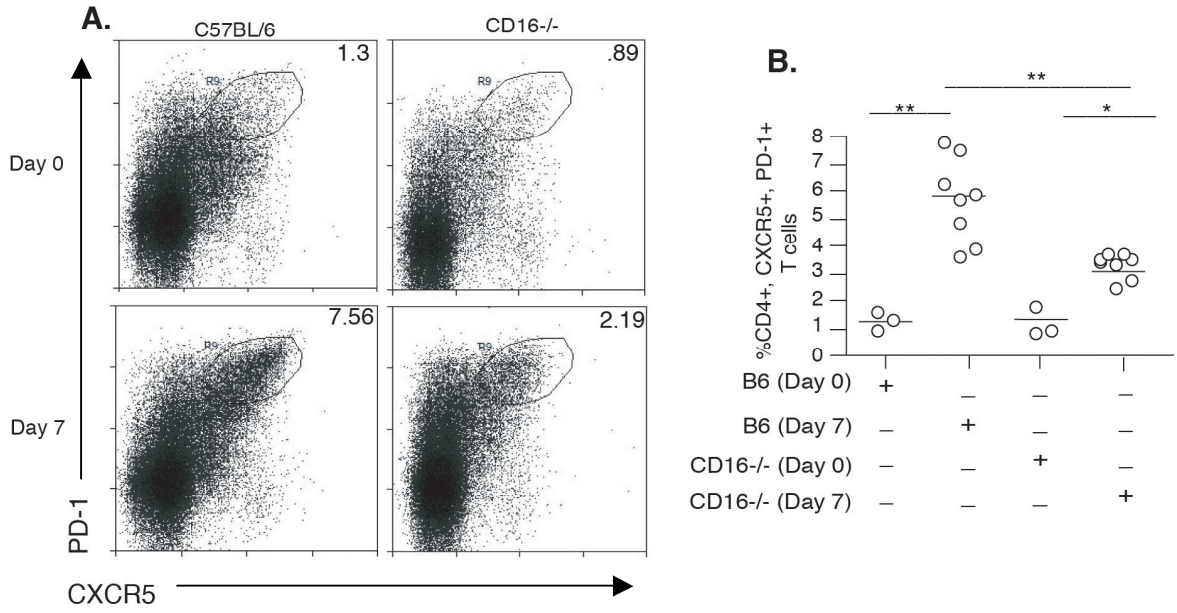


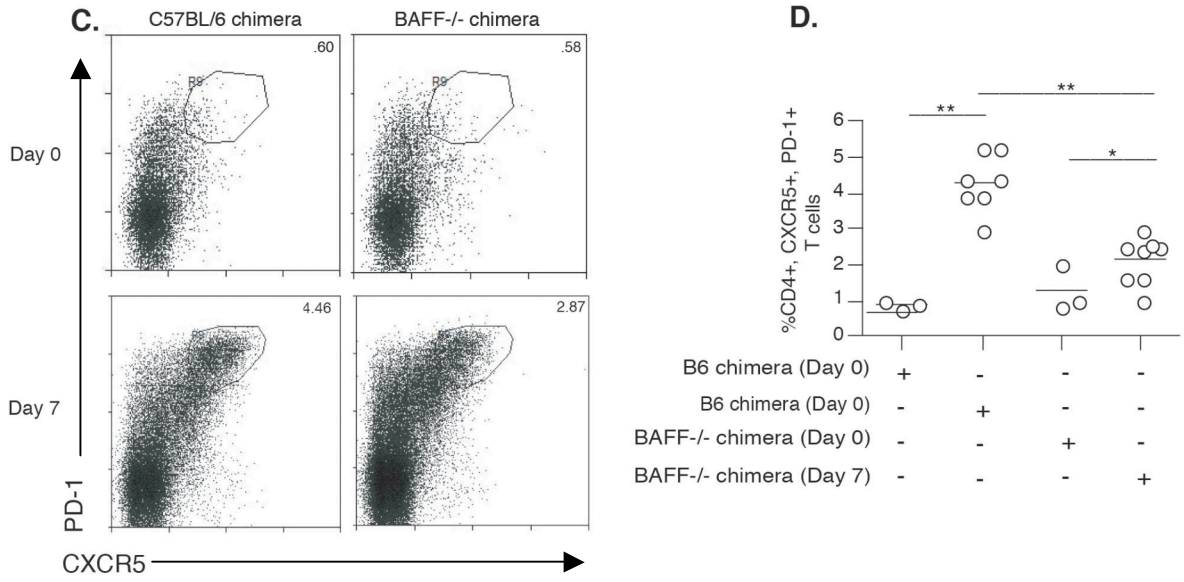
Fig. 2.10 DC-derived BAFF restores GC response in CD16$^{-/-}$ mice. BMDCs were prepared from B6 and BAFF Tg mice. B6 or BAFF Tg BMDCs were subcutaneously injected into CD16$^{-/-}$ mice, immunized with NP-$_{14}$-KLH. Inguinal lymph nodes were harvested seven days post-immunization, and assessed for the presence of CD19^{+}, GL-7^{+}, CD95^{+} GC B cells by flow cytometry. *$p \leq 0.05$, n.s: not significant, unpaired *t* test



2.11 Immunized BAFF$^{-/-}$ chimeras have fewer memory and GC B cells. Reconstituted B6 and BAFF$^{-/-}$ chimeras were immunized with NP_{14}-KLH precipitated in alum. **(A).** At 28 days post-immunization, spleens were harvested and CD19^{+}, AC-38 binding, IgG^{+} memory B cells were enumerated by flow cytometry. **(B. and C.)** At seven days post-immunization, CD19^{+}, GL-7^{+}, CD95^{+} GC B cells were enumerated by flow cytometry. * $p \leq 0.05$, **$p \leq 0.01$, unpaired *t* test



2.12 Immunized CD16$^{-/-}$ mice and BAFF$^{-/-}$ chimeras have fewer T_{FH} cells. 8-10 week old B6 and CD16$^{-/-}$ mice were immunized with NP-$_{14}$-KLH precipitated in alum. **(A and B)** At 7 days post-immunization, spleens were harvested and CD4^{+}, CXCR5^{+}, an PD-1^{+} T cells were enumerated by flow cytometry. * $p \leq 0.05$, ** $p \leq 0.01$, unpaired *t* test



2.12 Immunized CD16$^{-/-}$ mice and BAFF$^{-/-}$ chimeras have fewer T_{FH} cells. Reconstituted B6 and BAFF$^{-/-}$ chimeras were immunized with NP-$_{14}$-KLH precipitated in alum. **(C and D)** At 7 days post-immunization, spleens were harvested and CD4^{+}, CXCR5^{+}, an PD-1^{+} T cells were enumerated by flow cytometry.* $p \leq 0.05$, ** $p \leq 0.01$, unpaired *t* test

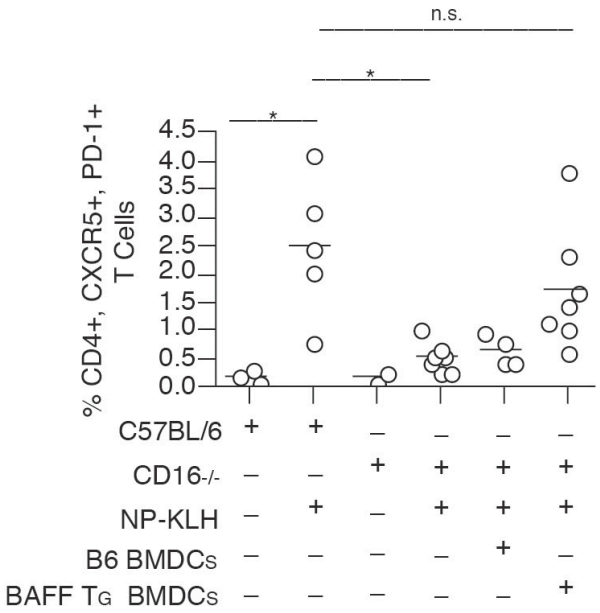
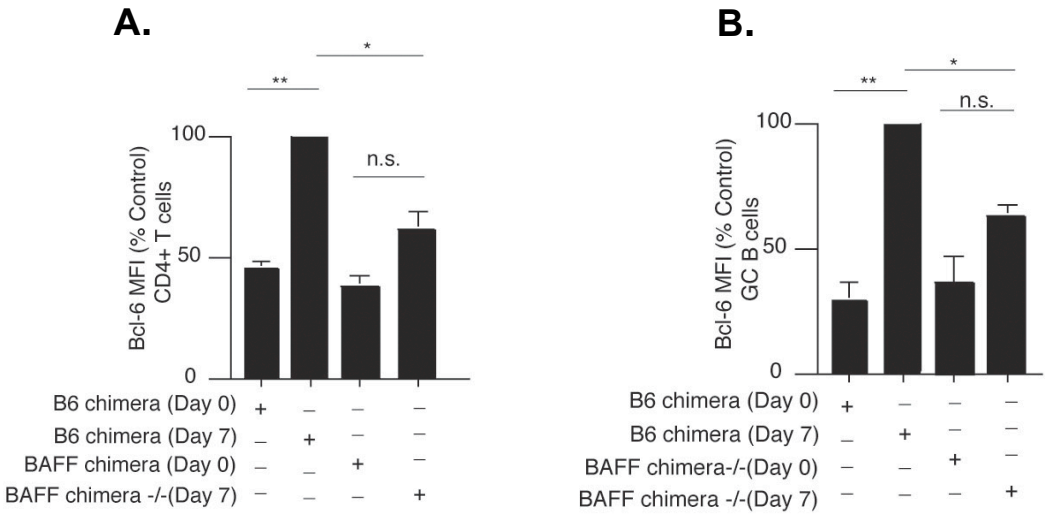


Fig. 2.13 DC-derived BAFF restores T_{FH} population in CD16$^{-/-}$ mice. BMDCs were prepared from B6 and BAFF Tg mice. B6 or BAFF Tg BMDCs were subcutaneously injected into CD16$^{-/-}$ mice, immunized with NP-$_{14}$-KLH. Inguinal lymph nodes were harvested seven days post-immunization, and assessed for the presence of CD4^{+} T_{FH} cells by flow cytometry. * $p \leq 0.05$, n.s: not significant, unpaired *t* test



2.14 Immunized BAFF$^{-/-}$ chimeras also have reduced levels of Bcl-6. Reconstituted B6 and BAFF$^{-/-}$ chimeras were immunized with NP-$_{14}$-KLH precipitated in alum. At 7 days post-immunization, spleens were harvested and intracellular Bcl-6 expression was assessed by flow cytometry in CD4^{+} T cells **(A.)** and GC (CD19^{+}, CD95^{+}, GL-7^{+}) B cells **(B.)** n =4. * $p \leq 0.05$, ** $p \leq 0.01$, n.s: not significant, one sample *t* test

