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Regulation of B cell Receptor Signaling: B cell activation through complement and the role of protein tyrosine phosphatase Shp2

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

Ravi V. Kolla

Committee in charge:

Professor Michael David, Chair
Professor Ananda Goldrath
Professor Robert C. Rickert
Professor Bartholomew M. Sefton
Professor Gregg Silverman

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Chair

University of California, San Diego

2006

DEDICATION

"…the end of education is character"
-Sathya Sai Baba

To my loving mother and father: thank you for being there throughout all the difficult times; I am truly the luckiest person in the world to have been blessed with parents as loving, supportive, and altruistic as the both of you. I hope for nothing else in this world but to make you proud. I love you both.

I would also like to thank the many friends who both endured my lowest moments and shared in my modest successes. Aristotle says that true friendship is among the most rare of precious gems; by these measures, I am among the most wealthy of men. My thanks and gratitude to the all of you in supporting me through this chapter of my life.

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ABSTRACT OF THE DISSERTATION

Regulation of B cell Receptor Signaling: B cell activation through complement and the role of protein tyrosine phosphatase Shp2

by

Ravi V. Kolla

Doctor of Philosophy in Biology

University of California, San Diego

Professor Michael David, Chair

My thesis is focused upon mechanisms regulating B cell receptor (BCR) activation and regulation. There are two branches of the immune system, the innate and adaptive. Proper cross-talk between the innate and adaptive immune system is crucial in initiating proper and effective immune responses and preventing inappropriate immune reactions such as autoimmunity and lymphoma.

Arguably the most crucial link between B cell function and the innate immune system is through the complement receptor CD21 (Cr2). CD21 is the receptor for the complement breakdown product C3d. Cross-linking of antigen with C3d has been shown to augment antigen specific antibody titers far above normal immune responses. In chapter 1, I have shown that conjugation of

protective antigen (PA) of anthrax with C3d produces 3-fold greater antibody titers as compared to PA in alum – the vaccine currently being developed for human administration. These antigen-specific antibodies are produced very rapidly during the primary response, are of all IgG isotypes, and are sustained long after primary immunization. Finally, I see that C3d may augment this response by enhancing the efficiency of germinal center formation and plasma cell differentiation.

In chapter 2, I focus upon the mechanism of CD21 function on B cells and follicular dendritic cells (FDCs). By immunizing WT and $CD21^{-/-}$ mice, I found that CD21 is important in initiating proper B cell function during the primary immune response but had little role in eliciting secondary or memory immune responses. I further examine CD21 function by examining the mechanisms by which CD21 functions on the surface of B cells. By using deconvolution microscopy and immunoprecipitation, I lend support to the sequestration model of CD21 function. This model implicates CD21 is an inhibitory receptor on B cells which actively sequesters CD19, the cardinal member of the BCR co-receptor complex.

Finally, in chapter 3, I look downstream of surface receptor regulation of BCR function and look at the role of the protein tyrosine phosphatase, Shp2, in B cell development, signaling and function. By using Cre/lox-p technology, I create a B cell specific deletion of Shp2 and show that Shp2 may be an important regulator of marginal zone versus follicular B cell development and is a crucial regulator in the GC reaction.

GENERAL INTRODUCTION

The immune system has two branches: the innate and the adaptive. Both branches collaborate in protecting an individual by recognizing pathogens, clearing these pathogens, and protecting the individual from future exposures. Coordination between the two arms is extremely intricate and still enigmatic. It is known however that a fine balance is necessary to maintain a healthy immune system - inappropriate activation or function of either branch of the immune system can lead to immunodeficiency, hypersensitivity, autoimmunity, leukemia and/or lymphoma.

The Innate Immune System

The Innate Immune System is essential in providing immediate, short-term protection against pathogen challenge. By definition, the innate branch of the immune system is incapable of memory – memory provides continued or prolonged protection from infection by specific pathogens. The innate immune system is generally activated in response to inflammation caused either by cellular damage, physical injury or through recognition of various microbes. Cells of the innate immune system include macrophages, dendritic cells, natural killer cells, and granulocytes. These leukocytes recognize repetitive motifs such as bacterial polysaccharides, carbohydrates, methylated-DNA or amino acids through evolutionarily conserved receptors known as Toll-like receptors or TLR's.

Alternatively, leukocytes may recognize foreign pathogens to which Ig is bound through Fcγ or Fcε family of receptors. These receptors recognize constant or Fc-portions of immunoglobulin-structured proteins secreted by B cells. The immunoglobulin (Ig) molecules secreted by B cells are also called antibodies and these molecules recognize a wide variety of proteins, nucleic acids, carbohydrates, or lipids. B cells are the key cell type involved in humoral adaptive immunity which will be discussed in further later in the text.

Specialized subsets of B cells secrete antibodies that are generally of low-affinity and varied specificity for common bacterial or mitogenic patterns. The Fc portion of the antibody will bind to Fcγ receptors (FcγR) only when antibodies are bound to an antigen; antibodies bound to antigen facilitates formation of immune complexes. Immune complexes will bind to leukocytes via the FcγR and TLRs which results in activation, phagocytosis, and degradation of antigens to allow further activation of the adaptive immune response. These innate antibodies are crucial to innate immune cell recognition.

Activation of leukocytes plays three general functions: pathogen clearance, inflammation, and presentation of antigens to cells of the adaptive immune response. Pathogen clearance entails either secretion of lytic particles (such as granzyme) or phagocytosis and consequent endosomal degradation of either foreign bodies or infected host-cells. Lysis of pathogens or pathogen infected cells will often lead to cytokine release causing further

inflammation triggering further leukocyte infiltration and activation (2). This process will continue until microbes and cellular debris are cleared and the release of inflammatory cytokines are ceased.

Dendritic cells and macrophages are in great part responsible for antigen presentation from sites of infection. These cells will take degraded peptides processed after phagocytosis, and migrate via the lymphatics or blood to lymphoid organs where peptides are presented to cells of the adaptive immune branch. Antigen presentation is mediated by the interaction of a diverse group of receptors called the major histobility type II (MHCII) receptors with an even more diverse group of receptors collectively called T cell receptors (TCR's). Binding of the TCR to peptide-bound MHC molecules results in the activation of T cells This process is essential for maintaining the immune responses and inducing long-lived memory responses which are provided solely by the adaptive arm of the immune system.

The other major component of the innate immune system is known as complement. Complement is a group of over 30 soluble proteins and receptors which are found as far back in evolution as invertebrates (3). Complement is canonically serum and cell surface components which when activated, aggregate to form the membrane attack complex (MAC) which will multiplex on a cell surface to form a lytic pore.

The Complement Pathway

The complement pathway (C') is highly regulated in mammalian organisms. There are three primary modes of C' mobilization or activation: 1. the classical pathway (CP), 2. the alternative pathway (AP), and 3. the lectin or MBP pathway (figure 1).

The Classical Pathway of Complement Activation

The classical pathway (CP) is initiated by the binding of the C1 complex to immune-complexes or closely aggregated antibodies (1). The C1 complex is composed of C1q – a complex multimeric molecule consisting of 3 distinct polypeptide chains – C1r and C1s. Multiple C1q molecules must bind to aggregated Fc portions of antibodies in order to allow docking of C1r and C1s.

Not all antibodies are capable of fixing C1q. In mice, only 4 of the 5 isotypes are capable of fixing C1q: IgM, IgG3, IgG2a and IgG2b, however not IgG1. In humans, C1q fixing isotypes are: IgM, IgG3, IgG1, and IgG2 (1). (Further discussion of B cells and sub-sets of B cells responsible for complement fixing antibodies will be discussed in later in the text.) IgM is unique among the soluble Ig in that IgM is secreted as a pentameric complex that efficiently fixes C1q only when bound to antigen. Conformational changes of IgM occur after antigen binding to expose the Fc portion of the antibody. IgG's are secreted as either a monomer or dimer and will only fix C1q when closely aggregated on a pathogen surface (1, 4).

C1q initiation of the C' cascade in response to IgM- or IgG- immune complexes (IC) leads to enzymatic cleavage of C4 and C2, which form the C3 convertase. C3-convertase initiates cleavage of C3 into C3a and C3b. C3b binds to C3-convertase and creates the C5 convertase that cleaves C5 into C5a and C5b. C5 is both the last component of the CP and first member of the MAC (1). Activation of the CP leads to either lysis of cellular organisms or precipitation of the IC from the blood and filtration and excretion of the IC by the kidneys (5).

Recent studies have also demonstrated additional roles for C1q. For example, C1q has been shown to bind directly to apoptotic cell surfaces and cell blebs via its globular head structure(1, 6, 7). Such antibody-independent function would allow for more efficient MAC formation and leukocytic infiltration to clear cellular debris which would in turn prevent exposure of lymphocytes to intracellular components which potentially can cause autoimmunity. C1q deficient mice and humans show a propensity to develop autoimmune disorders such as SLE (5). Other studies have recently shown C1q can complex at the B cell surface when IgM positive B cells recognize highly-valent antigens (8). Though these studies potentially reveal new roles, additive function, and novel regulation of CP activation, the relevance of C1q binding to surface IgM has yet to be clearly demonstrated.

The Alternative Pathway of Complement Activation

The alternative pathway (AP) of C' activation is antibody independent and functions to rapidly mark and destroy invading micro-organisms. C3

initiates the AP and utilizes three unique AP associated proteins: factor B (fB), factor D (fD) and properdin (1). These unique C3 associating proteins complex to create a different but functionally identical C5 convertase that specifically targets C5 deposition at C3b bound surfaces which, as with the CP, initates the MAC (1).

The requirement for specific targeting of the AP requires C3b binding to a pathogen or cell surface. The covalent attachment of C3b to cell surfaces is arguably the most crucial event in C' mediated innate immunity as well as a key event in the activation of the adaptive immune system. Initiation of C3 cleavage in the AP occurs by a process referred to as *tickover* that is the spontaneous cleavage of serum C3 and random deposition of C3b on surfaces both self and foreign. However non-specific C3b deposition on both "self" and "non-self" cells alike represents a significant potential problem in autoimmunity and tolerance. Several mechanisms regulate this process. First, C3b deposed on self cells is inactivated by factor I, which irreversibly cleaves C3b into iC3b, preventing formation of the C5 convertase (1). Factor I cleavage requires another protein, Factor H, as a co-factor (1).

In mice, Crry is another regulatory protein that is secreted into the serum. Crry has a high affinity for soluble C3b and prevents deposition of C3b on cell surfaces (1). Interestingly, Crry knockout mice have various autoimmune phenotypes and also suffer from infertility due to C3b deposition on the placental surface, which provokes leukocytic infiltration and destruction

of the placenta and neonates (9). Humans lack Crry but utilize other common C3 inhibitors such as DAF, CD59 and MCP that have also been shown to prevent autoimmunity, transplant rejection and miscarriage in mice (1, 9, 10). Figure 2 represents a summary of complement inhibitors in the alternative and classical pathways.

Complement proteins outside of the MAC

C' proteins have functions other than formation of the MAC; C' break-down products and associating factors have been shown necessary for proper activation of the adaptive immune response (11-13). The first studies in identifying the role of C' in humoral immunity were done by transient depletion of C3 levels in mice with cobra venom factor (CVF). Mice treated with CVF revealed a markedly impaired antibody response (14). The importance of complement in humoral immune response was later corroborated by characterization of knock-out mice deficient in C1q, C3 and C4. These mice share similar defects in fending-off bacterial infections, poor to no formation of immune complexes, and have impaired humoral responses. Furthermore, C1q and C4 deficient-mice show amazing propensities to develop autoimmunity such as lupus associated with an inability to clear apoptotic cellular debris (4, 6, 7, 15).

Table 1: Review of C' (1, 5, 16-21)

	Function	Inhibitors	KO phenotype
C1q	Initiates CP through binding of IgM	C1inh	Spontaneous B cell mediated autoimmunity and glomerulonephritis.
C3	Necessary of creating C5 convertase. Essential for AP and CP. Breakdown products augment adaptive immune responses.	CVF, Crry, Factor H, Factor I, sCR1,	Increased susceptibilities to various bacterial infections. Decreased T dependent humoral responses.
C4	Involved in CP and MBL pathway.	CVF	Defective clearance of bacterial infection, some instances of autoimmunity.
C5	Initiiates MAC, breakdown products promote leukocytic infiltration	NA	High susceptibilities to various pathogens, Inability to efficiently activate adaptive immune responses
Factor B	Necessary for C3 cleavage to form C3b	mAB	Susceptibility to various pathogens
Daf (CD55)	Dissociates C3 & C5 convertases from cell surfaces, prevents C5a binding to CD97	NA	KO not available
MCP (CD46)	Expressed on many cell types, most notably on maternal side of placenta	NA	Spontaneous abortion of pups
Crry	Soluble inhibitor of C3b deposition onto cell surfaces	NA	Spontaneous abortion of pups, disposition to SLE like symptoms
CR1/CR2	Expressed on B and FDCs receptor for C3b, C3d, C4b, promotes activation of humoral responses	Crry, 7G6 mAb	Decreased humoral response, decreased B1a cells, predisposition to SLE
C5aR	Widely expressed on leukocytes, binds C5a. Promotes CD8 T cell activation	NA	Decrease in T cell activation and susceptibility to various pathogenic infection.
C4bp	Prevents function of C3 convertase by inhibiting binding to cell surfaces, cofactor for Factor I,, putative role in survival via CD40 signaling	NA	KO not available. Various pathogens utilize C4bp to prevent recognition by C' system.

C4-null mice also show a role for C4 in the establishment of central tolerance via unclear mechanisms (16, 22). Fischer *et al* provided further evidence of the importance of complement activation in the adaptive response by showing C3 produced locally by bone-marrow derived macrophages was more important in augmentation of the humoral response than serum derived C3 (23).

The discovery of C' receptors on macrophages, dendritic cells, B cells, and follicular dendritic cells provided even further evidence for the necessity of C' in activation of the humoral response. Complement receptor deficient mice show similar phenotypes as observed from C3, C4, C5, and C1q knockouts, most notably increased susceptibility to various bacterial and parasitic infections as well as marked decreases in T dependent immune responses (16). Complement receptors CD35, CD21, CD11b, CD11c, FDC-M1 and FDC-M2 play major roles in not only facilitating B cell responses but in maintaining or promoting follicular structure in the spleen so as to allow efficient activation of the appropriate B and T cells (5, 11, 12, 16, 22, 24).

The Adaptive Immune Response

The adaptive immune response is responsible for providing both pathogen-specific and long-lived immunologic (memory) responses. The adaptive response is further divided into two parts: cellular (T cell mediated) and humoral (B cell mediated) immunity; both B and T cells are collectively called lymphocytes. Lymphocytes develop primarily in the bone marrow and

thymus from hematopoietic stem cell precursors and undergo a unique series of DNA rearrangements of the variable V, D and J segments of the TCR. Successful rearrangement and selection of a self-tolerant cell provide each with an antigen-specific surface receptor able to recognize a single foreign epitope or antigen. The DNA rearrangement of each lymphocyte is unique such that collectively, B and T cells are able to recognize a nearly limitless array of antigens. Antigen recognition by the T or B cell receptor leads to clonal expansion and differentiation of the responding cell, which eventually leads to the pathogen specific and long-lived memory response of the adaptive immune system.

As mentioned briefly above, T cells utilize their diverse repertoire of T cell receptors to bind peptide loaded MHC class I or class II molecules. There are two primary types of T cells: cytotoxic T cells (T_c) which function primarily in providing viral immunity, and helper T cells (T_h) which function in promoting Tc cell, Th cell and B cell activation through the release of cytokines and expression of activation molecules.

Also mentioned above, the primary function of the B cell is in the secretion of antibodies which will neutralize antigens in one of several ways: by the initiation of antibody dependent cell-mediated cytotoxicity (ADCC), activation of the CP, marking of pathogens to phagocytic or immune surveillance cells, or in precipitation of antigen from the blood to be filtered by the kidneys. There is considerable dependence of the humoral and cellular

immune responses on one another. Proper interaction between the two is not only for efficient adaptive immune responses but in also preventing inappropriate immune responses such as autoimmunity and lymphoma.

B cell development and the B cell Immune Response

In order to fully understand how B cells function in the immune response, one must understand the complicated array of signals which govern their development and subsequent function. B cells develop in the bone marrow of adult mice. The development of B cells from hematopoietic progenitors occurs in stages defined by the expression of distinct surface markers, the expression of regulatory genes and the functional rearrangement of heavy and light chain V, D, and J variable genes (25). A summary of B cell development in the bone marrow is represented in figure 3.

The first B cell progenitor is termed a pro-B cell. These cells are characterized by the expression of B220, AA4.1 and CD43. These cells are dependent on signals from the cytokine IL7, c-kit, and interactions with the bone marrow stroma to cycle and proliferate (26, 27). As pro-B cells develop, expression of CD19, CD79a (Igα) and CD79b (Igβ) is observed. Pro-B cells also begin to express Rag 1 & 2 (recombinase activating genes) and terminal deoxynucleotidyl transferase (TdT) (28). The Rag proteins are responsible for nicking DNA at RSS sequences upstream of V, D, and J heavy and light chain genes. TdT adds nucleotides at V(D)J joints to increase diversity of the antibody repetoire (29). During pro-B cell differentiation, rearrangement of the

$D_H \rightarrow J_H$ heavy chain genes begins and by the end of pro-B cell development, $V_H \rightarrow (D_H)J_H$ is completed (29).

Pre-B cells have a rearranged heavy chain protein product expressed on their surface which is stabilized by the surrogate light chain (SLC) (30). This is termed the pre-B cell receptor. It associates with surface Igα, Igβ and CD19 to make a functional signaling complex which is important in signaling allelic exclusion that prevents further heavy chain V(D)J rearrangements as well as giving proliferative signals to the B cell. Pre-B cells which have turned off heavy chain rearrangement, and cycled or proliferated correctly, then begin rearrangement of light chain locus genes through Rag-mediated V-J of either the kappa or lambda locus (29).

Checkpoints controlling the production of functional B cells first occur at the pre-B cell stage. Non-functional pairing of heavy chain with SLC or inappropriate proliferative signals mediated by CD19 and Igα/β both lead to apoptosis of the pre B cell (31, 32).

Successful rearrangement of light chain genes and successful pairing of the light chain product with surface heavy chain characterize the next stage of bone marrow development called the immature B cell. Immature B cells express IgM, which is the product of successful heavy and light chain products with the u sub-class constant region. The heavy and light chain pair is also called the B cell receptor (BCR). The BCR constitutively associates with Igα/β receptors and CD19 (33). Unsuccessful pairing of the heavy and light chain

products results in down-regulation of the light chain product and in a process called receptor editing, a new light chain product is produced and paired to the existing heavy chain product (34, 35). Cells unable to create a signal transducing B cell receptor will undergo apoptosis (36).

Immature B cells also become sensitive to negative selection, a process in which self-reactive B cells are prevented from exiting the bone marrow (35, 37). B cells which are auto-reactive, or non-tolerant, are signaled to undergo receptor editing. If they are not self-tolerant, the B cell will undergo apoptosis (38). The cell types and processes governing receptor editing is a widely studied area however it is clear that B cell negative selection is antigen and BCR dependent (37, 39, 40).

Peripheral B cell Subsets

Immature B cells travel to the spleen through the blood to continue their development into naïve mature B cells. Immature B cells upregulate IgM after emigration into the spleen and become transitional type 1 (T1) cells. These cells are characteristically high for IgM and HSA, intermediate or positive for 493 and B220 and low or negative for IgD, CD21, CD23 AA4.1 and CD5 (39, 41, 42). A summary of peripheral B cell subsets and their characteristic markers is shown in figure 4 (41).

T1 cells begin to upregulate IgD is they differentiate into transitional type 2 (T2) cells. T2 cells have high IgM and IgD, begin to reduce expression of 493 and HAS, and also express complement receptor CD21 and the Fcε

receptor CD23. Recent work has shown that T2 cells may also include cells undergoing some sort of further negative selection (31, 43-45). Much of this work has been done with the ARS transgenic model. In these mice, auto-specific T2 cells down regulate surface Ig and become anergic or non-responsive. These anergic cells have subsequently been called Transitional 3 (T3) cells (46, 47).

T2 cells that do not undergo further negative selection, will finally mature into follicular B cells (FOB), also called naïve mature B cells or B2 cells. These cells are characterized by being IgM intermediate, IgD high, CD21 and CD23 positive or intermediate. These cells are the dominant type of B cells in the B cell zone of the follicle directly surrounding the T cell zone (48) and function primarily in T cell dependent (TD) immune responses. The TD response depends on activation of T_H cells to upregulate markers such as CD40, B7 family members, ICOS and cytokines such as IL4 which lead to antigen specific B cell activation and differentiation.

FOB cells hone to signals produced by follicular dendritic cells (FDCs), most notably the chemokine B lymphocyte chemoattractant (BLC*)* which maintains B cells in the proper follicular location (49, 50). Recent work by the Browning lab has shown that FDCs may not be as important as previously thought because ablation of these cells through administration of excess lymphotoxin Beta (Ltβ) did not significantly alter localization of B cells in the follicle nor formation of activation dependent germinal center structures (51-

55). The importance of FDCs in the germinal center immune response will be discussed further in the later sections.

Alternative B cell Subsets

Two types of B cells are thought to function primarily in creation of natural antibodies involved with innate immune responses: marginal zone B cells and B-1 cells. Marginal zone B cells (MZB) are found in a ring surrounding the marginal sinus. They characteristically express high levels of IgM and CD21, intermediate levels of HSA, low levels of IgD, and are CD23 negative. Recent work has shown that they are derived from T2 cells via an AA4.1 positive progenitor (56).

Marginal zone cells typically express IgM which are low-affinity but specific for bacterial or innate immune antigens (57). Their position outside the marginal sinus makes them the first to screen for blood borne pathogens. Upon ligation of the BCR, these cells will move through the marginal sinus into the follicle where they may begin IgM secretion near the central arteriole or in extra-follicular regions as determined by SDF secreting cells. They may also abet the activation of other B, T and FDC cells in initiation of the germinal center (57-59).

B1 cells primarily reside in the peritoneum however they are also present in the spleen. They characteristically express high levels of IgM, intermediate levels of CD11b, CD21 and CD43, and are low or negative for IgD, CD23 and 493. There are two types of B1 cells – B1a and B1b. CD5 is

the distinguishing marker present on B1a type B1 cells. B1a and B1b cells can also be differentiated by the rearrangements of Vh and Vk expressed. B1 cells, like MZBs, have been shown to have specific specificities for bacterial-type antigens utilizing specific rearrangements of V_H and $V\kappa$ genes; they carry low-affinity IgM for antigens such as phosphatidyl choline (PtC) which is a lipid present in many bacterial cell walls (60-63).

The origin of B1 cells is intensely debated however evidence shows that they are initially derived from fetal-liver precursors and are slow-cycling (or rather slow to undergo cell division) and long-lived (64, 65). However new evidence shows B1 precursors may also reside in the bone marrow and can be characterized by expression of CD138 or Syn1 (66). Knockout mice lacking CD19, CD21, Vav, Btk, or PI3K show significant reductions in one or both subsets of B1 cells often also have impaired TD and TI immune responses indicating that B1 cells are important mediators in humoral immunity (15, 67-72).

B cell Antigens

B cell antigens fall into three categories: Thymus-independent type 1 (TI-1), Thymus-independent type 2 (TI-2) and Thymus-dependent (TD) antigens. TI-1 antigens induce polyclonal activation of B cells independent of antigen specificity. These B cell mitogens most often function by ligation of TLRs (29, 73). TI-1 antigens will not normally induce class switch or initiate the germinal center reaction (74), (29, 75). TI-2 antigens are characteristically

repetitive sequences or patterns and have low BCR affinity; B1 and MZ B cells have BCR rearrangements which are specific to TI-2 type antigens (74, 76-80). These antigens promote formation of antigen-specific IgM secreting cells (plasma cells) and can induce class switch to make IgG plasma cells independent of germinal center formation. TD antigens require T cell priming and will promote antigen specific B cells through the germinal center (figure 6).

The Germinal Center and Plasma B cells

The function of the germinal center (GC) is to produce higher affinity antibodies for neutralization of antigens. The germinal center reaction initiates activation of T cells, B cells, and FDCs. The germinal center forms at the edge between the B and T cell zone in-between the FDC network (see figure 6). The initiation of the GC requires antigen-activated T cells to release cytokines such as IL4 and express markers such as CD40L and B7 family members which all serve in activation of B cells (81-83). FOB cells migrate to the B/T border inside of the FDC network and become activated independent of antigen specificity to downregulate their IgM and undergo somatic hypermutation (SHM). SHM is the process in which B cells undergo further Ig recombination events and class switch to generate higher specificity IgG, IgA or IgE (84-86). SHM occurs within the dark zone of the GC. B cells that have finished SHM upregulate their modified surface Ig and move toward the FDC network or the light zone (48, 52, 84, 86).

Isotype switch (a.k.a class switch recombination) also occurs within the GC. The isotype to which the BCR will switch is dictated by both the type of antigen and the nature of the cytokines released during the immune response (87). As touched upon previously, various antibody subclasses have different functional properties based primarily on their structure. For example, IgM and IgG3 fix C' efficiently whereas other isotypes do not. The mechanism of class switch recombination is not well understood however recent work by Omori et al has implicated PI3K in the regulation of AID function (manuscript under review). AID is a nuclear protein that is necessary for isotype switch through a heavily debated RNA or DNA editing mechanism (88, 89).

FDC's are presumed to mediate negative selection within the GC; activated FDCs express novel receptors such as FDC-M1 and FDC-M2 that bind C4 breakdown products (1). Negative selection is important after SHM because inability to delete B cells which carry deleterious or auto-specific BCRs may cause development of various types of autoimmunity or lymphoma (90-92). The role of FDCs in the GC has become obfuscated by recent data showing that ablation of FDCs does not abrogate the GC response, class switch, or subsequent antibody responses (55, 93). However no evidence has been found to show FDCs do not play a role in the GC or to show FDC function outside of the GC. Furthermore, no alternative cell-types or mechanisms have been proposed to explain how negative selection may be mediated in the GC reaction in the absence of FDCs.

The high affinity antibodies generated by SHM and isotype switch in the GC are secreted by plasma cells. Plasma cells are B220 low, surface Ig low, Syn1 high immunoglobulin-secreting cells. Differentiation into plasma cells requires expression of the transcription factor Blimp1 and downregulation of Pax5 (94-96). Plasma cells will exit the follicle through the poorly understood bridging channel in response to SDF. Plasma cells form foci outside of the follicle and secrete large quantities of specific antibody until they eventually die (49, 97-99). Differentiation into plasma cells is thought to be through a fate decision in which unknown signals induce GC B cells to change into plasma cells rather than memory B cells (97, 99-102). Recent data shows that concentration of antigen or antigen recognition strength regulates plasma cell development (102).

Not much is known about memory B cells except they are characteristically surface Ig positive and are long lived. These mature recirculating B cells will often hone back to the bone marrow and continue to recirculate where they can quickly differentiate into plasma cells upon re-introduction of antigen and therefore promote rapid secondary (memory) responses (101, 103-107).

B cell activation by complement

B1 cells and marginal zone B cells are primarily responsible for the natural antibody which activates the CP of C' which as previously discussed, abets TD responses. However activation of C' can directly activate B cells

through C'-receptors and these interactions are important in TI and TD immune responses.

The primary C' receptors on B cells are CD21 and CD35, collectively called CR2. In mice, these receptors are splice variants of the same gene but in humans these proteins are encoded by separate genes (1, 108, 109). In both mice and humans, CR2 is expressed on both FDCs and B cells however humans have been shown to additionally express CD35 on red blood cells and a small subset of T cells.

CR2 is expressed late in B cell development on T2 cells. It is expressed most highly on marginal zone B cells and it associates with CD19, CD81 and the BCR on the B cell surface. The CD19-CD21-CD81 co-receptor complex lowers the threshold of B cell activation through the BCR and provides an important survival function. The signaling events mediated by the co-receptor complex will be discussed later in the text. Three different labs have generated CR2 knockout mice. All labs have reported a decrease in TD humoral responses and defects in B cell survival however one group also showed a selective decrease in B1a cells (11, 15, 110-112). How CD21 affects the development of these cells is still not clear.

