ROLES OF IGH INTRONIC ENHANCER Eμ IN CLONAL SELECTION AT THE PRE-B TO IMMATURE B CELL TRANSITION AND IN THE ELIMINATION OF AUTOREACTIVE B CELLS

by

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ABSTRACT

Roles of *Igh* Intronic Enhancer Eμ in Clonal Selection at the Pre-B to Immature B Cell Transition and in the Elimination of Autoreactive B Cells

By

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The immunoglobulin heavy chain locus (*Igh*) intronic enhancer, Eμ, enhances transcription of recombined *Igh* genes. We have previously shown that in mice with an Eμ-deficient *Igh* allele ($V_H\Delta^a$), Igμ is expressed at half of the wild-type levels in pre-B cells. We also described an Eμ-dependent "check-point", operating at the pre-B to immature B cell transition, for heavy chain allelic exclusion. We now show that deletion of Eμ results in a smaller immature B cell compartment, and the pre-BCR/BCR signaling is diminished in pre-B cells as a result of the reduced Igμ levels, making it difficult for emerging BCRs to reach the signaling threshold required for positive selection of pre-B cells to the immature B cell stage.

Our hypothesis is that, to circumvent the problem of inadequate signaling, Eμ-deficient B cells either 1) expand the rare precursor B cells seemingly breaking the rules of allelic exclusion to express a second IgH allele as "double-producers", to achieve higher levels of Igμ-chain and hence higher pre-BCR and BCR levels, or 2) undergo heightened light-chain editing to create an IgH/IgL combination with superior signaling properties to make up for the lower Igμ-chain (lower BCR) levels and signaling. To test these hypotheses and to determine whether escape

from the developmental defects in Eμ-deficient B cells is dependent upon light chain, we provided the Eμ-deficient mice with a pre-assembled V_L gene (3-83Vκ). This led to not only a larger immature B cell compartment, but also a decrease in "double-producers". We suggest that an IgH/IgL combination with superior signaling properties may compensate for the reduced BCR levels and eliminate the selective advantage of "double-producers".

We also find that "double-producers" in Eμ-deficient heterozygous mice ($V_H\Delta^a/WT^b$), include a subpopulation with autoreactive BCRs. We infer that the BCRs with IgH from the $V_H\Delta^a$ allele are ignored during negative selection at the pre-B to immature B cell transition, due to their low density. Instead, the double-producers are both positively and negatively selected on the basis of BCRs with IgH from the alternate allele (with Eμ intact). Taken together, these results suggest that Eμ functions to ensure sufficient Igμ (IgH) levels at the pre-B to immature B transition, which has an important impact on the maintenance of heavy chain allelic exclusion, the breadth of the BCR repertoire, and the elimination of autoreactive B cells.

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TABLE OF CONTENTS

LISTS OF FIGURES AND TABLES

ABBREVIATIONS

AID: activation-induced cytidine deaminase

BCR: B cell receptor

BM: bone marrow

bp: base pairs

C: constant region gene segment

CDR: complementarity-determining region

CLP: common lymphoid progenitor

CSR: class switch recombination

D: diversity gene segment

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetate

ELISA: enzyme-linked immunosorbent assay

FACS: fluorescence-activated cell sorter

FBS: fetal bovine serum

FO: follicular

H: heavy

HSC: hematopoietic stem cell

IFN: interferon

Ig: immunoglobulin

Igh: immunoglobulin heavy chain locus/gene

IgH: immunoglobulin heavy chain protein/recombined *Igh*

IgL: immunoglobulin light chain

IL: interleukin

J: joining gene segment

L: leader

LPS: lipopolysaccharide

MAR: matrix attachment regions

MHC: major histocompatibility complex

mRNA: messenger RNA

MZ: marginal zone

NeoR: neomycin resistance gene

NHEJ: non-homologous DNA end-joining

NK: natural killer (cell)

P: palindromic

PBS: phosphate buffered saline

PCR: polymerase chain reaction

pre: precursor

pre-BCR: pre-B cell receptor

pro: progenitor

RAG: recombination activating gene

RNA: ribonucleic acid

RS: recombining sequence

RSS: recombination signal sequence

RT: reverse transcription

RT: room temperature

S: switch

sIg: surface immunoglobulin

sIgM: surface IgM

SLC: surrogate light chain

SLE: systemic lupus erythematosus

TAE: Tris-acetic acid-EDTA

TCR: T cell receptor

TdT: terminal deoxynucleotidyl transferase

V: variable region gene segment

V_H: heavy chain variable region gene

WT: wild-type

Chapter 1 Introduction

1.1. Basics of the immune system

Living in a microbial and complex environment, we have a highly evolved immune system which is able to distinguish between foreign and self molecules and protect us from diseases. Our immune system effectively and efficiently detects and fights against a wide variety of pathogens, including viruses, bacteria, fungi, or parasitic worms. In some cases, the immune system also functions to destroy abnormal (like cancerous cells) or dying body cells. Due to the enormous number of invasive agents and various ways of infection, multiple defense mechanisms have been adopted and numerous cells and molecules participate in the host's immune response (1, 2).

Physical barriers are the first line of defense when the body is invaded. These include 1) mechanical barrier which expels or traps microbes, like the skin, or mucous membrane; 2) chemical barrier consists of substances that lyse or break down infectious cells, such as lysozyme, gastric acid, proteases, or collectins; and 3) biological barrier competitively limits the food or space supply for the pathogenic bacteria, for example the commensal flora in the gastrointestinal tract.

Once the host's physical barriers are passed, the innate immune system immediately starts to defend non-specifically against the invaders (3). With invariant receptors, the innate immune system recognizes the common features of pathogenic components. Microbes or other foreign molecules can be engulfed and destroyed by phagocytes (including macrophages, granulocytes, dendritic cells, etc). Natural killer cells (NK cells) are a type of cytotoxic lymphocyte critical to the innate immune system. NK cells do not directly attack invading microbes. Instead, they

destroy infected cells or tumor cells (4, 5). Inflammation recruits immune cells (mainly leukocytes) to the site of infection by cytokine signals to fight against the invasions (6-8). Complement system, as the humoral component of innate immune system, "complements" the abilities of antibodies and phagocytic cells to clear pathogens (9, 10). The complement system consists of a series of small proteins, which function to 1) enhance phagocytosis of antigens (opsonization) by marking the antigens, 2) attract inflammatory cells (chemotaxis), such as macrophages and neutrophils, to the site of infection, and 3) form membrane-attack complex (MAC) to rupture the cell membranes and lyse the cells (11). The innate immune system does not confer long-lasting protection to the host. Rather, it provides immediate defense against infections.