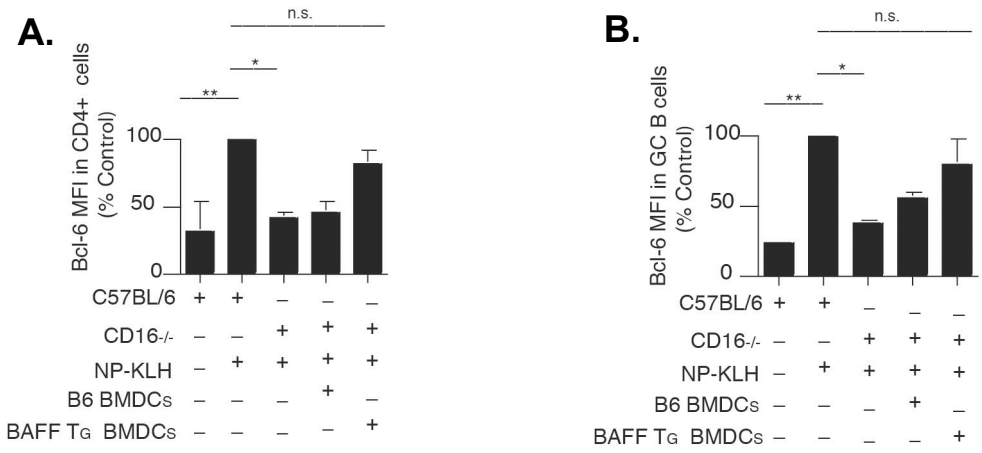


Fig. 2.15 DC-derived BAFF restores Bcl-6 expression in CD16$^{-/-}$ mice. BMDCs were prepared from B6 and BAFF Tg mice. 8 X 10^6 wild type or BAFF Tg BMDCs were subcutaneously injected into CD16$^{-/-}$ mice, immunized with NP-$_{14}$-KLH. Inguinal lymph nodes were harvested seven days post-immunization, and intracellular Bcl-6 expression in CD4$^+$ T cells **(A.)** and GC B cells **(B.)** was assessed by flow cytometry. n=5 *p $\leq$ 0.05, **p $\leq$ 0.01, one sample *t* test

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CHAPTER 3

BAFF is essential for memory B cell reactivation

3.1 Introduction

Antigen-specific recall responses initiated by memory B cells promote accelerated clonal expansion, rapid differentiation, and also give rise to high-affinity plasma cells as well as the replenishment of the memory B cell pool (1-3). The cellular and molecular basis for the faster and more robust response after antigen re-exposure remains unclear. Many studies have been aimed at understanding the intrinsic characteristics of memory B cells that enable robust secondary responses. Studies have found that the class-switched, IgG B cell receptors (BCRs) of memory B cells have enhanced signal initiation and microclustering compared to the BCRs of naïve B cells (4). The cytoplasmic tails of the BCRs of memory B cells also contribute to the burst of clonal expansion associated with antigen re-encounter (5). There is also evidence that there are significant changes in BCR signaling pathways of memory B cells. CD22-mediated signaling inhibition is prevented in memory B cells, thus enhancing BCR activation. (6). Increased affinity for antigen could also contribute to rapid reactivation of memory B cells and enhanced sensitivity to low doses of antigen that are otherwise unable to induce a primary immune response.

Much less is known about the extrinsic factors that promote the expansion of antigen-experienced plasma and memory B cells during recall responses. Circulating, high-affinity antibodies also contribute to memory B cell expansion, as rapid presentation of immune complexes to memory B cells is enhanced (1). There is also some evidence that memory B cells require cognate interaction with antigen-specific T helper cells to initiate secondary immune responses (1, 7). When the initial priming antigen and antigen presented during recall responses are identical, memory T_{FH} cells emerge and are reactivated preferentially over their naïve counterparts. The organization and kinetics of this process remain poorly understood. In addition, the specific role of T_{FH} cells in enhancing secondary immune responses has not been well studied.

There has also been recent evidence that memory B cells can re-initiate a GC reaction following antigen recall (8). The type of antigen appears to have an impact on the persistence of the primary-response GC, with particulate antigens (sRBCs) more likely than soluble antigens (NP-KLH), to sustain germinal centers (8). Whether the germinal centers observed during secondary responses are a continuation and re-expansion of a primary GC remains to be determined. More importantly, it remains to be seen whether these secondary germinal center structures support the replenishment of the memory B cell pool, the further diversification of affinity-matured BCRs, and the selection of B cell clones with higher affinities. The role of additional extrinsic factors, including cytokine production, in promoting antigen recall responses also requires further investigation. IL-21 plays a role in the accumulation of memory B cells and plasma cells following secondary immunization (9); however, it is not the sole factor responsible for memory B cell reactivation. Furthermore,

the mechanism by which IL-21 enhances memory B cell responses remains unclear; perhaps IL-21 synergizes with additional factors to promote memory B cell expansion.

Although a role for BAFF in B cell survival and homeostasis is well defined, its impact on B cells during memory responses remains unclear. Our studies reported herein demonstrate that during antigen recall responses, BAFF is essential for the expansion of memory B cells. The reduction of serum BAFF levels by BR3-Fc treatment resulted in decreased secondary NP-specific antibody responses. This deficit in secondary humoral responses was also associated with the failure of NP-specific memory B cells to expand into effector, antibody-secreting cells. This defect in secondary immune responses was also associated with a reduction in germinal center B cells and T_{FH} cells.

3.2 Materials and Methods

Animals

Animals were maintained in an accredited animal facility at University of North Carolina. C57BL/6 (B6) mice were bred in house and used at 8-12 weeks of age.

Antibodies and Reagents

Antibodies to CD19, CD4, GL-7, PD-1, and ICOS were purchased from Biolegend. Antibodies to murine IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and CXCR5 were purchased from BD Biosciences. Streptavidin Alexa 488 and Streptavidin Alexa 647 were purchased from Invitrogen. Purified antibodies from the hybridomas H33L and B1-8 were obtained from Dr. Garnett Kelsoe of Duke University. BR3-Fc and control antibody IgG-Fc were obtained from Dr. Robert Benschop of Eli Lilly. NP-Osu was purchased from Biosearch Technologies, and KLH was purchased from Sigma Aldrich. Alum was purchased from Pierce.

NP-KLH immunization

B6 mice (8-12 weeks of age) were immunized i.p. with 100 μg $NP-_{14}$-KLH precipitated in alum for the induction of primary responses. For secondary immunization, mice were boosted (i.v. injection) with 100 μg of soluble $NP-_{14}$-KLH at least 30 days after the primary immunization. Antibody levels were measured 4, 7, and 14 days after boost to quantitate memory recall responses.

BR3-Fc Treatment

Twenty-eight days following the initial NP_{14}-KLH immunization, the mice were injected (i.v) with 5 mg/kg of either control IgG-Fc or BR3-Fc at bi-weekly intervals. *In vivo* BAFF depletion was assessed by flow cytometry in the enumeration of $CD19^+$ B cells.

NP ELISA

Sera were tested for IgG anti-NP antibodies by ELISA. Microtiter plates were coated with 5 μg/mL NP_{13}-conjugated BSA (Biosearch Technologies). Serially diluted serum samples were incubated overnight at 4^oC. The anti-NP hybridoma, H33L was used as a standard for quantitation. Anti-NP IgG levels were detected using an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1/1000 dilution) followed by phosphatase substrate (Sigma-Aldrich). Optical density (OD) values were converted to concentration based on standard curves, using a four-parameter logistic equation (Softmax Pro 3.1 software; Molecular Devices).

NP ELISpot

Splenocytes from immunized mice were washed and transferred to plates coated with 10 μg/mL NP_{13}-conjugated BSA and incubated for 48 hrs (0.25×10^6 cells/well). The ASCs were detected using biotinylated anti-IgM and IgG followed by streptavidin-horseradish peroxidase. The plates were analyzed using an ImmunoSpot Analyzer and ImmunoSpot software package (Cellular Technology).

T_{FH} *Cell Enumeration*

B6 mice (8-10 weeks of age) were immunized and treated with BR3-Fc as described. At day 14 post-immunization, spleens were harvested, single cell suspensions were prepared, and $CD4^+$, $CXCR5^+$, $PD\text{-}1^+$, and $ICOS^+$ T cells were enumerated by flow cytometry.

Statistical analysis

The student's *t* test was used to compare Ig secretion following the boost in IgG-Fc control-treated and BR3-Fc-treated mice. The unpaired *t* test was used to compare ELISpot results, GC B cell and T_{FH} cell numbers in IgG-Fc and BR3-Fc treated mice. Statistical analyses were performed with GraphPad Prism.

3.3 Results

3.3.1 BAFF is required for memory B cell reactivation

Our studies thus far show that $CD16^{-/-}$ mice and $BAFF^{-/-}$ bone marrow chimeras fail to mount secondary immune responses, as indicated by reduced antibody levels following boost with antigen, as compared to immunized B6 mice. This defect in secondary immunity has been attributed to a reduction in memory B cell formation during the primary immune response. Alternatively, the reduced levels of anti-NP antibody during recall responses could result from the failure of the existing antigen specific memory B cells to expand and secrete antibody after secondary antigen exposure due to the lack of localized BAFF production. To assess the role of BAFF in memory B cell reactivation, wild type B6 mice were immunized with the T-dependent antigen NP-KLH and subsequently dosed with a 5 mg/kg dose of BR3-Fc to reduce serum BAFF levels. BR3-Fc treatment was initiated at 28 days post-immunization to allow for a normal primary immune response to NP-KLH immunization. By day 28 post-immunization, the primary response has contracted (10), and only the role of BAFF in memory B cell expansion and antigen recall was assessed. Following the bi-weekly dosing of B6 mice with BR3-Fc, we found that the percentage of $CD19^{+}$ B cells was decreased by 50%, indicating that the mature B cell compartment was sensitive to the reduction of serum BAFF levels (Figure 3.2). No significant changes in B cell numbers were found in IgG-Fc treated mice (Figure 3.2). Following the final dose of BR3-Fc, the mice were boosted with soluble NP-KLH and antibody responses were assessed by ELISA. We found that BR3-Fc treated mice had 2-fold lower anti-NP IgG antibody responses when compared to the IgG-Fc-treated control mice at 14 days post-boost (Figure 3.3). We also observed a slight, but not statistically significant, decrease in anti-NP IgM levels in the BR3-

FC injected mice at 7 days post-boost (Figure 3.3). In addition, the BR3-Fc treated mice displayed 50% fewer anti-NP IgG antibody secreting effector cells at 14 days post-boost, as indicated by an ELISpot assay (Figure 3.4). Overall, these results suggest that BAFF is necessary for the expansion of memory B cells and robust humoral responses during re-encounter with antigen.

