Regulation of anti-dsDNA B-cells in mice transgenic for the heavy and light chains of an anti-dsDNA antibody

By

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ABSTRACT

Regulation of anti-dsDNA B-cells in mice transgenic for the heavy and light chains of an Anti-dsDNA antibody

By Rita H. Lewis

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The diversity of the B-cell repertoire is important for the development of antibodies to a multitude of pathogens. However, in the process of generating antibody diversity, B-cells arise that produce antibodies to self-antigens. These autoreactive B-cells must be kept in check lest they secrete autoantibodies that can induce autoimmune disease. There are several mechanisms inherent in the immune system for regulating autoreactive B-cells. I have been using a transgenic mouse model, in which mice were made transgenic for the R4A IgM heavy and $V_\kappa 1$ light chain genes of an anti-double stranded DNA (anti-dsDNA) antibody, to study the regulation of anti-dsDNA B cells. Anti-dsDNA antibodies are the hallmark of the autoimmune disease Systemic Lupus Erythematosus (SLE). I observed that the transgenic anti-dsDNA B cells are targeted to anergy in the R4A-Cμ/$V_\kappa 1$ mice as evidenced by arrested development, receptor down modulation, and functional unresponsiveness and reduced calcium flux in response to B-cell receptor stimulation. I also observed a relatively high frequency of transgenic B cells in the T1 but not the T2 or mature stages of development. In addition, transgenic T1 B cells were observed to display features of anergy suggesting that the T1 stage may be a regulatory checkpoint where anti-dsDNA B cells are anergized and subsequently

eliminated. Interestingly, transgenic B-cells that were able to transition to the T2 and T3 stages of development tended to co-express an endogenous heavy chain while the majority of mature B cells in these mice expressed the endogenous heavy chain only. Surprisingly, however, B-cells expressing only the endogenous heavy chain on their membrane were also observed to express transgenic heavy chain transcripts. These results have led me to propose a model whereby autoreactive B cells can escape a regulatory checkpoint if they express more than one heavy chain. These dual receptor expressing B-cells may have diminished autoreactivity if the level of membrane expression of the transgenic heavy chain is low relative to the level of expression of the endogenous heavy chain. Subsequent down-regulation of the transgenic heavy chain as a B-cell matures in the periphery may be a novel mechanism for averting autoreactivity.

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Dedicated to

The memory of my mother
Claudia E. Lewis and my brother Samuel C. Lewis

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CHAPTER 1

1. INTRODUCTION

The immune system functions to protect the body from invasion by pathogenic organisms, such as bacteria and viruses as well as environmental agents that are foreign to the body r(1). In addition, it is able to distinguish these harmful agents from the molecules that make up the body. The property of the adaptive immune system, which allows lymphocytes to distinguish foreign antigens from self-antigens, is called self-tolerance. Self tolerance prevents the immune system from producing antibodies against components of one's own body. However, there are occasions when the body produces autoantibodies and mounts an attack against itself; attacking healthy tissue. This is referred to as autoimmunity. Systemic Lupus Erythematosus (SLE), commonly referred to as lupus, is an autoimmune disease that arises as a consequence of a breakdown in self-tolerance. SLE can affect any organ, or group of organs in the body; the skin, the central nervous system (CNS), the skeletal system and various internal organs, such as the kidney. Renal pathology is evident by the deposition of IgG immune complexes and components of complement in the kidney. The hallmark of SLE is the production of IgG antibodies against nuclear antigens specifically antibody to double stranded DNA (dsDNA).

Studies in transgenic mouse models have revealed several mechanisms for regulating the production of anti-dsDNA antibody that operate in both the bone marrow during B-cell development and in the spleen during B-cell maturation. Transgenic mouse studies have demonstrated that self-reactive B-cells are either deleted, anergized (rendered functionally unresponsive to antigen), or receptor edited where the specificity

of the receptor may be altered. These mechanisms are not mutually exclusive; together they effectively regulate autoreactive B-cells that are produced in the body. SLE may arise from a breakdown in a regulatory mechanism(s) during B-cell development.

Our laboratory has been studying B-cell regulation in mice transgenic for the IgM heavy chain of anti-dsDNA antibody designated R4A and observed that anti-dsDNA B-cells in these mice are regulated by anergy and deletion. In these mice the transgenic heavy chain can pair with a variety of light chains to produce both anti-dsDNA and non anti-dsDNA B-cells. A subset of anti-dsDNA B-cells targeted to anergy has previously been observed to utilize the transgenic R4A heavy chain and an unmutated endogenous $V_\kappa 1$ light chain. However the frequency of this subset is low in these mice, therefore the fate of these autoreactive B-cells is difficult to follow in a diverse B-cell environment. To increase the frequency of B-cells targeted to anergy, we therefore bred R4A-C_μ heavy chain transgenic mice with mice transgenic for the unmutated $V_\kappa 1$ light chain gene (a gift from the laboratory of Dr. Martin Weigert) to generate double transgenic R4A-$C_\mu/V_\kappa 1$ mice. In the present study, I have used this double transgenic mouse model, to further our understanding of how, where, and at what stage of development anti-dsDNA B-cells are regulated. I have observed that B-cells are targeted to anergy in the transitional, T1 stage of development in these mice but that some B-cells may escape this regulatory checkpoint by co-expressing an endogenous heavy chain. In addition, evidence suggests that many of these B-cells downregulate expression of their transgenic heavy chain as they mature. The persistence of these autoreactive B-cells in the periphery may be dangerous, since they can potentially be activated by environmental stimuli. Studies on the regulation of anti-dsDNA B-cells are important because understanding normal

regulation will help us gain further insights into how defects in regulation may arise thus leading to SLE and other autoimmune diseases.

1.1 BACKGROUND

1.1a ANTIBODY STRUCTURE

Antibodies are encoded by immunoglobulin genes and are the antigen-specific products of B cells. The production of antibodies in response to infection is an important contribution of B cells to immunity. An antibody binds specifically to a pathogen that elicits the immune response and recruits other cells and molecules to destroy the antibody coated pathogen. Antibodies that bind a pathogen can prevent the pathogen from binding to target cells, thereby neutralizing the pathogen. Antibodies can also enhance phagocytosis by coating a bacteria and then binding via their Fc region to Fc receptors on macrophages. This is referred to as opsonization. Binding of an antibody to a pathogen can also activate the complement cascade, leading to complement mediated cell lysis (1). All antibodies consist of two heavy chains and two light chains held together by non-covalent interactions and stabilized by disulfide bonds (Figure 1). Both the heavy and the light chains are composed of a constant region and a variable region. The overall shape of an antibody is a Y conformation. Each arm of the ″Y″ contains the antibody-binding site formed by the association of the amino terminal ends of the light chains with the amino terminal ends of the heavy chains. The amino terminal ends contain the variable regions. The variable regions are joined to the constant region by a flexible hinge region, and the pairing of the carboxy-terminal halves of the two heavy chains form the Fc constant region.

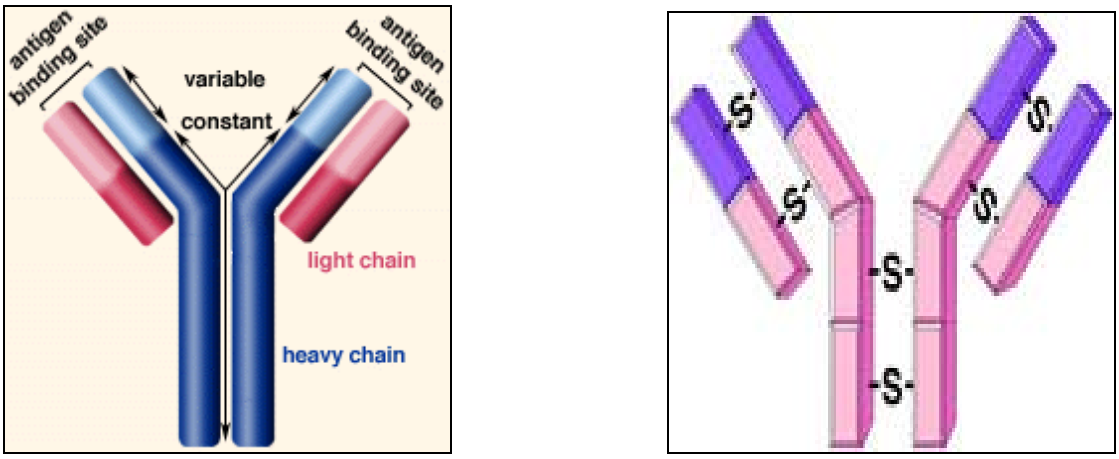


Figure 1. Antibody structure
Right panel shows a schematic of the general structure of a soluble antibody molecule (http//:www.bio.davison.edu/antibody). Left panel shows disulphide bonds that stabilizes the antibody (http//:www.fotosearch.com/antibody).

Within the variable regions of the heavy and light chains, there are three hyper-variable regions approximately 6-10 amino acids in length. These regions are called the complementarity-determining regions (CDR1, CDR2, and CDR3), and comprise the antibody binding site and give the antibody its specificity. Each CDR is separated by a relatively constant amino acid sequence, known as the framework region (FR) of which there are three FR1, FR2 and FR3 (1, 2).

Antibody molecules can be divided into different classes or isotypes based on differences in the structure of their heavy chain constant (C) regions. There are 5 heavy chain constant regions μ, δ, α, ε, and γ which give rise to the five classes of antibodies; IgM, IgD, IgA, IgE and IgG. Within the IgG isotype, there are four murine subclasses, IgG-1, IgG-2a, IgG-2b and IgG-3 (2). The classes and subclasses of an antibody determine the effector function of the antibody. Antibodies may vary slightly between different mouse strains due to allelic differences in amino acids sequences within the constant regions. These are known as allotypic differences. Antibodies also differ in

determinants that are unique to their variable regions. These determinants are referred to as idiotopes. Some idiotopes may be located within the antigen-binding site while others are located outside of the binding site. The sum of these idiotopes is called the idiotype of the antibody. An anti-idiotype antibody can be generated that is specific for an idiotypic determinant of an antibody by injecting that antibody into an animal (3).

1.1b IMMUNOGLOBULIN GENES, REARRANGMENT AND ALLELIC EXCLUSION

Three gene loci encode for the immunoglobulin (Ig) heavy and light chain genes. The heavy chain locus (HC) in the mouse is located on chromosome12, while the kappa (κ) light chain locus is located on chromosome 6 and the lambda (λ) light chain locus is located on chromosome 16 (4). The HC locus consists of four gene clusters 3 Mbp in size, the variable (V_H) gene region, the diversity (D_H) region, the joining (J_H) region and the constant region (C_H). In mice there are 195 V_H segments, 10-13 D_H regions, 4 J_H regions and eight constant (C_H) region gene segments that encode heavy chain isotypes; C_μ, C_δ, $C_{\gamma1}$, $C_{\gamma2a}$, $C_{\gamma2b}$, $C_{\gamma3}$, C_α, and C_ε (Figure 2) (5).

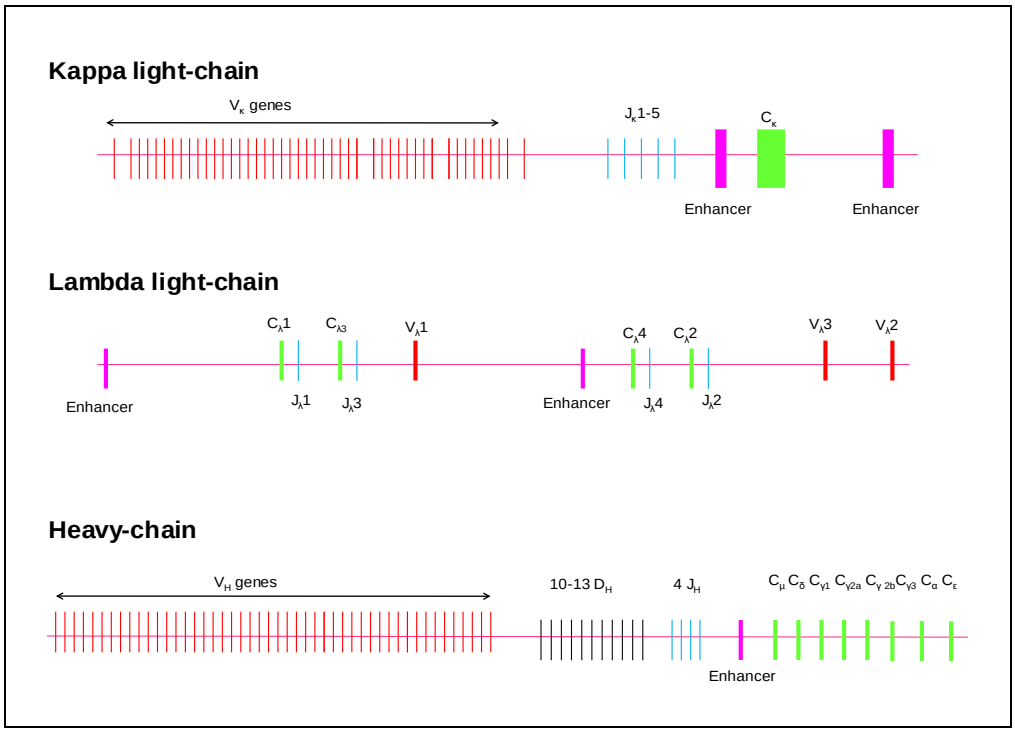


Figure 2. Representative schematic of the germ line immunoglobulin gene loci (not drawn to scale)

In the mouse there are seventeen V genes families, encoding approximately 110 V genes and 85 pseudogenes, genes that have a structure similar to other genes but cannot be expressed due to mutations. Each gene segment is approximately 500bp. The largest gene family J558 is located at the 5′ end and of the V_H loci. The middle region of the heavy chain variable gene locus contains the J606 gene family interspersed with members of the 36-60, and the S107 V_H family. At the 3′ end of the variable region gene are the Q52 and 7183 gene family members (6, 7).

The kappa light chain gene locus is 3,200 kb containing 174 immunoglobulin V kappa genes; ten are orphan genes. Orphan gene are genes that based on homology cannot be connected to any know gene family. There are eighteen subgroups and 93 IgV_κ genes, five J_κ-segments and one kappa constant gene (C_κ) (8). The lambda locus is 240kb

and has three variable segments, and four constant region genes. Each constant region gene has its own J-region segment (5, 9).

The V, D and J segment genes of the immunoglobulin heavy chain and the V and J segments of the light chain genes are flanked by conserved sequence recombination signal sequences (RSSs) that consist of conserved heptamer (5′-CACAGTG-3′) and nonamer (5′ACAAAAACC3′) motifs separated by a non-conserved spacer region of 12bp, or 23bp (10). Gene segments with a 12bp spacer can only join to a segment with a 23bp spacer, the 12 /23 rule (1). These RSSs specify the site of DNA cleavage by the recombination activating gene (RAG) proteins, RAG-1 and RAG-2. If the gene segments being recombined are in the same transcriptional orientation, then DNA is deleted as a result of recombination. If the gene segments being recombined are in the opposite transcriptional orientation, then the DNA is not deleted, but is inverted (11-13). RAG-1 recruits RAG-2 protein and produces a double-stranded break in the DNA at the boundary of the 5′ end of the heptamer and the gene coding region. This produces a hairpin at the coding end of the DNA, and a blunt 5′ phosphate at the signal (RSS) end. After cleavage the DNA is joined by non homologous end-joining, adding or removing nucleotides at the site (14, 15).

Immunoglobulin gene rearrangement occurs in an ordered sequence of events in which the heavy chain immunoglobulin DNA rearranges before the light chain DNA (16). The first step in immunoglobulin rearrangement is the joining of a D segment to a downstream J-segment (D-J recombination), and the deletion of the intervening DNA (Figure 3). Then a V-segment is rearranged next to this D-J unit. The rearranged VDJ segment is then transcribed along with the downstream C region. The primary RNA

transcript undergoes splicing removing introns bringing the constant region and the VDJ regions together. This mRNA is then translated into the IgM heavy chain polypeptide, and alternate splicing produces IgD heavy chain polypeptide.

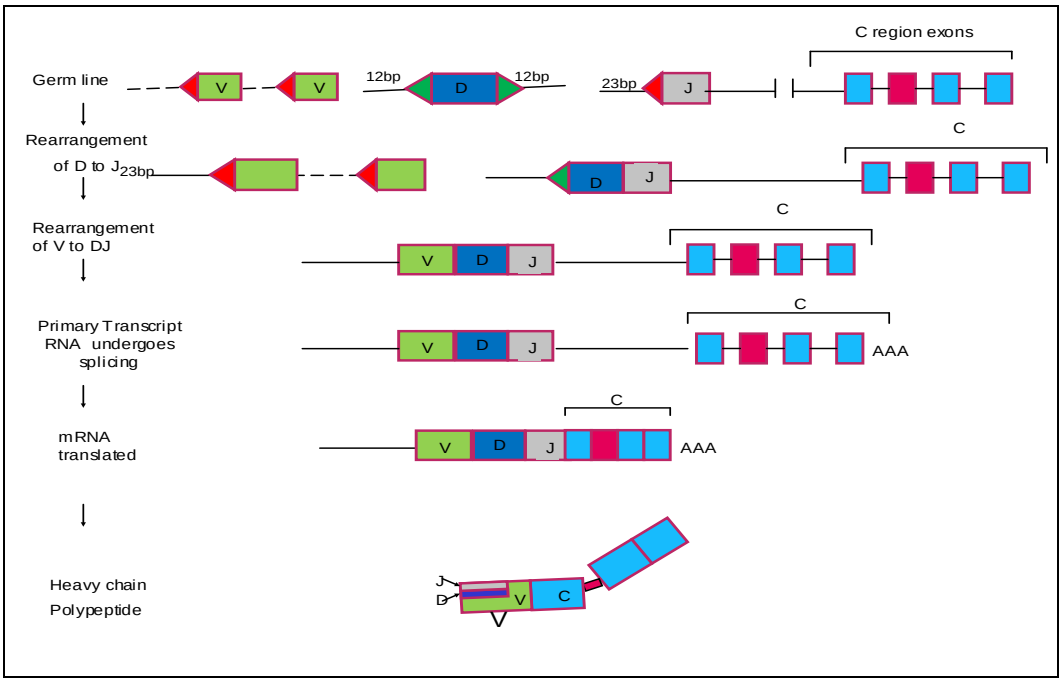


Figure 3. Schematic of heavy chain gene rearrangement of Diversity (D), joining (J), variable (V) and constant(C) regions genes; showing gene segments RSS and simplified to show one allele (not drawn to scale).

Gene rearrangement at the kappa light chain locus involves the joining of a V segment to a downstream J_k segment (Figure 4). Functional heavy chain and light chain polypeptides associate with one another in the endoplasmic reticulum (ER) to produce a functional antibody molecule.

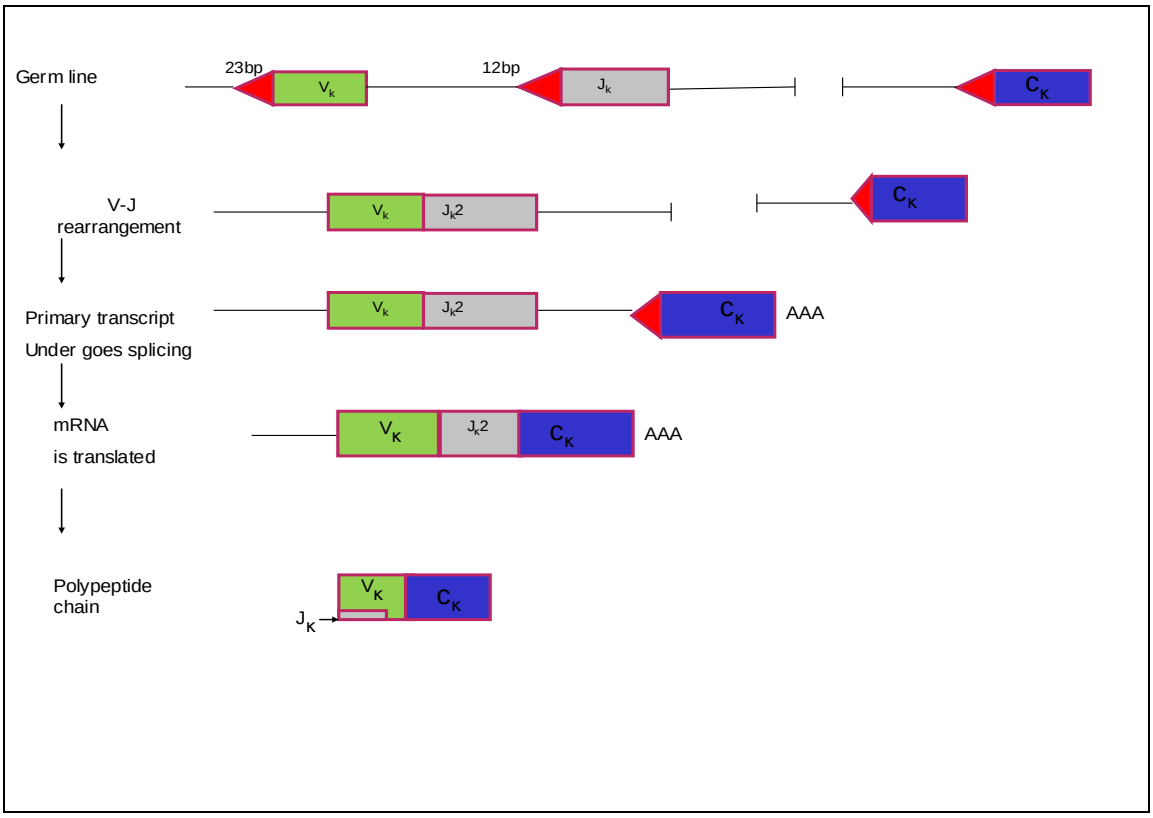


Figure 4. Immunoglobulin gene rearrangements at the kappa light chain locus. Figure simplied to show rearrangemnt on one allele variable (V), joining (J) and constsnt (C) region genes. Not drawn to scale.

1.1c Allelic Exclusion

Allelic exclusion is the phenomena whereby a B cell expresses only one functional heavy chain, and one functional light chain generating B lymphocytes with a receptor of a single specificity. In heavy chain allelic exclusion, the production of a functionally rearranged heavy chain that is paired with the surrogate light chain prevents the rearrangement of the other HC allele. Likewise productive rearrangement of the light chain gene and association with a functional HC prevents rearrangement on the other light chain alleles (1). Feedback mechanisms play a role in maintaining allelic exclusion. Immunogloblulin gene rearrangement leads to membrane signaling and the subsequent down regulation of recombination factors like RAG-1 and RAG-2 (17).

1.2 B-CELL DEVELOPMENT

1.2a B-cell development in the bone marrow

The major subset of B-cells is the conventional B-2 B-cells subset (18). During early fetal development, B-2 cells develop in the liver, but later on and throughout adulthood they develop and differentiate in the bone marrow, the primary lymphoid organ. B-lymphocytes are detected in the bone marrow at day 17 of gestation (19). They complete their differentiation to mature B-cells in secondary lymphoid organs in the periphery, such as the spleen, lymph nodes and Peyer's patches (20) **.**

B-cells arise from hematopoietic stem cells in the bone marrow, in response to interactions with the bone marrow microenvironment such as adventitial reticular cells referred to as stromal cells. Stromal cells have extensive processes that adhere to many hematopoietic cells via adhesion molecules, for example the vascular cell adhesion molecule-1(VCAM-1 or CD106). Stromal cells are the only cells in the marrow to produce the cytokine growth factor interleukin-7 (IL-7), and both stromal cell contact and IL-7 are needed for B-lymphopoiesis (21). IL7 also mediates B- cell differentiation (22).

The successive stages of B-cell development are identified by the temporal expression of certain cell surface proteins called clusters of differentiation (CD) antigens that can be identified by monoclonal antibodies. Rumfelt et al. showed that hematopoietic stem cells produce multi-lineage progenitors (MLP) that are CD93/AA4.1 ^{high}, CD43^{medium} (leukosialin), cKit ^{high} (receptor tyrosine kinase), and CD24/HSA ^{low} (Heat Stable Antigen, a cell surface glycoprotein involved in cell adhesion) (23). The multi-lineage progenitor can generate erythroid/ myeloid and lymphoid lineage cells. Macrophages, dendritic cells and granulocytes and megakaryocyte and red blood cells

arise from the common myeloid progenitor (CMP), while the common lymphocyte progenitor (CLP) gives rise to B cells, T-cells and nature killer cells (1, 23, 24). Hardy et al. used flow cytometry and the expression of surface molecules in normal bone marrow cell populations, to delineate the early stages of B lineage lymphocyte development (25, 26). Hardy and colleagues showed that B-cells develop through a series of ordered steps (Figure 5). They observed that pre-pro-B cells ($B220^{+}CD43^{+}$ $CD24^{low}$), differentiate into pro-B cells ($B220^{+}CD43^{+}$ $CD24^{+}$). These cells then develop into pre-B cells ($B220^{+dull}$ $CD43^{-}$ IgM^{-}) followed by newly generated immature B-cells ($B220^{+dull}$ $CD43^{-}$ IgM^{+}). Immature B-cells then develop into mature B-cells that are $B220^{+bright}$ $CD43^{-}$ IgM^{+} IgD^{+} (25).

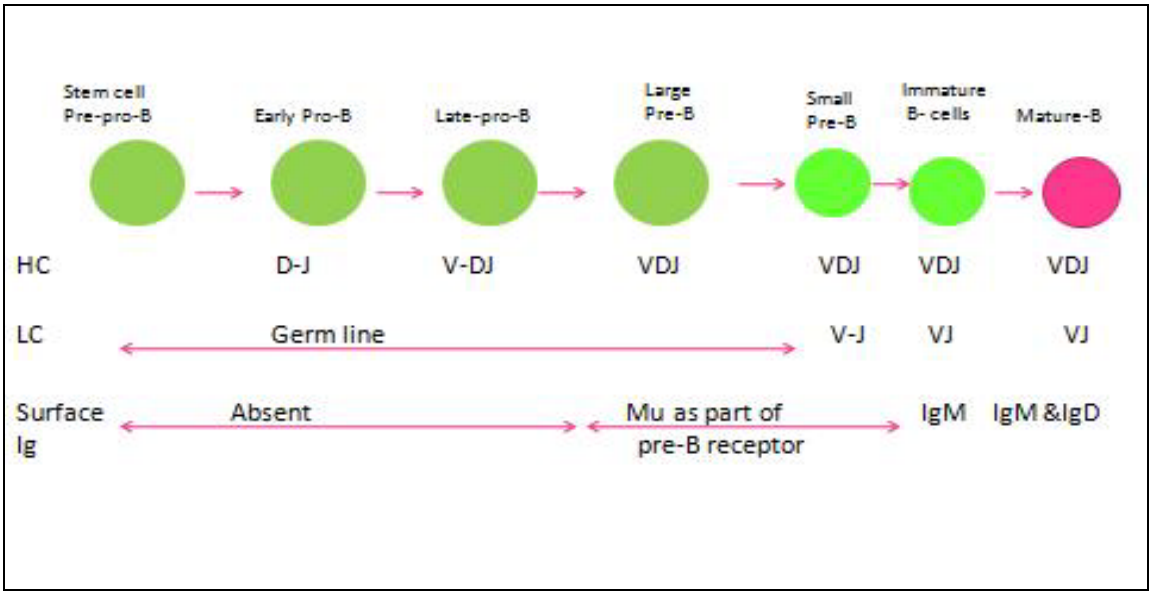


Figure 5. Stages of B-cell development and stage related gene rearrangement.

The earliest recognizable B-cell progenitor, is the pro-B cell which expresses B220/CD45R, CD43, c-kit, and the IL-7 Receptor (CD127). These cells also express

CD19, a B cell-specific member of the immunoglobulin (Ig) supergene family and its expression continues until the B-cell differentiates into a plasma cell (23, 25, 27, 28). Pro- B cells are subdivided into the early pro-B cell stage in which the immunoglobulin heavy chain gene locus undergoes D to J rearrangement, and the late pro-B cell stage in which V to DJ rearrangement takes place.

