THE ROLE OF BAFF OVEREXPRESSION IN THE LOSS OF ANTI-DSDNA B CELL TOLERANCE

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2010

This manuscript has been read and accepted for the
Graduate Faculty in Biology in satisfaction of the
dissertation requirement for the degree of Doctor of Philosophy

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Abstract

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Overexpression of BAFF is believed to play an important role in Systemic Lupus Erythematosus and elevated levels of serum BAFF have been found in lupus patients. Excess BAFF also leads to overproduction of anti-dsDNA antibodies and a lupus-like syndrome in mice. In the present study, we use mice transgenic for the R4A-Cμ (IgM^a) heavy chain of an anti-dsDNA antibody, to study the effects of BAFF overexpression on anti-dsDNA B-cell regulation. We observe that overexpression of BAFF promotes anti-dsDNA B cell maturation and secretion of antibody and enriches for transgenic anti-dsDNA B cells in the marginal zone and follicular splenic compartments. In addition, our data suggests that BAFF rescues a subset of anti-dsDNA B cells from a regulatory checkpoint in the transitional stage of development. The subset of B cells identified as transitional type 3 (T3) subset present in R4A-Cμ and R4A-Cμ/BAFF mice, exhibits an anergic phenotype and contains a high frequency of anti-dsDNA B cells. Our findings suggest that BAFF may enhance survival of this subset and promote anti-dsDNA Ab secretion in synergy with environmental stimuli such as CpG.

Acknowledgments

I would like to express my deepest gratitude to my mentor Dr. Linda Spatz who has made an enormous investment of patience, time and intellectual energy for over five years to enable me to become a scientist. You have unstintingly allowed me to mine the wealth of knowledge you possess, and taught me how to think critically, how to take a broad view of scientific problems, how to analyze results and write in a clear fashion. These and many other qualities I have learned from you are the qualities that will serve me for the rest of my life both in science and in my private sphere.

I would also like to thank the members of my committee: Dr. Laurel Eckhardt, Dr. Christine Grimaldi, Dr. Paul Gottlieb and Dr. Mark Pezzano for bringing their diverse perspectives into my research and providing constant encouragement, Alice Mumbey for her precision, consistency and speed with which she manages our vast colony of transgenic mice, Mark Pezzano and Masako Osada, our neighbors next door, for giving me technical advice, sharing their scientific knowledge, and for making their laboratory available for my use, Jeffrey Walker for spending countless hours sorting my cells and helping me with flow cytometry, Pragya Yadav and Rita Lewis for creating an intellectually stimulating environment in our laboratory. Pakeeza Alam and Stefan Kantrowitz, my students, who have worked hard on my projects, and who have since have embarked on successful careers as medical professionals, and Harry Acosta and the

staff of the City College animal facility for taking care of the smallest and most important subjects of my research, The New York Chapter of the Arthritis Foundation for funding part of my research and inviting me to give a presentation at the annual Paul Klemperer award at the New York Academy of Medicine and the organizers of the American Association of Immunologists conferences in 2006 and 2008 and Keystone Symposum in 2010 for giving me an opportunity to present my work.

I would also like to thank Dr. Susan Kalled at Biogen, Cambridge, MA, for providing us with the C57BL/6 BAFF Tg mice.

My research was supported by NIH/NCRR/RCMI Grant # 5G12 RR03060-24 and by NIGMS/SCORE Grant # SO6 GM 08168. A grant from the City University of New York from the PSC-CUNY Research Award Program also aided this work. In addition, I was supported in part by a grant from the New York Chapter of the Arthritis Foundation

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Chapter 1: Background

1.1 Antibody structure and the B cell receptor

B cells play a crucial role in adaptive immunity by generating circulating antibodies (Ab) that bind and neutralize antigen, opsonize or prepare antigens for clearance, or elicit complement mediated lysis. A large variety of pathogens that pose a threat to the organism requires that the antibody repertoire must be considerably diversified, and it is estimated that 10^{11} different specificities of Ab are present in the B cell repertoire (1). In order to understand how this diversity is achieved we need to take a closer look at the building blocks that make up a mature immunoglobulin (Ig) molecule, at the genomic organization of the Ig loci, the variety of gene segments that make up each locus, and the mechanisms whereby these components are assembled and modified at the level of DNA and RNA. The simplest Ig molecule consists of two identifal light chains (LCs) covalently bound to two identical heavy chains (HCs). The two HCs are in turn covalently linked to one another by disulfide bonds, ultimately resulting in one molecule composed of two Ig HCs and two LCs. The N-terminal portion of both Ig HCs and LCs form the variable region of the Ab responsible for binding to an antigen (Ag). The C-terminal portion of the Ab (also known as the constant region) is not involved in Ag binding but has important effector functions because it interacts with Ab receptors known as Fc receptors on some cells. The constant region also determines the Ab class: μ, δ, γ, α or ε.

The gene segments that encode the Ig HC locus are located on chromosome 12 in

the mouse and chromosome 14 in humans. The Ig HC is comprised of three segments: variable (V), diversity (D), and joining (J). V(D)J recombination refers to the process by which the three gene segments are brought together at the DNA level into a fully assembled Ig variable region capable of encoding Ig HC RNA. The HC RNA V region is subsequently spliced to the constant region and this mRNA can then be expressed as an Ig HC protein (2). About 150 V_H gene segments encompass 14 V_H segment families occupying approximately 2.7 Mb of DNA, about 100 kb upstream of 12-13 D_H segments (3). Downstream of the D_H segments are located four J_H gene segments. Downstream of the J_H segments there are regulatory elements such as the iEμ enhancer and the 3' Regulatory Region (3' RR), as well as an array of constant region exons that encode antibody isotype (4).

There are two Ig light chain loci: kappa and lambda which lack D regions. Mouse κ V, J and C regions, reside on chromosome 6 in the mouse and chromosome 2 in humans. There are about 30 different variable LC genes that may be expressed as functional proteins, although there may be as many as 300 pseudo genes that are present in the locus which occupies about 3,000 kb (5). The murine lambda LC locus is on chromosome 16. The human lambda locus is on chromosome 22. Lambda variable gene segments are followed by four sets of J lambda gene segments each of whom is linked to a different C lambda gene (6).

Immunoglobulin gene rearrangement is an ordered process in which the heavy chain rearranges first, followed by LC rearrangement. The rearrangement sequence is

summarized schematically in Figure 1. Two important lymphoid proteins needed for recombination are RAG-1 and RAG-2. RAG-2 deficient mice lack mature B and T cells because of the inability to undergo V(D)J recombination (7). The RAG-1/RAG-2 complex is responsible for making double stranded DNA breaks which in turn allows the rest of the recombination process to take place (8). D to J joining occurs first and is followed by V to DJ rearrangement. D to J rearrangement and joining takes place on both alleles while V to DJ recombination occurs on one of the HC alleles. The HC assembly thus proceeds according to the following order: D to J and V to DJ, resulting in a V(D)J exon which will be joined to the constant region after the HC mRNA is processed by splicing. The proper order of assembly of V, D, and J regions is ensured by the recombination signal sequences (RSs) which flank each Ig gene segment. V and J gene segments are flanked by 23 base pair (bp) RSs, while D gene segments are flanked by 12 bp RSs. The 12/23 rule allows the rearrangement of the segments flanked with 12 RSs to the segments flanked by 23 RSs, but not 12RSs to 12RSs, or 23RSs to 23RSs. This permits the rearrangements of D to J and V to D segments, but not V to J. The dominant mode of D to J joining is accompanied by deletion which removes the intervening D and J sequences (9). The complete V(D)J region encodes three complementarity determining regions (CDRs) that are responsible for antigen binding, thus largely determining antibody specificity and affinity. Transcription of the Ig HC with a complete VDJ region is promoted by the strong enhancer element (iEμ) which occupies the intronic space between the J and Cμ exons. RNA splicing joins together the V(D)J exon and the constant regions. Alternative mRNA splicing is also responsible for generating both the membrane bound and the secreted forms of the Abs.

How is Ab diversity made possible? The multiplicity of V, D and J segments affords some degree of diversity when they are randomly assorted. Junctional diversity contributes to Ab diversity by several mechanisms. The process of D_H to J_H and V_H to D_HJ_H, and V_L to J_L is imprecise and generates additional diversity. Non-template nucleotides are added randomly by terminal deoxynucleotidyl transferase (TdT) at the joints between D-J and V-DJ segments thereby increasing the Ab diversity further. Palindrome "P" nucleotide addition expands the junctional diversity further. Nucleotides may also be deleted in a random fashion by exonuleases prior to the joining of the Ig gene segments. Nucleotide additions and deletions may change the reading frame of the resulting mRNA and introduce stop codons which prematurely terminate the protein. Those rearrangements that produce functional Ig HCs and LCs are said to be productive. Since the genetic code is based on DNA base triplets, hypothetically, only about 33% of rearrangements are productive, however the real number of productive rearrangements is lower. The pairing of the HC with the LC provides another level of Ab diversity. Finally, when B cells move into the peripheral lymphoid organs and participate in germinal center reactions, mature B cells undergo hypermutation and affinity maturation, which represent the final mechanism of Ab diversification.

Generally, the Ig expressed on the surface of a B cell (termed the B cell receptor or BCR) is made up of only one kind of HC and LC. Each B cell may express several hundred BCRs but they are all the same and have the same specificity. The principle that each B cell expresses only one unique BCR is termed allelic exclusion. It is believed that

the molecular machinery required to continue Ig gene segment rearrangement on the other allele is downmodulated and shut off via a negative feedback inhibition after a productively rearranged BCR is generated. Because D to J rearrangement takes place on both alleles, a productive rearrangement of V to DJ on one of of the alleles will effectively shut off a similar rearrangement from taking place on the other allele. A similar mechanism may be operational for the LC although the timing of the expression of the Ig HC and LC is different. The order in which the Ig HC and LC are rearranged with relation to early B cell development will be discussed in the next section.

The goal of Ig gene rearrangement and HC and LC expression and association is to generate membrane bound Igs or B cell receptors (BCRs) and/or secreted antibody molecule. The BCR is anchored on the surface of a B cell and consists of a complex of not only the IgM heterotetramer composed of two Ig HCs and two LCs but, also, the associated Ig α and β heterodimer whose function is to transduce the signal received by the IgM upon antigen engagement to the cytoplasm of the cell.

The heavy chain constant region determined the antibody's class. The first antibody produced by any B cell is always an IgM antibody. Naive mature B cells express both IgM and IgD on their surface. IgM and IgD double expression is accomplished through alternative splicing of the Cμ messenger RNA transcript. Subsequently in the immune response, the HC class may switch depending on different cytokine signals that the B cell receives. Antibody class switching to γ, ε, or α (resulting in IgG, IgE and IgA respectively) is achieved through the rearrangement between the V(D)J region and the

gamma, epsilon or alpha constant regions, accompanied by a deletion of the intervening HC constant regions. The loss of the Cμ region is achieved by class switch recombination that replaces the Cμ constant region with one of the downstream constant regions: γ, α and ε which produce IgG, IgA and IgE respectively. There are several subclasses of IgG in mice. They are IgG1, IgG2a, IgG2b and IgG3 produced by the pairing of by γ1, γ2a, γ2b and γ3 HCs respectively . Humans have four IgG isotypes, IgG1, IgG2, IgG3 and IgG4. Human IgA molecules are of two subclasses: IgA1 and IgA2. Antibody isotypes have different effector function and participate in various immune responses. IgA is mainly responsible for blocking antigens at mucosal surfaces, IgG is the predominant serum antibody involved in secondary immune responses to the pathogens that enter the bloodstream. IgE is involved in allergic responses and is also produced in response to parasitic worms.

1.2 B cell development

Immunoglobulin heavy chain rearrangement occurs prior to light chain rearrangement during early B cell development in the bone marrow (10). D to J rearrangement takes place at the pro-B cell stage on both alleles, while V to DJ rearrangement usually occurs on one or the other allele (11). There is some evidence from IgM transgenic mouse models, however, that V to DJ rearrangement may occur on both IgH alleles even in the setting where allelic exclusion is maintained and only one unique heavy chain is expressed (12). The intronic enhancer mu (iEμ) is thought to be

important in enforcing allelic exclusion (12). The pro-B cell stage is subdivided into early and late phases, with D to J rearrangements occuring at the early pro-B cell stage, and V to DJ rearrangements occurring in late pro-B cells. B cell developmental stages are summarized in a flowchart in Figure 2.

At the large pre-B cell stage of development prior to immunoglobulin LC rearrangement, a functional HC is expressed in association with the surrogate LC which is encoded by lambda5 (λ5) and VpreB genes (13). λ5 is similar to the constant region of the lambda light chain, while VpreB resembles the variable region of a light chain. The resulting pre-BCR is the basis for assessing the HC fitness and for proceeding with the LC rearrangement. The immunoglobulin LC is rearranged during the small pre-B cell stage (14). Immunoglobulin LC recombination occurs first between Vκ and Jκ gene segments of the light chain kappa locus to form the LC, and, if this rearrangement is non-productive then the Vκ Jκ gene rearranges on the other allele. If this does not result in a productive LC, rearrangement occurs on the lambda light chain locus (15). The fully assembled BCR is expressed on immature IgM+/IgD- B cells in the bone marrow which then emigrate to the periphery and undergo further maturation (16). The earliest type of peripheral B cell is the transitional type 1 (T1) B cell subset, followed by transitional types 2 and 3 (T2 and T3) B cells (17). The three transitional B cell populations are classified according to their surface maker expression (Table 1). Some of the markers used to identify B cell subsets are: CD19 (BCR co-receptor), B220 (CD45 receptor), CD43 (CD54 receptor, adhesion molecule), CD21 (complement C3d receptor), CD23 (low affinity IgE receptor), CD24 (CD62P receptor), CD93 (also known as AA4.1,

phagocytic complement C1q receptor) and CD138 (extracellular matrix receptor). It has been demonstrated that T1 B cells undergo maturation and become T2 B cells, whereas the origin of the T3 population is less clear. Transitional B cells undergo maturation and give rise to mature follicular (FO) and marginal zone (MZ) B cells. Traditionally, it is thought that T2 B cells give rise to both FO and MZ B cell, however, a study by Tan et al describes the T1 subset as a possible reservoir for MZ B cells (18). Therefore, there may be multiple B cells subsets that may give rise to mature B cells. Naive mature B cells express both IgM and IgD on their surface and functionally are more sensitive to antigen stimulation than transitional B cells. Only a small fraction of B cells generated in the bone marrow ever reaches the mature B cell stage. It is estimated that over 90% of B cells are eliminated during maturation (19). The few mature B cells that are allowed to enter the follicle or MZ are relatively long lived and may become activated and proliferate upon encountering antigens. Mature B cells can be activated by an encounter with a specific antigen and an interaction with T cells (referred to as T cell help). During this activation process, the affinity of the B cell is fine-tuned by somatic hypermutation. In the process of somatic hypermutation some residues in the Ig variable regions are changed and the B cell with the highest affinity for the antigen is selected. MZ B cells do not require T cell help for activation and express high levels of receptors that recognize innate immune signals such as bacterial components and unmethylated DNA. The interaction of MZ B cells with innate immune signals leads to MZ B cell activation and antibody secretion. Because of their physiological location, MZ B cells are poised to interact with blood-borne pathogens and may provide the first line of adaptive immune defense against these pathogens.

Mature B cell interaction with an antigen and T cells ultimately results in the formation of either antibody secreting cells (ASC) also termed plasma cells or memory B cells. Plasma cells no longer express surface Ig but can be identified by expression of a plasma cell maker CD138. Plasma cells also produce large quantities of secreted Ig of the IgM, IgG, IgA or IgE classes. Another B cell type produced as the result of B cell interaction with antigens is the memory B cell. Memory B cells may persist for the organism's lifespan and provide long-term immune protection. Upon interaction with an antigen, memory B cells get re-activated.

1.3 B cell Regulation

The B cell repertoire is capable of generating antibodies to a vast array of antigens, thus providing adequate protection against nearly any possible pathogen. This enormous variability is made possible by several mechanisms including: the random recombination of immunoglobulin variable, diversity and joining gene segments, random nucleotide additions and subtractions during segment joining, and somatic hypermutation. Due to the essentially unpredictable nature of V(D)J recombination and somatic hypermutation, some of the BCRs generated by these processes will have affinity for self-antigens. Because autoreactive B cells present a potential risk to the organism, they need to be regulated. There are several mechanisms for maintaining B cell regulation or self-tolerance: clonal deletion, receptor editing, anergy, and follicular exclusion (20-24). Clonal deletion permanently removes B cells from the repertoire by initiating cellular

apoptosis and causing cell death. Receptor editing enables B cells to undergo additional rounds of HC and LC rearrangement in order to generate a non-autoreactive BCR. Anergy is defined as functional unresponsiveness and the inability of autoreactive B cells to be activated by BCR crosslinking and T cell help. Follicular exclusion is the tolerance mechanism that prevents self-reactive B cells from physically entering B cell follicles where B cell immune responses are usually initiated.

Receptor editing in B cells was demonstrated by Tiegs et al who generated transgenic mice that expressed functionally rearranged Ig HC and LC transgenes that encoded an antibody against an MHC class I H-2K molecule (23). The antibody was designated 3-83 and was initially expressed in mice whose MHC I locus haplotype was H-2d. Since the transgenic antibody was not specific for the MHC of the H-2d haplotype, it did not react with H-2d expressing cells. Therefore, H-2d mice expressing transgenic anti-H-2d Ab had significant levels of the Tg Ab in their serum. However, when the anti-H-2d transgene was crossed unto the H-2K background, the Tg anti-H-2K B cells were deleted because the presence of the autoantigen (H-2K) delivered a negative signal. In addition, it was observed that many of the B cells in these mice underwent secondary rearrangement of their kappa LC transgene in an attempt to avert autoreactivity. In support of this, elevated levels of RAG-1 and RAG-2 were detected in the bone marrows of these mice. Further support was provided by the observation that in the bone marrow of H-2d x H-2k mice transgenic for 3-83, the expression levels of the lambda LC on Tg B cells was unusually high, presumably because the autoreactive kappa light chains were being replaced with the lambda light chains as a result of receptor editing. The lambda light chain is more common in the setting where receptor editing has

exhausted potential kappa LCs in an effort to avert autoreactivity.

The mechanism for LC editing was also elucidated in a murine model in which mice were transgenic for the 3H9 HC and the Vk4Jk4 LC of an anti-DNA Ab (24). It was observed that these mice had few transgenic anti-dsDNA B cells. It was also observed that the LC transgene could undergo editing and could be replaced by an endogenous LC which often abrogated autoreactivity. The absence of Tg anti-DNA B cells was shown by an idiotype specific antibody that recognized the 3H9 HC in combination with the Vk4 LC. This was shown by isolating 3H9 HC hybridomas and analyzing their LC usage. The Tg Vk4 LC was replaced with endogenous editor light chains such as Vk23, Vk22 and Vk19. The resulting antibodies no longer bound dsDNA.

One of the most well-characterized models of B cell regulation by deletion and anergy is the hen egg lysozyme (HEL) mouse model. HEL was first used in the 1970s by the laboratory of Eli Sercarz to study T and B cell responses to foreign antigens (25, 26). HEL was initially administered by intraperitoneal (i.p.) injections, however, a transgenic mouse expressing HEL as a self antigen was later developed by Brink et al to study responses to self Ags (27, 28). Goodnow et al generated lymphocyte cell lines that expressed membrane bound or soluble HEL in order to study antigen presentation, and soon after made a transgenic model which expressed membrane bound or soluble HEL as well as B cells that expressed IgM and IgD antibodies to HEL (29, 30). HEL specific B cells were shown to undergo anergy or deletion in the bone marrow depending on the affinity of the B cell receptor (BCR) to various forms of HEL (30-32). High avidity

interaction with membrane bound HEL induced deletion of HEL B cells, whereas soluble HEL induced anergy in HEL specific B cells (30). HEL specific B cells adoptively transfered into sHEL Tg mice along with non-autoreactive B cells were shown to be excluded from the splenic follicles and exhibited a shortened lifespan due to their autoreactivity (20) . It was found that autoreactive B cells are at a competitive disadvantage for gaining entry into the follicles when non-autoreactive B cells are also present. Later it was hypothesized that autoreactive B cells are competing with non-autoreactive B cells for the survival factor BAFF (33).

Another Tg mouse model that demonstrated that anergy was a mechanism of B cell regulation was established in the laboratory of Dr. Betty Diamond (34). Offen et al generated mice transgenic for the IgG2b HC of an anti-dsDNA antibody designated R4A (35). In these mice the Tg HC could pair with endogenous light chains to form anti-DNA as well as non-self-reactive Abs. There was no spontaneous anti-dsDNA Ab production in these Tg mice, however, anti-dsDNA B cells were not completely purged from the B cell repertoire. A population of transgenic anti-dsDNA B cells targeted to anergy was identified in these mice. These B cells could not be activated to secrete Ab following BCR stimulation and T cell help, and were therefore demonstrated to be functionally unresponsive. The specificity of these Tg anti-dsDNA B cells could be shown by rescuing them as hybridomas (36).

Similarly, Erikson et al demonstrated anergy in mice transgenic for only the 3H9 HC of an anti-dsDNA Ab (37). In this model the Tg HC could also pair with endogenous LCs (37, 38). While many B cells specific for DNA were produced in 3H9 transgenic mice, no anti-DNA Ab was present in the serum. because these B cells were unable to be

activated to secrete antibody. Despite the lack of anti-DNA Ab production, the presence of DNA specific B cells in the in 3H9 mice was demonstrated by rescuing them as hybridomas (24).

Immune tolerance operates both centrally and peripherally. Deletion, receptor editing and anergy regulate the developing B cell in the bone marrow. In the periphery, deletion and anergy are the more common models of immune tolerance. One of the important features of immune tolerance is the progressive elimination of autoreactive B cells during their maturation. This ensures that those B cells that participate in the immune response are less likely to give rise to autoreactive antibodies. In the bone marrow, where B cell development begins, as much as 50% of B cells are autoreactive (39). The frequency of autoreactive B cells decreases throughout B cell maturation to approximately 15% in the mature compartment, and despite the presence of some mature autoreactive B cells, no significant titer of high affinity autoreactive antibodies is present in healthy individuals (40). Regulatory checkpoints are the stages in B cell development at which autoreactive B cells are eliminated. Multiple regulatory checkpoints exist both centrally and peripherally and their presence is commonly ascertained by measuring the frequency of autoreactive B cells before and after they transit through a particular stage of development. A reduction in the frequency of autoreactive B cells at a stage of development indicates that some B cells have been eliminated as a result of one of the tolerance mechanisms. In autoimmune diseases such as SLE and Rheumatoid Arthritis (RA), regulation imparted by a regulatory checkpoint may be lost, allowing autoreactive B cell to escape tolerance (41, 42). It is thought that the disruption of regulatory

checkpoints plays an important role in autoimmunity.

B cells encounter their first regulatory checkpoint during their development in the bone marrow. While over 50% of developing B cells are autoreactive, only 35% B cells exiting the bone marrow are autoreactive, indicating that many autoreactive B cells are eliminated in the bone marrow (43). The developmental stages of B cell maturation in the periphery also function as regulatory checkpoints. Phan et al showed that the T2 stage may serve as a regulatory checkpoint in mice transgenic for sHEL and anti-HEL. They observed that autoreactive anti-HEL B cells are arrested at the T2 stage of development and then eliminated by apoptosis before further maturation (44, 45). In wild type mice, the frequency of autoreactive transitional and mature B cells is 35% and 15% respectively, which implies that another regulatory checkpoint exists at the transitional/mature B cell interface (39).

Autoreactive B cells have also been shown to be regulated prior to a germinal center reaction. A recent study by Tsuiji et al has shed light on a late regulatory checkpoint between naïve and memory IgM B cells. Broadly reactive mature B cells were found to be removed from the repertoire before the formation of memory B cells (46). In this study human B cells were examined to determine the frequency of autoreactive B cells present. Antibodies were cloned from single B cells and expressed in order to test their autoreactivity. The frequency of autoreactive B cells was higher in naive mature B cells than in memory B cells, suggesting that the autoreactive B cells were eliminated prior to somatic hypermutation and memory B cell formation.

1.4 The R4A model of B cell regulation

Systemic Lupus Erythematosus (SLE) is characterized by the production of a variety of autoantibodies, specifically antinuclear antibodies (47). IgG anti-dsDNA antibodies are believed to be involved in SLE pathogenesis and nephritis (48). These antibodies may deposit in multiple anatomical locations and organs including the skin, joints, kidneys, and brain where they may lead to rashes, arthritis, glomerulonephritis, and cognitive impairment respectively.