3.3.2 BR3-Fc treated mice have reduced germinal center B cells and T_{FH} *cells*

Studies have shown that activated B cells can re-enter germinal center structures in secondary immune responses (8). Thus, it is possible that the failure to secrete antibody during recall responses could result in the lack of germinal center re-formation or re-utilization. To assess germinal center B cell responses following antigen boosting, spleens of immunized, BR3-Fc treated mice were sectioned and analyzed by immunofluorescence for the presence of germinal centers. We found that BR3-Fc treated mice did indeed show clustering of PNA^+, $B220^+$ B cells, indicative of germinal center structures (data not shown). However, in contrast to IgG-Fc treated control mice, BR3-Fc treated mice displayed germinal centers of a smaller size, most likely due to a reduction in the numbers of mature B cells (data not shown). In addition, the absolute number of $CD19^+$, $CD95^+$, $GL\text{-}7^+$ germinal center B cells was reduced by approximate 55% at 14 days post-boost in the BR3-Fc treated mice, compared to control mice, as indicated by flow cytometric analysis (Figure 3.5). Because germinal center formation requires proper T_{FH} cell function and maintenance, we assessed the numbers of $CXCR5^+$, $PD\text{-}1^{-/-}$, $CD4^+$ T cells within the spleens of immunized B6 mice 14 days post boost. Corresponding with a change in germinal centers, we also found that the reduction in BAFF serum levels resulted in a 40% reduction in $CXCR5^+$, $CD4^+$ B cells (Figure 3.6). No changes in PD-1 or ICOS expression were observed. Taken together, these

data suggest that the presence of BAFF in secondary immune responses influences germinal center responses and CXCR5 upregulation of $CD4^+$ T cells during secondary immune responses.

Our results demonstrate that BAFF is a required factor for memory B cell reactivation during secondary immune responses. This suggests that BAFF is necessary for plasma cell differentiation from activated memory B cells after re-encounter with antigen. In addition to fewer anti-NP ASCs following the boost in BR3-Fc injected mice, we also found that there were fewer GC B cells and T_{FH} cells present in the spleens of BAFF-depleted mice. Overall, these data suggest that BAFF has a novel role in productive B and T cell responses upon antigen recall responses.

3.4 Discussion

During the secondary immune response, re-encounter with antigen results in the expansion of memory B cells into effector antibody-secreting cells and the generation of additional memory B cells. Our studies indicate that BAFF has many regulatory roles in adaptive immunity, including modulating the expansion of B cells during secondary immune responses. This expansion results in a robust antibody response that protects the host from secondary infection by the pathogen. Our data indicate that BAFF is critical for memory B cell expansion and antibody responses following re-encounter with antigen. As a result of *in vivo* BAFF reduction, IgG antibody responses are significantly attenuated following the boost with NP-KLH.

The exact mechanism by which BAFF regulates memory B cell reactivation and secondary immunity remains unclear. Because there is some literature to suggest that naïve B cells can participate in secondary immune responses (11), it is possible that the depletion of naïve B cells from the BR3-Fc treatment is indirectly responsible for the lack of secondary responses. Alternatively, BR3-Fc treatment could directly inhibit the BCR activation of memory B cells once they have encountered antigen. There is also some evidence of signaling cross-talk between the BAFF receptor and the BCR and of BAFF enhancing BCR signaling (12). Thus, loss of BAFF signaling may also interfere with the threshold of B cell receptor activation and memory B cell function.

It has also been proposed that germinal centers contribute to secondary immune responses (8). Studies have also shown germinal centers can be reutilized by newly activated B cells (13). Our studies would suggest that BAFF inhibition by BR3-Fc treatment might

disrupt the seeding of secondary germinal centers and T_{FH} cell function during secondary immune responses, thereby preventing the rapid antibody response that is required to neutralize the intruding pathogen. Recent studies have shown that memory T_{FH} cells reside in close proximity to IgG memory B cells, which surround contracted germinal centers in the spleen (7). These studies propose that the close proximity of memory B and T_{FH} cells contribute to the rapid kinetics of secondary immune responses. It is also possible that additional factors such as BAFF are required for the localization of memory B cells and T_{FH} cells during antigen re-encounter. Our studies show that BAFF is required for the upregulation of the chemokine receptor CXCR5 on $CD4^+$ T helper cells. Thus, BAFF may promote the homing of memory T_{FH} cells into the B cell follicle, which allows for successful memory B cell activation. Because B cells are required for T_{FH} cell maintenance (14), the lack of CXCR5-expressing helper T cells could also be the product of partial B cell depletion resulting from BR3-Fc treatment. Future studies are required to further delineate the direct role of BAFF in T_{FH} cell function during secondary immune responses.

Our studies show that BR3-Fc treatment results in a reduction in total T_{FH} cell numbers during antigen recall. This would suggest that B cell depletion by *in vivo* BAFF neutralization also directly impacts T cell maintenance and the generation of T cell memory. Perhaps the reduced secondary responses following BR3-Fc treatment is also a result in the loss of function of memory T cells. Numerous reports have indicated that B cells can regulate $CD4^+$ memory T cell responses. In one study, using infection with *Heligmosomoides polygyrus* as a model, B cells were required to maintain and reactivate antigen-specific memory T helper cells (15). Similar results were found in a model of LCMV infection where LCMV-specific memory $CD4^+$ T cells failed to survive in B cell-

deficient mice (16). Combined, these studies indicate that B cells have a role in establishing and maintaining antigen-specific memory T cell populations. Thus, failure to mount robust antibody responses could not only reflect the inability to activate memory B cells in the absence of BAFF, but also failure to activate antigen-specific memory T cells as a result of partial B cell depletion.

The role of BAFF in memory B cell activation has vast implications in the regulation of memory to self-antigens in the setting of autoimmune disease. Our studies show that anti-BAFF immunotherapy has the potential to prevent autoreactive memory B cell activation in response to recognition of self-antigens. BAFF is known to provide a survival mechanism for autoreactive B cells, and BAFF over-expression has been linked to autoimmunity (17). Specifically, BAFF transgenic mice develop lupus-like disease as they age (18). In addition, high serum levels of BAFF have been found in a select group of SLE patients (17, 19). This clearly demonstrates a pathogenic role for BAFF initiating and sustaining autoimmune diseases. Our data reported herein demonstrate that the reduction of serum BAFF levels *in vivo* by BR3-Fc treatment results in the failure to reactivate memory B cells during secondary immune responses. Although previous reports have shown that memory B cell survival is independent of BAFF (20, 21), previous studies have not addressed the role of BAFF during antigen recall. Our data indicate that BAFF is indispensable in the activation of memory B cells during re-exposure to antigen in secondary immune responses. Thus, anti-BAFF therapies to treat autoimmune disease may contribute to the amelioration of disease by preventing memory B cell responses to self-antigen.

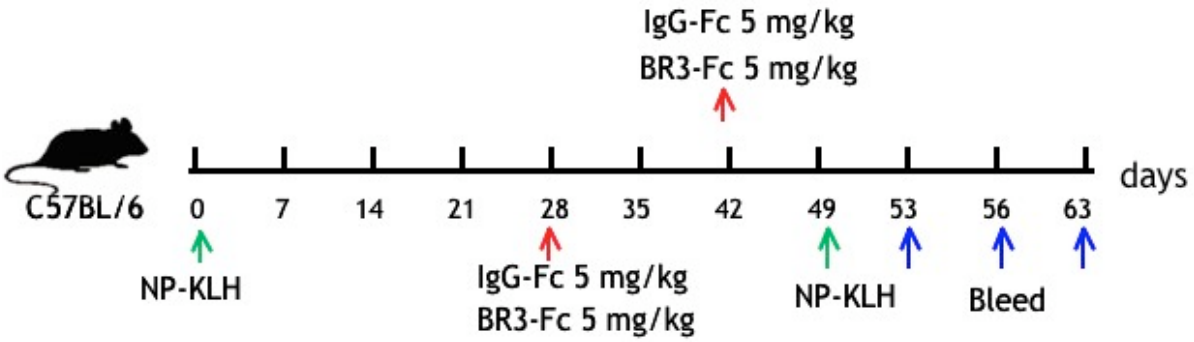

Fig. 3. 1 Reduction of serum BAFF levels by BR3-Fc treatment in B6 mice. 8-12 week old female B6 mice were immunized with 100 μg NP-$_{14}$-KLH (i.p.). At 28 days post-immunization, mice were treated with 5 mg/kg of the control antibody, IgG-Fc, or the BAFF depleting reagent, BR3-Fc. Mice were boosted one week after the 2nd dose of BR3-Fc. Mice were bled 4, 7, and 14 days following boost with NP-KLH. Serum anti-NP levels were assessed by ELISA.

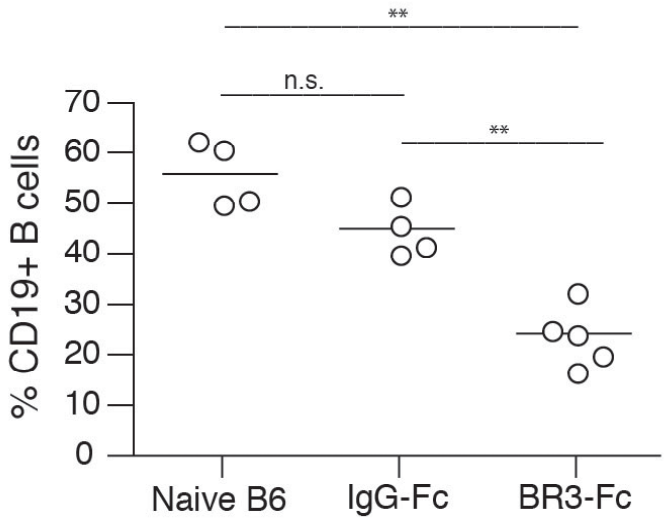

Fig. 3. 2 Treatment with BR3-Fc results in fewer CD19$^+$ B cells in B6 mice. 8-12 week old female B6 mice were immunized with 100 μg NP-$_{14}$-KLH (i.p.). At 28 days post-immunization, mice were treated with 5 mg/kg of the control antibody, IgG-Fc, or the BAFF depleting reagent, BR3-Fc. CD19$^+$ B cells were enumerated by flow cytometry. **p ≤ 0.01, n.s: not significant, unpaired *t* test