If the innate immunity is also evaded, the adaptive immune system then takes place in an antigen specific manner (12, 13). The adaptive immune system works together with the innate immune system, but is highly specific to the particular pathogen which induced it and may provide long-lasting protection for the host due to the immunological memory (14) (discussed in section 1.2). These three layers of defense with increasing specificity allow us to survive in the potentially pathogenic world.

1.2. The adaptive immune system

The adaptive immune response is carried out by a subset of leukocytes: the lymphocytes (15). T and B lymphocytes are the two major cellular members of the adaptive immune system, and they are involved in the cell-mediated and humoral immune responses, respectively. Both T and B lymphocytes have diverse specific receptors on their surfaces so that they can recognize and target the pathogens (16). According to the clonal selection theory, each lymphocyte has a single

type of receptor on its surface with single antigen specificity (17, 18). Cells with specificity to self-molecules are removed from the repertoire during early stages of their development, while those that recognize only foreign molecules survive and are activated once they encounter their matching antigens (19). When activated by the cognate antigens, T and B lymphocytes develop into memory cells and are able to mount a faster and stronger reaction once re-infected by the same pathogen. Activated T cells react against antigens that are presented by the host cells, whereas activated B cells secrete antibodies which bind to antigens for destruction (16).

1.3. Antibody expression

Antibody, also known as immunoglobulin (Ig), is one of the crucial components of the adaptive immune system (20). Antibodies, produced by B lymphocytes, can bind and neutralize pathogens to block their access to the host cells. Antibodies may also coat pathogens for opsonization to facilitate phagocytosis. In addition, natural antibodies produced without previous infection, are able to trigger the classical complement pathway to destroy and eliminate the pathogens (21, 22).

To fight against a large variety of antigens, antibody molecules are highly diversified, but sharing some common structural features (23). Antibody molecules are either secreted (except for IgD), or expressed on B cell surface (sIg) in a membrane-bound form. The sIg complex associates with the signal transduction molecules Igα and Igβ to form B cell receptors (BCR), which actively control the selection processes during B cell development (24). Secreted Ig fight against antigens as monomers (like IgG and IgE) or multimers (like IgA and IgM). IgM molecules are usually secreted and synthesize pentamers, whereas IgA molecules form dimers. Each subunit in these multimers are linked by disulfide bonds and joined to a joining (J) chain peptide molecule (22).

1.3.1 Structure of immunoglobulin

Each Ig molecule is a Y-shaped, symmetrical glycoprotein consisting of two identical heavy (H) chains and two identical light (L) chains, held together by inter-chain and intra-chain disulfide bonds (Figure 1.1). Each chain has a variable (V) and a constant (C) region. The V regions at the amino (N)-terminal are the antigen-binding domains that display variability and specificity. V regions differ in antibodies produced by different B cells, but are the same for all antibodies produced by a single B cell. The variable region of an Ig molecule contains 4 framework regions (FRs) and 3 complementarity determining regions (CDRs). The FRs are β-pleated sheet structures which support in position the CDRs (the hypervariable region, in contact with antigens) (25). The V region of an IgH or IgL is composed of one immunoglobulin domain, and is ~110 amino acids long. The C regions at the carboxy (C)-terminal of the heavy chain loci are the determinants of Ig isotypes or classes, and therefore determine the effector functions of the antibodies (26). The C regions are identical in all antibodies of the same isotype, but differ in antibodies of different isotypes. The C regions of IgH contain three or four immunogobulin domains depending on the class of the antibody, and C regions of IgL contain only one immunoglobulin domain. Each of the constant immunoglobulin domain is also ~110 amino acids long.

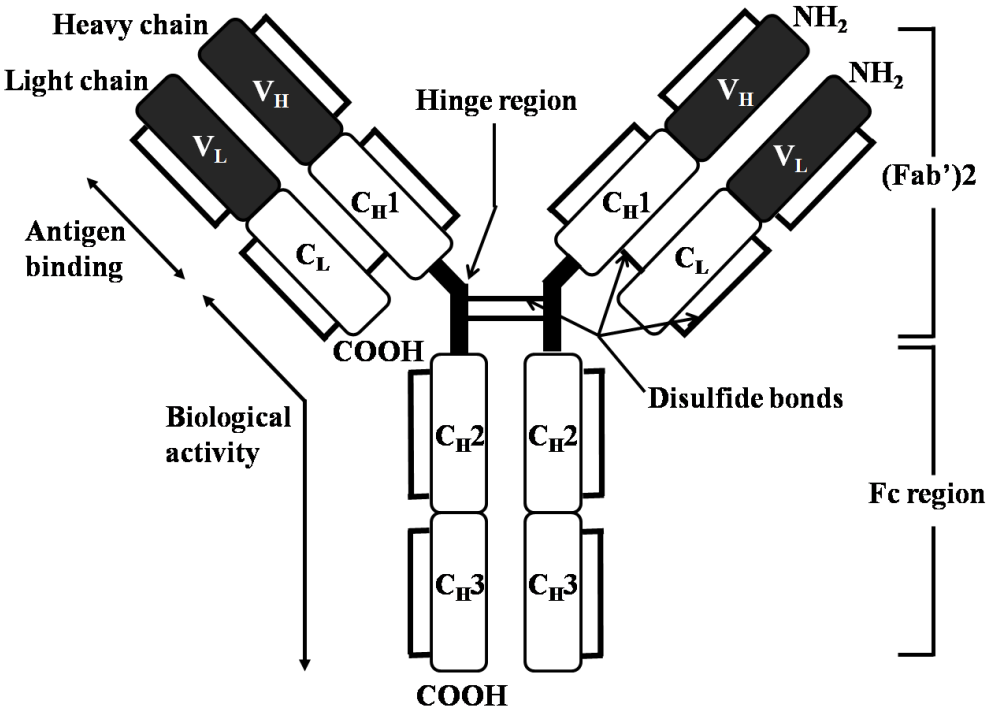
There are five known types of Ig heavy chain in human and murine: Igμ, Igδ, Igγ (subdivided to Igγ1, Igγ2a, Igγ2b and Igγ3 in mice), Igε and Igα. They define the classes (or isotypes) of immunoglobulins: IgM, IgD, IgG (subdivided to IgG1, IgG2a, IgG2b and IgG3 in mice), IgE and IgA, respectively. Antibodies of different classes possess their own structures and functional activities. For example, the C regions of Igγ, Igα and Igδ, consisting of three tandem