Both of the CR2 splice variants are differentially expressed on B cells and FDCs: B cells express predominantly CD21 whereas FDCs express predominantly CD35. The structural differences between CD21 (145kD) and CD35 (190kD) are few; CD35 consists of 24-25 short consensus repeat (SCR)

domains while CD21 consists of 15-16 SCR domains and both proteins share the same 35 amino acid cytoplasmic tail and transmembrane domains. The splicing event that dictates production CD21 over CD35 in mice is between exon 1 and exon 6 to create CD21 (1, 113, 114). The underlying mechanisms that dictate production of CD21 and CD35 are not completely clear however Oct-1 and Oct-2 are known to be involved (115, 116).

Though these splice variants are structurally similar, they have different affinities for complement C3 breakdown products. Both receptors bind breakdown products of C3 but CD35 also binds C4 breakdown products. The order of affinity of CD35 ligands are C3b > C4b > iC3b > C3d. CD21 only binds C3d > C3dg with high affinity (1, 15, 117).

Ligation of C3b to CD35 has several functions. CD35 is first a cofactor that allows C3b cleavage by membrane bound factor B, factor D, factor I and factor H. On FDCs, ligation of CD35 to C3b coated antigen or C3b containing immune complexes leads to retention of the IC on the FDC surface which presumably promotes activation of B cells (12, 118). Lastly, CD35 promotion of factor B and D function in C3b cleavage creates C3dg and C3d which will bind CD21 with high affinity to further activate B cells through direct binding of C3d labeled antigen or C3d containing IC.

The function of the C3d-CD21 interaction became appreciated after Dempsey *et al* reported that conjugation of Hen Egg Lysozyme (HEL) to trimeric-copies of C3d led to a 10,000 fold increase in specific IgG immune

response. They showed that this augmentation was caused in part by lowering of the threshold for Ca^{2+} activation induced by BCR engagement (72, 119). The role of C3d in promoting B cell immune response and BCR signaling is the major focus of the research discussed in chapters 1 and 2 of this dissertation. The role of CD21 in maintaining tolerance has also become an interesting issue. CR2 KO mice show a propensity to develop SLE-like symptoms. Additionally human lupus patients often have a point mutation in the CD21 locus which changes the glycosylation of CD21 and abrogates binding of C3d (120, 121). Recent work has also shown that inappropriate deposition on C3d can cause collagen-induced arthritis in mice (122). One recent report has shown a role of C3d in promoting humoral immunity independent of CR2 however this is controversial because functional studies have shown that CD21 is required for C3d-mediated protection from streptococcus pneumoniae infection (123). The use of the C3d-CD21 interaction as a natural adjuvant in augmenting the B cell response has been a major focus of several labs during the past decade. Chapter 1 of this dissertation utilizes C3d as an adjuvant for augmenting humoral responses against protective antigen of anthrax and further examines functional applications of C3d as compared to the widely used, and FDA approved adjuvant, alum.

B cell Signaling

Ligation of the BCR by antigen causes a signaling cascade which determines B cell fate and function. CD79a/b (Igα/β) are required players in BCR-mediated signaling. CD79a/b heterodimerize and are constitutively associated with the surface BCR (124). These receptors have multiple ITAMs that become phosphorylated mediated by a protein tyrosine kinase, possibly Lyn (124-126). However the BCR co-receptor complex is also a major player in BCR signaling.

As mentioned above, the co-receptor complex is composed of CD19, CD21 and CD81. BCR proximal signal transduction is summarized in figure 7. Two of the first events in BCR signal transduction are the activation of Lyn and Syk. Both kinases act in phosphorylation of many downstream targets but Lyn activation specifically phosphorylates tyrosines on CD19, CD32, and CD22. Lyn phosphorylation of CD19 leads to a deluge of signaling events that subsequently result in B cell proliferation and differentiation (127, 128)

CD19 in B cell signaling

CD19 is a 95kD transmembrane protein that is expressed very early in development and is cardinal for proper maturation and function of the B cell. It is made up of 2 extracellular Ig-like domains and a very long cytpolasmic tail, which contains 9 tyrosines that become phosphorylated upon ligation of the B cell receptor (BCR) by antigen (67, 129). CD19 knockout mice show a variety of severe phenoyptes, most notably: decreased B cell numbers, lack of marginal zone and peritoneal B1 cells, decreased immune responses to T-

dependent antigens, lack of germinal center formation, and reduced memory responses (67). CD19 has been shown to interact with a variety of signaling proteins including the Src kinases Lyn and Fyn, PI3K, Btk, PLCγ2, Grb2, Sos BCAP, and Vav (72, 130). These signaling molecules have downstream function in everything from Ca^{2+}mobilization, actin polymerization and cell motility, proliferation, and activation of inhibitory proteins and receptors (67, 131-133).

However the major function of CD19, the activation of PI3K, was determined in two ways. The first entailed creating CD19 transgenic mice with specific mutated tyrosines in CD19 cytoplasmic tail. Mutation of tyrosines 482 and 513 in the cytoplasmic tail almost completely imitated the phenotype of the CD19 knockout (134). Tyrosines 482 and 513 are phosphoryalted by Lyn and are specifically responsible for activation of PI3K (127, 134-136). Another study performed by Anzelon et al created a B cell specific deletion of the Pten phosphatase. PTEN dephosphorylates the product of PI3K activation, $PI_{(3,4,5)}P$. Deletion of Pten resulted in rescue of the phenotypes observed in the CD19 knockout and therefore shows Pten as a major negative regulator of CD19 function. From these data, it seems clear that the primary role of CD19 is in activation of PI3K and its downstream effectors.

CD21 in BCR signaling

The role of CD21 in the immune response has been discussed above. Here, I will discuss the role of CD21 in promoting B cell signaling. CD21 has a

very short cytoplasmic tail with 4 conserved cytoplasmic tyrosines (figure 8). These tyrosines are putatively phosphorylated rapidly and transiently after BCR stimulation and several targets are purportedly activated downstream of this activation but the significance in these results has not been confirmed (137, 138).

The usual model of CD21 signaling requires extracellular association with CD19. CD21 does not have signaling function of its own but functions through promoting CD19 signaling. Several studies have shown that stimulation of B cells by cross-linking BCR and CD21 leads to increased translocation of BCR, CD19 and CD21 into cholesterol enriched micro-domains known as lipid rafts (139-144). Most signal transduction is thought to occur in these lipid rafts as they become enriched with signaling molecules and aggregate in stimulated cells (142, 145). This model is further supported by the phenotype of the CD21 KO whose phenotype is generally similar to the CD19 KO though less severe. Further dissection of the role of CD21 cell specific function and in B cell signaling will be shown in chapter 2.

Inhibition and down regulation of B cell Responses and Signaling

The inhibition of BCR signaling is mediated by several major cell surface receptors. One of which touched upon earlier is FcγRIIb, also referred to as CD32. CD32 binds the Fc portion of IgG isotype antibodies which are often components of immune complexes (ICs). CD32 knockout mice reportedly are susceptible to age-onset autoimmune disorders such as SLE

(154-156). CD32 contains one cytoplasmic ITIM motif that is primarily responsible for activation of the inositol phosphatase SHIP (92, 146-149). This ITIM is initially phosphorylated by the src family kinase Lyn and this ITIM. The ITIM of CD32 has also been shown to associate with src homology tyrosine phosphatases Shp1 and Shp2 however it is unclear whether this association is significant (150-153). SHIP is one of two inositol phosphatases whose activity specifically antagonizes the $PI_{(3,4,5)}P3$ production mediated by PI3K function downstream of BCR signaling. SHIP dephosphorylates $PI_{(3,4,5)}P3$ into $PI_{(3,4)}P2$. Pten is the other major negative regulator of PIP3. Pten produces $PI_{(4,5)}P2$ which is the substrate for PI3K activity. Pten regulation is not as well characterized.

The other major inhibitory receptor on B cells is CD22. CD22 has a long cytoplasmic tail with 3 ITIM motifs. These ITIMS become phosphorylated after BCR ligation and recruit SHP1. Lyn is responsible for phosphorylation of these ITIMs. SHP1 is widely expressed in hematopoietic cells and plays a major role in the dephosphorylation of many tyrosine phosphorylated proteins that are important in promoting BCR signaling. A natural mutation of Shp1 has been found in humans and mice known as the motheaten viable. This point mutation creates a phosphatase-dead enzyme (157-160). Motheaten mice have a variety of physical abnormalities due in part to severe autoimmunity {Bignon, 1994 #1295; Tsui, 1993 #1297}. CD22 knockout mice show B cell

hyperproliferation, increases in B cell numbers and some cases of autoimmunity (161, 162).

Other inhibitory receptors on B cells include CD72, PIR-A, PIR-B, and Shps-1. These receptors do not have well characterized functions or effector molecules in B cells however study of new phosphatases in lymphocytes is an active area of research. One major phosphatase with unexplored function in B cells is the src homology phosphatase-2 (SHP2). SHP2 is a widely expressed phosphatase and knockout mice are embryonic lethal at day 10.5. Chapter 3 will focus on the role of SHP2 in B cell development, activation, function and signaling.

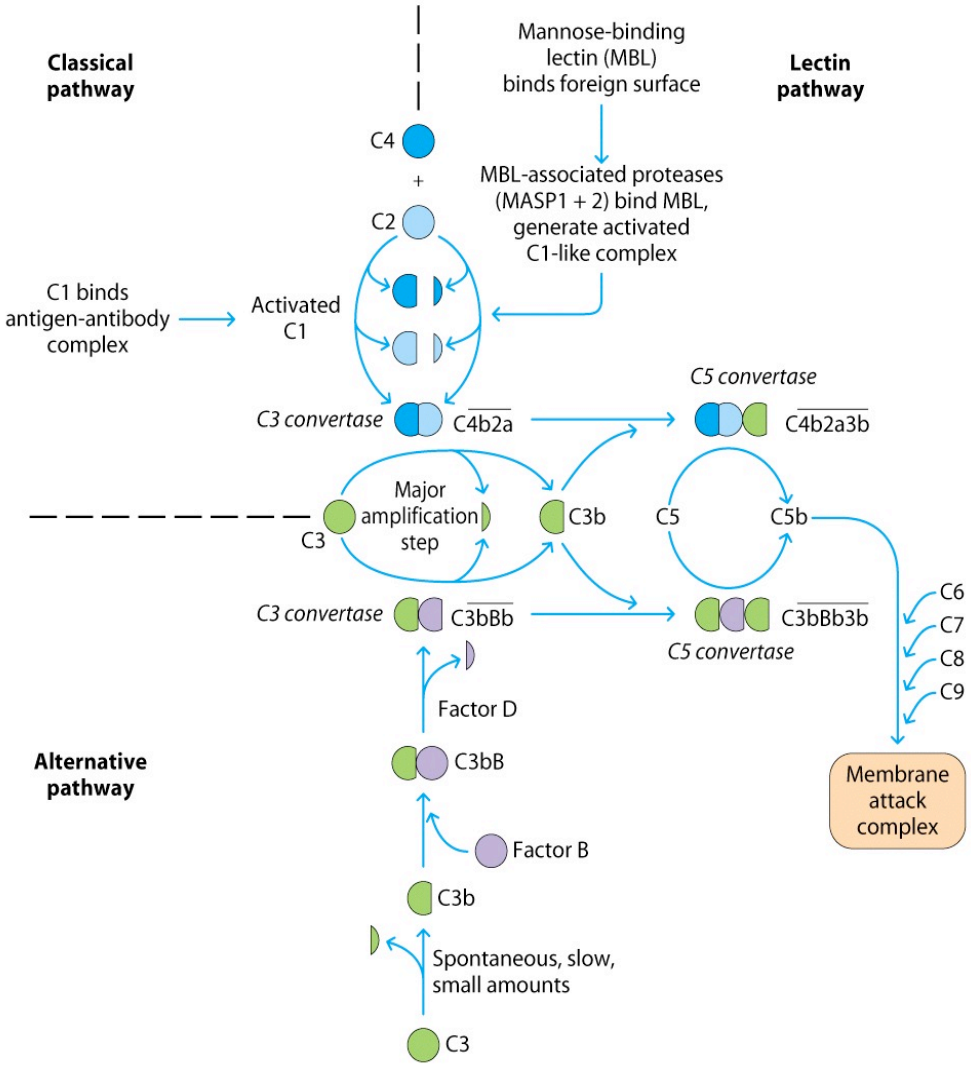


Figure 1: Overview of the Complement System
There exist three pathways in complement activation, the classical, alternative and lectin pathways. The classical pathway requires immune complexes, the alternative pathway is canonically activated by C3 binding to bacterial cell surfaces, and the lectin pathway requires MBL binding to foreign surfaces. C3 and C5 are necessary for pepetuation of all complement pathways in formation of the MAC complex.

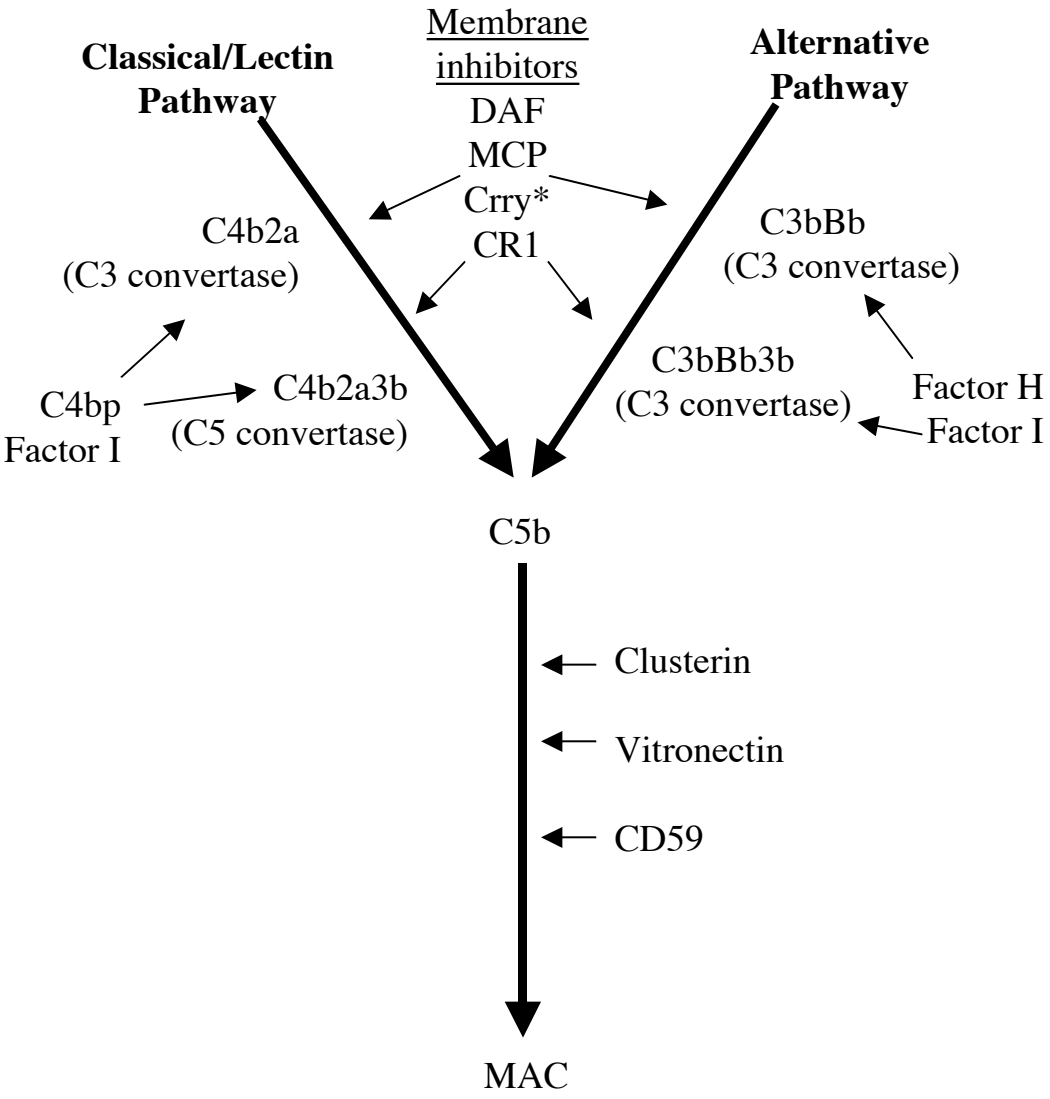


Figure 2: Summary of Complement inhibitors involved in regulation of MAC assembly (1).
* Crry is both membrane bound and soluble.

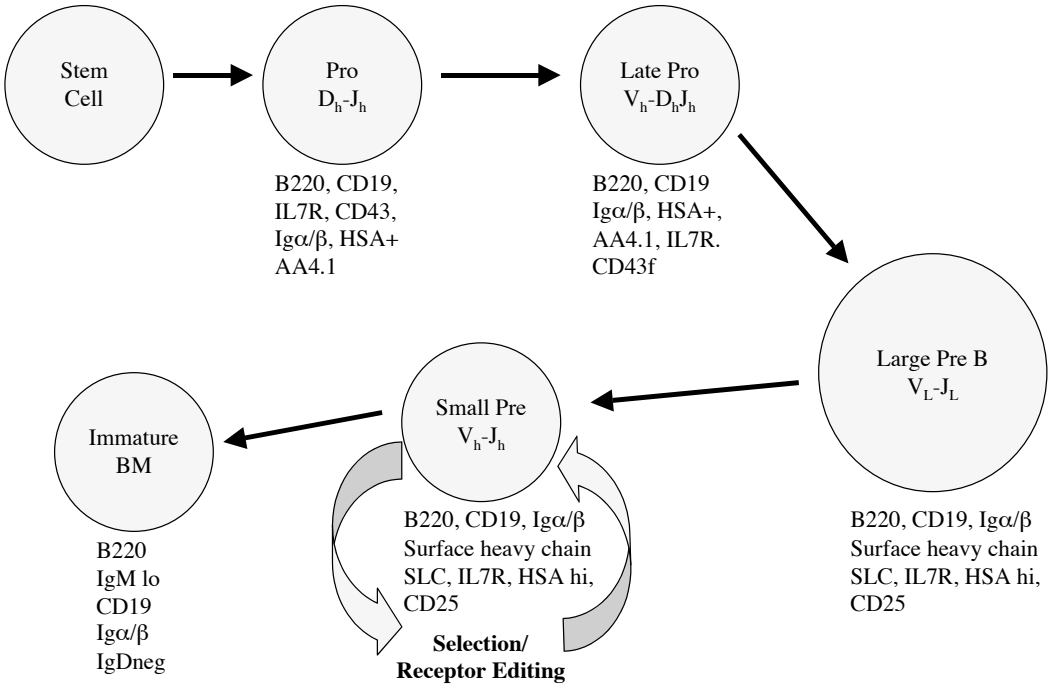


Figure 3: Summary of B cell Development in the Bone Marrow.

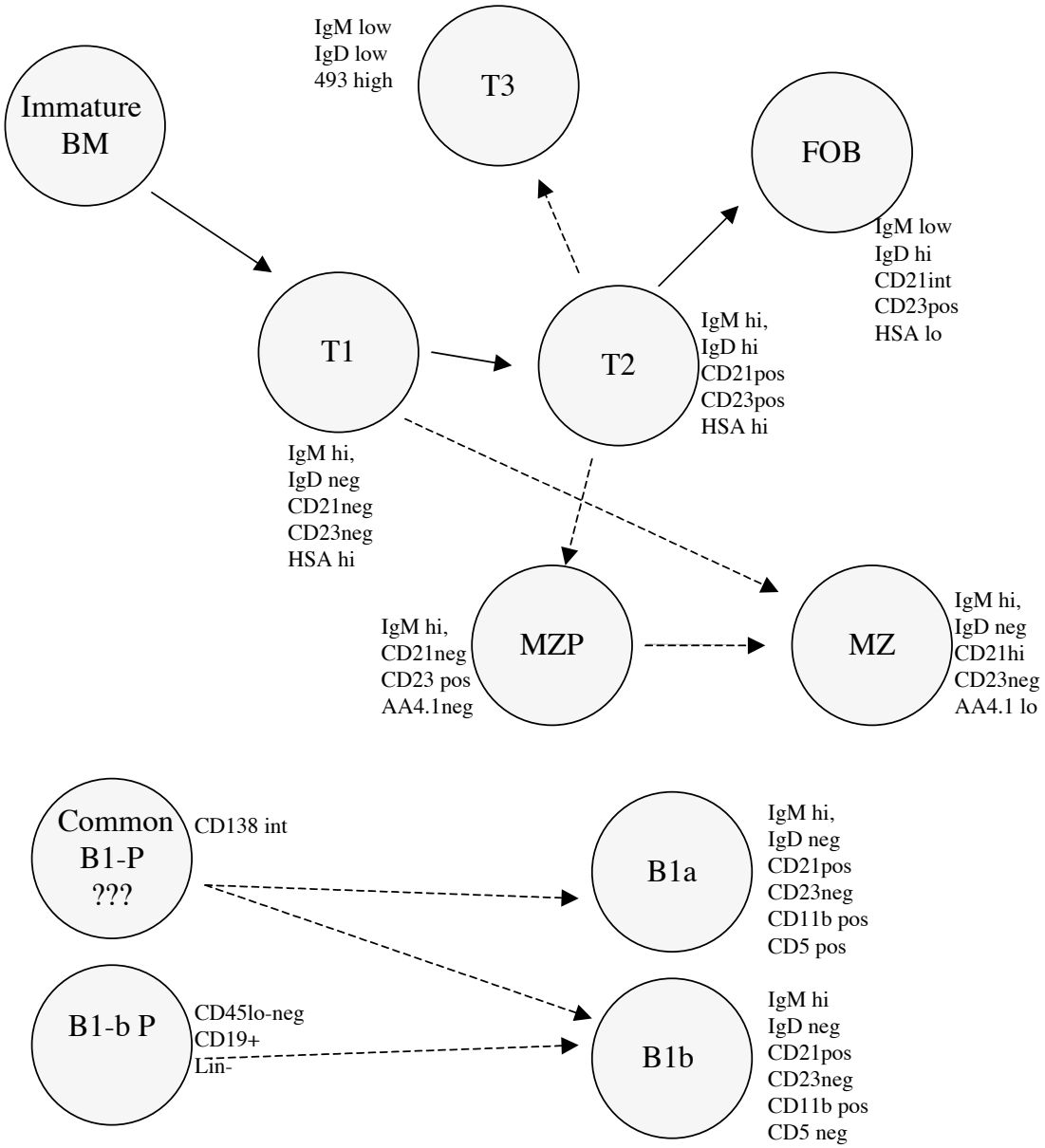


Figure 4: Development of B cells in the periphery. Dotted lines shown represent unclear lines of development or recently discovered.

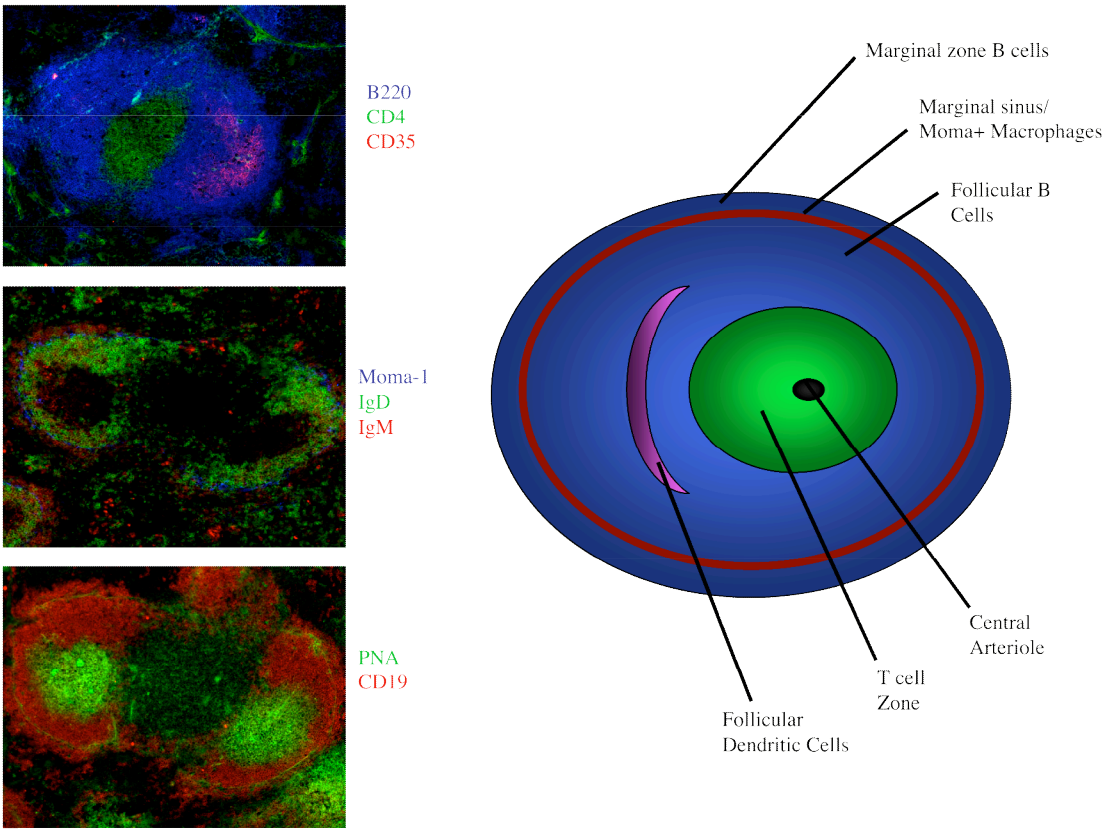


Figure 5: Overview of follicular structure in the spleen. The panels on the left are histological stains for the markers labeled.

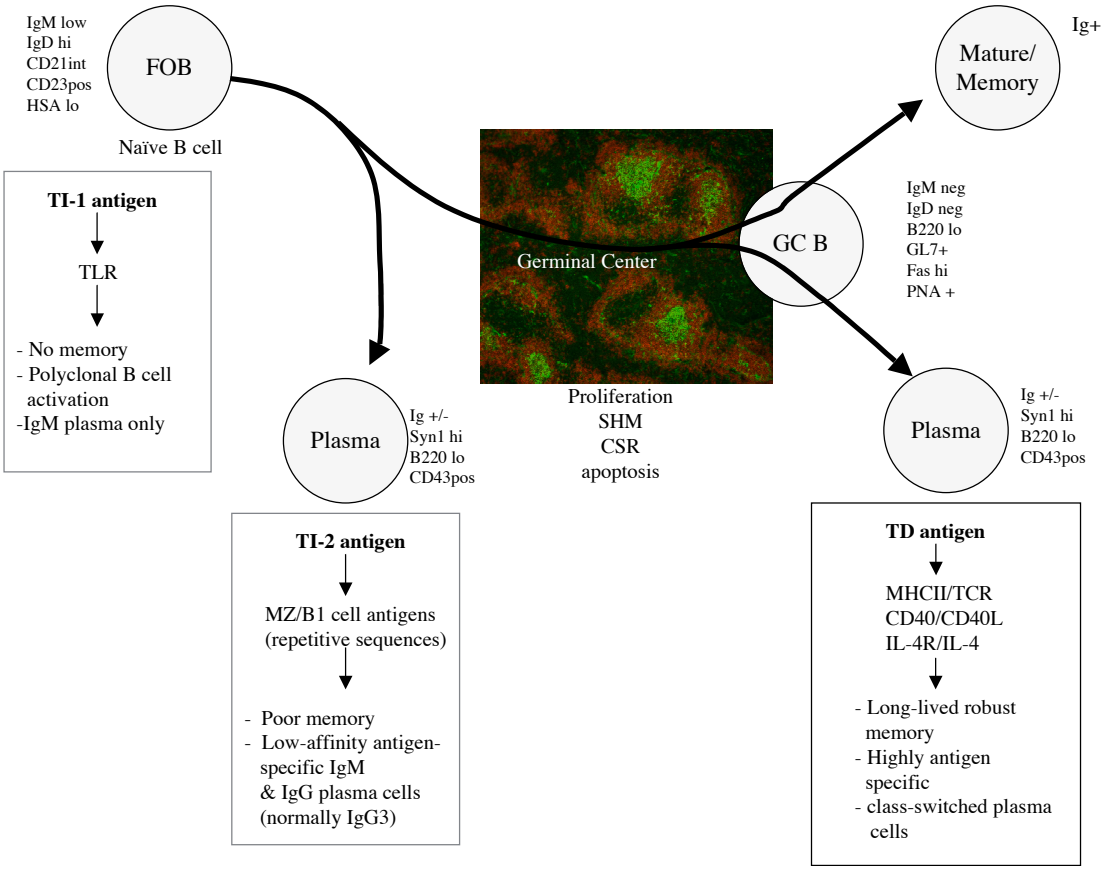


Figure 6: Summary of activated B cell subsets and types of B cell antigens.