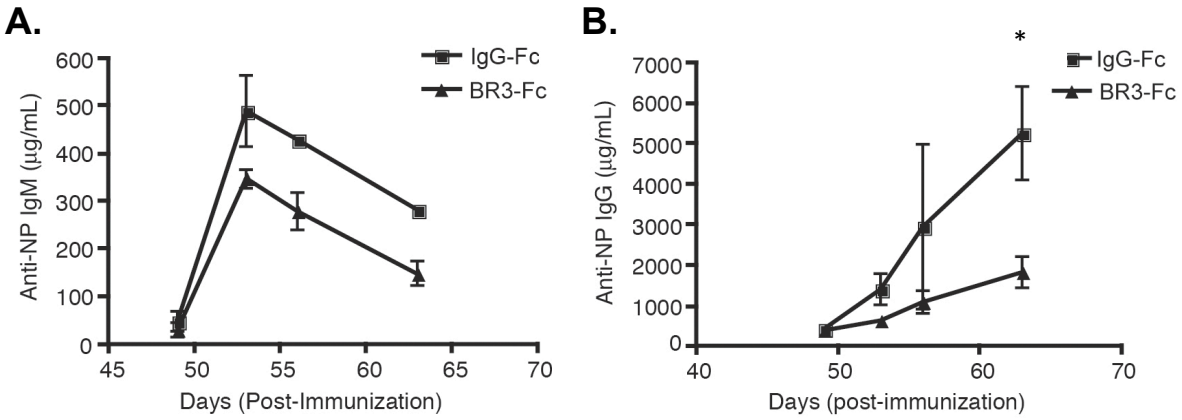

Fig. 3.3 BAFF is required for memory B cell reactivation. 8-12 week old B6 mice were immunized with 100 µg NP-$_{14}$ KLH (i.p.). 28 days post-immunization mice were dosed with 5 mg/kg IgG-Fc or BR3-Fc at bi-weekly intervals. Following BR3-Fc treatment, both cohorts of mice were boosted with soluble NP-$_{14}$-KLH. Serum IgM **(A.)** and IgG **(B.)** anti-NP levels were measure by ELISA 4, 7, and 14 days post-boost. n=8 mice per treatment group. *$p \leq 0.05$, Student's *t* test

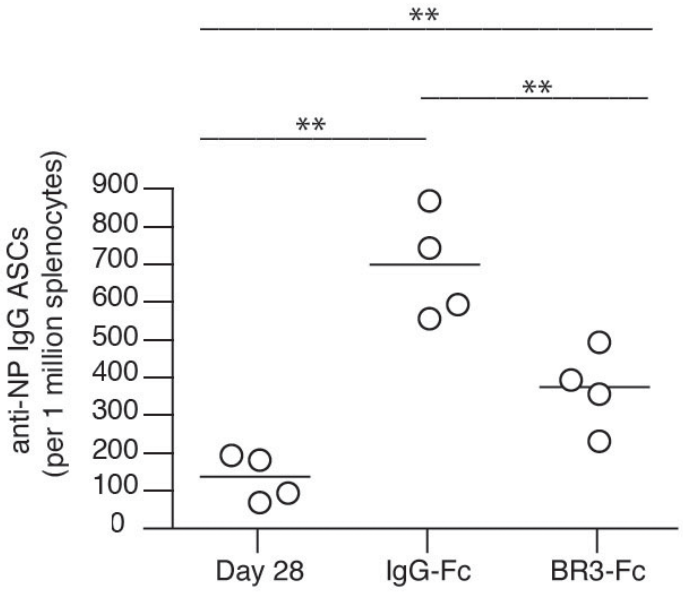

Fig. 3.4 BAFF is required for memory B cell expansion. 8-12 week old B6 mice were immunized with 100 μg NP-$_{14}$ KLH (i.p.). 28 days post-immunization mice were dosed with 5 mg/kg IgG-Fc or BR3-Fc at bi-weekly intervals. Following BR3-Fc treatment, both cohorts of mice were boosted with soluble NP-$_{14}$-KLH. Anti-NP ASCs were enumerated at day 28 post-immunization and 14 days, post-boost by ELISpot. *$p \leq 0.05$, unpaired *t* test

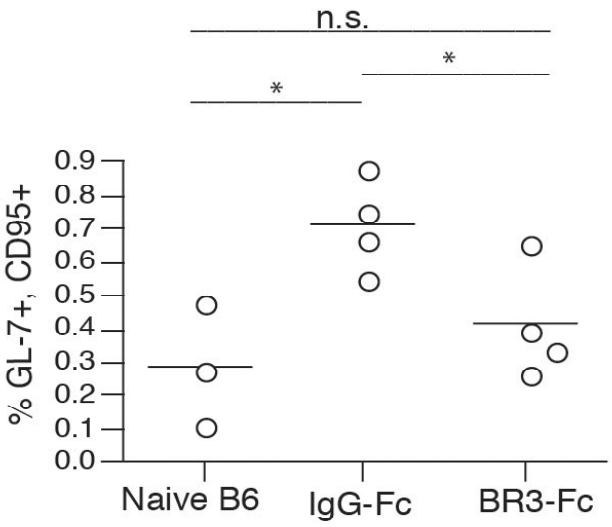

Fig. 3. 5 BR3-Fc treated mice have fewer GC cells, 14 days post-boost. 8-12 week old B6 mice were immunized with 100 μg NP-$_{14}$-KLH (I.p.). At 28 days post-immunization, the mice were treated with 5 mg/kg of the control antibody, IgG-Fc, or the BAFF depleting reagent, BR3-Fc. Following treatment with IgG-Fc or BR3-Fc, mice were boosted with 100 μg soluble NP-$_{14}$-KLH. CD19^{+}, GL-7^{+}, CD95^{+} GC B cells were enumerated by flow cytometry 14 days post-boost. *$p \leq 0.05$, **$p \leq 0.01$, unpaired *t* test

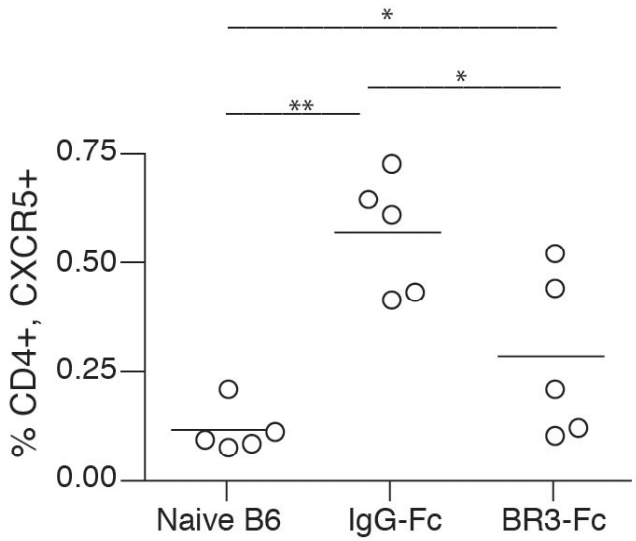

Fig. 3. 6 BR3-Fc treated mice have fewer cells 14 days post-boost. 8-12 week old B6 mice were immunized with 100 μg NP-$_{14}$-KLH (I.p.). At 28 days post-immunization, mice were treated with 5 mg/kg of the control antibody, IgG-Fc, or the BAFF depleting reagent, BR3-Fc. Following treatment with IgG-Fc or BR3-Fc, mice were boosted with 100 μg soluble NP-$_{14}$-KLH. 14 days following the boost, $CD4^+$, $CXCR5^+$ T follicular helper cells were enumerated by flow cytometry. *$p \leq 0.05$, **$p \leq 0.01$, unpaired *t* test

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CHAPTER 4

B cells secrete cytokines to regulate antigen-induced Ig secretion

4.1 Introduction

The production of high-affinity, antigen-specific antibodies during the adaptive immune response is critical in the host's survival during infection. The proper contraction of antibody production by activated B cells is also important in preventing inappropriate immune responses, which could lead to excess inflammation and, in extreme instances, malignancies and autoimmunity. Several regulatory mechanisms have been described that contribute to the regulation of B cell activation thresholds, including immune complexes binding FcγRIIb on the B cell surface. FcγRIIb , along with other inhibitory receptors such as PD-1, CD-5, and CD22, belong to the family of ITIM-containing immune inhibitory receptors. Fc receptor ligation on the surface of B cells results in SHIP phosphatase recruitment and attenuated BCR signaling (2, 3). Studies in FcγRIIb $^{-/-}$ mice have demonstrated its role in enhancing humoral immunity. However, the absence of FcγRIIb does not result in uncontrolled antibody production, indicating the presence of additional regulatory mechanisms. Other mechanisms of immune regulation include the generation of regulatory T and B cells, whose main function is to secrete regulatory cytokines that dampen the immune response. There is a large body of evidence demonstrating the role of regulatory T cells and their production of TGF-β in modulating T helper cell responses, and in some

cases, the function of activated B cells (4-6). Although they are mostly characterized by their production of immunoglobulins and their role in secondary antigen presentation, B cells are also capable of additional immunoregulatory functions. Regulatory IL-10-producing B cells can suppress $CD4^+$ T cell responses and can prevent the induction of autoimmune disease in several mouse models (7). Although there is emerging evidence of a key role for regulatory B cells in regulating T cell responses and maintaining tolerance, little is known about the role of antigen-stimulated B cells in actively regulating effector function of newly emerging naive B cells and antibody secretion via autocrine mechanisms. It also remains unclear whether B cells secrete other regulatory cytokines, in addition to IL-10.

Our data reported herein support a model in which antigen-stimulated B cells, independent of Fc receptor ligation, secrete the soluble regulatory factors TGF-β and CD40L, which negatively impact effector functions of newly stimulated B cells *in vitro*. We propose that the production of regulatory cytokines by B cells functions as a feedback mechanism that limits Ig production following a productive immune response. In addition, our studies suggest the production of TGF-β and CD40L by B cells also plays a role in the maintenance of tolerance to self-antigens, as B cells from lupus-prone mice fail to regulate Ig secretion. Overall, our studies demonstrate that in addition to immunoglobulin production and antigen presentation, B cells have an important role in regulating antigen-induced B cell responses.

4.2 Materials and Methods

Animals

Animals were maintained in an accredited animal facility at University of North Carolina. C57BL/6 (B6), FcγRIIb $^{-/-}$, B6.*lpr*, and MRL/*lpr* mice were bred in house and used at 10-12 weeks of age. OTII TCR transgenic mice and B6 Mer^{tkd} were obtained from Jenny Ting and Glenn Matsushima, from the University of North Carolina.

B and T cell purification

Splenic B cells were isolated from B6, FcγRIIb $^{-/-}$, MRL/*lpr*, Mer^{tkd}, and B6.*lpr* mice by negative selection (StemCell Technologies). B cells were 95-99% pure, as determined by flow cytometry (with fewer than 5% DCs and MFs). In some experiments, marginal zone (MZ) B cells were depleted by the addition of biotinylated anti-CD9. Splenic T cells were isolated from OTII TCR transgenic mice by negative selection (StemCell Technologies). T cells were 90-99% pure, as determined by flow cytometry.