The production of an in-frame functional V (D) J gene segment marks the beginning of the pre-B cell stage. The pre-B stage is subdivided into the large pre-B and the small pre-B stages (1, 27). The heavy chain is produced in the cytoplasm of the pre-B-cell. At this stage the cells up-regulate the expression of CD19. Pre-B-cells also express the developmental markers CD25, the IL2 receptor also found on activated T-cells B220, Igα (CD79a) and Igβ (CD79b), transmembrane proteins which together form a heterodimer that transmits activation signals from the surface of the B-cell to the cytoplasm (2). At the large pre-B cell stage the heavy chain polypeptide pairs with a surrogate light chain, which is encoded by two non-rearranged genes; lambda 5 (λ5) that resembles the constant region of the lambda light chain, and V-pre B which looks like an immunoglobulin variable region gene (1). This heavy chain-surrogate light chain pair forms the pre-B cell receptor (pre-BCR) and is expressed in the cytoplasm and on the cell surface in association with the α/β heterodimer. The IgMα/β heterodimer contains long cytoplasmic tails which contain ITAMS that are involved in signaling. Signaling through the pre-BCR allows large pre-B cells to progress to the small pre-B stage (1, 29). In the small pre-B cell stage, light chain V to J gene rearrangement occurs. If a functional light chain polypeptide is produced it displaces the surrogate light chain and pairs with the μ heavy chain, and the complete IgM molecule is expressed on the surface of the cell in

association with the α/β heterodimer. This marks the beginning of the immature stage of B-cell development (1).

Immature B cells are IgM ^{high}, IgD $^{-}$, CD24 ^{high}, and CD23^{-}. Ten percent of the B cells that develop in the bone marrow exit the bone marrow at the immature stage and travel to secondary lymphoid organs. Only one third of these B-cells will complete their development into mature B-cells, which express both IgM and IgD (1). Upon exiting the bone marrow and entering the peripheral circulation immature B-cells are referred to as transitional B cells (30, 31). These cells migrate via the blood to the spleen and enter through the terminal branches of the central arteriole. Five to ten percent of peripheral B-cells in adult mice are immature CD24^{high} (32).

In the periphery there are two populations of B-cells, an immature/ transitional population and a mature population. Loder et al. showed that the transitional B-cell compartment in the spleen is subdivided into two populations transitional -1 (T1) and transitional -2 (T2). The T1 population is IgM ^{high} IgD ^{dull} CD21^{-} CD23^{-}. It gives rise to the T2 B-cells which are IgM ^{high} IgD ^{dull} CD21^{-} CD23^{+}. T2 B-cells were shown to develop into mature B-cells and marginal zone B-cells (31).

Allman et al. subdivided the transitional B-cell population in the spleen into three subsets based on the expression of B220, AA4, IgM and CD23. According to Allman, T1 B-cells are AA4^{+} IgM ^{high} CD23^{-}, T2 B-cells are AA4^{+} IgM ^{high} CD23^{+} and T3 B-cells are AA4^{+} IgM ^{low} CD23^{+} (26). In the spleen transitional B- cells eventually migrate to the outer periarteriolar lymphoid sheath (PALS), and many enter the B-cell follicle where they differentiate and become mature B-cells. Mature B- cells are IgM ^{low}, IgD ^{high}, and HSA ^{low} (32). A mature B-cell, as a result of alternate RNA splicing expresses both IgM

and IgD molecules on its surface (1). Mature B-cells are IgM^{low} IgD^{high}. The mature B-cell compartment is comprised of two populations of B-cells based on anatomical location; marginal zone (MZ) B-cells and follicular B-cells (FO) (33). MZ B-cells are IgM^{high} $IgD^{low}CD1d^{high}$ (MHC class 1b protein), $CD21^{high}$ (complement receptor type II (CR2), $CD23^{low}$. FO B-2 cells are IgM^{low} IgD^{high} $CD21^{inter}$ $CD1d^{low}$ and $CD23^{high}$ (33, 34). In order for a mature B-cell to undergo further differentiation, it must come in contact with an antigen. Then it can become either an antibody secreting plasma cell or a memory B-cell, depending on the cytokines being secreted in the environment (1, 35). A linear model of B-cell development from bone marrow to the spleen is summarized in figure 6.

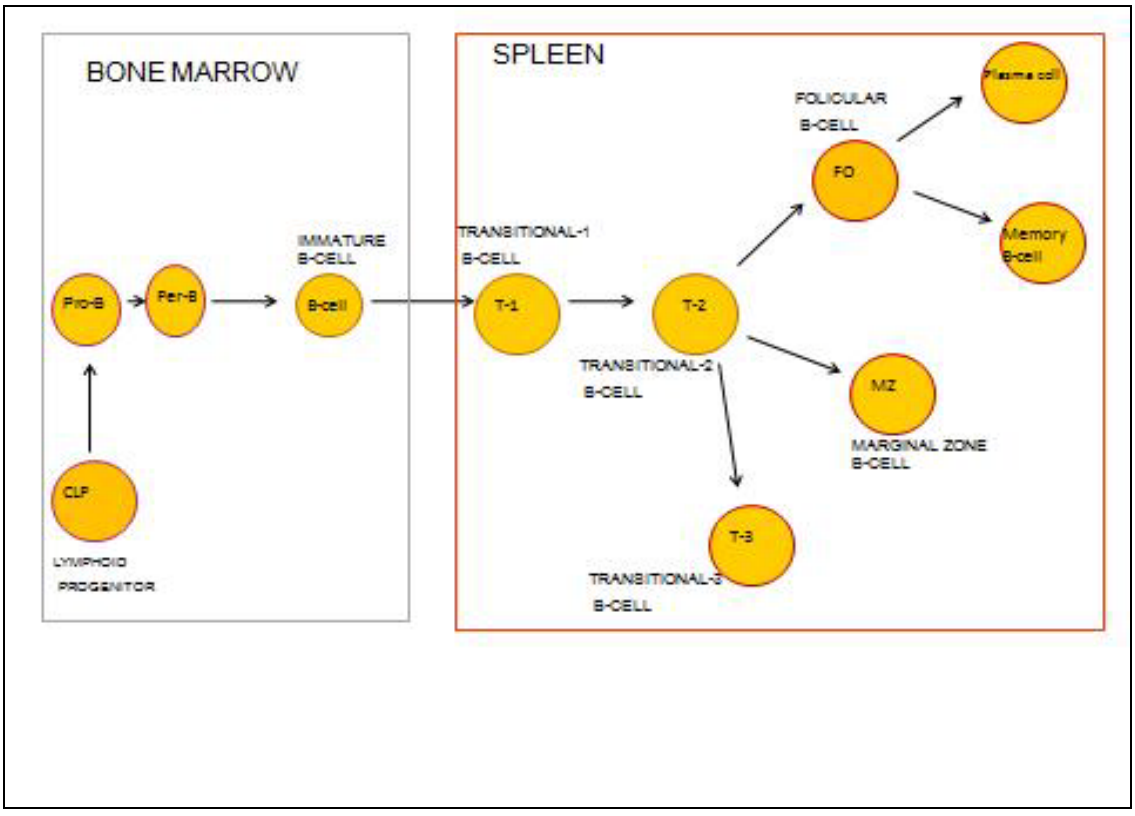


Figure 6. Linear model of B-cell development.
The different stages of B-cell development in the bone marrow and the spleen.

1.2b B-cell maturation in the spleen

The spleen is an important filter for the blood, and it is the major site of immune responses to blood-borne antigens (1). The spleen receives blood from a single splenic artery of the aorta. This splenic artery divides into tiny blood vessels or arterioles that carry the blood to the cords in the red pulp; from the cords to the venous sinuses then to the efferent splenic vein and onto the liver (36). Under the influence of cytokines and chemokines different types of lymphocytes migrate to and are segregated in the spleen. The small arterioles of the spleen are surrounded by a sleeve of T-lymphocytes referred to as the periarteriolar lymphoid sheath (PALS) or T-cell zone. Attached to the PALS are lymphoid follicles containing B-cells. Some follicles contain germinal centers (GC), which are areas of intense B-cell proliferation that develop in response to antigen stimulation (Figure 7). Lymphoid follicles that contain germinal centers are referred to as secondary follicles; those without germinal centers are primary follicles.

Surrounding the B-cell follicle is a region containing some macrophages, splenic dendritic cells, reticular cells (stromal cells), and B-cells. This region is called the marginal zone (MZ). B-cells residing here are called MZ B-cells. A high frequency of auto reactive B-cells have been found to reside in the MZ. The outer border of the MZ is marked by the marginal sinus which branches from the arteriole. The MZ separates the white pulp (a region which contains most of the lymphocytes) from the red pulp (a region containing senescent red blood cells) (1). Arteriole blood carrying antigen enters the marginal sinus en- route to the white or red pulp. Cells that reside in the MZ are positioned to respond rapidly to blood-borne pathogens. MZ B-cells display an activated phenotype and are IgM^{high}, IgD^{low}, $CD23^{lo}$, and $CD21^{high}$ (CR2). MZ B-cells also express

high levels of CD9, a scavenger receptor family protein, CD25, and CD38, an ADP-ribosylcyclase. MZ B-cells are not part of the recirculating B-cell population in mice. Although they are found in the spleen they are not found in other tissues (33, 35). Antigens are filtered by macrophages and immature dendritic cells in the marginal zone. When MZ dendritic cells get activated, they migrate to the T-cell zone of the spleen, where they activate T-cells, which in turn activate B-cells (1).

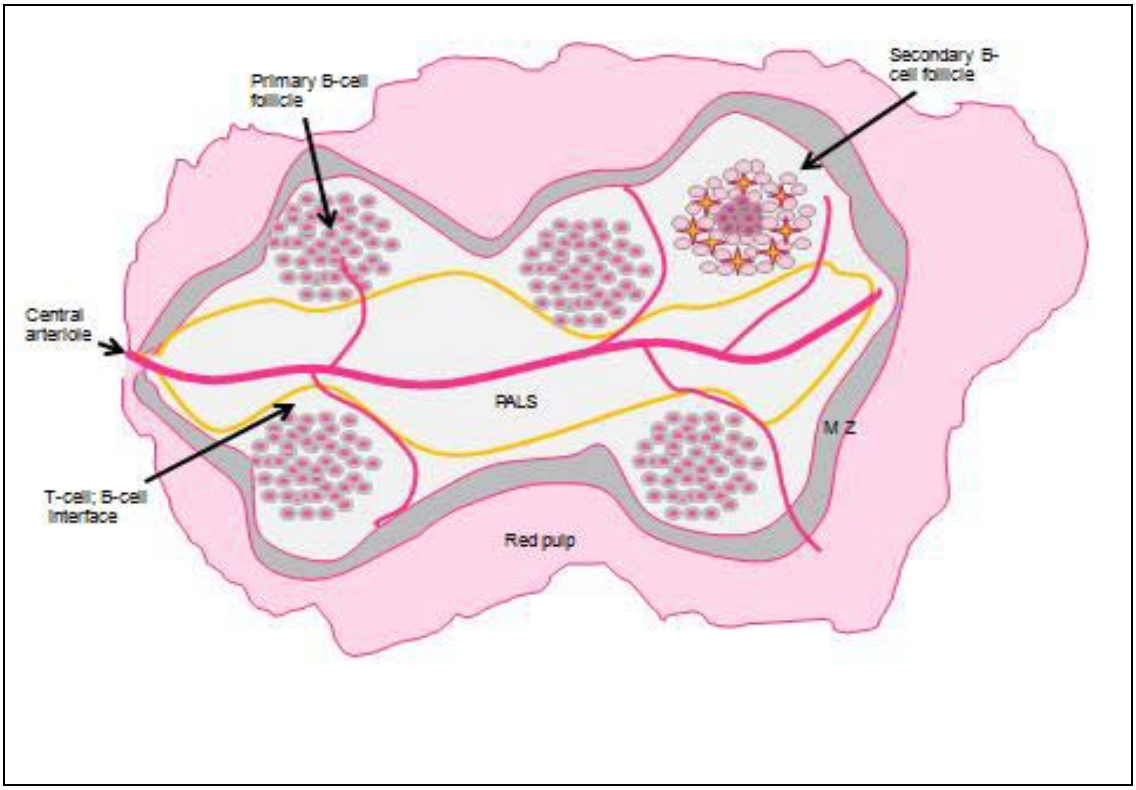


Figure 7. Schematic of a longitudinal view of the mouse spleen showing a section with red and white pulp, periarteriole lymphoid sheath (PALS) or T-cell zone, marginal zone (MZ), secondary B-cell follicle with germinal center and primary B-cell follicles (not to scale).

In germinal centers (GC) of the spleen (Figure 8), activated mature B-cells undergo the processes of somatic mutation, affinity maturation and selection. The initial

B-cell response to a protein antigen occurs at the boundary between the lymphoid follicle and the T-cell zone. Several days after antigen exposure some of the activated B-cells migrate deep into the B-cell zone and begin to proliferate forming a GC. These proliferating B-cells known as centroblasts comprise the dark zone of the germinal center. They undergo somatic mutation. These point mutations occur in the CDRs of the V-region at a very high rate. These regions of high mutations are referred to as the hypervariable regions. Some of these mutations may generate antibodies with a higher affinity for the antigen than the original antibodies, while others may result in a reduction or loss of antigen affinity and /or specificity. B-cells that undergo somatic hypermutation migrate into the follicular dendritic cell (FDC)-rich zone of the germinal center, known as the light zone where the FDCs display antigens. B-cells in this zone are known as centrocytes and they proliferate much less frequently then the centroblasts. B-cells that recognize antigen displayed by FDCs are selected to survive, those that do not, die by apoptosis. In an immune response, as more antibodies are produced, the amount of antigen available in the germinal center for capture by the antibodies is reduced. Those B-cells that are able to specifically bind antigen need to express antigen receptors with increasingly higher affinity. This process is called affinity maturation and it occurs in the light zone of the germinal center. After selection, high affinity antibodies undergo heavy chain class switching in response to the interaction of CD40 receptor on B-cells with CD154 (CD40 ligand) expressed on T cells and cytokines.

The enzyme, Activation Induced Cytidine Deaminase (AID), is responsible for both somatic mutation and class switching. AID converts cytosines in the switch and immunoglobulin variable regions to uracils. (37). Following affinity maturation and class

switching, some of the B-cells become antibody secreting plasma cells which leave the spleen and circulate to the BM, and some of these cells become memory B-cells (35). Cytokines secreted in the environment influence whether the B-cells become plasma cells or memory B-cells. B-cells are attracted to the follicle by the B-lymphocyte chemokine, CXCL13 which directs the organization of B-cells into discrete B-cell areas around FDC (38).

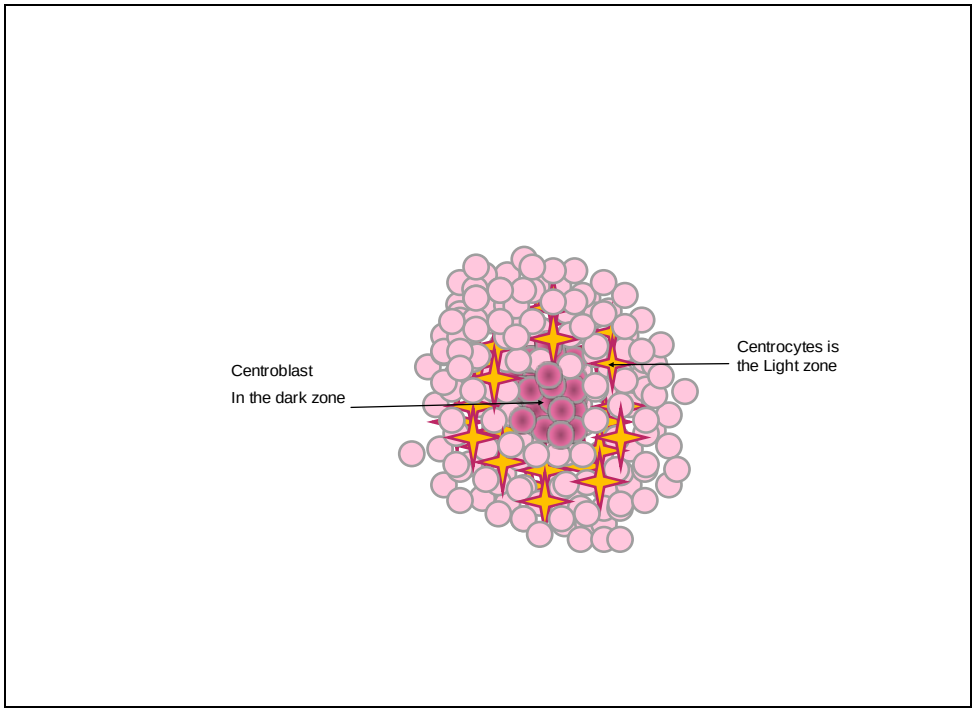


Figure 8. Germinal center showing centroblast in the dark zone, an area of intense cell proliferation and somatic mutation, and centrocytes the light zone where cell proliferation a reduced and where affinity maturation takes place.

1.3 MECHANISMS OF B-CELL TOLERANCE

B-cell tolerance is a property by which B cells distinguish “self” from “non self” and is defined by a B-cell’s inability to be activated in response to self-antigen. Loss of tolerance results in autoimmunity whereby the immune system attacks self components

and tissues. Studies using transgenic mouse models have revealed that several mechanisms are employed to maintain B cell tolerance. These include clonal deletion, receptor editing, anergy, and T cell suppression (1, 39). Mechanisms of B-cell tolerance operate centrally, in the bone marrow and peripherally, in secondary lymphoid organs such as the spleen and lymph nodes (39, 40).

In central deletion, autoreactive B-cells such as anti-DNA B-cells are removed from the bone marrow during the immature stage of development (41). Evidence for central deletion was obtained by Nemazee and Burki in mice transgenic for an IgM anti H-2^{k} MHC class I antibody. They found that the sera of H-$2^{d/d}$ mice transgenic for antibody to H-2^{k} contained high serum concentrations of anti-H-2^{k} antibody, but the sera of H-$2^{d/k}$ mice transgenic for anti-H-2^{k} did not contain transgenic anti H-2^{k} antibody. Although the percentages of B-cells in the spleen of both mice were similar, no H-2^{k} antibody producing cells were present in the spleen of the H-$2^{d/k}$ transgenic. This was due to the complete elimination of B-cells producing the transgenic antibody in the bone marrow of these mice (42) . Hartely et al. demonstrated that HEL B-cells were deleted in mice transgenic for membrane hen egg lysozyme (mHEL) and antibody to HEL (IgHEL) (43).

Receptor editing is another mechanism for regulating autoreactive B-cells. Receptor editing alters the specificity of an immunoglobulin receptor in an attempt to avert autoreactivity. It occurs at the level of DNA by replacement of a rearranged V_H or V_L chain gene encoding an anti-self receptor with an upstream V gene. On the κ light chain, if a Vκ- Jκ rearrangement results in autoreactivity, the kappa light chain gene may

undergo a secondary rearrangement, bringing an upstream Vκ gene next to a more downstream Jκ gene.

Evidence for receptor editing was originally demonstrated in mice transgenic for 3H9 heavy chain and $V_\kappa 4$ light chain (44). In the 3H9/Vk4 model, Gay et al. showed receptor editing of the transgenic light chain. Hydridoma studies from 3H9/ Vκ4 transgenic mice showed that the H/ L chain combination was not detected by anti-idiotypic antibody for 3H9/ Vκ4 combination (44). Using PCR assay on hybridoma lines obtained from $V_\kappa 4$ and 3H9/ Vκ4 mice, they showed that the transgenic light chain was replaced by endogenous light chains .By sequencing the light chain from many hybridoma lines, they observed that many used the endogenous light chain rather than the transgenic $V_\kappa 4$ light chain (44).

Tiegs et al, showed secondary gene rearrangement at the light chain loci in mice transgenic for the heavy and light chain genes of anti-$H2K^{k,b}$ antibody. They showed that B-cells that bound autoantigen attempted to alter their receptor specificity by receptor editing (45). Transgenic bone marrow B-cells encountering K^b or K^k proteins modified their receptors by re-expressing RAG-1 and RAG-2 genes and rearranging endogenous LC genes. In these mice a high frequency of B-cells were found to express the λ light chain rather than the kappa transgene. This resulted from secondary rearrangement at the endogenous λ locus (45).

Prak et al. showed receptor editing in a light chain transgenic model. In these mice unlike previous models generated by random insertion of the rearranged transgene into the genome, a pre-rearrange kappa light chain was inserted by homologous recombination into the immunoglobulin kappa locus. V_κ replacement (VkR) mice

simulate the genotype of normal B-cells in that it contain one copy of the functional pre-rearranged gene on the targeted allele and an un-rearranged kappa locus on the other allele. In these animals a rearranged L chain $V_\kappa4$-$J_\kappa4$ replaced the un-rearranged J_κ genes at the locus while retaining upstream Vs and the downstream $J_\kappa5$ segments (46).

Studies in $V_\kappa4$-$J_\kappa4$ mice, demonstrated secondary rearrangement at a productively rearranged immunoglobulin locus. In these mice the $V_\kappa4$-$J_\kappa4$ transgene was either replaced by an upstream V-gene that rearranged to $J_\kappa5$ segment on the targeted allele, or the $V_\kappa4$-$J_\kappa4$ replacement gene was loss by inversion being relocated upstream of its origin point due to the rearrangement of an upstream V, or deletion of the $V_\kappa4$-$J_\kappa4$ by rearrangement on the untargeted allele to $J_\kappa1$ or $J_\kappa2$ (46).

Anergy is another mechanism for maintaining B-cell tolerance. Anergy is defined as the functional inactivation of an autoreactive B cell. Anergic B-cells cannot secrete antibody in response to BCR. Goodnow et al. generated mice transgenic for soluble hen egg lysozyme (sHEL), and an antibody to HEL. They observed that the double transgenic mice had no detectable serum anti-HEL antibody. In contrast, mice transgenic for the anti-HEL antibody only, spontaneously secreted high serum titers of anti-HEL antibody. FACS analysis revealed that the majority of B-cells expressing the transgene encoded receptor were not clonally deleted, and that the amount of IgM expressed on these B-cells was dramatically reduced. These B-cells were found to be functionally unresponsive to antigen (47). Furthermore the life span of these self-reactive B-cells was found to be shorter (3- 4 days) than the life span of B-cells from non-transgenic mice (5- 6 weeks) (48).

Erikson et al. made mice transgenic for an antibody to single stranded DNA (anti-ssDNA), which utilizes the VH3H9 heavy chain and a $V_{\kappa}8$ light chain. They observed that these mice have an expanded population of transgenic B-cells that are arrested in development and that these B-cells do not spontaneously secrete anti-DNA antibodies. Furthermore, they demonstrated that these B-cells are anergic or functionally silent in response to antigen (49).

Spatz et al. observed evidence of anergy in a subset of anti-dsDNA B-cells in mice transgenic for the IgG2b HC of an anti-ds DNA (dsDNA) antibody designated R4A. These B-cells could not be activated to secrete autoantibody following BCR cross-linking (50, 51). However, they could be activated by lipopolysaccharide (LPS).

Noorchashm et al. demonstrated that autoreactive dsDNA binding B-cells targeted to anergy are arrested in development and they are incapable of differentiating into antibody secreting cells (52). They also demonstrated that the arrest in development of anergic anti-dsDNA binding B-cells is marked by a reduction in the expression of B220, CD21/35, CD22, CD23, and CD40 and an increase in expression of HSA, CD44 and MHC class II molecules. Normally as a B-cell matures the expression of certain cell surface developmental markers, such as B220, CD21/35, CD22, CD23, and CD40 increases while others, such as HSA and CD44 decrease. This phenotype is characteristic of B-cells chronically exposed to antigen. These observations suggested that the developmental arrest is antigen-mediated. These B-cells could not be stimulated to proliferate when treated with anti-IgM or (LPS), and were found to localize at the T-cell/B-cell interface of the splenic follicle (52).

CHAPTER 2

2.1 R4A Transgenic Mice

Because of tolerance, autoreactive B-cells constitute a very small percentage of the B-cell population in non- autoimmune mouse strains. Transgenic technology has allowed us to develop mouse model systems in which a given population of clonal B-cells expressing a single antibody is increased. This approach forces B-cells to express auto-antibodies and the fate of these autoreactive B-cells can be followed. Furthermore, it provides a means by which the mechanisms of tolerance can be studied.

One mouse model that has been used to study the regulation of autoreactive B-cells, is a mouse transgenic for the heavy chain of an anti-dsDNA antibody designated R4A. The R4A antibody was generated from a hybridoma obtained from a BALB/c mouse immunized with phosphorylcholine (PC) (53). It utilizes an S107 V11 heavy chain gene which is frequently used in the protective immune response in BALB/c mice, and in the anti-dsDNA response in both NZB/NZW F1 and MRL/lpr mice. The original R4A antibody uses a germline $V_{\kappa}1$ light chain gene like most pathogenic lupus autoantibodies. R4A is of the IgG isotype. It binds dsDNA with moderate affinity. It is encoded by an un-mutated heavy and light chain gene (53, 54).

It is believed that the presence of anti- dsDNA antibodies in the serum of patients with lupus is due to a defect in B-cell regulation resulting in a breakdown of tolerance. The R4A-Cγ2b transgenic mouse was generated in order to study the regulation of IgG anti-dsDNA B-cells. The R4A-Cγ2b transgene was constructed from a 3.5-kb DNA fragment of the rearranged VDJ region of the R4A HC gene, and an enhancer region ligated to a 6.8 kb DNA fragment containing both the membrane and secreted form of the

Cγ2b constant region gene. Studies in R4A-Cγ2b mice demonstrated that tolerance is maintained in these mice via anergy and deletion. R4A-Cγ2b mice do not spontaneously secrete transgenic anti-dsDNA antibody. An anergic dsDNA binding B cell population has been identified in these mice, which can be induced to secrete anti-dsDNA antibody upon stimulation with LPS only, but not by BCR cross-linking (54).

Since it is not physiological for transgenic B-cells to express IgG early in development, mice transgenic for an R4A IgM heavy chain gene were subsequently generated. The R4A-C_μ transgene contains the same 3.5 kb rearranged VDJ fragment as R4A-Cγ2b, but it is ligated to a 9.9 kb C_μ constant region containing the secreted and membrane exons of the Mu heavy chain and a downstream enhancer region (51). Studies in R4A-C_μ transgenic mice demonstrated that tolerance is also maintained in these mice by anergy and deletion. The sera of these mice do not contain transgenic anti-dsDNA antibody (51). Stimulation of the R4A-C_μ B-cells with $F(ab')_2$ anti-IgM in the presence of anti-CD40, which mimics the action of CD40 ligand, fails to induce these B-cells to produce anti-ds DNA antibody, indicating that like their γ2b counterparts these transgenic dsDNA binding B-cells are anergic. Although R4A-C_μ B cells cannot be activated by BCR cross linking, they can be activated following LPS stimulation. These B-cells display the phenotype of anergic B-cells; they exhibit reduced expression of surface IgM, are developmentally arrested and have a shortened lifespan (51).

Our laboratory previously bred mice transgenic for the R4A-C_μ HC to mice overexpressing human CD19 (hCD19) (55). CD19 is a component of the B-cell co-receptor complex that is involved in lowering the threshold for B-cell activation. Taylor et al. investigated whether overexpression of CD19 could alter tolerance of ds-DNA

binding B-cells in R4A-C_μ mice. They observed that in R4A-C_μ mice overexpressing CD19, tolerance was broken, leading to the spontaneous secretion of IgM^a anti-dsDNA antibody. The B-cells secreting transgenic anti-dsDNA antibody in the R4A-C_μ/CD19 mice were shown to be derived from the conventional B2 B-cell population, and not from $CD5^+$ B-1 cells found in the peritoneal cavity. Furthermore, the anti-dsDNA antibody secreting B-cells in these mice seemed to originate from the B-cells with a MZ phenotype rather than those with a FO phenotype (55). Grimaldi et al. have also reported the existence of a dsDNA reactive B-cell population in the marginal zone of R4A-Cγ2b transgenic after treatment with 17β-estradiol, the predominant form of estrogen (56). Spontaneous secretion of IgM^a anti-dsDNA antibody in the R4A-C_μ/ CD19 mice was found not to require T-cell help. This is consistent with the properties of B-cells that reside in the MZ which have been shown to respond to T independent antigens (57). A subset of Tg anti-dsDNA B- cells present in R4A-C_μ/ CD19 mice were still found to maintain an anergic phenotype. These B-cells were found to be hyperresponsive to activation by LPS and stimulatory CpG sequences. Specific CpG sequences have been shown to activate B-cells via the Toll 9 receptor (TLR9) (58, 59). The observation that anergic B- cells from the R4A-C_μ/ CD19 are more responsive to LPS and CpG signaling than B- cells from R4A-C_μ mice, suggests that innate signaling pathways may converge with the intrinsic CD19 pathway to amplify the signal for activation of anergic B-cells in a BCR independent manner.