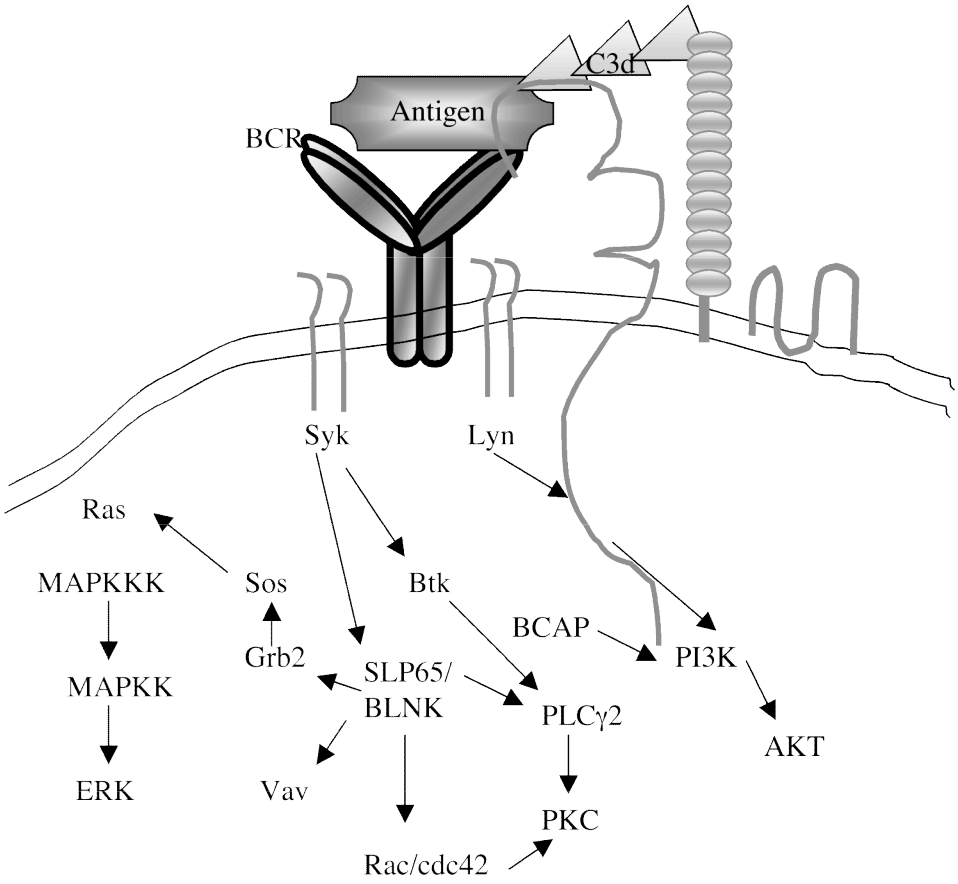


Figure 7: Overview of BCR downstream signaling.

Murine

KHRESNYYTKTRPKEGALHLETREVYS IDPYNPAS
KHRERNYYTDTSQKE AFHLEAREVYSVDPYNPAS

Human

Figure 8: Homology between CR2 cytoplasmic tail between mouse (murine)mice and human cytoplasmic tail of CR2.

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CHAPTER 1

Complement C3d conjugation to anthrax protective antigen promotes a rapid and sustained antibody response

Introduction

Anthrax, an acute infectious disease caused by the spore-forming bacteria *B. anthracis*, has come to recent prominence as an agent of bioterrorism. Acute exposure to anthrax can be treated effectively with antibiotics, passive antibody transfer and biological or chemical inhibitors of anthrax toxin. However, protection from future exposure or germination of residual spores can only be attained by vaccination and elicitation of a strong humoral response. The currently licensed anthrax vaccine (Biothrax™) is a cell-free filtrate derived from an avirulent unencapsulated strain of *B. anthracis* and adsorbed to an aluminum hydroxide salt (alum). While limited case studies indicate that Biothrax™ is protective, concerns remain regarding efficacy and safety (1). Approximately 30% of patients who are vaccinated with Biothrax™ suffer from mild skin irritation and cutaneous-anthrax-like symptoms, which are thought to be due to toxins present in the filtrate (2-4). The vaccination regimen requires six injections over the course of 18 months and additional early boosters to achieve and maintain its full effectiveness (4, 5). Therefore, there is an acknowledged need for an improved vaccine that exhibits prompt and sustained effectiveness and is free of reactogenic properties (6). Thus, next generation anthrax vaccines aim to: 1) induce rapid

neutralizing antibody titers, 2) maintain high levels of this neutralizing antibody, 3) be capable of inducing robust memory responses, and 4) be free of reactogenic properties that may cause illness or discomfort. In addition, vaccines for use in the general public need to impart protection in infants and the elderly.

Many of the new generation anthrax vaccines target specific components of *B. anthracis*, such as the capsule carbohydrate poly-γ-d-glutamic acid, or subunits of anthrax toxin in attempts to provide, at minimum, equivalent protection as *Biothrax* while additionally meeting the above criteria (4, 7, 8).

The major virulence factors of *B. anthracis* provide the most promising targets for vaccine development and are encoded by 2 plasmids, pXO1 and pXO2. Plasmid pXO1 encodes for the toxin proteins lethal factor (LF), edema Factor (EF) and protective antigen (PA), which work collaboratively to promote anthrax toxicity (7, 9, 10). Plasmid pXO2 encodes the capsule carbohydrate poly-γ-D-glutamic acid that prevents phagocytosis but is not toxigenic. Toxin pathogenesis first requires PA binding to TEM8 or CMG2 on the macrophage cell surface to allow heptamerization of PA and subsequent association with LF or EF (9, 11). The PA heptamer and a single LF/EF molecule form a large complex which induces receptor-mediated internalization into endocytic vesicles which mature into acidic lysosomes. Low pH within the vesicle changes the conformation of PA to create a pore

that allows exit of LF or EF from the vesicle into the cytoplasm before degradation can occur (10). Edema toxin (EdTx), the combination of PA and EF, primarily affects neutrophil function by disregulating water homeostasis leading to edema (10). Lethal toxin (LeTx), the combination of PA and LF, causes cleavage of MAPK family members leading to apoptosis of the infected cell (10). In mouse, macrophage sensitivity to LeTx-induced apoptosis does not always correlate with strain susceptibility to anthrax infection, indicating that additional understanding of the pathophysiology is needed.

Protective antigen is so named for its ability to elicit a protective immune response to anthrax infection, consistent with evidence that protection provided by Biothrax™ vaccination is attributed to PA-specific antibodies (1, 4). PA has no intrinsic enzymatic activity or pathogenic function, but is essential for the cellular entry of LF and EF. It contains four domains however deletion of domain 4 (amino acids 587-735) completely abrogates binding to cell surface receptors and consequently negates pathogenicity of the toxins (12, 13). Domain 4 has also been shown to be the most immunogenic of the four domains in mice (14). With the intent of curtailing side effects of Biothrax™ and eliciting a more robust PA-specific IgG response,one promising new approach for an improved vaccine is to utililize recombinant PA (rPA) instead of cell-free filtrates (1). However, phase I trials using aluminum-adsorbed rPA (rPA102) were disappointing in

that PA-specific antibody titers were significantly lower than with Biothrax™ (15).

Relatively little work in anthrax vaccine development has focused specifically on augmenting B cell immune responses by the design and testing of alternative adjuvants. The only approved adjuvants in the U.S. contain aluminum hydroxide or phosphate salts (alum) and therefore much of the work in testing new vaccine targets have not strayed away from these conventional adjuvants (16-18). In seminal work, Dempsey *et al* reported the use of C3d, a breakdown product of complement serum protein C3, as a natural molecular adjuvant that significantly augmented antigen-specific antibody titers (19). C3d has since been used in mice for a variety of vaccine applications including HIV, measles, streptococcus pneumoniae and influenza (15, 20-24). C3d function is primarily mediated through its binding to complement receptors CD21/CD35 that are expressed on both B cells and follicular dendritic cells (FDCs). Though the mechanism of C3d function has not been fully elucidated, work from our group and others suggests that cross-linking of complement receptors leads to retention of antigen on either FDC or B cell surfaces and prolonged signaling by the B cell receptor complex, leading to augmented B cell activation (25-31).

In this study, we evaluated the use of C3d as an adjuvant for PA. We demonstrate that conjugation of PA to trimeric C3d ($C3d_3$) induces a more robust specific-IgG response to intact PA or domain 4 as compared to PA

adsorbed to alum (PA-Alum). The response provided by C3d conjugation occurs more rapidly and is at least as durable as PA-alum after just a single immunization. Furthermore, $C3d_3$-PA is effective when administered by various routes of immunization and is a functional adjuvant in adolescent mice lacking a fully developed B cell compartment. Lastly, we find that increased efficacy of $C3d_3$-PA may derive in part from its ability to augment the germinal center (GC) response and enhance differentiation into plasma cells and memory B cells.

Results

Cloning of $C3d_3$-bio

C3d was cloned from murine cDNA in tandem trimeric copies into the XA3 vector (Promega) as described in the materials and methods (**Figure 9**). Three clones were produced that contained 1, 2, and 3 copies of C3d respectively. This construct was transformed into the BL21 strain BL21-A1 for bacterial expression (**Figure 10**).

$C3d_3$ conjugation is superior to alum adsorption in eliciting antibody responses to intact PA and domain 4 after immunization with PA.

To determine if $C3d_3$ is an effective adjuvant for PA vaccination, we compared B cell immune responses in mice from several strains immunized with either PA-alum, PA-$C3d_3$ or PA alone. For preparation of the immunogen, rPA was *in vitro* biotinylated and either conjugated to biotinylated-$C3d_3$ via an avidin-bridge (PA-Av-$C3d_3$) and emulsified in IFA,

complexed with avidin and adsorbed to alum (PA-Av-Alum) or complexed in the presence of avidin and emulsified in IFA. Groups of A/J, BALB/c, and C3H/HeJ mice were immunized intraperitoneally (i.p.) with equivalent amounts of PA (20 μg) and PA-specific IgG was measured by ELISA (**figure** 11, A).

In all strains of mice, PA-Av-C3d$_3$ elicited a markedly greater IgG response than PA-Av-Alum during the primary response (fig. 11, A). Elevated titers of PA-specific IgG was observed just 7 days after initial immunization with PA-Av-C3d$_3$– approximately 2.5- to 3-fold enhanced over PA-Av-Alum immunization. Peak IgG titers were observed between days 14 and 21 in PA-Av-C3d$_3$. At day 42, all groups received a boost of PA in saline. Mice receiving PA-Av-Alum showed a clear secondary response indicating that although alum did not provide a robust primary IgG response, it was capable of priming a secondary response. In contrast, secondary immunization of PA-Av-C3d$_3$-primed mice with soluble PA maintained the high specific-IgG titers observed during the primary immunization. Results from immunization in C3H/HeJ mice also demonstrated that preparation of the C3d adjuvant was free of LPS endotoxin contamination as these mice express a nonfunctional TLR4 protein (32). Curiously, PA-Av alone evoked strong IgG responses in A/J mice in both the primary and secondary responses. However, PA alone (non biotinylated, no avidin) elicited similar IgG responses (data not shown), and BALB/c and C3H/HeJ mice did not respond well to PA-Av and. Moreover,

immunization of A/J mice with PA-Av and. Therefore, we conclude that A/J is a high-responder strain to PA and that the C3d-adjuvant effect is not due to multimerization of PA or the use of IFA. Most importantly, regardless of the strain used in these experiments, C3d elicited a consistent 2.5 - 3 fold greater antibody response after primary immunization compared to alum.

Antibodies specific for domain 4 of PA are capable of neutralizing toxin by blocking binding of the toxin to the macrophage cell surface receptors (11, 14, 33). Indeed, passive protection from anthrax exposure is conferred by injection of monoclonal antibody 14B7 which binds to this region with high affinity (33). We therefore tested whether immunization with PA-Av-$C3d_3$ efficiently produced domain-4 specific antibodies (fig. 11, B). In all strains of mice tested (A/J, BALB/c, and C3H/HeJ), PA-Av-$C3d_3$ elicited an approximately 3-fold enhancement in domain 4-specific IgG titers relative to PA-Av-Alum and PA-Av in the primary response (fig. 11, B). Secondary immunization of A/J and C3H/HeJ mice with soluble PA alone did not further elevate the existent high titers of domain 4-specific antibody present in PA-Av-$C3d_3$ immunized mice. However, BALB/c mice did show a modest increase in domain 4-specific IgG after the boost (fig 11, B). Altogether, these data indicate that $C3d_3$ is a more effective adjuvant than alum in eliciting a rapid and sustained antibody response to PA and, in particular, neutralizing antibodies to domain-4 after a single immunization.

PA-Av-C3d$_3$ augments isotype switch and provides equivalent antigen-specific long-lived IgG titers relative to PA-Av-Alum

Though no comprehensive studies of protective Ig isotypes has been performed in anthrax challenged mice, IgG1 has been associated with efficacy of some vaccination regimens which utilize alum (34). To determine adjuvant-dependent differences in generating neutralizing class-switched domain 4-specific antibodies, antigen-specific ELISAs were performed to measure IgM and IgG titers in A/J mice immunized with PA-Av, PA-Av-Alum or PA-Av-C3d$_3$ (**figure 12**, A). We observed that C3d$_3$-conjugation dramatically enhanced domain 4-specific IgM and all IgG subtypes during the primary response. At day 14, PA-Av-C3d$_3$ induced, at minimum, a 2-fold increase in serum IgM, IgG1, IgG2a, IgG2b and IgG3 titers compared to PA-Av-Alum. However, following the boost at day 49, alum and C3d$_3$ induced equivalent IgG1, IgG2a, and IgG2b serum titers (fig. 12, A), but C3d$_3$ promoted augmented IgM and IgG3 (fig. 12, A). A similar isotype distribution was observed for antibodies binding intact PA (data not shown).

To test adjuvant dependent effects on the duration of the PA- and domain 4 antibody responses, immunized A/J and BALB/c mice were bled 9 weeks after secondary immunization with either PA-Av, PA-Av-Alum or PA-Av-C3d$_3$ (fig 12, B). A/J mice showed statistically equivalent serum PA- and domain 4- specific titers after secondary immunization (fig 12 B, left panel). In contrast, C3d$_3$ was more effective than alum in inducing a durable response

against domain 4 in BALB/c mice (fig 12 B, right panel). Altogether, these data indicate that $C3d_3$ is more effective than alum in promoting a rapid and sustained, high-titer, pan IgG antibody response.

PA-Av-C3d is effective when administered via intraperitoneal or subcutaneous routes.

The adjuvant function of C3d is primarily mediated through its high affinity interaction with complement receptor CR2 (CD21/35) expressed on B cells and FDCs (35, 36). However, B cells and FDCs are not likely to be present at sites of subcutaneous (s.c.) or intramuscular (i.m.) delivery, which are the routes of administration for the Biothrax™ and rPA102 vaccines (4, 37). Therefore, we determined whether delivery of $C3d_3$ by these routes would lower the efficacy observed with i.p. injection. Interestingly, we found that PA-Av-$C3d_3$ produced equivalent titers of anti-PA IgG antibodies when administered via i.p. or s.c. routes (**figure 13**, A). Anti-PA titers from mice immunized s.c. with PA-Av-$C3d_3$ were elevated 3-fold over PA-Av-Alum-immunized mice (fig. 13, C). Domain 4-specific IgG was most effectively induced by i.p. immunization, however s.c. injection also induced significant IgG titers (fig. 13, B). I.M. administration of PA-Av-$C3d_3$ did not appear as effective as the other routes in eliciting PA- and domain-4- IgG (fig. 13 B), but was similarly effective as PA-Av-Alum. The rapid kinetics of the PA-Av-$C3d_3$–induced response was unchanged, regardless of the route of administration (fig. 1-3 A, B).

Mikszta *et al* conducted studies to test the efficacy of PA in alum when administered by various routes and showed high efficiency when delivered subcutaneously (37). These data indicate that C3d retains effectiveness as an adjuvant when administered s.c and is comparable to alum when injected i.m.

PA-Av-C3d is not effective in augmenting secondary responses

Due to observations that $C3d_3$ elicits augmented primary responses however not better secondary responses when given simply PA alone, we wanted to see whether administration of PA-Av-$C3d_3$ would increase boost responses. Therefore, 12 A/J mice were immunized with PA-Av-Alum and 28 days later, were boosted with: PA-Av, PA-Av-Alum, PA-Av-C3d, or PBS respectively. Blood was collected every seven days up to 49 days after primary immunization and domain-4 specific IgM and IgG was measured by ELISA (**Figure 14**).

Analysis of domain-4-IgG responses (fig. 14, A) shows that $C3d_3$ is unable to augment alum mediated primary responses over PA alone or PA-Av-Alum alone. $C3d_3$ does not initiate a second primary response during this experiment shown lack of IgM production after boost at day 28 (fig. 14, B). This additively shows alum's ability to prime the memory response for secondary response. Taken together, C3d is most effective in creating strong primary antibody responses but does not likely abet secondary immune or recall responses.

PA-Av-C3d elicits strong antibody responses in adolescent mice

Another key parameter in vaccine development is safety and efficacy in infants and children. We therefore investigated whether C3d would be an effective adjuvant in adolescent mice, which lack fully mature B cell compartments. Marginal zone B cells (MZBs) line the splenic marginal sinus and respond to blood born antigens, but are underrepresented in the young. This is an important consideration since MZBs express high levels of CD21 and may be key mediators of C3d function (35, 36). Characterization of B cell compartments in 3-week old A/J mice showed an over-abundance of immature-mature "transitional" B cells in the spleen, however follicular structure and B/T segregation was intact (data not shown). As expected, MZBs were present but at a lower frequency than in adult mice (data not shown). Twenty-two day old A/J mice were immunized i.p. with equivalent amounts of PA (10 μg) in the form of PA-Av, PA-Av-Alum, or PA-Av-$C3d_3$. Serum was collected every 10 days and PA- and domain 4-specific IgG titers measured by ELISA (**figure 15**). As seen in adult mice, PA-Av-$C3d_3$ elicited greater PA- and domain 4-specific IgG than PA-Av alone or PA-Av-Alum (fig. 15 A, B). The kinetics of the response was also similar to adult mice insofar as significant levels of IgG were detected by day 10 and increased through day 20 (fig. 15, A, B). Alum-immunized mice mounted minimal PA- and domain 4-specific antibody responses. PA-Av alone did induce PA-specific

IgG, however minimal domain 4-specific IgG was detected. Thus $C3d_3$ is an effective adjuvant in adolescent mice.

$C3d_3$ augments immune responses by abetting the formation of germinal centers and plasma cell differentiation

To address how $C3d_3$ mediates enhanced immune responses, we asked whether and how $C3d_3$ affects formation of germinal centers (GCs) and plasma cells. GCs are specialized structures within follicles where B cells undergo somatic hypermutation and class switch recombination of rearranged immunoglobulin genes to generate high-affinity plasma cells or memory B cells (37). Groups of mice were immunized with PA-Av-Alum or PA-Av-$C3d_3$ and analyzed by flowcytometry and immunohistology 0, 2, 4, 8 and 12 days post-immunization to enumerate splenic GCs, plasma cells and activated T cells. Serum was collected from all mice and PA- and domain 4 -specific IgG titers measured by ELISA (**Figure 16**).

Analysis of PA–Av-Alum and PA-Av-$C3d_3$ immunized mice showed that immunization with $C3d_3$ augmented the generation of $GL7^+$, Fas^+ GC B cells (fig 16 A). Peak GC B cell production occurred at day 8 (0.16%) in PA-Av-$C3d_3$ immunized mice and was approximately 2-fold greater than PA-Av-Alum immunized mice (0.05%) (fig. 16 A). At day 12, differentiation into GC B cells increased in PA-Av-Alum immunized mice while PA-Av-$C3d_3$ immunized counterparts maintained the high percentage of GC B cells seen at day 8. Immunofluorescent staining of splenic histological sections

corroborated the flow cytometric data and showed that PA-Av-$C3d_3$ immunization elicited larger and more frequent GCs than PA-Av-Alum immunized counterparts (fig. 16 H). Taken together, these findings suggest that $C3d_3$ conjugation hastens the onset and promotes the maintaintenance of the GC reaction.

In addition to GC formation, $C3d_3$ immunization also promoted plasma cell generation more efficiently than alum, as evidenced by the increased (4-fold) generation of $B220^{lo}$, $Syn1^{hi}$ B cells as soon as 2 days post-immunization (fig 16, B). Peak plasma cell formation in PA-Av-Alum immunized mice (5.2%) was observed at day 4 after immunization, whereas PA-Av-$C3d_3$ induced peak plasma cell levels at day 8 (8%) (fig. 16, B). Furthermore, a high percentage of plasma cell numbers were maintained through day 12 in PA-Av-$C3d_3$ immunized mice. Serum PA- and domain 4-specific IgM and IgG titers corroborated the plasma cell formation data as PA-Av-$C3d_3$ immunization induced a 3-fold increase in PA-specific IgM and IgG at day 8 as compared to alum. Domain 4-specific IgM and IgG showed a similar 2-fold increase at day 8 (fig 16, D-G).

Analysis of *in vivo* T cell activation in PA-Av-$C3d_3$ and PA-Av-Alum immunized mice show no major alteration in the overall percentage of activated T cells ($CD62L^{lo}$, ICOS+)(fig 16 C), though the subset of PA-specific T cells was not assessed. Indeed, we speculate that the augmented GC reaction and plasma cell formation is through direct B cell activation by co-

ligation of $C3d_3$ and the BCR as well as CD21-facilitated uptake of antigen for presentation to T cells. Finally, no evidence of elevated TNFα, IL1-β or IL-6 was observed in the serum of PA-Av, PA-Av-Alum, or PA-Av-$C3d_3$ immunized mice at 1.5, 6, or 24 hours after immunization nor during any of the time points shown in figure 5, indicating that $C3d_3$ is not generally pro-inflammatory (data not shown).

Discussion

To avoid a calamitous outcome from B. *anthracis infection*, the humoral immune systems needs to respond rapidly and effectively to negate pathogenic effects of anthrax toxins. If administered prior to the symptomatic stage, antibiotics are effective in stemming disease. However, since these symptoms are nonspecific, effective prophylactic vaccines are necessary to prevent spore germination, block vegetative replication and/or neutralize exotoxin function. The currently licensed Biothrax™vaccine consists of aluminum hydroxide-adsorbed culture supernatant material from toxigenic noncapsulated strains of *B. anthracis*. In response to the acknowledged threat of *B. anthracis* spore dissemination as a biowarfare agent, usage of Biothrax™ for active vaccination programs as well as prospective stockpiling have accelerated. The resurgent need for an anthrax vaccine(s) has also refocused concerns regarding the specific efficacy and nonspecific reactogenicity associated with vaccination. In response to the stated need to develop a well characterized and efficacious vaccine that is also well tolerated

(6), we generated and characterized a novel subunit vaccine that employs C3d as a natural adjuvant and PA as the target antigen for neutralizing antibodies. In comparison to PA-Av-Alum, injection of PA-Av-$C3d_3$ by various routes of administration induced a more rapid and augmented PA-specific antibody response. Production of effector antibodies of all IgG isotypes was sustained, and no evidence of pro-inflammatory cytokines was observed.

Second generation anthrax vaccines currently in clinical trials consist of recombinant PA (rPA) adsorbed to aluminum hydroxide. Hence, the adjuvant is the same as that used in Biothrax™, but lot variability in PA content as well as residual toxicity are avoided. Protective antigen is an attractive neutralization target since binding, uptake, and cytosolic release of LeTx and EdTx are dependent upon PA. We found that coupling of C3d to PA elicited a rapid PA-specific antibody response in adult and adolescent mice that was superior to the response elicited by equivalent amounts of PA adsorbed to alum. All IgG isotypes were efficiently induced by PA-Av-$C3d_3$, indicating that sufficient T cell help was recruited to promote class switch recombination and continued differentiation into plasma cell effectors. Correspondingly, C3d conjugation enhanced the generation of GC B cells and the coincident generation of $Syn1^+$ plasma cells. Rechallenge with PA in saline induced similar titers of PA-specific antibody in mice receiving primary immunizations of PA-Av-$C3d_3$ or PA-Av-Alum. These results could indicate the equivalent generation and survival of PA-responsive B cells from the

primary response. However, we find it more likely that the sustained levels of anti-PA titers at 6 weeks post-immunization with PA-Av-C3d$_3$ leads to rapid binding and clearance of PA by Fcγ receptor-bearing effector cells. Unlike PA-Av-Alum, PA-Av-C3d$_3$ immunization was also capable of inducing rapid and sustained IgG titers against domain 4 of PA. This is an important hallmark as domains 2 and 4 interlace to bind the anthrax receptor and thus are indispensable for anthrax toxicity (11). These findings imply that PA-Av-C3d$_3$ should be effective against natural or engineered variants of *B. anthracis*.

Soluble protein antigens such as PA are poorly immunogenic, therefore adjuvants are necessary to augment the immune response by promoting lymphocyte activation and preventing rapid clearance ("depot effect") of the immunogen. Despite the fact that aluminum salts have been in use for decades and are the only approved vaccine adjuvants, little is known about how they act. Although some proteins adsorb to aluminum hydroxide with high efficiency, several reports have shown rapid desorption *in vivo* (38). Such findings argue against a strong depot effect and suggest that adjuvancy may be achieved by the release of concentrated antigen. Microbial pathogens activate the innate immune system by engaging Toll-Like Receptors (TLRs), resulting in cytokine release and the upregulation of costimulatory molecules on antigen-presenting cells. It has been observed that aluminum compounds can promote the maturation or activation of

myeloid antigen-presenting cell subsets, however since TLR signaling does not appear to be involved it is unclear how this occurs (38-41).

In contrast to aluminum hydroxide, the adjuvancy effect of C3d is better understood (42). Soluble proteins generally do not activate the complement cascade, but need to be opsonized via antibody binding or in the context of local inflammation - as may occur with other adjuvants that activate innate immunity. C3d facilitates antigen retention by binding to CD21/35 on FDCs, which do not efficiently endocytose antigen and may protect bound antigen from proteolysis, thus creating a depot effect. In addition to focusing antigen to the B cell surface, C3d promotes B cell activation by co-aggregating the BCR and CD21, resulting in enhanced signaling via CD21-associated CD19. In addition, C3d-fixation facilitates the uptake and presentation of antigen to recruit T cell help (29, 31). It is known that CD19 and CD21 are crucial for germinal center formation and resultant memory B cell generation. Therefore, in addition to promoting the recruitment of PA-specific B cells into the primary response, C3d-conjugation promotes the generation of high affinity antibody-secreting cells and the maintenance of the memory B cell compartment.

Results of a phase I trial comparing Biothrax™ and rPA/aluminum hydroxide (rPA102) was recently reported (43). Toxin neutralizing assays revealed similar efficacy with either vaccine and reduced reactogenicity with rPA102. However, PA-specific antibody titers were significantly reduced in

subjects receiving rPA102. Extrapolating from these findings and our own studies, we conclude that PA-C3d conjugates hold great promise as a next generation vaccine. Unlike Biothrax™ or rPA102, PA-Av-C3d$_3$ elicits rapid and sustained PA-specific antibody titers when administered i.p., s.c. or i.m., suggesting that protection against anthrax can be achieved and maintained with a single vaccination. Altogether, our data suggests that PA-linked-C3d would be more efficacious than alum-adsorbed PA as both a preventative vaccine and for treatment upon acute anthrax exposure.