Antibodies and Reagents

Recombinant murine IL-4 and IL-5 were purchased from Peprotech. Monoclonal antibodies 54.1 (3-83 idiotype), Y-12 (anti-Sm), and 33-60 and B7.6 (anti-mu) were purified from hybridoma culture supernatants. Streptavidin microbeads were purchased from Miltenyi. Whole ovalbumin protein was purchased from Sigma Aldrich. Neutralizing antibodies (anti-TGF-β and anti-CD40L) were purchased from R&D Systems.

Preparation of B cell conditioned media (CM)

2 X 10^5 purified B cells were stimulated with 30 μg/mL anti-mu (B7.6 complexed to streptavidin microbeads), rIL-4 and rIL-5 (10 ng/mL). After seven days of culture, supernatants were harvested, and stored at -20^{o}C for future use.

B6 B cell and OTII T cell co-culture

2 X 10^5 purified B6 B cells were co-cultured with 2 X 10^5 T cells purified from OTII TCR transgenic mice. Cells were cultured for 7 days in the presence of 100 μg ovalbumin. Supernatants were harvested on day 7, and IgM was measured by ELISA, or stored at -20^{o}C for future use.

Total IgM ELISAs

IgM levels from B6 mice were detected using anti-mouse IgM (33-60) and biotin-labeled anti-mouse IgM (B7.6).

Neutralization of B cell CM

B cell CM were pre-incubated with anti-TGF-β, anti-CD40L , or isotype control antibodies (54.1 and Y-12) for one hour prior to addition to purified B cells and cultured with anti-mu stimulated B cells for seven days. Supernatants were harvested, and secreted IgM was quantitated using ELISA.

Statistics

The one sample *t* test was used to compare secreted IgM levels in anti-mu stimulated controls and treatment groups. Statistical analyses were performed using Graph Pad Prism.

4. 3 Results

4.3.1 Antigen-stimulated B cells secrete repressive factors

B cells primarily function in antigen-specific adaptive immune responses by secreting high-affinity antibodies to neutralize invading pathogens. Few studies have addressed the role of B cells in directly regulating B cell effector responses, including the production of immunoglobulin. It is unclear whether B cells produce factors during antigen stimulation that negatively regulate B cell responses through a feedback mechanism. To assess whether B cells possess the ability to regulate Ig secretion through an autocrine mechanism, wild type B6 B cells were stimulated with anti-mu, rIL-4, and rIL-5 for seven days to mimic a T-dependent, adaptive immune response. After 7 days, supernatants from the stimulated B cells (B cell conditioned media (CM)) were harvested and tested for repressive capabilities. Subsequently, additional B6 B cells were purified, stimulated with anti-mu, and cultured with the B cell CM. We found that anti-mu stimulated B cells cultured with B cell CM showed a 95% reduction in Ig secretion compared to the anti-mu stimulated control B cells (Figure 4.1). We also found that while Ig secretion was significantly inhibited by culture with B cell CM, cell proliferation and viability remained unaffected (data not shown).

4.3.2 The production of regulatory factors by activated B cells is independent of Fc receptor ligation

Because B cells express the inhibitory Fc receptor FcγRIIb, which negatively regulates B cell activation, it is possible that the B cell CM inhibits Ig production through an Fc-dependent mechanism, involving immune complex binding by FcγRIIb . To test this, B cells were purified from FcγRIIb $^{-/-}$ mice, stimulated with anti-mu and rIL-4/5, and cultured with B cell CM from B6 mice. The repressive ability of the CM was independent of Fc

receptor ligation since Ig secretion of B cells from FcγRIIb deficient mice was also inhibited by the B cell CM (Figure 4.1). Furthermore, CM prepared from wild type B cells using anti-mu $F(ab)_2$ also inhibited Ig production (Figure 4.1), indicating that the presence of the Fc portion of antibody is not required for B cell-mediated repression of Ig secretion.

4.3.3 Ovalbumin-stimulated B cells secrete factors that negatively impact Ig secretion

To confirm that the mechanism by which B cells auto-regulate Ig production is not unique to anti-mu stimulation, an *in vitro* culture system was designed to stimulate B cells using bona fide antigen and T cell help. Purified wild type B6 B cells were stimulated with ovalbumin protein in the presence of OTII T cells, which express an ovalbumin-specific T cell receptor (TCR). In the presence of ovalbumin and antigen-specific T cells, B6 B cells proliferated (Figure 4.2) and secreted Ig, indicating a productive immune response to ovalbumin stimulation. Conditioned media from these stimulated B cells was harvested and tested for its repressive abilities (OVA B cell CM). Freshly isolated B6 B cells were ovalbumin-stimulated and cultured with the OVA B cell CM. We found that Ig secretion was inhibited by 60% (Figure 4.2), indicating that these repressive effects of the B cell CM are not unique to anti-mu B cell stimulation, but also impact B cells stimulated with bona fide antigen.

4.3.4 CD9 expressing B cells secrete soluble factors that inhibit Ig secretion

During the process of B cell development in bone marrow, naive B cells differentiate and migrate to different microenvironments, so that peripheral lymphoid tissues contain mature B cell subsets with varying phenotypes and functions (8). Although the majority of B cells in the spleen are located in the follicular (FO) area, B cells of slightly larger size are

located in the marginal zone and are more readily activated (9, 10). Because B cells with an origin similar to the marginal zone have been ascribed regulatory functions (11), we wanted to identify the B cell subset secreting the regulatory factors. Studies have shown that regulatory B cells play a major role in maintaining immune tolerance and that the function of these B cells is dysregulated in SLE patients (12). To identify the B cell subset, we purified B cells from B6 mice and subsequently depleted the marginal zone subset using an anti-CD9 antibody. Marginal zone depletion was confirmed by flow cytometry, as demonstrated by the near absence of $CD21^{high}$, $CD23^{low}$, $CD19^{+}$ B cells (13). The remaining B cells were then anti-mu stimulated and CM was harvested after seven days. We found that B cells stimulated with CM from marginal zone-depleted B cells failed to inhibit Ig secretion (Figure 4.3), demonstrating that the source of the repressive factors was the marginal zone subset. Because B cells of the B-1 subset and some plasma cells also express CD9, we cannot rule out the contribution of these B cell subsets in regulation Ig secretion (13).

4.3.5 Ag-stimulated B cells secrete TGF-β and CD40L, which regulate Ig production

We have now identified a novel mechanism by which B cells secrete soluble factors that regulate Ig production, independent of Fc receptor ligation. We next wanted to identify the soluble factors secreted by B cells upon stimulation. We began by neutralizing the CM with a panel of antibodies against cytokines that are secreted by B cells and are known to have regulatory roles in the immune response. Ig secretion by stimulated B cells cultured with CM neutralized with either anti-TGF-β or anti-CD40L alone remained inhibited (Figure 4.4 A). We found that when the B cell CM was neutralized with both anti-TGF-β and anti-CD40L, Ig secretion by the B cells was fully restored to levels similarly observed in anti-mu stimulated controls (Figure 4.4 A). These results were not due to non-specific antibody

binding because restoration of Ig secretion was not observed when CM treated with isotype controls. These results indicate that B cells secrete TGF-β. and CD40L, which limit Ig production by other antigen-stimulated B cells. Furthermore, when B cells were cultured with both recombinant TGF-β and CD40L combined, Ig secretion was reduced to 90% of the anti-mu stimulated controls (Figure 4.4), while proliferation remained unaffected (data not shown).

4.3.6 B cells from lupus-prone mice fail to repress Ag-induced Ig secretion

Our studies suggest that B cells secrete TGF-β and CD40L, which negatively regulate Ig secretion by other antigen-stimulated B cells. It is also possible that these regulatory factors may play a role in maintaining tolerance to self-antigen and preventing activation of self-reactive B cells, as previous studies in our lab have shown that CD40L prevents TLR-activation of Sm-specific B cells (14). Furthermore, DCs and MFs from lupus-prone mice fail to secrete regulatory factors in response to LPS stimulation (15). If TGF-β and CD40L play a significant role in maintaining tolerance, one would predict that B cells from autoimmune-prone mouse models, in which tolerance has been breached, would show decreased production of these regulatory cytokines. To test whether these factors have a role in immune tolerance to self-antigen, B cell CM were prepared from the lupus-prone mouse model MRL/*lpr* and from mouse models with known clearance defects and an increased propensity for autoimmunity (B6.*lpr* and Mer^{tkd}). We found that compared to CM from wild-type mice, B cell CM from MRL/*lpr*, Mer^{tkd}, and B6.*lpr* mice failed to inhibit IgM production by anti-mu stimulated B6 B cells (Figure 4.5). B cells cultured with these CM secreted levels of IgM that were comparable to anti-mu stimulated controls. These data suggest that B cells from lupus-prone mice fail to secrete the regulatory products. Failure to

maintain tolerance to self in these lupus-prone mouse models could be partially attributed to the lack of TGF-β and CD40L production by antigen stimulated B cells.

4.4 Discussion

Robust B cell activation is critical in providing the host with protective humoral immunity against invading pathogens. Tightly regulated B cell activation and subsequent antibody production allows for the effective neutralization of foreign antigen, while maintaining unresponsiveness to self-antigens. Mechanisms to induce proper contraction of the immune response must also exist to prevent uncontrolled antibody responses and inflammation. Dysregulation of B cell responses can lead to several pathological conditions, including lymphoid cancers, autoimmunity, and immunodeficiency. Historically, the activation of ITIM-containing inhibitory cell surface receptors, including FcγRIIb , has been recognized as an essential mechanism in regulating BCR signaling thresholds and B cell proliferation and Ig secretion (16). However, additional mechanisms must exist, as FcγRIIb$^{-/-}$ mice can still attenuate BCR-derived antibody responses (1). In summary, we have found that antigen-stimulated B cells, predominantly of the marginal zone subset, produce the regulatory factors TGF-β and CD40L, which negatively impact Ig secretion *in vitro*. These data suggest that B cells themselves have an active role in regulating BCR-derived immune responses. Our data also highlight the importance of these regulatory cytokines produced by B cells in models of autoimmunity because B cells from lupus prone mice fail to secrete factors that regulation Ig secretion.