As B-cells develop and mature they receive signals from their environment. These signals come from interactions with other cells in the stroma as well as with soluble substances in the vicinity of the cell. One such signal comes from the cytokine B cell

activating factor of the TNF family (BAFF), also known as BlyS, TALL-1, THANK, 2TNF4, which promotes survival and maturation of immature and mature B-cells in the spleen. BAFF induces immature B-cells to differentiate into mature B-cells (60). Patients with SLE have significantly higher levels of BAFF than normal individuals (61). Normal serum concentration of BAFF is less than 5ng/ml. In SLE patients the serum concentration of BAFF is greater than 5 ng/ml, and may even be as high as 40 ng/ ml. Patients with levels of BAFF in excess of 15 ng/ ml show significantly higher levels of IgG, IgM and IgA anti-dsDNA antibodies in their serun compared to patients with low BAFF (<5 ng/ml), suggesting that BAFF may play a role in the loss of tolerance (61). Mice that overexpress BAFF show an increase in the total number of B -cells, with an excess of mature B-cells in particular. BAFF-Tg mice secrete autoantibodies and have elevated serum concentration of IgG, IgM and IgA compared to wild type mice (62)

Recently our laboratory examined the effect of BAFF overexpression on the regulation of anti-dsDNA B-cells (63). We found that more than 50 % of R4A-C_μ mice homozygous for BAFF (R4A-C_μ $BAFF^{+/+}$) secreted the transgenic IgM^a anti-dsDNA antibody compared to only 10 % of R4A-C_μ mice heterozygous for BAFF (R4A-C_μ $BAFF^{+/-}$). It was shown that a threshold level of BAFF (>300 ng/ ml) needs to be expressed to break tolerance of B-cells targeted to anergy. BAFF was shown not to affect B-cell tolerance in the bone marrow, but to promote survival and maturation of transgenic anti-dsDNA B-cells in the periphery of R4A-C_μ $BAFF^{+/+}$ mice. Transgenic dsDNA binding B-cells were found to be enriched in the MZ and follicular zone of R4A-C_μ/ $BAFF^{+/+}$ mice, although more were found in the MZ.

A comparison of dsDNA binding B-cells in the immature AA4.1$^+$ and the mature AA4.1$^-$ B-cell populations of R4A-C$_\mu$/BAFF$^{+/+}$ mice, suggests that BAFF overexpression enables B-cells to escape a regulatory checkpoint in the transitional stage of development. The frequency of Tg anti-dsDNA B-cells is significantly higher in the AA4.1$^+$ transitional population compared to the AA4.1$^-$ population in R4A-C$_\mu$ mice suggesting that the transitional stage of development operates as a regulatory checkpoint. However in R4A-C$_\mu$/BAFF$^{+/+}$ mice, the frequency of Tg anti-dsDNA B-cells is not significantly different in the AA4.1$^+$ and AA4.1$^-$ populations, suggesting that BAFF enables some Tg anti-dsDNA B-cells to escape a regulatory checkpoint in the transitional stage of development (63).

2.2 R4A-C$_\mu$/V$_\kappa$1 DOUBLE TRANSGENIC MICE

Previous studies in R4A-Cγ2b and R4A-C$_\mu$ heavy chain transgenic mice demonstrated that tolerance is maintained by anergy and deletion. B-cells that are targeted to anergy in these mice were observed to preferentially utilize a V$_\kappa$1 light chain (50, 51). To enrich for a population of anergic B-cells and to gain insight into how and when anti-dsDNA B-cells are targeted to anergy, we generated mice transgenic for both the R4A-C$_\mu$ HC and the V$_\kappa$1 LC on a C57BL/6 background. C57BL/6 V$_\kappa$1 targeted knock-in light chain transgenic mice were obtained from the laboratory of Dr. Martin Weigert (University of Chicago) and were mated to C57BL/6 R4A-C$_\mu$ heavy chain transgenic mice. This present study was undertaken to gain further insight into the mechanism of regulation of anti-dsDNA B-cells and to more thoroughly understand how and at what stage of B-cell development this regulation occurs.

CHAPTER 3

MATERIALS AND METHODS

3.1 Generation of the R4A-Cμ/Vκ1 double transgenic mice on a C57BL/6 background

We generated R4A-C_μ/V_κ1 double transgenic mice on the C57BL/6 background by mating C57BL/ 6 V_κ1 knock-in transgenic mice, obtained from the laboratory of Dr. Martin Weigert (University of Chicago), with C57BL/6 R4A-C_μ transgenic mice to generate C57BL/6 R4A-C_μ/V_κ1 double transgenic animals.

The Vk1 transgenic mice were generated by placing an unmutated pre-rearranged V_κ1 J_κ1 gene segment from BALB/c mouse, into the Ig kappa locus while maintaining the upstream V_κs and downstream J_κs gene segments. The 11kb V_κ1 fragment with its promoter and downstream J_κ 2, 3, 4, and 5 segments was placed in to a replacement type targeting vector containing the neomycin and thymidine genes for transfection and selection (appendix 2). This construct was transfected into mouse ES cells (personal communication). Homologous recombination replaces the sequence in the chromosomal gene with the pre-rearranged transgene. Crossing V_κ1 knock-in lines to J_κ deficient mouse and performing PCR and southern blot confirmed the presence of a single copy of the transgene and a single endogenous kappa allele (personal communication).

R4A-C_μ heavy chain transgenic mice were generated by microinjecting a construct consisting of a 3.5 kb rearranged VDJ segment of the R4A antibody, ligated to a 9.9 kb genomic fragment of the C_μ constant region from a BALB/c mouse containing the secretory and membrane exons, and a downstream enhancer region (appendix 1), into male pronucleus of C57Bl/6 x CBA F1 fertilized eggs (51). R4A-C_μ mice contained

five copies of the transgenes (51). R4A-C_μ transgenic was backcrossed onto a C57Bl/6 background for at least ten generations.

3.2 Genotyping

The presence of the intact R4AC_μ heavy chain and the V_κ1 light chain transgenes in the double transgenic animals was confirmed by PCR, on tail DNA using puRe Taq™ Ready-To-Go™ PCR beads from Amersham Bioscience (Piscataway N.J.). To obtain tail DNA, 1-2 mm biopsies of mouse tails were placed in a 1.5 ml micro-centrifuge tubes containing 100-200ul NaOH (50 mM) and incubated for 20 min at 100^{o}C on a heating block. Tubes were immediately placed on ice and 30ul Tris buffer (1M) was added.

3.3 PCR to Detect R4A-Cμ Heavy chain

Two microliters of genomic tail DNA was added to 23ul of a rehydrated illustra™ puRe Taq™ Ready-To-Go™ PCR beads (GE Healthcare Piscataway NJ), containing 2.5units puRe Taq DNA polymerase, 200 uM dNTP in 10 mM Tris HCl (pH9.0), 50 mM KCl and 1.5 uM $MgCl_2$, 10 pmol of the R4A- V11FR1 heavy chain forward primer BD272 ($^{5'}$GGT GAA GCT GGT GGA ATC TGG $^{3'}$), and 10 pmol of the heavy chain reverse junctional oligo BD243 ($^{5'}$CAT AGG GAT CCT ATC TCT $^{3'}$) (Intergrated DNA Technologies Coralville, IA). DNA was amplified under the following conditions, 94^{o}C for 30 seconds, 51^o C for 30 seconds, 72^o C for 40 seconds for 30 cycles, with an extension at 72^o C for 5 minutes. Under these conditions a 350 bp product was amplified and detected on a 1% agarose gel.

3.4 Detection of the Vκ1 Light chain by PCR

To detect the V_κ Light chain, 50 ng/ml of DNA was added to 23ul of a mix containing rehydrated illustra™ puRe Taq™ Ready-To-Go™ PCR beads (GE Healthcare Piscataway NJ), 10pmol of the $V_\kappa 1$ forward primer, ($^{5'}$GTA ATG CCT AGA AGT GAC TAG ACA CTT$^{3'}$) and 10pmol of the JKB3 reverse primer, ($^{5'}$GAT TCT TTG CCT TGG AGA GTG CCA GAA$^{3'}$) (Integrated DNA Technologies Coralville, IA). The DNA was amplified under the following PCR conditions: 94^{o} C for 30 seconds, 62^{o} C for 30 seconds, 72^{o} C for 40 seconds for 30 cycles followed by an extension at 72^{o}C for 5 minutes. A 600bp product was amplified.

3.5 IMMUNO- LINKED ENZYME ASSAYS (ELISAs)

3.5a Anti-Double stranded DNA ELISA

The presence of IgM^{a} anti-dsDNA- antibody in the sera of R4A-$C_\mu/V_\kappa 1$ transgenic mice was determined by an anti ds-DNA ELISA. Immulon-2 HB polystyrene 96 well plates (Thermo eletro Corporation Milford MA), were coated with calf thymus dsDNA (150 ug/ml) in 1X phosphate buffer saline (1X PBS) (1.9 mM NaH_2PO_4, 1.8mM Na_2HPO_4 and 5.4 mM NaCl pH 7.2), that has previously been filtered through a 0.45 micron nitrocellulose filter to remove single stranded DNA (Millipore Bedford MA). ELISA plates were dried for 48 hours at 37^{o}C. Plates were washed, by rocking for 5 minutes, with 200 ul distilled water and blocked for 2 hours at room temperature with PBS/ 1% BSA. Then they were washed 3 times with wash buffer (1X PBS plus 0.05% Tween-20®). Sera from transgenic mice were diluted (1/100) in PBS/ 0.1% BSA and

100 ul was applied to wells. After incubating at 37^{o}C for 2 hours, the plates were washed six times with wash buffer and 100 ul of biotin mouse anti-mouse IgM^{a} (1/200 dilution) (0.5 mg/ml) (BD Pharmingen San Diego, CA) in 1XPBS/ 0.1 % BSA was added to the wells. The plates were incubated for 1 hour at 37^{o} C. Next plates were washed six times and 100 ul of Streptavidin-AP (Southern Biotechnology Birmingham, AL) (1/1000 dilution) was added to wells and incubated for 1 hour at 37^{o} C. After six washes with wash buffer, 100 ul of alkaline phosphatase substrate buffer containing phosphate substrate 5 mg tables (Sigma) (1mg/ml), 0.5M Na_2CO_3, and 0.01 M $MgCl_2$ (pH 9.8) and H_2O at a 1:1:8 ratio (pH 9.4- 9.8) was added. The plates were kept overnight at room temperature and read on a Titertek Multiscan ELISA reader at 405 nm.

3.5b Anti-IgM^{a} Antibody ELISAs

Flacon® ProBind™ 96 well microtiter plates (Becton Dickinson Franklin Lake N.J.) were coated with goat anti-mouse IgM antibody (Southern Biotech) diluted 1/1000 in 1XPBS and incubated for 1 hour at 37^{o}C. Plates were blocked for 2 hours at room temperature with 200 ul of PBS/1% BSA. Next, 100ul of 1/100 dilution of sera were applied to wells, and plates were incubated for 1 hr at 37^{o}C. After 6 washes with wash buffer, 100 ul of biotin conjugated mouse anti-mouse IgM^{a} (1/200 dilutions) was added to wells and allowed to react for 1 hour at 37^{o}C. Following 6 washes with wash buffer, 100 ul of streptavidin-AP (1/1000 dilution) was added and the plates were incubated for 1 hour at 37^{o}C. After washing, 100 ul of phosphatase substrate buffer (pH adjusted), was added to wells and plates were incubated for 30 minutes before reading on a Titertek Multiscan ELISA reader at 405 nm wavelength.

3.6 Harvesting of lymphocytes from spleen and bone marrow

To ascertain the phenotype of bone marrow and splenic B cells in the R4A-Cμ/Vκ1 double transgenic mice, B cells were harvested from both the spleen and bone marrow. The spleen was removed from the animal, rinsed in 5 ml of 3% FCS /PBS. The organ was transferred to a fresh petri dish containing 5 ml of 3% FCS/PBS, and the cells were extracted by pressing them through a cell strainer and then re-suspended in 3% FCS/PBS. The sample was at room temperature for 5 minutes to allow large particles of debris to settle. The supernatant was then transferred to a new 15 ml falcon tube, spun at 1200 rpm for 7 minutes at 4^{o}C, and the cell pellet was resuspended in 1 ml red blood cell (RBC) lysis buffer, (15.5 mM NH_4Cl, 1.0 mM $KHCO_3$,0.01 mM EDTA pH 7.2). RBCs were lysed at room temperature for 4 minutes. Cells were then washed twice in 10 ml of 3% FCS/PBS, spun at 1200 rpm for 7 minutes at 4^{o}C, and the remaining lymphocytes were resuspended in 1% FCS/PBS and counted using a hemocytometer.

To isolate bone marrow B cells, two hind limbs were amputated washed in a 20 mm petri dish containing 12 ml of 3% FCS/PBS and all the surrounding muscle tissue was removed from the femur, tibia and fibula. The cartilage was removed to facilitate extraction of the marrow. The cleaned bones were transferred to a petri dish containing 5 ml of 3% FCS/PBS. The marrow was flushed out using buffer delivered with a 10 ml syringe attached to a 27-gauge needle. The cells were re-suspended in 3% FCS/PBS and transferred to a fresh tube, and allowed to stand for 5 minutes at room temperature for the large particles of debris to settle. After this point, the cells was treated the same as the splenic cells.

3.7 In-vitro activation and ELISPOT assay

The number of splenic B-cells in the R4A-$C_\mu/V_\kappa 1$ mice that produce IgM^a antibody or IgM^a anti-dsDNA antibody was determined by ELISPOT. Spleens were harvested and rinsed in RPMI 1460 containing 10% FCS. Splenocytes were depleted of RBCs as previously described. They were enriched for B-cells by depletion of $CD43^+$ cells using anti-CD43 microBeads and magnetic bead column. Cells were first washed in PBS (pH 7.2) supplemented with 0.5% BSA with 2 mM EDTA and resuspended in 90 ul of buffer per 10^7 cells. Next microBeads were added to cells (10 ul per 10^7 total cells) and cells were incubated for 15 minutes at 6-12^{o}C. After washing and centrifuging at 1200 rpm for 5 minutes, the cells were re-suspended in 1 ml of column buffer (PBS/ 0.5% BSA) before applying to the column. The column was washed with 9 mls of buffer and the flow through; containing the B-cell enriched population was collected.

The flow through was centrifuged at 1200 rpm for 5 minutes at 4^{o}C, re-suspended at $2x10^6$ cells/1 ml in RPMI 1640 supplemented with 10% FCS, 50 uM beta mercaptoethanol, 1% sodium pyruate, 2% L-glutamine, 1% penicillin-streptomyicin, and activated *in-vitro* by with 1ug/ml stimulatory ODN 1826 phosphorothioate modified CpG (5´-TCCATGACGTTCCTGACGTT-3´) (Oligo Etc, Inc, Wilsonville, OR) (58) or 10 ug/ml of purified anti-CD40 (Pharmingen), plus 10 ug/ml goat anti mouse -$F(ab´)_2$ IgM (Thermo Scientific, Rockford, IL), or medium alone then incubated for 48 hrs in a 5% CO_2 incubator at 37^{o}C. Following *in-vitro* activation, cells were then plated on Falcon plates coated with anti- IgM antibody (1/1000), or on Immunlon-2 coated with dsDNA (150 mg/ ml). Cells were plated at $2x10^5$ cells per 50 ul in the first row and serially diluted two-fold across the plate. After incubating at 37^{o}C for 8 hrs, plates were

washed 5 times with ELISPOT wash buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.2). A 1/300 dilution of biotin mouse anti-mouse anti-IgM^{a} was added to wells and plates were incubated overnight at 4^{o}C. Following five washes with wash buffer, 50 ul of streptavidin-AP (1/1000 dilutions) was applied and plates were incubated at room temperature for 2-3 hours. Following washes, 5́ BCIP 1mg/ml in AMP (2-amino-2 methyl-1-propanol) buffer was applied to wells. Plates were washed and examined for spots 7-8 hours later. Spots were counted under a dissecting microscope.

3.8 Fluorescence Activated Cell Sorting (FACS)

FACS analysis was performed on mouse bone marrow and splenic cells from R4A-C_{μ}, R4A-$C_{\mu}/V_{\kappa}1$, $V_{\kappa}1$ and WT C57BL/6 mice to determine B-cell development and phenotype. Cells were resuspended in 1% FBS/PBS at a concentration of 1X10^{6} cells/ 100 ul and aliquoted into 5 ml FACS tubes and immunostained with various combinations of fluorescent-conjugated antibodies to cell surface proteins at approximately 0.4-2 ug per 10^{6} cells for 30 minutes in the dark at 4^{o}C. The following antibodies were used; APC conjugated anti-mouse CD93 clone AA4.1 (eBioscience), APC-Cy7 anti-mouse B220, or PerCP-Cy5.5 rat anti- mouse CD45 or Cy-Chrome™ anti-mouse B220, FITC anti-mouse CD21, PE anti-mouse CD23, PE anti-mouse IgM^{a}, PE or FITC anti-mouse IgM^{b}, FITC anti-mouse Ig lambda, PE anti-mouse IgM, biotin anti-mouse CD24 (HSA), biotin anti-mouse CD43, biotinylated anti-mouse Ig kappa light chain (BD Pharmingen San Diego, CA). When Biotinylated antibodies were used the cells were counter stained with APC Strepavidin (BD Pharmingen San Diego, CA). Cells were then washed with 2 ml of 1 % FCS/PBS, and spun at 1200 rpm for 7 minutes

at 4^{o}C; and fixed with 500 µl of PBS 1% para-formaldehyde. Data was acquired on a BD LRS II flow cytometer using BDFACS diva software, and analyzed using Flowjo software version 4.5.8.

3.9 Flow Cytometry and Cell sorting

Splenocytes from 12-wk-old transgenic R4A-$C_{\mu}/V_{\kappa}1$ mice were harvested in FACS sorting buffer (1% FBS/PBS and 5uM EDTA) and treated with RBC lysis buffer to deplete erythrocytes. Cells were then washed with FACS sorting buffer and centrifuged at 1200 rpm for 7 minutes at 4^{o}C. The supernatant was aspirated and the remaining lymphocytes were resuspended in 1% FBS/PBS at 1 x 10^{7} cells/ml. Lymphocytes were immunostained for 30 min on ice in the dark with the following antibodies: Per Cp-Cy5.5 rat anti-mouse B220, PE anti-mouse IgM^{a}, and FITC anti-mouse IgM^{b} (BD Bioscience). Sorted $B220^{+}$ IgM^{a+} cells and $B220^{+}$ IgM^{b+} cells were collected in separate 5 ml FACS tubes containing 20% FBS/PBS with 5 mM EDTA. Cell sorting was conducted using the BD FACS Aria cytometer and BD FACSDIVA software.

3.10 Isolation of genomic DNA and total RNA from sorted IgM^{a} and IgM^{b} B-cell populations

Purification of genomic DNA and total RNA from sorted IgM^{a} (2.5 x 10^{5} cells) and IgM^{b} (3.4 x 10^{5} cells) B cells were performed using the Qiagen AllPrep DNA/RNA Micro Kit (Qiagen, Inc Valencia, CA), according to the manufacturer's protocol. B lymphocytes were initially centrifuged at 375 rpm for 5 minutes and the supernatant

aspirated. The pellet was gently loosened and cells were lysed with 350 ul buffer RLT Plus containing guanidine thiocyanate. To homogenize the samples, each lysate was passed 5 times through an RNase-free syringe equipped with a 20-gauge needle. The homogenized lysate was transferred to an Allprep DNA spin column, capped, and centrifuged for 30s at 10,000 rpm. The previous centrifugation step was repeated to ensure that no liquid remained on the membrane column. The flow through was collected for RNA purification and the AllPrep DNA spin column was stored at 4°C for further DNA processing.

To purify total RNA, 350 ul of 70% ethanol was added to the above flow through and the solution was mixed thoroughly by pipetting. The sample, including any precipitate that may have formed, was transferred to an RNeasy MinElute spin column and centrifuged for 15s at 10,000 rpm. The flow through was discarded and 700 ul RW1 buffer containing guanidine hydrochloride was added to the RNeasy MinElute spin column. The column was capped and its contents centrifuged for 15s at 10,000 rpm. The flow through was again discarded and 500 ul of RPE buffer containing 96-100% ethanol, was added to the spin column. The column was centrifuged for an additional 15s at 10,000 rpm and the flow through discarded. To wash the column membrane, 500 ul of 80% ethanol (prepared with RNase-free water) was added to the RNeasy MinElute spin column and the sample was centrifuged for 15s at 10,000rpm. Following centrifugation, the RNeasy MinElute spin column was transferred to a clean collection tube and centrifuged at 14,000 rpm for 5 minutes. The spin column was again placed into a new collection tube, and the RNA was eluted by adding 14 ul of RNase free water and centrifuging for 1 minute at 14,000 rpm. The above procedures were performed at

ambient temperature (20-25°C). All centrifugation steps were performed at 25°C using a standard microcentrifuge.

To purify genomic DNA, 500 ul of AW1 buffer was added to the AllPrep DNA spin column and the column centrifuged for 15s at 10,000 rpm. The flow through was discarded and 500 ul of AW2 buffer, containing ethanol was added to the column. The spin column and its contents were centrifuged for 2 minutes at 14,000 rpm. The AllPrep DNA spin column was transferred to a new collection tube and 50 ul of elution buffer EB) (preheated to 70°C) was added directly to the column membrane. The column was incubated at room temperature (25°C) for 2 minutes, and then centrifuged for 1 minute at 10,000 rpm to elute the DNA.

3.11 Two step RT-PCR for diction of the R4A heavy chsin transcript

Two-step Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed on RNA isolated from sorted IgM^a and IgM^b B-cells obtained from splenocytes using the Invitrogen *Superscript* kit RNA (Invitrogen, Carlsbad, CA). RNA isolated from sorted IgM^a and IgM^b B cells, was initially treated with deoxyribonuclease I (DNase) (Invitrogen, Carlsbad, CA) to remove any trace of DNA. As per the manufacturer's protocol, 200 ng of RNA was incubated with 1ul of 10X DNase Rxn buffer (200 mM Tris-HCl_2 (pH 8.4), 20 mM $MgCl_2$ and 500 mM KCl) and 1ul of DNase 1 for 15 minutes at room temperature. DNase 1 was inactivated with 1ul of 25 mM EDTA and heating for 10 minutes at 65^oC. Reverse transcription of the RNA was accomplished using 50- 250 ng of random primers, 1ul of 10 mM dNTP mix, and 100 ng RNA and water to total volume of 20 µl (Invitrogen, Carlsbad, CA). The reaction was

incubated at 65^{o}C for 5 minutes, to open secondary structures in the RNA, and then placed on ice for 1 minute. Next 4 ul of 5X buffer 1ul of 0.1M DTT, 1ul of RNase-OUT™ 40 units/ul and 1ul of Superscript™ III RT (200 units/ul) was added. Complementary DNA (cDNA) was then generated under the following conditions 25^{o}C for 5 min, 50^{o}C for 45 mins and 70^{o}C for 15 min. PCR was then performed on 2 ul of template cDNA in a mix containing 5 ul of 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 1.5 ul $MgCl_2$, 1 ul of 10 mM dNTP, 10 ρmol of R4A-V11 heavy chain FR1 forward primer BD272 ($^{5'}$-GGT GAA GCT GGT GGA ATC TGG-$^{3'}$), and (10 ρmol) R4A heavy chain reverse junctional primer BD243 ($^{5'}$-CAT AGG GAT CCT ATC TCT-$^{3'}$), and 0.4 ul of Taq polymerase (5 U/ul) and autoclaved distilled water to a final volume of 50 ul. PCR was performed as follows: 94°C for 2 min followed by forty cycles of (94°C for 30 s, 51°C for 30 s, 72°C for 40s). Next tubes were kept at 72°C for 5 min, and overnight at 4°C.

3.12 PCR amplification of the R4A VDJ heavy chain transgene

DNA isolated from IgM^{a} and IgM^{b} B-cells was amplified using Illustra Pure-Taq Ready-To-Go PCR Beads (GE Healthcare, Piscataway, NJ), containing the following: 200 um dNTPs in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 units puRe Taq DNA polymerase. Two microliters of the DNA template, 20 ρmol of HC forward and reverse primers (BD 272 and BD 243) were added to reaction tube containing a single PCR bead. PCR on a control housekeeping gene (G3PDH) was also performed using the forward primer ($^{5'}$-ACC ACA GTC CAT GCC ATC AC-$^{3'}$) and the reverse primer ($^{5'}$-TCC ACC ACC CTG TTG CTG TA-$^{3'}$) the volume of the tube was

then brought up to a total volume of 25 ul with autoclaved distilled water. The DNA template was amplified in a thermal cycler set at the following conditions: 94°C for 9 min, thirty cycles of (94°C for 30 s, 51°C for 30 s, 72°C for 40 s), 72°C for 5 min, and 4°C for ∞.

3.13 Single cell sort of transitional and mature B-cell subsets

Splenocytes from 12 to 20 wk old transgenic R4A-$C_\mu/V_\kappa 1$ mice were harvested in FACS sorting buffer and treated with RBC lysis buffer to deplete erythrocytes. Splenocytes were then washed and resuspened in 1% FBS/PBS, and immunostained with the following antibodies or single cell sort of immature and mature B-cell subpopulations, cells were stained immunostained and AA4.1-APC (eBioscience) Per Cp-Cy5.5 rat anti-mouse B220, PE anti-mouse IgM^a, FITC anti-mouse CD23 (BD Pharmingen) for 30 min on ice in the dark (0.2-5 ug/1x10^6 cells). To differentiate the three transitional populations of B-cells: T1 ($AA4.1^+$, $B220^{+,}$ $IgM^{a\ hi}$, $CD23^-$), T2 ($AA4.1^+$, $B220^{+,}$ $IgM^{a\ hi}$, $CD23^+$), T3 ($AA4.1^+$, $B220^+$, $IgM^{a\ lo}$, $CD23^+$), and the mature B-cell population ($AA4.1^-$, $B220^+$, IgM^{a+}). These lymphocytes were sorted one cell per well directly into 0.2 ml thin wall Template 1 PCR plates (USA Scientific Inc.) into a solution consisting of 0.2 ul of 10X PBS, 0.2µl of RNAsin® Plus RNase Inhibitor (10,000 U) (Promega), 0.128 ul of Stop RNAse Inhibitor (2000U) (5 Prime), 0.4 ul DTT (100 mM) in sterile H_2O to a final volume of 4 ul. After sorting cells were immediately placed on dried ice. Cell sorting was conducted using the BD FACS Aria cytometer and BD FACSDIVA software.

3.14 Single cell RT-PCR to detect the $V_\kappa 1$ light chain transgene

Single-cell Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed using Invitrogen SuperScript III Reverse Transcriptase™ RT (Invitrogen) and the Roche Fast Start PCR Master (2 X Enzyme mix) (Roach Applied Science Mannheim Germany), containing 2U of FastStart Taq DNA polymerase, magnesium chloride, double concentrated reaction buffer and nucleotides dATP, dCTP, dGTP, and dTTP 0.4 mM each. Cells were lysis in 3.5 ul of a mix containing 0.5 ul of 10% v/v NP-40, 0.5 ul of a random hexamer primers (Qiagen) (0.4 ug/ul), 0.03 ul Stop RNAse Inhibitor (Promega), and 2.47 ul of sterile H_2O.

For first strand cDNA synthesis, 2.3 ul of 5 X RT Buffer, 3.125 ul of 10 mM dNTPs, 1 ul of 100 mM DTT, 0.17 ul of sterile water (RNAse free), 0.125 ul of RNAsin (Promega), 0.03 ul of Stop RNAse Inhibitor, and 0.25 ul of RT Superscript III (200 U/ul) was alloquted into wells containing a single cell each. Plates were then incubated at 60^{o}C for 1 minute followed by 42°C for 5 min, 25°C for 10 min, 40°C for 60 min and 94°C for 5 min.

Two successive rounds of PCR were then performed on the above single stranded cDNA. $V_\kappa 1$ light chain cDNA (1 µl) was first amplified in a 30 ul reaction containing 15 ul of 2 X enzyme mix, 10 ρmol of light chain forward primer, BD273 ($^{5\prime}$-CCAGCAGGATGTTGTGATGACCC-$^{3\prime}$), and 10 ρmol Ck reverse primer, Ck1 ($^{5\prime}$-TGGATGGTGGGAAGATG-$^{3\prime}$) and 12 ul of distilled H_2O; PCR conditions for the first round amplification were; 95°C for 4 min, followed by 95°C for 30 s, 55°C for 30 s, 72°C for 1 min for 50 cycles, then 72°C for 7 min, and 4°C for ∞.