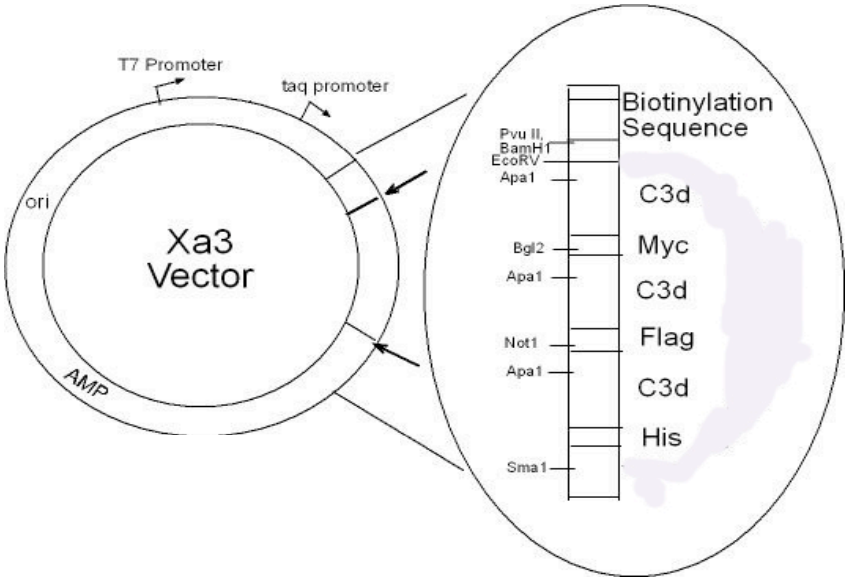


Figure 9: C3d cloning strategy.
Individual copies of C3d were tagged with various markers and cloned in tandem into the XA3 Promega vector. Biotinylation sequence was at the N-terminus.

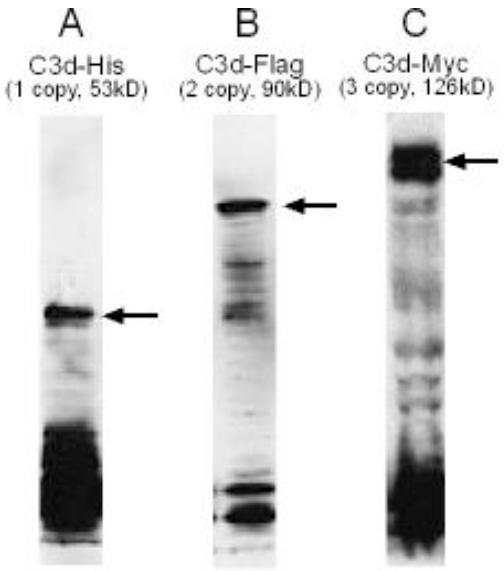


Figure 10: C3d-bio bacterial expression. Each of the C3d constructs(1,2, and 3 copy) were transformed into BL21 DE RIL e-coli (Stratagene) and subsequently grown in 5ml cultures. Each of the cultures were lysed by sonication and verified by western blot for expression of biotinylated protein via Sav-HRP detection. A. C3d-his (monomeric protein), B. C3d-flag (dimeric protein), and C. C3d-Myc (trimeric protein).

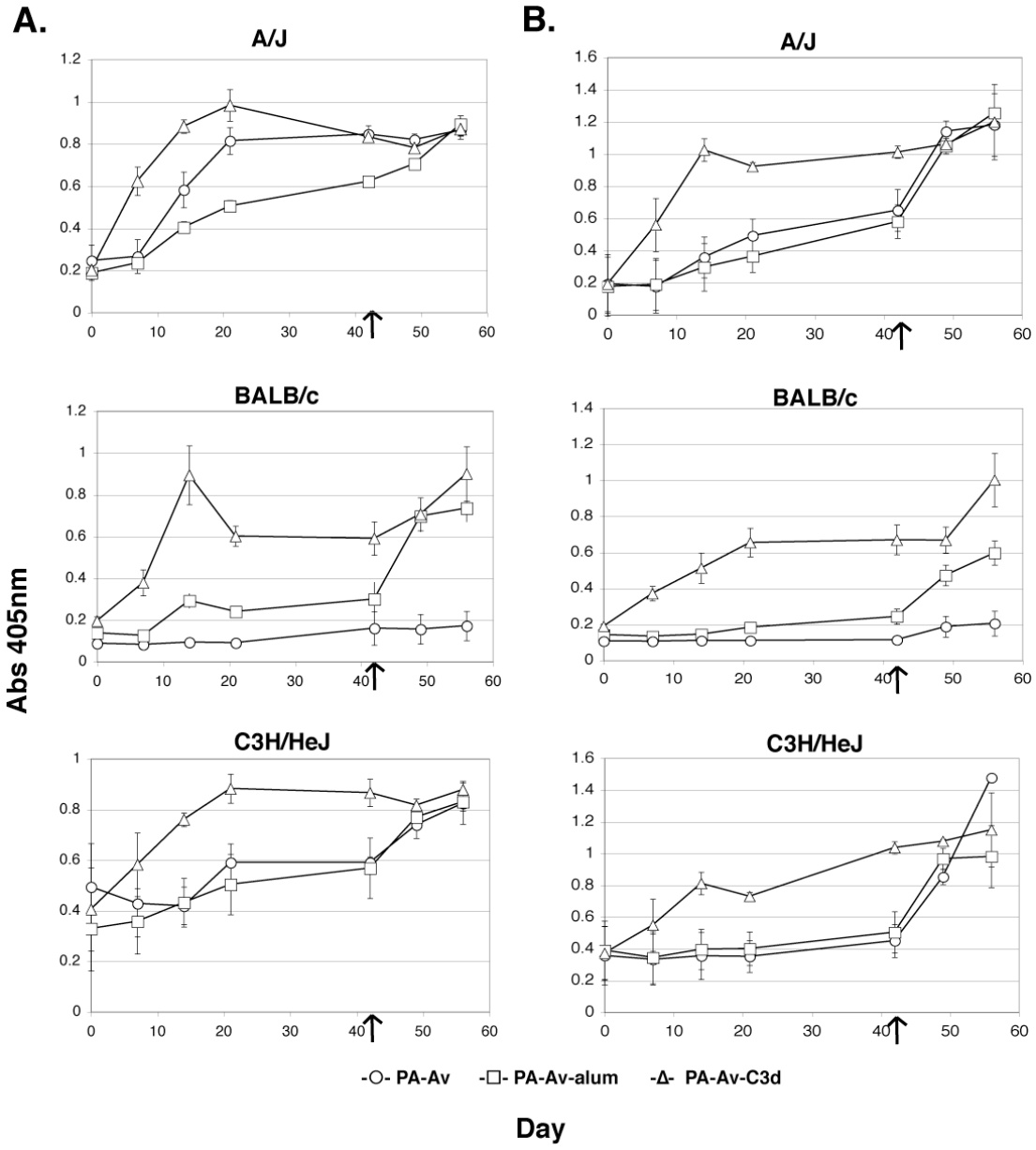


Figure 11: Immunization with PA-Av-C3d$_3$ augments production of PA- and Domain-4 specific antibody relative to alum. Groups of A/J (top), BALB/c (middle), and C3H/HeJ (bottom) mice were immunized with PA-Av (○), PA-Av-Alum (□), or PA-Av-C3d (△) and serum PA- (A) and Domain-4- (B) specific IgG was measured by ELISA (abs 405nm). All mice were boosted at day 42 with PA in saline as indicated by the arrow. Error bars indicate SEM of 5 mice/group.

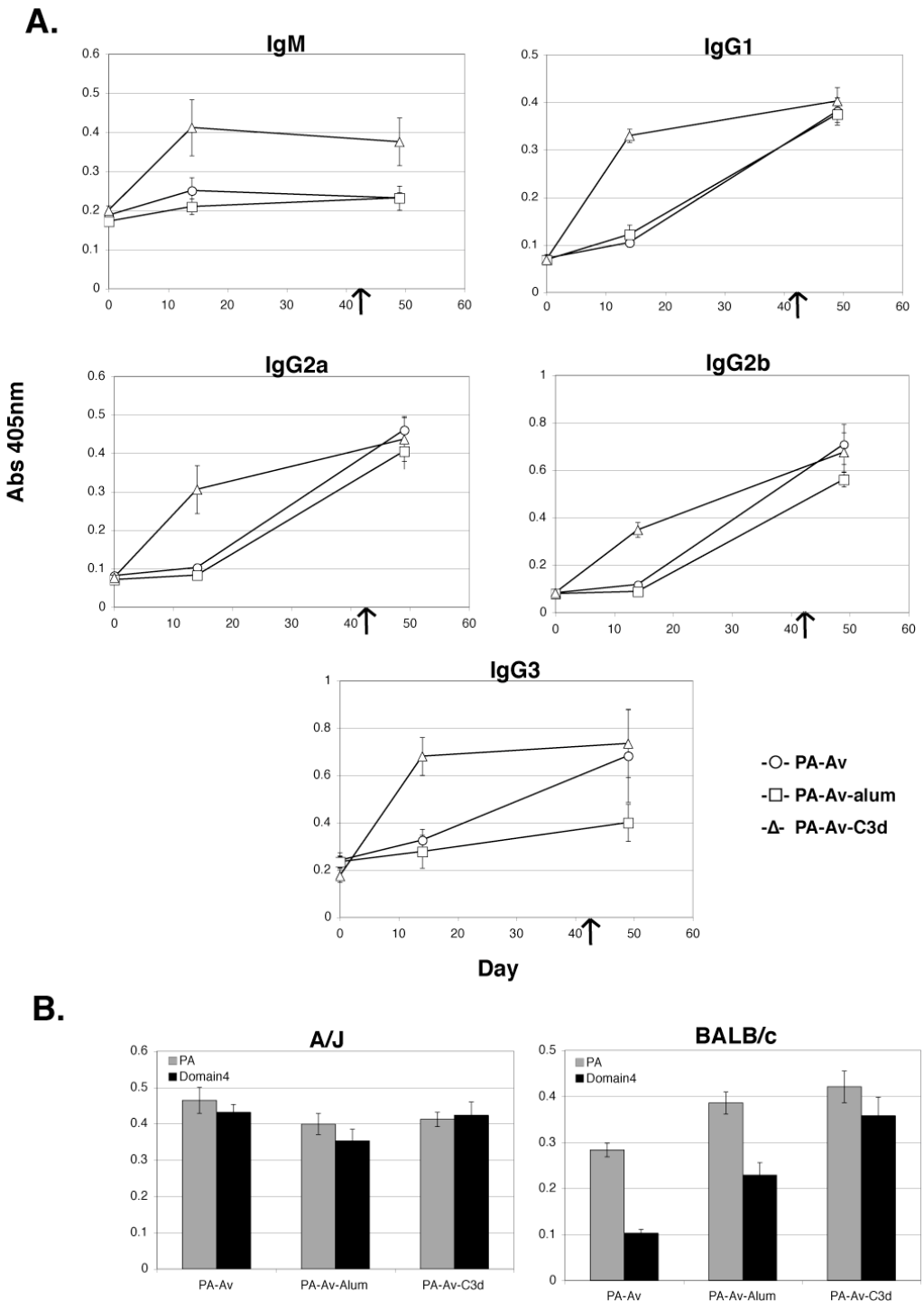


Figure 12: Immunization with PA-Av-C3d$_3$ augments isotype switch and provides equivalent long-lived titers as PA-Av-alum. (A) Domain-4- specific IgM, IgG1, IgG2a, IgG2b and IgG3 ELISAs (abs 405nm) were performed from PA-Av (○), PA-Av-Alum (□), or PA-Av-C3d$_3$ (△) immunized A/J mice. (B) Serum PA- and Domain-4 specific IgG titers were measured from immunized A/J (left) and BALB/c (right) mice 63 days after secondary immunization (105 days after primary immunization). All error bars indicate SEM of 5 mice.

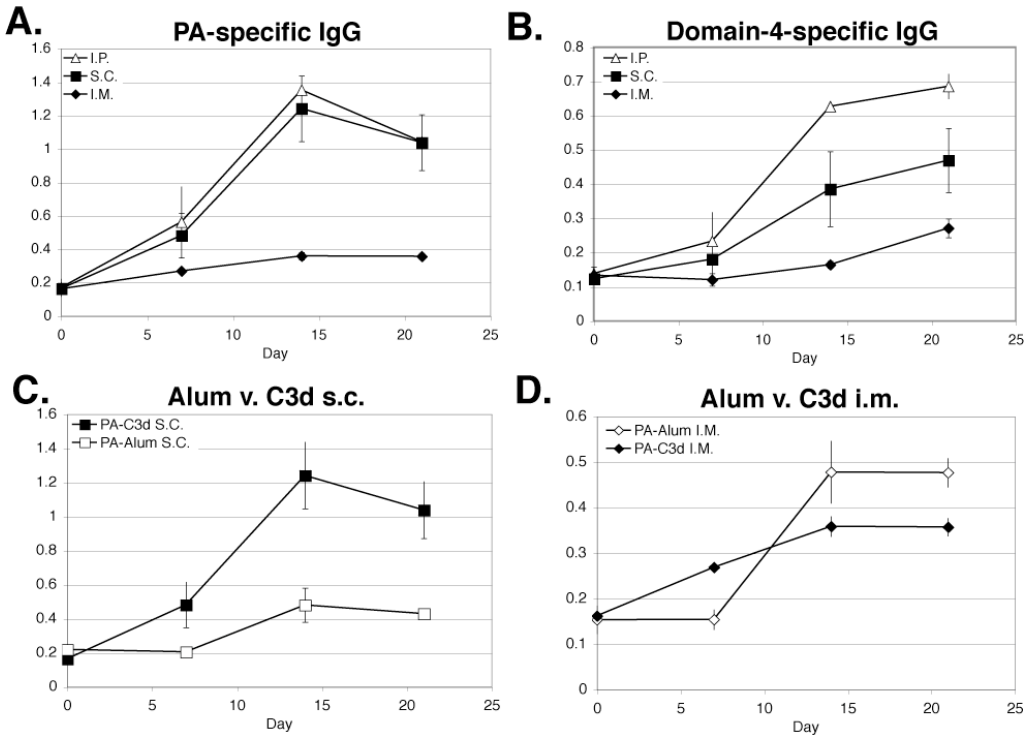


Figure 13: $C3d_3$-mediated humoral responses are augmented relative to alum when administered i.p. and s.c., and equally effective to alum when delivered i.m. PA-Av-$C3d_3$ was injected i.p., s.c., and i.m. and PA- (A) and Domain-4 (B) specific IgG was measured by ELISA (405nm). Efficacy of PA-Av-$C3d_3$ and PA-Av-alum was further compared when administered s.c. (C) and i.m. (D). Error bars indicate SEM of 3 mice/group.

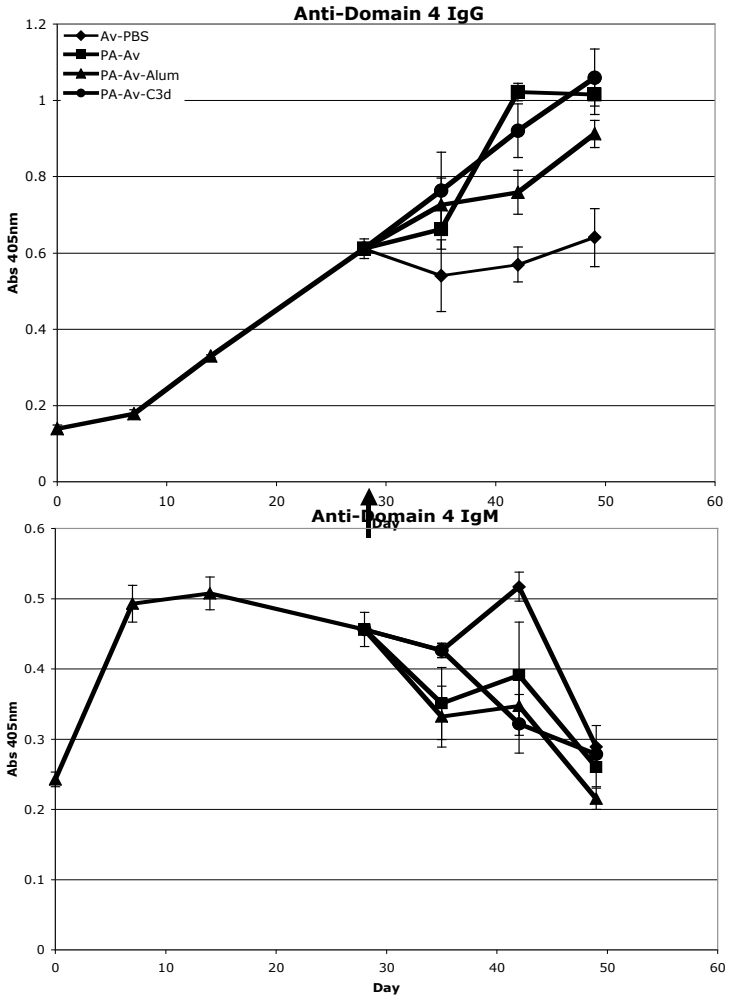


Figure 14: $C3d_3$ does not augment secondary responses. PA-Alum immunized mice were boosted with either Av-PBS, PA-Av, PA-Av-Alum, or PA-Av-C3d and IgG (A) and IgM (B) domain-4 specific responses measured.

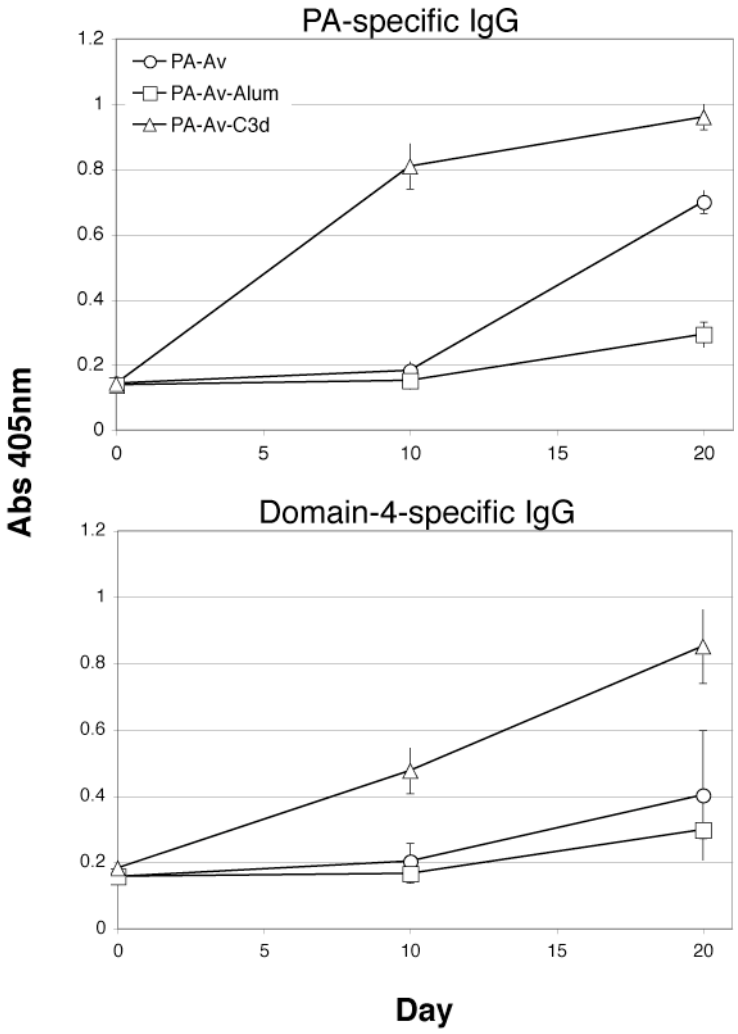


Figure 15: C3d is a functional adjuvant in adolescent mice. 3-week old A/J mice were immunized with PA-Av (○), PA-Av-Alum (□), or PA-Av-$C3d_3$ (△). Data is representative of multiple experiments. (A) PA-specific IgG or (B) domain-4 specific IgG was measured by ELISA (405nm) up to 20 days after primary immunization. Error bars indicate SEM of 2 mice/group.

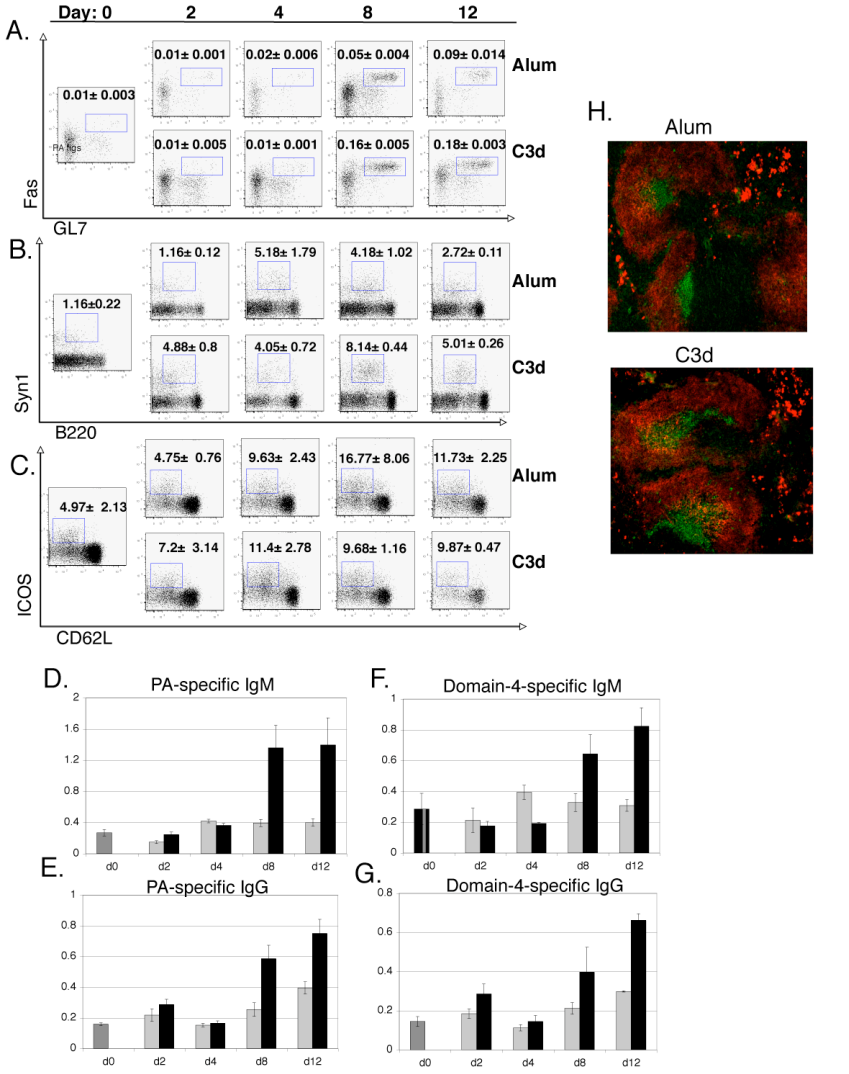


Figure 16: C3d augments the formation of germinal centers and plasma cells over alum. A/J mice were immunized with either PA-Av-Alum or PA-Av-C3d$_3$ at 12, 8, 4, 2 or 0 days prior to analysis. Flow cytometry was used to analyze (A) percent splenic germinal center B cells (CD3$^-$, CD11b$^-$, IgD$^-$, B220$^+$, GL7$^+$, Fas$^+$) of total B cells, (B) percent plasma cells (CD11b$^-$, CD3$^-$, IgD$^-$, B220^{lo}, Syn1^{hi}) of IgD$^-$ B cells, and (C) percent activated T cells (B220$^-$, CD11b$^-$, CD4$^+$, CD62L^{lo}, ICOS$^+$) of CD4$^+$ cells. Representative FACS plots are shown with average and standard deviation of 3 mice/group placed above the respective gate. ELISAs were performed to measure PA-specific IgM (D) and IgG (E) and domain-4 specific IgM (F) and IgG (G) from serum of immunized and naïve mice. Error bars represent SEM of 3 mice/group. (H) Splenic sections were fluorescently stained from alum and C3d-immunized mice with IgM (red) and GL7 (green). Representative images of day 8 are shown.

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CHAPTER 2

Elucidating the role of CD21 in immune responses,

B cell activation, and signaling

Introduction

CD21 (CR2) is the cardinal complement receptor on B cells and binds the C3 breakdown products C3d and C3dg. In chapter 1, the role of C3d in augmentation of B cell immune responses and potential as a potent adjuvant was established. In this chapter, I will focus on the mechanism of CD21 function and its role in B cell activation, homeostasis and signaling.

As mentioned in the general introduction, the CR2 gene encodes two distinct protein products: CD21 and CD35. Reference to CR2 includes both CD21 and CD35; deletion of either CD21 or CD35 is actually a knockout of both receptors. CD35 is the longer isoform of CR2 expression. Both receptors are expressed on B cells and follicular dendritic cells (FDCs); B cells predominantly express CD21, while FDCs predominantly express CD35 (1). The understanding of complement receptor function on FDCs is incomplete. There are two types of immune complexes: those which are complexes of both antigen and antibody and those which have antigen, antibody, and deposed complement products C3b/d. Complement deposition on IC's is thought to abet retention of antigen on the surface of FDCs. This antigen retention promotes the activation of B cells and mediates negative selection of post-germinal center B cells. However experiments

directed at elucidating FDC mechanism and function are difficult and therefore little concrete evidence has been obtained o reveal the purpose of complement receptors on the FDC surface (2-5).

On B cells, CD21/35 is part of the BCR co-receptor complex which also includes CD19 and CD81. The BCR co-receptor complex functions in lowering the threshold of BCR activation; co-ligation of the BCR with the co-receptor complex leads to robust activation of many downstream signaling molecules including PI3K, Erk, Vav, NFκB, PLCγ, and src family kinases. CD19 is the main arbiter in promoting these responses through its long, tyrosine-rich cytoplasmic tail that may either directly interact with the above signaling molecules or facilitate upstream signaling events which leads to their activation.

CD19 is expressed very early in B cell development. CD21, in contrast, is expressed late in B cell development as the cells transit to the periphery. Association of CD21 and CD19 is thought to be constitutive, depending on interactions between the extracellular and transmembrane regions of CD19 and the extracellular region of CD21 (6, 7). However there is a greater abundance of CD19 on the B cell surface than CD21, meaning that some CD19 may not be associated with CD21, but most CD21 molecules are likely associated with CD19 (7). Frade *et al*. reported additional roles for CD21 in the activation of downstream molecules nucleolin, p85 and Grb2. However the region of CD21 responsible for these interactions is unidentified and the

strength of this interaction is brief as the physical association is seen at 30 seconds after stimulation and absent as soon as 1 – 2 minutes after stimulation (8).

The most popular theory describing co-receptor association with the BCR is the recruitment model in which BCR and co-receptor association is mediated through C3d-labeled antigen. In this model, antigen bound by C3d cross-links both the BCR and CD21, bringing them into close-proximity. This also promotes subsequent association of the BCR with CD19 which results in enhanced tyrosine and serine/threonine phosphorylation of many downstream signaling molecules; CD21 is integral in augmenting CD19 proximity to the BCR (9). Accordingly, co-cross-linking of CD21 and the BCR with either monoclonal antibodies or natural ligand increases Ca^{2+} flux by lowering the threshold of activation (10).

Initial BCR signaling events are thought to occur within the lipid rafts. Lipid rafts are cholesterol-enriched, detergent-insoluble membrane fractions in which acylated and phosphoinositide bound proteins congregate to transduce signals. Cheruki *et al* reported that antigen binding is unable to translocate the BCR, CD19, and CD21 into lipid rafts unless it is conjugated to C3d (11, 12). Concurrently, Cherukuri et al also published that B cells lacking CD81, a tetraspanin receptor which has poorly characterized function, show a distinct inability to translocate CD19 and CD21 into lipid rafts (13). Therefore, it appears CD21 is not likely the sole mediator of raft translocation.