B cells secreting anti-dsDNA antibodies are believed to arise in SLE patients because of a defect in tolerance of anti-dsDNA B cells. To study anti-dsDNA B cell regulation, mice transgenic for the R4A heavy chain of an anti-dsDNA antibody were generated in the laboratory of Dr. Betty Diamond. The R4A transgene was derived from a hybridoma cell line generated by immunizing BALB/c mice with phosphorylcholine, a bacterial antigen which induces anti-DNA antibodies (49). The cell line designated R4A.12 was found to produce a high affinity anti-dsDNA antibody endoced by the VH11 heavy chain gene and the Vκ1 light chain gene. A 3.5-kilobase (kb) DNA fragment containing the rearranged V(D)J portion of the R4A heavy chain and an upstream heavy chain enhancer region was subsequently ligated to a 6.8-kb γ2b constant region sequence

containing both the membrane bound and secreted form of γ2b (35). The entire 10.3-kb R4A γ2b construct was microinjected into C57BL/6 x CBA hybrid mouse eggs to generate R4A-γ2b Tg mice expressing both membrane bound and secreted R4A-γ2b antibodies. In the R4A-γ2b Tg mice, the R4A-γ2b heavy chain can pair with a full spectrum of endogenous light chains to produce dsDNA binding and non-dsDNA binding Abs. The transgene was bred for greater than 10 generations onto the NZW background. No significant levels of Tg IgG2b anti-dsDNA antibody was observed in the serum of NZW R4A-γ2b Tg mice because Tg anti-dsDNA B cells were found to be regulated by deletion and anergy in these mice (35). The spleens of NZW R4A mice had very few B cells suggesting deletion. However, the presence of a subset of Tg anti-dsDNA B cells targeted to anergy was identified in these mice. Tg B cells were unresponsive to BCR cross-linking. However, they could be activated by lipopolysaccharide (LPS) and subsequently rescued as hybridomas. LPS is a component of the cell wall of gram negative bacteria. It was shown that anergic R4A-γ2b anti-dsDNA B cells could be activated to secrete Ab by LPS in a BCR-independent manner (35). LPS activates B cells by engaging Toll-like receptor 4 (TLR4). Analysis of Ig LCs utilized by the Tg anti-DNA Abs secreted by these hybridomas revealed that moderate to high affinity Tg anti-dsDNA antibodies predominantly utilized Vk1 light chains many of which were mutated (36). Further studies were undertaken to determine how Tg anti-dsDNA B cells were regulated in the NZB/W F1 autoimmune strain of mice. The expression of R4A-γ2b in NZB/W F1 mice led to the loss of anti-dsDNA B cell tolerance as evidenced by significant levels of Tg anti-dsDNA antibody (50, 51). B cells rescued as hybridomas from NZB/W F1 mice were found to preferentially utilize germline encoded non-Vk1

LCs. The absence of anti-dsDNA B cells using the germline non-Vk1 LC from NZW mice suggested that these B cells were deleted in the non-autoimmune mouse strain.

Anti-dsDNA Tg B cells in R4A-γ2b mice were also shown to be rescued when an anti-apoptotic proto-oncogene bcl-2 was constitutively expressed (52). Bcl-2 protects B cells from undergoing apoptosis due to a variety of death inducing signals. Bcl-2 promotes B and T cell survival, and overexpression of bcl-2 is associated with high titers of autoreactive antibodies. It was shown that bcl-2 overexpression in R4A-γ2b non-autoimmune mice induced Tg anti-dsDNA Ab production. Both moderate and high affinity Tg anti-dsDNA Abs were produced by hybridomas derived from R4A-γ2b/bcl-2 mice. The light chains utilized by Tg anti-dsDNA B cells in these mice were reminiscent of the mutated non-Vk1 LCs utilized in R4A-γ2b NZB/W F1 mice, suggesting that bcl-2 rescued B cells targeted to deletion in R4A-γ2b mice.

Women are ten times more likely to develop SLE than men, and it is thought that hormones play a significant role in SLE pathogenesis. Estradiol is a synthetic equivalent of estrogen and could be used to study the effects of hormones on the regulation of autoreactive B cells. The effect of estradiol treatment on autoreactive B cells has been studied in R4A-γ2b mice. Estradiol delivered subcutaneously as sixty-day time-release pellets in R4A-γ2b mice has been shown to allow high affinity anti-dsDNA B cells to escape tolerance in the bone marrow and in the periphery (53, 54). R4A-γ2b Balb/C mice treated with 17 beta-estradiol (E2) produced Tg anti-dsDNA Abs. The E2 treatment induced significant changes in the frequencies of peripheral B cells, such as an expansion

of Tg CD138 plasma cells and $CD24^{low}$ mature B cells in the spleen. The T1 and MZ B cell populations were also expanded in E2 treated mice. The MZ subset contained 10-fold more Tg dsDNA binders than the FO subset. Subsequent studies revealed that E2 treatment rescued high affinity Tg anti-dsDNA B cells both at the immature and transitional stages, possibly by disrupting regulatory checkpoints in the bone marrow and in the periphery. The marginal zone has been found to be enriched in self-reactive B cells in other studies (55, 56). In the lupus-prone (NZBxNZW)F1 mouse model, MZ B cells have been shown to produce large amounts of anti-DNA antibodies, suggesting that MZ B cells may play a role in autoimmunity (57, 58). Thus it appears that in many cases autoreactive B cells are selected into the MZ compartment.

Mice transgenic for an IgG transgene are not physiologically normal since the first antibody produced during development is an IgM not IgG. In addition, the regulation of B cells producing an IgG antibody may be different than B cells producing IgM. To represent a more physiologic system to study anti-dsDNA B cell regulation Chu et al generated mice transgenic for an IgM HC that utilized the same R4A V(D)J as R4A-γ2b, but a μ constant region. The rearranged VDJ region and the upstream heavy chain enhancer portion of the R4A HC were ligated to the constant Cμ region to generate the R4A-Cμ transgene (59). The μ constant region was cloned from a BALB/C mouse which bears the "a" allotype. The R4A-Cμ transgene was bred onto the C57BL/6 background which bears the “b” allotype. Therefore, the transgenic B cells and antibodies could be conveniently detected using allotype specific antibodies. It was observed that the transgene is expressed in 20-40% of peripheral B cells and the

transgene is unable to undergo editing because the Tg is not targeted to the Ig HC locus. R4A-Cμ Tg mice displayed similar characteristics to their γ2b counterparts in that no significant level of Tg anti-dsDNA Ab was detected in the serum of R4A-Cμ Tg mice (59). Tolerance was shown to be maintained in these mice by anergy and deletion. Evidence for anergy was demonstrated by the presence of Tg anti-dsDNA B cells that failed to secrete antibody upon BCR stimulation. Futhermore, these B cells had an anergic phenotype characterized by BCR downregulation and developmental arrest at an immature $CD24^{hi}$ stage. Tg anti-dsDNA B cells targeted to anergy could be activated to secrete antibody following stimulation with LPS or CpG (59). CpG is a synthetic oligonucleotide that activates B cells through the Toll-like receptor 9 (TLR9). TLR9 is not expressed on the surface of the B cells, but in endocytic vesicles (60). CpG is internalized via the BCR or pinocytosis and engages intracellular TLR9 in the endosome.

R4A-Cμ Tg B cells were shown to secrete Tg anti-dsDNA antibody in mice overexpressing CD19 (59). R4A-Cμ mice were bred to mice transgenic for CD19 to see if this would lead to a loss of tolerance (61). CD19 is part of the BCR complex and is responsible for lowering the threshold for B cell activation. Therefore, B cells expressing excess levels of CD19 tend to be hyper-responsive to BCR activation. R4A-Cμ mice overexpressing CD19 were observed to have elevated levels of transgenic anti-dsDNA antibodies in their serum. Tg B cells secreting IgM anti-dsDNA antibody in these mice displayed a marginal zone like phenotype although they did not reside in the MZ. Interestingly, the splenic architecture was disrupted in these mice and B cell follicles and the MZ were disorganized. Tg anti-dsDNA B cells in R4A-Cμ mice overexpressing

CD19 could be activated to secrete antibody following BCR crosslinking *in vitro*, however they were hyper-responsive to innate signaling mechanisms. This suggested that CD19 overexpression may promote anergic B cells to escape tolerance by converging with BCR independent pathways.

1.5 BAFF and BAFF receptors

B cell activating factor of the TNF family of ligands (BAFF) was identified in 1999 based on the homology of its sequence with TNF superfamily members (62). Also known as BlyS, TALL-1, THANK, zTNF4 and TNFSF13b, BAFF was demonstrated to be required for the survival and maturation of B cells (63, 64). Human BAFF protein is 285 amino acids long while mouse BAFF is slightly longer (309 amino acids long) and there is a high degree of homology between the human and murine BAFF proteins (86% identity, 93% homology) (62, 65). BAFF is expressed as a trimer on the surface of BAFF producing cells before being proteolitically cleaved and released as a soluble trimer (62, 66). Human and more recently mouse BAFF have been shown to oligomerize into 60-mers comprised of twenty BAFF trimers *in vitro* (67, 68). BAFF 60-mers have also been detected in the sera of BAFF transgenic mice, raising the possibility that BAFF 60-mers may be relevant in human disease. BAFF 60-mers were shown to be 100-fold more potent than BAFF trimers in signaling through TACI (one of BAFF receptors) supporting survival of activated B cells and plasmablasts (68).

BAFF is mainly expressed by myeloid cells such as macrophages, dendritic cells and monocytes (66, 69, 70). In addition, BAFF is expressed by the stromal cells of the bone marrow, some T cells, and recently has been shown to be produced by B cells themselves (62, 71, 72).

BAFF engages three distinct receptors, BAFF-R (also known as BR3), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and B cell maturation antigen (BCMA) (73-76). BAFF-R is expressed predominantly on T2 and mature B cells as well as a small subset of T cells (64, 77). BCMA is found on antibody secreting cells as well as T1 B cells (78, 79). TACI is expressed on both resting and activated B cells as well as a population of activated T cells (80). Of the three BAFF receptors, BR3 and BCMA are generally thought to promote B cell survival, while, TACI is thought to have both positive and negative regulatory functions. BR3 and BCMA promote the survival of mature and plasma B cell respectively. TACI is believed to inhibit B cell proliferation because the number of B cells in TACI knockout mice is elevated (81). Moreover, it is thought that the survival action of BAFF may be counteracted by TACI (82). In addition, TACI is important for Ig class switching to IgG, IgE and IgA and for T independent B cell responses (83, 84). A study by Erikson et al hypothesizes that it is the relative ratio of BR3 to TACI expression on B cells that determines whether negative regulation or survival takes place (85). Therefore, a high BR3/TACI ratio is postulated to promote B cell survival, whereas a low BR3/TACI ratio may lead to B cell tolerance.

B cell survival and maturation in the spleen depends on BAFF. BAFF knockout mice undergo normal bone marrow B cell development and generate T1 B cells but fail to generate normal numbers of T2 B cells and are deficient in mature B cells (86, 87). As a result of mature B cell depletion in BAFF-/- mice, normal antibody responses are inhibited for all IgG subclasses as well as for IgM (86). Mice with a mutated BAFF-R have a phenotype similar to BAFF-/- mice (88).

BAFF signaling promotes B cell survival through the classical and non-clasical NF-kB pathways (89). Signal transduction via BAFF receptors occurs via engagement of trimeric soluble BAFF with BAFF-R which, in turn, allows for the interaction of the intracellular portion of the BAFF receptor with one of the TNF-receptor-associated factors (TRAFs). BR3 interacts with TRAF3, whereas TACI and BCMA interact with TRAF2, TRAF5, TRAF6 and TRAF1, TRAF2, TRAF3, respectively. The non-classical arm of the NF-kappa B signaling responsible for the promotion of B cell survival is mediated by the phosphorylation of p100 initiated by the NF-kappa B inducing kinase (NIK). The binding of the BAFF trimer to BR3 receptors initiates the oligomerization of BR3 and recruitment of TRAF3 to the receptor's TRAF-binding sequences (90-92). Through its association with BR3, TRAF3 is prevented from interacting with the cytoplasmic TRAF2. In the absence of BAFF signaling TRAF3 and TRAF 2 form a complex (TRAF2/TRAF3) which normally inhibits NIK. However, when TRAF3 is sequestered on BR3, TRAF2/TRAF3 complex is not formed and NIK is allowed to accumulate in the cytoplasm. The stabilization of NIK induces NF-kB phosphorylation and activation which in turn promotes B cells survival (93, 94). The phosphorylated form

of p100 is targeted for ubiquitination and degradation, resulting in the formation of p52 and RelB, both of which translocate to the nucleus and activate prosurvival programs. Thus, TRAF3's binding to BR3 appears to be involved in promoting NIK induced of NF-kB degrdation, thus promoting B cell survival (90, 92). This means that when BR3 is not engaged by soluble BAFF, TRAF3 is repressing survival signaling by suppressing NF-kappaB activation. BR3 engagement by BAFF also activates the classical NF-kappa B pathway, although less robustly than the alternative NF-kappa B pathway. The classical NF-kappa B pathway is Bruton's tyrosine kinase (Btk) dependent and requires sustained cRel activation (95). The anti-apoptotic mediators of BAFF induced survival are A1/Bfl1 and Bcl-XL (96, 97). Btk has recently been shown to couple pro-survival signaling through BAFF-R and BCR and activate the classical NF-kB pathway (98).

1.6 BAFF overexpression and autoimmunity

Physiological levels of BAFF are required for normal B cell homeostasis and are crucial for normal antibody responses. However, excess BAFF is harmful because it may promote the survival of autoreactive B cells. Elevated serum levels of BAFF have been found in patients with rheumatoid arthritis, Sjögren's Syndrome and SLE (99-101). An association between elevated serum BAFF levels and autoreactive antibodies in individuals with autoimmune disease has recently been shown (102). Moore et al showed that soluble BAFF administration in mice led to an increase in the serum IgM although the study did not address the autoreactivity of serum IgM (66). Three murine models of

BAFF overexpression have been generated to study the effects of BAFF overexpression on B cell regulation (73, 103, 104). These mice have a similar phenotype characterized by an increased peripheral B cell numbers, hyperglobulinemia, elevated titers of total and autoreactive antibodies and immune complex deposition in the kidneys.

Mackay et al observed in the C57/BL6 mice Tg for BAFF that these mice develop splenomegaly, have elevated numbers of mature B cells and develop some features of lupus-like disease (103). In addition, these mice have increased levels of total immunoglobulin as well as high levels of rheumatoid factors, anti-single standed and anti-double stranded DNA autoantibodies, and circulating immune complexes (103). Antibody deposition in the kidneys and proteinuria was also detected in BAFF Tg mice although these mice did not develop overt renal disease. However, when BAFF was overexpressed on an autoimmune background such as in lupus prone B6.Sle1 and B6.Nba2 strains, these mice did develop glomerular disease (105).

Despite the general agreement that BAFF promotes the loss of B cell tolerance, there is controversy regarding the correlation of serum BAFF and autoantibody production. High levels of serum BAFF have not always been shown to correlate with anti-dsDNA IgG antibodies, or with the severity of glomerular disease. In addition, high levels of serum anti-DNA IgG antibodies have not always correlated with the extent of kidney disease. It is therefore clear that BAFF alone is insufficient for the development of autoimmunity and other factors are actively involved in SLE pathogenesis.

An observation that overexpression of BAFF increases the frequency of marginal zone B cells (MZ B cells) may be relevant to BAFF's role in autoimmunity because a high frequency of autoreactive B cells is found in the MZ (103, 106-108). In a recent study, it has been proposed that excess BAFF may drive late transitional T2 B cells to become MZ B cells (109). In addition, it has been postulated that BAFF overexpression may rescue autoreactive B cells from negative selection in the transitional stage (64). MZ B cells express high levels of BAFF receptors which may explain why BAFF promotes survival of this B cell subset (85).

The role of BAFF overexpression in the loss of B cell tolerance has been studied in several transgenic models of B cell regulation. In a study by Thien et al, mice transgenic for mHEL and anti-HEL Tg/BAFF Tg were studied to determine the effect of excess BAFF (110). In the absence of excess BAFF, high affinity anti-HEL B cells were deleted in the bone marrow and were eliminated. Excess BAFF could not alter this and could not rescue these high affinity autoreactive B cells from deletion. However, anti-HEL B cells with lower affinity for HEL were able to be rescued by excess BAFF and allowed to enter a follicular and marginal zone compartments. Lesley et al showed in another study that when lethally irradiated sHEL Tg mice were reconstituted with a mixture of BM B cells from anti-HEL Ig mice and WT mice, the anti-HEL B cell were quickly eliminated (33). They proposed that the WT and anti-HEL B cells were competing for BAFF, and in the course of this competition, the autoreactive B cells lost the competition and were eliminated. Furthermore, they speculated that autoreactive B cells have reduced BAFF receptor signaling and are therefore at a competitive

disadvantage for follicular entry in the presence of a diverse B cell repertoire.

In another study the role of excess BAFF in the regulation of anti-dsDNA B cells was examined. Mice transgenic for the 3H9 , HC of an anti-chromatin antibody were injected intraperitoneally (i.p.) with recombinant BAFF for 9 days. This resulted in an increase in the frequency of mature anti-chromatin B cells, but failed to induce significant levels of anti-chromatin antibody in the sera (85). This study suggested that BAFF overexpression could influence autoreactive B cell distribution and maturation, but could not lead to autoantibody secretion. The amount and duration of BAFF administered intraperitoneally may have been insufficient to induce a complete loss of anti-chromatin B cell tolerance in the 3H9 Tg mice.

Chapter 2: Introduction I

The B cell activating factor, BAFF also known as BLyS is a member of the TNF family of cytokines. BAFF is produced as a membrane bound protein that is cleaved and released as a soluble ligand, which is the active form of BAFF. BAFF has been shown to play an indispensable role in B cell survival and maturation (111-113). Mice deficient in BAFF or mice in which the action of BAFF is blocked, have abnormally low numbers of mature peripheral B cells and a severe reduction in total serum immunoglobulin (113, 114). BAFF is predominantly produced by dendritic cells, monocytes, macrophages, neutrophils and bone marrow stromal cells (115-117). More recently BAFF production has also been observed by activated T and B cells (118, 119). BAFF can bind and deliver signals through three receptors, BAFF-R, TACI and BCMA, which are differentially expressed during B cell development.

Three independent BAFF transgenic (Tg) mouse models have been generated and each exhibits a profound increase in peripheral B cell number, hypergammaglobulinemia, elevated titers of anti-dsDNA antibody, and immune complex deposition in the kidneys, characteristic of Systemic lupus erythematosus (SLE) (120-122). In one of these BAFF Tg mouse models, mice also develop sialadenitis, decreased saliva production, and submaxillary gland destruction as they age, resembling the autoimmune disease, Sjögren's syndrome (SS) (123).

Elevated serum levels of BAFF, increased titers of anti-dsDNA antibodies, and proteinuria have also been observed in autoimmune NZB/W F1 and MRL-lpr/lpr mice (120). Treatment of these lupus prone mice with BAFF blocking agents has been shown to prevent lupus like disease and prolong survival (120, 124, 125). Elevated levels of BAFF have also been observed in the sera of patients with SLE, Rheumatoid Arthritis (RA) and SS and these levels are associated with high titers of serum anti-dsDNA antibodies (126-128). The association between increased autoantibody production and BAFF overexpression has led to investigations of whether BAFF overexpression alters B cell tolerance.

The maintenance of B cell tolerance has been shown to occur at several regulatory checkpoints throughout B cell development and maturation. The earliest checkpoint that has been identified occurs in the bone marrow at the immature stage of B cell development. A number of well-established Tg mouse models have been used to study B cell tolerance and have identified three major mechanisms by which autoreactive B cells are regulated in the bone marrow: receptor editing, deletion, and anergy (129-133). B cell tolerance has also been observed to occur in the periphery at multiple regulatory checkpoints, although the mechanisms of tolerance at these checkpoints are less clearly defined. One peripheral regulatory checkpoint that has been observed occurs as newly emigrant transitional B cells become mature B cells and a second checkpoint has been observed when mature naïve B cells transition to IgM memory B cells (134, 135).

Recent studies have begun to address whether BAFF overexpression can rescue autoreactive B cells from central and/ or peripheral deletion and anergy (112, 136-138). The effects of excess BAFF were first examined in a model in which the neo-self antigen, hen egg lysozyme (HEL) was presented in either membrane-bound (mHEL) or soluble form (sHEL) to HEL specific B cells. It was observed that overexpression of BAFF could not rescue high affinity self-reactive B cells from central deletion but could rescue them from peripheral deletion if there was negligible competition from non-self reactive B cells for BAFF. However, in a more diverse B cell environment, high affinity anti-HEL B cells could not effectively compete with non self-reactive B cells for BAFF and were therefore eliminated (138). It was further demonstrated that self reactive anti-HEL B cells are more dependent on BAFF for survival than non self-reactive B cells (137).

More recently, we and others have been interested in studying the role of BAFF overexpression on the regulation of more physiologic autoantigens, such as double stranded DNA (dsDNA) and chromatin since these are the hallmark autoantigens in SLE (136). We previously demonstrated that tolerance is maintained by anergy and deletion in mice transgenic for the germline encoded S107 V_{11} heavy chain of an anti-dsDNA antibody, designated R4A, regardless of whether the transgene contained an IgG2b (R4A-γ2b) or an IgM (R4A-Cμ) constant region (132, 139). In both R4A-Cμ and R4A-Cγ2b transgenic (Tg) mice, the transgenic heavy chain can pair with a variety of light chains to produce a diverse B cell repertoire, including dsDNA binding B cells with a range of affinities for dsDNA as well as non dsDNA binding B cells. We demonstrated in R4A-Cμ Tg mice, that anergic anti-dsDNA B cells display a phenotype characterized

by downmodulation of surface IgM, arrest in development, and inability to respond to activation by BCR crosslinking and T cell help. However, we also showed that these B cells could be activated to secrete Tg anti-dsDNA antibody by T cell independent B cell signaling via TLR4 and TLR9 receptors (139). The R4A Tg mouse model has become a very successful model to study genetic and hormonal influences on anti-dsDNA B cell regulation (140). Our laboratory recently demonstrated that overexpression of CD19, a molecule involved in lowering the threshold for B cell activation, could lead to a loss of B cell tolerance and the spontaneous secretion of Tg anti-dsDNA antibody (141).

The present study was undertaken to examine the effect of BAFF overexpression on the regulation of anti-dsDNA B cells. We now demonstrate that high levels of BAFF expression in R4A-Cμ mice, promotes the maturation of a subset of transgenic anti-dsDNA B cells and the secretion of antibody. In addition, our data suggests that BAFF enables these B cells to escape a regulatory checkpoint in the transitional stage of development.

Chapter 3: Results and Discussion

3.1 Overexpression of BAFF promotes the secretion of transgenic anti-dsDNA antibody

We have previously shown that tolerance is maintained in C57BL/6 mice transgenic for the R4A-Cμ (IgM^a) heavy chain gene. Because anti-dsDNA B cells are regulated in these mice by anergy and deletion, they do not spontaneously secrete Tg IgM^a anti-dsDNA antibody. Overexpression of the B cell survival factor, BAFF has been shown to lead to the secretion of anti-dsDNA as well as other autoantibodies and to the development of lupus in mice as well as humans. To study the effect of BAFF overexpression on anti-dsDNA B cell tolerance, we therefore bred C57BL/6 R4A-Cμ mice to C57BL/6 mice transgenic for BAFF, to generate R4A-Cμ mice heterozygous for the BAFF transgene (R4A-Cμ/$BAFF^{+/-}$). We then backcrossed these mice to BAFF Tg mice to generate R4A-Cμ mice homozygous for BAFF (R4A-Cμ/$BAFF^{+/+}$). Transgenic IgM antibody can be distinguished from endogenous antibody in these mice, by an IgM allotype specific antibody. The IgM transgene is of the a allotype since it was originally obtained from a BALB/c mouse, while endogenous IgM in C57BL/6 mice is of the b allotype. Mice were screened by ELISA for the presence of Tg (IgM^a) anti-dsDNA antibody in their serum. Serum samples from R4A-Cμ mice heterozygous or homozygous for BAFF were considered positive for IgM^a anti-dsDNA antibody if the

OD was at least 4 standard deviations above the average OD value obtained from the serum of R4A-Cμ mice. We observed that overexpression of BAFF contributed to the secretion of Tg IgM^a anti-dsDNA antibody in greater than 50% of R4A-Cμ mice homozygous for BAFF, but only 10% of R4A-Cμ mice heterozygous for BAFF (Figure 3A). The concentration of Tg anti-dsDNA antibody was measured by a quantitative ELISA and we observed that the majority of R4A-Cμ/$BAFF^{+/+}$ mice had much higher serum concentrations of IgM^a anti-dsDNA antibody than R4A-Cμ/$BAFF^{+/-}$ mice although both mice heterozygous and homozygous for BAFF had significantly higher concentrations of Tg anti-dsDNA antibody than R4A-Cμ mice (R4A-Cμ/$BAFF^{+/-}$ to R4A-Cμ $p < 0.05$) (Figure 3B).