The second round of amplification was performed using 2 ul cDNA (from the first round PCR), 20 ul of 2X enzyme mix, 10 ρmol of the same forward primer as above, 10 ρmol of a more upstream Ck reverse primer (Ck2 primer) (^{5}′-AAGATGGATACAGTTGGT-^{3}’) and dH_2O to bring the final volume to 40 ul; second round PCR employed the same cycler conditions as the first round, with the exception that the annealing temperature used was of 60°C instead of 50^{o}C.

3.15 Fluorescent and Confocal microscopy

Lymphocytes were isolated from R4A-Cμ/$V_κ$1 splenocytes and immunostained with PerCP-Cy 5.5 rat anti-mouse B220, PE anti-mouse IgM^{a}, plus FITC anti-mouse IgM^{b}. For fluorescent microscopy, sorted cells were centrifuged at 1200 rpm for 7 mins the supernatant was aspirated, and cells were transferred to a microcentrifuge tube and spun at 1200 rpm for 7mins. After descanting supernatant, approximately 100 ul was placed a slide. Slides were examined on Nikon Eclipes TE 2000S at magnification of 400X. For confocal imaging, unsorted splenocytes ($1x10^{6}$ cells/ 100 ul) were first stained as above then cytospun at 800 rpm for 5 mins onto slides and image data acquired on Ziess LSM 510 Confocal Microscope at a magnification of 40X.

3.16 Calcium flux

Splenocytes were isolated from R4A-$C_μ$/$V_κ$1, WT and R4A-Cμ mice, depleted of red blood cells (pervoiusly described), and resuspended at $1x\ 10^{7}$ cells/ ml in Hanks Balance Salt Solution (HBSS) supplemented with 0.5% BSA (GIBCO). Cells were loaded with Indo-1-AM (Molecular Probes) at a final concentration of ug/ml and incubated at

37^{o}C for 30 min in the dark. They were then washed in HBSS, centrifuged at 1200rpm for 7 minutes at 37^{o}C and then resuspended at 1x 10^{7}cells/ ml. The cells were then labeled at for 20 min at room temperature with the following antibodies: APC anti-mouse AA4.1 (eBioscience), PerCP-Cy 5.5 rat anti-mouse B220, PE anti-mouse IgM^{a} or PE anti-mouse IgM^{b} (in WT), and FITC anti-mouse CD23 (BD Bioscience). A baseline reading was recorded for 1 minute. The cells (5x10^{7}/500 ul) were then stimulated with either F(ab′)$_2$ IgM$_\mu$ antibody (10 ug/ml) (Pierce Thermo Scientific) and data recorded for 1 minute, cells were rested at 37^{o}C for 15 minutes prior to data collection. Mean Ca^{2+} was measured with a LSR flow cytometer

3.17 HYBRIDOMA STUDIES

3.17a Generation of IgM^{a} hybridomas

Hybridomas from R4A-C$_\mu$/V$_\kappa$1mouse were generated and their supernatants were screened for expression of antibodies that utilize the IgM^{a} heavy chain transgene. The supernatants were then tested by ELISA for the presence of transgenic IgM^{a} and transgenic IgM^{a} anti-dsDNA antibody. IgM^{a} positive clones were be subcloned two times in soft agar. At least 20 different subclones were picked and their RNA was extracted. cDNA was made by RT-PCR and cDNA was amplified for V$_\kappa$1 light chains, and sequenced to see if the V$_\kappa$1 transgene was utilized, or if here was editing of this LC.

To generate hybridomas, splenocytes from R4A-C$_\mu$/V$_\kappa$1 mice were fused with NSO cells in a 2:1 ratio using 50% PEG 4000 (64). Cells were then plated at 2x10^{5} splenocytes/ ml in DME, HAT selection medium supplemented with 20% fetal calf

serum (Hyclone Laboratories, Logon, UT), 10% NCTC 109, 1% pen/strep and 1% non-essential amino acids. Hybridomas were screened for the presence of IgM^a antibody and IgM^a anti-dsDNA antibodies by ELISA as previously described. Positive clones were selected for expansion and sub-cloned on soft agar consisting of DME media containing 10% J774, 20% FCS, 10% NCTC 109, and 1% Pen-Strep and 36% agarose (Seaplaque Bioproducts Rockland Maine). Sub-clones were screened again for IgM^a and IgM^a anti-dsDNA antibodies. Positive clones were expanded, and RNA isolated.

3.17b Hydridoma RNA isolation

Total RNA was extracted from hybridomas using TRIZOL® Reagent (Invitrogen, Carlsbad, CA). Cells were centrifuged at 1200 rpm. The supernatant was removed and the cell pellet lysed by pipetting up and down in TRIZOL® Reagent (1ml per 3-10 $x10^6$ cells). Cells were incubated for 5 to 15 minutes at 37^oC. After adding 0.2 ml of chloroform per 1ml of TRIZOL® Reagent, the samples were centrifuged at 1200 x g for 14 minutes at 4^0C. The top, aqueous phase containing the RNA was transferred to a fresh tube; the RNA was isolated by adding 0.5 ml of isopropyl alcohol per 1ml of TRIZOL®. The sample was incubated at 15-30^oC for 10 minutes, and centrifuged at 1200 x g for 10 min at 4^oC to pellet the RNA. The pellet was washed with 1 ml of 75% ethanol. Tubes were vortexed and centrifuged at 7,500 x g for 5 mins at 4^oC. The RNA pellet was air dried for 5-10 mins and redissolved in RNase free DEPC water. Protein purity was determined by measuring the OD at 260 nm and determining the ratio of OD 260/ 280 ratio close to 2.0).

3.17c Hydridoma RT-PCR and gene cloning

Complementary V_κ DNA (cDNA) was generated and amplified in a one step RT-PCR procedure using total hydridoma mRNA and Ready-To-Go RT-PCR beads (Amersham Bioscience, Piscataway NJ). The generation of cDNA by RT was achieved using Moloney Murine leukemia virus (MMLV) reverse transcriptase. PCR amplification was performed using *Taq* DNA polymerase, 20 pmol of BD 273 and a reverse primer specific for the mouse kappa LC constant region (ACACTCATTCCTGTTGAA) (50). The reverse transcription was conducted under the condition of 42^{o}C for 30 minutes, 95^{o}C for 10 minutes. PCR was performed under the following conditions of 95^{o}C for 30 seconds, 50^{o}C for 40 seconds, and 72^{o}C for 7 minutes for 35 cycles, and a final extension at 72^{o}C for 10 minutes. The PCR products generated were cloned into pCR®2.1 TA cloning vector (Invitrogen, Carlsbad CA) and transformed into One Shot® chemically competent E coli cells. The cells were then plated on LB ampicillin plates (1 % Tryptone, 0.5 % Yeast extract, and 1% NaCl pH 7.0). White colonies were selected and screened for the presence of recombinant plasmid by digesting with EcoR1.

Plasmids were isolated using QIAprep® mimiprep kit according to manufacturer's protocol. Bacterial cells pellets were resupended in microcentrifuge tubes with 250 ul of P1 buffer containing RNase A. An equal volume of P2 buffer was then added. After mixing 350 ul of N3 buffer was added and tubes were centrifuged for 10 min at 13,000 rpm. The supernatants were then transferred to QIAprep columns and centrifuged for 30-60 sec at 13,000 rpm, then washed with 500 ul of PB buffer, centrifuge for 1 min at 13,000 rpm and DNA collected in the flow through. Plasmid containing the appropriate size insert was sent

to Gene Wiz sequencing facility (North Brunswick, NJ) for sequencing using M13 universal primer.

CHAPTER 4

RESULTS

4.1 Tolerance is maintained in R4A-Cμ/Vκ1 Mice

Anti-dsDNA antibodies are the distinguishing characteristic of the autoimmune disease Systemic Lupus Erythematosus (SLE). These antibodies are generally of the IgG isotype, and are produced following class switching of an IgM antibody upon B-cell activation in peripheral lymphoid organs. Studies reveal that in humans, 55-75 % of immature B-cells produced in the bone marrow are autoreactive (65). In the peripheral blood 40 % of the immature B-cells produced are autoreactive, and 20 % of mature B-cells in humans are autoreactive and recognize single stranded DNA (ssDNA) and double dsDNA (66)

We wanted to examine the regulation of anti-dsDNA B-cells during development in mice transgenic for the heavy and light chain genes of an anti-ds DNA antibody. Previous studies in R4A-C_μ heavy chain only transgenic mice showed tolerance is maintained by deletion and anergy in these mice (51). It was also observed that most of the Tg anti-dsDNA B-cells targeted to anergy in these mice utilized the R4A-C_μ HC and an unmutated $V_\kappa 1$ LC. However, the frequency of these anergic B-cells was less than 2 %. To better study the features of this anergic population of B-cells, we decided to generate mice that were enriched for anergic B-cells by generating C57BL/6 mice transgenic for both the R4A-C_μ HC and an umutated $V_\kappa 1$ LC. The LC knock-in transgenic mice were a gift from the laboratory of Dr. Martin Weigert. These mice were bred to R4A-C_μ transgenic mice on a C57BL/6 background for a minimum of 10 generations. Mice heterozygous for both the R4A-C_μ HC and the $V_\kappa 1$ transgene were

used for all studies. We wanted to determine if Tg anti-dsDNA B-cells in these mice were down regulated by anergy and were therefore unable to secrete anti-dsDNA antibody. I used an IgM^a anti-ds-DNA ELISA to test whether serum from R4A-$C_\mu/V_\kappa1$ mice contained Tg anti-dsDNA antibody. I observed that Tg B-cells were not spontaneously secreting Tg anti-dsDNA antibody suggesting that tolerance was being maintained (Figure 9). The antibody content was compared to that of sera from R4A-C_μ and wild type mice. There was no significant difference in the serum anti- dsDNA antibody content from R4A-$C_\mu/V_\kappa1$ mice compared to R4A-C_μ heavy chain only transgenic animals ($p = 0.714$).

4.2 Reduced frequency of bone marrow B-cells in R4A-Cμ/Vκ1 and R4A-Cμ transgenic mice relative to wild type mice

To determine whether tolerance is induced in the bone marrow of R4A-$C_\mu/V_\kappa1$ mice I first examined the frequency of $B220^+$ B-cells generated in the bone marrow of these mice. The frequency of $B220^+$ cells in the bone marrow of R4A-$C_\mu/V_\kappa1$and R4A-C_μ mice was compared to that of $B220^+$ cells in the bone marrow of wild type and $V_\kappa1$ light chain only transgenic mice. I observed a reduction in the frequency of total B220 B-cells in the bone marrow of R4A-$C_\mu/V_\kappa1$ mice (44 %) compared to wild type (64 %) and $V_\kappa1$ (61 %) mice. This reduction in B-cell frequency is similar to that observed in R4A-C_μ heavy chain only transgenic mice (41 %) (Figure 10). The first immunoglobulin receptor expressed on newly formed immature B-cells is the IgM immunoglobulin. The frequency of total IgM^+ B-cells in the bone marrow of R4A-$C_\mu/V_\kappa1$ mice is reduced relative to the frequency of IgM B-cells in wild type mice and $V_\kappa1$ light chain transgenic mice.

While 25 -35 % of B- cells in wild type and $V_\kappa 1$ mice express the IgM receptor, <15 % of B-cells in R4A-$C_\mu/V_\kappa 1$and R4A-C_μ mice express surface IgM, suggesting deletion of B-cells in R4A-$C_\mu/V_\kappa 1$ and R4A-C_μ animals (Figure 11). The absolute number of bone marrow B-cells was diminished 4.5 fold in R4A-C_μ mice relative to wild type mice (Table 1). However, it could not be ruled out that the decrease in bone marrow B-cells in R4A-$C_\mu/V_\kappa 1$ mice is not due to a developmental bottle neck.

To determine whether B-cells are arrested in an early stage of development in R4A-$C_\mu/V_\kappa 1$ mice, I examined the profile of the developing B-cells in the bone marrow of these mice. Newly formed B-cells express high levels of the heat stable antigen (HSA) also known as CD24 (32). I first compared the frequency of immature ($CD24^{hi}$) and mature ($CD24^{lo}$) B-cells in the bone marrow of R4A-$C_\mu/V_\kappa 1$ mice to that of these subsets of B-cells in $V_\kappa 1$ transgenic and wild type mice. Unlike wild type and $V_\kappa 1$ mice, the majority of IgM^+ B-cells in the bone marrow of R4A-$C_\mu/V_\kappa 1$ mice as in R4A-C_μ mice are immature (Figure 12A). R4A-$C_\mu/V_\kappa 1$ mice, display an approximately two- threefold increase in the frequency of immature, $CD24^{high}$ B-cells compare to wild type mice. Assessment of the frequency of immature and mature B-cell using an alternative developmental marker for early B cell lineage, AA4.1 results in a similar observation. The frequency of immature, $AA4.1^+$ B-cells in the bone marrow of R4A-$C_\mu/V_\kappa 1$ mice is greater than in WT mice (Figure 12B). Likewise the frequency of re-circulating mature ($AA4.1^-$) B-cell is much lower in R4A-$C_\mu/V_\kappa 1$ mice than WT mice.

I then looked at the frequency of B-cells in various stages of B-cell development in the bone marrow of R4A-$C_\mu/V_\kappa 1$ mice, and compared this to the frequency of B-cells in corresponding stages of B-cell development in the bone marrow of R4A-C_μ heavy

chain only transgenic mice, wild type and $V_\kappa 1$ mice. The stages of B-cell development can be defined according to their surface expression of various cell surface molecules, for instance, pro-B cells are $B220^+ CD43^+ IgM^-$, while pre-B-cells are $B220^+ CD43^- IgM^-$and both immature and mature B-cells are ($B220^+ CD43^- IgM^+$) (26). Mature B-cells can be distinguished from immature B-cells by the down regulation of CD24 as previously described and the expression of IgD. However, Tg B-cells do not express IgD since the transgene only encodes a μ HC and is not homologously inserted in the HC locus. R4A-$C_\mu/V_\kappa 1$ mice exhibit a similar frequency of pro-B-cells relative to WT and $V_\kappa 1$mice (Fig.13). However, the frequency of pre B-cells in R4A-$C_\mu/V_\kappa 1$ mice is not significantly different from that of WT mice. The frequency of pre-B-cells ($B220^+CD43^- IgM^-$) in R4A-$C_\mu/V_\kappa 1$ mice is lower than that in R4A-C_μ and wild type mice, while the frequency of the immature/ mature IgM^+ B-cells is increased in R4A-$C_\mu/V_\kappa 1$ mice relative to R4A-C_μ and WT mice (Figure 13). This may be because the presence of the pre-rearranged transgenic HC and LC accelerate the maturation of these B-cells from pre- B cells to immature B-cells. The IgM^+ compartment in the bone marrow likely reflects a mixed population of cells containing both immature and re-circulating mature IgM^+ B-cells. Interestingly we observed that the majority of IgM^+ B-cells in the bone marrow of R4A-$C_\mu/V_\kappa 1$ mice express intermediate levels of CD43 whereas the majority of IgM^+ B-cells in WT mice are $CD43^{lo}$. This may be because the presence of a pre-rearranged LC causes these B-cells to accelerate their progression to immature B-cells before CD43 expression gets completely downregulated. $V_\kappa 1$ mice also have a high frequency of CD43 intermediate B-cells.

4.3 Majority of bone marrow B-cells in R4A-Cμ/Vκ1 mice express the IgM^a transgene

The transgenic μ heavy chain is from a BALB/c mouse and is of the a-allotype. It can be distinguished from the endogenous IgM, which is of the b-allotype in C57BL/6 mice, using allotype specific antibodies. Examination of the frequency of B-cells expressing the IgM^a transgene in the bone marrow of R4A-$C_\mu/V_\kappa1$ mice by flow cytometry reveals that the majority of IgM$^+$ B-cells express the transgenic IgM^a (> 85 %) (Figure14). This indicates that the HC transgene is preferentially expressed in the bone marrow of R4A-$C_\mu/V_\kappa1$ mice. I was unable to use flow cytometry to determine the frequency of transgenic B-cells expressing the transgenic $V_\kappa1$ LC since a reagent to detect $V_\kappa1$ LCs is not available. However, I did immunostain bone marrow B-cells with antibodies to kappa and lambda light chain to demonstrate that the majority of B-cells in R4A-$C_\mu/V_\kappa1$ mice express a kappa and not a lambda light chain (Figure 15).
This observation does not prove that the V_κ light chain utilized by most bone marrow B-cells is encoded by the transgenic LC, since most (95 %) of mouse B-cells in wild type mice normally express kappa LC. However, the observation that the λ LC frequency is not increased in R4A-$C_\mu/V_\kappa1$ mice ruled out the possibility that editing to the lambda LC occurs in these mice.

4.4 Reduction in the frequency of splenic B-cells in R4A-$C_\mu/V_\kappa1$ mice

Immature B-cells leave the bone marrow and transit to the spleen to complete their development into mature immune competent cells, which when activated by specific antigen, differentiate into antibody secreting plasma cells. To determine the frequency of

B-cells in the spleen of R4A-$C_\mu/V_\kappa1$ mice, I immunostained splenic B-cells with antibodies to B220 and then analyzed the frequency of $B220^+$ B-cells by flow cytometry. I observed that the frequency of splenic $B220^+$ cells is reduced two-fold in R4A-$C_\mu/V_\kappa1$ mice compared to wild type mice and $V_\kappa1$ mice (Figure 16). The absolute number of B-cells is reduced 3 fold in R4A-$C_\mu/V_\kappa1$ mice relative to WT mice (Table 2). A similar reduction in peripheral B-cells was observed in R4A-C_μ (17%) mice.

I also examined the frequency of B-cells expressing IgM in the spleen of R4A-$C_\mu/V_\kappa1$ mice by flow cytometry. I observed a reduction in the frequency of total splenic IgM B- cells in R4A-$C_\mu/V_\kappa1$ relative to wild type and $V_\kappa1$ mice (Figure 17 and Table 2). This reduction is similar to that observed in R4A-C_μ mice, which were previously shown to have a reduced frequency of peripheral Tg B-cells due to anergy and deletion (51).

A comparison of the frequency of splenic B-cells expressing the IgM^a transgene to B-cells expressing endogenous IgM^b (Figure 18) revealed that a greater frequency of B-cells express endogenous IgM^b than IgM^a in the periphery of R4A-$C_\mu/V_\kappa1$ mice. This may be because endogenous IgM^b B-cells are preferentially selected over the transgenic B-cells in the periphery, or transgenic IgM^a B-cells are preferentially deleted due to their autoreactivity. This was also previously observed in R4A-C_μ mice (55). A population of B- cells co-expressing IgM^a and IgM^b was also observed in R4A-C_μ and R4A-$C_\mu/V_\kappa1$ mice. These dual expressing ($IgM^{a/b}$) B- cells were not observed in the bone marrow of these animals (Figure 14).

4.5 Generation of IgM^a hybridomas from R4A-$C_\mu/V_\kappa1$ mice and analysis of LC usage

In order to look for evidence of LC chain editing, we decided to generate IgM^a producing hydridomas and determine whether the IgM^a monoclonal antibody produced by these hybridomas utilizes the transgene encoded $V_\kappa1$ LC or other LCs. We hypothesized that dsDNA binding IgM^a antibodies obtained from these hybridomas were likely to utilize the $V_\kappa1$ $J_\kappa1$ transgene encoded LC, while non dsDNA binding IgM^a antibodies were likely to utilize mutated $V_\kappa1$ LC or non $V_\kappa1$ LCs as a consequence of LC editing. I generated IgM^a secreting hybridomas from an R4A-$C_\mu/V_\kappa1$ mouse and analyzed the LC usage of 3 IgM^a dsDNA binding antibodies and 6 non dsDNA binding antibodies. The 3 dsDNA binding antibodies and 5 of 6 non-dsDNA binding Abs was found to utilize the unmutated $V_\kappa1$ LC. This suggested that LC receptor editing does not play a major role in the regulation of Tg anti-dsDNA B-cells in R4A-$C_\mu/V_\kappa1$ mice.

4.6 High frequency of transgenic B-cells are arrested in the immature/ transitional stage of development in R4A-$C_\mu/V_\kappa1$ mice

I examined the distribution of immature (CD24^{high}) versus mature (CD24^{low}) IgM^a B-cells in the periphery of R4A-$C_\mu/V_\kappa1$ mice (Figure 19). In R4A-$C_\mu/V_\kappa1$ mice the majority of transgene expressing B-cells are CD24 ^{high}, compared to wild type mice, where conversely the majority of IgM expressing B-cells are CD24 ^{low}. Staining with the early B-cell lineage marker, AA4.1 corroborates these observations. Greater than 80 % of B cells in R4A-$C_\mu/V_\kappa1$ and R4A-C_μ mice stain positively for AA4.1, while less than 40 % are AA4.1$^+$ in WT mice (Figure 20). The high frequency of immature B-cells in

R4A-$C_\mu/V_\kappa1$ mice suggests developmental arrest of transgenic B-cells in the periphery of these mice.

4.7 Transgenic B-cells are arrested at the T-1 stage in R4A-$C_\mu/V_\kappa1$

Using flow cytometry I examined the frequency of the transitional IgM^a T1, T2, and T3 B-cell in R4A-$C_\mu/V_\kappa1$ mice, and found that these mice display an expanded T1 (IgM^+CD23^-) compartment (64 %) relative to wild type mice (32 %) (Figure 21). R4A-C_μ mice also have an expanded T1 (47 %) compartment, but not as large as that in R4A-$C_\mu/V_\kappa1$ mice. The frequency of IgM^a B-cells in the T-1 compartment of R4A-$C_\mu/V_\kappa1$ mice is approximately 2 fold greater than the T-1 compartment in wild type mice. In addition, the T2 compartment of R4A-$C_\mu/V_\kappa1$ mice is significantly reduced relative to the T2 compartment of wild type mice. This suggests that IgM^a B-cells in R4A-C_μ mice are arrested in the T1 stage of development.

Despite my observation that very few IgM^a B-cells mature in the periphery of R4A-$C_\mu/V_\kappa1$ mice, I also observed by flow cytometry that some do mature and acquire a marginal (MZ) or a follicular (FO) B-cell phenotype. MZ B-cells are IgM^+ $CD21^{hi}$ $CD23^-$ while FO B-cells are IgM^+ $CD21^{inter}$ $CD23^+$ according to Loder et al. (31). Both R4A-$C_\mu/V_\kappa1$ and R4A-C_μ mice have a larger frequency of MZ B-cells than WT mice (Figure 22). This is a feature observed in other mouse models where mice are transgenic for autoreactive B-cells and may be because autoreactive B-cells often reside in the MZ (67-69).

4.8 Transgenic B-cells in R4A-$C_\mu/V_\kappa 1$ mice display evidence of anergy

The majority of splenic IgM^a B-cells in R4A-$C_\mu/V_\kappa 1$ mice appear to be arrested in development which is a feature of anergy (52). I therefore wanted to determine whether the IgM^a B-cells in R4A-$C_\mu/V_\kappa 1$ mice displayed other features consistent with anergy, such as receptor down regulation, unresponsiveness to BCR cross-linking, reduced calcium mobility and shortened life span (47). To examine the level of IgM expressed on Tg B-cells relative to endogenous B-cells, I first immunostained B-cells with antibodies to B220, IgM^a or IgM^b and kappa LC and then gated on Tg (IgM^a) or endogenous (IgM^b) B-cells and measured kappa expresion by flow cytometry. I observed that the mean fluorescent intensity (MFI) of kappa light chain expression on transgenic B-cells was much lower than that on endogenous B-cells in R4A-$C_\mu/V_\kappa 1$ mice (Figure 23). The extent of reduction of surface IgM (sIgM) on transgenic B-cells in R4A-$C_\mu/V_\kappa 1$ mice was similar to that previously observed on transgenic B-cells in R4A-C_μ mice (55) (Figure 23).

B-cell anergy is also characterized by a B- cell's failure to respond to activation via BCR cross-linking along with T-cell help (47). B-cell enriched splenocytes from R4A-$C_\mu/V_\kappa 1$ mice were stimulated *in- vitro* with an $F(ab')_2$ anti-IgM antibody plus anti-CD40 antibody as a surrogate for T-cell help. I found that the transgenic B-cells were refractory to stimulation through their BCR and were not induced to secrete transgenic IgM^a anti-dsDNA antibody (Figure 24A) and therefore were functionally anergic. However, I also observed there were some IgM^a B- cells in the periphery of R4A-$C_\mu/V_\kappa 1$ mice that were capable of secreting IgM^a antibody when stimulated through their BCR (Figure 24B). It is likely that these B-cells were non-dsDNA binding B-cells that utilized

the Tg IgM^a HC and an endogenous LC that when paired with the R4A HC did not confer specificity for dsDNA.

4.9 R4A-$C_\mu/V_\kappa1$ B-cells exhibit reduced Ca^{2+} flux upon BCR stimulation

Activation of B-cells via their BCR leads to inositol-1, 4, 5- triphosphate (IP_3) binding its receptor (IP_3R) on the endoplasmic reticulum (ER), triggering the release of intracellular calcium ions into the cytosol. Anergic B-cells exhibit a sub-optimal Ca^{2+} flux (70). I wanted to determine whether IgM^a B-cells in R4A-$C_\mu/V_\kappa1$ mice exhibited a reduction in Ca^{2+} flux relative to B-cells in WT mice. I examined Ca^{2+} flux in IgM^a B-cells from R4A-$C_\mu/V_\kappa1$ mice, and compared this to those in WT mice and observed that IgM^a B-cells showed a reduction in Ca^{2+} flux (Figure 25).

I next examined calcium flux within the transgenic T1, T2 and mature B-cell populations in R4A-$C_\mu/V_\kappa1$ mice and compared this to Ca^{+} flux of respective B-cell subsets in WT mice. I observed that Tg T1 B-cells do not mobilize Ca^{2+} as well as T2 and mature B-cells (MB) in R4A-$C_\mu/V_\kappa1$ mice, whereas in WT mice T1 mobilizes Ca^{2+} equally as well as MB and T2 B-cells (Figure 26). Mature and T2 B-cells flux Ca^{2+} similarly in both R4A-$C_\mu/V_\kappa1$ and WT mice. The diminished Ca^{2+} flux in the T1 subset of R4A-$C_\mu/V_\kappa1$ mice suggests that this subset may be a reservoir for anergic B-cells.

4.10 Does T-1 represent a regulatory checkpoint in R4A-$C_\mu/V_\kappa1$ mice?

Since transgenic B-cells in R4A-$C_\mu/V_\kappa1$ mice display an expanded T1 compartment that shows reduced Ca^{2+} flux upon B-cell cross-linking, we wondered whether this stage represents a regulatory checkpoint for autoreactive B-cells in these

mice. We hypothesized that if T1 was a regulatory checkpoint in R4A-$C_\mu/V_\kappa 1$ mice, then the T1 subset would contain a higher frequency of Tg anti-dsDNA B-cells than the T2 subset, as the autoreactive B-cells would be eliminated prior to progressing to T2. Yurasov et al. found that there was no significant difference in the frequency of immature peripheral autoreactive B-cells in SLE patients and healthy donors (66). However, the frequency of mature peripheral autoreactive B-cells in SLE patients was much higher (41-50%) than that in healthy donors (20.4 %) (66). This suggested that in SLE patients there is a defect in the elimination of autoreactive B-cells at a peripheral regulatory checkpoint between the immature and mature stage of B-cell development.

To determine the frequency of Tg anti-dsDNA B-cells present in the T1 and T2 subsets of R4A-$C_\mu/V_\kappa 1$ mice, I FACS sorted on IgM^a T-1 and T-2 B-cells from R4A-$C_\mu/V_\kappa 1$ mice and stimulated these B- cells *in-vitro* with CpG sequence to recues them as antibody secreting cells. Our laboratory has previously shown that stimulatory CpG can activate anergy anti-dsDNA B-cells to become antibody secreting cells (51, 63). ELISPOT assay was then used to determine the frequency of anit-dsDNA secreting B-cells within the transitional population of these mice. The number of dsDNA binding B-cells in both the T1 and T2 compartments of R4A-$C_\mu/V_\kappa 1$ mice was greater than that in wild type mice (Figure 27). However, there was no significant difference in the number of transgenic anti-dsDNA B-cells in the T1 and T2 compartments of R4A-$C_\mu/V_\kappa 1$ mice, suggesting that the autoreactive transgenic anti-dsDNA B-cells were not being eliminated at the T1- T2 transition.