In addition to immunoglobulin production and providing co-stimulation to T cells, it has been well documented that B cells can also secrete cytokines and chemokines in response to antigen and other stimuli (17, 18). Although it has been known for decades that B cells can secrete cytokines, the idea that B cell-derived cytokines are involved in regulating various components of the adaptive immune response is relatively new. The role of TGF-β

and CD40L production by marginal zone B cells in regulating adaptive immune responses *in vivo* has yet to be determined. Marginal zone B cells are strategically located in the periphery of the B cell follicle and are the first cells to encounter antigen from the bloodstream (19). As a result of their location, marginal zone B cells are more readily activated than follicular B cells and do not require T cell help (8, 19). MZ B cells preferentially secrete antibodies of the IgM and IgG_3 isotypes, while follicular B cells preferentially secrete IgG_1 or other Ig isotypes after stimulation, reflecting the role of MZ B cells in the primary T-independent response (19). Because our studies show that the $CD9^+$ marginal zone B cells are the predominant producers of TGF-β and CD40L, it is possible that the primary function of B cell-derived TGF-β and CD40L *in vivo* is to modulate T-independent, extra-follicular antibody responses. Conditional knockout of TGF-β signaling in B cells results in exaggerated IgG_3 responses, which is indicative of its role in T-independent B cell responses (20-22). Additional studies in our lab show that mice containing a conditional knockout of the TGF-β receptor on B cells have an expanded population of $CD138^+$ pre-plasma cells at 3 days post-immunization, thus supporting the role of TGF-β in regulating the early extra-follicular events in the adaptive immune response.

The role of CD40L in regulating B cell responses and in the maintenance of immune tolerance also requires further investigation. Several studies have demonstrated that CD40 signaling by CD40L-expressing T cells promotes germinal center formation, isotype switching, affinity maturation, and the formation of long-lived plasma cells and B cell memory (23, 24). The role of CD40-CD40L ligand interactions in inducing T-dependent immunity is well established; however, CD40 can also have an opposing function in regulating B cell responses. Studies have shown that excessive signaling by CD40 can

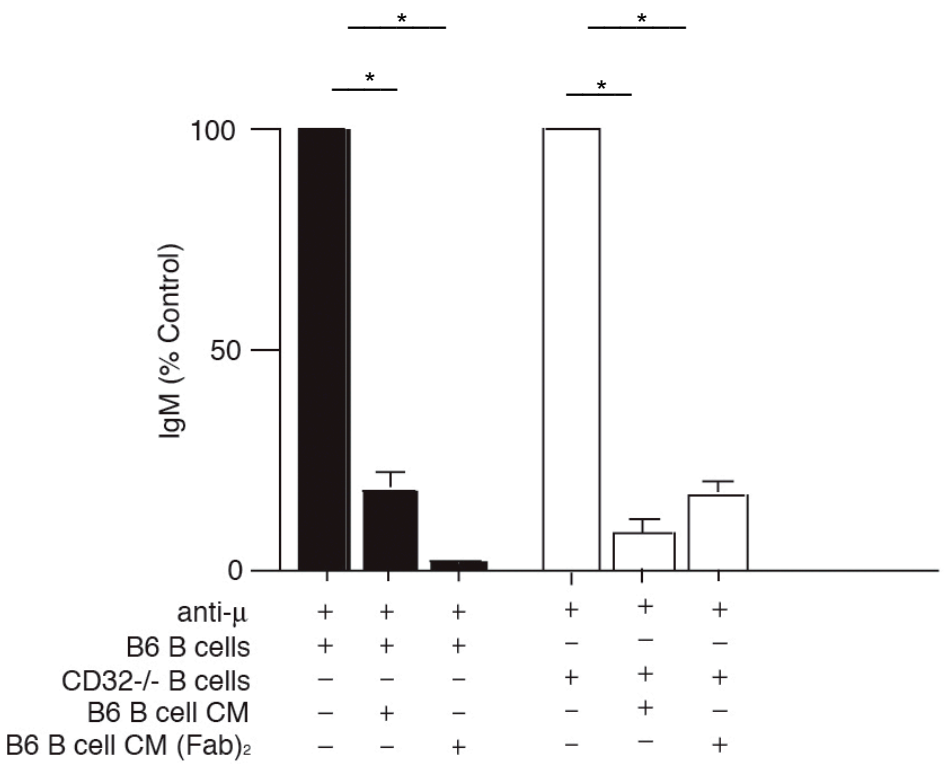
impede the ability of B cells to differentiate to long-lived plasma cells (25). Similarly, enhanced CD40 signaling attenuates long-lived immunity by inducing antigen-specific B cells to differentiate to short-lived plasma cells at the expense of germinal center formation and induction of B cell memory (26). Studies have also shown that the administration of a CD40 agonist along with a T-dependent antigen ablates long-lived humoral immunity in favor of short-lived extra-follicular plasma cells (23, 26). Thus, enhanced CD40 signaling may also impact extra-follicular B cell responses, similar to TGF-β.

Because B cell CM from lupus-prone mice fail to inhibit Ig secretion *in vitro*, TGF-β and CD40L also contribute to the maintenance of immune tolerance by preventing autoantibody production. Our lab has previously demonstrated a role for CD40L in maintaining immune tolerance by preventing polyclonal activation of autoreactive B cells during TLR-induced innate immune responses (14). Our current studies reported herein also implicate CD40L in an additional role of regulating BCR-derived immune responses to self-antigen. The role of TGF-β preventing autoimmunity has been highlighted in several studies using TGF-β^{-} mice, which succumb to severe systemic autoimmune disease shortly after birth (27). In several other mouse models of autoimmunity, autoantibody production originates from B cells that reside outside of the follicle. In MRL/*lpr* mice, for example, anti-DNA antibody production occurs in extrafollicular sites (28-30). Furthermore, AM14 rheumatoid factor-positive B cells on the MRL/*lpr* background can be activated by self-antigen, independently of T cell help and outside of germinal centers (31). Because our *in vitro* studies suggest that lupus-prone mice fail to secrete repressive factors, one should consider that the inappropriate extrafollicular antibody responses observed in *lpr* deficient (MRL/*lpr* and B6.*lpr*) mice are a result of the failure to secrete repressive factors.

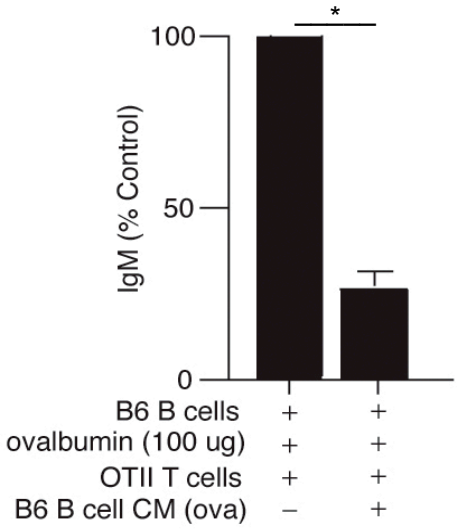
Further studies are required to link increased apoptotic burden resulting from the lack of Fas(*lpr*) or Mer expression to decreased production of regulatory factors by activated B cells, as indicated from our studies in Mer^{tkd} and B6.*lpr* mice. There is a demonstrated relationship between failure to clear apoptotic debris and the development of autoimmunity in humans (32). It is also believed that the increased availability of apoptotic cells caused by Mer and *lpr* mutations in mice could contribute to the induction of autoreactive B cell responses (33, 34). It has been demonstrated that similar to defective Mer function (35), *lpr* mutations induce the activation of autoreactive marginal zone and peritoneal B-1 B cells by an antigen-specific mechanism (36). The similar outcomes of Mer and Fas (*lpr*) defects could be explained by the observations that both proteins have roles in apoptotic cell clearance (36). The increased activation of MZ B cells and the B1-subset in Mer^{tkd} and B6.*lpr* mice could be attributed to both the increased apoptotic burden and a subsequent lack of TGF-β production by marginal zone B cells. Perhaps the increased apoptotic burden triggers production of TGF-β by marginal zone B cells. If TGF-β and CD40L do indeed negatively regulate extrafollicular IgM responses by marginal zone B cells, the lack of TGF-β production will lead to enhanced T-independent extrafollicular antibody responses that should promote non-pathologic clearance of the burden of apoptotic debris. Additional studies are required to further define the link between apoptotic clearance and the secretion of regulatory factors by B cells.

TGF-β is a well-known immunosuppressive cytokine, mainly produced by $Foxp3^{+}$ T regulatory cells and is critical in limiting pro-inflammatory responses (4, 37). Our studies identify an additional source of TGF-β, and highlight a novel role for B cells in regulating BCR-derived humoral immune responses. It is not surprising that B cells are able to

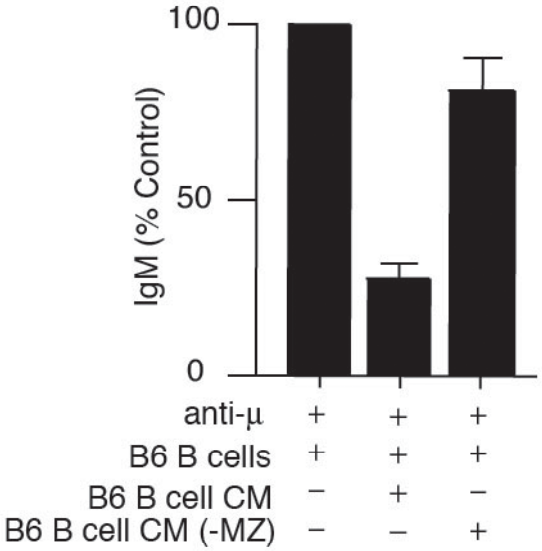
regulate humoral immune responses by an autocrine mechanism involving TGF-β because members of the TGF-β family of proteins usually act in an autocrine or paracrine manner because the biological half-life of TGF-β is very short (38). Thus, the close proximity of activated TGF-β -secreting B cells within extrafollicular sites to newly emerging, naïve B cells is critical in maintaining tolerance and preventing unnecessary activation of additional B cells. There is increasing evidence to suggest that cytokines produced by B cells are also involved in mediating T cell responses. One group has shown that Foxp3-expressing T regulatory cells proliferate in response to co-culture with resting B cells (39). Thus, B cells may provide an additional source of TGF-β, which is likely to promote regulatory T cell expansion. Additional studies have also reported that B cell co-culture can induce anergy in both $CD4^+$ and $CD8^+$ T cells (40, 41). Perhaps TGF-β derived from B cells has multiple functions in maintaining immune tolerance: first, to limit Ig production during extrafollicular responses; second, to suppress effector T cell function by inducing anergy; third, by enhancing the development and maintenance of regulatory T cells. Overall, our studies highlight the importance of B cell derived cytokines in regulating B cell effector function *in vitro*. Future studies are aimed at addressing the role of TGF-β and CD40L in regulating various elements of adaptive immunity *in vivo*, particularly extrafollicular responses.