immunoglobulin domains and one hinge region for flexibility, are ~330 amino acids long. The C regions of Igμ and Igε, both composed of four immunoglobulin domains, are ~440 amino acids long (27). IgG is the major class of antibody in circulation whose functions include but not limited to coating pathogen surface for the recognition by phagocytes, activating the classical complement pathway to eliminate pathogens, and immobilizing antigens by binding them together via agglutination. Whereas IgE plays an essential role in immunity to parasites, or to trigger mast cells or basophils to release histamine. IgA, found in mucous secretions, protects the hosts against microbes growing in body secretions. There are two types of Ig light chains in mammals, kappa (κ) and lambda (λ) light chains, in association with the Ig heavy chains (28). As discussed before, a light chain has two successive domains: one variable domain and one constant domain, ~220 amino acids long (Figure 1.1). The overall structures of *Igh*, *Igκ* and *Igλ* loci are discussed below (section 1.3.2 and 1.3.3).

The two arms of the Y-shaped antibody are also called the Fab (fragment, antigen binding) region. It is composed of the V region and one constant immunoglobulin domain at the N-terminal from each heavy chain and each light chain (25). The base of the Y-shaped antibody is called the Fc (fragment, crystallizing) region. It consists of the remaining two or three of the constant immunoglobulin domains at the C-terminal from the two heavy chains (Figure 1.1). Fab and Fc regions can be cleaved by proteolytic enzyme papain or pepsin. Fab region, which contains the V region with varied antigen specificity, binds to antigens. Fc region interacts with Fc receptors or proteins of the complement system to mediate different physiological effects of antibodies, for appropriate immune responses to different antigens (27, 29).

Figure 1.1: The schematic structure of an IgG antibody molecule.

An antibody (Ig) molecule consists of two identical heavy chains and two identical light chains. Each heavy chain and each light chain consists of an antigen-binding variable region (V_H or V_L, shown in dark angled boxes as indicated), and a constant region (C_H or C_L, shown in open angled and vertical boxes as indicated). The V region determines the specificity of an antibody, and the C region determines the effector function of an antibody. Function domains are shown, with interchain and intrachain disulfide bonds. Of an IgG antibody, each V region has one immunoglobulin domain, each C_H region has three immunoglobulin domains (C_H1, C_H2, and C_H3) and a hinge region, and each C_L has one immunolobulin domain. Each immunoglobulin domain is depicted as a rectangle. Fab, Fc, and hinge regions are indicated.



1.3.2 The heavy chain locus

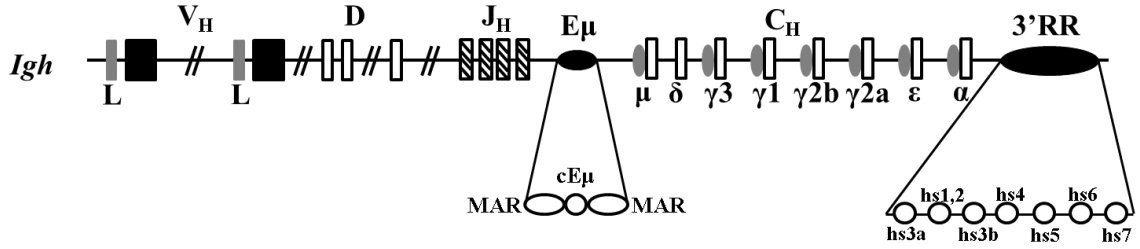
The mouse heavy chain locus (*Igh*) spans around 3 megabases (Mb) on chromosome 12 (30, 31). The *Igh* comprises V-region in the 5' part and C-region in the 3' part. The V-region includes arrays of gene segments: the variable (V_H), diversity (D) and joining (J_H) gene segments. The C-region includes a cluster of C_H exons encoding different constant domains (Figure 1.2).

Mouse *Igh* locus contains hundreds of V_H gene segments (i.e. approximately 150 V_H genes in C57BL/6J mice, spanning ~2.5 Mb) (32, 33), 9-12 D_H gene segments according to mouse strains, and 4 functional J_H gene segments (J_H1-J_H4) (32, 34, 35). There are different families of the V_H, D and J_H segments classified by sequence similarities (32, 34, 35). Among the 14 V_H family gene clusters, at the 5' most lies the V_HJ558 family as distal V_H genes. The most downstream V_H gene family is V_H7183, which are referred to as the proximal V_H genes and are separated from the first D gene segment $D_{FL16.1}$, by a 98 kilobase (kb) intergenic region (36). There are four D gene families. The most downstream D gene segment is D_{Q52}, lying less than one kb upstream of the J_H gene cluster. A leader (L) sequence exists 5' of each V_H gene segment, which encodes a leader peptide (i.e. a signal sequence) to signal the transportation of the newly synthesized IgH chain into the endoplasmic reticulum for surface expression or secretion. This leader peptide is excised in the final IgH chain (discussed in section 1.3.5).

There is a cluster of *Igh* C-region (C_H) gene segments (~200 kb), lying downstream of the J_H genes. Different Ig classes (isotypes) and subclasses (allotypes) are encoded by the different C_H exons, including Cμ, Cδ, Cγ3, Cγ1, Cγ2b, Cγ2a, Cε and Cα in murine (Figure 1.2).