4.11 The frequency of IgM^a B-cells utilizing the $V_\kappa 1$ LC does not decrease as B-cells mature in the periphery

We were concerned that the *in-vitro* activation and ELISPOT assay may not have been the best way to quantitate the frequency of anti-dsDNA B-cells in the T1 and T2 populations because some anergic T1 B-cells may not have been susceptible to activation by CpG. I therefore decided to quantitate the number of anti-dsDNA B-cells in the T1 and T2 subsets by determining the frequency of B-cells utilizing the R4A-C_μ heavy chain and the unmutated $V_\kappa 1$ light chain since this heavy and light chain combination was previously shown to encode dsDNA specificity (51). If T1 was a regulatory checkpoint then I would have expected to see a higher frequency of IgM^a B-cells utilizing the unmutated $V_\kappa 1$ LC, in the T1compartment than the T2 compartment, because the autoreactive B-cells would have been eliminated before entering T2.

I therefore anticipated that as B-cells matured in the periphery there would be a reduction in the frequency of IgM^a B-cells utilizing the $V_\kappa 1$ LC and an expansion of B-cells utilizing endogenous LCs. To determine frequency of $V_\kappa 1$ usage among Ig IgM^a B-cells at different stages of development, I first sorted on immature (AA4.1$^+$) IgM^{a+} T1, T2, and T3, and mature B-cells from R4A-C_μ/$V_\kappa 1$ mice. I then performed single cell RT-PCR on cells from each population using a $V_\kappa 1$ specific forward primer and a reverse C_k primer. I then electrophoresed the PCR products on an agarose gel, purified a 350 bp products and sequenced them to confirm if products were $V_\kappa 1$ (Figure 28). I observed that the majority of B-cells at each stage of development expressed the transgene encoded $V_\kappa 1$ LC mRNA. I found no decrease in $V_\kappa 1$ LC usage as transgenic B-cells progressed through the T1 to the T2 to the mature stages of development. Eight out of ten T1 B-

cells, ten out of ten T2 B-cells and nine out of ten mature B-cells examined gave the expected PCR product of 350 bp, corresponding to the size of the rearranged $V_\kappa 1$ light chain. PCR products were sequenced and shown to be identical to the germline $V_\kappa 1$ and $J_\kappa 1$ regions. The single cell PCR results confirmed the ELISPOT results, and demonstrated that IgM^a B-cells utilizing the Tg $V_\kappa 1$ LC are not preferentially eliminated in the transition from T1 to T2, nor are B-cells that have edited their LC preferentially expressed in subsequent stages of maturation.

4.12 Allelic inclusion and dual receptor expression in R4A-$C_\mu/V_\kappa 1$ mice

As previously mentioned, I observed by flow cytometry that a high frequency of B-cells in the periphery of R4A-$C_\mu/V_\kappa 1$ mice co-express IgM^a and endogenous IgM^b HCs. This is known as allelic inclusion. To examine allelically included peripheral B-cells microscopically, splenic B-cells were immunostained with antibodies to IgM^a and IgM^b and then sorted on $IgM^{a/b}$ cells. The cells were then fixed on a glass slide and examined by fluorescent microscopy (Figure 29). I observed that these cells stained positively for both IgM^a and IgM^b. To confirm the presence of double expressers within the splenic population and to obtain images of better resolution, I used confocal microscopy. I observed that B-cells expressing dual receptors, IgM^a and IgM^b are readily detected, and I observed that the surface expression of transgenic IgM^a and endogenous IgM^b was not evenly distributed and resulted in a speckled pattern (Figure 30). Both techniques clearly show dual receptor expression on some splenic B-cells obtained from R4A-$C_\mu/V_\kappa 1$mice.

4.13 The frequency of dual receptor expressing B-cells increases as they mature in R4A-C_μ/V_κ1mice

Since co-expression of transgenic IgM^a and endogenous IgM^b antibodies were not observed in the bone marrow but only in the spleen of R4A-C_μ/V_κ1 mice, I wondered whether the frequency of these dual expressing B-cells increased as the B-cells underwent maturation in the periphery. I therefore investigated the frequency and expression of the endogenous IgM^b receptor on peripheral B-cells as they progressed from the immature ($AA4.1^+$) to mature ($AA4.1^-$) stage in R4A-C_μ/V_κ1mice. I found that the frequency of IgM^a B-cells decreased and the frequency of IgM^b B-cells increased as they mature from $AA4.1^+$ to $AA4.1^-$ B-cells (Figure 31). While, the total frequency of B-cells expressing dual receptors (IgM^a and IgM^b) seemed to decreased as they mature, when we calculated the frequency of IgM^a B-cells co-expressing IgM^b, I observed that this increased slightly as they mature (46%) in $AA4.1^+$ compared to 52% in the $AA4.1^-$ population of R4A-C_μ/V_κ1mice. I then looked at IgM^b and IgM^a expression on Tg B-cells as they passed through each of the transitional stages of development (Figure 32).

I observed that the frequency of IgM^a B-cells co-expressing the endogenous IgM^b receptor increased as these transgenic B-cells passed through the T1 (25 %), T2 (37 %) and T3 (71 %) stages of development (Figure 32 top panel). The T3 compartment exhibited the highest frequency of double expressers among the immature splenic B-cell populations. A similar phenomenon was also observed in R4A-C_μ mice but the frequency of B-cells expressing dual receptors was reduced (Figure 32 bottom panel). In wild type mice, the T-3 compartment has been shown to contain cells that display an anergic

phenotype (70). Our observation that these B-cells have a high frequency of dual receptor expression may be a novel characteristic of T 3 anergic B-cells.

I also examined dual receptor expression on mature AA4.1$^-$ IgM^a B-cells. IgM^a B-cells in the marginal zone displayed a higher frequency of dual receptor expression (63 %), than follicular B-cells (33 %) (Figure 33 top panel); a similar trend was observed in R4A-C_μ heavy chain only transgenic, although at reduced frequencies (Figure 33 bottom panel).

4.14 Endogenous IgM^b B-cells express low levels of IgM^a heavy chain transgene

Since a much higher frequency of B-cells expressing IgM^b only was observed in the mature AA4.1$^-$ population than in the immature AA4.1$^+$ population (figures 14 and 15), I wondered whether these B-cells were derived from the dual receptor expressing B-cells, or arose as a result of selection and expression of the IgM^b B-cells in the bone marrow. I first sorted on IgM^a and IgM^b splenic B-cells from R4A-C_μ/V_κ1 mice by flow cytometry. I then did PCR on DNA from each population of cells to confirm that they contained the R4A VDJ transgene and had not deleted it (Figure 34). I observed that the IgM^b B-cells did contain the rearranged R4A VDJ transgene.

I then performed RT-PCR to on the sorted IgM^a and IgM^b B-cell population determine whether the IgM^b B-cells contained R4A transcripts (Figure 35). I observed that IgM^b B-cells expressed R4A VDJ transcripts but at a lower level than in IgM^a B-cells. Future studies will require the use of Real Time PCR to quantitate the level of R4A- VDJ RNA transcrpts in the IgM^a and IgM^b populations.

4.15 Discussion

The present study was undertaken to exam the regulation of anti-dsDNA B-cells in mice transgenic for the R4A, V_H11, IgM^a heavy chain and the $V_\kappa1$ LC of an anti-dsDNA antibody. I observed that a subset of transgenic B-cells was targeted to anergy in the R4A-$C_\mu/V_\kappa1$ double transgenic mice. These B-cells were arrested in the periphery in an early stage of development and displayed reduced expression of their $sIgM^a$ receptor. In addition, they were functionally anergic as they were unable to secrete transgenic anti-dsDNA antibody upon BCR cross-linking. Furthermore, these B-cells exhibited reduced calcium flux upon BCR cross-linking with anti-IgM antibody. Similarly, a subset of B-cells targeted to anergy was previously observed in mice transgenic for the R4A-C_μ heavy chain only (51). One major difference between anergic B-cells in the R4A-C_μ mice and those in the R4A-$C_\mu/V_\kappa1$ mice was that at least some anergic B cells in R4A-C_μ mice could be activated to secrete anti-dsDNA antibody following incubation with stimulatory CpG sequences or LPS while anergic B-cells from R4A-$C_\mu/V_\kappa1$ mice could not. This suggests that the anergic B-cells responsive to innate immune signaling in the R4A-C_μ mice were not ones that utilized the $V_\kappa1$ LC as in the R4A-$C_\mu/V_\kappa1$ mice and were therefore likely to be less stringently regulated.

I observed that the transgenic T1 compartment was significantly expanded in R4A-$C_\mu/V_\kappa1$ mice relative to wild type mice suggesting that the transgenic B-cells were arrested in development at this stage. Developmental arrest at an immature stage is a feature of anergic B-cells (49). This suggested that the HC transgenic B-cells may be targeted to anergy in the T1 stage of development. Other evidence consistent with anergy of the transgenic T1 B-cells was that they displayed diminished Ca^{2+} flux, relative to T1

B-cells from WT mice. In addition, the IgM^a T1 B-cells showed reduced Ca^{2+} flux relative to IgM^a T2 B-cells in the double transgenic mice, while in WT mice both T1 and T2 B-cells showed comparable Ca^{2+} flux (71). These results led us to conclude that T1 might not only represent a stage of B-cell development, but might also represent a regulatory checkpoint whereby anti-dsDNA B-cells are first anergized and subsequently eliminated since anergic B-cells have a shortened lifespan.

Although most IgM^a B-cells were arrested in the T1 stage, some were observed to progress through the T2 and T3 stages and become mature B-cells. I initially hypothesized, that the only B-cells that progressed through development were those that had lost specificity for dsDNA by editing their LC. Since B-cells utilizing the R4A-C_μ HC and the $V_\kappa 1$ LC have specificity for dsDNA, I assumed that these autoreactive B-cells were anergized in T1. However, I did not observe evidence of LC editing, such as, use of a downstream J_κ gene segment in the more mature IgM^a HC B-cells. In fact the frequency of dsDNA binding B-cells utilizing the IgM^a HC and the $V_k 1$ LC seemed to remain relatively constant among the B-cells that had escaped T1 and progressed to the T2 and more mature stages of development, although the absolute number of IgM^a B cells decreased.

As B cells passed through the transitional stage of development, many seemed to express both the IgM^a HC transgene and the endogenous IgM^b HC. The frequency of these dual HC expressing B-cells increased from T1 to T2 to T3 and was also high in MZ B-cells. The expression of a second heavy chain on the IgM^a B-cells may have facilitated the escape of these B cells from regulation in T1 because it diluted the level of expression of the autoreactive receptor. In addition, the frequency of B-cells expressing IgM^b only,

increased as B-cells became more mature. This increase may have been a result of deletion of the HC transgene due to intrachromosomal homologous recombination between the several copies of the transgene followed by expansion of these B cells. Since the R4A HC transgene is not targeted in the HC locus, it cannot undergo successful receptor editing by V gene replacement. However, it has been postulated that deletion of the transgene by intrachromosomal recombination may occur as a consequence of editing in an attempt to avert autoreactivity. Following deletion of the transgene, endogenous HCs can undergo rearrangement. This has previously been reported to occur by Chen et al. in another conventional transgenic model where mice were made transgenic for the non targeted 56R HC transgene and the Vk8 LC transgene (72). Like our R4A transgene, the 56R transgene was present as several copies in the genome. In the 56R/Vκ8 mice, examination of non dsDNA binding hybridomas revealed complete or partial deletion of the transgenic heavy chain by homologous recombination. However, in R4A/Vκ1 mice, the IgM^a transgene was still present in the genome of IgM^b B cells suggesting that it had not been deleted by intrachromosomal recombination although I could not exclude the possibility that some copies of the transgene were deleted. Interestingly, I observed that IgM^a mRNA was expressed at low levels in IgM^b B cells suggesting that the dual HC expressing B-cells were precursors of the IgM^b B-cells and as the dual expressers matured, they downregulated expression of the autoreactive transgene and up regulate expression of IgM^b.

The expression of more than one heavy or light chain in any given B-cell, also known as allelic inclusion has been observed in several other mouse models (67, 68, 73, 74). It has been proposed that allelic inclusion is actually a form of receptor

editing, and not a result of incomplete allelic exclusion (67). Generally in receptor editing an autoreactive V gene is replaced by a non autoreactive V gene which in effect eliminates the autoreactive receptor. In allelic inclusion, however, the autoreactive V gene is retained and is expressed along with a 2^{nd} V gene that has been productively rearranged on another allele. Casellas et al observed that under physiological conditions, editing occurs by deleting the autoreactive V gene or allelic inclusion at equal probability (67). Li et al observed that mice transgenic for the 56R HC of a high affinity anti-dsDNA antibody have a relatively high frequency of MZ B-cells expressing both a κ and λ LC (69). Autoreactive B-cells expressing dual receptors may be retained in the repertoire because they have a weaker avidity for the self antigen since the expression levels of the self reactive receptor are diluted by the expression levels of the non autoreactive receptor.

Chen et al. observed in 56R HC transgenic mice, that in addition to B-cells that had lost expression of the transgenic HC, some B-cells were present that expressed both the V_H transgene and endogenous V_H mRNA (72). However, the transgenic mRNA was present at a greatly reduced level and it was thought that the endogenous Ig RNA was silencing the expression of the transgene. Heavy chain allelic inclusion has also been demonstrated in site directed knock in mice transgenic for the HK171 heavy chain of an anti-arsonate antibody (75). Dual heavy chain expressing B-cells were found to predominate in the MZ of these mice. Interestingly, the HK165 HC transgene which differs from HK171 by only eight amino acids was found to promote efficient allelic exclusion. It was postulated that the structure of the VH domains, the specificity of the BCR encoded by the HC, and the level of surface expression of the BCR affects allelic exclusion. Li and Eckhardt also observed evidence of HC allelic inclusion in mice

lacking the Eμ heavy chain intronic enhancer (73). They observed that in these mice, IgM levels were at or below the threshold for effective positive selection and only B-cells expressing two heavy chain alleles, or B-cells with strong enough signaling strength would have a selective advantage for survival.

In most mouse models where allelic inclusion has been studied, it is thought that receptor editing in the bone marrow is responsible for the development of dual receptor expressing B-cells. However, in some of these mouse models such as in the site directed 56R HC transgenic mice, a high frequency of dual expressing B-cells is not seen in the bone marrow but only in the spleen (69). It is hypothesized that a small subset of allelically included B-cells in the bone marrow are expanded in the periphery. R4A-$C_\mu/V_\kappa 1$ mice do not appear to have any dual expressing B-cells in the bone marrow although there is a relatively high frequency in the spleen. While I cannot exclude the possibility that a small fraction of dual expressing B-cells are present in the bone marrow of these mice and are subsequently expanded in the periphery, I hypothesize that HC editing may actually be occurring in the periphery of R4A-$C_\mu/V_\kappa 1$ mice by rearranging a VH gene at the endogenous locus. Rice et al. demonstrated receptor editing in peripheral B-cells of mice immunized with a peptide mimetope of dsDNA (76). They observed RAG 2 expression in mature B-cells in response to the peptide mimetope, which correlated with co-expression of kappa and lambda LCs. Future studies will look for evidence of peripheral RAG expression to determine whether peripheral editing may be occurring in R4A-$C_\mu/_{V\kappa 1}$ mice.

In summary, I propose a model to explain the regulation of transgenic anti-dsDNA B-cells in the R4A-$C_\mu/V_\kappa 1$ mice (Figure 36). Transgenic anti-dsDNA B cells that

utilize the R4A-IgM^a heavy chain and the $V_\kappa 1$ light chain become arrested in the T1 stage of development when they exit the bone marrow and are subsequently anergized. Some IgM^a B-cells may undergo heavy chain receptor editing and rearrange the endogenous chromosome in an attempt to avert autoreactivity. As a consequence of editing some B-cells may express both IgM^a and IgM^b. Others may delete the HC transgenes altogether by intrachromosomal recombination. Co-expression of a 2nd Ig heavy chain may enable dual receptor expressing B-cells to escape the regulatory checkpoint in T1 and enter T2 and subsequently mature. Some of the dual receptor expressing B-cells may become marginal zone B-cells and others may downregulate expression of IgMa and express IgM^b only. The dual receptor expressing B-cells may have diminished autoreactivity if the level of membrane expression of the transgenic heavy chain is low relative to the level of expression of the endogenous heavy chain. Subsequent down-regulation of the transgenic heavy chain as the B-cell matures in the periphery may be a novel mechanism for averting autoreactivity.

FIGURES

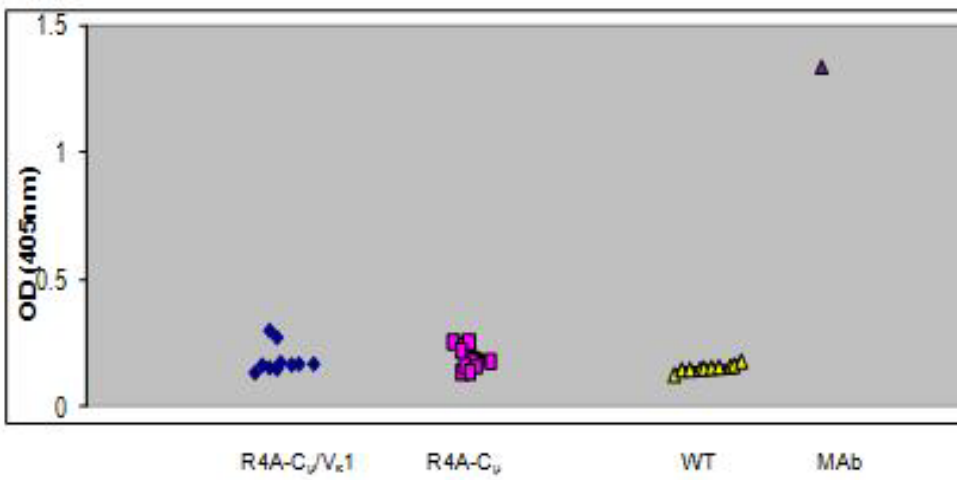


Figure 9. Tolerance is maintained in R4A-$C_\mu/V_\kappa 1$ mice.

Transgenic anti-dsDNA B-cells fail to secrete antibody in R4A-$C_\mu/V_\kappa 1$ mice. There is no significant difference in serum anti- dsDNA antibody content of R4A-$C_\mu/V_\kappa 1$ mice compared to R4A-C_μ heavy chain only transgenic animals (R4A-$C_\mu/V_\kappa 1$ vs: R4A-C_μ $p = 0.714$). IgM^a anti-dsDNA monoclonal antibody is the positive control ($n = 10$).

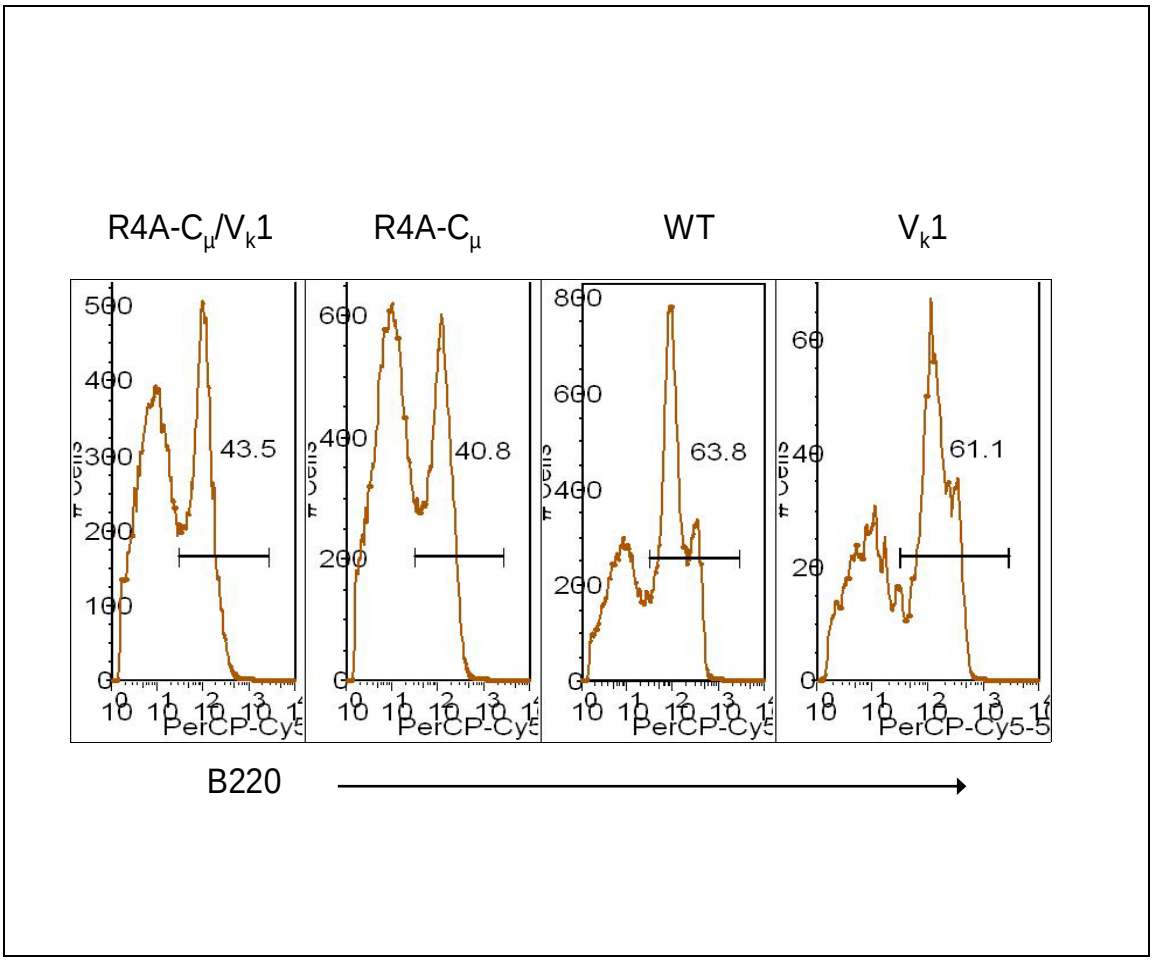


Figure 10. Reduced frequency of bone marrow B-cells in R4A-$C_\mu/V_\kappa1$ as well as R4A-C_μ mice relative to wild type and $V_\kappa1$ mice.

Bone marrow (BM) B-cells were immunostained with antibody to B220, gates were set on lymphocytes, immature B-cells ($B220^{low}$), mature B-cells ($B220^{high}$) in R4A-$C_\mu/V_\kappa1$ R4A-$C_\mu$wild type (WT) and $V_\kappa1$mice (n=4).

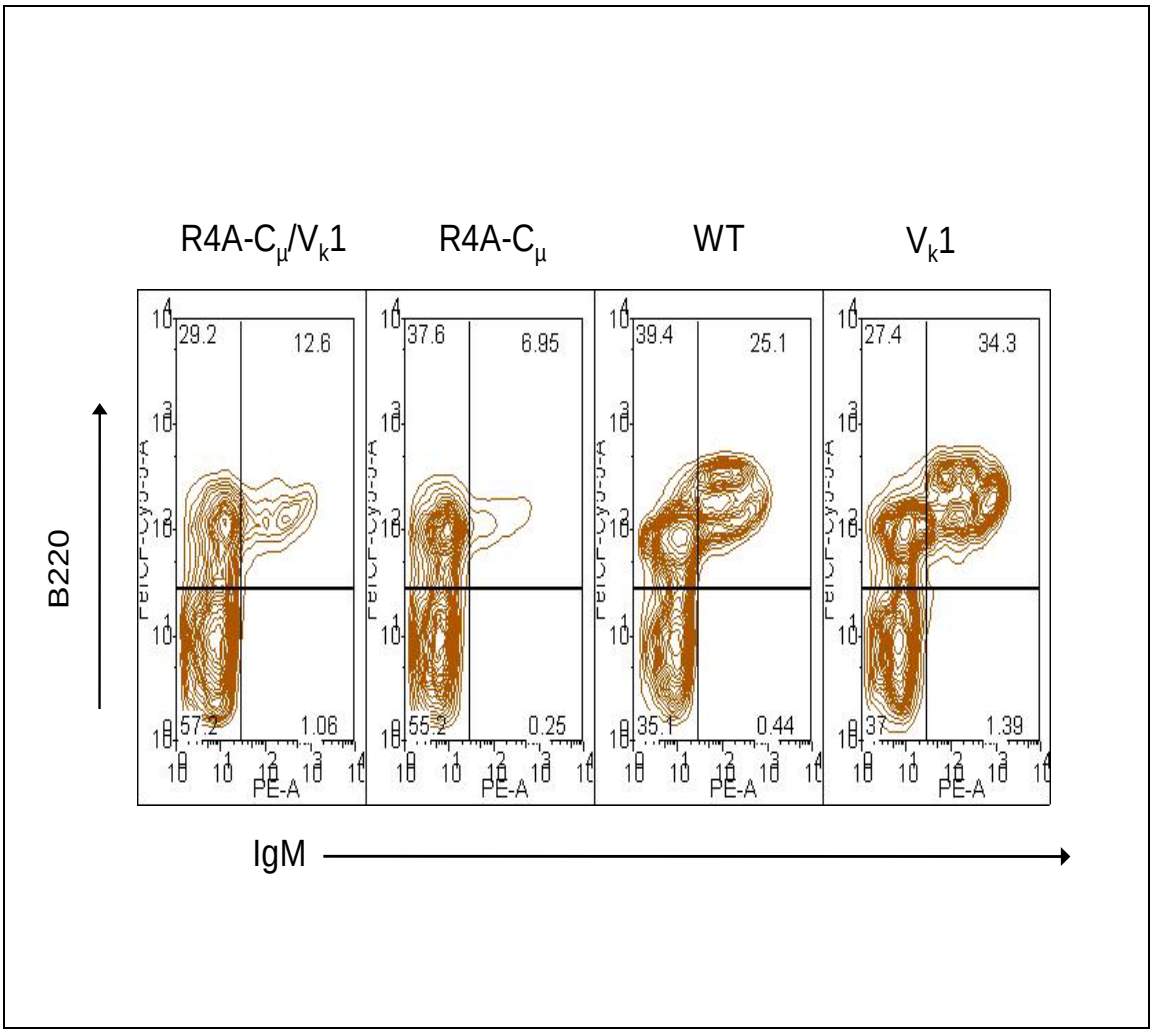


Figure 11. Reduced Frequency of total IgM B-cells in the bone marrow of R4A-$C_\mu/V_\kappa 1$ mice relative to wild type, bone marrow (BM) B-cells were immunostained with antibody to B220, and IgM. Gated on lymphocytes(n= 3).

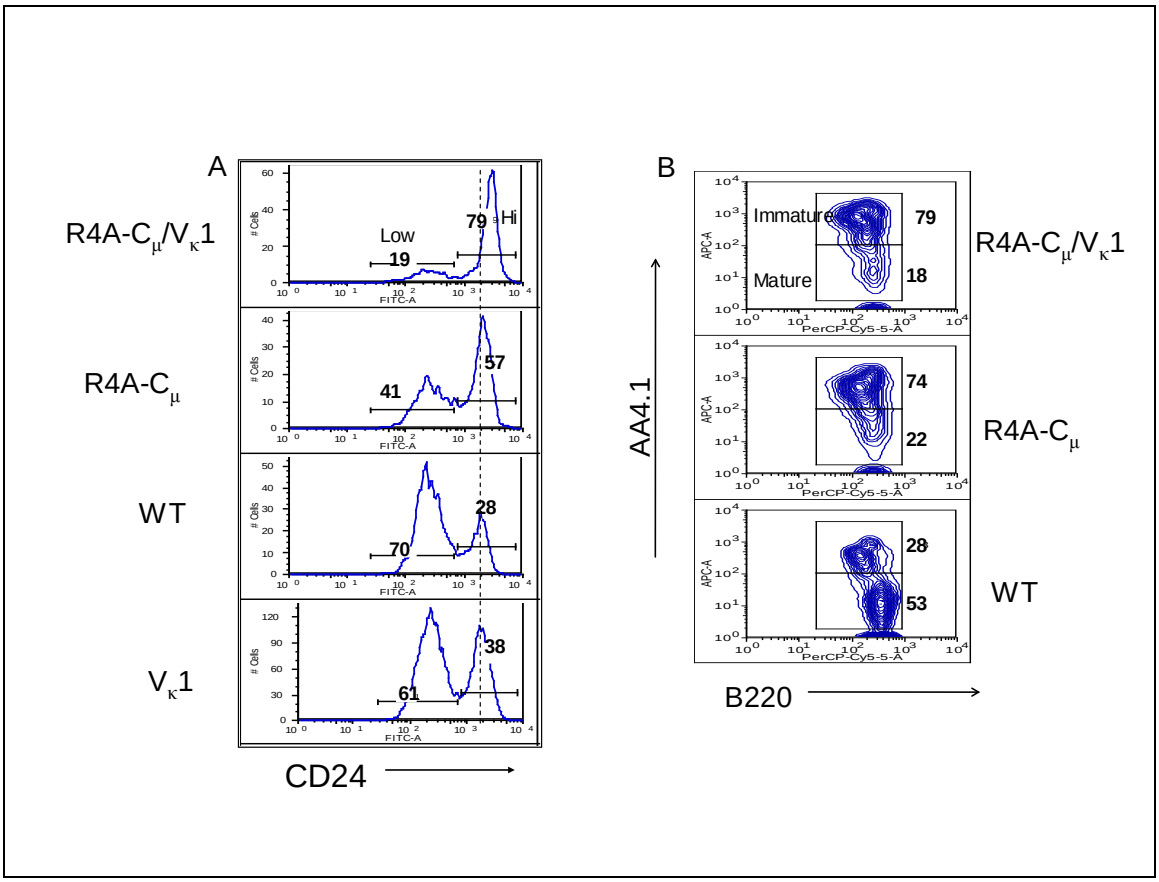


Figure 12. High frequency of IgM B-cells in the bone marrow of R4A-$C_\mu/V_\kappa1$ mice are immature.