The recruitment model of CD21 function implies that CD21 passively prevents association of CD19 with the BCR in the absence of complement-deposed antigen. This passive inhibition may be mediated through several different mechanisms. First, either iC3b or C3d bound on the surface of B cells in the absence of antigen may prevent BCR-CD19 association. One possible mechanism which may explain basal inhibition is that the rapid clearance of iC3b from the cell surface mediated by CD35 may cause internalization of the entire co-receptor complex. iC3b may therefore augment receptor turnover to prevent basal association with the BCR. This theory adds importance to CD35 on B cells even though this isoform of CR2 is expressed at a low level in comparison to CD21. No evidence exists to corroborate augmented CD19 turnover with iC3b ligation to CR2 but we contend that passive inhibition of CD19 association with the BCR is not mutually exclusive with the recruitment model.

A second theory of CD21 function in B cell activation implicates CD21 as an inhibitory receptor that actively prevents CD19 and BCR co-association. In this model, complement bound antigen functions to dissociate CD21 from CD19 to allow BCR-CD19 association and promote canonical signaling. In other words, CD21 functions to actively sequester CD19 away from the BCR therefore preventing activation of the B cell. This last theory is referred to as the sequestration model.

Circumstantial evidence supporting the sequestration model is provided by experiments performed by Marchbank *et al* in which expression of CD21 is prematurely forced in stages of B cell development in the bone marrow (14). This results in very early deletion of B cells and reduction in mature B cell numbers in the periphery (14). This Early B cell deletion could be due to inhibition of the tonic signals mediated by CD19 and the pre-BCR which promote progression and differentiation of Pre-B cells into immature B cell stages (15, 16). Furthermore, CD21 lacks any known myristoylation or acylation motifs that would contribute to CD21 mediation BCR/CD19 association in lipid rafts. Lastly, Chakravarty *et al* reported that depletion of CD21 from B cell membranes reduced levels of the src kinase Lyn which is necessary to promote BCR activation (17). Activation of Lyn is a membrane proximal event which is an early arbiter of CD19 and subsequent B cell activation (18, 19). Therefore, CD21 may inhibit Lyn and CD19 association with the BCR in the absence of C3d-labeled antigen.

In this chapter, I further examine the function of CD21 in promoting B cell responses and examine CD21 function on B cells and FDC's. Utilizing $CD21^{-/-}$mice, I elucidate the role of CD21 in B cell survival, proliferation and the immune response. I will also address CD21 function in recruitment and sequestration models of BCR co-receptor complex signaling.

Results

Comparison of various adjuvants in WT and CD21-/- mice

$CD21^{-/-}$ mice have been reported to have impaired humoral responses and defects in memory responses to T-dependent antigens such as NP-KLH (20). Published studies have used CFA as an adjuvant that activates complement to promote immune cell recognition and subsequent activation. We wanted to ascertain whether the defect in $CD21^{-/-}$mice was intrinsic to the adjuvant used or whether $CD21^{-/-}$ B cells exhibited a general defect in mounting antibody responses. Therefore, we immunized C57B/6 WT and $CD21^{-/-}$ mice with sub-optimal doses of NP_{12}-KLH i.p and surveyed the lambda-NP-specific immune response for 10 weeks and the secondary response for an additional 3 weeks (**Figure 17**). Several adjuvants were used in this study: alum, CFA, C3d, and C3dg (21). C3dg is a predecessor to C3d cleavage and also binds CD21 with high affinity (22).

As previously reported, WT mice immunized with NP-KLH in CFA show a strong primary and secondary immune response. $CD21^{-/-}$ mice mount an appreciable, albeit diminished, response in comparison to WT. This result corroborates previous reports that CD21 abets primary and memory immune responses (9, 20). In addition, the kinetics of this response is altered during the primary response in $CD21^{-/-}$ NP-KLH-CFA immunized mice; serum titers peak later than the WT counterpart.

Comparison of WT and $CD21^{-/-}$ alum-immunized groups shows a similar defect to NP-KLH-CFA in the primary immune response in $CD21^{-/-}$ mice showing that the defect in humoral responses is a general feature of

$CD21^{-/-}$ mice and not an adjuvant-specific defect. It should be noted however, that memory responses seem equivalent between WT NP-Alum and $CD21^{-/-}$ NP-Alum immunized mice indicating that CR2 may not play as significant a role in the secondary response. Though it is possible the mode of alum action may generate memory B cells more efficiently than CFA immunization, our data implicates that CD21-deficiency may only affects primary immune responses. Defects observed in secondary responses by other labs may be more dependent on the type of adjuvant used and less intrinsic to CR2-deficiency.

NP-C3d elicits roughly equivalent secondary or memory immune responses as NP-KLH-alum or NP-KLH-CFA in WT mice, however it does not appear as effective in creating strong primary immune responses. This result is in contrast to data shown in chapter 1 where C3d is a far more robust adjuvant for PA. This difference can probably be best explained by efficient solubility properties of NP-KLH in alum and/or poor C3d labeling of NP-KLH. Also noteworthy, is the immune response observed by NP-C3dg immunization appears equivalent to NP-C3d. This is important as many other studies utilize C3dg as an adjuvant and this proves that our reagent is at least equivalent in immunogenic properties (21, 23). Lastly, NP-KLH-C3d immunization of $CD21^{-/-}$ mice, elicit poor immune responses, showing the requirement of CD21 in C3d based augmentation of B cell responses.

CD21/35 function synergistically on FDCs and B cells to promote efficient humoral response

To determine the role of CR2 on B cells and FDCs, we lethally irradiated WT and $CD21^{-/-}$ mice and reconstituted these mice with bone marrow stem cells to create chimeric mice. These chimeric mice expressed: CR2 on both B cells and FDCs (W-W), CR2 on FDCs only (W-2), CR2 on B cells only (2-W), or complete CR2 deletion on both B cells and FDCs (2-2). These mice recovered for 8-weeks to allow repopulation of their peripheral immune system and were subsequently immunized with 10ug NP-KLH to assay for B cell immune responses (**Figure 18**).

We observed that CD21 functions synergistically on both FDC's and B cells in promoting the primary immune response. NP30 lambda-specific Ig titers show that loss of CD21 on either B cells or FDC's similarly lowers the Ig-response by immunized WT mice. The secondary responses in the chimeric mice show that CD21 may not play an important role in promoting the secondary or memory immune response. Data from figures 17 and 18 both demonstrate normal secondary immune responses in $CD21^{-/-}$ mice.

One possible explanation for the similar decrease in both chimeric mice is that CD21 on FDC's may function in *trans* to interact with CD19 and the BCR on B cells and vice versa. An extension of this theory is that since CD35 is the dominant form of CR2 on FDC's, perhaps CD35 can substitute for CD21 function in *trans*. It is additionally possible that C3b deposition on

antigen serves a similar function as C3d. However, most data indicate that C3b turnover by factor B and D is relatively quick to produce C3d which may indicate that a low level of CD21 is sufficient on FDC's to promote efficient B cell responses. Further studies are necessary to clarify this mechanism.

Whether memory cells express reduced levels of CR2 is unknown. However the memory response may be co-receptor independent and ligation of antigen to high affinity BCR may be sufficient to mount this response. Moreover, memory B cells generally express class-switched Ig and whether IgG, as opposed to IgM, requires co-receptor association to signal is unclear. This is an active area of research but the $CD21^{-/-}$ mice should provide a tractable system with which to study co-receptor association with IgG expressing B cells.

CD21 does not effect formation of long-lived plasma cells

In the bone marrow, Long-lived plasma cells help to maintain protection from previous immunogens. Chimeric mice from Figure 18 were sacrificed approximately 40 days after secondary immunization to quantify the number of long-lived plasma cells in the bone marrow (**Figure 19**). We found that no significant difference in the number of long-lived plasma cells in WT (W-W), $CD21^{-/-}$ (2-2) or mice lacking CD21 on FDCs (W-2).

CD21 expression regulates production of Baff which may lead to altered B cell survival

Since CD21$^{-/-}$ mice showed significant defects in producing primary immune responses, I asked whether there may be a defect in B cell survival that could explain lower antibody titers. Fischer et al reported a defect in survival of germinal center B cells in the absence of CR2 (24). B cell activating factor (BAFF) is a TNF family member that is secreted by various stromal cell types and is essential in promoting proper B cell development and survival (25-27). BAFF has also been implicated in various autoimmune diseases such as SLE, which is also associated with point mutations in CD21 (25, 28). Furthermore, Baff$^{-/-}$ mice were initially reported to have decreases in marginal zone and transitional type 1 and 2 B cells. However, further analysis showed that these cells do in fact exist, but have lower levels of CD21 and CD23 that are generally used as markers in characterizing these B cell subsets (29). Since BAFF is important for GC B cell survival, we asked whether deletion of CD21 affects Baff-R expression or soluble secretion of Baff in the serum (**Figure 20**).

Lymphocytes from 3 WT and 3 CD21$^{-/-}$ mice were isolated from either the blood (figure 20, A) or spleen (figure 20, B). Flow cytometry was performed staining for B220, CD21 and BAFF-R through incubation of fluorescently labeled recombinant murine BAFF (mBAFF-PE). Our data show that CD21$^{-/-}$ B cells in both the blood and spleen bind higher levels of mBAFF-

PE. CD19$^{-/-}$ B cells however bind equivalent Baff-PE as WT (data not shown). This indicates that CD21$^{-/-}$ B cells express higher levels of membrane bound Baff-R, BCMA or TACI. Alternatively, mBAFF-PE may bind more efficiently to CD21$^{-/-}$ B lymphocytes due to decreased endogenous BAFF present within the respective micro-environment from which the B cells were isolated, such that fewer Baff-R are occupied by ligand upon isolation.

Therefore, we assayed levels of soluble BAFF in the serum of naïve and immunized WT and CD21$^{-/-}$ mice. Our results indicate that CD21$^{-/-}$ mice secrete higher levels of BAFF both basally and 7 days after immunization (figure20, C). This result indicates that there is no paucity of BAFF in the serum of CD21$^{-/-}$ mice, therefore CD21$^{-/-}$ mice must express higher levels of either Baff-R, BCMA, or TACI.

Co-ligation of CD21 with the BCR prevents BCR capping and alters co-association of CD19 and surface Ig

Our data shown to this point have shown that defects due to CD21 deficiency mimic defects seen in CD19 knockout mice. There are however stark differences: 1) CD21$^{-/-}$ mice form efficient memory responses, 2) they exhibit no differences in long-lived plasma cell formation and 3) CD21$^{-/-}$ B cells express altered Baff-receptor levels on their surface. Therefore, we inferred that CD21 function on B cells is not simply a redundant function for CD19. Therefore, to determine the role of CD21 in recruitment or sequestration of CD19 from the BCR, we used purified splenic B cells from

MD4 transgenic mice, which have a monoclonal population of B cells specific for hen egg lysozyme (HEL) (30). Utilizing biotinylated HEL and $C3d_3$-bio, we stimulated cells for 0', 10', 30' or 60' with either: HEL-Avidin (HEL-Av), HEL-Avidin-C3d, or HEL+C3d (HEL-C3d) respectively (**Figure 21**). Cells were fixed with and stained with Sav-Cy3 to recognize HEL-bio (red), IgM-fitc (green), and CD19-APC (blue). 3-D images were taken using deconvolution microscopy and representative projection images are shown.

HEL-Av-stimulated B cells efficiently formed caps beginning at 10 minutes and peaking at 30 minutes. By 60 minutes, internalization of antigen was observed as evidenced by the appearance of larger, more intense caps and staining inside of the membrane ring. The efficiency of capping and internalization is likely augmented through the hyper-crosslinking of antigen via the avidin bridge. This hypothesis is supported by the analysis of the HEL-C3d samples in which neither HEL nor C3d is highly aggregated and minimal capping or polarization of IgM, HEL or CD19 was seen. C3d-Av or HEL alone do not cause capping or re-localization of IgM, CD19 or HEL (data not shown). It should also be noted that although a significant percentage of the surface CD19 does participate in capping and subsequent internalization events, complete co-localization of CD19 does not occur.

HEL-Av-C3d stimulated samples however have a much different surface phenotype over the same time-period. No caps form in these samples. Rather a more diffuse polarization occurs. This polarized area does not

internalize and includes almost all detectable surface IgM and approximately 50-60% of the CD19. Polarization is maintained for the duration of the one hour stimulation retaining HEL on the surface of the cell. The idea that C3d retained antigen on the surface of B cells or FDCs was proposed by Fischer et al and later corroborated by work from the Holers lab.

We further quantified the co-association of CD19 and the BCR by calculating the Pearson Correlation coefficient (**Figure 22**), which measures the statistical relationship between two independently assorting variables. In these calculations, a value of (-1) indicates inverse correlation or active non-association, a value of (0) indicates no correlation, and a value of (1) indicates perfect correlation or complete co-association.

From these calculations, we determined coefficients of 0.3 to 0.45 for basal co-association of CD19 and the BCR in non-stimulated B cells. We did not expect a value of (0) in these samples as IgM and CD19 are likely to reside in membrane microdomains which may both inhibit independent movement of these molecules and represent functional basal association for tonic signaling to provide survival signals to resting B cells.

Furthermore, we tested the correlation coefficients of HEL and IgM (MD4 antigen and antigen receptor respectively) and found an average value of 0.98 (data not shown) indicating complete co-association and adding validation to the values discussed below.

Stimulation of MD4 B cells with HEL-Av (black bar) resulted in strong and persistent association of CD19 and the BCR; Pearson values increase to 0.9 after stimulation and remain above 0.8 for the duration of the experiment. However, stimulation with HEL-Av-C3d (grey bar) starkly changes the kinetics and the apparent relationship of CD19 and BCR. At 10 minutes after BCR-CD21 engagement, CD19 and IgM association increased to 0.5. This shows that although co-association is increased over resting B cells, the total surface BCR that is functionally associated with total CD19 is decreased almost 2-fold compared to BCR ligation alone. At 30 minutes, correlation values are similar to HEL-Av-stimulated samples showing that strong association of CD19 and IgM still occurs although the kinetics of this association are slower when CD21 and BCR are co-aggregated. It should be noted that the duration, or perhaps stability, of CD19 and IgM co-association is also decreased at 60 minutes as evidenced by a lower Pearson value of 0.4 in CD19-association. Aggregation of BCR and CD21 independently (HEL-C3d, white bar) also increases CD19 and BCR association. However this seems transient as strong correlation exists at 10 minutes and decreases over time. The shorter life-span of the interaction may be in part due to non-Avidin aggregation of the BCR antigen which prevents the prolonged association seen in the HEL-Av samples.

CD21 prevents association of CD19 with Igβ

To further test models of CD21 function in either the recruitment or sequestration of CD19 to or from the BCR, we performed immuno-precipitation experiments (i.p's) to analyze differences in CD19 association with Igβ (CD79b) in the absence of CD21. Igβ is part of a hetero-dimer which is thought to constitutively associate with the BCR to promote BCR mediated signaling events via its cytoplasmic ITAM motifs.

We therefore purified splenic B cells from WT and $CD21^{-/-}$ mice and stimulated them with 10 ug/ml of anti-IgM $F(ab)_2$ for either 0 or 2 minutes, lysed cells in Brij-96 lysis buffer, performed CD19 ip's and subsequent immunoblot analysis for Igβ relative to WT (**Figure 23, A**). Immunoprecipitation of CD19 in the $CD21^{-/-}$ B cells shows increased association of Igβ. These data likely mean that CD21 may prevent basal co-association between CD19 and Igβ. Alternatively, more CD19 and Igβ may differentially assort into the detergent insoluble membrane fractions such as lipid rafts, suggesting that CD19/Igβ association may be dependent upon the lysis detergent. Therefore, we utilized deconvolution microscopy to address this question.

We performed Pearson Correlation analysis with B cells of WT and $CD21^{-/-}$ mice and measured CD19 association with Igβ and MHC class I respectively (Figure 23, B, C). In resting $CD21^{-/-}$ B cells, we observed a significantly higher co-association of CD19 and Igβ compared to WT cells. As a control, we assayed CD19 and MHC Class I association; CD19 and

Class I are not thought to have a functional interaction on the B cell surface (31). We observed a Pearson Correlation value of approximately 0.2 for both WT and CD21$^{-/-}$ cells (Figure 23, C). Comparing this value to that seen in Figure 2-7 B we conclude that in untreated B cells from WT mice, CD19 does not associate with Igβ. However significant aggregation of these molecules occurs in CD21$^{-/-}$ cells. This is preliminary evidence showing that CD21 may sequester CD19 from the BCR signaling molecule Igβ.

The role of CD21 in BCR mediated signaling

To further our observation that increased CD19 associates with the BCR complex in the absence of CD21, we investigated whether CD21 deficiency has defects on BCR- and CD19- mediated signal events. Therefore, we stimulated purified splenic B cells with 10 ug/ml anti-IgM F(ab)2 fragments and immunoblotted for phosphorylation of CD19 (Y482/513) as well as Erk1/2, and AKT (ser 473) (**Figure 24**).

Comparison of WT and CD21$^{-/-}$ samples showed prolonged phosphorylation of Y482/513 of CD19 in CD21$^{-/-}$ B cells. Phosphorylation of these tyrosine residues have been shown to be crucial for proper activation of PI3K downstream of the BCR (32). We observed similar kinetics for phosphorylation of Erk in CD21$^{-/-}$ splenic B cells (Figure 24, A). However, the degree of phosphorylation appears unaltered in CD21$^{-/-}$ B cells. Analysis of AKT activation by phosphrylation of serine 473 shows no apparent difference between WT and CD21$^{-/-}$ mice (Figure 24, B).

Figure 23 and 24 combined provide a scenario in which CD21 may negatively regulate signaling events that combine to prevent activity of CD19. CD21 appears to inhibit association of CD19 with the primary BCR signaling molecule CD79b and may have an additive role in inhibition of CD19 signaling. This inhibition of signaling may be due to specific activation of a tyrosine phosphatase which dephosphorylates CD19 and Erk. This could possibly explain why AKT is unaffected as it is the substrate of the serine/threonine kinase PDK1 (33, 34). These data provide some preliminary evidence to question the tenets of the recruitment model and lend support to the idea which CD21 may actively sequester CD19 from the BCR.

Discussion

In this chapter, we study CR2 (CD21/CD35) function in both the humoral response and its molecular function on B cells as a member of the BCR co-receptor complex. Using knockout mice provided by the Holers' lab, we find that immunization of $CR2^{-/-}$ mice with T-dependent antigen shows a significant role for CD21/CD35 in propagating primary immune responses specifically. Our data indicate that CD21/CD35 does not play a role in initiating secondary or memory B cell responses. These conclusions differ from previously published reports that CD21 deficiency affects both primary and secondary B cell responses (35). An obvious explanation for the differences in our conclusions could be due to differences in the knockout mice used; the $CD21^{-/-}$ mouse generated by the Carroll lab has been shown to

express a truncated protein product. It is unclear whether this product has any residual CD21 function (35-38). However ignoring the potential differences between the source of knockout mice, our studies have gone beyond previous reports in characterizing CD21 function. All of our results are consistent in showing that CD21's role in only the primary response using various adjuvants (CFA, alum, C3dg and C3d), quantifying plasma cell formation and in creating chimeric mice where CD21 expression is restricted to either FDCs or B cells respectively. Furthermore, data in chapter 1 recapitulates that C3d functions to significantly augment the primary response however cannot elicit stronger secondary responses. This likely indicates that CD21 may function differently on memory cells than on naïve mature B cells in the periphery.

Evidence from several other groups support the notion that CD21 or co-receptor function may differ on memory B cells from follicular B cells. It is well accepted that CD21 plays an important role on GC B cells in promoting survival within the GC (24). However, recent data also indicates that high affinity B cells preferentially accumulate in the GC and undergo more SHM than lower affinity B cells (39). It is therefore possible that the co-receptor complex specifically abets activation, signaling, and survival of B cells with low-affinity BCRs and does not abet function of B cells with high affinity BCRs. Stated more simply, high-affinity BCRs may not require the co-receptor complex to survive, differentiate, and proliferate.

Presuming this idea to be true, ligation of C3d-CD21 would then augment the number of B cells with low-affinity BCRs to participate and survive within the GC. In contrast selection events governing differentiation of high-affinity, non-autoreactive B cells into memory B cells would be unaltered. Therefore, the rapid secondary humoral response is primarily dependent upon the rapid differentiation of high-affinity memory cells into plasma cells.

Our data may further repute this notion as we used congenic C57/B6 WT and $CD21^{-/-}$ mice in our experiments. Therefore, the number of B cells with natural high-affinity NP-specific BCR's should not differ between the WT and $CR2^{-/-}$ mice used in our immunizations experiments. The lowered NP-specific Ig we observe in $CR2^{-/-}$ mice may be due to a specific decrease in activation of low-affinity B cells. High-affinity B cells must differentiate normally in $CR2^{-/-}$ mice providing for the equivalent memory responses. Our data combined with previous evidence suggest that the co-receptor complex may serve prominent function only in the activation of low-affinity BCR containing B cells.

However it should be stated that the defects in $CD19^{-/-}$ B cells may confound this theory. $CD19^{-/-}$ B cells do not elicit efficient primary or secondary humoral responses and have a variety of severe B cell defects, including decreases in B cell numbers, lack of marginal zone and B1 B cells, and inability to form germinal centers (15, 40-42). However recent data from Del Nagro *et al* show that reconstitution of innate antibody in $CD19^{-/-}$ mice

actually rescues germinal center formation (manuscript in preparation). This indicates that the lack of B1 and MZ B cells may precede issues relating to formation of GC. Further studies are necessary to show whether $CD19^{-/-}$ mice are able to mount normal memory responses when GCs are reconstituted.

As mentioned above, CD21 has a well-established role in promoting survival within the GC (24). The altered levels of BAFF-R, BCMA, or TACI on $CR2^{-/-}$ B cells may be to compensate for poor survival of $CR2^{-/-}$ GC B cells (35, 43, 44). BAFF deficient mice have been clearly shown to have normal induction but attenuated function of GC responses (43). FDCs are canonically key regulators of B cell survival, especially within the germinal center, and this is likely mediated through production of BAFF. However it is important to note however that FDCs are not likely the only source of Baff (27, 45, 46). Therefore, secretion of BAFF may not be the sole means by which FDCs promote B cell survival in the GC. Work by the Browning lab and others have shown that B cells can form germinal center-like structures in the absence of FDC (46, 47). It is therefore possible that FDC's may mediate GC B cell survival through complement receptors directly. Antigen retention on FDC's is likely mediated through both Fcγ- and complement receptors (2, 48, 49). The absence of CR2 may lower antigen retention and abrogate the efficiency of B cell interactions with antigen deposed on FDCs leading to decreased B cell survival within the germinal center. Furthermore, CR2

seems to work collaboratively in B cell immune responses as deletion of CR2 on either B cells or FDCs similarly lower antigen-specific antibody titers. These data indicate that CR2 may function in *trans* to promote efficient B cell activation. Stated differently CD21 expressed on FDCs may associate with CD19 molecules on B cells to augment BCR signaling and subsequent survival and differentiation.

The current model of CD21 function is that CD21 recruits CD19 to the BCR through complement-linked-antigen and that CD21 promotes BCR activation as a positive signaling receptor (50). Significant support for this idea comes from lipid raft studies where antigen linked-C3d recruits and maintains CD19 and CD21 in lipid rafts with the BCR for prolonged signaling. One difficulty with these experiments is separation of caveolae, clathrin-coated pits and endocytic vesicles from lipid-rich domains is difficult and the isolation of lipid rafts through sucrose-density gradients suffers from contamination of these other cellular compartments (51-53). Therefore, some of the “lipid-raft” associated CD19 may actually be internalized receptors. Data indicates that antigen-linked C3d retains antigen, BCR and CD19 on the surface of the B cell. Further studies are necessary to determine whether these surface receptors are contained within lipid rafts. Other evidence that is used to support the recruitment model of CD21 function is circumstantial. For example, Dempsey *et al*. showed that ligation of antigen-C3d lowers the

threshold of Ca^{2+} flux however these data do not address the question of CD19 and CD21 are associated (10).

Our data indicate that CD21 may not function as previously thought. We have shown that CD21-deficiency both alters basal association of the BCR and Igβ to each other and prolongs phosphorylation of CD19 and Erk. These results indicate CD21 may be an inhibitory receptor on B cells and lend some support to the sequestration model for CD21 function on B cells. The sequestration model states that CD21 actively inhibits CD19 association with the BCR and inhibits co-receptor mediated signaling. No concrete data has been published to support this model however these data indicate that further experimentation is needed to reliably determine the mode of CD21 function on B cells.

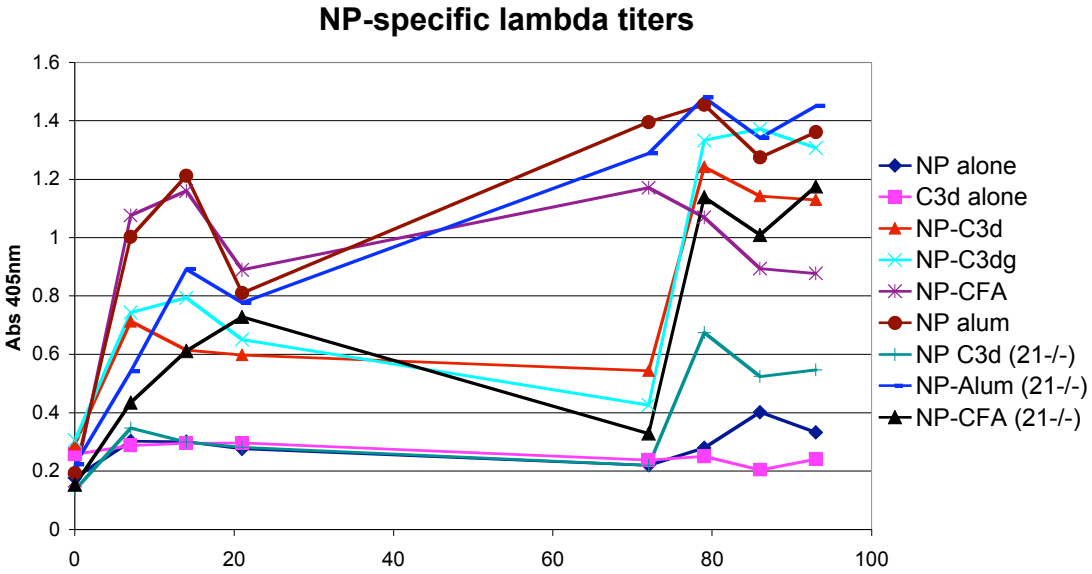


Figure 17: Comparison of various adjuvants in WT and CD21-/- mice. 3 mice/group were immunized with NP-KLH as listed in the legend above right. Responses shown represent lambda specific NP30 serum titers.