To determine whether BAFF overexpression altered the frequency of Tg B cells in the bone marrow and the spleen of R4A-Cμ mice, the cells were isolated from both sites and immunostained with antibodies to B220, IgM^a and IgM^b (Figure 4A and 4B). The cells were gated on B220 before enumerating the frequency of IgM^a and IgM^b B cells. The frequency of Tg B cells was similar in R4A-Cμ and R4A-Cμ/$BAFF^{+/+}$ mice, suggesting that BAFF overexpression does not lead to an increase in the frequency of total Tg B cells. In the spleen, the frequency of double negative (IgM^a-/IgM^b-) B cells was increased, reflecting the expansion of non-IgM B cells (IgA and IgG) induced by BAFF overexpression (Figure 4B).

We also observed that the total number of $B220^+$ B cells in the BM of R4A-Cμ/$BAFF^{+/+}$ was similar to that in R4A-Cμ mice but reduced relative to the number of

$B220^+$ B cells in C57BL/6 WT and BAFF overexpressing mice (Table 2). The number of Tg IgM^a B cells in the BM of R4A-Cμ and R4A-Cμ/$BAFF^{+/+}$ mice was similar as well (Table 2).

Since not all mice overexpressing BAFF secreted Tg anti-dsDNA Abs, we wanted to determine whether the serum concentration of BAFF correlated with Tg anti-dsDNA antibody secretion. We therefore measured the serum concentration of BAFF in R4A-Cμ, R4A-Cμ/$BAFF^{+/-}$ and R4A-Cμ/$BAFF^{+/+}$ mice and observed that both mice heterozygous and homozygous for BAFF had variable concentrations of serum BAFF, although BAFF concentrations were generally higher in mice homozygous for BAFF (Fig. 2A). Variations in the serum concentration of BAFF in BAFF transgenic mice have been reported by others as well (142). One potential reason for this is that some BAFF Tg mice may develop antibodies to BAFF which either facilitates the clearance of BAFF or masks its detection by anti-BAFF antibodies used in the ELISA (143). Another reason is that excess BAFF may get excreted in the urine (143). We also observed that elevated BAFF concentrations did not correlate very strongly with high concentrations of Tg anti-dsDNA Ab suggesting that BAFF alone is not sufficient for Tg autoantibody secretion and that other factors may play a role (Figure 5C). However, we did observe that a threshold concentration of BAFF (~300 ng/ml) was required for Tg B cells to secrete anti-dsDNA antibody (Fig. 5B). Below this threshold, few R4A-Cμ/$BAFF^{+/+}$ mice secreted Tg anti-dsDNA antibody while above it 60% of R4A-Cμ/$BAFF^{+/+}$ mice, secreted Tg anti-dsDNA antibody. Since not all peripheral B cells in R4A-Cμ/$BAFF^{+/+}$ mice expressed the Tg antibody but expressed non Tg IgM instead (paralleling observations in

R4A-Cμ mice), the effect of BAFF overexpression on the secretion of non Tg anti-dsDNA antibody was also examined. Interestingly, we observed that almost 90% of R4A-Cμ/BAFF$^{+/+}$ mice secreted non Tg anti-dsDNA antibodies even at low concentrations of serum BAFF (10-250 ng/ml) (Fig. 5B). It is not clear why the Tg anti-dsDNA B cells required higher levels of BAFF to secrete antibody than the non Tg B cells but differences in the affinity or fine specificity of the Tg and non Tg anti-dsDNA antibodies may play a role. The Tg anti-dsDNA antibodies may have a higher affinity for dsDNA or they may be more broadly cross-reactive with other autoantigens than the non Tg anti-dsDNA B cells which may cause them to be more stringently regulated and therefore require more BAFF to become antibody secreting cells. In addition, the extent of B cell receptor signaling (BCR) on autoreactive B cells could affect their level of expression of BAFF receptors, which could influence their responsiveness to BAFF signaling.

Since secretion of transgenic anti-dsDNA antibodies was predominantly observed in mice expressing high concentrations of BAFF (>300 ng/ml), we chose to use only R4A-Cμ mice homozygous for BAFF for all subsequent experiments in this study.

3.2 Excess BAFF does not alter development of bone marrow B cells in R4A-Cμ/BAFF$^{+/+}$ mice

Several studies have reported that bone marrow (BM) B cells are unresponsive to BAFF (113, 138, 144). Mackay et al observed that there were no significant differences

in the frequency and absolute number of pro, pre, and IgM^+ B cells in the BM of BAFF Tg versus WT type mice (122). Furthermore, Thien et al observed that excess BAFF failed to rescue BM HEL B cells from deletion following adoptive transfer into mHEL, BAFF Tg mice (138). We were interested in determining the effect of BAFF overexpression on the development and regulation of anti-dsDNA B cells in the BM of R4A-Cμ mice. Using flow cytometry, we examined the frequencies of pro ($B220^+$ $CD43^+$ IgM^-), pre ($B220^+$ $CD43^-$ IgM^-) and IgM^+ ($B220^+$ $CD43^-$ IgM^+) B cells in the BM of R4A-Cμ, R4A-Cμ/$BAFF^{+/+}$, WT, and BAFF mice (Figure 6A). We observed that the frequencies of pro, pre, and IgM B cells in R4A-Cμ/$BAFF^{+/+}$ mice paralleled those in R4A-Cμ mice. The higher frequency of pro B cells observed in R4A-Cμ and R4A-Cμ/$BAFF^{+/+}$ mice compared to WT and BAFF mice is due to the presence of the pre-rearranged VDJ transgene which accelerates entry into this early stage of development. However, the reduced frequency of IgM^+ B cells in the BM of R4A-Cμ and R4A-Cμ/$BAFF^{+/+}$ mice relative to WT and BAFF mice suggests that some Tg anti-dsDNA B cells may be undergoing deletion in the BM even when BAFF is overexpressed.

3.3 Transgenic B cells display a reduced level of expression of BAFF-R and TACI relative to endogenous B cells

We were interested in determining whether the Tg, IgM^a B cells expressed altered levels of the BAFF receptors, TACI and BR3, on their surface relative to non Tg, IgM^b B cells, since this could influence their susceptibility to BAFF signals. We therefore

examined the levels of TACI and BR3 on IgM^a and IgM^b B cells from R4A-Cμ and R4A-Cμ/$BAFF^{+/+}$ mice by flow cytometry. There are three BAFF receptors; BR3, TACI, and BCMA which are differentially expressed on B cells as they mature (145, 146). BR3 also known as BAFF-R, is the dominant receptor that promotes B cell survival (147, 148). TACI (transmembrane activator and calcium modulating cyclophilin ligand interacting protein) is believed to have both a positive and negative regulatory role and is believed to be involved in T-cell independent type II antibody responses, isotype switching and B cell homeostasis (148, 149). BCMA (B-cell maturation antigen) promotes plasma cell survival (149). We did not examine expression levels of BCMA in this study since its expression has been shown to be negligible on transitional and mature peripheral B cells (136). Figure 7 depicts the levels of BR3 (A) and TACI (B) on transitional ($AA4.1^+$), mature follicular (FO) ($AA4.1^-$ $CD23^{hi}$ IgM^{int}) and marginal zone (MZ) ($AA4.1^-$ $CD23^{lo}$ IgM^{hi}) B cells expressing the IgM^a transgene (solid black line) or endogenous IgM (IgM^b) (grey line) in R4A-Cμ/$BAFF^{+/+}$ mice. Consistent with reports by others, we observed that the levels of both BR3 and TACI were highest on MZ B cells compared to FO and transitional B cells (150). High levels of TACI and BR3 on MZ B cells, could explain why MZ B cells are exceptionally responsive to BAFF survival signals. In addition, we observed that the levels of BR3 (Figure 7A) and TACI (Figure 7B) were consistently much lower on transitional B cells and slightly lower on follicular B cells expressing the IgM^a transgene than non Tg IgM^b B cells, while they were comparable on Tg and non-Tg MZ B cells. B cells with a reduced level of BR3 and TACI are likely to have a lower probability of engaging BAFF when they are competing with B cells that express higher levels of BAFF receptors. This could explain why higher levels of BAFF were required

for Tg B cells to secrete anti-dsDNA antibody than non Tg B cells. No difference was observed in the level of expression of TACI and BR3 on Tg B cells obtained from R4A-Cμ/BAFF$^{+/+}$ and R4A-Cμ mice (data not shown).

The relative expression levels of BR3 and TACI have been observed to vary in different peripheral niches during B cell development, regulation, and activation and the ratio of expression of BR3 to TACI has been reported to have important implications in the role of BAFF in B cell survival (148). Hondowicz et al observed that anti-chromatin B cells in the transitional stage had a lower ratio of BR3 to TACI than non Tg B cells due to an elevated level of TACI expression, implying that TACI levels may be higher on autoreactive B cells (136). We therefore determined the ratios of the MFIs of BR3 to TACI on transitional and mature Tg and non-Tg B cells in both R4A-Cμ/BAFF$^{+/+}$ and R4A-Cμ mice to see if there was a relative increase in TACI expression particularly on transitional Tg B cells. We did not observe a lower ratio of BR3 to TACI on Tg B cells compared to non-Tg B cells in R4A-Cμ/BAFF$^{+/+}$ mice (Figure 7C). In fact the ratio of BR3 to TACI was slightly higher for IgM^{a} than IgM^{b} transitional and follicular B cells and there was relatively no difference in the ratio for IgM^{a} and IgM^{b} MZ B cells.

3.4 Overexpression of BAFF promotes the maturation of Tg B cells in the periphery of R4A-Cμ/BAFF$^{+/+}$ mice

We previously reported a reduction in the frequency and absolute number of total B220^{+} and IgM^{+} B cells in the spleens of R4A-Cμ mice relative to wild type mice (139). In the present study, a similar reduction in the frequency and number of splenic B cells was observed in R4A-Cμ/BAFF$^{+/+}$ mice (Figure 6B and Table 2). This was surprising since we assumed that excess BAFF would promote the survival of peripheral B cells and therefore lead to an increase in B cell number. The frequency of total splenic IgM B cells in R4A-Cμ/BAFF$^{+/+}$ (17%) mice was similar to that in R4A-Cμ mice (20%) and much less than that of BAFF mice (65%) and WT mice (42%) (Figure 6C). In addition, the frequency of B cells expressing the IgM^{a} transgene was similar in R4A-Cμ (10%) and R4A-Cμ/BAFF$^{+/+}$ (10.6%) mice (Figure 6C).

The absolute number of B cells expressing the transgene was slightly elevated in R4A-Cμ/BAFF$^{+/+}$ relative to R4A-Cμ mice but this was due to a preferential expansion of the non Tg, IgM^{b} subset of B cells rather than the IgM^{a} population (Table 2).

Although the total number of Tg B cells was not increased in the spleens of R4A-Cμ/BAFF$^{+/+}$ mice, we wondered whether the number of Tg B cells with a mature phenotype was increased since BAFF has been reported to promote B cell maturation (112, 114). We previously observed that the majority of Tg B cells present in R4A-Cμ

mice were arrested in the immature/early transitional stage of development (139). We used flow cytometry to determine the frequencies of transitional and mature splenic B cells in R4A-Cμ/BAFF$^{+/+}$ and R4A-Cμ mice and compared this to that in wild type mice. Splenocytes were immunostained with antibodies to IgM^{a} and B220 and the heat stable antigen (HSA), also known as CD24. High levels of CD24 expression (CD24^{hi}) have been shown to be characteristic of immature and transitional B cells while mature B cells are CD24^{lo} (151). We observed that the frequency of mature, IgM^{a} CD24^{lo} Tg B cells was much higher in R4A-Cμ/BAFF$^{+/+}$ mice (63%) than in R4A-Cμ mice (35%) (Figure 8A). More recently, immature/transitional B cells have also been shown to express the cell surface type I transmembrane protein, AA4.1 (CD93) (152). By immunostaining splenocytes with antibody to AA4.1 we demonstrated that R4A-Cμ/BAFF$^{+/+}$ mice have a lower frequency of AA4.1^{+} transitional B cells (30%) and a higher frequency of mature, AA4.1^{-} B cells (70%)) than R4A-Cμ mice (61% and 39% respectively) (Figure 8B).

While BAFF has been shown to be necessary for normal B cell development, (112-114, 122, 153) it has been difficult to discern whether BAFF directly plays a role in B cell differentiation, or if BAFF simply enhances survival of transitional B cells which consequently go on to mature. Therefore to demonstrate that BAFF can promote the maturation of transitional Tg B cells to mature B cells, we isolated AA4.1^{+} IgM^{a} B cells from R4A-Cμ mice by cell sorting and adoptively transferred them into either C57BL/6 WT or C57BL/6 BAFF Tg mice (Figure 8C). Following the cell sort, the purity of AA4.1^{+} Tg B cells was almost 95%. One hundred thousand cells were then transferred into each recipient mouse and 48 hours later, splenocytes were collected and

immunostained with antibodies to AA4.1, IgM^a, and B220. Cells were gated on IgM^a and the frequency of $AA4.1^+ B220^+$ and $AA4.1^- B220^+$ Tg B cells were determined by flow cytometry. We observed that a higher frequency of IgM^a $AA4.1^+$ B cells matured to $AA4.1^-$ cells in BAFF recipients (79%) than in wild type recipients (51%) indicating that excess BAFF promoted their maturation (Figure 8D).

Transitional $AA4.1^+$ B cells have been classified into three subgroups, T1, T2 and T3, according to the classification of Allman et al: T1 cells are $AA4^+$, IgM^{hi}, $CD23^-$. T2 cells are $AA4^+$, IgM^{hi}, $CD23^+$ and T3 cells are $AA4^+$, IgM^{lo}, $CD23^+$ (152). T1 cells are the precursors of T2 cells, but it is unclear whether T3 represents a developmental stage between T2 and mature B cells or an anergic population of B cells as recently described by Merrell et al (152, 154). A study by Hsu et al suggested that BAFF promotes the survival of late transitional B cells but not T1 B cells (144). Furthermore, several studies reported that full responsiveness to BAFF is not achieved until B cells reach the T2 stage (113, 144, 153). To determine the effect of excess BAFF on transitional Tg B cell populations, splenic B cells from R4A-Cμ and R4A-Cμ/$BAFF^{+/+}$ mice were immunostained with antibodies to B220, IgM^a, AA4.1, and CD23 and the frequency of T1, T2, and T3 B cells examined by flow cytometry. Although the overall number of transitional B cells was reduced in R4A-Cμ/$BAFF^{+/+}$ mice, the relative frequency of T2 and T3 B cells was increased and the frequency of T1 B cells decreased in R4A-Cμ/$BAFF^{+/+}$ mice relative to R4A-Cμ mice (Figure 9A). This is consistent with reports that BAFF promotes the survival of late, but not early transitional B cells. However, our

data cannot exclude the possibility that BAFF promotes the maturation of T1 to T2 B cells or enables autoreactive B cells to escape arrested development in the T1 stage.

3.5 R4A-Cμ mice overexpressing BAFF have an enlarged MZ and an increased frequency of Tg B cells displaying a MZ phenotype

Mice overexpressing BAFF have previously been shown to have an enlarged MZ (122). Several laboratories have demonstrated that the splenic MZ is a reservoir for autoreactive B cells (111, 140, 155-157). We were interested in determining whether R4A-Cμ/BAFF$^{+/+}$ mice have an increased frequency of MZ precursor cells and an enlarged MZ compartment relative to R4A-Cμ mice. To determine the frequency of B cells with a MZ phenotype, we immunostained splenocytes from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice as well as BAFF overexpressing and wild type mice, with antibodies to B220, CD21, CD23, IgM^{a}, or IgM^{b}. We then examined by flow cytometry, the frequency of Tg, IgM^{a} B cells from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice, or non Tg, IgM^{b} B cells from BAFF or WT mice, that displayed a MZ (CD21^{hi}, CD23^{lo}) or FO (CD21^{int}, CD23^{hi}) phenotype according to Loder B cell phenotype classification. Consistent with reports by Mackay et al, we observed that the frequency of splenic IgM B cells with a MZ phenotype was greater in BAFF overexpressing mice than WT mice (Figure 9B, bottom panel) (122). In addition, we observed that R4A-Cμ/BAFF$^{+/+}$ mice had an increased frequency of Tg B cells with a MZ phenotype compared to R4A-Cμ mice (Figure 9C,

top panel). No significant difference was observed in the frequency of B cells with a FO phenotype in R4A-Cμ or R4A-Cμ/BAFF$^{+/+}$ mice.

There are several possible MZ precursor populations, one of which is hypothesized to be the late transitional CD21^{hi}, CD24^{int} B cell population (Meyer-Balhburg, Andrews, Rawlings JEM 2008). In R4A-Cμ/BAFF$^{+/+}$ mice the frequency of CD21^{hi}, CD24^{int} B cells was elevated, indicating that BAFF overexpression may be responsible for the expansion of MZ B cell precursors in our model (Figure 9C).

Next, by immunohistochemical staining, we examined the splenic B cell compartments in R4A-C*μ*/BAFF$^{+/+}$ and R4A-Cμ mice for the localization of Tg B cells. Frozen spleen sections were immunostained with PE conjugated antibodies to IgM or IgM^{a} to visualize total IgM B cells and Tg IgM^{a} B cells respectively, and biotin labeled anti-MOMA-1 antibody followed by streptavidin FITC, to visualize metallophilic macrophages that appear as a ring in the marginal sinus. The marginal sinus demarcates the boundary between the MZ and the follicle. We observed that the follicles were larger and the MZ expanded in R4A-Cμ/BAFF$^{+/+}$ mice compared to R4A-Cμ mice (Figure 10A). We also observed that Tg B cells were distributed in both the FO and MZ of R4A-Cμ and R4A-Cμ/BAFF/$^{+/+}$ mice but that there were significantly more Tg B cells in the MZ and follicular compartments of R4A-Cμ/BAFF$^{+/+}$ than R4A-Cμ mice (Figure 10B).

Without an anti-idiotypic antibody specific for Tg anti-dsDNA B cells, we were unable to determine by immunohistochemical staining whether any of the Tg B cells that

localized to the MZ and FO compartments were specific for dsDNA. A previous report by Lesley et al demonstrated that B cells normally compete for limiting amounts of BAFF in order to gain entry into the follicles and that autoreactive B cells require more BAFF for entry than non self-reactive B cells (137). However, in the presence of excess BAFF, high affinity autoreactive B cells have been reported to enter follicles when competition with non self-reactive B cells is limited (138). Excess BAFF has also been shown to rescue autoreactive B cells of intermediate affinity and facilitate their entry into the MZ as well as the follicles (138).

3.6 Follicular and marginal zone B cell populations in R4A-Cμ mice overexpressing BAFF are enriched for Tg anti-dsDNA B cells

We were interested in determining whether the frequency of Tg anti-dsDNA B cells with a MZ or follicular phenotype was increased in R4-Cμ/BAFF$^{+/+}$ mice relative to R4A-Cμ mice. Therefore splenocytes from R4-Cμ/BAFF$^{+/+}$ and R4A-Cμ mice were sorted by flow cytometry into MZ and FO B cell subsets. They were then cultured for 48 hours in vitro, in media alone or in media supplemented with stimulatory CpG sequences, to induce antibody secretion. The number of IgM^{a} anti-dsDNA secreting B cells per 10^5 cells (Figure 11A) and the number of total IgM^{a} secreting B cells per 10^5 cells (Figure 11B) were then enumerated by ELISPOT. Hypomethylated CpG sequences have been reported to activate dsDNA binding B cells via TLR 9 receptors and we have previously demonstrated that CpG sequences could activate Tg anti-dsDNA B cells isolated from R4A-Cμ mice overexpressing CD19 (141, 158, 159). In the present study, we observed

that the number of IgM^a secreting B cells specific for dsDNA was higher in both the MZ and FO compartments of R4A-Cμ/$BAFF^{+/+}$ mice than R4A-Cμ mice. Unstimulated Tg B cells were not observed to spontaneously secrete anti-dsDNA antibodies in either R4A-Cμ or R4A-Cμ/$BAFF^{+/+}$ mice.

3.7 BAFF overexpression promotes the escape of Tg anti-dsDNA B cells from regulatory checkpoints in the transitional stage of development

The increased frequency of Tg anti-dsDNA B cells in the MZ and FO compartments of R4A-Cμ/$BAFF^{+/+}$ relative to R4A-Cμ mice suggested that BAFF might enable some Tg anti-dsDNA B cells that normally undergo negative selection in the transitional stages of development, to escape regulatory checkpoints and transition to more mature B cells in the periphery. To address this, we decided to sort on $AA4.1^+$ (transitional) and $AA4.1^-$ (mature) B cells from R4A-Cμ and R4A-Cμ/$BAFF^{+/+}$ mice and determine the frequency of Tg dsDNA binding B cells in each of these populations by ELISPOT. Sorted B cells were activated in vitro with stimulatory CpG sequences to induce antibody secretion. We reasoned that if regulation was intact in the transitional population, then the percent of dsDNA binding B cells in this population would be higher than in the mature B cell population, while if there was a defect in regulation at the transitional stage in R4A-Cμ/$BAFF^{+/+}$ mice then the percent of Tg dsDNA binding B

cells would not be significantly different in the transitional and mature B cell populations. We observed that in R4A-Cμ mice, the percent of Tg anti-dsDNA B cells was significantly higher in the $AA4.1^+$ population compared to the $AA4.1^-$ population ($p=0.0056$) indicating that at least some Tg anti-dsDNA B cells were unable to progress from the transitional stage to the mature stage of development (Figure 12). In contrast, in R4A-Cμ/$BAFF^{+/+}$ mice, the frequency of Tg anti-dsDNA B cells was not significantly different in the $AA4.1^+$ and $AA4.1^-$ populations ($p= 0.414$), suggesting that BAFF enables some Tg anti-dsDNA B cells to escape a regulatory checkpoint in the transitional stage of development.

3.8 B1 cells are unlikely to be the source of Tg anti-dsDNA B antibody in R4A-Cμ/$BAFF^{+/+}$ mice.

Mice overexpressing BAFF have been shown to have an expansion of B cells belonging to the B1 subset as well as MZ B cells and antibodies produced by B1 cells in these mice have been shown to contribute to autoreactivity and disease (160). We previously demonstrated that in R4A-Cμ mice overexpressing CD19, B cells secreting the Tg anti-dsDNA antibody were not of the B1 lineage (141). To determine whether B1 B cells are a potential source of secreted Tg anti-dsDNA antibody in R4A-Cμ/$BAFF^{+/+}$ mice, we isolated peritoneal B cells from both R4A-Cμ/$BAFF^{+/+}$ and R4A-Cμ mice by lavage and immunostained these B cells with antibodies to B220, CD5 IgM^a , or IgM^b and measured the frequency of $CD5^+$ and $CD5^-$ B cells expressing the transgene or

endogenous IgM. R4A-Cμ/BAFF$^{+/+}$ mice were observed to have an elevated frequency of CD5^{+} B1 cells relative to R4A-Cμ mice (Figure 13A). However, when gates were set on B220, the frequency of IgM^{a} CD5^{+} and IgM^{a} CD5^{-} B1 cells was relatively low in R4A-Cμ/BAFF$^{+/+}$ mice (Fig. 10B, upper panel). Most of the CD5^{+} or CD5^{-} B1 cells in these mice expressed IgM^{b} (Figure 13B, lower panel). Since negligible numbers of B1 B cells expressed the transgene in R4A-Cμ/BAFF$^{+/+}$ mice, it is unlikely that these B1 cells were the source of Tg anti-dsDNA antibody. Absolute proof of this would require in vitro activation of peritoneal B cells to see if they secrete Tg anti-dsDNA antibody. However, this is technically very difficult to do since the number of peritoneal B cells expressing the transgene is so low.

3.9 Discussion

This study demonstrates that BAFF overexpression can alter B cell regulation and promote the maturation of a population of Tg anti-dsDNA B cells and lead to their secretion of antibody. These anti-dsDNA B cells can enter MZ and FO niches and be activated to secrete antibody following CpG stimulation. In contrast to the results in this study, Hondowicz et al observed that Tg anti- dsDNA/chromatin binding B cells in VH3H9 heavy chain Tg mice, failed to enter splenic follicles following daily ip injections of BAFF (136). Similarly, Lesley et al observed that in a diverse B cells environment in HEL/anti-HEL mice, anti-HEL B cells, failed to enter splenic follicles after being given daily BAFF injections (137). They concluded that autoreactive B cells have a higher dependence on BAFF for survival and for entry into the follicles than non-self reactive B

cells and that in a diverse microenvironment they are at a competitive disadvantage for follicular entry. One possible explanation for the difference in their observations and ours is that exogenous BAFF treatment may not have provided enough BAFF to enable self-reactive B cells, in the context of a heterogeneous B cell repertoire, to enter splenic compartments, while in mice homozygous for the BAFF transgene, sufficient BAFF may have been present for self-reactive B cells to traffic to the follicles as well as the MZ. Another potential reason for the disparate observations is the likely differences in the affinities of the self-reactive B cells in each of these transgenic mouse models. The anti-chromatin/dsDNA B cells and the anti-HEL B cells in the other studies may have been of higher affinity than the R4A-Cμ anti-dsDNA B cells in our study. Thien et al observed that while high affinity anti-HEL B cells could not enter follicles in the presence of high intercellular competition in HEL/anti-HEL Tg mice, intermediate affinity B cells could (138). Furthermore, they observed that excess BAFF enabled intermediate affinity HEL B cells to enter the MZ as well. While we do not know the dsDNA binding affinities of the Tg anti-dsDNA B cells that enter the FO and MZ compartments in R4A-Cμ/BAFF$^{+/+}$ mice, we suspect that they have a range of affinities from intermediate to low. A future study is underway to resolve this.