(A). Histographs display $CD24^{high}$ (immature) and $CD24^{low}$ (mature) B-cells in the bone marrow. Bone marrow B-cells were immunostained with antibodies to B220, CD24 and IgM^a or IgM^b. Gates were set B220 IgM^a (R4A-$C_\mu/V_\kappa1$ and R4A-Cu) or B220 IgM^b (WT and Vk1 mice) (n= 5). (**B**) Contour of immature ($AA4.1^+$) B-cells and mature ($AA4.1^-$) B-cells in R4A-$C_\mu/V_\kappa1$, and R4A-C_μ and wild type (WT) and $V_\kappa1$mice. Cells were immunostained with antibodies to B220, AA4.1 and IgM^a or IgM^b B-cells. Gates were set on B220 IgM^a or B220 IgM^b B-cells (n=2).

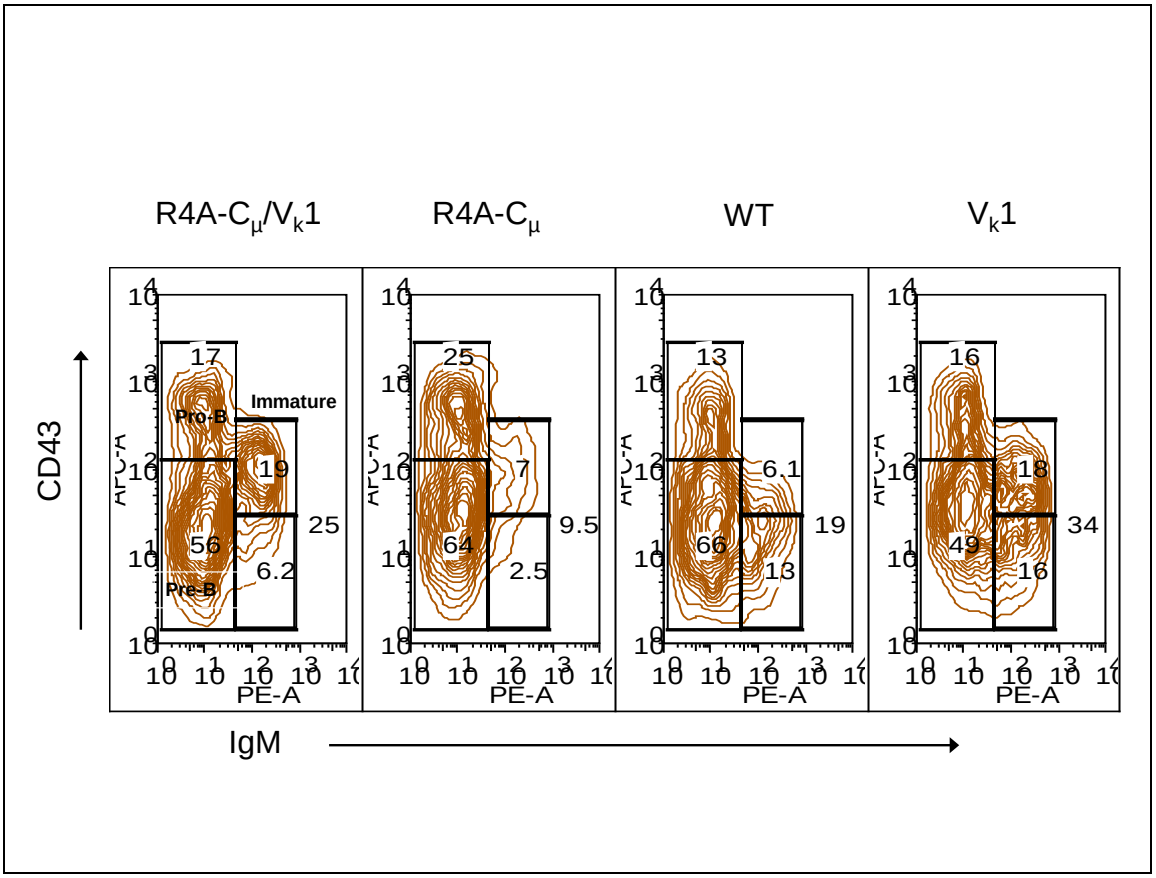


Figure 13. Developmental stages of B-cells in the BM of transgenic mice.

BM cells were immunostained with B220, IgM and CD43. Gates were set on $B220^{+}$ cells, Pro-B ($CD43^{high}$ IgM^{-}), pre- B ($CD43^{low}$ IgM^{-}) and immature/IgM B-cells ($CD43^{-}$ IgM^{+}) (n =3).

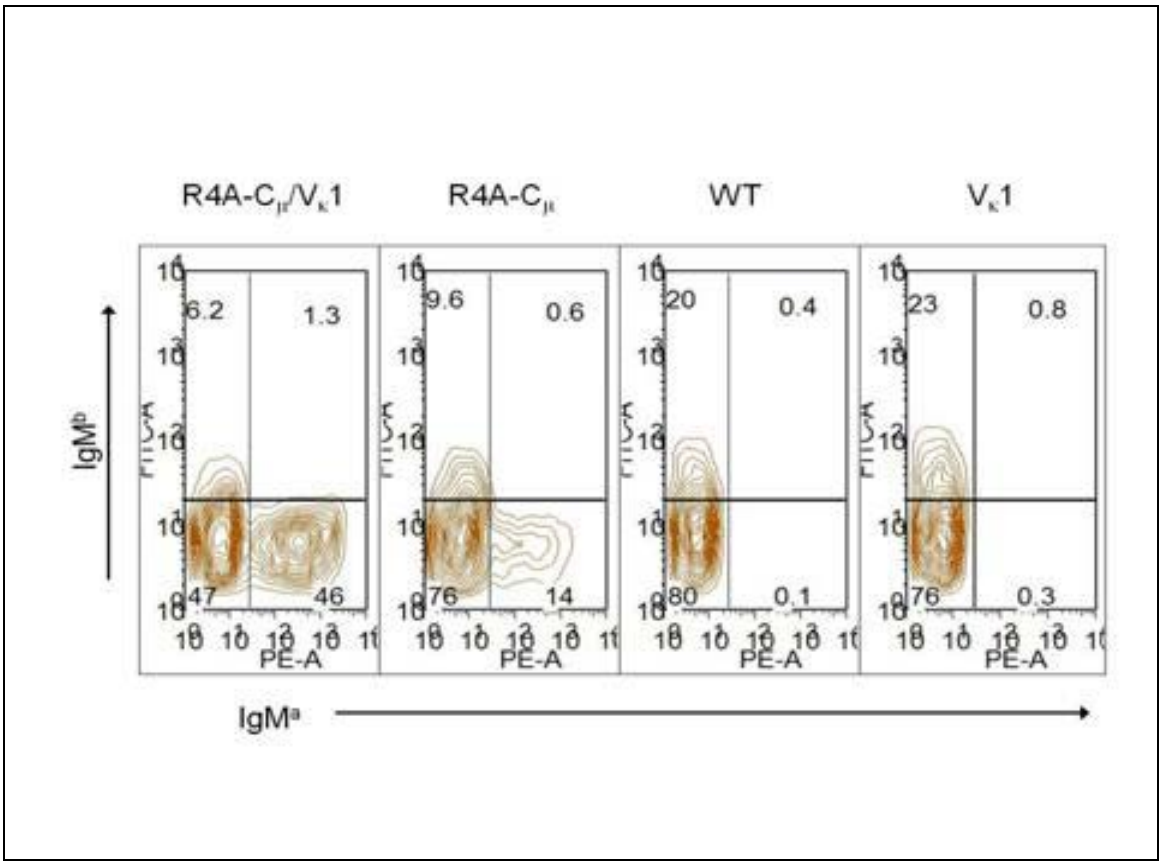


Figure 14. Majority of bone marrow B-cells express the IgM^a transgene.

BM cells were inmmunostained with B220, IgM^a, and IgM^b. Lymphocyte gated then B220 gated. Allelic exclusion in the BM of R4A-$C_\mu/V_\kappa1$ mice similar to that of WT mice are controls (n=4).

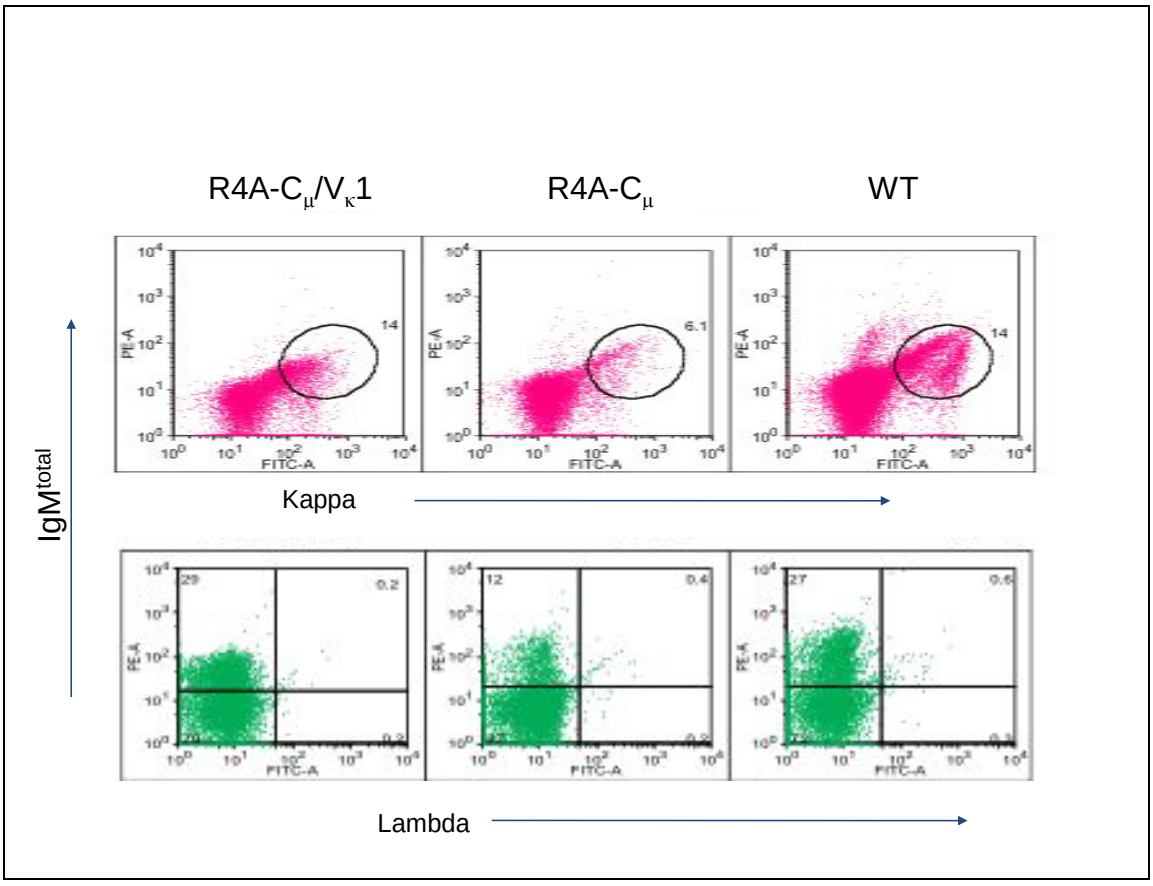


Figure 15. Majority of bone marrow B-cells express kappa light chains.

Bone marrow B-cells were inmmunostained with antibodies to B220, IgM and the kappa light chain or lambda light chain. Gate set on $B220^{+}$ B-cells (n=3).

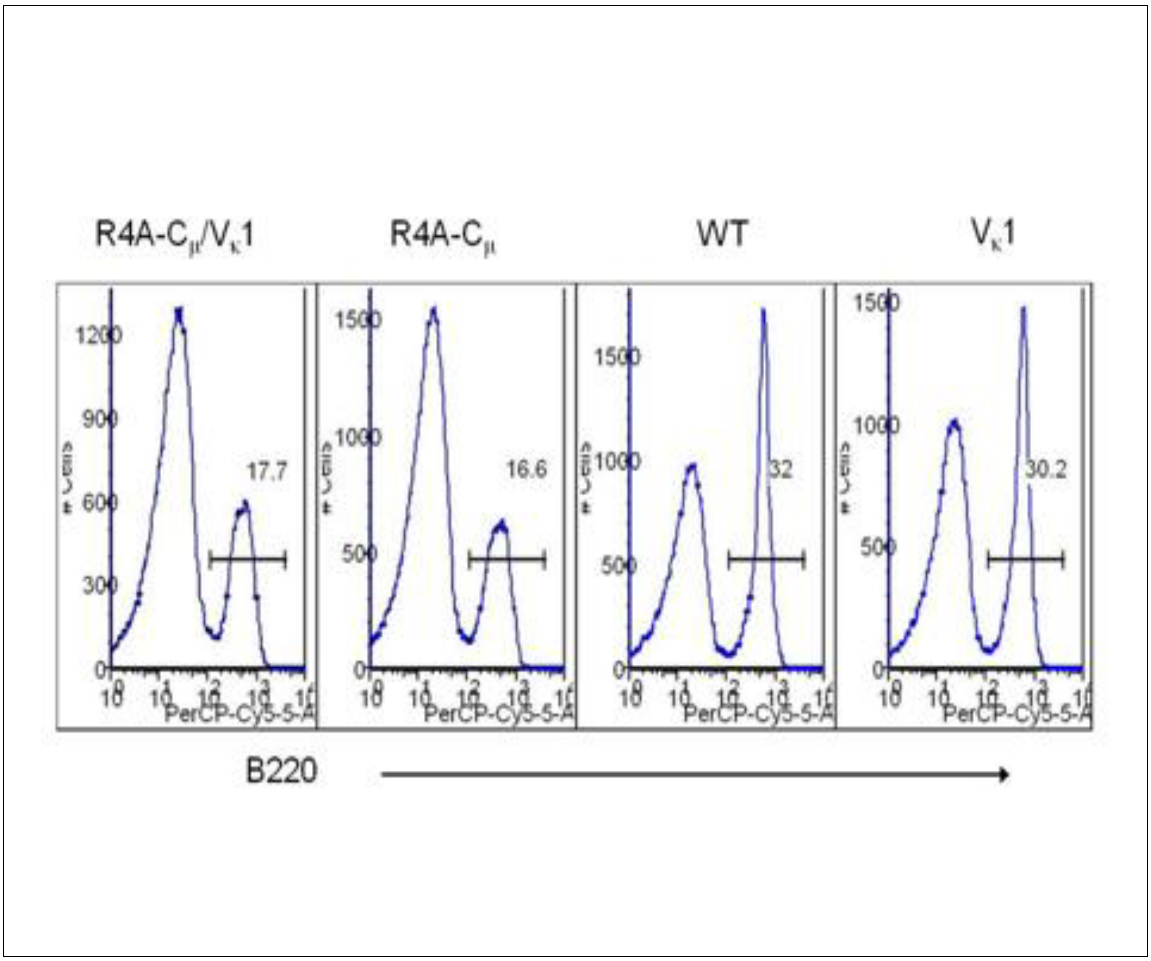


Figure 16. Reduced frequency of splenic B-cells in R4A-C_μ/V_κ1 relative to wild type mice, splenic B-cells were immunostained with antibody to B220, gate set on lymphocytes (n=4).

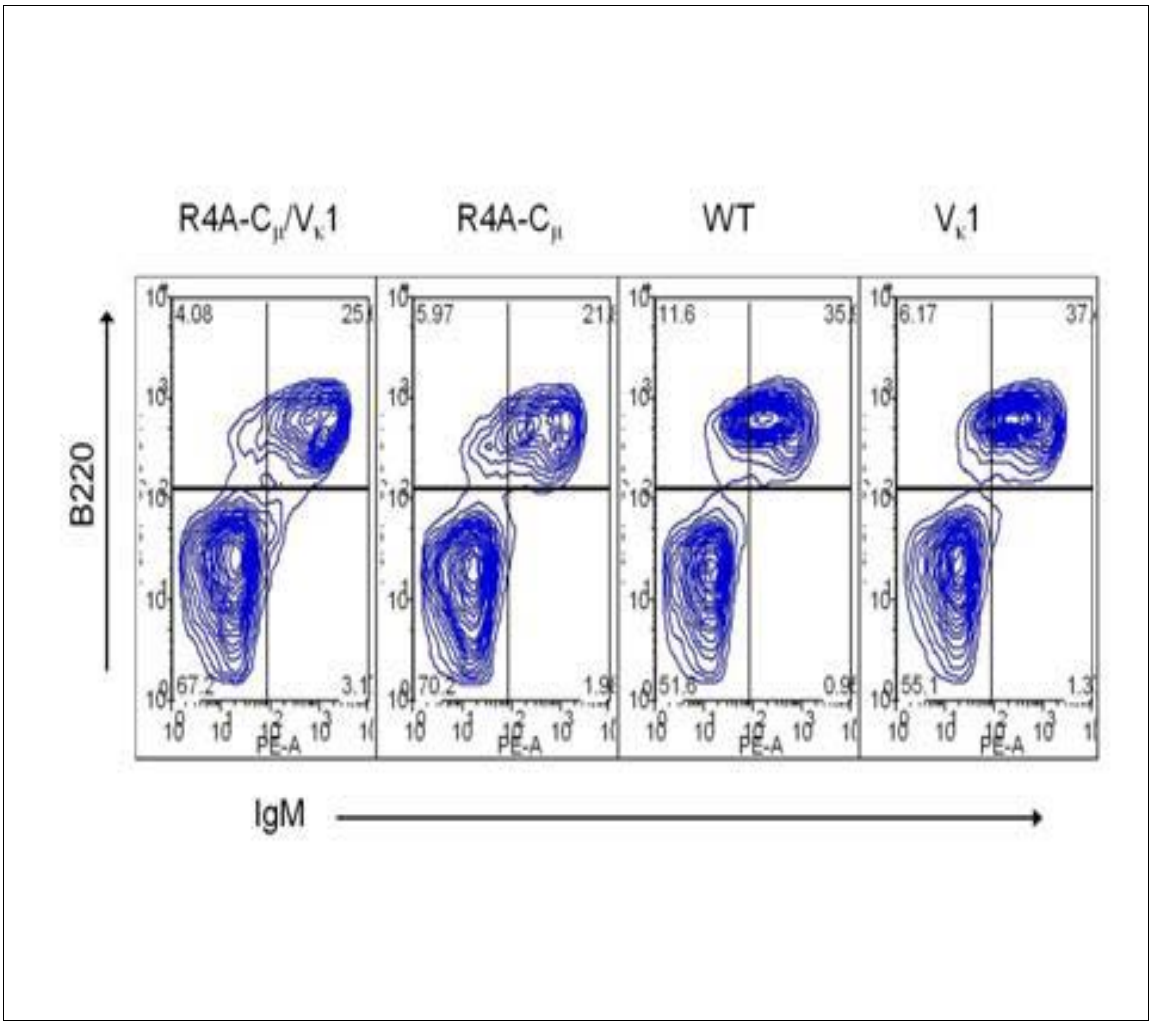


Figure 17. Reduction in the frequency of splenic IgM B-cells in R4A-C_μ/V_κ1mice. Splenic B-cell from R4A-C_μ/V_κ1, R4A-C_μ, WT and V_κ1 mice were immunostined with antibodies to B220 and IgM and were analyzed by flow cytometry (n=3).

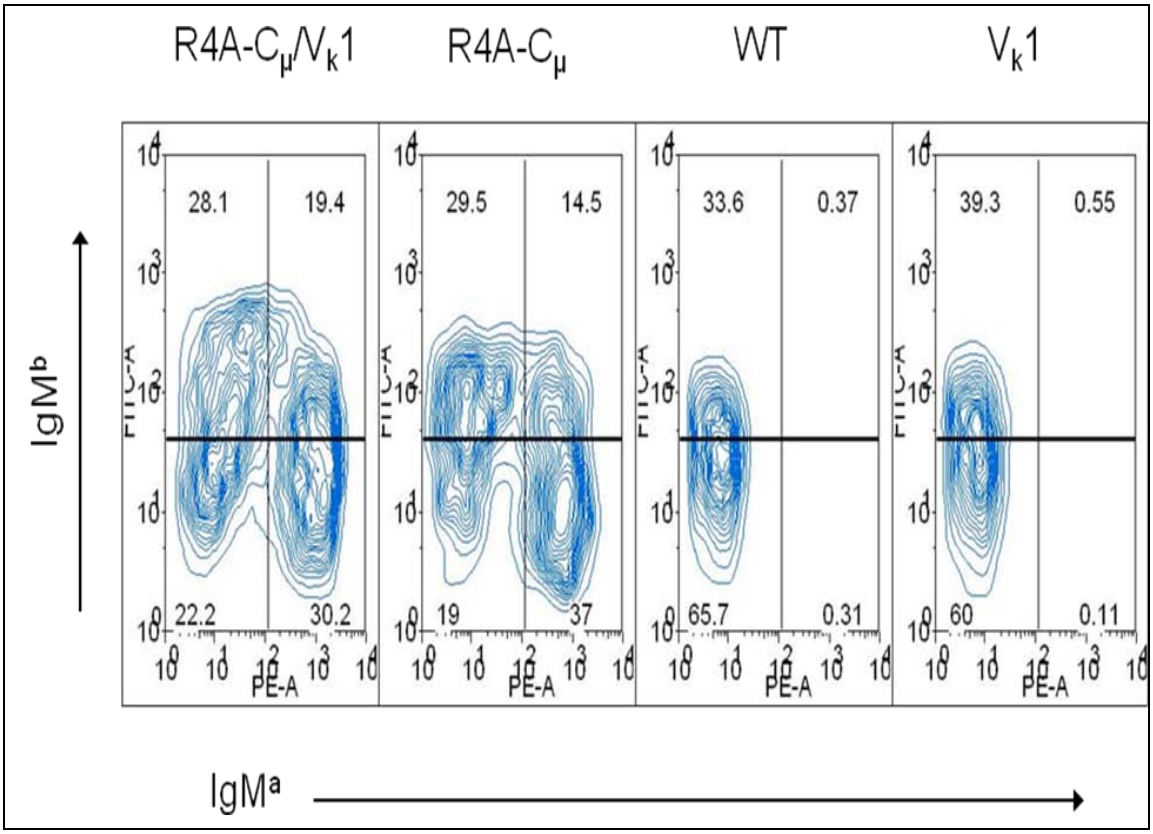


Figure 18. Frequency of splenic B-cells expressing the IgM^a transgene.

Splenic B-cells were immunostained with antibodies to B220, IgM^a and IgM^b. Gates were set on B220$^+$ B cells (n=4).

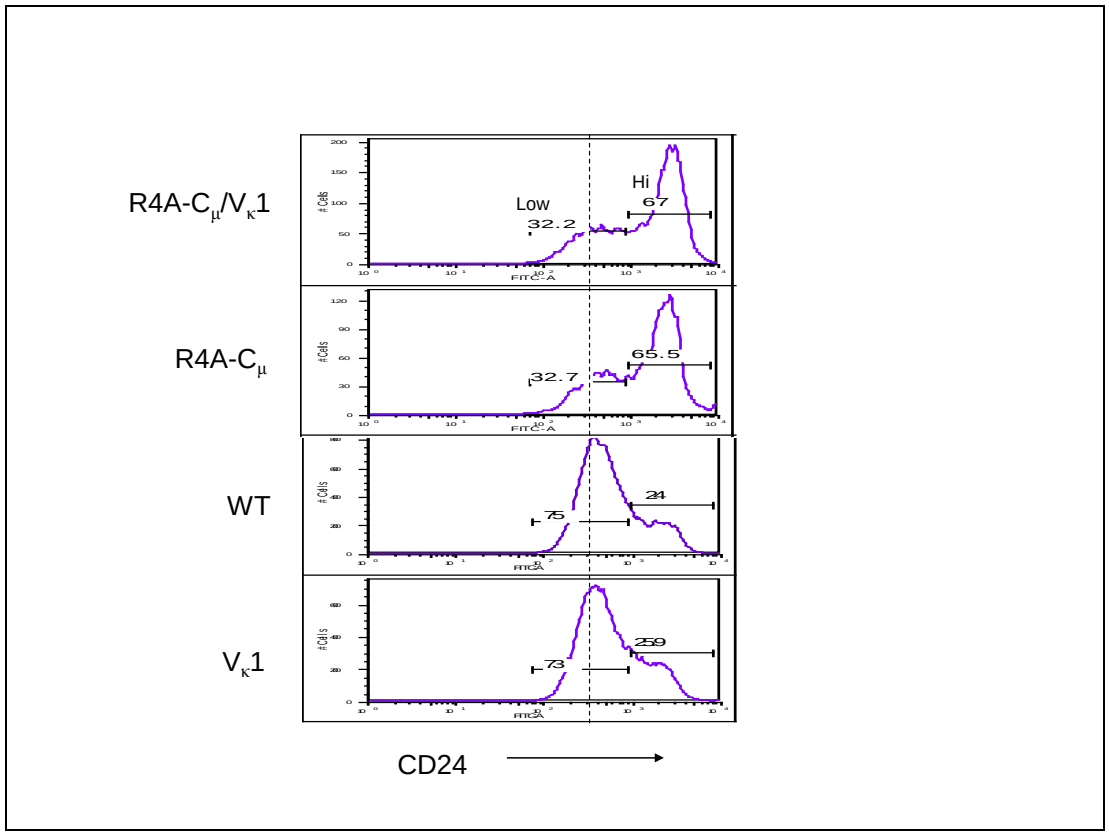


Figure 19. High frequency of transgenic B-cells are arrested in the immature/ transitional stage of development in the periphery, $CD24^{high}$ (immature), $CD24^{low}$ (mature). Gates set on B220 IgM^{a} for R4A-$C_{\mu}/V_{\kappa}1$ and R4A-C_{μ} mice or on B220 IgM^{b} for WT and $V_{\kappa}1$ mice (n=4).