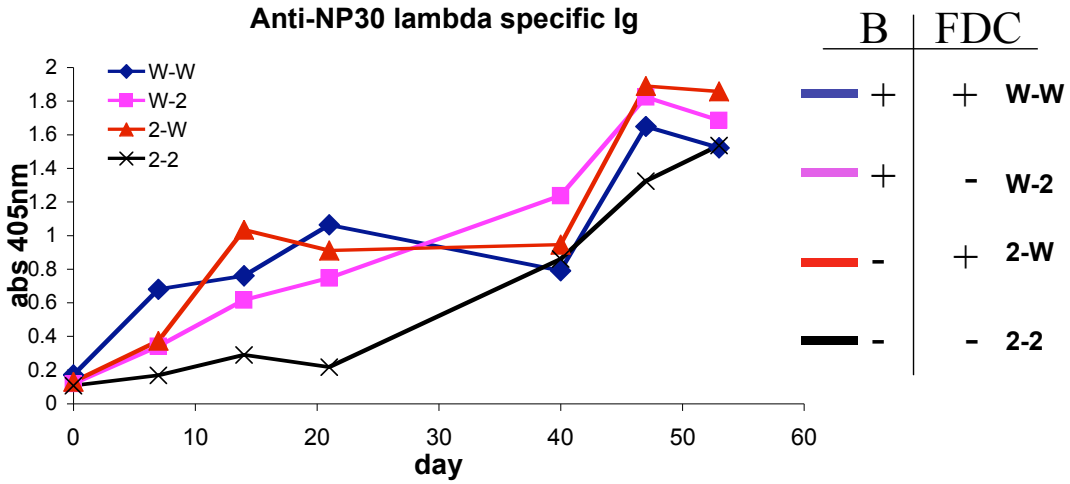


Figure 18: CD21 expression functions synergistically on B cells and FDCs to promote efficient immune responses. WT and CD21-/- mice were lethally irradiated and reconstituted to create chimeric mice which either expressed CD21 on both B cells and FDC's (W-W), CD21 on just B cells (W-2), CD21 on just FDCs (2-W) or were CD21 deficient (2-2). These mice were immunized after 8 weeks of recovery with NP12-KLH I.p and serum taken up to 56 days. NP30-lambda Ig responses are measured.

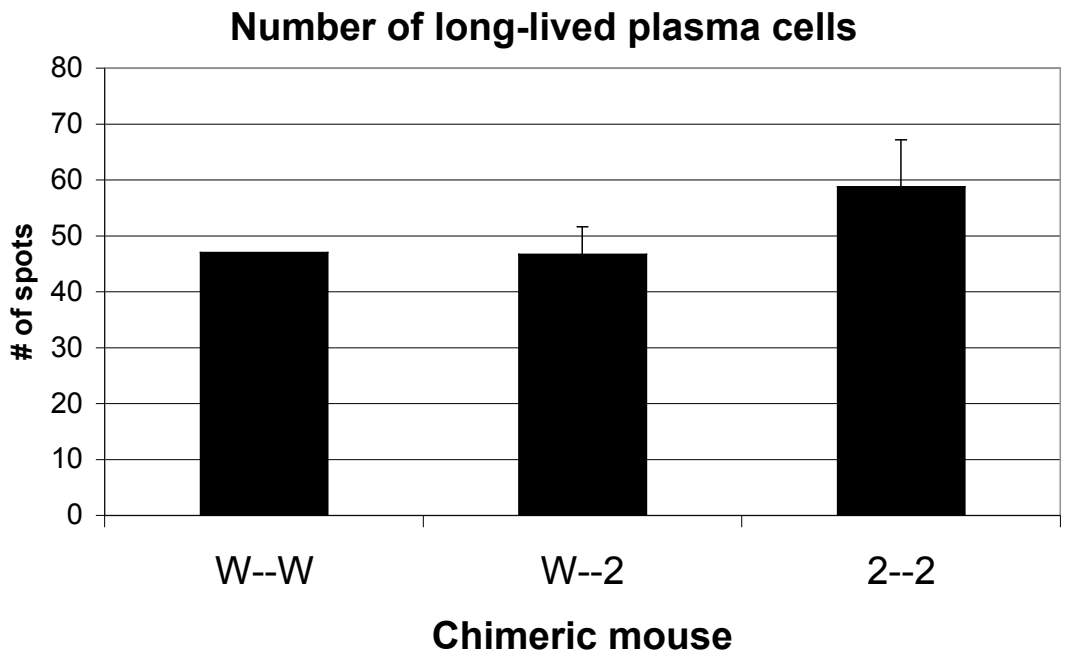


Figure 19: Quantification of long-lived plasma cells in WT, CD21 chimeric and CD21 deficient mice. ELISPOTs were performed on bone marrow isolated from the chimeric mice used in figure 2-2. Error bars represent standard deviation of 3 mice/group.

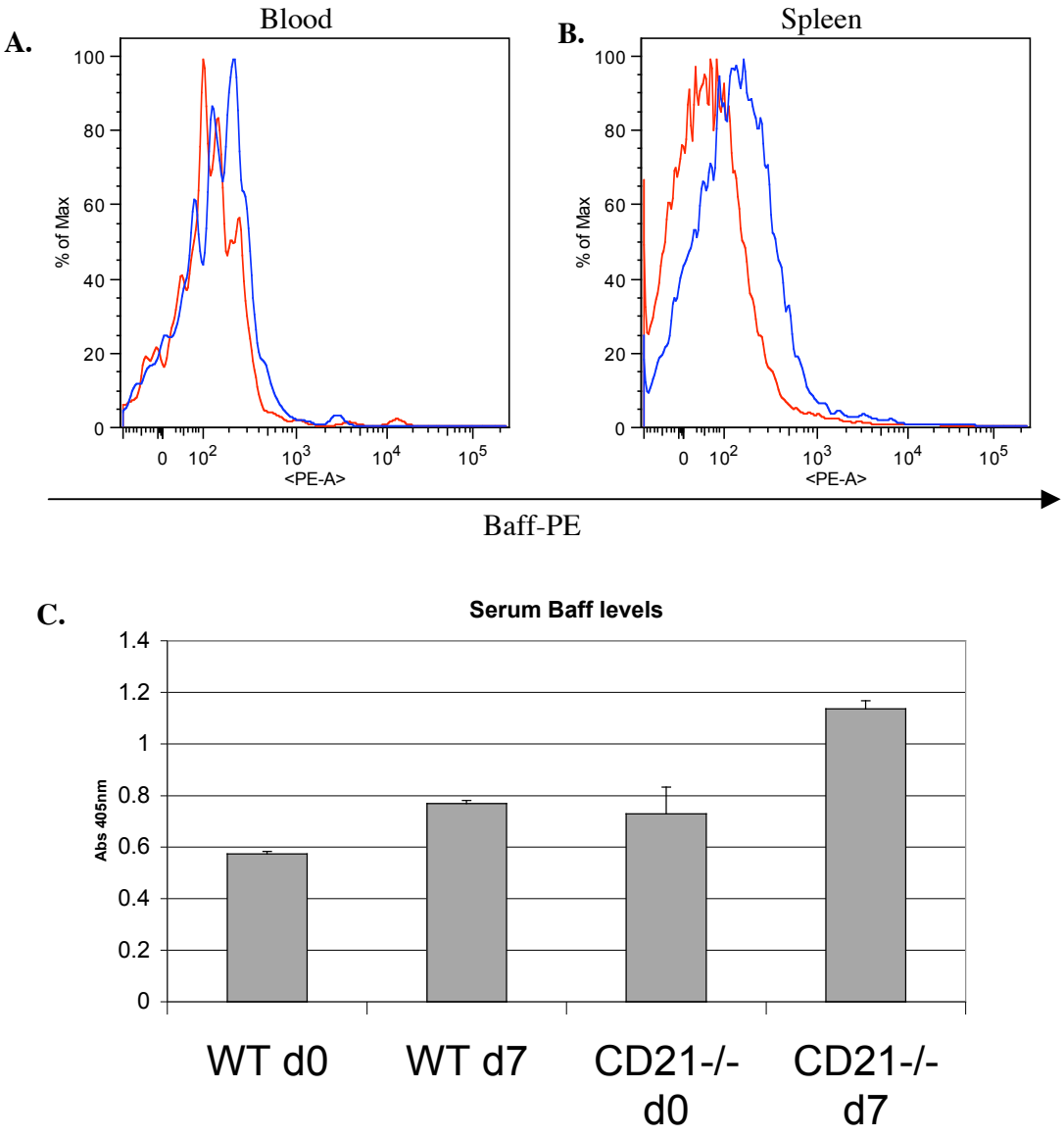


Figure 20: Altered soluble BAFF in CD21-/- mice. WT and CD21-/- lymphocytes were isolated from blood (A) or spleen (B) and stained for B220, CD21, and with mBAFF-PE. (C) 3 WT and CD21-/- mice were immunized with NP_9-KLH in CFA respectively and ELISAs were performed using BCMA-Fc to capture and ascertain levels of sBAFF in the serum from days 0 and 7 respectively.

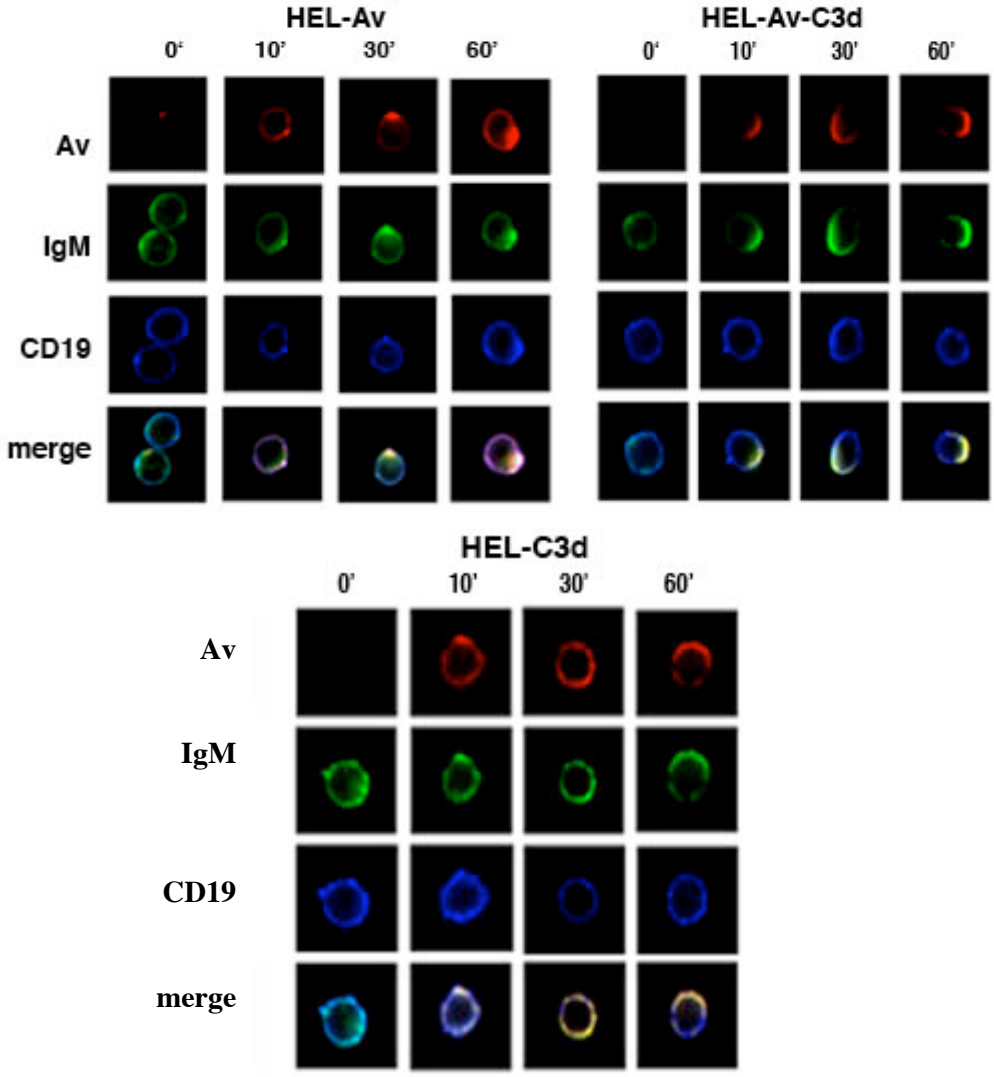


Figure 21: Surface localization of antigen, IgM and CD19 in BCR and BCR+CD21 stimulated B cells. Splenic B cells were isolated from MD4+ B cells. These cells were stimulated either HEL-bio + C3d-bio. After stimulation, cells were plated on poly-L-Lysine coated coverslips, fixed in paraformaldehyde, and stained with IgM (green), CD19 (blue), and Sav-Cy3 (red). Images were taken with an oil-immersion 63x objective and representative images are shown.

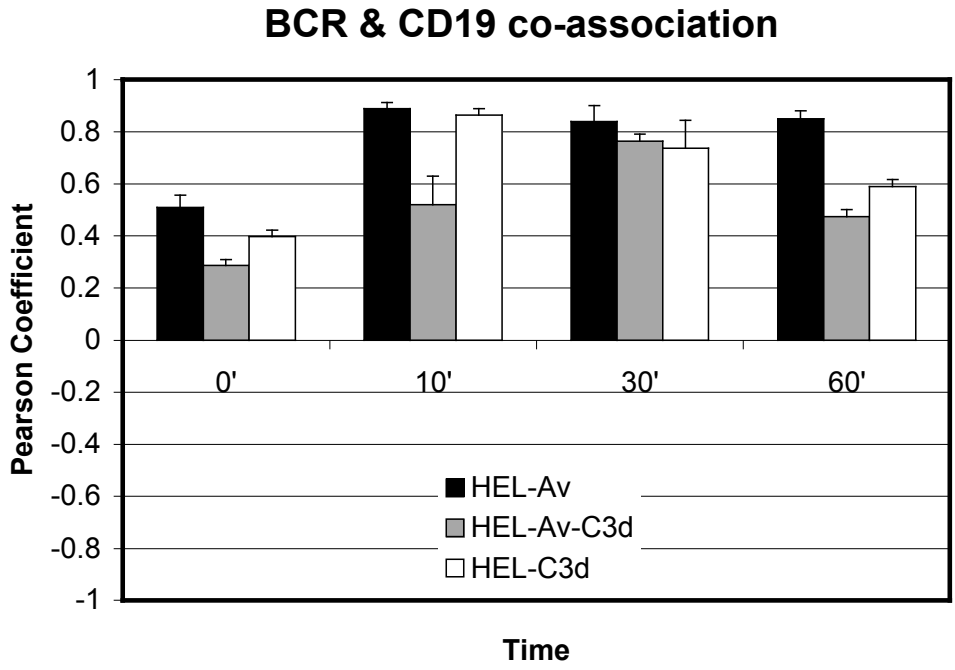


Figure 22: Quantification of CD19 and BCR co-association under BCR and BCR-CD21 stimulation. Pearson correlation coefficients were calculated from microscopy images of 20-25 individual cells and averages shown above.

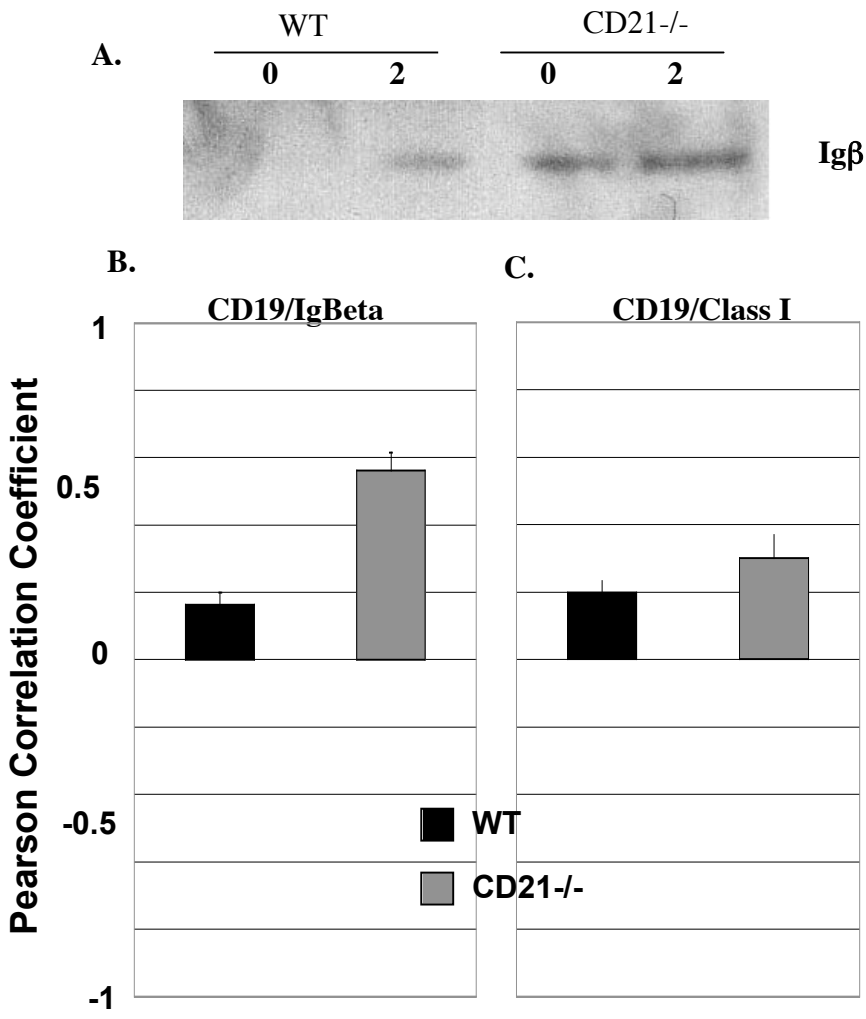


Figure 23: Increased co-association of CD19 and Igβ in CD21-/- mice. (A) Immunoprecipitation of CD19 and immunoblotting for CD79b from WT and CD21-/- purified splenic B cells stimulated with IgM Fab2 fragments for either 0' or 2'. (B & C) Pearson Correlation coefficient values from unstimulated splenic B cells assaying correlation of (B) CD19 and CD79b or (C) CD19 and MHC class I respectively.

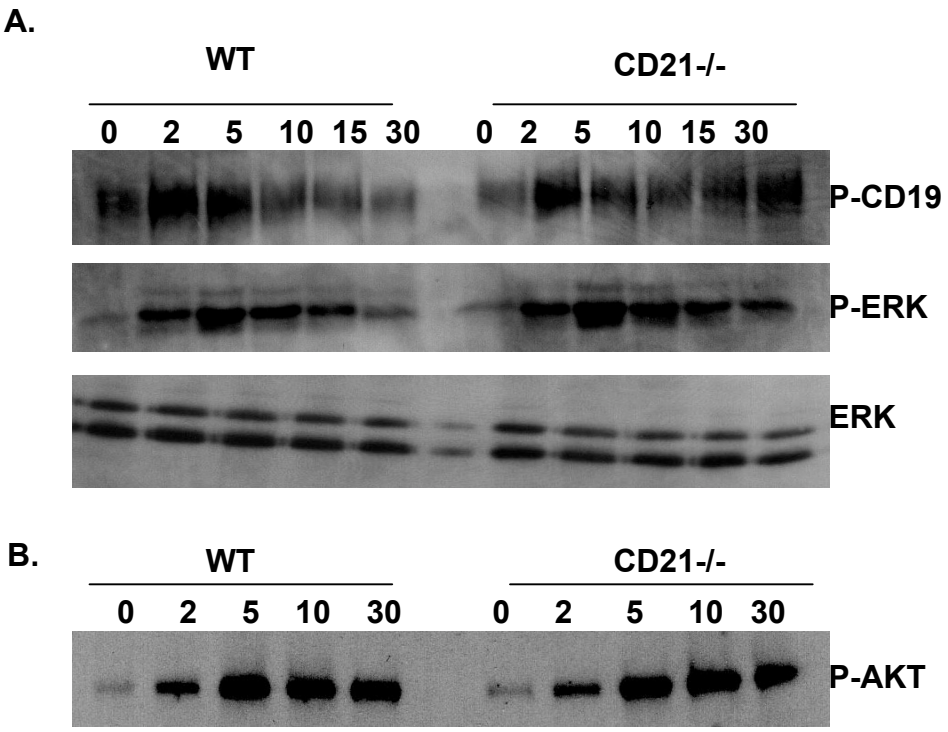


Figure 24: BCR stimulation in WT and CD21-/- splenic B cells. Purified splenic B cells were stimulated with anti-IgM Fab2 fragments for the times listed above. Immunoblotting was performed to look at phosphorylation of (A) CD19, Erk and total Erk and (B) P-AKT ser 473.

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CHAPTER 3

Shp2 functions as both a positive and negative regulator in B cell development, migration and activation.

Introduction

Chapters 1 and 2 emphasized the regulation of B cell activation through manipulation of BCR co-receptors - primarily the complement receptor CD21. Chapter 2 additionally discussed a role for CD21 in coordinating association of the BCR with CD19 and subsequent CD19 activation. CD19 activation leads to a series of signaling events mediated in large part by tyrosine phosphorylation of its cytoplasmic tail (1). The role of tyrosine phosphorylation in regulating B cell function has been well established in many signaling studies. However, the role of phosphatases in regulating co-receptor activation and B cell function is poorly understood. In this chapter, we study the role of the protein tyrosine phosphatase Shp2 in B cell development, activation, and signaling.

Src homology protein tyrosine phosphatases 1 (Shp1/PTP1C) is probably the best studied phosphatase expressed in B cells. A closely related family member of Shp1 is Shp2 (PTP2D). These phosphatases are similar in structure, but appear to play very different functions *in vivo*. Both proteins contain a PTP domain and two src homology 2 (SH2) domains (3). Shp1 is expressed primarily in cells from hematopoietic lineages and serves an important function in the inhibition of lymphocyte and leukocyte activation. A

natural mutation characterized in mice as *motheaten*, has been traced to a point mutation in *Shp1* that causes a truncated non-functional isoform to be produced (4-6). There are two types of *motheaten* mutations, *motheaten* (*me/me*) and *viable motheaten* (*mev/mev*), caused by differences in RNA splicing events of the mutated Shp1 gene on chromosone 6 (7). *Motheaten* mice normally die within 2-4 weeks of birth whereas *mev/mev* die at 8-10 weeks due to severe autoimmunity mediated in large part by inappropriately activated macrophages and leukocytes (8), (9). Viable motheaten mice typically are disheveled in appearance, having malformed ears, patchy fur, and severe dermatitis (10). Beyond obvious roles for Shp1 in regulating macrophage activity, *me/me* mice also have defects in B cell lymphopoiesis, showing abnormal TdT activity and approximately 30% fewer peripheral B cells than WT controls (10). Furthermore, these mice exhibit B cell-dependent autoimmune abnormalities including hypergammaglobulinemia, production of autoantibodies, and eventual glomerulonephritis (10). Finally, analysis of Shp1 in Burkitt Lymphoma tumors shows down-regulated expression in both germinal centers and Burkitt lymhoma cells (11). These data underscore an important role for this phosphatase in preventing inappropriate B cell activation.

Molecular studies have further shown that Shp1 activation is mediated through direct interaction with the ITIM of CD22. CD22 is a well-characterized inhibitory receptor of BCR signaling (12). Studies indicate that

activation of Shp1 by CD22 causes dephosphorylation of the src kinase Lyn, which is necessary for phosphorylation of CD19 and through inhibition of Syk (13), (14). Finally, more recent studies have also implicated the inhibitory receptor FcγRIIB (CD32) as necessary for efficient Shp1 activation (15-17).

Shp2, in contrast to Shp1, is expressed early during mammalian development and ubiquitously in both hematopoietic and non-hematopoietic cell types (18). Mutations of *Shp2* in humans causes Noonan syndrome. Noonan syndrome has several classical hallmarks, most notably: physical deformities of the face and head, mental retardation, congenital heart disease, short stature and chest deformity (19). There are no known natural mutations in *Shp2* in mice, however gene-targeted Shp2-deficient mice are embryonic lethal at day 10.5, showing a major role of Shp2 in fetal development (20). Rag complementation experiments also showed that Shp2-deficient embryonic stem cells were unable to undergo lymphopoiesis, which indicates that Shp2 is likely involved in lymphoid cell development (21, 22).

Shp2 has a well-defined role in the promotion of Erk signaling through the adapter protein Grb2-associated binder 2 (Gab2) and Grb2, though the precise mechanism seems to vary between cell types (20, 23). However, it is unclear what role Shp2 plays in B cells. Several reports show that Shp2 may directly interact with src family kinases to promote Erk activation, though the nature or validity of this interaction is undetermined (20, 24-27). In

contrast to this stimulatory role for Shp2, it has been reported that Shp2 may mediate the antagonistic function of FcγRIIB on BCR activation (17). Data verifing this conclusion have also been reported but were performed *in vitro*, thus the significance of a Shp2-FcγRIIB association *in vivo* is unclear (16). Altogether, it remains to be reported whether Shp2 and Shp1 play redundant inhibitory roles or instead have distinct functions in regulating BCR activation.

In contrast to Shp2's ambiguous role in BCR signaling, it has a clear role in inhibiting interleukin receptor function, where it has been shown to be important downstream of the IL-1, IL-2, IL-3, and all gp130-family cytokine receptors, such as IL-6R (18, 28). Work of several groups has lead to the fine characterization of Shp2-mediated inhibition of Stat3 activation downstream of the IL-6 receptor (28). These studies extol a complex coordinate interaction of both Shp2 and SOCS3 in regulating the phosphorylation of gp130 at position Y759. Phosphorylation of this tyrosine is necessary for Jak2 activation and subsequent Stat3 dimerization and transcription factor activity (29-31).

Shp2 has also been implicated in lymphocyte migration toward both SDF and BLC (26, 32). In one report, Shp2 was shown to inducibly associate with the src family kinase Fyn, which became activated after SDF stimulation *in vitro*. Constitutive association with the adaptor protein c-cbl was also necessary for efficient migration (26). Relatedly, Shp2 has also

been shown to co-associate with FAK family kinases to facilitate integrin-mediated adhesion (33, 34).

In this chapter, I explore the function of Shp2 in B cell development, activation, and signaling. By utilizing conditional gene-targeting technology, we show that Shp2 is required for the proper development of B cells in the bone marrow. In the periphery, we find that Shp2 is required for the maturation of marginal zone B cells and B2 cells and to maintain the proper proportions of B1a and B1b cells in the peritoneum. Analysis of B cell migration shows that Shp2 is necessary for SDF signaling through CXCR4. We also show a novel role for Shp2 in inhibiting the germinal center reaction, perhaps by attenuating CD40 signaling. Finally, we find that Shp2 is necessary for efficient Erk activation downstream of BCR signaling and is additionally necessary for proper tyrosine phosphorylation of CD19.

Results

B cell specific deletion of Shp2

Mice expressing a conditional Shp2 allele were generated as previously described by insertion of Cre recombination recognition (*loxP*) sites into the introns flanking exon 4 of the *Shp2* gene (**Figure 25,** A) (35). To conditionally inactivate *Shp2* in B cells, these mice were crossed to CD19-Cre mice, which express Cre throughout B cell development due to targeted insertion of the *Cre* gene into the *CD19* locus, thus utilizing the endogenous *CD19* promoter (36). Both WT and B cell-specific Shp2-

deficient (Shp2f/f) mice are therefore heterozygous for CD19 expression (CD19+/-). Specific loss of Shp2 protein in B cells was confirmed by immunoblot analysis of CD43-depleted MACS sorted fractions from the spleen (~95% B cells) and total bone marrow cells (figure 25, B). *Shp2* is not deleted in CD43+ cells (composed of T cells and macrophages), but is totally ablated in B cells. In the bone marrow, an apparent splice-variant of *Shp2* is recognized in non-B cells (figure 25, B). Splenic B cells were also purified from 3 WT and 3 Shp2f/f mice and compared for Shp2 expression (figure 25, C). In all Shp2f/f mice, Shp2 is completely abrogated in splenic B cells.

Role of Shp2 in B cell development

Germline Shp2-deficient mice have defects in c-kit signaling, which is considered to be the main reason for embryonic lethality (20). Therefore, we examined the role of Shp2 in early B cell development. Bone marrow was isolated from 8-week old adult mice from WT and Shp2f/f littermates and performed FACS analysis to examine defects or alterations in B cell development (**figure 26**). Shp2f/f mice had an increase in CD43+, B220- cells (figure 26 A, B), indicating a relative or absolute increase in CD11b+ macrophages in the bone marrow. Relative proportions of pro & pre-B cells ($CD43^+$, $B220^+$) appeared normal, however there is a decrease in B220+ CD43- immature cells. This decrease in immature cells is slight but may either represent a developmental block or pre-mature emigration from the

bone marrow. Relatively normal populations of mature recirculating B cells (B220 ^{high}, CD43 ^{neg}) are also present in WT and Shp2f/f mice.