In R4A-Cμ/BAFF$^{+/+}$ mice, we observed that the Tg anti-dsDNA B cells require higher levels of serum BAFF for antibody secretion than the non Tg B cells. This may be because they express lower levels of both TACI and BR3, especially in the transitional stage of development, than their non Tg counterparts. In a diverse B cell environment, we hypothesize that B cells with reduced levels of BAFF receptors would have a lower

probability of engaging BAFF than B cells with higher levels of BAFF receptors, especially if the amount of BAFF is limiting. However, when serum BAFF levels are high, then B cells with low levels of BAFF receptors would have a better probability of engaging BAFF. Hondowicz et al observed a higher level of TACI expression on immature anti-chromatin Tg B cells compared to immature non Tg B cells although, no difference was found in the levels for BR3. They concluded that TACI is upregulated on autoreactive B cells and plays a negative role in B cell survival and homeostasis. It is unclear what accounts for the difference in TACI levels observed on the Tg B cells in our Tg mice and in the Hondowicz model but differences in the signaling strength of the autoreactive BCRs in both transgenic mouse models may play a role. Another reason why higher concentrations of BAFF may have been required for R4A-IgM^{a} anti-dsDNA B cells to become antibody secreting cells than IgM^{b} anti-dsDNA B cells, may be because of differences in their affinity and/or fine specificity for dsDNA and their cross-reactivity with other autoantigens. These factors have been shown to affect the stringency of B cell regulation (139, 161). If the Tg anti-dsDNA B cells are more tightly regulated than the non Tg anti-dsDNA B cells because they cross-react more strongly with other self antigens or because they have a higher affinity for self antigen than the non Tg anti-dsDNA B cells, then they may require more BAFF in order to escape peripheral regulation.

BAFF does not enhance the absolute number of bone marrow B cells in R4A-Cμ/BAFF$^{+/+}$ mice relative to R4A-Cμ mice. As previously demonstrated, the number of bone marrow B cells is reduced in R4A-Cμ mice relative to WT mice and overexpression

of BAFF does not seem to alter this. In addition, the frequency and absolute number of immature IgM B cells is similarly reduced in R4A-Cμ/BAFF$^{+/+}$ and R4A/Cμ mice relative to WT mice. This is consistent with a report by Thien et al that excess BAFF does not alter central deletion (138).

Surprisingly, R4A-Cμ/BAFF$^{+/+}$ mice do not have enlarged spleens, enriched in B cells as observed in BAFF mice. Instead they have a reduced number of total splenic B cells. We previously demonstrated that a subset of high affinity anti-dsDNA B cells present in an autoimmune strain of mouse transgenic for the R4A-Cγ2b heavy chain gene, are deleted from R4A-Cγ2b mice on a wild type background (161). The reduced number of splenic B cells found in R4A-Cμ mice relative to WT mice similarly suggests deletion of a population of anti-dsDNA B cells. The lack of expansion of peripheral B cells in R4A-Cμ/BAFF$^{+/+}$ mice may be due to BAFF's inability to rescue high affinity anti-dsDNA B cells targeted to deletion. This is consistent with a study by Thien et al demonstrating that excess BAFF fails to rescue high affinity anti-HEL B cells in sHEL/anti-HEL BAFF Tg mice (138).

If BAFF is unable to rescue high affinity R4A-Cμ anti-dsDNA B cells, then what is the source of the Tg anti-dsDNA antibody secretion in R4A-Cμ/BAFF$^{+/+}$ mice? B1 cells are unlikely to be the source since the frequency of B1 cells expressing the Tg is negligible in these mice. We speculate that BAFF may rescue intermediate to low affinity anti-dsDNA B cells from regulation in the transitional stages of development. It has recently been reported that the transitional stages of development also function as

regulatory checkpoints (134, 135). We observed that in R4A-Cμ mice, the percent of Tg anti-dsDNA B cells in the transitional stage is significantly higher than in the mature stage suggesting that many of the autoreactive B cells are undergoing regulation in the transitional stage. However, in R4A-Cμ mice overexpressing BAFF, a greater frequency of Tg anti-dsDNA B cells are able to enter the mature B cell stage and the ratio of mature to transitional Tg anti-dsDNA B cells is much higher in R4A-Cμ/BAFF$^{+/+}$ mice than in R4A-Cμ mice suggesting that BAFF enables some of the autoreactive B cells to escape regulatory checkpoints in the transitional stage. The exact mechanism of regulation at these checkpoints is unclear, however, arrested development and/or deletion have been proposed. In addition, Merrell et al suggests that anergy may play an important role as they have demonstrated that transitional, T3 B cells display an anergic phenotype and that a high frequency of B cells in this population are autoreactive (152, 154). Interestingly, we recently observed that the frequency of Tg anti-dsDNA B cells is significantly higher in the T3 than the T2 and T1 populations in R4A-Cμ mice and that these Tg T3 anti-dsDNA B cells appear to have an anergic phenotype (data not shown). While BAFF may rescue autoreactive B cells from regulatory checkpoints in the transitional stages of development, it is not known how these B cells get activated to become antibody secreting cells. We propose that BAFF may promote the survival and maturation, of intermediate affinity anti-dsDNA B cells targeted to anergy as well as low affinity anti-dsDNA B cells in R4A-Cμ/BAFF$^{+/+}$ mice and that this increases the opportunity for an antigen independent, environmental trigger such as CpG to activate these autoreactive B cells. Studies are underway to investigate this.

In summary, this study demonstrates that high levels of serum BAFF promotes the survival and maturation of a subset of anti-dsDNA B cells and facilitates their secretion of antibody. Furthermore, our data suggests that BAFF enables anti-dsDNA B cells to escape a peripheral regulatory checkpoint in the transitional stage. These results are important as they illustrate the role of BAFF in altering peripheral B cell tolerance and they emphasize how excess BAFF poses a risk that can lead to the development of anti-dsDNA antibodies that arise in SLE.

Chapter 4: Introduction II

Autoreactive antibodies play a significant role in B cell driven autoimmune disorders including Systemic Lupus Erythematosus (SLE) and Sjogren's Syndrome (162-164). An array of self-reactive antibodies detected in the sera of patients with SLE includes anti-chromatin, anti-RNA, anti-ssDNA and anti-dsDNA antibodies. Autoreactive antibodies in SLE patients deposit in the kidneys and may be partially responsible for SLE pathogenesis (165). Although autoreactive B cells are produced naturally in the course of normal B cell development, they may be regulated by receptor editing, deletion or anergy in the bone marrow, or by deletion or anergy at multiple stages in the periphery. Furthermore, there are multiple stages during development in both the bone marrow and periphery where autoreactive B cells can be eliminated. These are known as regulatory checkpoints (21-24). Autoimmunity may arise when autoreactive B cell escape one or more of these regulatory checkpoints due to defective tolerance mechanisms. The identification of B cell developmental stages in which autoreactive B cells escape tolerance, may result in the development of therapeutic strategies that can specifically target autoreactive B cells subsets.

Anergy is an immune tolerance mechanism responsible for the functional silencing of autoreactive B cells in the bone marrow and in the periphery as postulated by several transgenic mouse models (22, 35, 166-169). Functionally, anergic B cells are believed to be incapable of normal B cell activation through their BCRs. Phenotypically, anergic B cells are characterized by B cell receptor downmodulation, shortened life span and developmental arrest. We have shown that anergic B cells can be activated via BCR

independent mechanisms resulting in autoantibody production (61, 170). Whether or not anergic autoreactive B cells contribute to the pool of anti-self antibodies in vivo is currently unknown. Recently, the transitional type 3 (T3) B cell population has been postulated to represent an anergic B cell subset (171). Originally identified by Allman as one of the three transitional B cell populations, the T3 subset has subsequently been characterized in transgenic and wild type mice, and, more recently, in humans (17, 172, 173). T3 B cells are AA4.1 (CD93) positive, IgMlow and CD23 positive. A high frequency of autoreactive cells has been found in the T3 subset (172). Functionally, T3 B cells are refractory to activation through the B cell receptor and flux intracellular calcium poorly (172).

It is unclear whether T3 B cells play a role in the development of autoimmunity. However, we have hypothesized that prolonged survival of T3 B cells could contribute to autoreactive antibody production *in vivo*. The factors that could promote the survival, maturation and antibody secretion of B cells derived from the T3 subset are unknown. Two studies failed to show that T3 B cells could undergo maturation in cell culture or when adoptively transferred (171, 172). In one of these studies, B cell activating factor of the TNF family of ligands (BAFF) failed to promote the maturation of T3 B cells stimulated in vitro with anti-IgM antibody. Purified T3 B cells from WT mice were cultured in the presence of BAFF and anti-IgM for 72 hours. The expression of AA4.1 on T3 B cells was assessed after culture by FACS and it was demonstrated that there was no increase in the frequency of AA4.1- (mature) B cells under these conditions, suggesting there T3 B cells did not undergo maturation (171). Although T3 B cells

failed to undergo maturation in this study, we were nevertheless interested in determining whether BAFF and non-BCR activation signals could induce T3 B cells to mature and secrete antibodies.

BAFF is required for normal B cell maturation, survival and antibody secretion, however, an excess of BAFF has been linked to SLE in humans and a lupus-like syndrome in mice (63, 103, 174). SLE patients have an elevated level of BAFF in their serum, and it is thought that excess BAFF may be involved in the breakdown of B cell tolerance (175, 176). Transgenic mice that overexpress BAFF develop excess autoantibodies, nephritis, proteinuria and immune complex deposits in the kidneys (103). In some circumstances, BAFF is thought to be able to rescue autoreactive B cells destined to die. In mice co-transgenic for the neo-self antigen, hen egg lysozyme (HEL) and antibody to HEL, excess BAFF can rescue low to moderate affinity anti-HEL B cells from deletion under conditions where competition with non-autoreactive B cells is negligible (110). Autoreactive B cells are thought to require high levels of BAFF for survival (33).

We have recently shown that overexpression of BAFF in mice transgenic for an anti-dsDNA IgM^a antibody designated R4A-Cμ leads to the breakdown of tolerance in over 50% of mice (177). We also observed that an increased frequency of anti-dsDNA B cells is present in the mature follicular (FO) and marginal zone (MZ) subsets of these mice. In addition, we observed that a high frequency of of Tg B cells present in the transitional subset of these mice display a late transitional T2 and T3 phenotype (Figure

9A). This observation prompted us to investigate whether the T3 subset is a source of anergic anti-dsDNA B cells in R4A-Cμ mice and whether this subset could be activated to become antibody secreting cells when BAFF is overexpressed.

In the present study we show that the frequency of dsDNA binding B cells is relatively increased in the T3 subset compared to the T1 and T2 subsets. Furthermore, this subset has the features of anergic B cells and cannot be activated by BCR signaling but can be activated by signaling through TLR receptors. We also show that BAFF overexpression can promote the survival of T3 B cells and can synergize with innate signals to lead to the activation of this population of B cells *in vitro* and the secretion of autoantibodies.

Chapter 5: Results and Discussion II

5.1 A high frequency of anti-dsDNA B cells is present in the T3 B cell subset

We have previously shown that mice transgenic for the heavy chain of an anti-dsDNA Ab designated R4A-Cμ do not spontaneously secrete anti-dsDNA Ab due to regulation by anergy and deletion (59). We also demonstrated that a population of B cells targeted to anergy exists in the periphery of R4A-Cμ mice. These B cells have a shortened life span and arrested development, and are refractory to antigen stimulation. However, we previously demonstrated that these anergic B cells can be activated by innate mechanisms of signaling such as stimulatory CpG engagement of TLR9, or LPS interaction with TLR4. It is also unclear at what stage of development Tg anti-dsDNA B cells are targeted to anergy in R4A-Cμ mice. The T3 B cell population has recently emerged as a possible anergic B cell subset that harbors autoreactive B cells (17, 171). We recently demonstrated the existence of a sizeable T3 B cells subset in R4A-Cμ mice (177).

We were interested in determining whether the T3 population is a reservoir for anergic anti-dsDNA B cells in R4A-Cμ mice. We therefore sorted to purify this population of B cells along with T1 and T2 B cells from R4A-Cμ mice (Figure 14A). I then determined whether these B cells could be activated *in vitro* with stimulatory CpG sequences to become antibody secreting cells. Purified T1, T2 and T3 cells were incubated *in vitro* with 1µg/ml of CpG for 2 days and then ELISPOT was performed to

enumerate Tg anti-dsDNA B cells. I observed that the T3 subset contained a high frequency of Tg anti-dsDNA B cells compared to the T1 and T2 subsets in which only a few dsDNA specific B cells were present (Figure 14B). I also examined whether T3 B cells could be activated to become antibody secreting cells via engagement of other Toll-like receptors. E. Coli LPS and P. Gingivalis LPS were used to stimulate T3 B cells *in vitro* through TLR4 and TLR2 respectively (Figure 15). I observed that T3 B cells were induced to secrete anti-dsDNA Ab in response to TLR9, TLR4 and TLR2 *in vitro* and it is therefore likely that T3 B cells may be sensitive to a variety of innate immune stimuli *in vivo*.

5.2 T3 B cells exhibit an anergic phenotype in R4A-Cμ mice

The observation that the frequency of anti-dsDNA B cells is very high amongst T3 cells prompted us to characterize the T3 subset. It has been hypothesized that T3 B cells represent an anergic population rather than a stage of B cell development (171). Evidence for anergy can be observed in several phenotypic and functional features which ultimately result in developmental arrest, inability to respond to activation and, shortened life span. To determine whether T3 B cells display an anergic phenotype in R4A-Cμ mice, I examined levels of surface IgM expression on these B cells and looked at their ability to flux intracellular calcium, and to secrete antibody upon BCR stimulation. By definition T3 B cells have been shown to express low levels of surface IgM (172). Identification of T3 B cells in R4A-Cμ mice by flow cytometry reveals that they express low levels of surface IgM^a relative to T2 and mature B cells (Figure 16A). In addition, by

gating on T1, T2 and T3 B cells and examining level of expression of kappa light chain I demonstrated T3 B cells have a reduced level of expression of surface immunoglobulin (Figure 16B). T3 B cells have also been shown to respond poorly to BCR stimulation (172). I examined the ability of T3 B cells from R4A-Cμ mice to flux intracellular calcium upon BCR stimulation with anti-IgM antibody and compared it to that of T1, T2 and mature AA4.1- B cells (Figure 17A). I observed that T1, T2, as well as mature B cells showed robust intracellular calcium mobilization upon BCR stimulation, while T3 cells were clearly impaired in BCR signaling, showing a reduction in calcium flux (Figure 17A). In order to asses the ability of T3 B cells to become Tg anti-dsDNA antibody secreting cells in response to antigen activation and surrogate T cell help, I purified T3 B cells by flow cytometry and stimulated them *in vitro* with antibodies to IgM and CD40. I observed that no anti-dsDNA antibody was produced by T3 B cells stimulated with anti-IgM/anti-CD40 Abs (Figure 17B). Lack of responsiveness to anti-IgM plus anti-CD40 antibody stimulation suggests that these dsDNA specific T3 B cells are functionally anergic. Stimulation with CpG was used as a positive control. These studies are consistent with previous reports that T3 B cells represent an anergic population.

5.3 A substantial fraction of T3 B cells exhibits a loss of allelic exclusion

Allelic exclusion is a mechanism which ensures that only one type of antibody is made by each B cells. The Ig expression in B cells is under tight regulation and the frequency of double expressors (B cells that express more than one type of Ig HC) is extremely low. Allelic exclusion may be lost in some autoreactive B cells and allow them to escape immune tolerance (36). Alternatively, double expression may lower the autoreactivity of self-reactive B cells by diluting the autoreactive receptors with a non-autoreactive one. We reasoned that in our model, the loss of allelic exclusion may manifest in the co-expression of transgenic IgMa and endogenous IgMb on B cells. Transgenic B cells were labeled with antibodies to resolve transgenic T1, T2, T3. Additionally, anti-IgMb antibody was added to identify double expressors amongst IgM^a B cells. A much larger proportion of T3 B cells were double expressors compared to T1 and T2 subsets, 24% versus 6% and 10% respectively, indicating that almost a quarter of T3 B cells exhibit allelic inclusion of both IgMa and IgMb alleles (Figure 18). We have previously shown that lack of allelic exclusion permits autoreactive B cells to escape deletion (36).

5.4 BAFF enhances survival and antibody production by T3 B cells in vitro

We previously observed that R4A-Cμ mice overexpressing BAFF display a loss of tolerance and spontaneously secrete Tg anti-dsDNA antibody(177). These mice were also observed to exhibit an increase in the frequency of T3 B cells in R4A-Cμ mice transgenic for BAFF (Figure 19A). We wondered whether excess BAFF could promote the survival of T3 B cells can lead to the secretion of Tg anti-dsDNA antibody. To study the effect of BAFF on T3 B cells survival, I sorted to purify T3 B cells by flow cytometry and incubated them with or without 1ug/ml of BAFF for four days. Cell viability was measured by staining the cells with AnnexinV and propidium iodide (PI) which label necrotic (AnnexinV$^+$, PI$^+$) and apoptotic cells (AnnexinV$^+$, PI$^-$). The percent of double negative (AnnexinV$^-$, PI$^-$) cells reflect the frequency of viable cells in cell culture (Figure 19B). I observed that T3 B cells treated with BAFF remained viable in cell culture longer than untreated cells, suggesting that BAFF may prolong survival of T3 cells *in vitro* and, possibly, *in vivo*. After three days of culture 40% of cells treated with BAFF were viable as compared to less than 10% of untreated cells. We also wanted to address the possibility that BAFF may have an effect on anti-dsDNA Ab secretion induced by CpG. It has been shown in one report that stimulatory CpG treatment increases BAFF receptor expression on B cells (178). T3 B cells were purified by flow cytometry and stimulated *in vitro* with 0.5 μg/ml of CpG in the presence of absence of 1μg/mL BAFF for five days. At the end of the incubation period the supernatants were collected and the amount of anti-dsDNA Ab produced by the cells was measured by a quantitative ELISA (Figure 19C). I observed that CpG induced anti-dsDNA antibody production by T3 B

cells, and that the addition of BAFF enhanced the amount of anti-dsDNA Ab produced by T3 B cells by approximately two-fold. This suggests that the effects of CpG and BAFF may synergize to enhance anti-dsDNA antibody production by T3 B cells.

5.5 Expression of BAFF receptors on T3 B cells is upregulated upon CpG stimulation

We have previously observed that the level of expression of BAFF receptors, BR3 and TACI are lower on Tg B cells than endogenous B cells (61). We hypothesized that this was a feature of anergy and could explain why Tg B cells required higher levels of BAFF for survival and Ab secretion than Tg B cells. T3 B cells represent an anergic population of B cells in R4A-Cμ mice, then we wondered whether this subset of B cells would express lower levels of BR3 and/or TACI than other B cell subsets.
I therefore immunostained transgenic B cells from R4A-Cμ mice with antibodies to BR3 and TACI and examined the levels of expression of these receptors by flow cytometry. I observed that the levels of both BR3 and TACI were lower on T3 B cells compared to T2 and (Figure 20A and 20B) and mature B cells (not shown). BR3 is the BAFF receptor mainly responsible for promoting B cell survival and maturation, whereas TACI may be involved in both positive and negative regulation of B cells as well as immunoglobulin class switching (83, 84, 86, 179, 180).

Since excess BAFF promotes T3 B cell survival, I wondered whether the levels of BR3 and TACI was increased on T3 B cells in R4A-Cμ mice overexpressing BAFF. I did not observe this by flow cytometry (not shown). CpG has been shown to induce

upregulation of BAFF receptors on B cells (178). I therefore sought to determine whether CpG could promote the expression of BAFF receptors on T3 B cells. FACS sorted T3 B cells were stimulated in vitro with CpG for 24 hours and BAFF receptor expression was determined using anti-BR3 and anti-TACI antibodies (Figure 20C and 20D). CpG treatment induced BAFF receptor upregulation on T3 B cells compared to unstimulated B cells. Mice may be exposed to CpG as a result of bacterial infection, ingesting fecal matter and as a byproduct of cell death. It is possible that T3 B cells in R4A-Cμ mice are exposed to CpG and other innate immunity agonists in vivo and upregulate BAFF receptors as a result. This may in turn sensitize T3 B cells to BAFF and promote their maturation and/or survival, enabling them to become antibody secreting cells.

5.6 Discussion

T3 B cells have recently been shown to represent an anergic subset of B cells (171). Our laboratory previously observed that a population of anti-DNA B cells targeted to anergy is present in R4A-Cμ mice (139). In the present study, I demonstrate that these anergic B cells, display a T3 phenotype as defined by Merrell et. al. These B cells are $AA4^{+}$, IgM low, and $CD23^{hi}$. In addition, they fail to flux calcium and fail to become antibody secreting cells upon BCR stimulation. I have observed that in R4A-Cμ mice, the T3 subset is significantly enriched for Tg anti-dsDNA B cells compared to the T1 and T2 subsets.

The T3 subset was initially reported to represent a normal late stage of transitional B cell development following the T2 stage (17). Subsequent reports, however, have characterized T3 B cells as an anergic subset of B cells, that do not undergo further maturation but rather are eliminated because of their autoreactivity (171, 172). T3 B cells fail to undergo maturation when cultured *in vitro* with antigen or when adoptively transferred into wild type mice. A recent study that uses a mathematical model based on BrdU incorporation, to describe the kinetics of B cells destined for elimination, suggests that the T3 subset represents a death niche for most B cells that are anergized at the immature, transitional, or mature stage of development (181).

In the present study, I observed that the frequency of T3 B cells is increased in R4A-Cμ mice that overexpress BAFF. I also observed that BAFF promotes the survival of T3 B cells. Based on these observations and our recent report of a loss of tolerance and the secretion of Tg anti-dsDNA antibody in R4A-Cμ/BAFF mice, I now speculate that Tg anti-dsDNA secreting B cells are derived from the T3 subset in these mice (177). The persistence of this population of anergic, autoreactive B cells can be potentially dangerous to the host if these B cells become activated and succeed in producing autoreactive antibodies. Although anergy induces quiescence in B cells to prevent their activation, it is reversible. Anergy has been shown to require chronic exposure to an auto-antigen and the anergic state can be reversed when self-antigen is removed. This was demonstrated by Merrell et al in the arsonate mouse model (172). When arsonate specific B cells were incubated with monovalent hapten to induce arsonate dissociation from the B cell receptor, T3 B cells which are AA4.1 positive,down-regulated their levels

of AA4.1. This demonstrated that anergic B cells could progress to a mature stage of development when self antigen was no longer available to chronically stimulate them. Anergy can also be reversed by certain environmental factors that stimulate B cells via BCR independent mechanisms. It has been shown by Offen et al that anergic IgG2b anti-dsDNA B cells can be activated via TLR4 receptors by lipopolysaccharide (35). Similarly, anergic IgM anti-dsDNA B cells were shown to be activated by LPS stimulation (139) . Activation of TLR9 via stimulatory CpG sequences may be particularly important for the production of anti-DNA antibodies by anergic B cells because autoimmune-prone mice that lack TLR9 do not develop anti-DNA antibodies (182). DNA specific B cells may bind cellular DNA internalize it in endosomes, where it can engage TLR9 and induce anti-DNA antibody production. In the present study, I demonstrated that Tg anti-dsDNA T3 B cells can be activated by LPS and stimulatory CpG sequences via TLR4 and TLR9 respectively. T3 B cells may therefore be sensitive to T-independent signals *in vivo* and may get activated by environmental stimuli to become autoantibody secreting cells.

I have observed that *in vitro* treatment of T3 B cells with BAFF, in combination with CpG, enhances anti-dsDNA Ab secretion relative to B cells treated with CpG alone. This may be because BAFF promotes intracellular expression of TLR9 as previously reported by Groom et al which may make B cells more responsive to stimulatory CpG (183). I have not yet determined whether TLR9 levels are increased in T3 B cells in R4A-Cμ mice overexpressing BAFF but these studies are underway. However, if BAFF does upregulate TLR9 levels in T3 B cells, it would further strengthen the association between excess BAFF and CpG-induced antibody production.