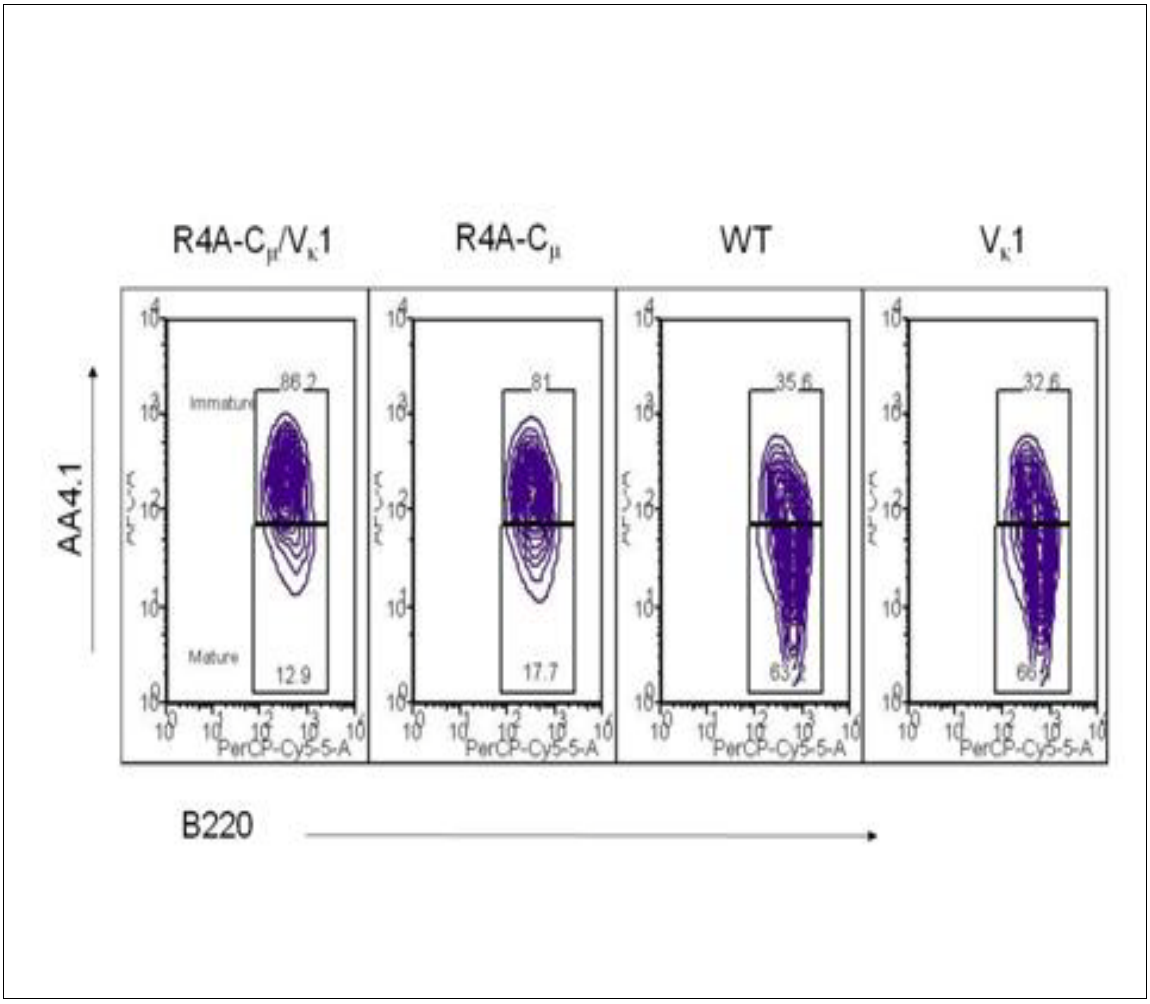


Figure 20. High frequency of transgenic B-cells are arrested in the immature /transitional stage of development in R4A-$C_\mu/V_\kappa1$ Mice.

Splenic B-cells were immunostained with antibodies to B220 and AA4.1 and IgM^a or IgM^b. Gates were set on B220 IgM^a for Tg, or B220 IgM^b for WT mice. $AA4.1^+$ (immature) $AA4.1^-$(mature) B-cells (n=3).

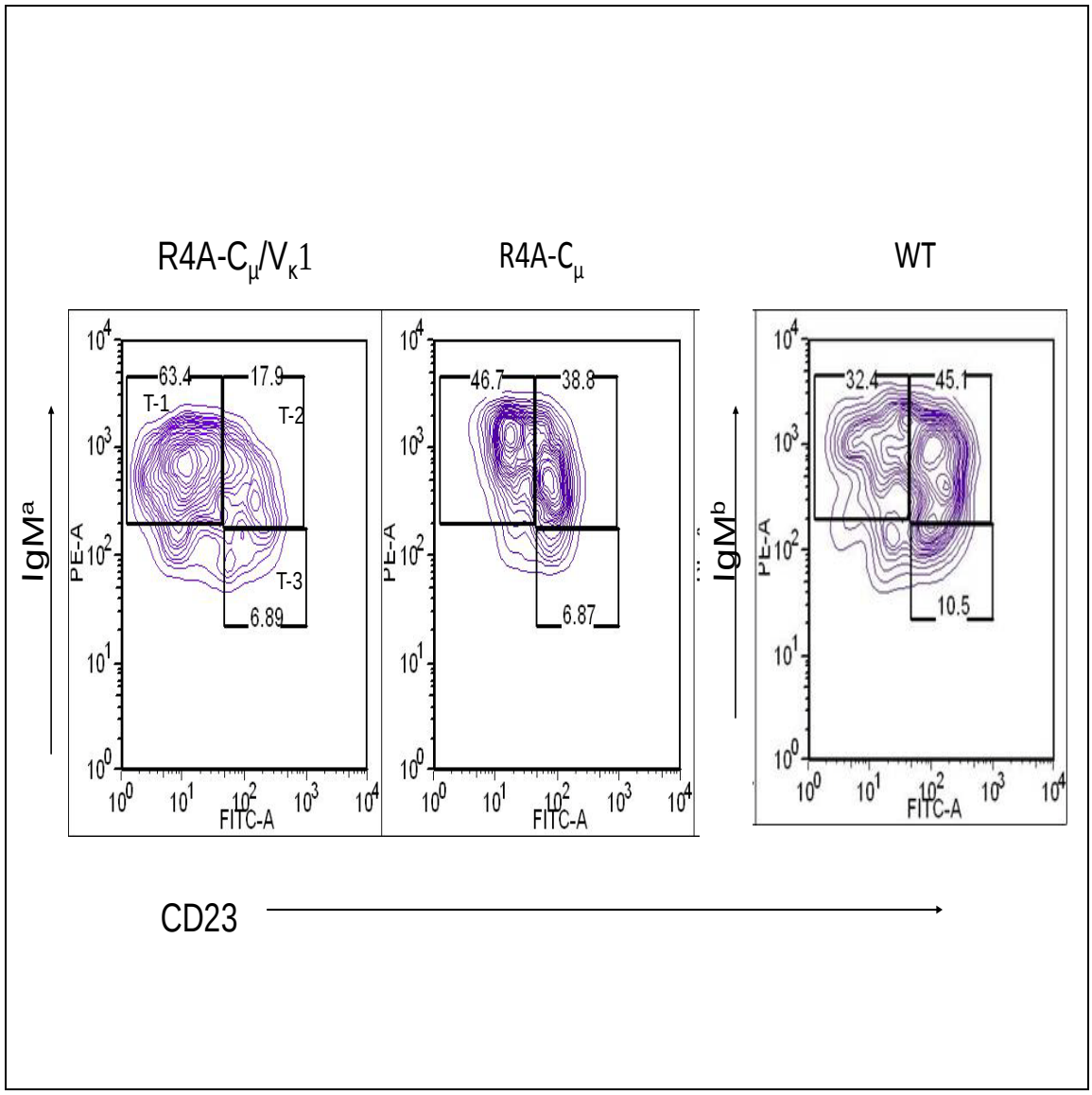


Figure 21. Expanded T1 compartment in R4A-$C_\mu/V_\kappa1$mice relative to wild type mice, splenic B-cells from R4A-C_μ, R4A-$C_\mu/V_\kappa1$, and WT mice were immunostained with antibodies toB220, AA4.1, IgM^a or IgM^b, and CD23.Gates were set on B220,$AA4.1^+$ IgM^a or IgM^b (n=4).

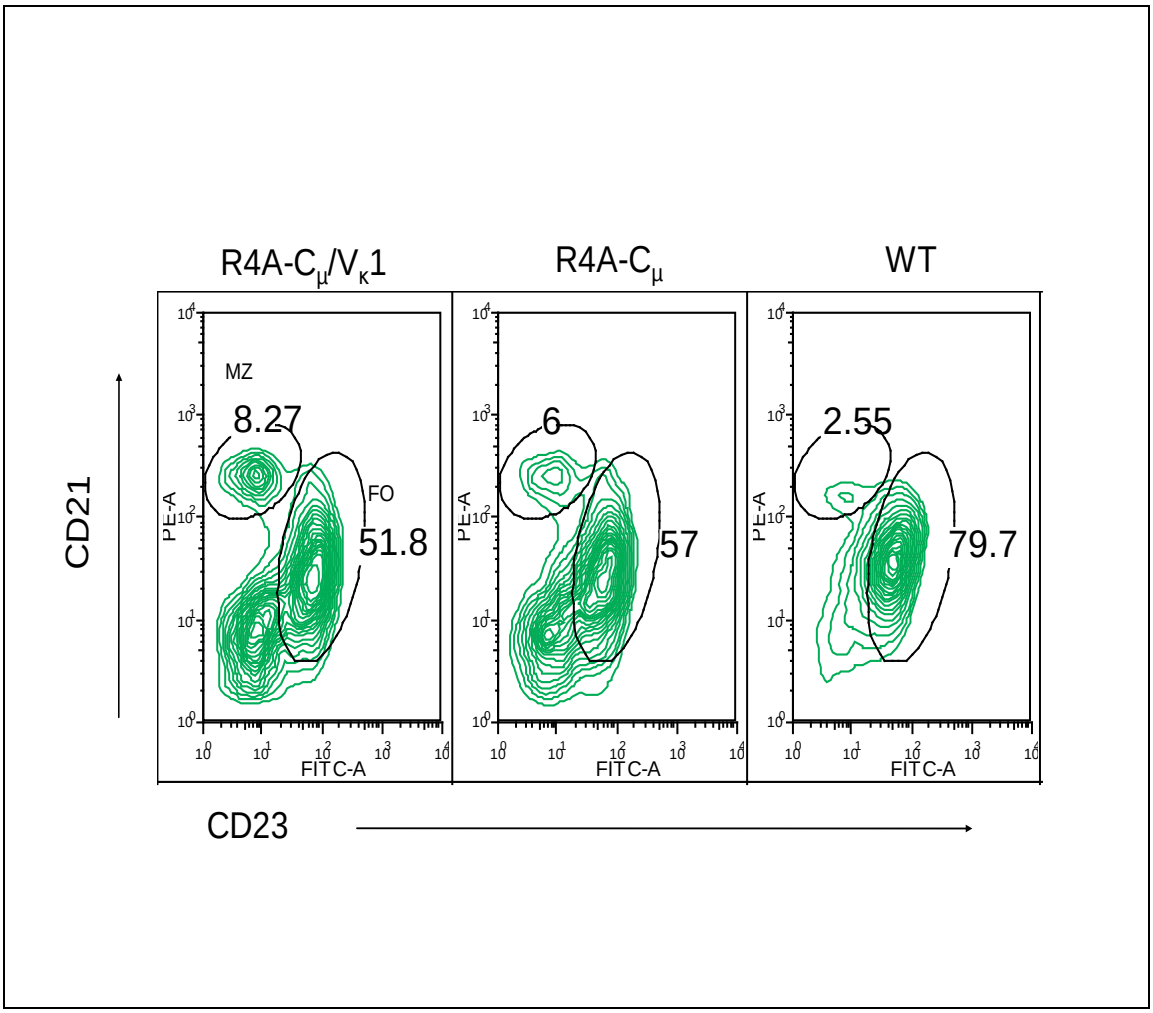


Figure 22. Increased frequency of marginal zone B-cells in R4A-$C_\mu/V_\kappa1$ mice.

Contour of mature B-cell populations in R4A-$C_\mu/V_\kappa1$, R4A-Cu and WT mice. Gates were set on B220 and IgM^a for transgenic mice, B220 and IgM^b for wild type mice (n=3).

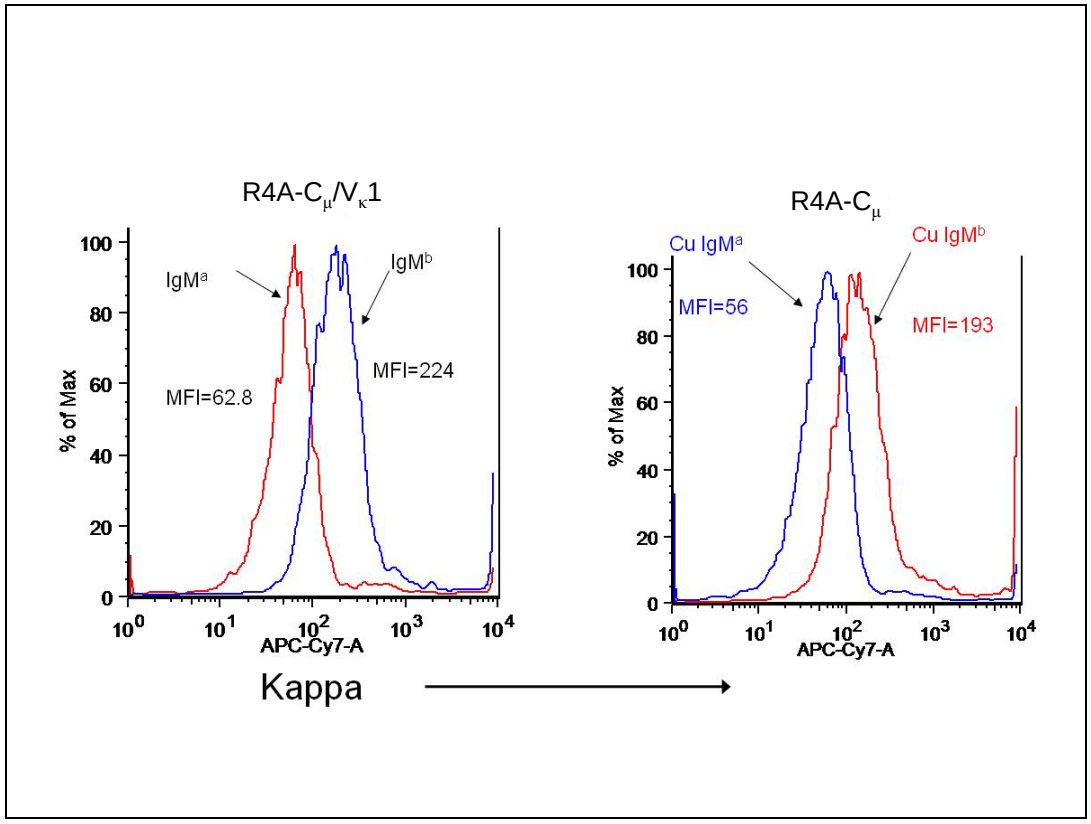


Figure 23. Receptor down regulation on transgenic B-cells.

The mean flouresence intensity (MFI) of sIgM on B-cells expressing IgM^a or IgM^b in R4A-$C_\mu/V_\kappa 1$ mice (n=3).

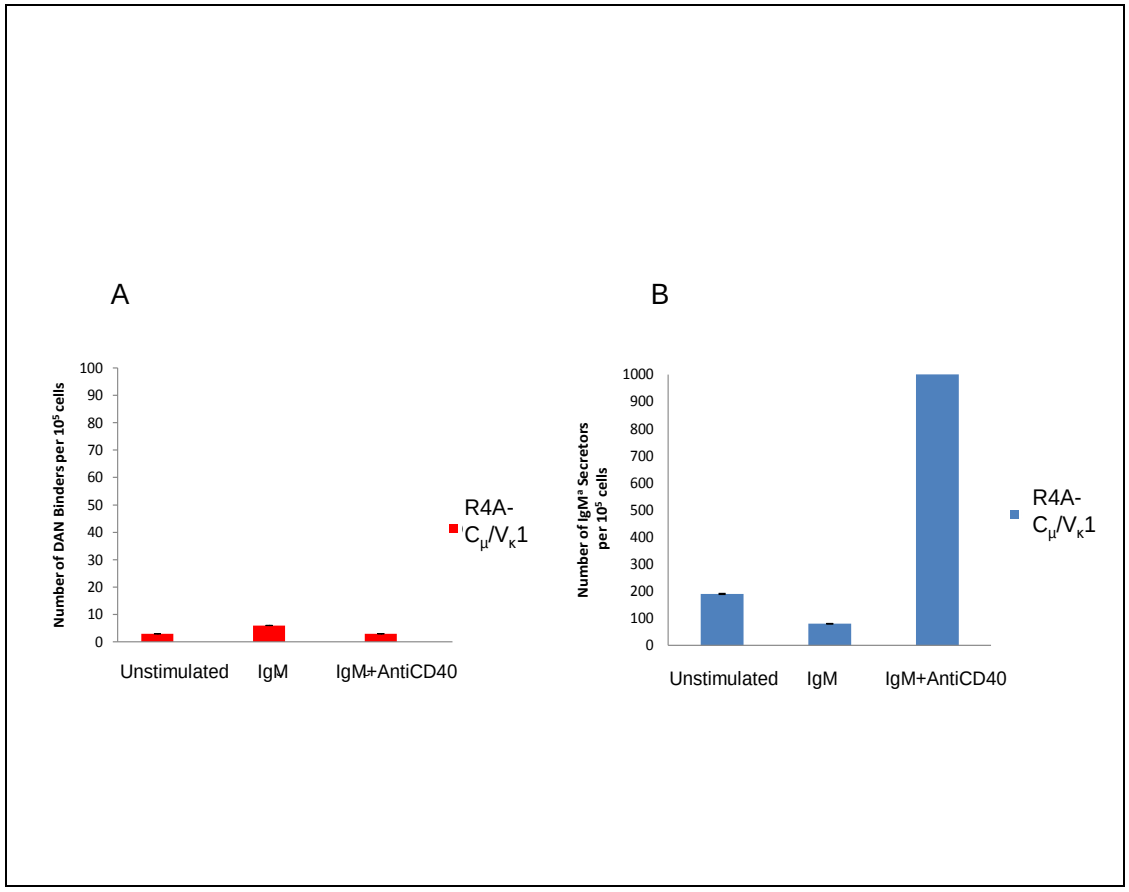


Figure 24. Transgenic ds-DNA binding B-cells in R4A-$C_\mu/V_\kappa1$ mice are not capable of being activated via their BCR to secrete anti-ds-DNA antibody.

(A). B-cells secreting IgM^a anti-dsDNA antibody were enumerated by ELISPOT.

(B). B-cells secreting total IgM^a antibody were enumerated by ELISPOT, (n=3).

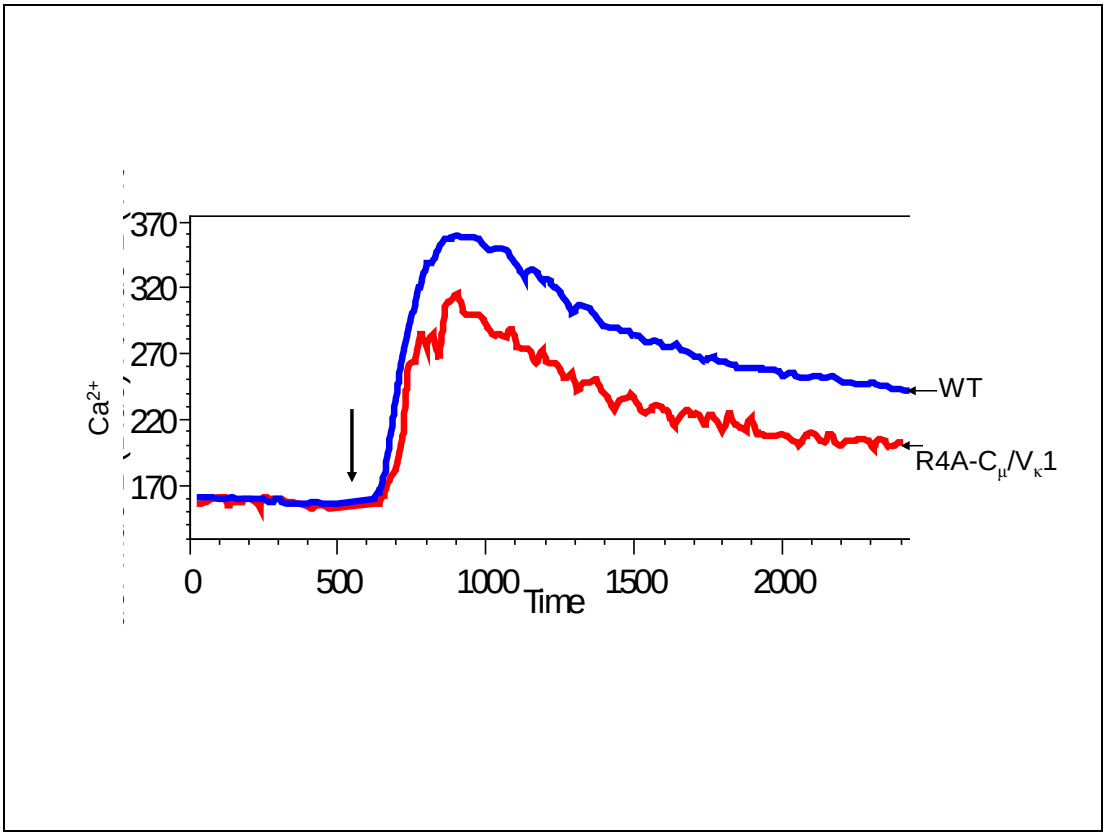


Figure 25. Reduced calcium flux in R4A-C_μ/V_κ1 IgM B-cells relative to wild type B-cells, IgM^a B-cells in R4A-C_μ/V_κ1 mice do not flux calcium as well as IgM^b B-cells from wild type mice. Gates were sets on B220 IgM^a or B220 IgM^b B-cells (n=3).

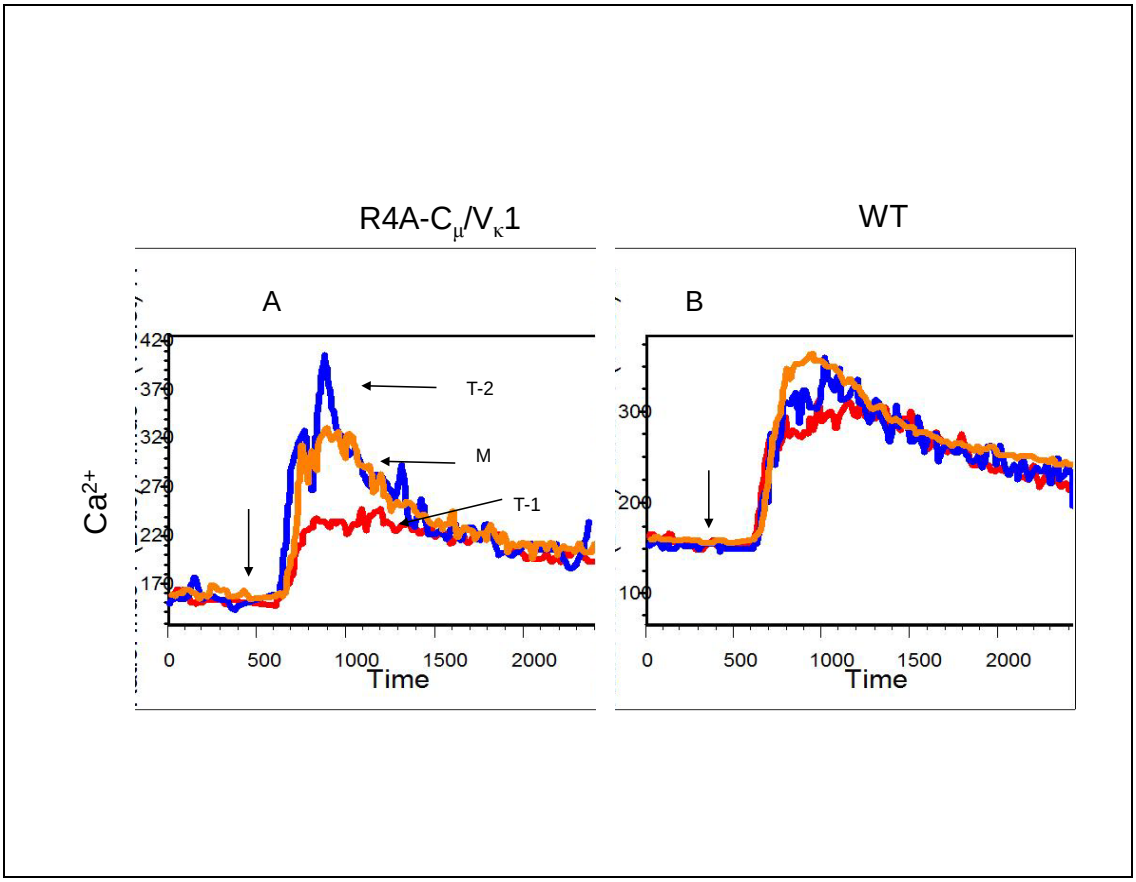


Figure 26. Reduced calclium flux in T-1 B-cells in R4A-$C_\mu/V_\kappa 1$ mice.

T1 B-cells (red), T2 B-cells (blue), and mature IgM^a B-cells (M) (orange) from R4A-$C_\mu/V_\kappa 1$ **(A)**, and the corresponding IgM^b population in WT **(B)** mice. The arrowhead indicates addition of stimulus (10 μg/ ml anti-IgM antibody) after 1 minute baseline reading; stimulus response taken for 4 minutes (n-3).

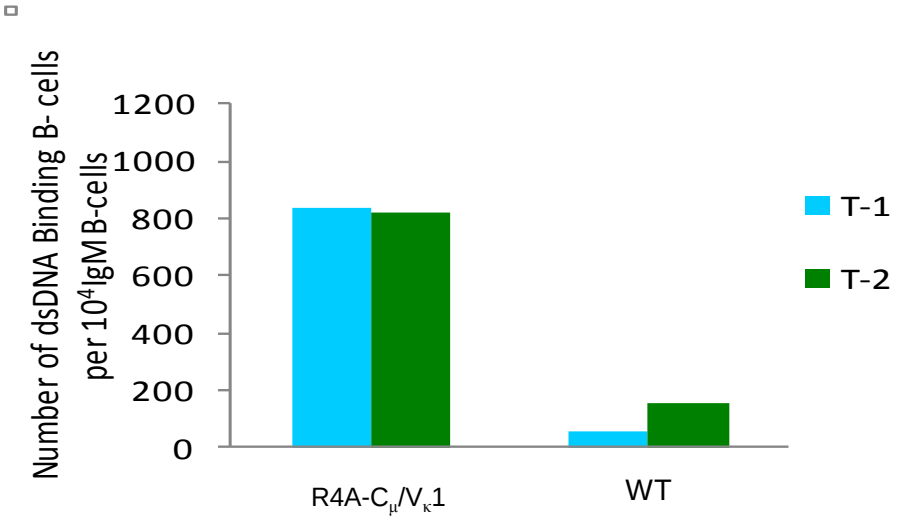
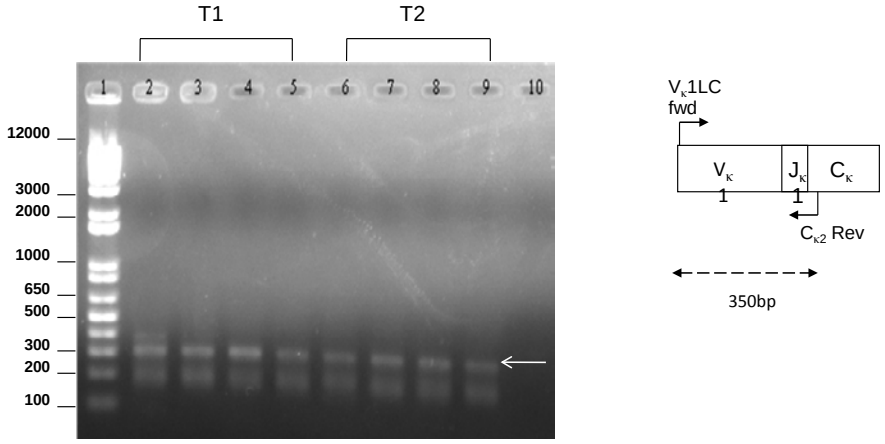


Figure 27. Increased number of anti-dsDNA secreting B-cells in the T1 and T2 subsets of R4A-$C_\mu/V_\kappa1$ mice relative to WT mice.

Splenic B-cells from R4A-$C_\mu/V_\kappa1$ mice were sorted by flow cytomety into T1 and T2 subsets. T1 and T2 B-cells were stimulated in vitro with 1ug/ml of CpG for 48hours and IgM^a anti-dsDNA B-cells were enumerated by ELISPOT (n=3).



Populations	T-1	T-2	Mature B-cells
Number of 350bp positive ($V_\kappa 1$)	8/10	10/10	9/10

Figure 28. Single cell RT-PCR of transgenic B-cell populations shows expression of rearranged $V_\kappa 1J_\kappa 1$ light chain transgene.

Lane 1- 1KB DNA ladder, lanes 2, 3, 4 and 5: T1 B-cells, lanes 6, 7, 8 & 9: T2 B-cells, lanes 11, 12, 13 mature B-cell, lane 9 & 11: negative controls (primers only) lane 10: positive control (R4A-$C_\mu/V_\kappa 1$ tail DNA).