In the periphery, analysis of B220+ CD23+ cells showed a significant decreases in the proportion and absolute number of T2 (IgM ^{high}, CD21^{+}) and mature B cells in Shp2f/f mice (IgM ^{int}, CD21^{+}) (figure 27 B-D). However, Shp2f/f mice have a specific increase in the number of marginal zone B cells by flow cytometry, which is also corroborated by histologic staining for IgM, IgD, and Moma-1 (figure 27 E). These data suggest that Shp2 negatively regulates the formation of marginal zone B cells, and is necessary in regulating the signaling events which promote FOB formation.

Shp2 promotes proper B1 cell development within the peritoneum

B1 cells are located primarily in the peritoneal cavity of mice, but can also be found in the spleen. The cells have distinctive phenotypic characteristics (CD23-, IgMhigh, IgDlow), and peritoneal B1 also express CD11b. The B1a subset is distinguished from the B1b subset by the expression of CD5. Comparison of WT and Shp2f/f peritoneal cells showed a slight increase in the total percent of B cells in the peritoneum (as compared to CD5+ T cells). This increase is due specifically to B1 cells as there is a decrease in the percent of B2 cells based on staining of IgM vs CD23 (**Figure 28**). In WT mice, approximately 2/3 of B1 cells are B1a (CD5 positive, IgM high, CD23 negative) and 1/3 are B1b cells (CD11b positive,

IgM high, CD23 and CD5 negative). Shp2f/f mice had relatively equal proportions of B1a and B1b cells (figure 28 B, C).

B1 cells are thought to be the major source of natural IgM in the serum. IL-6 is an important cytokine that helps to maintain B1 cells in the peritoneum and promote B1 cell function (37). Shp2 has been shown to inhibit IL-6 signaling through either dephosphorylation of Y759 of gp130 or direct inhibition of JAK. Comparison of naïve serum of WT and Shp2f/f showed significantly higher levels of basal IgM (figure 28, D), but normal levels of IgG. This increase in IgM may be due to increased activity of Stat3 downstream of IL-6 signaling in Shp2f/f mice. These data indicate a role for Shp2 in normal B1 cell development and in negatively regulating IgM secretion by these cells.

Shp2 negatively regulates T-dependent immune responses

Due to the observation that Shp2f/f mice have altered peripheral B cell subsets, I asked whether antibody responses may be altered in these mice. Therefore, we immunized groups of WT and Shp2f/f mice with TNP-ovalbumin in CFA and monitored TNP-specific Ig serum antibody over the course of 49 days after immunization (**Figure 29**). We found that both WT and Shp2f/f mice produced equivalent levels of low-affinity TNP26-specific IgG titers - both during the primary response and after the boost at day 28 (Figure 29, A). However, Shp2f/f B cells produced greater amounts of high-affinity TNP3-specific IgG (figure 29, B), which is apparent at day 28 and

continues flollowing the boost through day 49. Figure 3-5, C further illustrates the increased affinity maturation in Shp2f/f mice as measured by the ratio of TNP3 over TNP26.

Affinity maturation occurs within the germinal center (GC) and typically peaks between 7 and 12 days after immunization. However, we did not observe the increased production of high-affinity antibodies until later in the immune response. This observation led us to investigate whether these antibodies were derived from a more efficient GC response and/or increased secretion of antibodies due to enhanced signaling of IL6. Therefore, immunized WT and Shp2f/f mice were sacrificed 56 days post-primary immunization and spleens analyzed for the presence of GCs. Comparison of WT and Shp2f/f splenic sections showed more frequent and larger GCs in the Shp2f/f mice (figure 29 D). The GC B cells were normal in phenotype, expressing lower levels of B220 and high levels of PNA. Furthermore, B cells within the GC upregulated CXCR4, expressed lower IgM and were negative for IgD (38). LN histology showed similar increase in frequency and size of GCs (data not shown). Therefore, continued secretion of high affinity antibodies may be due at least in part to of the sustained GC response in Shp2f/f mice. These data further indicate that Shp2 serves an inhibitory role to promote the attrition of the GC reaction.

Shp2 inhibits proliferation to CD40 and BCR stimulation

The increase in affinity maturation and GC formation observed in Shp2f/f mice led us to address the role of Shp2 in proliferation to both BCR and CD40 signaling. CD40 is a TNF receptor family member that is important in promoting GC formation and B-T cell collaboration (39). Therefore, we performed thymidine incorporation assays to determine the role of Shp2 in regulating proliferation to various stimuli (**figure 30**). The most striking observation was the 3-fold increase in H^3-incorporation in Shp2f/f B cells responding to CD40 stimulation. Furthermore, Shp2f/f B cells had enhanced proliferation to CD40 + IL4 and anti-IgM (Fab_2) + CD40 stimulation. Analysis of IL4 stimulation alone shows that Shp2 does not likely play a regulatory role in IL4 signaling. Stimulation with anti-IgM Fab_2 alone showed a modest increase in thymidine incorporation. Therefore, the augmented proliferation seen with CD40+IL4 and Fab2+CD40 is more likely due to enhanced CD40 signaling in the absence of Shp2. These experiments reveal a novel role for Shp2 in regulating germinal center formation, likely through the inhibition of CD40 signaling.

Shp2 is necessary for efficient B cell migration to SDF

Spleen sections from Shp2f/f mice showed high expression of CXCR4, the chemokine receptor for SDF, within the GC (Figure 31, D). This led us to ask whether defective CXCR4 signaling could contribute to the increase in affinity maturation observed from the immunization shown in

figure 29. Therefore, we used transwell migration plates to test the ability of Shp2f/f splenic B cells to migrate to both BLC and SDF, which are the major chemokine directing B cell homing and residence in the spleen (12, 40-42) (**Figure 31**). We found that Shp2f/f B cells had a specific defect in migration to SDF (figure 31, A). Approximately 60% of the WT B cells migrated through the chamber filter to 750 ng/ml of SDF. However only 30% of the Shp2f/f B cells migrated to the same concentration of SDF (figure 31, A). Defective chemokinesis in Shp2f/f B cells was further illustrated in the SDF control in which equal concentrations of SDF was placed in both the upper and lower chambers of the transwell plate. BLC did not induce migration of either WT or Shp2f/f B cells, which may indicate the low activity of this reagent.

In order to determine whether the migration defect was due to altered expression of CXCR4, we tested surface expression of CXCR4 by flow cytometry (figure 31, B). Shp2f/f B cells expressed lower levels of CXCR4 than WT B cells. The lower expression was not specific to any particular B cell subset (data not shown). Surface levels of CCR5 and CCR7 were unaltered between WT and Shp2 f/f B cells (data not shown), suggesting that Shp2 plays a specific role in CXCR4/SDF signaling.

Shp2 role in BCR signaling

To further elucidate the role of Shp2 in B cell function and activation, we stimulated purified splenic B cells from WT and Shp2f/f mice and

examined downstream BCR-mediated signaling events (**figure 32**). We first examined phosphorylation of Erk 1 and 2, since many reports have shown an important role for Shp2 in promoting Erk activation (43). As expected, Fab_2 stimulation of Shp2f/f splenic B cells showed decreased Erk phosphorylation. We also observed a decrease in phosphorylation of tyrosines 482 and 513 of CD19, which are primarily responsible for activation of PI3K (2). Curiously, we did not observe a change in the phosphorylation of AKT on either serine 473 or threonine 308, indicating that PI3K function was unaltered in the absence of Shp2.

We also checked phosphorylation of Btk, Jnk and p38 and found increased phosphorylation upon Fab_2 stimulation in the Shp2f/f B cells. Analysis of p38 tyrosine phosphorylation was particularly interesting as basal phosphorylation was high in knockout B cells. These data indicate that Shp2 likely inhibits Btk, JNK, and p38 function, while simultaneously promoting Erk activity. This indicates that these pathways may be distinct and may have opposing function in B cell activation.

Lastly, induced tyrosine phosphorylation of STAT3 downstream of BCR activation was determined. Tyrosine phosphorylation of STAT family proteins is necessary for dimerization and subsequent nuclear translocation of STAT proteins (44). Phosphorylation of STAT3 was not observed until 10 minutes post-IgM-Fab_2 stimulation and peaked at 30 minutes in WT B cells. However Shp2f/f B cells do not show any appreciable tyrosine

phosphorylation of STAT3. It is unclear whether Shp2 acts directly to promote STAT3 phosphorylation or is an indirect function of Erk (45, 46).

Discussion

In this chapter, we show novel and important roles for Shp2 in B cell development, activation and signaling. These data clearly demonstrate that the functions of Shp2 are distinct from Shp1 in B cells. However, in contrast to previous speculations, Shp2 is not uniformly stimulatory (47), but appears to act in a receptor-specific fashion to modulate B cell development and function (47).

One of the most striking phenotypes we observed in Shp2f/f B cells was the increased generation of MZB cells. By FACS, we see that these cells express canonical MZ markers and appear to develop at the cost of FOB cells, particularly T-2 type B cells. Total B cell numbers were not significantly altered between WT and Shp2f/f splenic lymphocytes. Furthermore, though the MZ compartment is enlarged in Shp2f/f mice, there are many IgM-high B cells that also inhabit the follicle. Therefore, it is likely that Shp2 contributes to the cell fate decision that determines MZ versus FOB development. Further support for this hypothesis comes from work published by Itoh *et al* who reported that Gab1 expression was restricted to MZ B cells (48). Shp2 is known to interact with Gab/daughter of sevenless family members to promote Shp2 function through Grb2 and the p85 subunit of PI3K (49). Therefore, Shp2 interaction with Gab1 may be necessary to

regulate the formation of MZB's either through promotion of Erk signaling or through selective activation of PI3K. Further experiments are necessary to clarify this mechanism. Analysis of the MZ compartment in Erk1,2 B cell specific-knockout mice would be crucial to determining whether Erk signaling promotes or inhibits MZ B cell development.

Other important players involved in cell-fate decisions for MZ development include Aiolos and Btk (50). In XID mice (in which Btk is nonfunctional) and $Btk^{-/-}$ mice, FOB cell survival and/or generation is impaired (50-52). The data presented in this chapter shows a role for Shp2 in inhibiting Btk phosphorylation downstream of BCR signaling. Therefore, the defect in FOB generation in Shp2f/f mice could, at least in part, be due to reduced activation of Btk. We have yet to analyze Aiolos expression in B cells of Shp2f/f mice.

Work from our lab and others have shown that tyrosine phosphorylation of CD19 on tyrosines 482/513 are essential for promoting PI3K activation (2, 53, 54). However, we observed that Shp2 was necessary for normal phosphorylation of Y482/513 on CD19, but not for serine/threonine phosphorylation of AKT. This finding likely indicates that Shp2 inactivation does not have a dramatic effect on BCR-induced PI3K activation. This postulate does not exclude a role for Shp2 in PI3K activation in a subset of B cells, such as MZBs, or downstream of other receptors.

The data in this chapter also present the first evidence of Shp2 as an inhibitor of the GC reaction. As stated above, Shp2 seems to play a primarily positive signaling function downstream of the BCR, however anti-CD40 stimulation elicits enhanced proliferation of Shp2f/f B cells. This defect is specific to CD40 signaling as proliferation to BAFF, another TNF receptor family member, is intact. Though the signaling mechanisms which mediate CD40 signaling have not been completely elucidated, several studies implicate Jak3 and STAT3 as key players (55, 56). However recent data from Fornek *et al* has shown that T-dependent immune responses are not impaired in B cell-specific STAT3-deficient mice, indicating that STAT3 is not necessary for the GC reaction (57). Furthermore, data from Lundin-Brockdorff *et al* showed that tyrosine phosphorylation of STAT3 and STAT5 is not dependent on Shp2 in T cells (58, 59). Therefore, it is possible that Shp2 may regulate Jak3 kinases directly to curtail GC function in the absence of STAT3, or that Shp2 may regulate the GC reaction by alternate means. Data from other labs have also shown that Btk plays a significant role in promoting the CD40 response through TRAF2 (56). Further work is necessary to determine how Shp2 regulates CD40 signaling pathways. Potential targets will first include Jak family kinases, Btk, TRAF family proteins, Bcl family members and NF-κB.

The importance of Shp2 in GC function may be relevant to several types of B cell lymphomas (11, 60-62). Moreover, abnormal Shp2 function

has already been connected with cases of human leukemias such as CML (63-65). Future studies will apply our understanding of Shp2-dependent signaling to specific B cell malignancies.

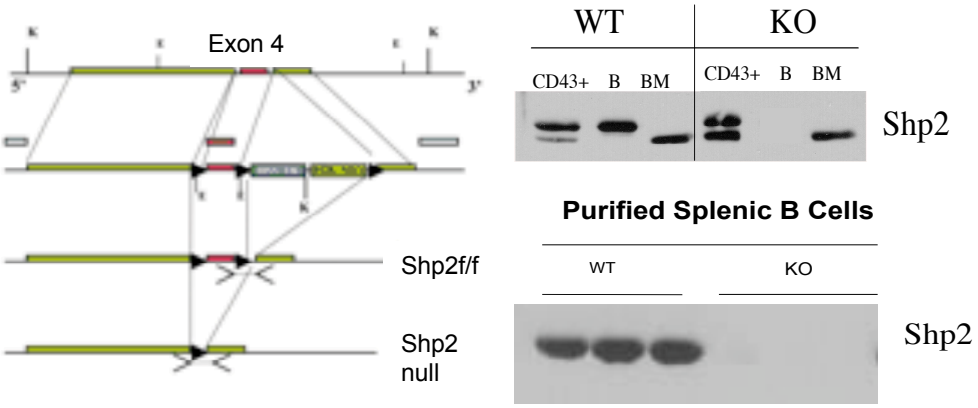


Figure 25: Generation of a B cell specific deletion of Shp2. (a) targeting of Shp2 exon 4 for Cre mediated deletion. (b) Immunoblot showing Shp2 protein expression in CD43+ (T cells and macrophages) and purified splenic B cells. Shp2 expression is not altered in BM though a different splice variant of Shp2 is expressed. (c) immunoblot analysis of Shp2 expression from 3 individual splenic B cell samples.

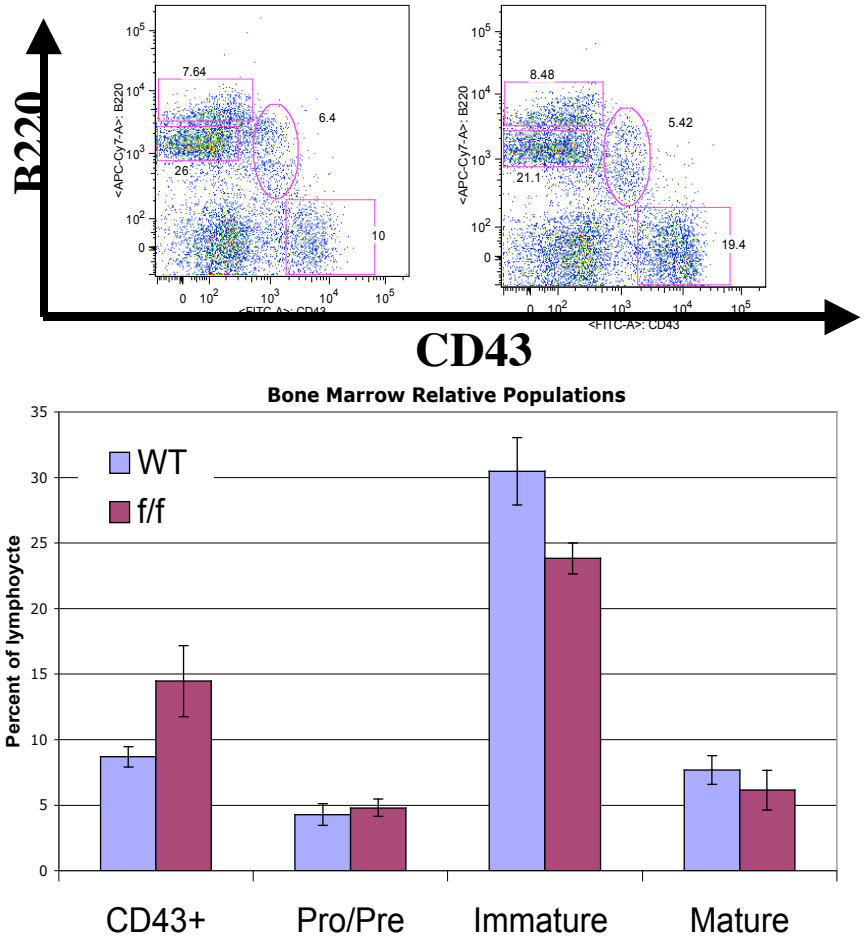


Figure 26: Analysis of bone marrow populations between WT and Shp2f/f mice. (A) Representative FACS plots comparing WT and Shp2 f/f. (B) Standard deviations of 5 mice are shown.

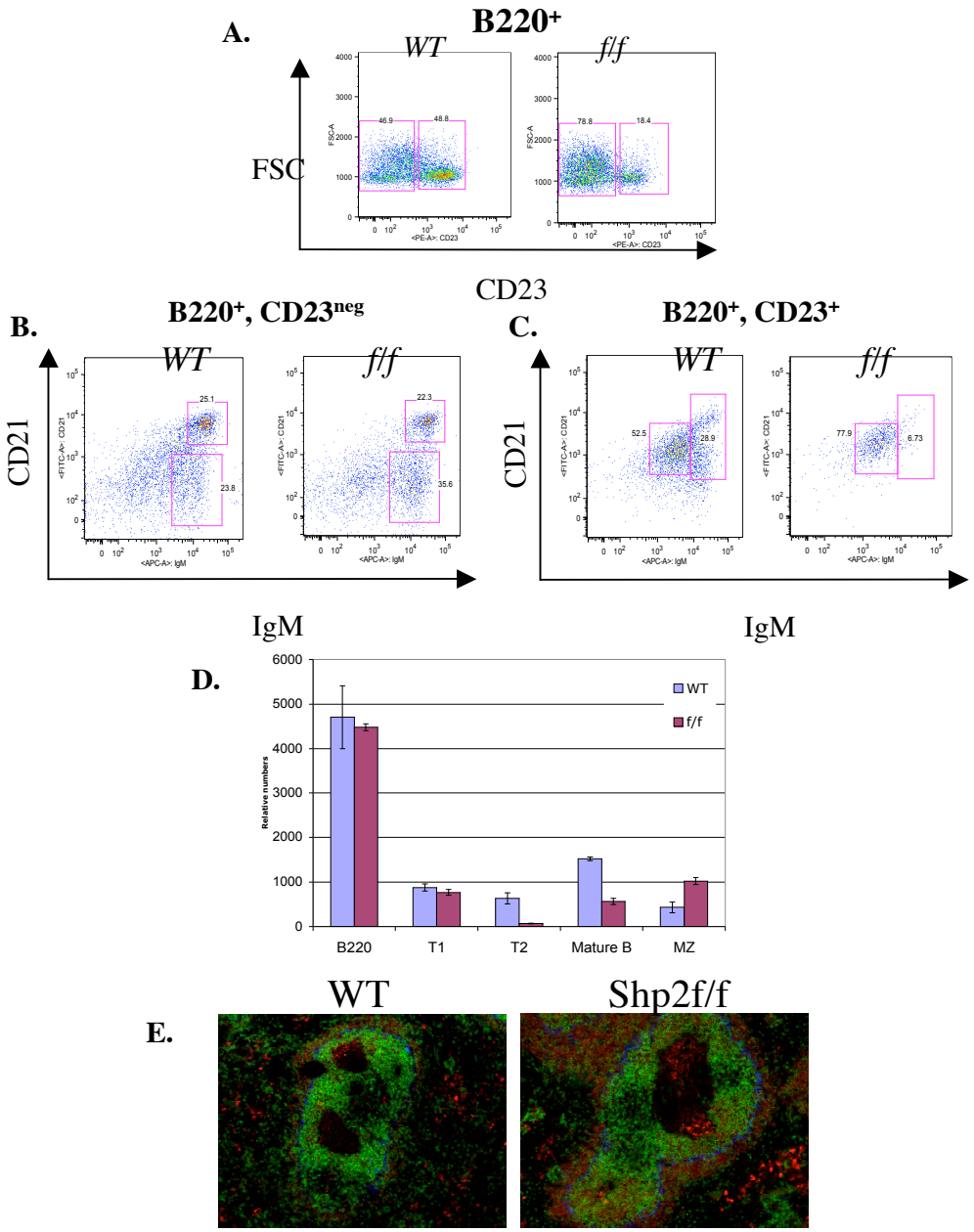


Figure 27: Shp2 promotes follicular B cell development and inhibits marginal zone B cell development. (A) B220+ cells were gated by FACS and analyzed CD23 v FSC. CD23 neg (B) and CD23 + (C) were gated and analyzed to ascertain formation T1, MZ, FOB, and T2 respectively. (D) Relative numbers of cells were obtained by counting number of events per millioni cells. (E) Immunofluorescent staining of splenic sections staining for IgM (red), IgD (green), and moma-1 (blue).

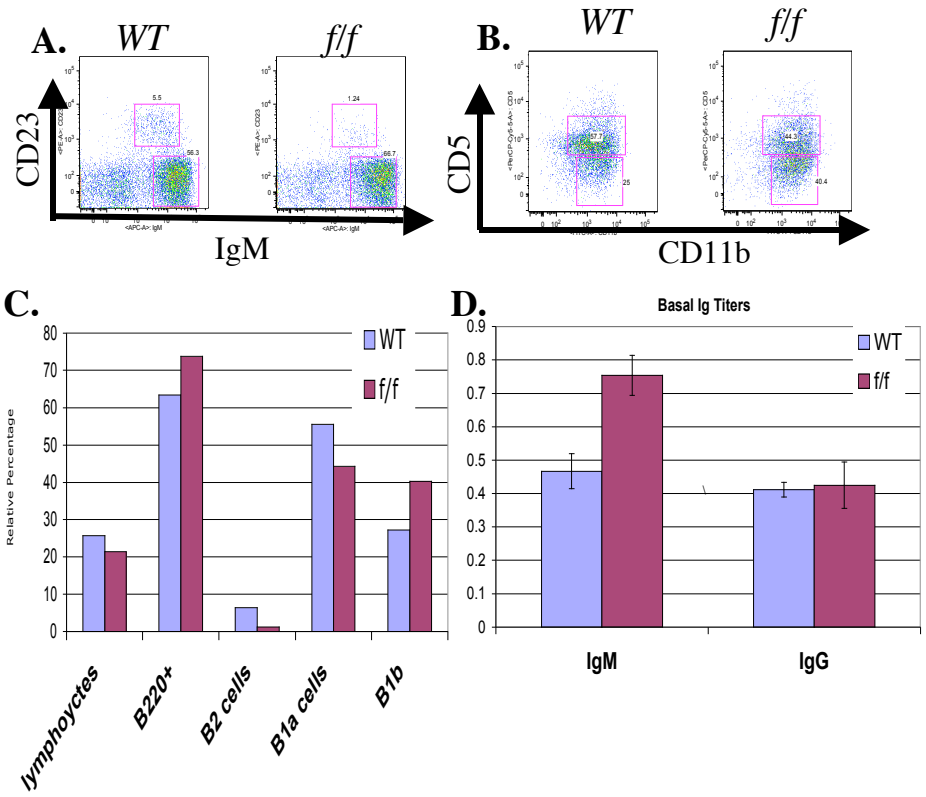


Figure 28: Altered peritoneal B cell populations in Shp2f/f mice. (A) Comparison of IgM v CD21 of total peritoneal cells. (B) IgM high, CD23 neg B1 cells were gated and examined by CD11b v CD5 to differentiate B1b and B1a cells respectively. (C) Percentages of peritoneal cells of 3 WT (blue) and Shp2 f/f (maroon) mice respectively. (D) Serum ELISA to measure naïve IgM and IgG from WT (blue) and f/f (maroon).

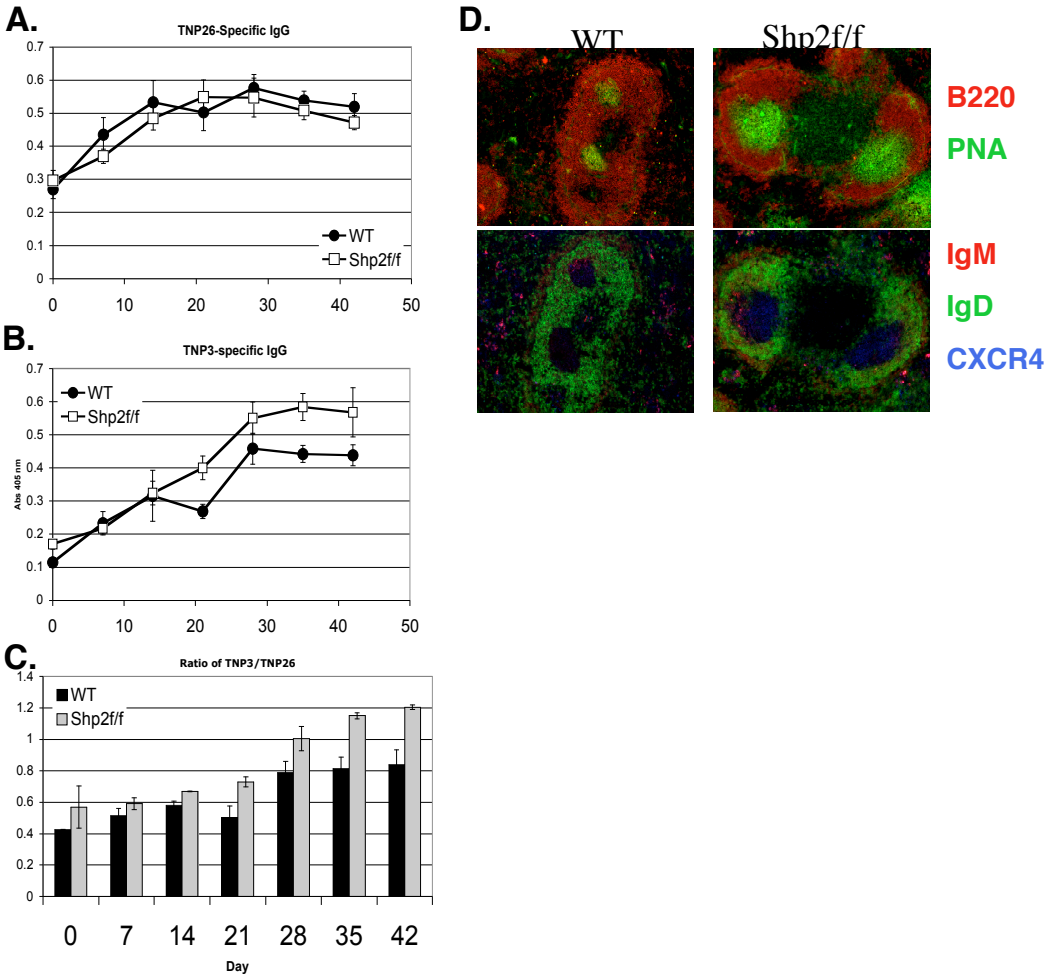


Figure 29: Shp2 negatively regulates GC formation and function. 3 WT and Shp2f/f mice respectively were immunized with 100ug TNP-Ova i.p and serum antibody titers were measured over the time course shown. (A) Low affinity TNP-26 or (B) high affinity TNP-3 IgG was measured by ELISA. (C) Ratio of high to low affinity IgG was calculated to measure affinity maturation. (D) Frozen splenic sections were immunofluorescently stained with the markers labeled to illustrate enhanced GC formation.