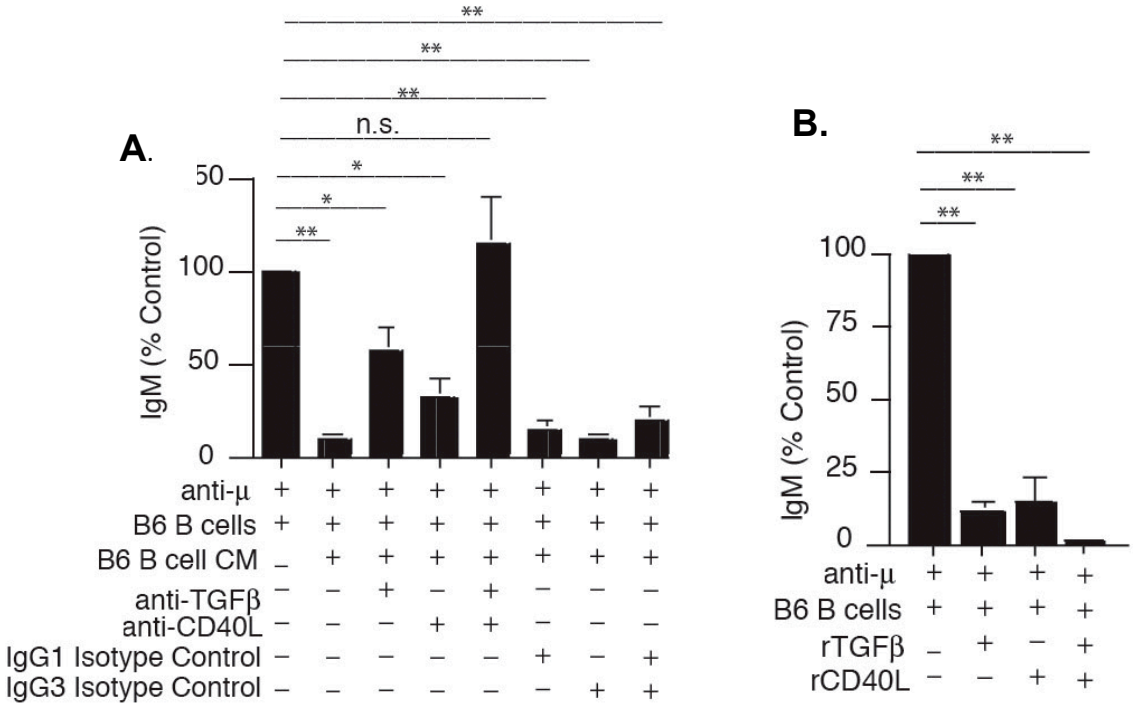
4.1 Repression of Ig by B6 B cell CM is independent of CD32. B cells were purified from B6 and $CD32^{-/-}$ mice and stimulated with anti-mu (in addition to rIL-4 and rIL-5). B cells were also cultured with CM from B cells stimulated with either anti-mu with intact Fc, or anti-mu $F(ab)_2$. IgM secretion was measured by ELISA. anti-mu stimulated controls secreted 15-31 μg/mL IgM. n=4 * $p \leq 0.05$, one sample *t* test



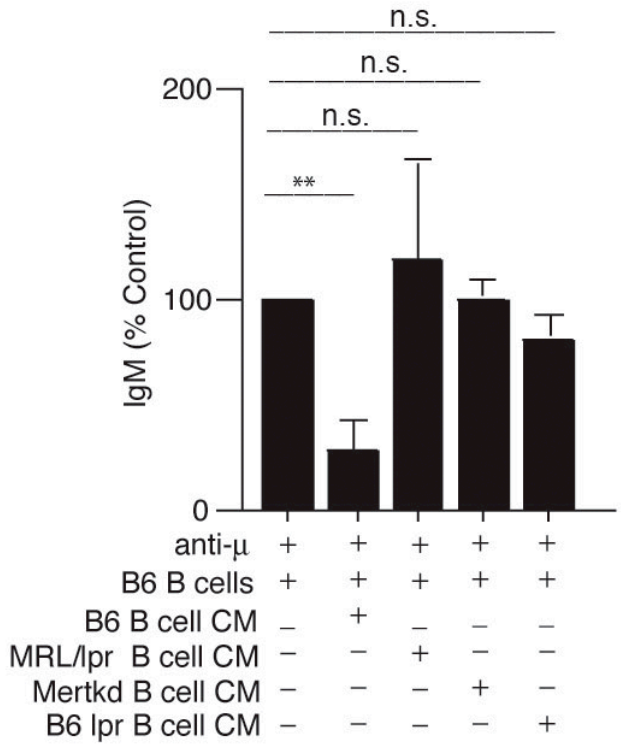
4.2 OVA-stimulated B6 B cells also secrete inhibitory factors. Purified B6 B cells were antigen stimulated with 100 μg/mL ovalbumin in the presence of OTII TCR Tg T cells. CM from these B cells were harvested and cultured with freshly isolated B6 B cells, stimulated with ovalbumin (in the presence of OTII T cells. Ovalbumin-stimulated B cells secreted an average of 1.2-2 μg/mL of IgM. n=3 * $p < 0.05$, one sample *t* test



4.3 MZ B cells secrete factors that inhibit Ig secretion. Splenocytes from B6 mice were depleted of marginal zone B cells by the addition of anti-CD9. Purified, marginal zone depleted B cells were stimulated with anti-mu, rIL-4 and rIL-5. CM were harvested seven days after culture. n=3 *$p<0.05$, n.s: not significant, one sample *t* test



4.4 Ag-stimulated B cells secrete TGF-β and CD40L (A). B6 B cell CM were neutralized with 10 μg/mL of anti-CD40L and anti-TGF-β for 1 hour at room temperature. Neutralized supernatants were added to freshly isolated B6 B cells, and cultured for 7 days, with additional anti-mu, rIL-4 and rIL-5. n=4. **(B)** B6 B cells were cultured with rCD40L (200 ng/mL) and/or rTGF-β (100 pg/mL) for 7 days with anti-mu, rIL-4, and rIL-5. Total IgM secretion was measured by ELISA. anti-mu stimulated controls secreted 15-31 μg/mL IgM. n=4 *$p<0.05$, **$p \leq 0.01$, n.s: not significant, one sample *t* test



4.5. B cells from lupus-prone mice fail to secrete repressive factors. B cells were purified from either B6, MRL/*lpr*, Mer^{tkd}, or B6.*lpr* mice and stimulated with anti- mu, rIL-4, and rIL-5 for 7 days. B6 CM were harvested and cultured with B6 B cells, in the presence of additional anti-mu, rIL-4 and rIL-5. n=4. ** $p \leq 0.01$, n.s: not significant, one sample *t* test

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CHAPTER 5

Final Discussion

5.1 Summary model

Memory B cells and long-lived plasma cells generated from germinal centers provide durable protection from infectious disease and are essential for the maintenance of human health. Plasma cells that develop in response to infection or vaccination secrete high levels of protective, high-affinity immunoglobulin (Ig) that persist for decades. Upon re-encounter with pathogen, memory B cells quickly respond and differentiate into plasma cells, which enhance the pre-existing titers of antigen-specific antibody. Although critical for host survival, the factors that govern these processes are not completely understood. It is important to understand how germinal center initiation and the differentiation of memory B cells and plasma cells are regulated, as dysregulation at any step in B cell activation can be detrimental to the health of the host.

Using both *in vitro* and *in vivo* models, we have now identified novel mechanisms that regulate B cells during T-dependent immune responses. We have found that dendritic cells sense the products of a productive immune response in the form of immune complexes (IC) and upon IC binding by Fc receptors, will secrete factors that influence critical events through the low-affinity IgG Fc receptor, CD16, and are induced to secrete the cytokine BAFF. Our studies show that DC-derived BAFF is required for Bcl-6 expression within the

germinal center, which is essential for T_{FH} cell maintenance and for the proper seeding and of the adaptive immune response. Specifically, dendritic cells can bind IgG_1-containing ICs initiation of the germinal center reaction. Our results from *in vitro* experiments indicate that the production of BAFF by DCs negatively regulates Ig production. This result may seem paradoxical at first, given that BAFF is known to promote B cell responses. We propose that BAFF serves to shut off plasma cell programming, by downregulating key transcription factors (XBP-1 and IRF-4), while promoting the formation of non Ig-secreting memory B cells. We have also identified roles for BAFF in the reactivation of memory B cells during secondary immune responses. Overall, our studies indicate that BAFF is not only essential for B cell survival and homeostasis (1), but it is also critical for key events during adaptive immunity, including memory B cell responses.

5.2 The role of additional Fc receptors in mediating adaptive immune responses

Our current studies highlight CD16 activation and BAFF production by DCs in regulating germinal center responses and memory B cell formation in response to immunization with the soluble protein antigen NP-KLH. However, additional studies are required to determine if this mechanism also promotes germinal center formation and B cell memory in response to infection. Immunization of mice with NP-KLH results in a primarily IgG_1-mediated antibody response (2). In contrast, bacterial and viral models of infection can produce antibodies of varying antigen specificities and IgG isotypes. For example, LCMV infection results in a primarily IgG2a-mediated response. It is possible that infections that give rise to antibodies of differing isotypes will highlight the role of additional Fc receptors. Preliminary studies with collaborators have demonstrated that B6 and $CD16^{-/-}$ mice mount comparable primary and secondary immune responses to LCMV infection, suggesting that

CD16 does not play a role in B cell memory responses to LCMV. Because this infection results in a primarily IgG_{2a} mediated response, and CD16 preferentially binds IgG_1, it is likely that other Fc receptors, particularly CD64, are involved. Alternatively, in the LCMV infection model, additional mechanism may exist (i.e. IFN-α production) to promote BAFF-induced memory B cell responses. Thus, future experiments are being designed to address the role of CD16 and additional Fc receptors in regulating secondary immune responses to infection.

5.3 Additional factors that induce memory B cell formation by BAFF

It must also be considered that the mechanisms by which BAFF regulates B cell memory and reactivation may require the priming of B cells by additional factors. Without additional levels of regulation, every B cell encountering BAFF would enter the memory B cell pool. Preliminary studies in our lab demonstrate that repression of B cell Ig secretion *in vitro* by BAFF requires the presence of TGF-b in culture. TGF-b has been implicated by other studies in our lab (Chapter 4) as a regulator of B cell responses *in vitro*. Perhaps the production of additional cytokines, including TGF-β may also impact BAFF secretion by antigen presenting cells. TGF-β signaling in macrophages is known to induce BAFF production (3). In addition to immune complex binding by Fc receptors, other factors may also enhance BAFF production by DCs, that overall, impact B cell responses and adaptive immunity.