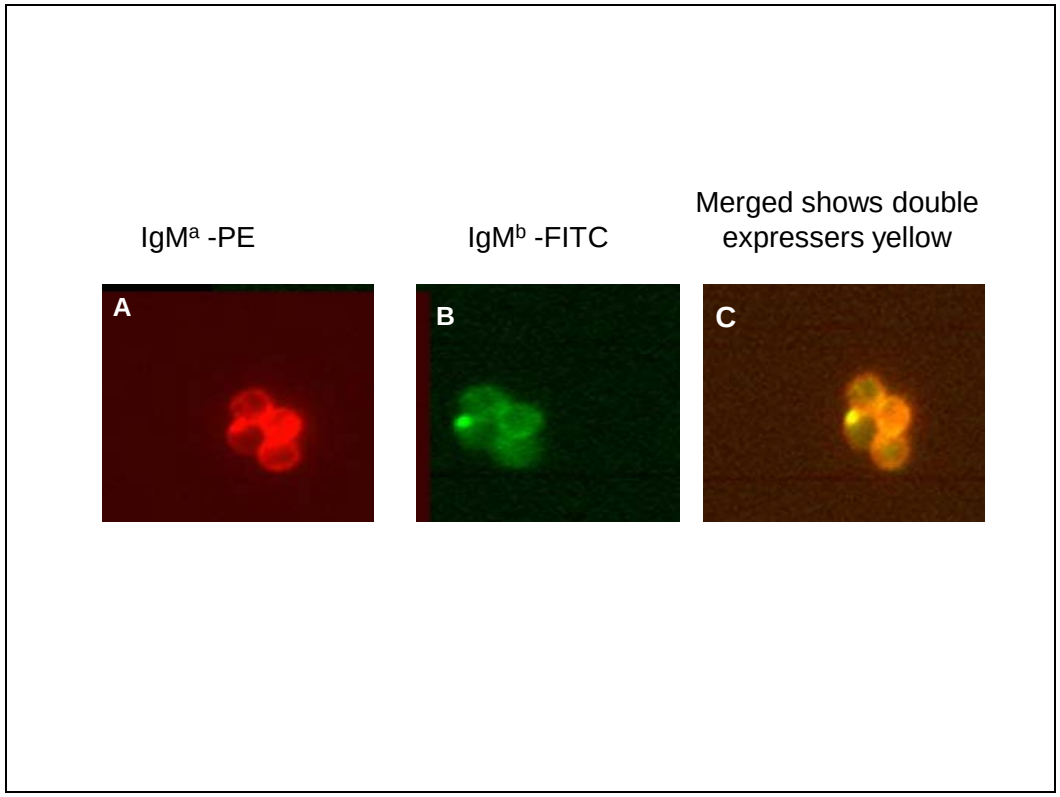


Figure 29. Dual receptor expression on transgenic B-cells.

Sorted IgM$^{a/b}$ B-cells from R4A-$C_{\mu}/V_{\kappa}1$ mice were immunostained with PE antibody to IgM^{a} and FITC antibody to IgM^{b}. These cells stained positively for both IgM^{a} (red), IgM^{b} (green). Cells were visualized using Nikon Eclipes TE 2000S Microscope, magnification 400X.

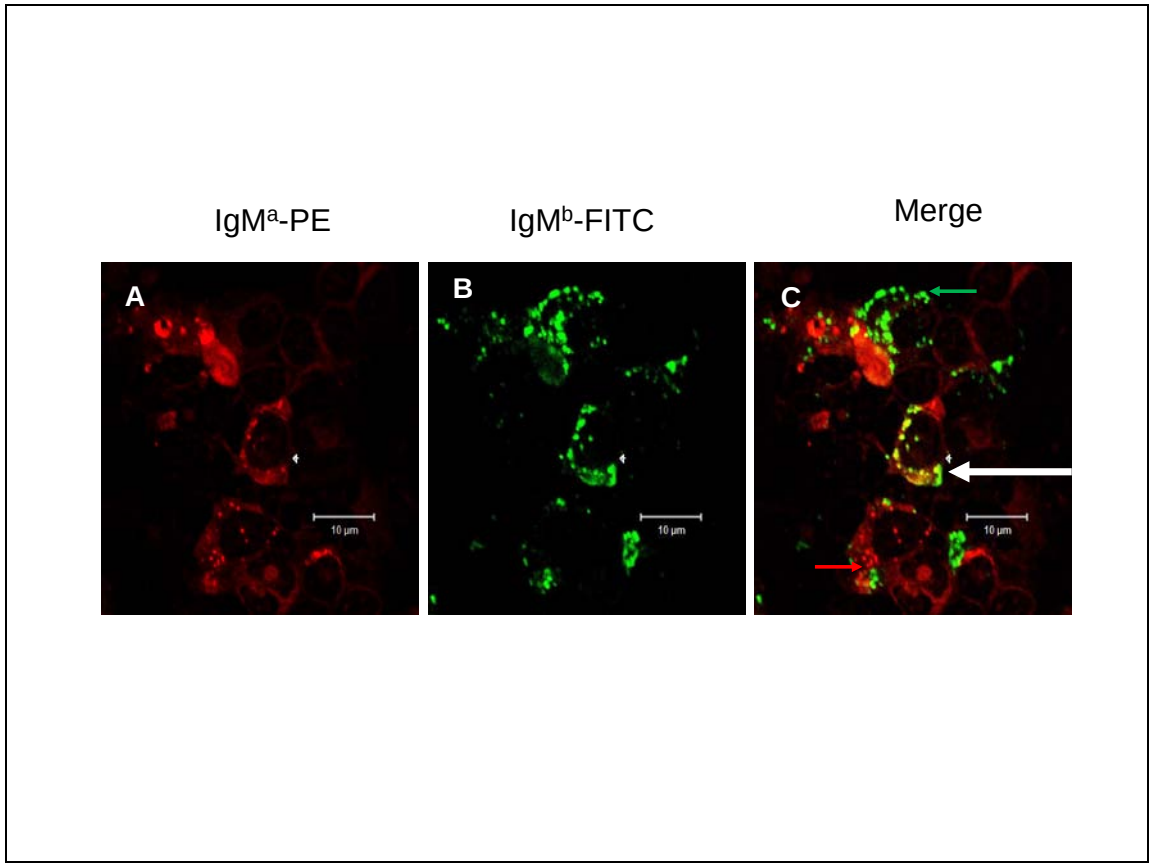


Figure 30. Dual receptor IgM$^{a/b}$ expressing cells among splenocytes in R4A-C$_{\mu}$/V$_{\kappa}$1 mice, total splenocytes from an R4A-C$_{\mu}$/V$_{\kappa}$1 mouse were immunostained with PE conjugated antibody to IgM^{a} (A), and FITC conjugated antibody to IgM^{b}(B). Merge of A and B (C), green arrow points to IgM^{b} single expresser cell, red arrow points to IgM^{a} single expresser cell and the white arrow points to cell co-expressing IgM^{a} and IgM^{b}. Cells were visualized by confocal microscopy using Zeiss LSM 510 microscope magnification 40X.

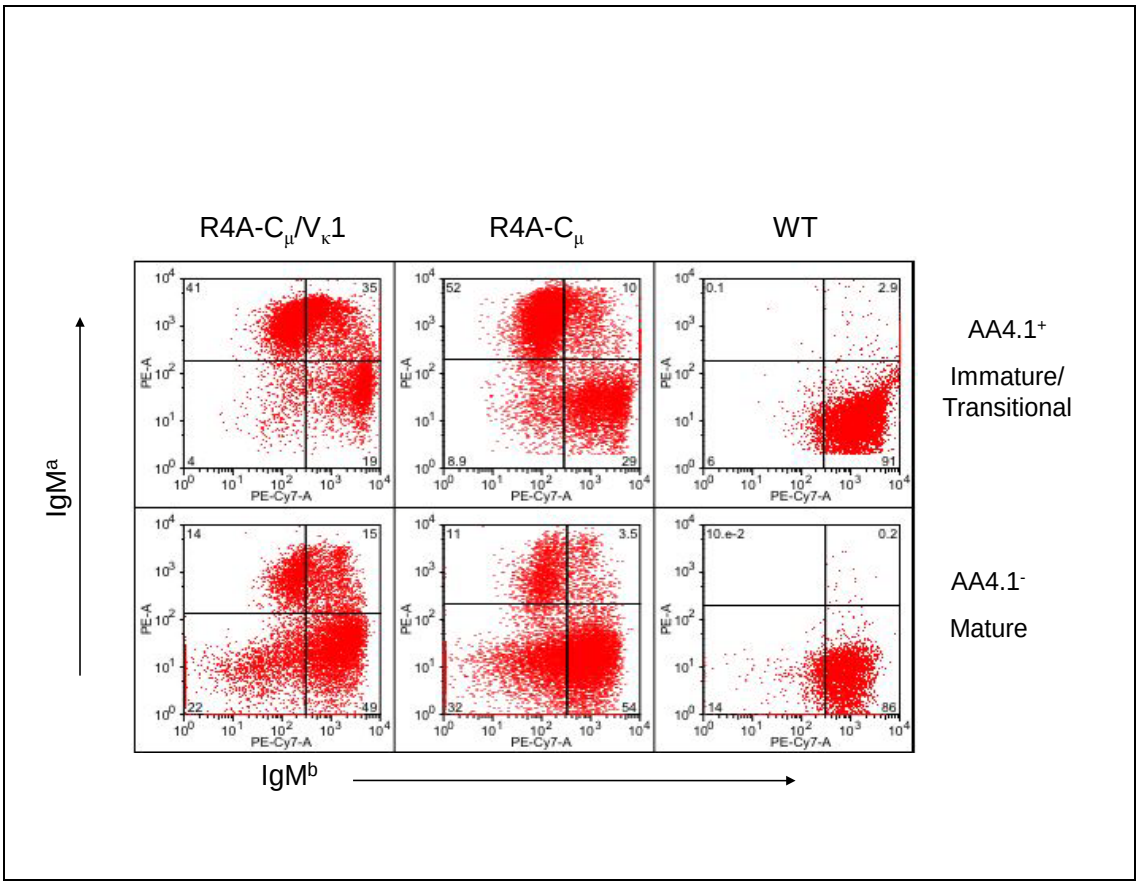


Figure 31. IgM^a and IgM^b expression on immature (AA4.1$^+$), and mature (AA4.1$^-$) B-cells, splenic B-cells from R4A-C$_\mu$/V$_\kappa$1, R4A-C$_\mu$ and WT mice were immunostained with antibodies to B220, AA4.1, IgM^a and IgM^b. Cells were gated on B220, AA4.1$^+$ (top panel), or B220, AA4.1$^-$ (bottom penal). The frequency of IgM^a B-cells decreased and the frequency of IgM^b B-cells increased as B-cells matured in R4A-C$_\mu$/V$_\kappa$1 as well as R4A-C$_\mu$ mice (n=2).

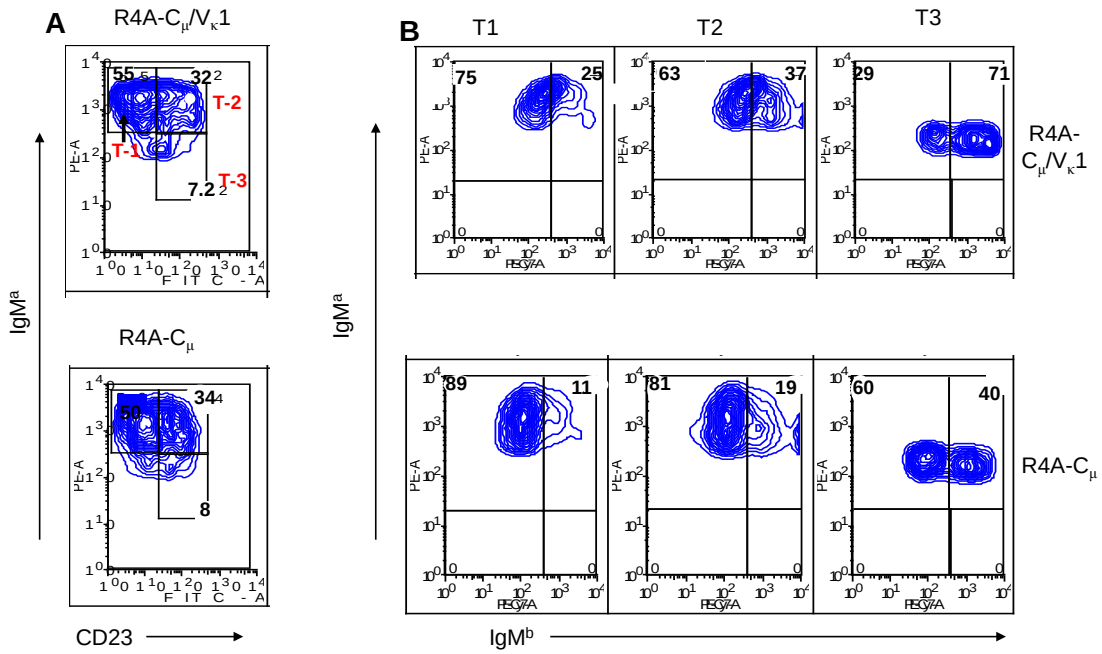


Figure 32. The frequency of transgenic B-cells expressing dual receptors increases as the cells mature from T1 to T3, (A) Splenic B-cells from R4A-C_μ/V_κ1mice were immunostained with antibodies to B220, AA4.1, IgM^a or IgM^b and CD23. Live lymphocytes were gated on B220, $AA4.1^+$, IgM^{a+} cells. Contours graphs indicate the frequency of $AA4.1^+$ T1 ($IgM^{a\ hi}$ CD23-) T2 (IgM^{hi} $CD23^+$) and T3 (IgM^{lo} $CD23^+$) B-cells in R4A-C_μ/V_κ (top panel) and R4A-C_μ (bottom panel) mice. (B) Cells were gated on IgM^a T1, T2, or T3 populations and the frequency of IgM^a T1, IgM^a T2, or IgM^a T3 B-cells co-expressing IgM^b was assessed by flow cytometry (n=3).

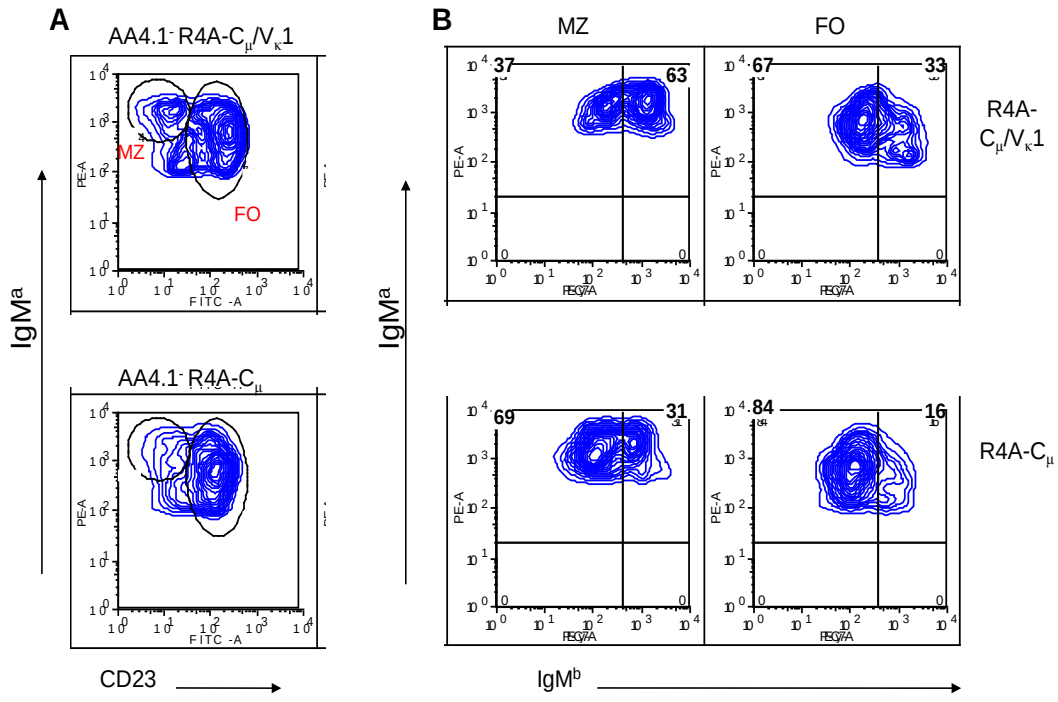


Figure 33. High frequency of dual receptor in B-cells in the MZ compartment.

B-cells were immunostained with antibodies to B220, AA4.1, IgM^a, IgM^b and CD23. (A) Gates were set on B220 $AA4.1^-$ IgM^{a+} B-cells. Contour graphs indicate the frequency of $AA4.1^-$ MZ (IgM^{a+} $CD23^-$), or FO (IgM^{a+} $CD23^+$) B-cells. (B) Cells were gated on IgM^a MZ or FO B-cells co-expressing IgM^b (n=3).

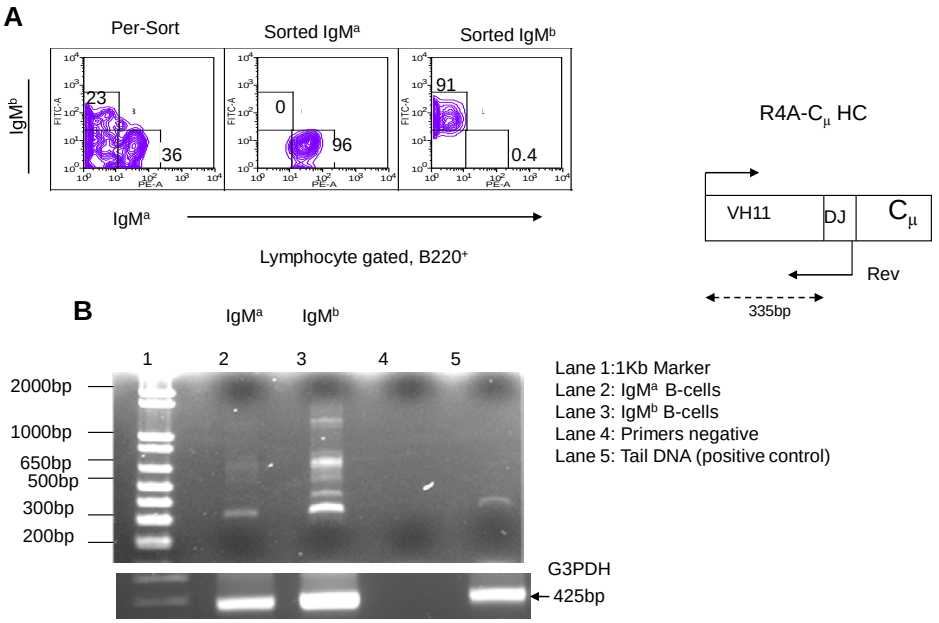


Figure 34. R4A-C_μ heavy chain Transgene is retained in endogenous IgM^b B-cells in R4A-C_μ/V_κ1 mice; (A) Splenic B-cells were sorted into IgM^a and IgM^b cells subsets by flow cytometry. Purity of IgM^a B-cells was 96%. Purity of IgM^b B-cells was 91%. **(B)** PCR was performed on DNA isolated from IgM^a and IgM^b B-cells subsets using a V_H11 forward primer and a J_H reversed primer to yield a 313bp product.

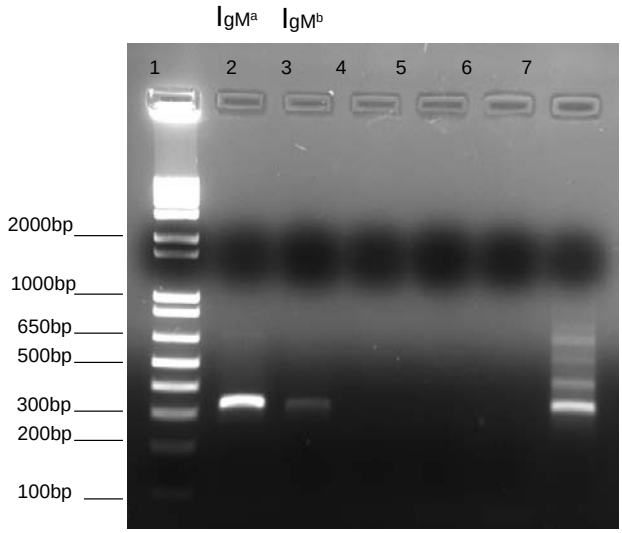


Figure 35. IgM^b B-cells express low levels of the heavy chain Transgenic.

RT-PCR was performed on RNA isolated from sort purified IgM^a and IgM^b splenic B-cells from R4A-C_μ/V_κ1 mice using a V_H11 forward primer and a reverse J_H junctional primmer. Lane 1; MW marker, lane 2; IgM^a RNA, lane 3; IgM^b RNA, lane 4; IgM^a RNA but no RT, lane 5; IgM^b RNA no RT, lane 6; Primers only, lane 7; positive control R4A-C_μ tail DNA.

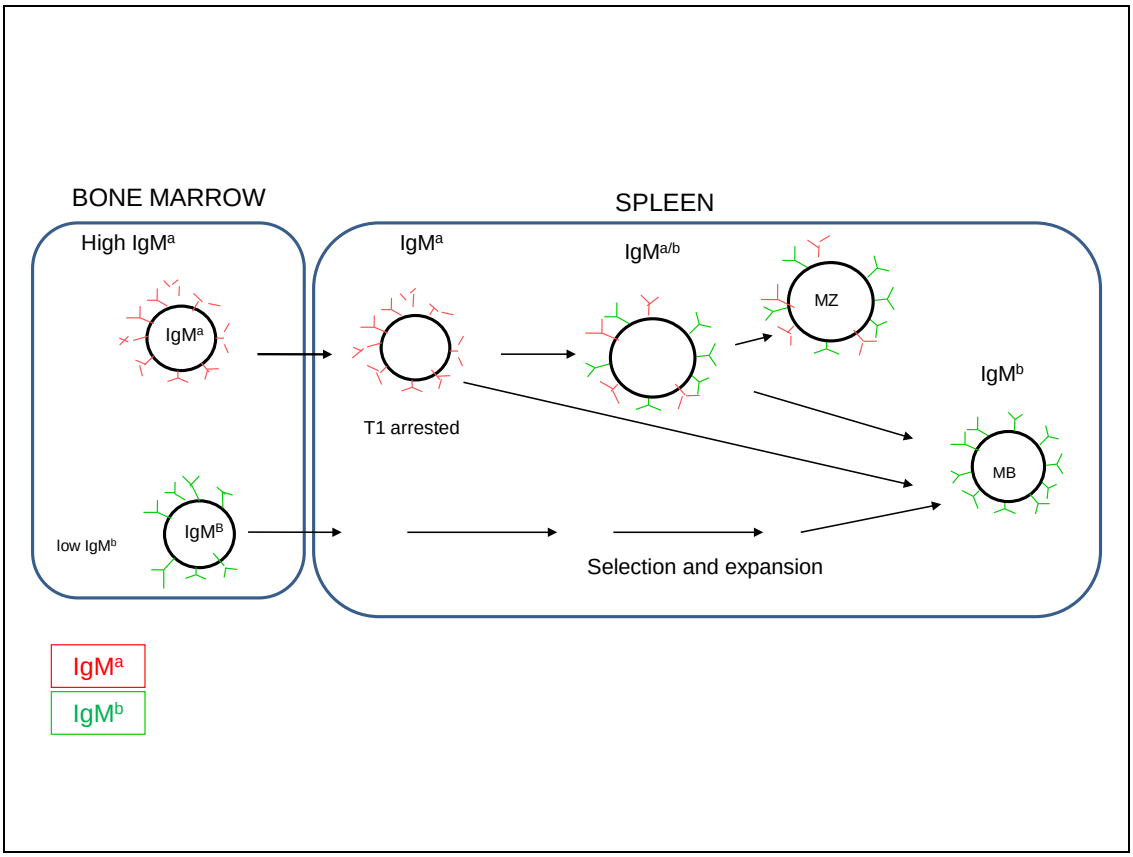


Figure 36. Model: Receptor down regulation as a mechanism for regulating autoreactive B-cells, auto reactive B-cells in the spleen begin to up regulate expression of endogenous IgM and down regulate IgM^{a} as they mature.Transitional-1 (T1), marginal zone (MZ) and mature B-cells (MB).

Table 1
The number of total B-cells in bone marrow of R4A-$C_\mu/V_\kappa1$, R4A-C_μ and WT mice

BONE MARROW		**WT**	**R4A-$C_\mu/V_\kappa1$**	**R4A-C_μ**
Total B220	B-cell x (10^6)	9± (0.5)	2.0 ± (1)	1.3± (1.3)
B220 IgM[a]	B-cell x (10^6)	N/A	0.8 ± (0.5)	0.3 ± (0.2)
B220 IgM[b]	B-cell x (10^6)	2 ± (0.7)	0.09± (0.05)	0.1 ± (0.1)
B220 IgM[a/b]	B-cell x (10^6)	N/A	0.01± (0.5)	0.01 ± (0.01)

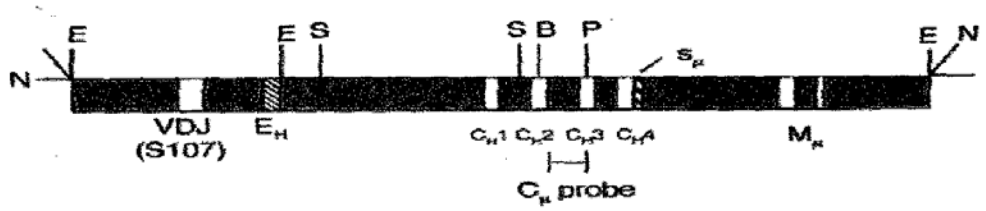
Results are the average of 3 experiments. Absolute number of B-cells was determined by flow cytometry.

Table 2
The number of total B-cells in spleen of R4A-$C_\mu/V_\kappa1$, R4A-C_μ and WT mice

SPLEEN		**WT**	**R4A-$C_\mu/V_\kappa1$**	**R4A-C_μ**
Total B220	B-cell x (10^6)	12± (9)	4.4 ± (3)	2 .0 ±(1.4)
B220 IgM^a	B-cell x (10^6)	N/A	1.5 ± (1)	0.3 ± (0.4)
B220 IgM^b	B-cell x (10^6)	28 + (39)	1.0±(1)	0.4 ± (0.3)
B220 $IgM^{a/b}$	B-cell x (10^6)	N/A	0.8 ± (0.5)	0.1 ± (0.1)

Results are the average of 3 experiments. Absolute number of B-cells was determined by flow cytometry.

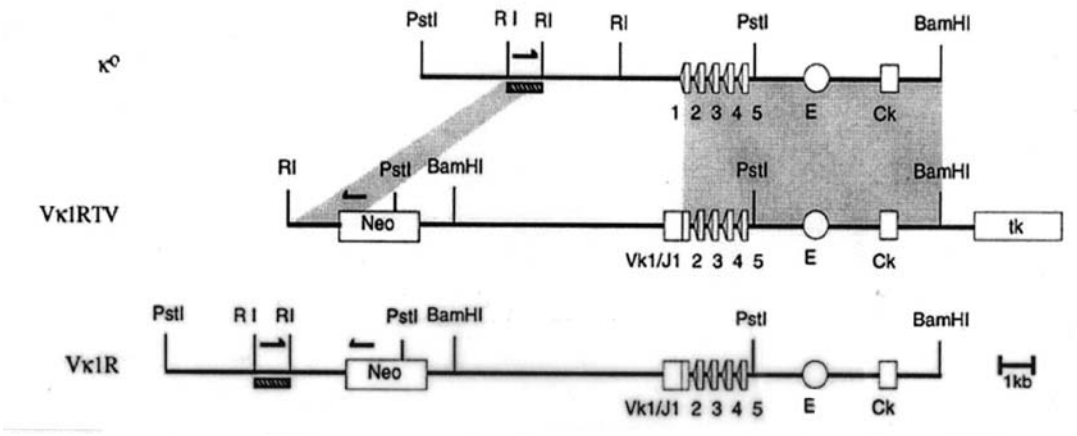
Appendix 1



R4AC$_\mu$ heavy chain transgene construct

The 3.5kb fragment containing the rearranged VDJ, ligated to a 9kb fragment containing the Mu constant region including the membrane exons (Mu), and the heavy chain enhance (E_H) region. The white boxes depicts exons of the constant region (C_H 1-4), Su secretion coding exon, the dark areas depicts introns. Constant region is from a BALB/c mouse and is allotype a.(51)

Appendix 2



Targeting strategy for Vk1 transgenic mouse.

(Courtesy Dr. Martin Weigert and Dr. Cindy Alderfer)

Rearranged $V_\kappa 1J_\kappa 1$ gene construct with its promoter, downstream $J_\kappa 1$, 2, 3, 4 and 5 segments along with C_κ exon was used to transfect mouse ES cells.

Shaded areas indicate points of homologous recombination. Transgenic mice contain one copy of transgene in the normal immunoglobulin locus.

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