B cells stimulated with TLR agonists have been shown to upregulate BR3 and TACI (118, 184). We have shown that the density of the BAFF receptors, BR3 and TACI are very low on T3 B cells in R4A-Cμ mice, however, CpG treatment upregulates the level of expression of these receptors. Whether reduced levels of these BAFF receptors is a general feature of anergic, T3 B cells is not yet clear. It is interesting to note that in R4A-Cμ/BAFF mice, very high levels of serum BAFF are required to lead to a breakdown in tolerance and secretion of antibody by transgenic anti-dsDNA B cells compared to non Tg anti-dsDNA B cells (177). This may be because the anergic Tg B cells have such low levels of BAFF receptors compared to the lower affinity non Tg anti-dsDNA B cells.

BAFF is not only a B cell survival factor but it has also been shown to be required for B cell maturation (90, 113). However, it is not known whether BAFF, in addition to CpG can promote the maturation of anergic T3 B cells. Two separate studies demonstrated that T3 B cells are unable to mature when adoptively transferred into wild type mice, however, the effect of excess BAFF on T3 B cell maturation following adoptive transfer was not examined (172, 185). In preliminary studies, I have adoptively transferred Tg T3 B cells from an R4A-Cμ mouse into a wild type or a BAFF mouse but I have not observed any evidence of Tg B cell maturation even in BAFF recipients. This suggests that BAFF may not promote the maturation of T3 B cells. Signaling through TLR4 and TLR9 may be required for T3 B cells maturation in R4A-Cμ/BAFF mice. Additional studies are needed to confirm this.

Based on our in vitro observations, we hypothesize that in an *in vivo* setting, BAFF and innate immune signals such as stimulatory CpG, may collaborate to promote the survival, activation, and secretion of anti-dsDNA antibody by T3 B cells. We propose the following model to explain the mechanism of tolerance loss and Tg anti-dsDNA antibody secretion in R4A-Cμ/BAFF mice (Figure 21): in the course of B cell maturation, autoreactive B cells of moderate affinity are anergized and acquire a T3 phenotype. T3 B cells have a shortened lifespan when BAFF is limited, and are normally cleared by apoptosis. However, it is likely that when BAFF is overexpressed, the lifespan of T3 B cells is prolonged. An extended lifespan may enable T3 B cells to have a greater window of time to become activated by environmental stimuli, via TLR4 or TLR9 in a T- independent manner. Potential sources of molecular patterns that can deliver activating signals to TLR4 and TLR9 are LPS from the cell walls of gram negative bacteria and unmethylated bacterial CpG sequences respectively. However, certain sequences of eukaryotic dsDNA released from dying cells may also be the source of stimulatory CpG oligonucleotides. In future studies, we will determine whether injection of mice with stimulatory CpG sequences can activate T3 B cells and lead to a loss of tolerance and whether this is enhanced in the presence of excess BAFF.

Chapter 6: Materials and Methods

6.1 R4A-Cμ and R4A-Cμ /BAFF transgenic mice

The R4A-Cμ transgene was originally derived by ligating a 3.5 kB fragment containing the rearranged S107 VDJ region encoding the variable heavy chain region from the anti-dsDNA antibody designated R4A, to a 9.9 Kb Ig fragment containing the μ heavy chain constant region. The region contained the secreted and membrane-bound exons of the Cμ region. The resulting transgene encodes anti-dsDNA IgM^a heavy chain that can pair with endogenous Ig light chains. The transgene was bred onto the C57BL/6 strain of mouse for 10 generations.

C57BL/6 BAFF transgenic mice used in this study were a generous gift from Dr. Susan Kalled (Biogen Idec Inc., Cambridge, MA). R4A-Cμ transgenic mice heterozygous for the BAFF transgene (R4A-Cμ/$BAFF^{+/-}$), were generated by crossing C57BL/6 mice transgenic for the R4A-Cμ (IgM^a) heavy chain transgene with mice homozygous for the BAFF transgene ($BAFF^{+/+}$). R4A-Cμ mice homozygous for the BAFF transgene (R4A-Cμ/$BAFF^{+/+}$) were generated by back crossing R4A-Cμ/$BAFF^{+/-}$ mice with $BAFF^{+/+}$ mice. The presence of the R4A-Cμ and BAFF transgenes were ascertained by PCR using Illustra PuReTaq Ready-To-Co PCR beads (GE Healthcare, Piscataway, NJ). The following primer pair; 5'GGTGAAGCTGGTGGAGTCTGG 3' (FR1 primer) and 5'CATAGGGATCCTATCTCT3' (reverse junctional primer) was used to amplify a 313 bp fragment of the rearranged R4A transgene. PCR conditions for the detection of the rearranged transgenic VDJ were as follows: 94ºC for 9 min, followed by 30 cycles of

94ºC for 30 sec, 51ºC for 30 sec and 72ºC for 40 sec, followed by an extension at 72ºC for 5 min. The following primer pair was used for detection of the BAFF transgene; 5'GCAGTTTCA CAGCGATGTCCT 3' (BAFF forward primer) and 5'GTCTCCGTTGCGTGAAAT CTG 3' (BAFF reverse primer). PCR conditions for detection of a 719 bp fragment of the BAFF transgene were as described by Mackay et al. (122). Mice homozygous for the BAFF transgene were identified by crossing the progeny from R4A-Cμ/BAFF$^{+/-}$ x BAFF$^{+/+}$ mice with C57BL/6 wild type mice. If all the progeny were heterozygous for BAFF then that indicated that the R4A-Cμ parent was also homozygous for BAFF.

6.2 ELISAs

6.2.1 Anti-dsDNA ELISA

ELISAs to detect the presence of anti-dsDNA antibody in mouse sera, were performed using calf thymus dsDNA coated 96 well plates (139). Immulon 2HB plates (Thermo, Milford MA) were coated with dsDNA by adding 100uL of 100ug/mL sonicated and purified calf thymus dsDNA (Sigma) diluted in PBS and incubating at 37°C for 48 hours until the wells were dry. DNA plates were washed with distilled water for 5 min and blocked with 1%BSA/PBS for 2 hours at room temperature (RT). Mouse serum samples were diluted 1:100 in dilution buffer (0.1% BSA/PBS) and added at 100μL/well in triplicates. The plates were then incubated for 2 hours at 37ºC. Transgenic (Tg) (IgM^{a}), or endogenous (IgM^{b}) anti-dsDNA antibody was detected by biotinylated goat anti-mouse IgM^{a} or IgM^{b} antibody respectively (BD Pharmingen, San Jose, CA) diluted 1:200, followed by the addition of 1:1000 dilution of streptavidin conjugated to alkaline

phosphatase (Southern Biotech, Birmingham, AL). Endogenous IgG anti-dsDNA antibody was detected by goat anti-mouse IgG conjugated directly to alkaline phosphatase (Southern Biotech) diluted 1:1000. P-nitrophenyl disodium phosphate (Sigma) was added as substrate. Color development was allowed to proceed at 37ºC for 1 to 3 hours and plates were read at 405 nm using a Titertek Multiscan ELISA plate reader.

To quantitate Tg IgM^a anti-dsDNA antibody by ELISA, two-fold serial dilutions of mouse serum samples (beginning with a 1:50 dilution), were applied to calf thymus dsDNA coated plates and incubated for 2 hours at 37ºC. The remainder of the anti-dsDNA ELISA was performed as described above. A purified mouse monoclonal IgM^a anti-dsDNA antibody, isolated in our laboratory was also serially diluted, beginning with a concentration of 1000 ng/ml and was applied to wells and used to generate a standard curve. The concentration of serum IgM^a anti-dsDNA antibody was extrapolated from this curve.

6.2.2 BAFF ELISA

Serum BAFF was quantitated using a BAFF ELISA kit (Apotech, Lausen, Switzerland) according to the manufacturer's protocol. The kit contained 8-well strips of anti-BAFF Ab coated wells. After blocking the wells with 300µl diluting buffer for 2 hours at RT, mouse serum was added to the wells at 1:10 and 1:30 dilutions, while the standards were added at 0, 5, 10 and 20 ng/ml in order to generate standard curve. Serum samples and standard were incubated in the well for one hour, washed and incubated with detection antibody for one hour at RT. The secondary Ab was detected with HRP-Streptavidin and

the color was developed using OptEIA TMB substrate reagent set (BD Pharmingen). The reaction was stopped with H_2SO_4 and the absorbance was measured using a Titertek ELISA plate reader. The reading was taken at an absorption of 450nm and the amount of serum BAFF was extrapolated from the standard curve each time the assay was carried out.

6.3 Flow Cytometry

To identify B cells in the pro and pre B cell stages of development, bone marrow cells from R4A-Cμ, R4A-Cμ/BAFF$^{+/+}$, C57BL/6 wild type (WT) and BAFF$^{+/+}$ mice were immunostained with anti-B220-PerCP-CY5.5, anti-CD43-APC and anti-IgM-PE antibodies (BD Pharmingen, San Jose, CA). Splenocytes from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice were immunostained with the following antibody combinations: anti-B220-APC-Cy7 (BD Pharmingen), anti-IgM-FITC, to detect total IgM B cells; anti-B220-APC-Cy7, anti-CD24-FITC, and either anti-IgM^a-PE or anti-IgM^b-PE (BD Pharmingen) to detect immature/transitional transgenic or endogenous B cells respectively; anti-B220-APC-Cy7, anti-IgM^a-PE, anti-AA4.1-APC (eBiosciences, San Diego, CA), and anti-CD23-FITC (BD Pharmingen) to detect transitional type 1, 2, and 3 B cells (T1—B220$^+$, AA4.1$^+$, IgMa^{hi}, CD23$^-$; T2— B220$^+$, AA4.1$^+$, IgMa^{hi}, CD23$^+$; T3—B220$^+$, AA4.1$^+$, IgMa^{lo}, CD23$^+$), according to the classification of Allman et al, (152); anti-B220-APC-Cy7, anti-CD21 followed by anti-rat-APC (BD Pharmingen), anti-IgM^a-PE, and anti-CD23-FITC to detect follicular (FO) (CD23$^+$, CD21^{lo}) and marginal zone (MZ) (CD23^{lo}, CD21^{hi}) transgenic B cells according to Loder et al (186). In many instances, splenocytes were pre-incubated with the FcR block, anti FcγR III/II (BD Pharmingen) to prevent binding of antibodies to B cells via their Fc receptors. To

examine the levels of BR3 and TACI on AA4.1^{+} and mature FO and MZ B cells, splenocytes from R4A-Cμ and R4ACμ/BAFF$^{+/+}$ mice were immunostained with the following antibody combinations: anti-B220-APC-Cy7, anti-AA4.1-APC, anti-CD23-FITC, anti-IgM^{a}-PE or anti-IgM^{b}-PE, and biotinylated antibody to either BR3 or TACI (R&D Systems, Minneapolis, MN) followed by streptavidin-PerCP-Cy5.5 (BD Pharmingen). Peritoneal B cells were immunostained with antibodies to IgM^{a} or IgM^{b}, B220 and anti-CD5-APC (BD Pharmingen) in order to detect B1 B cells. Immunostained cells were fixed in PBS/paraformaldehyde and analyzed using a BD LSR II FACS machine with FACS Diva software. Additional analyses were performed using FloJo software.

6.4 FACS sorting

Splenocytes from R4A-Cμ mice were immunostained with the following antibody combinations: anti-B220-PerCP-Cy5.5 (BD Pharmingen, San Jose, CA), anti-IgMa-PE, anti-AA4.1-APC (eBiosciences, San Diego, CA), and anti-CD23-FITC (BD Pharmingen) to detect transitional type 1, 2, and 3 B cells (T1, T2, T3), according to the classification of Allman et al (17). Anti-kappa-Biotin followed Streptavidin-APC-Cy7 in addition to the staining for T1, T2, T3 and mature IgMa and IgMb B cells was used to determine the levels of B cell receptor. B cells were also immunostained with biotinylated antibodies to the BAFF receptors, BR3 and TACI (R&D Systems, Minneapolis, MN) followed by streptavidin-PerCP-Cy5.5 (BD Pharmingen). Immunostained cells were fixed in PBS/paraformaldehyde and analyzed using a BD LSR II FACS machine with FACS Diva software. Additional analyses were performed using FloJo software.

6.5 Adoptive transfer

Splenocytes from an R4A-Cμ mouse were immunostained with antibodies to B220, IgM^a, and AA4.1 and then cell sorted using a BD FACS ARIA to isolate transitional Tg B cells ($B220^+$ IgM^a $AA4.1^+$). Approximately 1 x 10^5 transitional B cells were resuspended in 200uL of 1%FBS/PBS and were then adoptively transferred retro-orbitally, into either a wild type C57BL/6 or BAFF Tg mouse. Forty eight hours post transfer, recipient mice were sacrificed and their splenocytes immunostained with antibodies to IgM^a, B220, and AA4.1 for detection of mature ($AA4.1^-$) and immature/transitional ($AA4.1^+$) Tg B cells.

6.6 Histology

Mouse spleens were harvested from mice and embedded in Tissue-Tek OTC compound (Sakura Finetek USA, Torrance CA). Six micrometer sections were cut on Leica CM1950 cryostat (Bannockburn, IL) and transferred onto BondRite slides (Waltham, MA) , fixed in undiluted ice cold acetone for 10 minutes, washed three times in PBS for 5 minutes and blocked with 3% FBS/PBS. Tissue was immunostained with PE conjugated antibodies to total IgM or IgM^a and biotin conjugated antibody to MOMA-1 (Fitzgerald Industries International, Concord, MA) followed by streptavidin-FITC (BD Pharmingen). Anti-IgMa-PE and anti-IgM-PE (BD pharmingen) were diluted 1:100 and added at 100ul per slide. Anti-MOMA1-Biotin Ab was diluted 1:50 and Streptavidin-FITC was diluted 1:100. All incubations were carried out in a humidified chamber at 4°C for at least 3 hours. Slides were then treated with ProLong Gold antifade reagent (Molecular Probes,

Carlsbad, CA) and examined under a Nikon Eclipse TE2000-5 (Nikon, Melville, NY) fluorescent microscope at 100 to 600x magnification.

6.7 B cell Enrichment

Mouse spleens were harvested and mechanically disrupted by grinding through 40μm cell strainers (BD Falcon, Bedford, MA). Cell were then pelleted at 1200rpm for 10 minutes and resuspended in RBC lysis buffer (15.5mM NH_4Cl, 1 mM $KHCO_3$, 0.01 EDTA pH 7.2) 2ml/spleen at RT for 5 minutes before being centrifuged again and resuspended in magnetic cell separation (MACS) buffer (filter sterilized 0.5% BSA PBS). Cells were counted and resuspended in MACS buffer at 10^7 cells/90μl. Ten μl of anti-CD43 magnetic beads (Miltenyi Biotec, Auburn CA) were added for every 90μl of cell suspension. The cells were incubated for 15min at 4°C and washed with MACS buffer. While the cells were incubated MACS LS columns (Miltenyi Biotec, Auburn CA) were washed by gravity with 3 ml of MACS buffer. The cells were resuspened in 1ml of MACS buffer and put through the LS column mounted on the Miltenyi magnet. The flowthrough was collected in a fresh tube, centrifuged and counted.

6.8 In vitro activation and ELISPOT

Splenocytes from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$, mice were immunostained with fluorochrome conjugated antibodies to B220, CD23, and CD21 and cell sorted using a FACS Aria, to isolate MZ (B220$^+$ IgM^{hi} CD21^{hi} CD23^{lo}) and FO (B220$^+$ IgM^{int} CD21^{int} CD23$^+$) B cells. Splenocytes from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$, mice were also immunostained with fluorochrome conjugated antibodies to B220, IgM^a, and AA4.1 and

sorted into $B220^+$ IgM^a $AA4.1^+$ (transitional) and $B220^+$ IgM^a $AA4.1^-$ (mature) B cell populations. Following cell sorting, purified B cell populations, at a concentration of 1 x 10^6 cells per ml, were cultured for 48 hours in B cell medium consisting of RPMI-1640 media (Sigma) supplemented with 10% Fetal Calf Serum (HyClone, Logan, UT), 2mM L-glutamine (Sigma), 100μM nonessential amino acids (Sigma), 100U/ml penicillin, 100 μg/ml streptomycin (Sigma), and 50 μM β-mercaptoethanol. B cells were incubated in media alone or activated with 1 μg/ml of stimulatory CpG oligonucleotide sequences (ODN 1826 TCC ARG ACG TTC CTG ACG TT) (Oligos Etc, Wilsonville, OR) for two days. After stimulation, B cells were harvested, counted and plated on dsDNA or anti-IgM coated 96-well-plates at a concentration of 10,000-100,000 cells/well. After an overnight incubation, plates were washed and incubated for 3 hours with goat anti mouse IgM^a antibody conjugated to biotin diluted 1:200 followed by a 3 hour incubation with streptavidin-AP (1:500 dilution) according to Taylor et al (141). 5-Bromo-4-chloro-3-indoyl phosphate (Sigma) was added as substrate at 1mg/ml. Antibody secreting cells were enumerated under a dissecting microscope using 40 X magnification. To study BAFF receptor expression upon CpG stimulation, sort purified T3 B cells were cultures at 100,000 cells/well on a 96-well plate stimulated with 1 μg/ml CpG for 24 hours. At the end of incubation, the cells were centrifuged and stained with anti-BR3 and anti-TACI antibodies as described in the section on FACS.

6.9 Calcium flux

To measure intracellular calcium flux, B cells were stained with antibodies to IgM^a, B220, CD23 and AA4.1 as described in the section on FACS. Prior to antibody staining, cells diluted to 10^7 per ml in Hank's Balanced Salt Solution (Gibco) were loaded with 4 μl per 1ml of cell of Indo-1 (Invitrogen) (1mg/ml) in the dark for 30 minutes at 37°C. The cells were then stimulated with 0, 1, 10 and 20ug/ml rabbit $F(ab')_2$ anti-mouse IgM (Zymed Invitrogen, Carlsbad, CA) and analyzed on BD LSR II FACS analyzer. Some cells were stimulated with ionomycin as a control to ensure that Indo-1 was properly loaded. Calcium flux curves were generated in FloJo and are based on the ratio of Indo-1(violet)/Indo-1(blue) over time.

6.10 *In vitro* activation and anti-dsDNA Ab production

For *in vitro* activation of B cells, transgenic T2, T3 and mature B cells were cell sorted as previously described (see FACS and FACS sorting). Cell were plated in B cell medium at 30,000 cells/100ul/well in a 96-well tissue culture plate and stimulated with 0.5ug/ml CpG or 0.5ug/ml CpG plus 1ug/ml recombinant mouse BAFF (R&D Systems, Minneapolis, MN). In addition to CpG, Escherichia coli lipopolysaccharide Serotype 055:B5 (Signa. St. Louis, MO) and Porphytomonas gingivalis lipopolysaccharide (InvivoGen, San Diego, CA) were used to stimulated B cells. After five days of culture, the supernatants were collected and assayed by a quantitiative ELISA to determine the concentration of Tg anti-dsDNA antibody as previously described (see ELISA).

6.11 Cell viability assay

To determine cell viability, Tg T3 B cells were sorted by flow cytometry and plated at 100,000 cells/100µl/well on a 96-well cell culture plate. Wells were either unstimulated or stimulated with 1µg/ml recombinant BAFF and incubated for one, two, three and four days. Cells were collected on each day and counted. Viability was assayed using the Annexin-V-FITC Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA). The proceduce was carried out according to the manufacturer's protocol. The cells were centrifuged and resuspended in 100µl phosphate binding buffer provided in the kit. Five µl each of anti-annexin-V-FITC and propidium iodide were added to the cells, mixed and incubated in the dark for 15 min at RT. At the end of the incubation period, an additional 400µl of binding buffer were added to the cells which were then analyzed using an LSRII FACS analyzer. Annexin-V and propidium iodide double negative cells were considered viable cells in this assay.

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Tables and Figures

Location	Designation	Surface Markers
Bone Marrow	Pro-B cell	IgM-, B220, CD19, CD93+, CD43+
Bone Marrow	Pre-B cell	IgM-, B220, CD19, CD93+, CD43-
Bone Marrow/Blood	Immature	IgMhigh, B220, CD19, CD93+
Spleen	T1	IgMhigh, B220, CD19, CD93+, CD23-, CD21-
Spleen/Lymph Node	T2	IgMhigh, B220, CD19, CD93+, CD23+, CD21int
Spleen	T3	IgMlow, B220, CD19, CD93+, CD23+
Spleen	Follicular	IgMint (IgD+), B220, CD19, CD93-, CD23+, CD21low
Spleen/Lymph Node	Marginal Zone	IgMhigh (IgD+), B220, CD19, CD93-, CD23-, CD21high
Spleen/Blood	Memory	IgM-, IgG, IgA, B220+, CD19
Spleen/Bone Marrow	Plasma	IgM-, CD138 (syndecan), B220-, CD19-

Table 1. Common antigenic markers expressed on the surface of B cells during development and maturation

	WT	BAFF	R4A-Cμ	R4A-Cμ/BAFF$^{+/+}$
Bone marrow				
Total B cells (x 10^6)	5.1 ± 1.4	4.3 ± 0.9	1.7 ± 0.7	1.8 ± 0.8
IgM^a B cells (x 10^6)	NA	NA	0.3 ± 0.1	0.4 ± 0.3
IgM^b B cells (x 10^6)	1.9 ± 0.9	2.8 ± 0.8	0.1 ± 0.02	0.1 ± 0.04
Spleen				
Total B cells (x 10^6)	21.6 ± 9.7	70.3 ± 26.0	2.8 ±1.1	4.2 ± 2.5
IgM^a B cells (x 10^6)	NA	NA	1.0 ± 0.5	0.9 ± 0.8
IgM^b B cells (x 10^6)	18.0 ± 7.6	61.1 ± 20.6	1.1 ±.0.5	2.1 ±1.3

Table 2. Absolute number of Tg B cells in R4A-C*μ*/BAFF$^{+/+}$ mice. Absolute numbers of total B cells and B cells expressing Tg (IgM^a) or non Tg (IgM^b) antibody were determined by flow cytometry. Results are the average of six experiments.