Figure 1.2: The mouse heavy chain locus.

Schematic view of murine *Igh* locus. There are four gene clusters on the *Igh* locus. All V_H, D, J_H and C_H gene segments are in germline configurations but not shown to scale. Dark filled boxes = V_H coding segments; hatched boxes = J_H coding segments; open boxes = D or C_H coding segments as indicated. Not all V_H genes nor all D genes are shown. Slash marks indicate DNA regions not shown. A leader (L) sequence upstream of each V_H gene segment is shown as a gray box. All C_H genes are shown as indicated in Greek letters. Swith regions upstream from C_H exons are shown as vertical gray ovals. Murine *Igh* locus consists of several *cis*-control elements: the intronic enhancer Eμ and the 3' regulatory region (3'RR), represented by horizontal black ovals. Eμ is composed of a core enhancer region (cEμ) and two flanking matrix attachment regions (MARs). Seven DNase I hypersensitive enhancers have been identified within the 3'RR, designated hs3a, hs1,2, hs3b, hs4, hs5, hs6 and hs7.

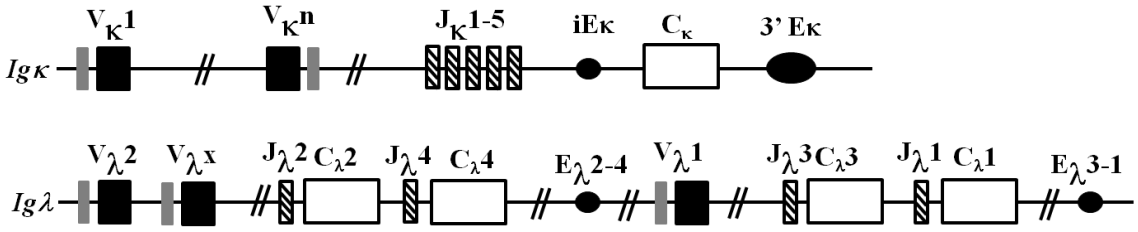


1.3.3 The light chain locus

The mouse kappa (κ) light chain locus is on chromosome 6, and the lambda (λ) light chain locus is on chromosome 16 (Figure 1.3). Igκ locus spans ~1.25 Mb, consisting of approximately 140 Vκ genes at the 5' of the locus, followed by 5 Jκ genes, and a single Cκ gene segment at the downstream of the locus. The germline λ chain locus has three Vλ gene segments, four Jλ gene segments and four Cλ gene segments. The Jλ and Cλ segments are arranged in pairs (Figure 1.3). There are many more Vκ genes as compared to the only three Vλ genes. However, there is only one kappa light chain class due to the single Cκ coding sequence, and three lambda chain classes (λ1-λ3) encoded by three of the four Cλ coding sequence (Cλ4 is a pseudogene and incapable of coding proteins) (28, 37). Murine express kappa light chains in most of their immunoglobulins. Only ~5% of immunoglobulins in most normal inbred mice carry the lambda light chains, of which ~80% are λ1 and the remaining ~20% are λ2 and λ3 (37, 38).

Figure 1.3: The mouse κ and λ light chain loci.

Schematic view of murine *Igκ* and *Igλ* loci. There are three gene clusters on each light chain locus. All V_κ (V_λ), J_κ (J_λ) and C_κ (C_λ) gene segments are in germline configurations but not shown to scale. Dark filled boxes = V_κ (V_λ) coding segments; hatched boxes = J_κ (J_λ) coding segments; open boxes = C_κ (C_λ) coding segments. A leader (L) sequence upstream of each V_κ (V_λ) gene segment is shown as a gray box. Not all V_κ genes are shown. Slash marks indicate DNA segments not shown. Enhancer regions within the *Igκ* and *Igλ* loci are indicated as black horizontal ovals, iEκ and Eλ, respectively.



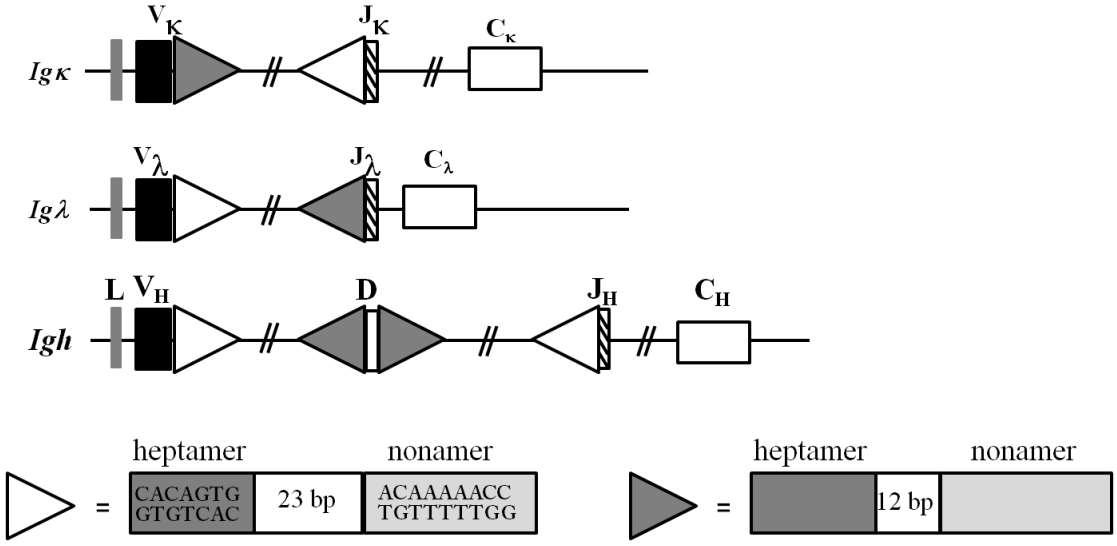
1.3.4 Recombination of immunoglobulin genes