5.4 Anti-BAFF therapies in the treatment of autoimmune disease

Historically, the treatment of autoimmune diseases such as SLE has been challenging because a balance must be maintained between the efficacy of immunosuppressive agents and the adverse side effects of immune suppression. Our studies have vast implications in using anti-BAFF pharmacotherapies to treat SLE and other autoimmune diseases. BAFF has been previously linked to autoimmune-related pathologies in mice and humans. Studies have demonstrated that mice over-expressing BAFF have expanded B cell populations, which contribute to spontaneous germinal center development, increased autoantibody titers, elevated numbers of effector T cells, and immunoglobulin deposition within the kidney (4). BAFF-Tg mice develop a severe autoimmune disease that is similar to SLE and Sjogren's syndrome in humans (4, 5). Thus, it is clear that BAFF overproduction in mice has a pathogenic role in autoimmunity by enhancing the survival and activation of autoreactive B cells. Additionally, increased levels of BAFF have also been detected in patients with various autoimmune conditions, and a correlation with disease progression has frequently been observed (6). It remains unclear whether the increased levels of BAFF found in human patients are a primary cause of autoimmunity or the result of increased production of pro-inflammatory cytokines (such as IFN-α), which are known to promote BAFF production (7, 8). Our studies would suggest that BAFF has a causative role in the clinical manifestations of SLE. Ongoing studies in our lab show that increased BAFF production by dendritic cells and macrophages, induced by pathogenic immune complexes, results in the migration of inflammatory cells into target organs and increased disease manifestation in lupus-prone mice. Our data reported herein would also suggest that in addition to increased B cell survival, excessive BAFF promotes the formation of autoreactive memory B cells,

reactivation of those B cells in response to self-antigen during secondary immune responses, and finally, the propagation of autoantibody production. Thus, BAFF may have multiple roles in initiating the breakdown in immune tolerance.

Within the last year, a BAFF-neutralizing monoclonal antibody, belimumab (Benlysta®), received FDA approval as the first new therapeutic to treat SLE in over 50 years (9, 10). Its success has been attributed to the depletion of self-reactive B cells, which require BAFF for survival. Depletion of B cells in Benlysta®-treated SLE patients occurs predominantly in the naïve and transitional populations, and maximum depletion is reached at 6-8 months after starting therapy (6). In light of our data, the success of anti-BAFF therapies could also be attributed to the failure to reactivate memory B cells that respond to self-antigens, as well the failure to generate additional self-reactive memory B cells. Because autoreactive B cells are chronically exposed to antigen (DNA, histones, Sm, etc.), reactivation of self-reactive memory B cells presents a challenge in the treatment of autoimmune disease. Studies have shown that memory B cell *survival* is independent of BAFF (11); however, our studies demonstrate that the *formation* and *reactivation* of memory B cells is dependent on BAFF. Our work highlighting the importance of BAFF in memory B cell formation in mice is supported by studies by Ettinger et al. demonstrating that BAFF and IL-21 can synergize to promote the reactivation of human memory B cells (12). Although memory B cells can persist following BAFF depletion, our studies herein demonstrate that their reactivation is prevented, which may contribute to amelioration of disease in SLE patients. Furthermore, the formation of additional autoreactive memory B cells will also be prevented as a consequence of anti-BAFF therapy. Because BAFF is critical for human memory B cell formation and expansion, one must also consider potential side effects of

Benlysta® therapy, such as failure to form memory in response to foreign antigens and vaccination. It is likely that any vaccinations (i.e., influenza vaccination) occurring after Benlysta® treatment has been initiated may fail to give rise to immunological memory. Future studies are needed to assess vaccine efficacy in Benlysta®-treated patients.

5.5 BAFF may improve vaccine efficacy

Most modern, successful vaccines have been developed to neutralize pathogens that possess few antigenic variations and cause acute infections, followed by long-term protective immunity (13, 14). Unfortunately, there are several instances where a primary infection does not lead to protective immunity, such as infections with highly variable pathogens, including dengue virus, influenza virus, HIV-1, and *Mycobacterium tuberculosis* (15-17). For unknown reasons, clearance of respiratory syncytial virus (RSV) infection is not followed by protective immune memory: re-infection with the same virus can occur multiple times (18). Other pathogens such as *Staphylococcus aureus* can evade the immune system and suppress the development of protective immunity in the infected host. It remains unclear why infections by these pathogens fail to yield protective immunity and why vaccines that target these pathogens have also failed. In light of our data, the failure to induce protective immunity and memory following some infections could occur if immune evasion by the pathogen results in failure to form immune complexes and promote BAFF secretion. However additional studies are required to determine if Fc binding and BAFF production modulates the immune response in these infections.

Because of its role in B cell homeostasis, germinal center maintenance, and the generation of T_{FH} cells as well as antigen-specific memory B cells, one would also

hypothesize that BAFF can be used to improve vaccine strategies. Indeed, a few studies have demonstrated a beneficial role of BAFF in enhancing vaccine protection. One specific study has shown that soluble recombinant BAFF enhanced vaccination against infectious bursal disease (IBDV) in chickens (19). The administration of soluble BAFF has also improved the efficacy of an HIV DNA vaccine in mice (20). An additional study has shown that the transient over-expression of BAFF resulted in increased antigen-specific antibody responses upon immunization with heat-killed *Pseudomonas aeruginosa* (21). Further, over-expression of BAFF also enabled immune protection following a lethal secondary challenge with *P. aeruginosa* in mice. Protective immunity was also improved when BAFF was administered four weeks after immunization, demonstrating that the temporary over-expression of BAFF can also augment an ongoing immune response following initial exposure to antigen. It is important to note that these studies used transient doses of BAFF, as long-term administration of BAFF would likely result in autoantibody production and autoimmunity. Together these data imply that BAFF may be considered as a molecular vaccine adjuvant that could support the induction of antigen-specific protective immune responses to infections in which there are currently no successful vaccine regimens. Many experimental vaccines and monoclonal antibodies directed against *Pseudomonas* have been tested in preclinical trials, but only a few have reached clinical phases and none has obtained FDA approval (22). BAFF may represent an advance towards successful vaccination programs for *Pseudomonas* infection. Interestingly, the expression of CD16 is also required for protective immunity against *P. aeruginosa* (23). Thus, both BAFF and CD16 seem to be important key factors in mediating immune responses against *Pseudomonas* infection. Based on our studies, one

would predict that by modifying BAFF levels *in vivo*, memory B cell formation during T-dependent immune responses could also be modulated to improve vaccine efficacy.

5.6 The immune system as a target organ system of toxicant action

There is increasing evidence that chronic exposure to certain toxicants at levels too low to be overtly toxic can harm human health by modulating the function of the immune system. Many compounds, including metals, pharmacological agents, and pesticides, are able to alter immune function (24). Additionally, the ability of toxicants to alter host resistance to a variety of bacterial and viral challenges suggest that there is a possible role of toxicants in increasing morbidity and mortality due to increased susceptibility to infection. This increased susceptibility may be the consequence of failure to induce immunological memory and protection following pathogen exposure. In addition, toxicant exposure may also adversely affect responses to immunization and the ability to initiate secondary immune responses. Several compounds have been shown to inhibit T-dependent secondary immune responses, including tetraethyl lead, methyl mercury, aflatoxin B1, and 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) (24). Although these toxic effects on secondary immunity have been observed, the mechanisms of action are still relatively undefined. Lead has been shown to inhibit antibody responses by impairing T cell function, similar to the effects of both dioxin and aflatoxin (25-27). Perhaps lead and toxicants with similar mechanisms of action impair recall responses indirectly by dampening overall antibody production. Lack of Ig secretion would result in decreased immune complex formation and according to our studies, a deficit in DC-derived BAFF production. Thus these toxicants may disrupt secondary immune responses by inhibiting IC-induced BAFF production. A single study has also implicated lead in downregulating the expression of

CD16 (28). This study in conjunction with our data implies that lead may exert its immunotoxic effects by limiting IC binding by CD16 and subsequent BAFF production.

Because of the effects of lead, aflatoxin, and other toxicants on T cell function, one would also predict an additional role in modulating other events in the adaptive immune response, including germinal center initiation, B and T cell memory generation, and isotype switching. More recent studies have further documented the role of toxicant exposure in the impairment of secondary immunity and have begun to elucidate the mechanism by which this impairment occurs. Some particular chemicals have generated renewed interest in their immunotoxic potential, including lead, dioxin, and fluorinated compounds. A few recent studies have demonstrated that dioxin exposure suppresses the formation of germinal centers induced by NP-CGG immunization, as well as the high-affinity antibody response (29). The authors assert that the suppression of germinal center formation was most likely due to the inhibition of B and T cell proliferation (29). Perhaps TCDD exposure also results in reduced BAFF levels, which, according to our data, could also explain the defect in germinal center formation observed in our studies. Subsequent studies by Lawrence et al. have demonstrated that TCDD exposure also impacts long-term immune memory. Specifically, the authors showed that activation of the aryl hydrocarbon (Ah) receptor by TCDD results in a diminished secondary immune response to influenza infection (30). The authors attribute this defect to the failure to generate functional $CD8^+$ memory T cells. Alternatively, the effects of dioxin exposure on secondary immune responses could also reflect the impairment of the DC's ability to bind immune complexes and secrete BAFF. Perfluorinated compounds (PFCs), such as PFOA and PFOS, can also impact secondary immune responses. Recently, the effects of PFC exposure on T-dependent immunity and responses to vaccination have

generated much interest (31). Ongoing studies are necessary to address additional roles of environmental toxicants in modulating immune responses.

Many environmental toxicants that affect the immune system in humans are immunosuppressive; however, other chemicals can be pro-inflammatory and contribute to autoimmunity. Although genetic predisposition may be a contributing factor for the development of spontaneous autoimmune disease, the incomplete concordance in SLE in twin studies would suggest that exogenous factors are also important in disease development (32, 33). There are a number of chemicals that clearly impact the immune system and can contribute to the exacerbation of autoimmune disease, including organic solvents, such as trichloroethylene (TCE), mercury, vinyl chloride, organic solvents, silica, and ultrafine particles (34). Many epidemiological reports and case studies have found that chronic, low-level exposure to the organic solvent TCE is linked to various autoimmune diseases including SLE, scleroderma, bullous pemphigoid, diabetes, and immune-mediated hepatitis (35-40). Even if overt disease is not diagnosed, signs of immune activation are evident, including increased T cell numbers and elevated levels of serum autoantibody (41, 42). In addition to human studies, mice chronically exposed to TCE showed increased incidence of autoimmune hepatitis (43) and increased production of antinuclear antibodies, indicative of lupus (44). Although alterations in T cell function have been implicated in the mode of action, the mechanisms by which these chemicals induce autoimmunity remain unclear. Perhaps toxicant exposure can modulate BAFF expression and contribute to pathologies found in SLE patients. Future studies are required to address the use of increased serum levels of BAFF as a biomarker of toxicant exposure, potentially leading to the onset of the breakdown of immune tolerance subsequent autoimmunity.

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