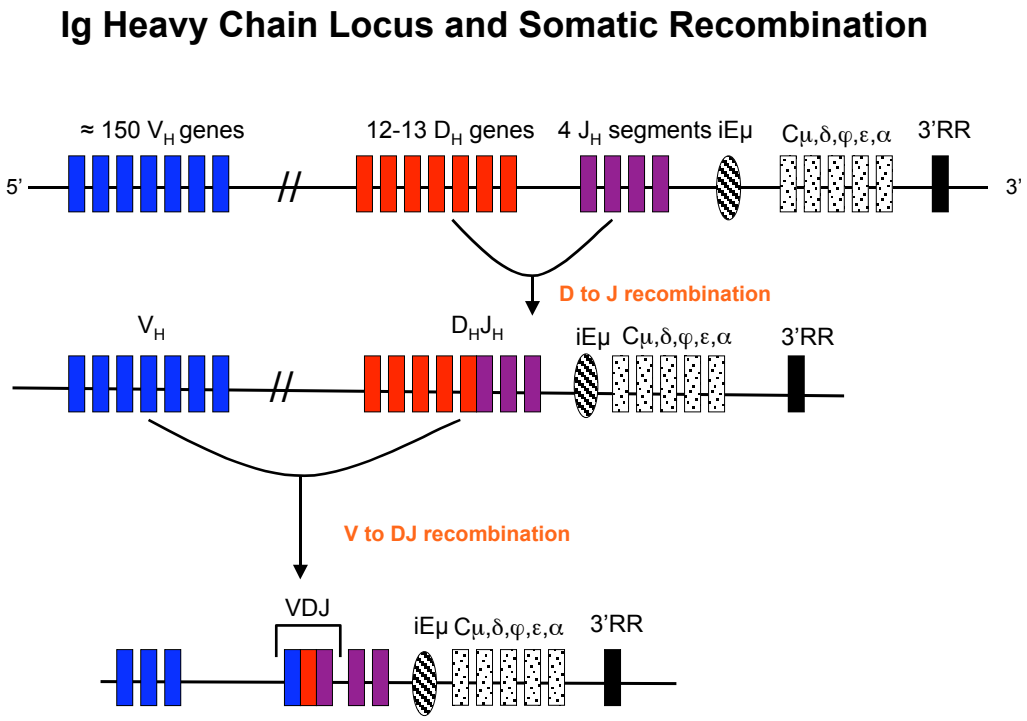


Figure 1. Immunoglobulin heavy chain rearrangement

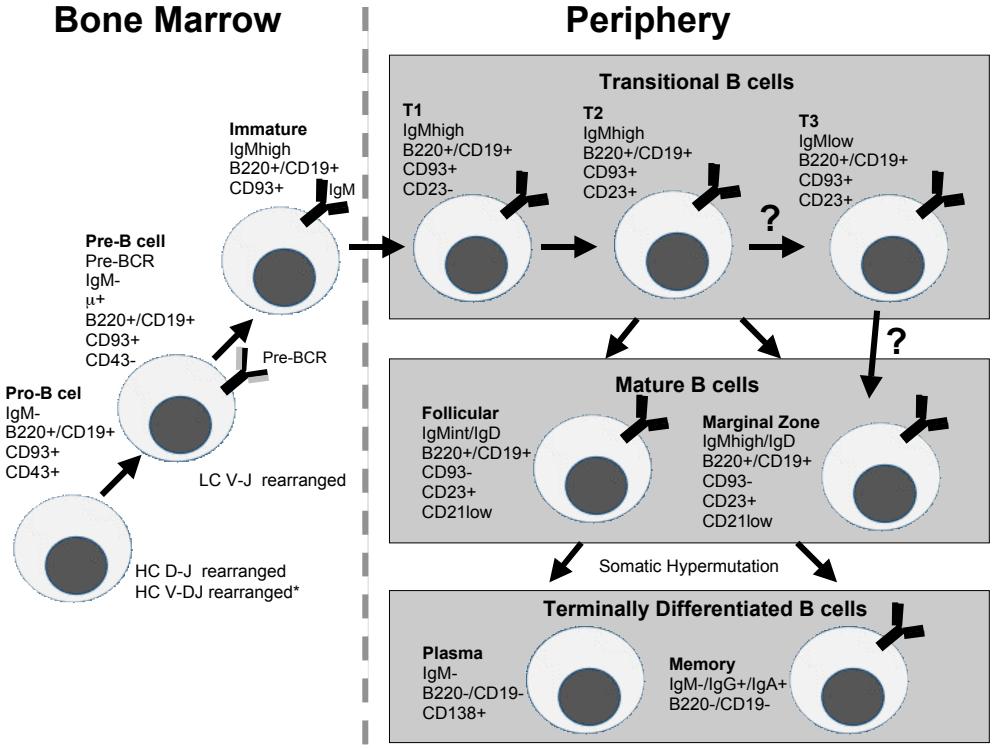


Figure 2. B cell development

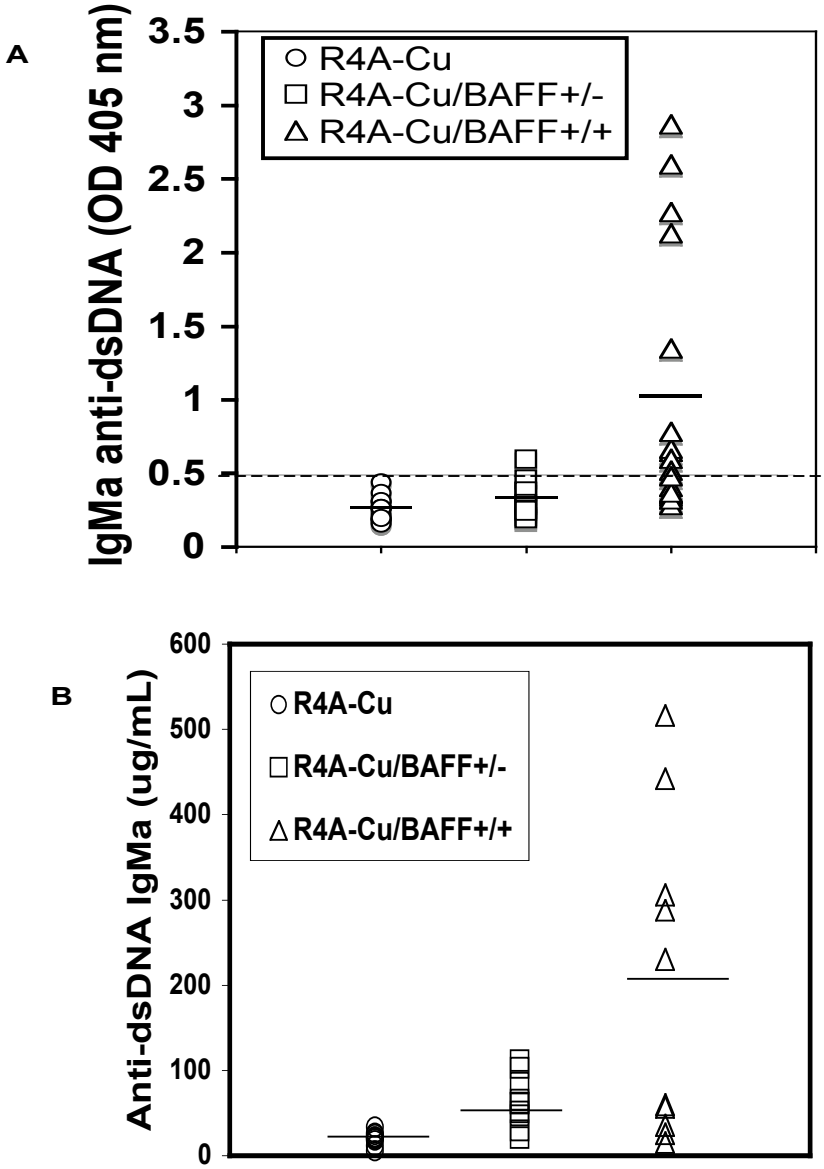


Figure 3. R4A-Cμ mice homozygous for BAFF secrete elevated levels of IgM^a anti-dsDNA antibody and have increased numbers of anti-dsDNA B cells. A. Sera from 13 R4A-Cμ mice, 18 R4A-Cμ/BAFF$^{+/-}$ mice and 18 R4-Cμ/BAFF$^{+/+}$ mice were tested by ELISA for Tg, IgM^a anti-dsDNA antibody. Horizontal lines represent average OD at 405 nm. Dotted line represents 4 standard deviation units above the average OD of serum samples from R4A-Cμ mice. **B.** IgM^a anti-dsDNA antibody concentrations were measured from the sera of 10 R4A-Cμ mice (circles), R4A-Cμ/BAFF$^{+/-}$ mice (squares) and R4-Cμ /BAFF$^{+/+}$ mice (triangles) by a quantitative ELISA.

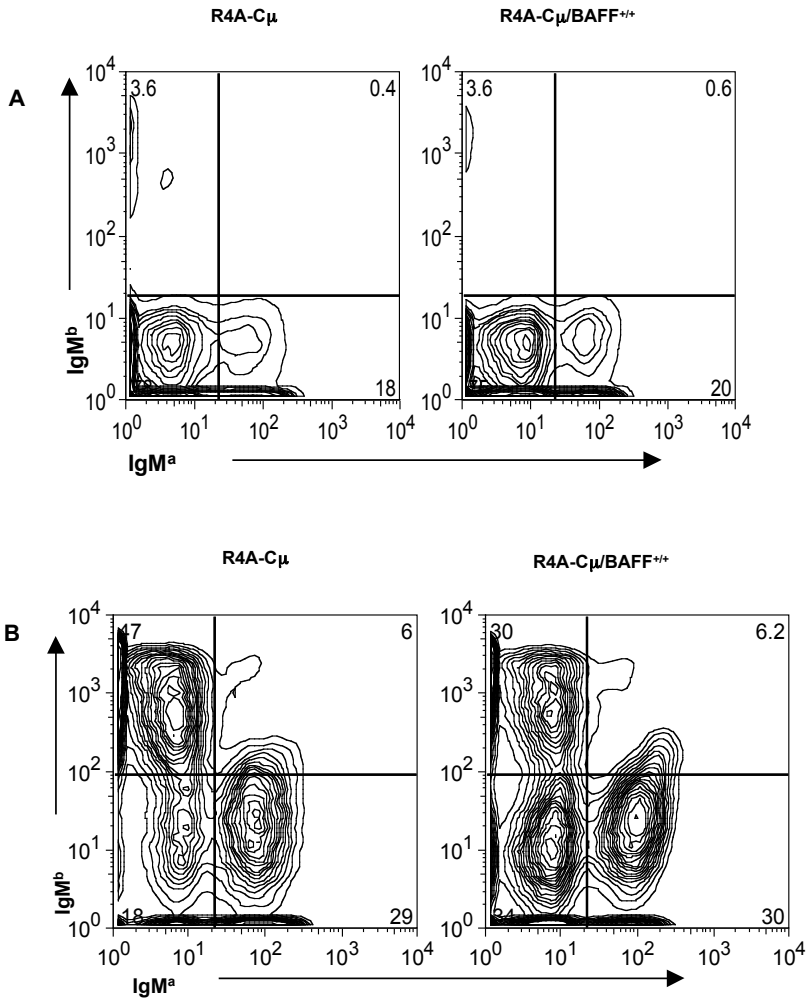


Figure 4. R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice have similar frequencies of Tg B cells in the BM and spleen. The frequency of Tg (IgM^a) and non-Tg (IgM^b) B cells was determined in R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice. Splenic and BM cells were stained with antibodies to B220, IgM^a, and IgM^b. The contour plots represent splenocytes gated on B220. The results are typical of at least 10 experiments.

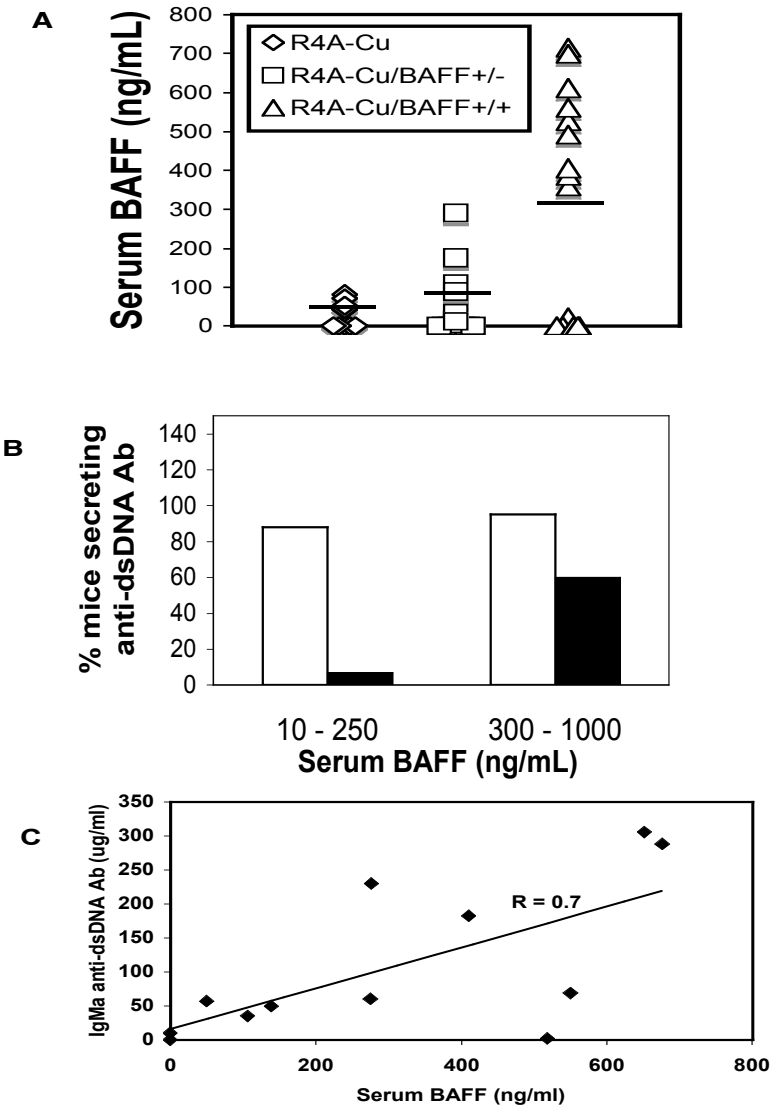


Figure 5. R4A-Cμ anti-dsDNA B cells require high concentration of serum BAFF for the secretion of antibody. A. Variations in serum BAFF concentrations in mice overexpressing BAFF. Serum concentrations of BAFF were measured by ELISA for 11 R4A-Cμ mice (diamonds), 15 R4A-Cμ/BAFF$^{+/-}$(squares) and 15 R4A-Cμ/BAFF$^{+/+}$ mice (triangles). Horizontal bars represent average concentrations of BAFF. **B.** A higher concentration of serum BAFF is required for the secretion of Tg versus non Tg anti-dsDNA antibody. Serum BAFF concentrations were measured for 30 R4A-Cμ /BAFF$^{+/+}$ mice. Mice were grouped into those with high serum concentrations of BAFF (300 - 1000 ng/ml) and those with lower concentrations of BAFF (10 - 250 ng/ml). Graph depicts percent of mice in each group secreting either non Tg (IgM^{b} or IgG) (open bar) or Tg (IgM^{a}) anti-dsDNA antibody (closed bar). **C.** Serum BAFF and anti-dsDNA IgMa concentration were determined in 12 R4A-Cμ/BAFF$^{+/+}$ mice and plotted against each other. The R value of 0.7 and the trendline represent the correlation of serum BAFF and serum anti-dsDNA IgMa.

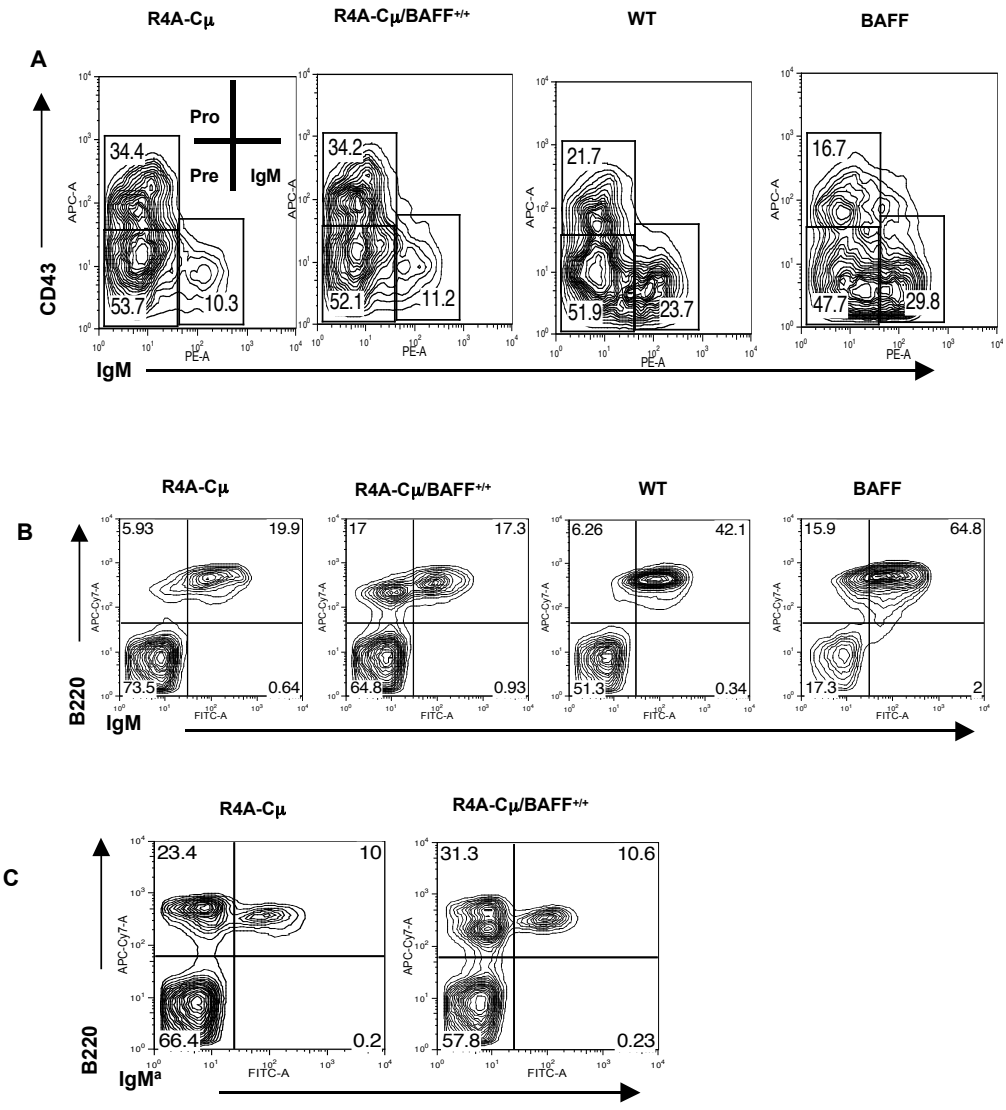


Figure 6. BAFF overexpression does not alter B cell development in the bone marrow. **A**. Bone marrow B cells from R4A-Cμ, R4A-Cμ/BAFF$^{+/+}$, WT, and BAFF mice were immunostained with antibodies to B220, IgM and CD43 and analyzed by flow cytometry. Gates were set on B220^{+} cells. Insert indicates windows for pro- (CD43^{+}IgM^{-}), pre- (CD43^{-} IgM^{-}) B cells, and immature/mature IgM (CD43^{-} IgM^{+}) B cells. **B.** Splenic B cells from R4A-Cμ, R4A-Cμ/BAFF$^{+/+}$, WT, and BAFF mice were immunostained with antibodies to B220 and IgM. Gates were set on live lymphocytes. **C.** Splenic B cells from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice were immunostained with antibodies to B220 and IgM^{a}.

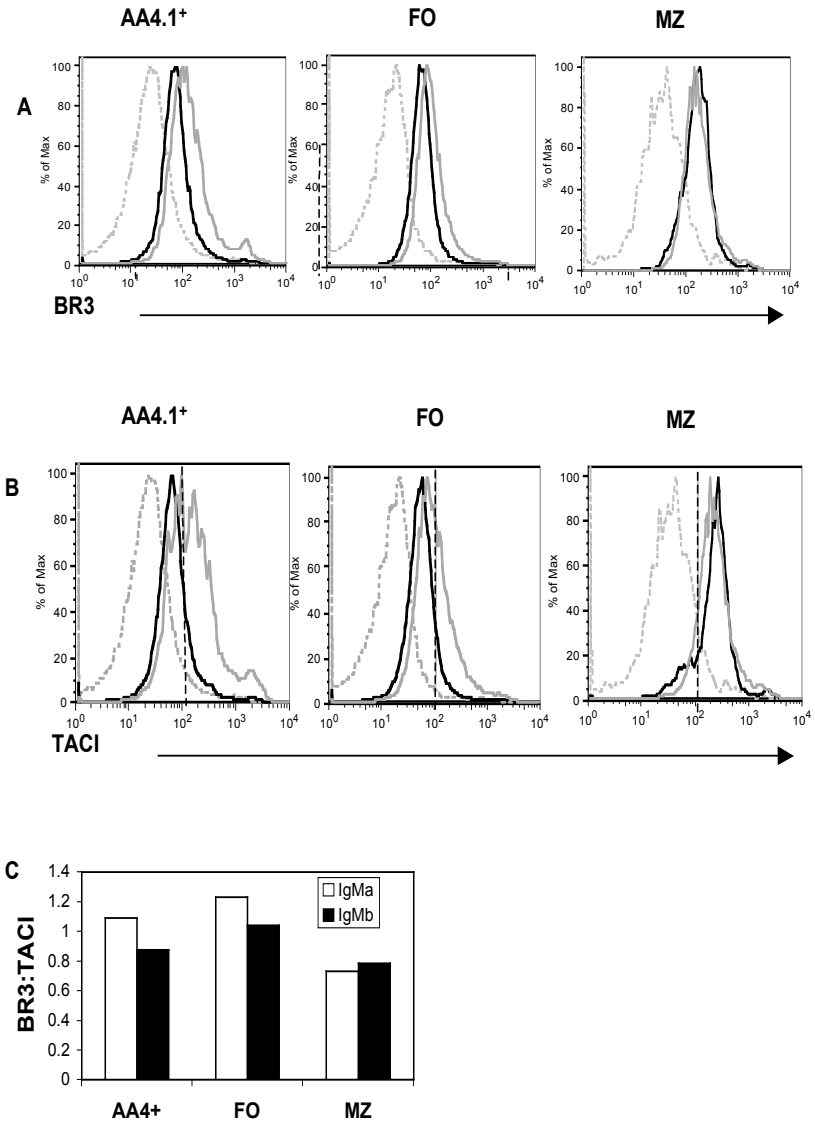


Figure 7. Expression levels of BR3 and TACI are reduced on R4A-Cμ Tg B cells. Splenocytes from R4A-Cμ/BAFF$^{+/+}$ mice were immunostained with antibodies to IgM^{a}, IgM^{b}, B220, AA4.1, CD23, TACI, and BAFF-R. Expression levels of BR3 **(A)** or TACI **(B)** were examined on transitional (B220^{+} AA4^{+}), FO (AA4.1^{-}, CD23^{hi} IgM^{int}), and MZ (AA4.1^{-} CD23^{lo} IgM^{hi}) B cells expressing the IgM^{a} transgene or endogenous IgM^{b}. Gates were set on IgM^{a} (solid black line) or IgM^{b} (solid grey line) B cells with a transitional, FO, or MZ phenotype. Dashed histogram indicates isotype control. Dotted vertical line depicts arbitrary reference point. Histograms are representative of at least 6 experiments. **C.** Ratios of the Mean Fluorescent Intensities (MFIs) of BR3 (in A) to TACI (in B).

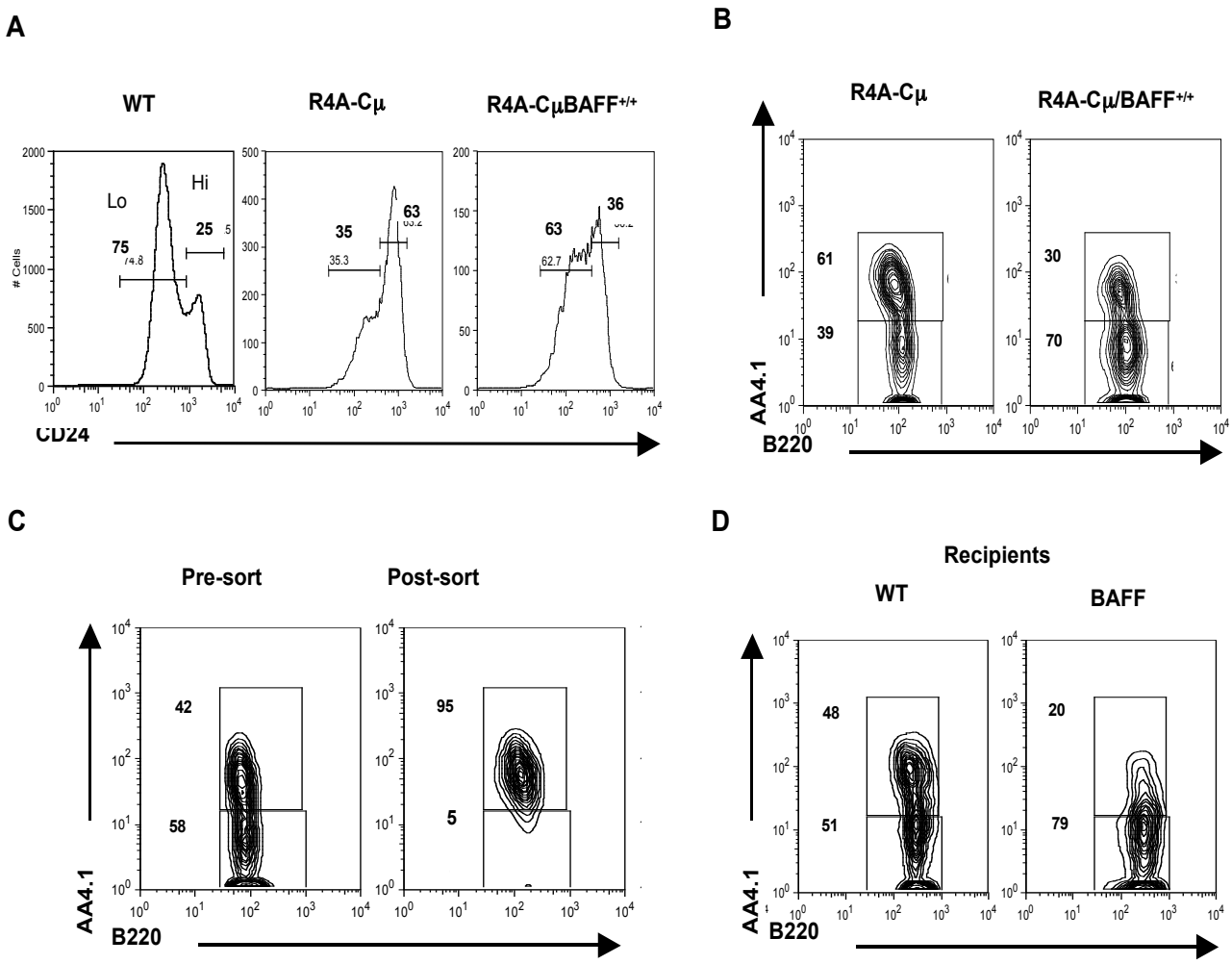


Figure 8. Overexpression of BAFF promotes the maturation of R4A-Cμ, Tg B cells in the periphery. Splenocytes from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice were immunostained with antibodies to IgM^a, B220, CD24, and AA4.1 and analyzed by flow cytometry. **A.** Histogram displays CD24^{hi} and CD24^{lo} IgM^a B cells. Gate set on live, IgM^a, B cells. **B**. Contour graph indicates frequency of AA4.1$^+$ and AA4.1$^-$ Tg B cells in R4A-Cμ and R4A-Cμ/BAFF mice. Gate set on live, IgM^a, B cells. **C.** Splenocytes from R4A-Cμ mice were sorted to collect AA4.1$^+$ Tg B cells, using a BD FACS, ARIA. Purity of the post sort population was 95% (right panel). **D.** Sorted AA4.1$^+$ IgM^a cells from **C** were transferred into wild type or BAFF mice, intravenously. Two days later, the spleens of recipient mice were harvested and immunostained with antibodies to IgM^a and AA4.1 to assess the maturation state of the transferred cells. Results in A and B are representative of 6 experiments and results in C and D are representative of 3 experiments.