There is only a limited number of V, (D), J and C coding genes on Ig loci in comparison to the numerous specificity of the antibody repertoire. The great diversity of antibody specificity is achieved through random rearrangement of the several arrays of *Igh* and *Igl* genes, a process termed V(D)J recombination which occurs exclusively in B lymphocytes. For example, the V region of IgH is assembled from V_H, D, and J_H gene segments by V(D)J recombination (39). These rearranging gene segments (V_H, D, and J_H) are flanked by recombination signal sequences (RSSs) for the recombination process. Each RSS consists of one highly conserved palindromic heptamer, and one moderately conserved A/T rich nonamer sequence, with a non-conserved 12 or 23 base pairs (bp) DNA spacer between them (Figure 1.4) (40). According to the 12/23 rule of recombination, only a pair of different spacer RSSs are able to recombine, so that the 12 bp RSS recombines only with the 23 bp RSS (40). The V_H and J_H gene segments are flanked by 23 bp RSS 3' and 5', respectively, whereas D segments are flanked by 12 bp RSS on both sides. Therefore, there is no direct V_H to J_H gene recombination since both of them are flanked by 12 bp RSSs, resulting in an ordered V_H-D-J_H assembly. Similarly, Vκ gene segments are flanked at the 3' by 12 bp RSS, and Jκ gene segments are flanked by 23 bp RSS at the 5' end. The Vλ gene segments are flanked by 23 bp RSS and the Jλ by 12 bp RSS (Figure 1.4). These complementary RSS pairs (12/23), therefore, ensure recombination between Vκ and Jκ, as well as Vλ and Jλ (35, 41, 42).

Figure 1.4: RSS of mouse heavy and light chain loci.

Schematic view of murine RSSs on the *Igh*, *Igκ* and *Igλ* loci. Angled lines denote that not all DNA segments are shown, so the coding segments may be very far apart on the chromosome. Dark filled boxes = V_H, V_κ or V_λ coding segments; hatched boxes = J_H, J_κ or J_λ coding segments; open boxes = D, C_H, C_κ or C_λ coding segments as indicated. A leader (L) sequence upstream of each V_H, V_κ or V_λ gene segment is shown as a gray box.

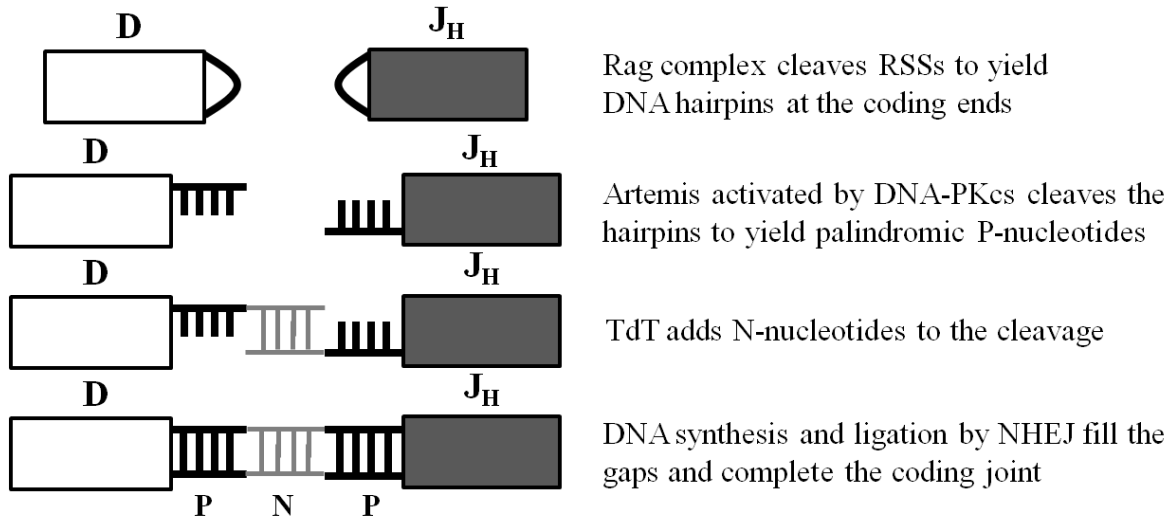
V, D and J gene segments are flanked by conserved heptamer and nonamer sequences with different spacers (drawn not to scale). Open and closed triangles denote Recombination Signal Sequences (RSSs) - their structures are indicated to the right (heptamer, spacer, nonamer).



The RSSs can be recognized by a common recombinase machinery including Rag1 and Rag2 proteins (gene products of the recombination activating genes RAG1 and RAG2 on chromosome 2 in murine). Rag1/2 proteins induce the DNA synapsis and double-strand DNA cleavage between the V, (D) and J coding gene segments and their flanking RSSs, forming hairpin loops at the coding ends (43, 44). These ends must be joined together to form contiguous coding DNA. Repair and joining of the DNA breaks require non-homologous end-joining (NHEJ) proteins: Ku70/80 proteins recruit the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) onto the coding end; DNA-PKcs activates artemis (a DNA endonuclease) to open the hairpins, which makes a random single cut (variable) near the tip of the hairpin, resulting in addition of a series of palindromic P-nucleotides at the coding end (45-47) (Figure 1.5). Addition of P-nucleotides generates alternative amino acids at the coding joint, therefore creates the junctional diversity (or junctional flexibility). Subsequently, terminal deoxynucleotidyl transferase (TdT), adds further random N-nucleotides to the P-nucleotides of the V, D and J exons before the newly synthesized strands anneal to one another. This establishes additional diversity in the antibody repertoire. TdT is expressed at the pro-B cell stage during heavy chain gene assembly, but is turned down during light chain gene assembly (48, 49). Therefore, N-nucleotide additions to the light chain gene segments are not as common as to the heavy chain gene segments, resulting in less abundant junctional diversity on light chains. End joining is completed by DNA synthesis and repair machinery (X-ray repair cross-complementing protein 4 (XRCC4), DNA ligase IV, etc) (41, 48, 49), so that unpaired nucleotides are removed and gaps are filled (50) (Figure 1.5).

Figure 1.5: P- and N-nucleotides are included in the coding joints between gene segments during lymphocyte gene rearrangement.