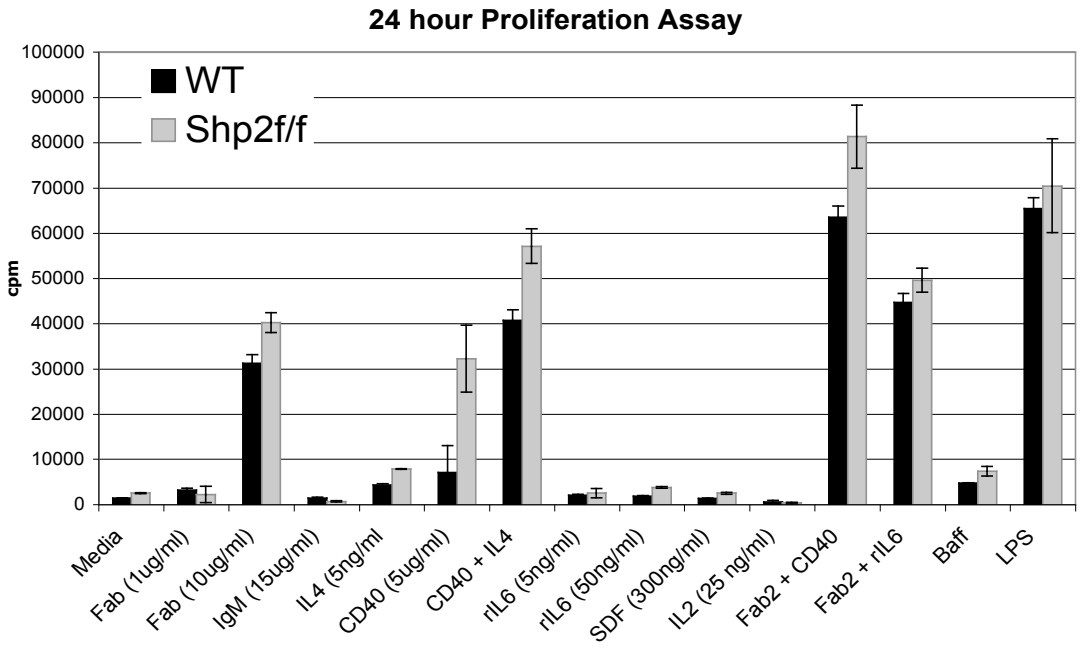


Figure 30: Shp2 negatively regulates proliferation to CD40. Purified splenic B cells from WT and Shp2f/f mice were stimulated with the mitogen shown. Thymidine incorporation was measured after 24 hours stimulation. Error bars shown are representative of triplicate wells.

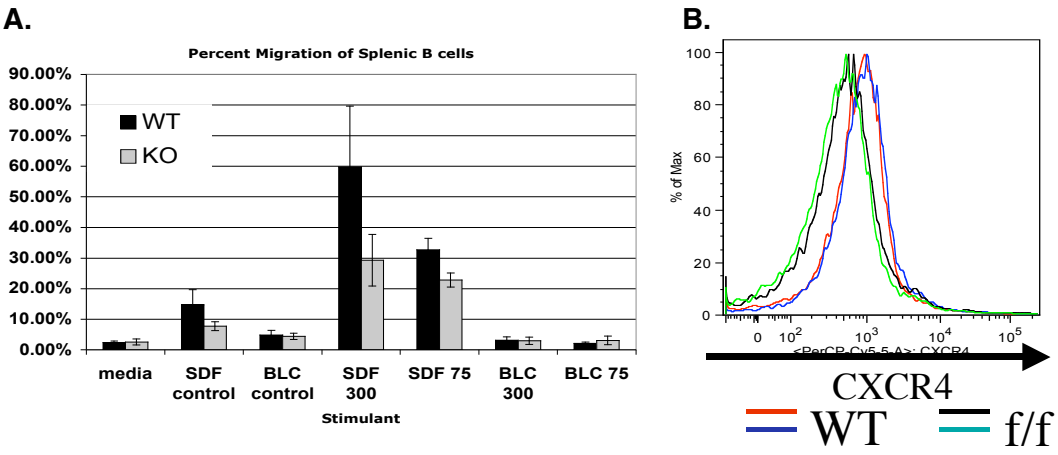


Figure 31: Shp2 promotes migration to SDF. (A) Purified splenic B cells were placed in 6 micron transwell migration plates. Percent of migrated cells was measured from total cells placed into each well. (B) Splenic B cells from 2 WT and 2 Shp2f/f mice were measured for surface expression of CXCR4 by flowcytometry.

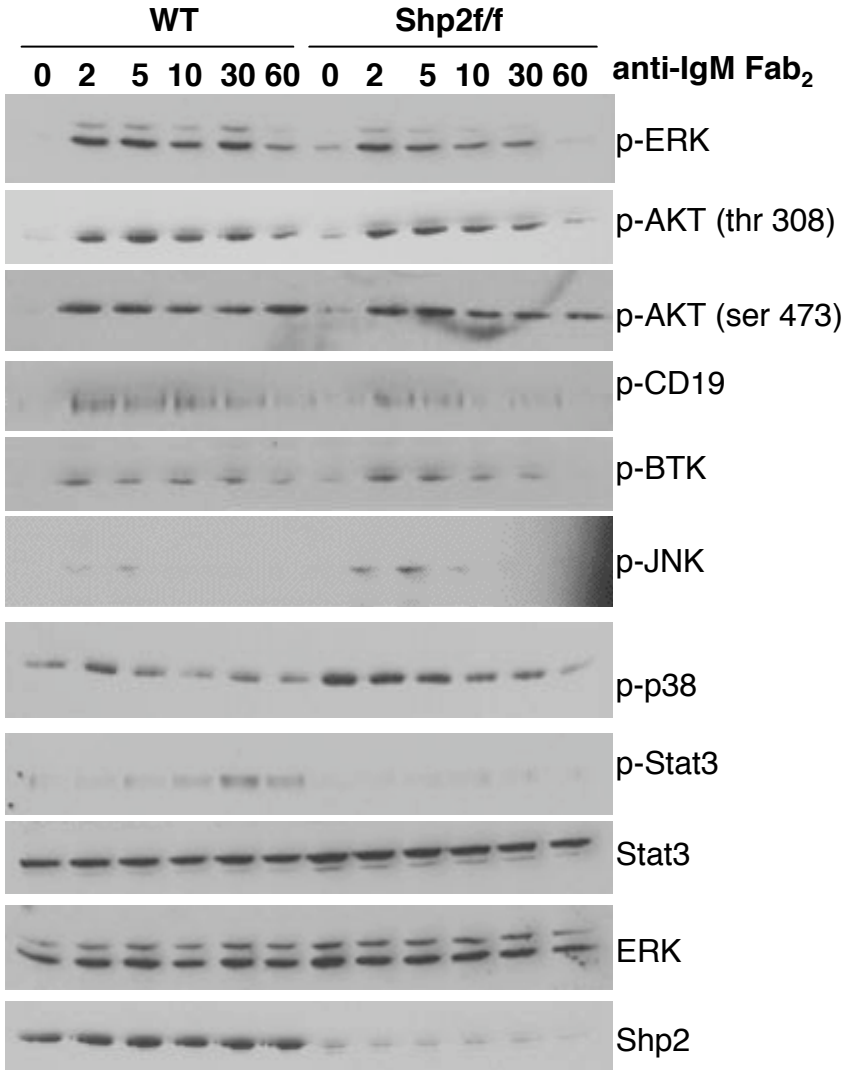


Figure 32: Shp2 is a major regulator of BCR signaling. Purified splenic B cells were stimulated with 10ug/ml of IgM Fab_2 for the time indicated above. Immunoblot analysis was performed to measure phosphorylation of the respective molecules shown.

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Cloning of C3d

The C3d3-bio reagent was created by PCR cloning three copies of murine C3d into the PinPoint Vector (Promega), which allows for *in vivo* biotinylation of C3d at its N terminus in *Eschereichia coli*. Copies of C3d were introduced into PinPoint sequentially using a common 5' primer (5'-ATCGGCGGCGGCGGCGCACACCTGATCGTGACCCCCCGCAAGGCG CTGGGG-3') and distinct 3' C3d primers encoding:

Myc (5'-TGCCAGATCTTCTTCGGAGATCACCTTCTCTTCGGCTCCTCC TCCTCCGGCGGGGAGTGGAAGGACA-3'), Flag (5'- AAGCTTATCATC ATCATCCTTGTAATCGGCTCCTCCTCCTCCGGCGGGGAGGTGGAA GGACA-3'), or His (5'-TCAGTGGTGGTGGTGGTGGGCTCCTCC TCCGGCGGGGAGGTGGAAGGACA-3') epitope tags.

Purification of C3d and conjugation to PA

Over-expressed protein was purified using Ni-NTA beads (Qiagen), dialyzed against PBS, concentrated (Amicon ultra; Millipore) and endotoxin removed (Endoclean; Biosource). PA (LIST Labs) was biotinylated *in vitro* (EZ link Sulfo-NHS-biotin; Pierce) and endotoxin was removed. PA was subsequently conjugated to 2.5 fold molar excess of $C3d_3$-bio via a molar equivalent of Avidin (Sigma).

Mice

8 week old A/J, C3H/HeJ and Balb/c mice were purchased from Jackson laboratories. $CD21^{-/-}$ mice were a generous gift from Holers *et al*. $MD4^{+}$ mice were a generous gift from Goodnow *et ai* All animals were maintained in a pathogen-free environment and handled in accordance with the animal subjects program at the UCSD and Burnham Institute for Medical Research and guidelines set by the NIH.

Immunizations

For PA experiments in chapter 1, three groups of A/J, BALB/c, or C3H/HeJ mice received PA-Av, PA-Av-C3d [both emulsified 1:1 in IFA (Sigma)] and PA-Av adsorbed to alum (Imject; Pierce). Injection volume was 200ul when injected i.p., 100ul when injected subcutaneously (s.c) and 50ul for intramuscular (i.m.) injections. For i.m. injections, PBS was used instead of IFA. All mice were immunized with either 10μg - 20mg of PA as noted in results. All secondary immunizations were given the respective amount of PA in PBS i.p. Immunizations of adolescent mice were performed on groups of 2-3, 21-25 day old A/J mice. These mice received 10ug PA and were injected i.p.

Experiments assaying germinal center, plasma cell formation, and activated T cells were performed by immunizing groups of 3 mice 12, 8, 4, 2, and 0 days prior flowcytometric splenic analysis. Non-immunized A/J mice were used to quantify day 0 analysis.

Immunizations using TNP-Ova or NP-KLH were performed via i.p administration and quantities were given as indicated in text. Immunogens were emulsified as indicated 1:1 in alum or CFA respectively. Peripheral blood serum was taken by retro-orbital bleed before immunization and at 7- to 10- day intervals as stated in the text and sandwich ELISAs performed as specified below.

ELISA

Serum antibody titers against PA or domain-4 were detected using either 10ug/ml PA or domain 4-coated 96 well plates (BD Biosciences) and alkaline phosphatase-conjugated rat anti mouse IgG, IgM, IgG1, IgG2a, IgG2b or IgG3 secondary antibodies (Southern Biotechnology). PNPP substrate (Southern Biotechnology) was used for quantification of absorbance at 405nm using a micro plate reader.

Serum antibody titers against TNP_{26}, TNP_3, NP_{30}, or NP_3 were performed when indicated and specific-antibody titers were measured by use of alkaline phosphatase-conjugated rat anti-mouse antibodies. PNPP substrate was used for quantification of absorbance at 405nm using a micro plate reader.

Purification of Primary Splenic B-cells

Splenic B-cells from 6-12-week-old wild-type BALB/c and CD19 null mice (BALB/c background) were isolated by depletion using the MidiMACS system with anti-CD43 (Ly-48) microbeads (Miltenyi Biotech, Auburn, CA)

as per the manufacturer's instructions. Isolated B-cells were greater than 97% pure by fluorescence-activated cell sorting analysis (anti-B220-PE, anti-CD3-fitc; BD Pharmingen). Cells were kept at room temperature at all times with the exception of the antibody/magnetic bead binding step, which was done at 4 °C for 15 min.

Adoptive Transfer

Recipient mice (C57B6 WT or $CD21^{/-}$) were lethally irradiated (1000 rads) and administered antibiotics (Neomycin 1mg/ml and Polymixin B 0.1mg/ml, Gibco) in the drininking water post-reconstitution. Bone marrow cells from WT and $CD21^{-/-}$ mice were harvested from femur, tibia, and/or humorous and $1X10^7$ bone marrow cells were injected into the lateral tail vein of irradiated mice. To generate chimeras, $CD21^{-/-}$bone marrow cells were injected into WT mice and vice versa. All mice were immunized as indicated eight weeks post-irradiation.

ELISPOT assays

96 well PVDF coated biological assay plates (Millipore) were coated with 10ug/ml NP_{30}-BSA in PBS. Plates were washed with PBS and blocked in 5% BSA in PBS. Bone marrow and splenic purified B cells were prepared as stated above and counted to ensure $4x10^5$ cells were plated per well. Triplicate wells were coated and cells allowed to secrete for 2 hours at 37°C and then washed with PBS + 0.5% tween. Anti-kappa + anti-lambda horseradish peroxidase conjugated secondary antibodies

(Southern Bitoechnology) were used to detect secreted antibodies. Spots were developed by dissolving 20mg AEC tablet (Sigma) in DMF (Fisher) and adding to 0.1M NaAc pH 5.0 solution with 25ul of 30% H_2O_2 (Fisher). AEC substrate solution was added to each well and let to develop until spots were seen. AEC was discarded into appropriate waste container and excess DI H_2O used to quench reaction. Plates were allowed to dry and spots counted with ELISPOT plate reader.

Flow Cytometry

Splenic cells were harvested and red blood cells removed by ACK lysis. Cells were counted and $1.5x10^6$ cells were assayed per FACS stain using a FACS Canto (BD biosciences). In chapter 1, stains for germinal centers included B220-APC-Cy7, IgD-bio, Sav-Pcp-Cy5.5, CD3-PE-Cy7, GL7-fitc, Fas-APC, CD11b-PE (BD pharmingen, eBiosciences). Stains for plasma cells substituted Syn1-PE, IgD-fitc, IgM-APC, and CD3-fitc. T cell activation stains included ICOS-PE, CD62L-APC, CD4-APC-Cy7, B220-APC.

For lymphocyte compartment analysis shown in chapter 3, erythrocyte-depleted splenocytes and cells from peripheral blood, peritoneal cavity and lymph nodes were harvested, washed in FACS buffer (PBS, 1% FCS, .01% Azide), and stained (20-45 minutes on ice) in this buffer with antibodies against IgM, IgD, CD5, CD19, CD23, B220, CD21, CD138 (syndecan.1), CD43, CXCR4, IgG-1/2a/b/3 (BD Pharmingen, San

Diego, CA), CD3, CD11b (eBiosciences, San Diego, CA), PtC-containing liposome reagent or PNA (VectorLaboritories, Burlingame, CA) labeled with PE, FITC, APC, PE-Cy7 APC-Cy7 and in some cases detected with streptavidin-PerCP (Caltag Laboratories, Burlingame, CA), streptavidin-FITC, APC (BD Pharmingen, San Diego, CA) or streptavidin PE-cy7(eBiosciences, San Diego, CA). For compartmental and activation marker analysis, FACS analysis was done with a live or lymphocyte gate on either the FACScalibur or FACSCanto (Becton Dickinson Biosciences, San Jose, CA), based on forward and side scatter.

Histology

Spleens were harvested and placed in OCT (Tissue-Tek) followed by snap-freezing in liquid nitrogen. 7-micron sections were cut and stained as indicated in the results using B220-APC, CD4-fitc, IgM-APC, IgD-fitc, CD35-bio, Moma-1 bio, streptavidin-Cy3, PNA-fitc, and GL7-fitc (BD-pharmingen; eBioscience). Images were taken using 10x objective fluorescence microscope.

Cell Stimulation, Imaging, and Deconvolution Microscopy

Splenic B cells were purified (as stated above) from $MD4^{+}$ mice and let to equilibrate to 37°C for approximately 30 minutes. $2x10^{6}$ Cells were stimulated with 10ug/mL of biotinylated HEL (EZ-link biotinylation kit, Pierce) conjugated to $C3d_3$-bio as indicated or to HEL alone with streptavidin-Cy3. Cells were stimulated for the length of time indicated and

kept at 37°C for the same amount of time. Prior to the end of the respective time point, cells were plated on poly-L-lysine (Sigma) coated coverslips (Fisher), let to adhere for 2 minutes and washed with excess PBS to remove stimulating antibodies. Cells were then fixed with 1% paraformaldehyde for 10 minutes at room temp. Slides were washed and then blocked with 5% FBS in PBS for 30 minutes and subsequently stained with Sav-Cy3, CD19 APC, and either CD79b-fitc, IgM-fitc, or CD21 fitc for 30-60 minutes at roomtemp. Cells were then visualized using a 63x oil-immersion object and deconvolution microscope to take 15-17 images of 1 micron separation. Pearson Correlation coefficients were then quantified with the program Slidebook Pro.

Stimulations and Immunoblotting

Purified splenic B cells were purified as stated above and 2-4 x 10^6 cells were stimulated as indicated with either 10ug/ml of HEL (Sigma) or 10ug/ml of goat-anti-mouse $F(ab)_2$ fragments (Jackson laboratories) for the time points indicated. Cells were spun down at 4,000rpm at 4°C for 3 minutes and resuspended in 1% Nonidet P-40 lysis buffer (Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 10 mM sodium fluoride, 1 mM pyrophosphate, 1 mM EDTA, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 1 mM microcystin).

15-25 μg of total cell lysate were loaded onto 7.5% SDS polyacrylamide gels, and separated proteins were transferred to PVDF (Millipore, Bedford, MA). Primary antibodies for Western blots were rabbit anti-phospho Akt (Thr^{308} and Ser^{473}), rabbit anti-mouse phospho p44/42, rabbit anti-mouse phopsho-CD19, rabbit anti mouse phospho-Btk, anti-mouse phospho-Stat3, rabbit anti-Akt, anti-mouse phospho-p38, anti-mouse phospho-Jnk, and anti-mouse p44/42 (Cell signaling technology). Western blots were probed with horseradish peroxidase-conjugated secondary antibodies (either goat anti-mouse IgG or goat anti-rabbit IgG) for 1 h at room temperature and developed with ECL+ (Amersham Pharmacia Biotech).

Cell Culture, Survival, Proliferation and Cell Cycle Assays

Splenic B cells were purified as described above and isolated into RPMI-1640, 10 % FCS. For 3H-thymidine incorporation assays, cells were added to 96-well polysterene round bottom tissue culture plates (Corning, costar, New York, NY) at 107 cells/mL, 100 μL volume with the stimulants shown and rabbit-mouse-intact IgM (IgM-intact), (high=10 μg/mL) (low=1 μg/mL) (Jackson Immunoresearch, West Grove, PA), recombinant IL-4 (5 ng/mL) (Sigma-Aldrich, St. Louis, MO), anti-CD40 (clone 3/23) (5 μg/mL) (BD pharmingen, San Diego, CA), recombinant human BAFF, rIL6 and SDF-1 (peprotech), and Lipopolysacharide (LPS) (25 μg/mL) (Sigma-Aldrich, St. Louis, MO) for 24 hours, 37°C. To assay DNA replication by ^{3}H thymidine incorporation, 3H-thymidine was added for an additional 12

hours, and incorporation was measured by plate measuring by plate harvesting and scintillation counting. CPM (counts per minute) are displayed as an average of triplicate wells.

For CFSE proliferation assays, CFDA-SE (Molecular Probes, Eugene, OR) was loaded into cells and allowed to label proteins before culture at 5 mM by addition to cells in PBS for 5', and subsequent washing with serum-containing media. Cells were cultured and cell division measured by flow cytometric determination of loss of CFSE intensity (fluorescence in FL1).

Generation of Shp2f/f Mice

B cell specific deletions of *Shp2f/f* mice were generated by crossing *CD19cre*$^{+/-}$ mice to either *Shp2f/f* mice (generated by the Gen Sheng-Feng laboratory) mice to achieve conditional deletion of Shp2 in B cells. PCR was used to assay mouse tail DNA samples for the presence of WT and *loxP* alleles and also for the presence of the *CD19cre* allele. Deletion of Shp2 in B lymphocytes was determined by western blotting for total Shp2 protein. PCR primers used for Shp2 were forward primer 5' ACGTCATGATCCGCTGTCAG 3', and reverse primer 5' ATGGGAGGGACAGTGCAGTG 3'. The UCSD / Burnham Institute Animal Subjects Committee approved all animal experiments.

Migration Assay

For migration assays, erythrocyte-depleted splenocytes resuspended in RPMI-1640, 10 %FCS at 10^7 cells/mL, 100 μL volume, were loaded into upper chamber of Transwell© 24 well plates with 5 μM polycarbonate inserts (Corning costar, New York, NY). 600 μL media alone or media + recombinant BLC (R&D Systems Inc. Minneapolis, MN), or SDF-1α (Peprotech Inc. Rocky Hill, NJ) in varying concentrations was added in the lower chamber. Migration took place for 3 hours, 37°C. Cells were taken from the lower chamber and counted by flow cytometry on the FACScalibur (Becton Dickinson Biosciences, San Jose, CA) for fixed time. Counts for migrated cells were compared to that for “input cells” in an equal volume, and percent migration was determined. Experiments were done using single wells per sample however triplicate mice.

GENERAL DISCUSSION

B cells do not express a complete co-receptor complex until relatively late in B cell development (1, 2). It is not until B cells pass through selective events which delete auto-reactive or non-functional BCRs that they take on the characteristics of follicular B cells (FOBs), marginal zone (MZ), or B1-type B cells. Co-receptor function has been shown necessary for this development in several ways, most notably through knockout studies which show ablation of CD19 or CD21 alters the maturation or formation of these peripheral B cell populations (3-5).

In this dissertation, I explore the function of the BCR co-receptor complex in the immune response. Co-receptor function is primarily assayed by co-ligation of complement breakdown product C3d with the B cell complement receptor CD21 (CR2). The association of CD21 with the cardinal member of the BCR co-receptor complex, CD19, is constitutive and genetic ablation of CD21 from mice causes diminished B cell responses and function (6), (5). Current theories concerning the mechanism of C3d function suggest that C3d recruits CD19 in proximity to the BCR to promote the signaling events that dictate B cell activation, differentiation, and proliferation (7, 8). The data presented in chapters 1 and 2 challenge some of the previously accepted roles for CD21.

Our data corroborate previous reports that ligation of CD21 with C3d abets primary immune responses (9, 10). Using anthrax as a model to test

the adjuvant properties of C3d, we clearly show that specific ligation of CD21 and the BCR by PA linked C3d enhances antibody responses to PA compared to the prominent new generation vaccine being tested for human use – rPA_{102} in alum. Though it remains to be explicitly tested, the rapid and enhanced antibody production from PA-C3d immunization appears able to provide acute protection against anthrax spore challenge. Previous work from Cheruki et al has shown that co-engagement of the BCR and CD21 augments CD19 recruitment to lipid rafts to potentiate BCR signaling (7). However co-engagement of these surface receptors also further augments class-switch recombination, GC formation, and plasma cell differentiation.

It is unclear whether the augmented B cell responses seen with antigen-linked-C3d are due to specific activation of B cells. C3d immunization does not significantly alter T cell activation more than other adjuvants such as alum. C3d does not appear to directly effect T cells. In mice, CD21 is expressed on both B cells and FDCs (11). The augmented GC formation and antibody secretion in the spleen may be at least in part due to antigen-linked-C3d deposed on the surface of FDCs. FDCs reside within the B cell zone of the follicle and are thought to promote both B cell survival, the GC reaction and B cell selection (12). The origin and development of FDCs is ambiguous however CD21/CD35 expression on the FDC surface is thought necessary for proper maturation and function of

these cells (13, 14). It is clear from our data that optimal B cell responses require coordinate expression and function of CD21 on both B cells and FDCs. Furthermore, our data indicate that deletion of CD21 increases levels of soluble BAFF. BAFF is a B cell survival factor thought to be secreted by stromal cells in the spleen, most likely by FDCs (15, 16). FDCs have a well-characterized function in promoting B cell survival and secretion of BAFF may be one of the ways in which FDCs accomplish this function (17). The elevated soluble BAFF produced in $CD21^{-/-}$ mice may be a compensatory mechanism to promote survival of B cells in $CD21^{-/-}$ mice. Alternatively CD21 expression may negatively regulate production of BAFF. Further experiments are necessary to distinguish between these theories. Altogether, our data indicate C3d and CD21 may serve dual functions in activating FDCs to promote B cell activation, and on B cells to promote BCR and/or BCR co-receptor function.

In contrast to previously published reports, our data clearly indicate CD21 has a minor or no role in promoting secondary immune responses (5, 18). Secondary immunization of $CD21^{-/-}$ mice with differing antigens and adjuvants shows equivalent antibody titers to WT counterparts. Even specific ligation of CD21 during secondary immunization is unable to augment antibody production in this regard. Furthermore, long-lived plasma cells are unaltered in $CD21^{-/-}$ mice after immunization. This indicates that CD21 – and possibly the entire BCR co-receptor complex

(CD19, CD81, Leu13, and CD21) – may function differently on memory and naïve B cells. Though further experiments are necessary to confidently assert this mechanism, I we believe these data lend support to a model in which high affinity BCRs may not require co-receptor function to initiate differentiation or BCR signaling whereas B cells with low-affinity BCRs utilize the co-receptor complex to participate in B cell immune reactions.

The current dogma explaining how CD21 exerts its function is through recruitment of CD19 to the BCR. This model is supported by compelling although indirect evidence. Alternative models for CD21 function have been given little consideration to date. The sequestration model suggests that CD21 may actively sequester CD19 away from the BCR in the absence of antigen. This is not exclusive from passive inhibition models that state inactive or non-antigen-linked C3d may prevent association of CD19 and the BCR in the absence of antigen. Using deconvolution microscopy and biochemistry, we provide compelling evidence that suggests that CD21 may in-fact, actively prevent CD19 and BCR association during B cell stimulation and activation.

Though I have not focused upon the role of co-receptors in the development of peripheral B cell subsets, it has been well established that CD19 and CD21 knockout mice have defects in formation of MZ and B1-type B cell differentiation. Ablation of CD19 completely abolishes the MZ and B1 cell populations. Deletion of CD21 reduces these populations

similarly to the CD19 knockout however is less severe (3-5). Further analysis of these phenotypes indicates that PI3K to be a major player in promoting development of these cell types; deletion of PI3K, PI3K subunits, or PI3K effectors similarly reduce or abolish differentiation of MZ and B1 cells. Furthermore, point mutation of CD19 tyrosines 482 and 513 which are required for CD19 mediated PI3K activation reproduce the phenotypes of the CD19 knockout and ablate MZ and B1 cell development. However PI3K is not the sole determinant of the development of these cells. We show that the protein tyrosine phosphatase Shp2 is an important regulator dictating the development of these cell types *in vivo*.

By creating a B cell specific-deletion of Shp2 using Cre-*lox*p technology, we find that Shp2 inhibits marginal zone B cell development and promotes FOB cell differentiation. From purification of CD43 negative B cells, we see that PI3K signaling is unaffected based on our findings that serine/threonine phosphorylation of AKT are unaffected. Shp2's primary role in many cell types appears to be in promoting activation of Erk through the Gab/Grb2 signaling complex (19). Our findings may indicate that Erk may be more important than previously expected in promoting proper B cell development. Alternatively, our results bring to light novel roles for Shp2 such as augmented Btk and p38 phosphorylation which may also contribute to the altered peripheral B cell development. Further studies are

necessary to elucidate Shp2's role in differentiation of MZ, B1 and FOB cells.

As eluded to above, BCR co-receptors are crucial in propagating B cell immune responses, specifically in the differentiation of B cells into GC and plasma cells – $CD19^{-/-}$ are unable to form GCs and also mount poor primary and secondary immune responses (3). Our data indicate that Shp2 is also crucial in regulating the GC response, specifically as a negative regulator of the GC. Further research is necessary to discover the substrates of Shp2 that may be the effectors in inhibition of the GC are necessary. All these data together appear to place Shp2 as both a promoter and inhibitor of BCR co-receptor complex function and may be regulated independently of co-receptor complex activation.

The work presented throughout this dissertation advances our understanding of B cells and B cell function in several novel ways. First, I have further elucidated the convoluted relationship between the innate and adaptive arms of the immune response. Secondly, we have advanced our understanding of the BCR co-receptor complex in both the functional outcomes of co-engagement and the mechanisms of co-receptor complex action. Finally, I am the first to address the roles of Shp2 in B cell development, immune responses, and signaling and to show that Shp2 is both a positive and negative regulator of B cell development and immune responses.

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