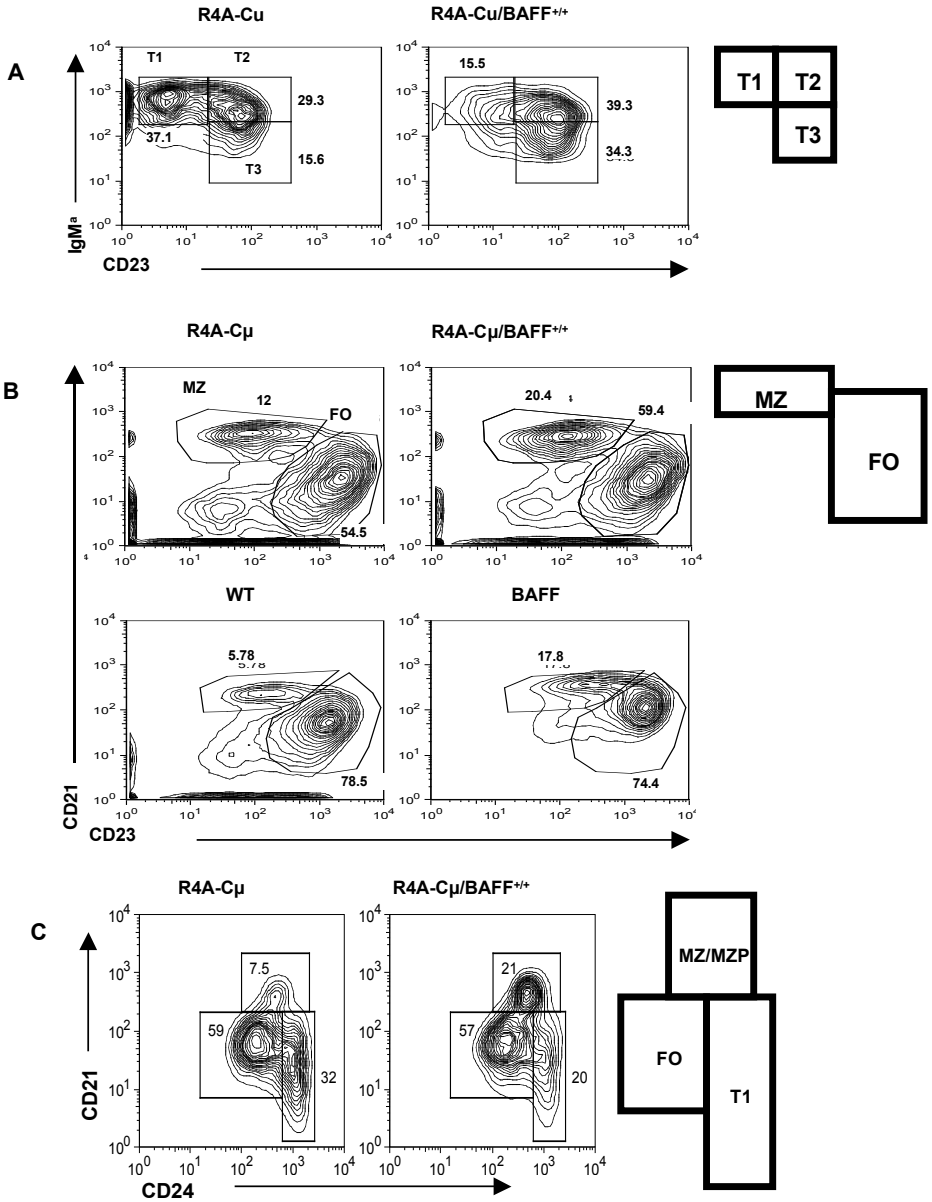


Figure 9. Increased frequencies of late transitional and MZ, Tg B cells in R4A-C*μ*/BAFF$^{+/+}$ mice. Splenocytes from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice were immunostained with antibodies to IgM^a, B220, CD23, and AA4.1 and analyzed by flow cytometry. **A**. Contour graph indicates the frequency of AA4.1$^+$, T1 (IgM$^{a\ hi}$, CD23$^-$), T2 (IgM$^{a\ hi}$, CD23$^+$) and T3 (IgM$^{a\ lo}$, CD23$^+$) B cells in R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice. Gates were set on live, IgM^a AA4.1$^+$ B cells. **B.** Splenocytes from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice (top panel), and WT and BAFF mice (bottom panel) were immunostained with antibodies to B220, CD21, CD23, and antibody to IgM^a (to detect Tg B cells, top panel) or antibody to IgM^b (to detect non Tg B cells from WT and BAFF mice, bottom panel) and analyzed by flow cytometry to determine the frequency of MZ (CD21^{hi} CD23^{lo}) and FO (CD21^{int} CD23^{hi}) B cells. Gates were set on live B220 IgM^a cells for R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice and live B220 IgM^b cells for WT and BAFF Tg mice. **C.** The frequency of MZ precursor IgM^a B cells was determined by CD24 and CD21 staining. MZ precursor B cells are CD21^{hi} and CD24^{int} based on the Rawlings classification, and are expanded in R4A-Cμ/BAFF$^{+/+}$ mice. Results are representative of 6 experiments.

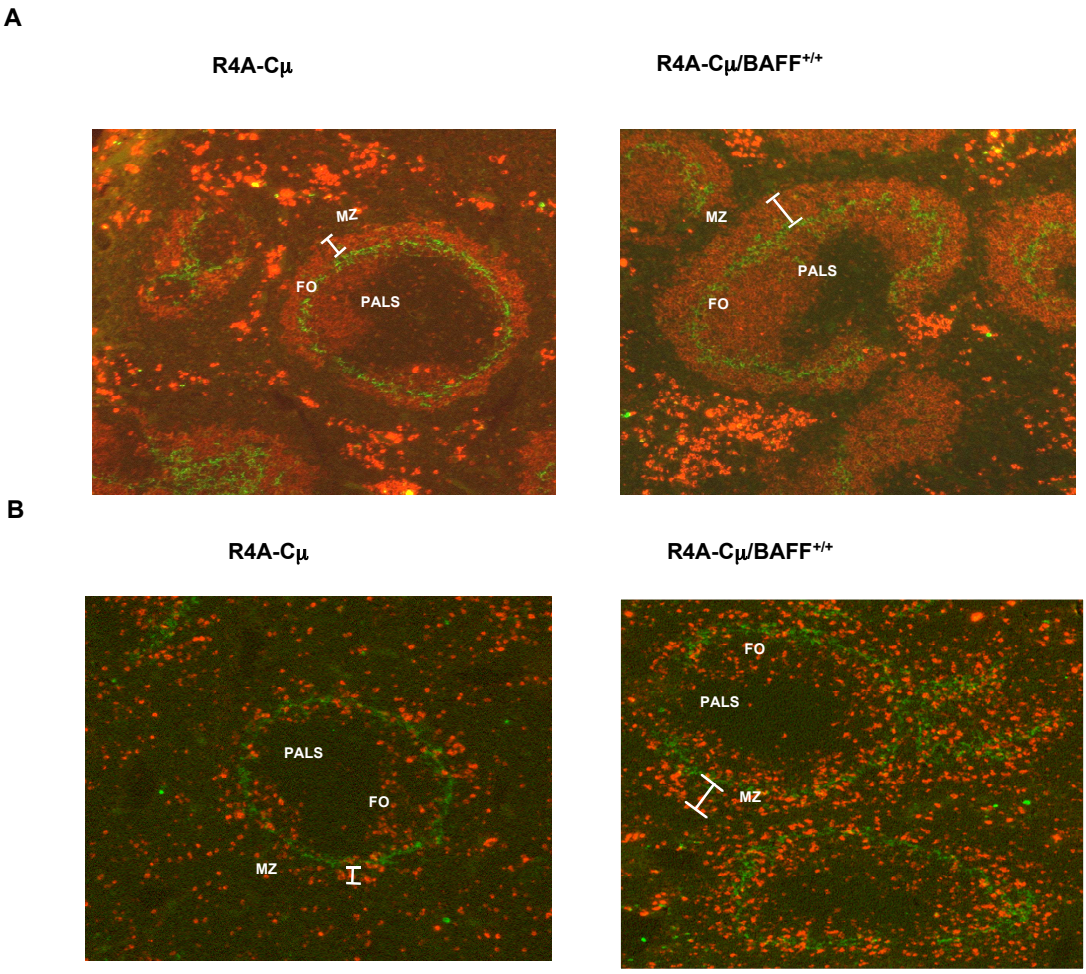


Figure 10. MZ and FO compartments are enriched with Tg IgM^a B cells in R4A-C*μ*/BAFF$^{+/+}$ mice. Frozen spleen sections (6 μm) from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice were immunostained with antibody to MOMA-1 (marker for metallophilic macrophages) and antibodies to total IgM **(A)** or IgM^a **(B)**. Immunostaining of marginal sinus is indicated by green fluorescence. Immunostaining of total IgM B cells in **(A)** or IgM^a B cells in **(B)** is depicted by red fluorescence. (PALS, Periarteriolar lymphatic sheath; MZ, marginal zone: FO, follicle). Crossbars indicate width of the MZ. Sections are representative of 4 experiments.

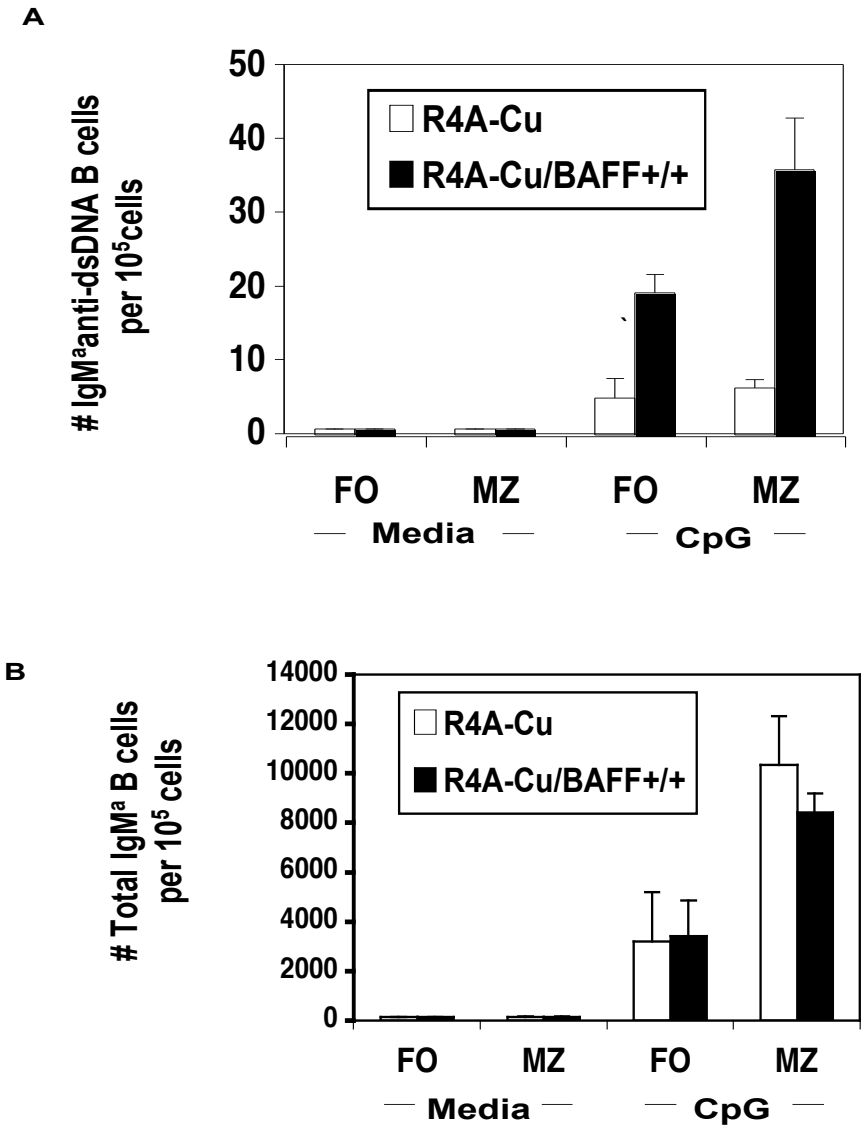


Figure 11. The number of R4A-C*μ*, Tg anti-dsDNA B cells is increased in the MZ and FO of R4A-Cμ/BAFF$^{+/+}$ mice. Splenic B cells from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice were immunostained with antibodies to B220, CD21, and CD23 and sorted to obtain MZ (B220^{+} CD21^{hi} CD23^{lo}) and FO (B220^{+} CD21^{int} CD23^{hi})B cells. Cells were then cultured in either media alone or activated in vitro with 1mg/ml of stimulatory CpG oligonucleotides for 48 hours and then IgM^{a} anti-dsDNA secreting B cells **(A)** and total IgM^{a} secreting B cells **(B)** were enumerated by ELISPOT.

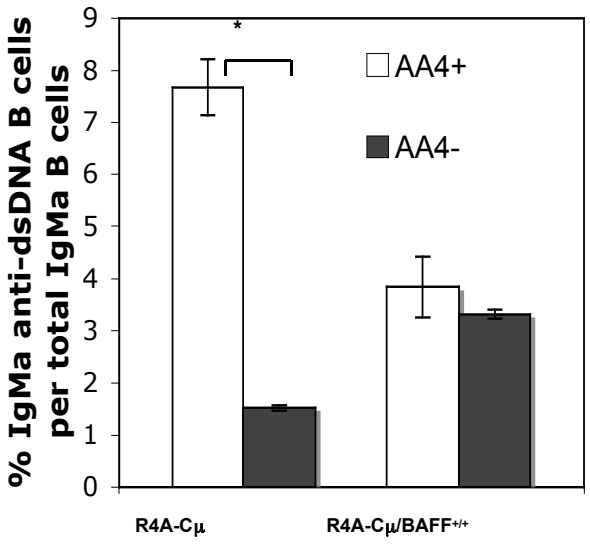


Figure 12. BAFF enables R4A-C*μ*, Tg anti-dsDNA B cells to escape the transitional stage of development. Splenic B cells from R4A-Cμ and R4A-Cμ/BAFF$^{+/+}$ mice were immunostained with antibodies to B220 and AA4.1 and sorted into B220^{+} AA4.1^{+} (transitional) and B220^{+} AA4.1^{-} (mature) populations. They were then activated in vitro with 1mg/ml of stimulatory CpG oligonucleotides and the number of IgM^{a} anti-dsDNA secreting B cells and the total number of IgM^{a} secreting B cells was determined by ELISPOT. Results are represented as the percent of IgM^{a} secreting B cells that are specific for dsDNA. * indicates $p< 0.005$.

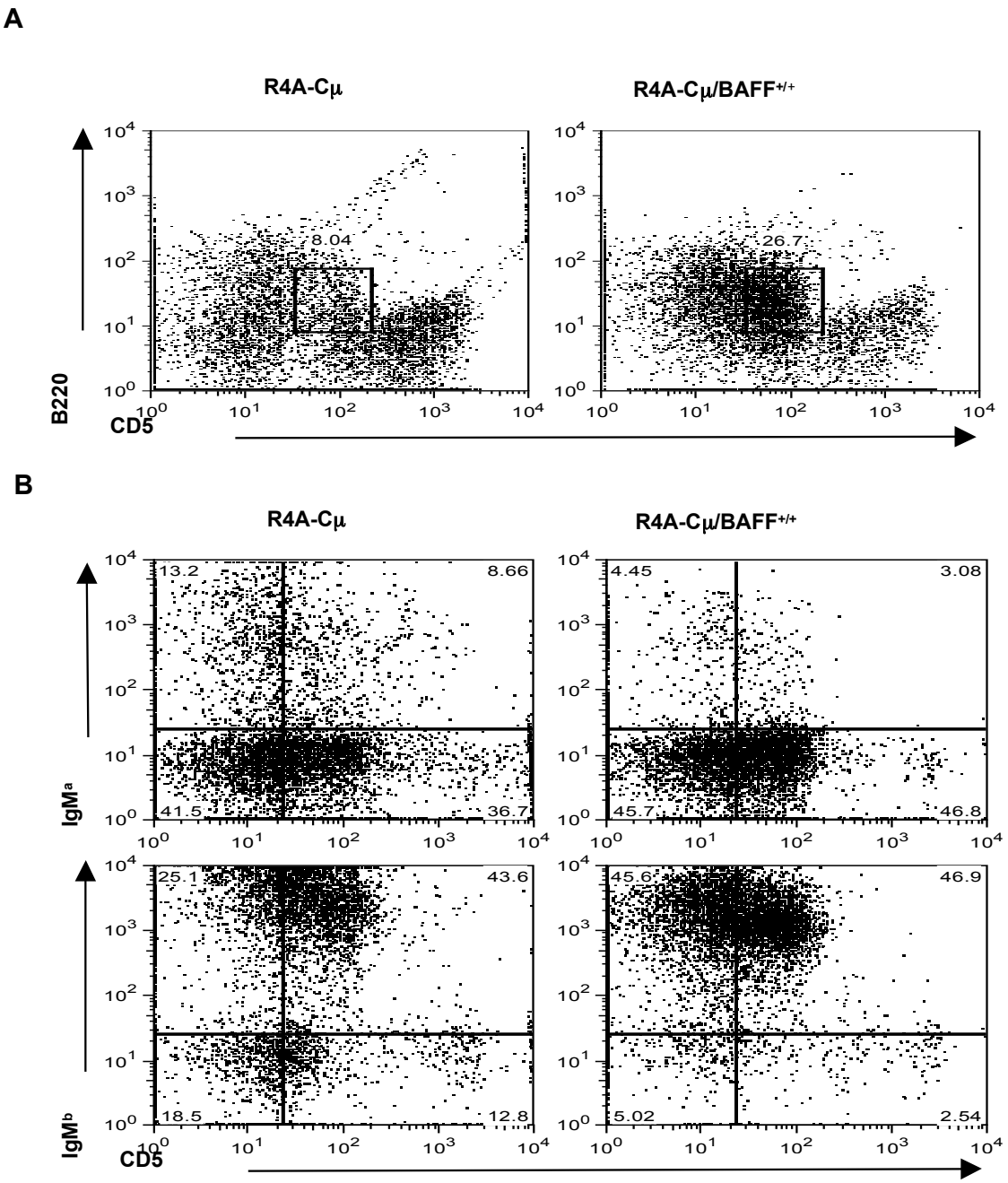


Figure 13. Negligible frequency of peritoneal B1 cells express the IgM^a transgene in R4A-Cμ/BAFF$^{+/+}$ mice. Peritoneal cells isolated by lavage from R4A-Cm and R4A-Cm/BAFF$^{+/+}$ mice were immunostained with antibodies to B220, CD5, and IgM^a to detect the frequency of B1 cells expressing the transgene and IgM^b to detect B1 cells expressing non Tg IgM and examined by flow cytometry. **A.** The frequency of B220$^+$ CD5$^+$ B cells is >3 fold greater in R4A-Cm/BAFF$^{+/+}$ than R4-Cm mice. **B.** Very few peritoneal B cells derived from the B1 lineage express the IgM^a transgene in both R4A-Cm and R4A-Cm/BAFF$^{+/+}$ mice (top panel). Most express the non Tg IgM^b (bottom panel). Gate was set on B220$^+$ B cells and the frequencies of CD5$^+$ and CD5$^-$ B cells expressing IgM^a (top panels) or IgM^b (lower panels) are indicated.

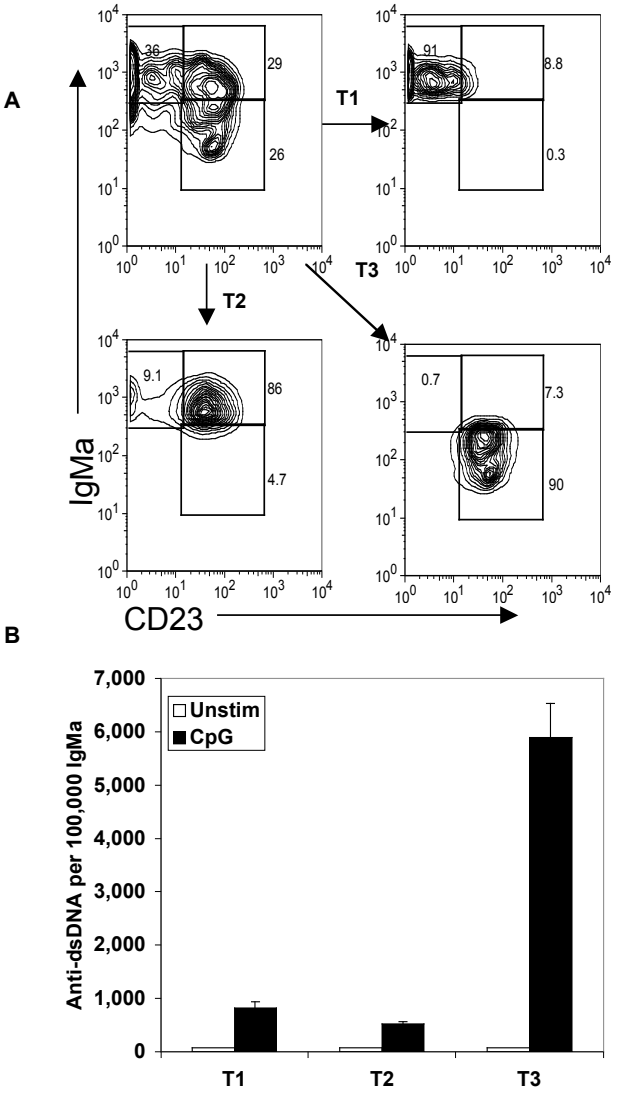


Figure 14. The frequency of Tg anti-dsDNA B cells in elevated in the T3 subset. FACS sorted T1, T2 and T3 B cells were stimulated *in vitro* with stimulatory CpG followed by ELISPOT to determine the frequencies of anti-dsDNA B cells in the transitional subsets. The purity of the sort to isolate T1, T2 and T3 populations was checked on LSRII FACS analyzer (A). The frequency of Tg anti-dsDNA B cells in the T3 subset was higher than in T1 or T2 subsets where low frequencies of Tg anti-dsDNA B cells were detected (B). Error bars in ELISPOT expreriments are based on the standard deviation of the experimental triplicate. The experiments represent typical results obtained in at least two ELISPOT experiments.

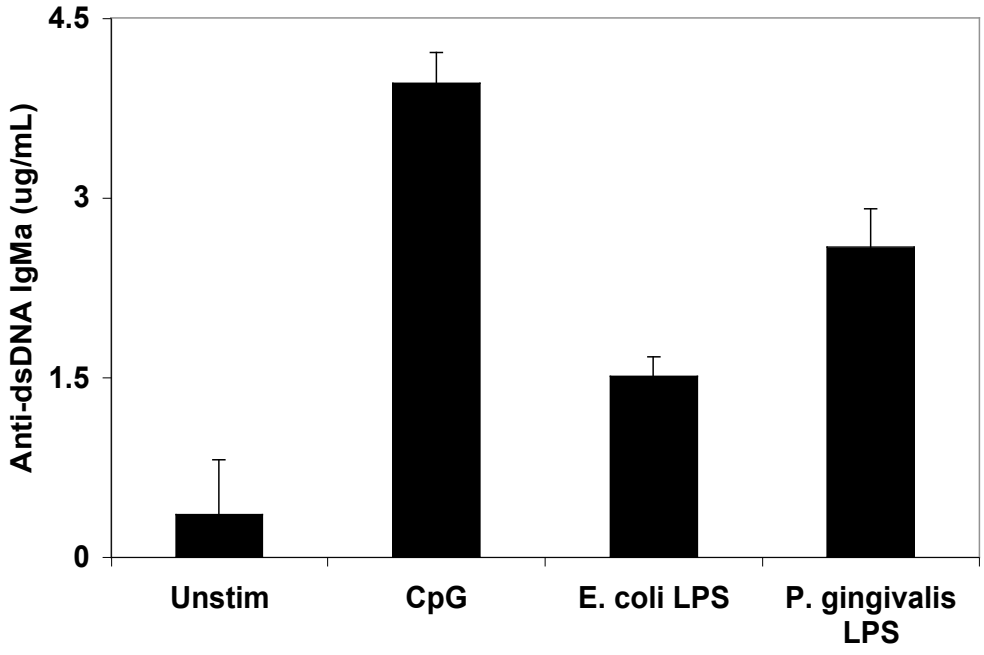


Figure 15. T3 B cells can be induced to secrete anti-dsDNA Ab by a variety of Toll-like Receptor agonists. The responsiveness of T3 B cells to innate immune signaling was tested by quantitating the concentration of anti-dsDNA IgM[a] following *in vitro* stimulation with various TLR agonists: CpG (TLR9), E. coli LPS (TLR4) and P. gingivalis LPS (TLR2).