During joining of heavy chain V region coding sequences, P- and N-nucleotide additions occur after the RSS is cut and cleaved by RAG enzyme to form hairpin structures. The cleavage yields a single-stranded DNA end. During the subsequent repair, complementary nucleotides are added, forming P-nucleotides. In pro-B cells, N-nucleotides are added randomly by TdT before complementary pairing occurs, increasing the variability of the recombined gene segments. Nucleotide addition event is exemplified by a D to J_H joining. Open boxes = D coding segments; gray filled boxes = J_H coding segments.



The recombination at the *Igh* loci (in pro-B cells, see section 1.4) precedes the recombination at the *Igl* loci (in pre-B cells, also see section 1.4). D-to-J_H gene recombination occurs on both the *Igh* loci, preceding V_H-to-DJ_H recombination which occurs on only one of the two *Igh* alleles. *Igκ* loci recombination precedes *Igλ* recombination. Both *Igκ* alleles are rearranged but not functional in Igλ-expressing B cells (51).

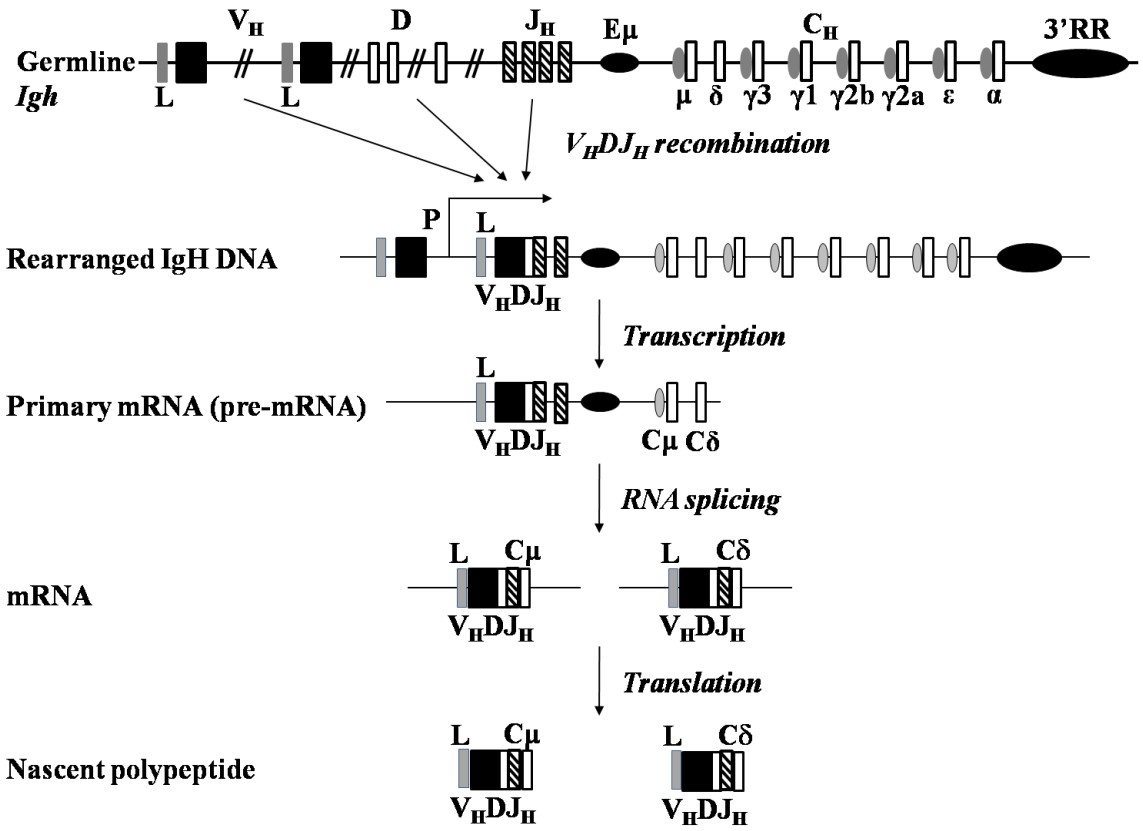
1.3.5 Expression of immunoglobulin genes

A human can produce more than 10^{12} different antibody molecules as a preimmune antibody repertoire. This enormous diversity of Ig specificities makes the antibody defense very formidable. The level of Ig proteins increases from precursor cells to the terminally differentiated (Ig-secreting) plasma cells. During the process of Ig gene expression, DNA rearrangement (through V(D)J recombination) is required, and only one of the two alleles is functionally expressed. After DNA rearrangement, the recombined gene becomes transcriptionally active because the promoter associated with the V_H or V_L gene segment is brought closer to the enhancer on *Igh* or *Igl* locus (Eμ, iEκ, or Eλ, Figure 1.2, 1.3). Transcription initiates from the promoter and produces a pre-mRNA which contains sequences of L, V(D)J and C exons as well as the introns between L and V(D)J and between V(D)J and C (also introns between subregions of the C_H clusters in the case of *Igh*). This pre-mRNA is then spliced in the nucleus such that the remaining introns are removed, resulting in mRNA with contiguous L, V(D)J and C exons (Figure 1.6). The mRNA is translated in the cytoplasm into a nascent peptide, which is then transported into the lumen of the endoplasmic reticulum (ER). In the ER, the leader peptide is removed so that mature IgH (μ or δ protein) and IgL proteins assemble and the Ig molecule is inserted in the membrane (except for plasmacytes) or secreted via the route of secretory proteins.

Figure 1.6: Expression of immunoglobulin heavy chain protein.

Schematic view of expression of murine *Igh* genes. There are four gene clusters on the *Igh* locus. All V_H, D, J_H and C_H gene segments are in germline configurations but not shown to scale. Dark filled boxes = V_H coding segments; hatched boxes = J_H coding segments; open boxes = D or C_H coding segments as indicated. A leader (L) sequence upstream of each V_H gene segment is shown as a gray box. Switch regions upstream of C_H exons are shown as vertical gray ovals. The intronic enhancer Eμ and the 3' enhancer (3'RR) are represented by horizontal black ovals. Not all V_H genes nor all D genes are shown. Slash marks indicate DNA segments not shown. All C_H genes are shown as indicated in Greek letters.

During the earlier stages of B cell development, the heavy chain variable region genes undergo V_HDJ_H recombination. The recombined IgH gene is transcribed from the promoter (P) upstream of the recombined V_HDJ_H gene, making a pre-mRNA with L, V_HDJ_H, and C_H exons as well as introns between them. This pre-mRNA is then processed (spliced) in the nucleus so that the introns are excised. The resulting mRNA with only L, V_HDJ_H, and C_H exons (with 5' and 3' untranslated regions) is translated in the cytoplasm. Although similar to transcription of light chain genes, transcription of heavy chain genes sometimes involves an alternative RNA splicing process, which generates different products (mRNAs for Igμ or Igδ heavy chains; Igδ not made in immature B cells). As the nascent polypeptide (protein) is transported into the lumen of the endoplasmic reticulum (ER), the leader peptide is removed, producing a mature μ or δ heavy chain (not shown).


Germline
Igh
L
V_H
L
D
J_H
Eμ
μ δ γ3 γ1 γ2b γ2a ε α
C_H
3'RR
V_HDJ_H recombination
Rearranged IgH DNA
P
L
V_HDJ_H
Transcription
Primary mRNA (pre-mRNA)
L
V_HDJ_H
Cμ Cδ
RNA splicing
mRNA
L Cμ
V_HDJ_H
L Cδ
V_HDJ_H
Translation
Nascent polypeptide
L Cμ
V_HDJ_H
L Cδ
V_HDJ_H

A complete V_HDJ_H recombination occurs on one of the two *Igh* loci. Transcription across the recombined IgH gene results in the formation of Igμ molecules. However, not all recombination leads to a functional Ig chain. In this case, the gene segments on the second allele recombine. After a functional heavy chain expression as an Igμ molecule, a Vκ gene segment starts to rearrange with a Jκ gene segment. Once a productive VκJκ gene is expressed and able to pair with the Igμ heavy chain, a Y-shape mature Ig molecule is assembled. Only if recombination on both the κ alleles fails, the λ locus rearrangement takes place.

As B cells mature, IgM^+ B cells migrate to the secondary lymphoid organs (such as spleen and lymph nodes), where antibody class-switch recombination (CSR) occurs in response to antigen recognition and costimulatory signals. CSR is a deletional-recombination process that exchanges the C_H (like Cμ) with one of a set of downstream C_H genes (such as Cγ3, Cγ1, or Cε, etc). CSR requires the enzyme activation-induced cytidine deaminase (AID), to make DNA breaks at switch (S) regions, which precede the C_H genes (52). dsDNA breaks are made within both the donor and acceptor S regions by a series of proteins, including AID, UNG, APE, etc, followed by the repair of DNA to make appropriate DNA ends for end joining. During the process of CSR, the intervening sequences between the two switch regions upstream of the two C_H coding sequences involved are deleted as an episomal circle. CSR leads to a rearranged C_H locus encoding a secondary isotype, retaining a functional antibody gene with the original specificity (the V region of IgH remains the same) but a distinct constant domain in their heavy chains. Cytokines stimulate transcription through the C_H gene and determine the immunoglobulin isotype that the B cell will switch to. As a result, different daughter cells from the same activated B cells produce antibodies that retain the same specificity and affinity for the same antigen, but

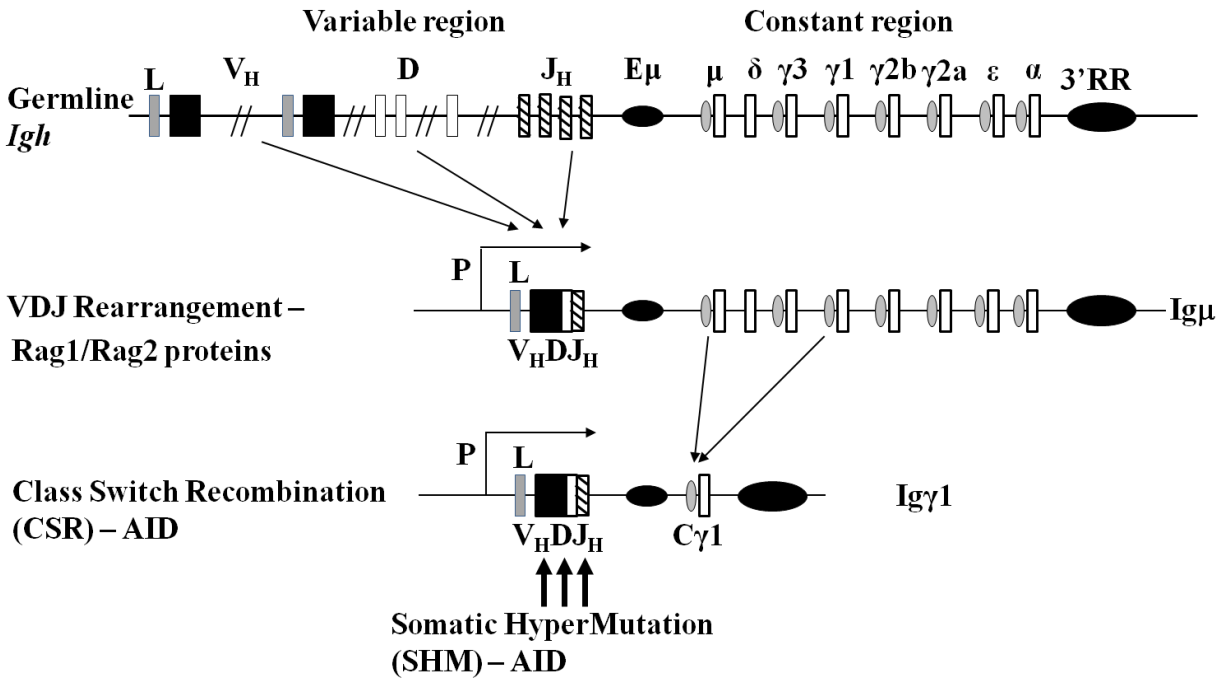
in response to different cytokine signals, the antibodies produced are of different isotypes (e.g. IgM, IgG, IgE, etc.) and can interact with different effector molecules (53) (Figure 1.7).