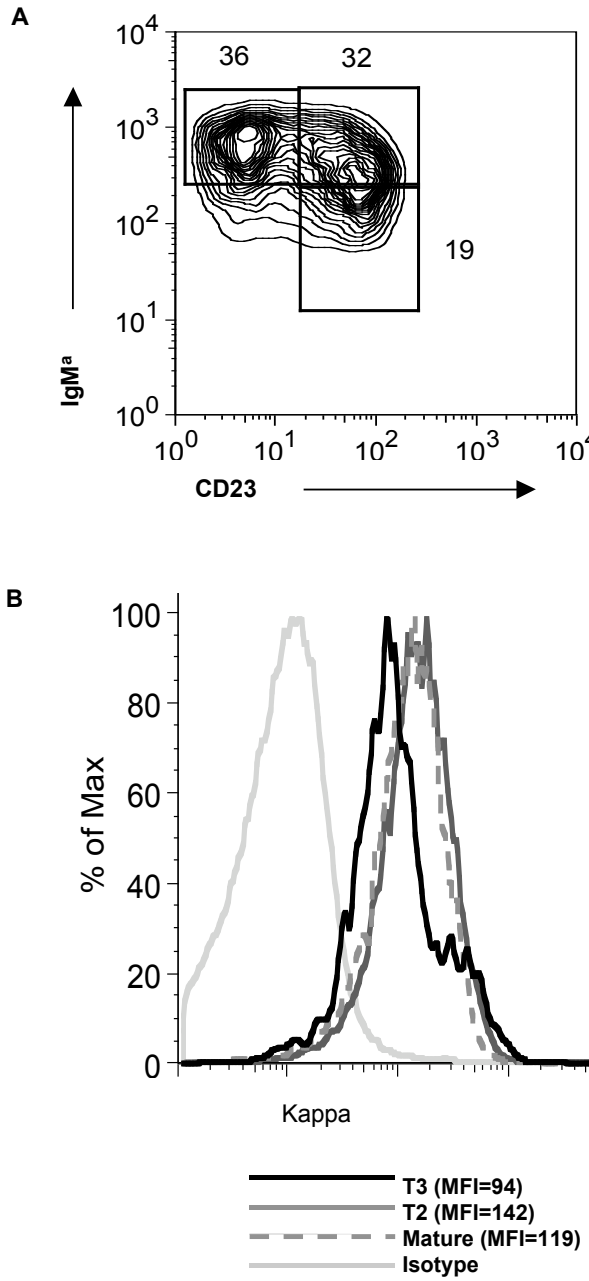
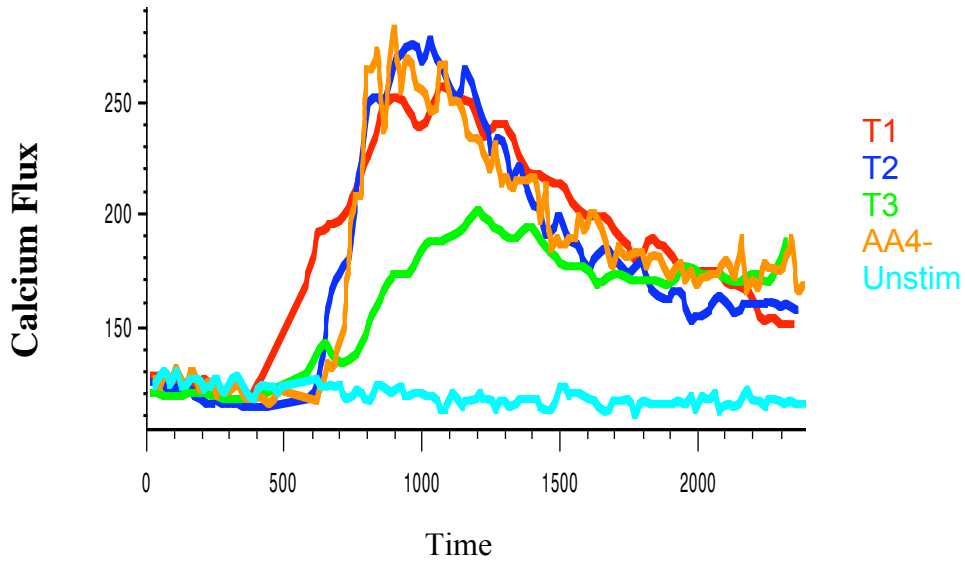


Figure 16. The B cell receptor is downmodulated on T3 B cells. T3 B cells are distinguished by by low IgM^a expression and high CD23 expression from T1 and T2 B cells when transitional (AA4.1+) B cells are resolved into three transitional population according to Allman phenotypes **(A).** In addition, the BCR expression on T3 B cells was assessed by using anti-kappa Ab which binds to the immunoglobulin light chain. T3 B cells expressed lower kappa levels compared to mature and T2 B cells **(B).**

A



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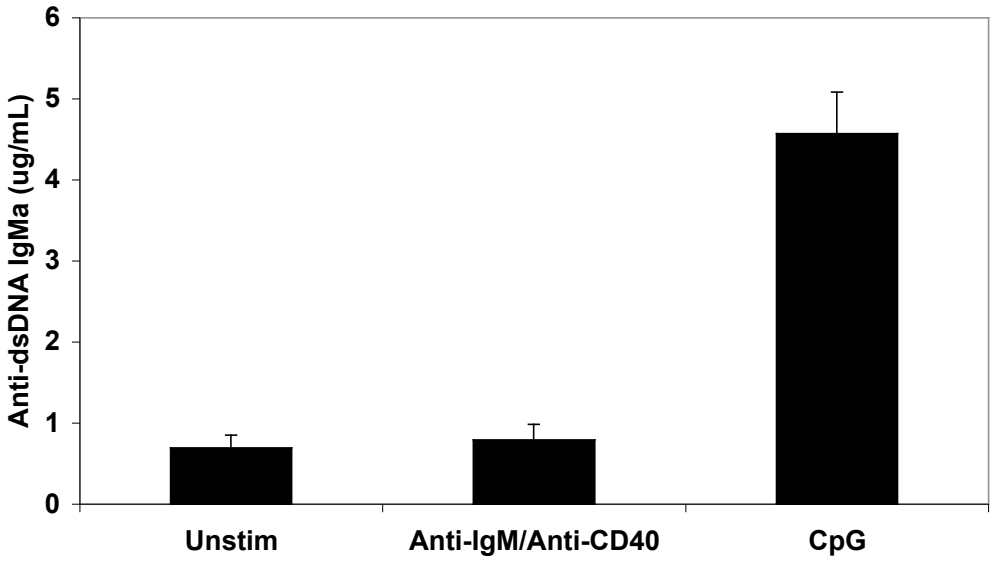

Figure 17. T3 B cells exhibit the characteristics of functional anergy. T3 B cells also showed impaired BCR signaling as indicated by a poor intracellular calcium flux upon the BCR stimulation with an anti-IgM antibody compared to T1 (red), T2 (blue) T3 (green) and mature B cells (orange) **(A).** ELISPOT revealed that while CpG induced anti-dsDNA production by T3 B cells, the stimulation by anti-IgM/anti-CD40 did not, suggesting that T3 B cells may be refractory to the stimulation by an antigen in the presence of T cell help **(B).**

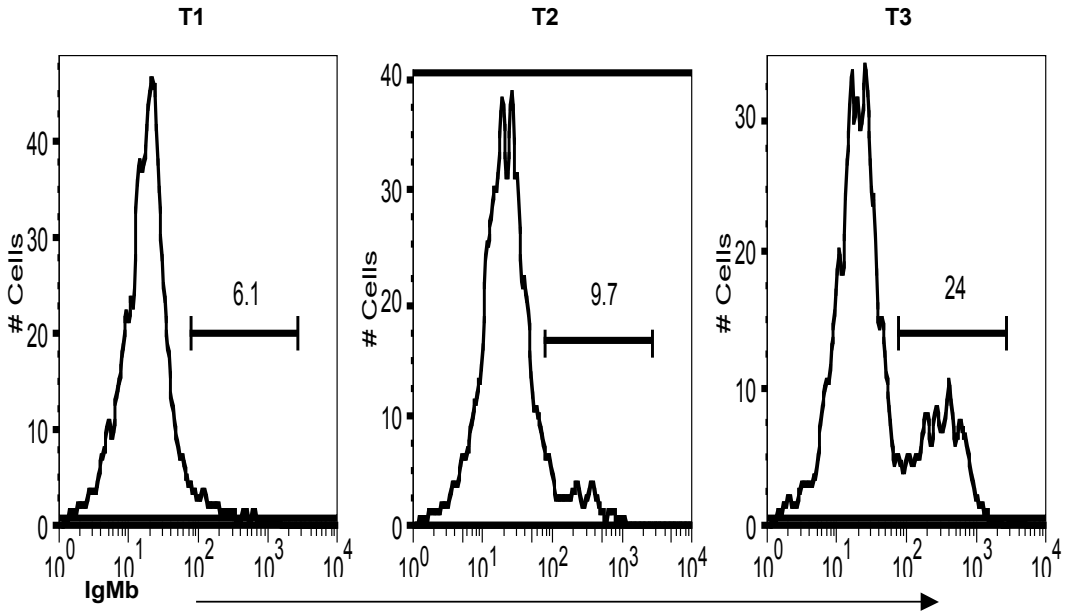

Figure 18. Allelic exclusion is lost in a significant fraction of T3 B cells. Transgenic IgM^a B cells were immunostained to resolved into three transitional populations according to Allman et al. In addition to the antibodies used to identify Tg transitional B cells, anti-IgM^b antibody was included in the staining. The expression of IgM^b on IgM^a T1, T2 and T3 B cells was assessed to determine whether Tg transitional B cell co-express ssurface IgM^a and IgM^b. The frequency of IgM^a and IgM^b dual expressors was elevated in T3 B cells compared to T1 and T2 B cells which constitute 24%, 6.1 and 9.7% of total B cells respectively.

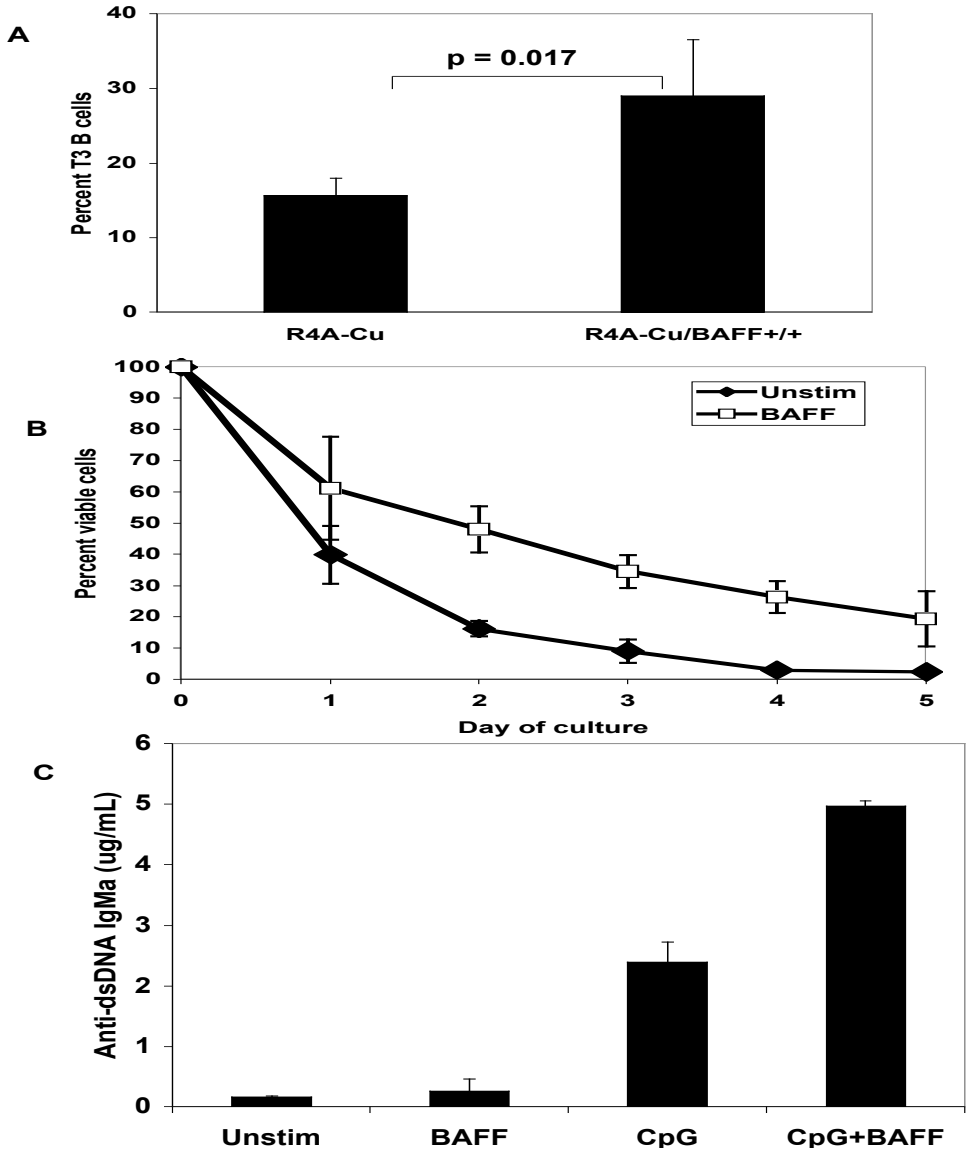

Figure 19. BAFF promotes T3 B cell survival *in vitro* and enhances anti-dsDNA Ab production by T3 B cells. The frequency of T3 B cells in R4A-Cμ and R4A-Cμ/BAFF mice were determined by FACS. R4A-Cμ/BAFF mice exhibited an increased percentage of T3 B cells compared to R4A-Cμ mice **(A).** Bars represent an average of four experiments. To determine whether BAFF could enhance T3 B cell survival, T3 B cells were cultured with (open squares) or without (black diamonds) 1 ug/mL BAFF for four days. Cell viability was measured each day using Annexin V and Propidium Iodide incorporation by FACS. T3 B cells cultured with BAFF showed increased survival **(B),** suggesting that BAFF may also enhance T3 B cell survival in vivo. To determine whether CpG and BAFF can synergize in inducing T3 B cells to secrete anti-dsDNA Ab, FACS sorted T3 B cells were stimulated *in vitro* with CpG or with CpG and BAFF. CpG together with BAFF enhances anti-dsDNA antibody production compared to CpG alone **(C).** In comparison, BAFF stimulation alone did not result in anti-dsDNA Ab production.

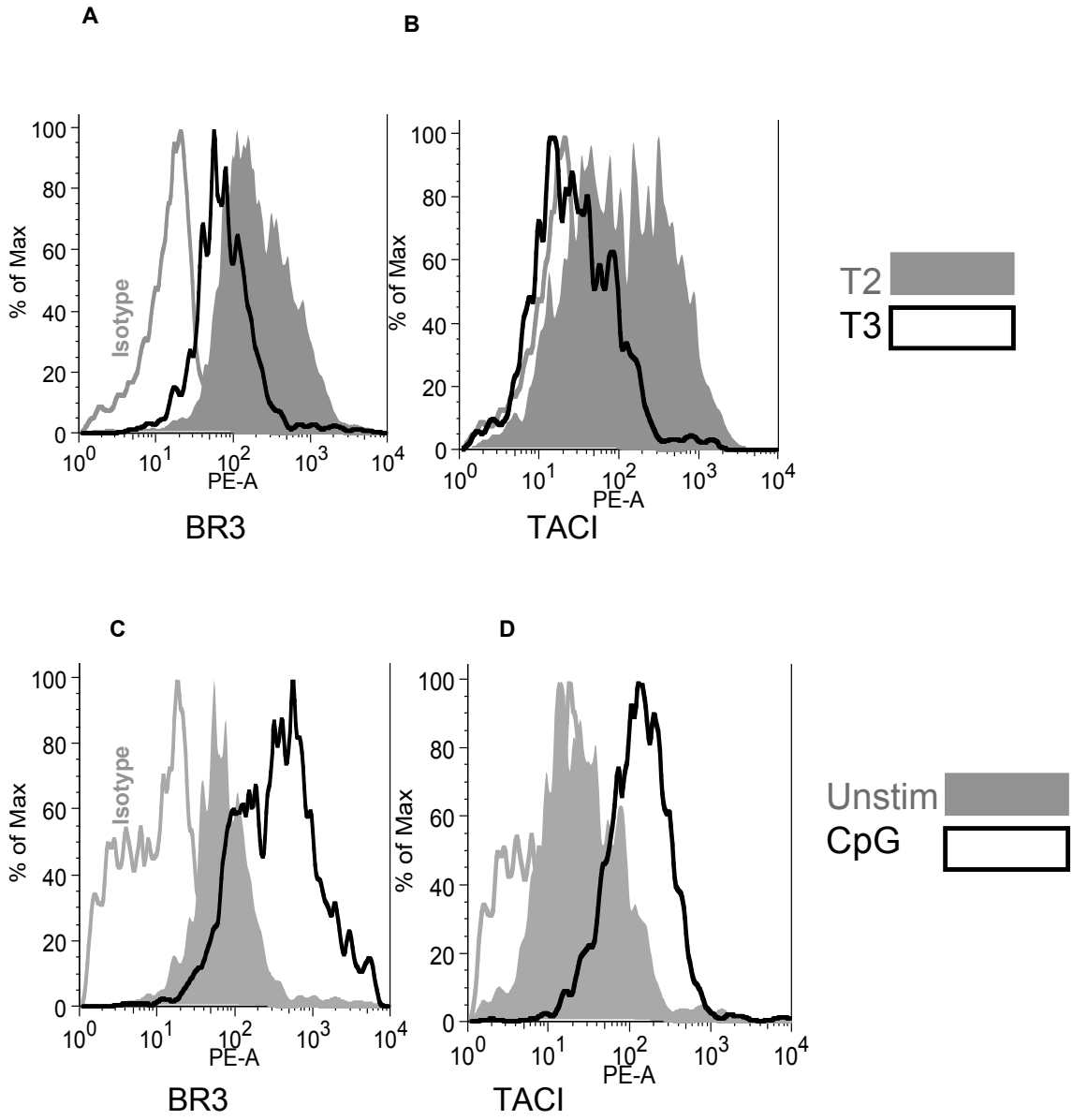

Figure 20. CpG stimulation upregulates BAFF receptor expression on T3 B cells. The expression of two BAFF receptors BR3 and TACI was assessed by FACS using anti-BR3 and anti anti-TACI antibodies. T3 B cells (black line) showed lower levels of BR3 and TACI compared to T2 cells (dark gray) **(A and B)** as well as mature B cells (not shown). To determine whether CpG activation would have an effect of BAFF receptor expression, FACS sorted T3 B cells were stimulated *in vitro* with CpG for 24 hours and BAFF receptors' expression determined by FACS. BR3 and TACI were upregulated by T3 B cells stimulated by CpG (black line) compared to the untreated T3 B cells (dark gray) **(C and D),** suggesting that CpG treatment may not only induce anti-dsDNA Ab production but may also sensitize T3 B cells bind more BAFF present in R4A-Cμ/BAFF mice.

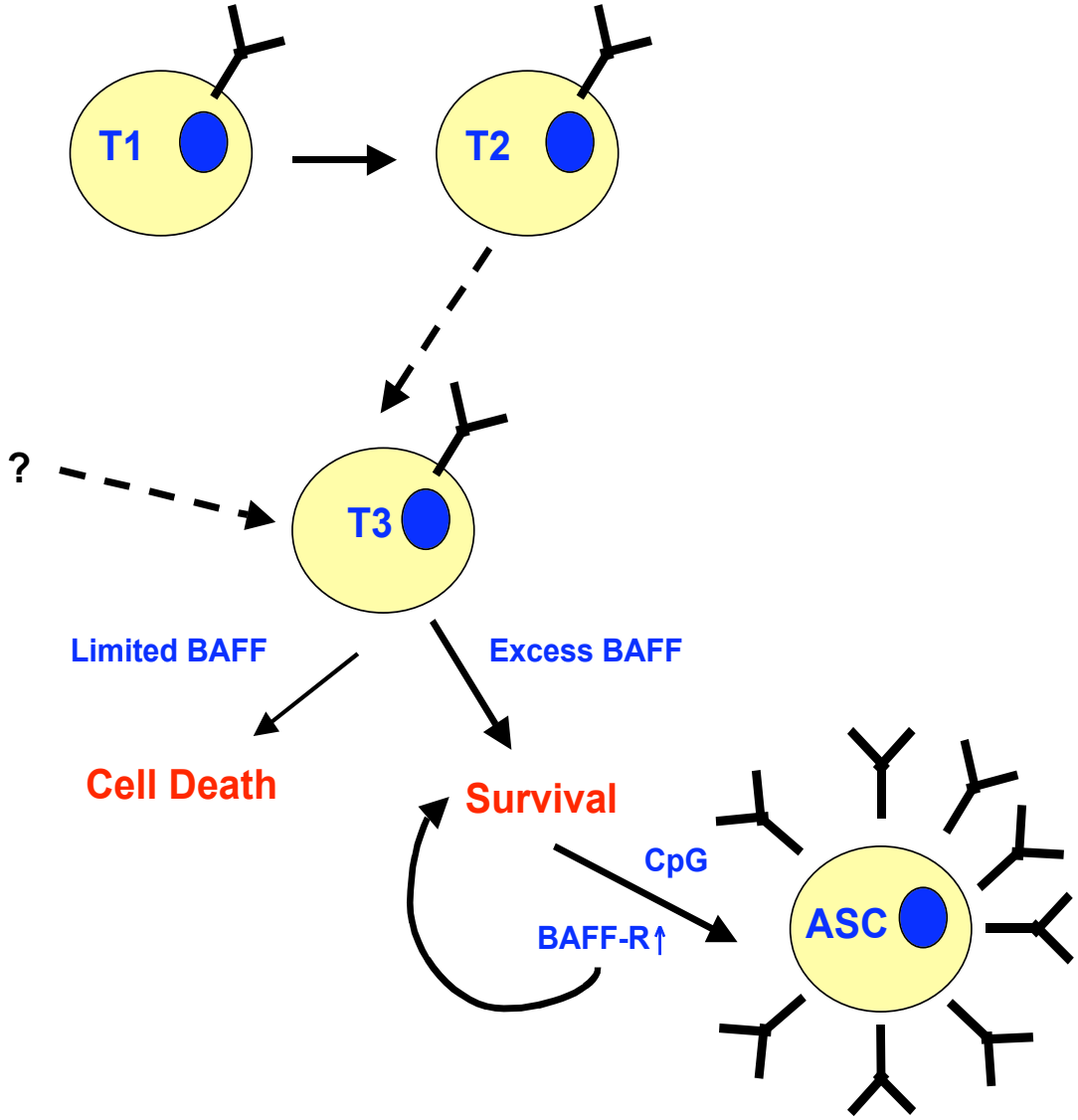

Figure 21. The mechanism of BAFF overexpression and CpG contributing to anti-dsDNA T3 B cell tolerance loss. Based on our results we are proposing a hypothetical model of how BAFF overexpression together with innate immunity signaling may contribute to the production of anti-dsDNA Ab production. During B cell maturation in R4A-Cμ mice a subpopulation of anti-dsDNA B cells is anergized to give rise to T3 B cells. T3 B cells display several characteristics of anergy including the downregulation of BCR and poor BCR signaling. Anergy, together with the downregulation of BAFF receptors may make T3 B cell vulnerable to cell death. In the setting where excessive BAFF is present, the lifespan of T3 B cells may be extended. Excess BAFF may therefore allow T3 B cells to become activated through innate immunity receptors and mature into anti-dsDNA Ab secreting cells. Furthermore, the presence of BAFF may enhance anti-dsDNA Ab production and result in a high concentration of serum anti-dsDNA Ab.