Besides CSR, somatic hypermutation (SHM) is another process that takes place within the germinal centers of secondary lymphoid organs. SHM is a largely random process which occurs in an individual B cell to adapt to antigens that confront it (54). SHM also involves the Activation-Induced Cytidine Deaminase (AID), causing high rate of point mutations (cytosine to uracil) in rearranged V region genes, following B cell exposure to antigens (Figure 1.7). Since uracil does not normally exist in DNA, to maintain the integrity of the genome, the uracil bases are removed by DNA glycosylase. Error-prone DNA polymerases then fill in the gaps which creates mutations at the mutated sites (55). SHM is generally considered as an affinity maturation process by which B cells produce antibodies with higher affinity for antigen.

Figure 1.7: The processes of heavy chain locus modification: V_HDJ_H recombination, CSR and SHM.

Schematic view of somatic modification events on murine *Igh* locus. There are four gene clusters on the *Igh* locus. All V_H, D, J_H and C_H gene segments are in germline configurations but not to scale. Dark filled boxes = V_H coding segments; hatched boxes = J_H coding segments; open boxes = D or C_H coding segments. A leader (L) sequence upstream of each V_H gene segment is shown asa gray box. Swith regions upstream of C_H exons are shown as vertical gray ovals. The intronic enhancer Eμ and the 3' enhancer (3'RR) are represented by horizontal black ovals. Not all V_H genes nor all D genes are shown. Slash marks indicate DNA segments not shown. All C_H genes are shown as indicated in Greek letters.

During the earlier stages of B cell development, the heavy chain variable region genes undergo V_HDJ_H recombination and the recombined *Igh* locus expresses Igμ protein. During later stages of B cell development, the heavy chain locus undergoes class switch recombination (CSR) through the switch regions upstream of each C_H gene segment, to produce antibodies of different classes but with the same antigen specificity. Individual B cells also undergo somatic hypermutation (SHM), during which process the *Igh* genes mutate to increase the affinity for an antigen. Both CSR and SHM involve the enzyme Activation-Induced Cytidine Deaminase (AID).


Variable region
Constant region
Germline
Igh
L
VH
D
JH
Eμ
μ
δ
γ3
γ1
γ2b
γ2a
ε
α
3'RR
P
L
VDJ Rearrangement –
Rag1/Rag2 proteins
VHDJH
Igμ
P
L
Class Switch Recombination
(CSR) – AID
VHDJH
Cγ1
Igγ1
Somatic HyperMutation
(SHM) – AID

1.3.6 Control of V(D)J recombination

The rearrangement of *Igh* and *Igl* loci is stringently regulated in a lineage, stage, and allele specific manner. This means that Ig molecules are expressed only in B lymphocytes, *Igh* gene expression precedes *Igl* gene expression, and only one of the *Igh* or *Igl* alleles is functionally expressed (35, 44, 56, 57). Regulation of V(D)J recombination in these contexts requires the collaboration of *cis*- and *trans*-acting factors, appropriate chromatin structure, as well as DNA breakage and repair mechamisms. Precise regulation of V(D)J recombination is crucial for Ig expression and ensures a competent adaptive immune system against invaders. Erroneous V(D)J recombination, in contrast, is a great threat to the integrity of the immune system.

Essential to the process of V(D)J recombination, RAG1 and RAG2 genes are expressed only in developing lymphoid lineage cells, to control the cell-type and stage-dependent rearrangement of Ig and TCR genes in a specific order (58). Mice deficient of RAG1 or RAG2 gene lack B and T lymphocytes because of their inability to rearrange their Ig and T cell receptor (TCR) genes (59, 60). Ig variable region gene recombination, by intrachromosomal deletion, is known to be an ordered process in which remodeling of *Igh* loci precedes *Igκ*, and then *Igλ* loci. V_H and J_H segments are both flanked with 23 bp RSSs, preventing them from direct recombination. It has been noted that D-to-J_H rearrangements generally occur as the first step of Ig recombination on both chromosomes, followed by V_H-to-DJ_H rearrangements on one of the two alleles (39, 41, 61). Although V_H to D rearrangement in the absence of D to J_H joining is possible according to the 12/23 rule, this is not observed in developing B cells (62). In addition, D-to-J_H joints were observed in developing T lymphocytes, whereas V_H-to-DJ_H joints were not found in T lymphocytes (63). Taken together, it has been suggested that the V_H to DJ_H recombination is a

step that is regulated in the context of lineage specificity and allelic exclusion (also see section 1.5) (44, 62).

Although the mechanisms of ordered, allelic excluded Ig gene rearrangement are not fully understood, an accessibility model has been proposed to explain the lineage and stage specific recombination of Ig and TCR genes, with controlled/restricted accessibility of Rag proteins to RSSs of the V, (D), and J gene segments (39, 42, 51, 64, 65). The hypothesis of accessibility model was based on the observation that germline transcription of unrearranged Ig gene segments occurred prior to V(D)J recombination, making the genes "open" and accessible to the recombination machinery (35, 39, 51, 66). It is well known that *Igh* genes are rearranged in pro-B cells but not in pre-B cells when *Igl* variable region genes are assembled. Correspondingly, the murine germline V_H gene segments are found to be transcribed and produce germline transcripts (noncoding and unrearranged) only in pro-B cells that are actively undergoing V_H to DJ_H joining, but not in B cells at other stages where there is no V_H to DJ_H recombination occurring (51, 64). This concomitant of *Igh* gene recombination and germline transcription was also described during *Igl* gene recombination (67), suggesting an open chromatin structure required and provided for efficient Rag binding to RSSs (66). It has been proposed that the germline transcription at Ig loci leads to supercoiling which is necessary for RSS recognition by Rag1/2 proteins (66).

A lot of studies have been done to uncover the roles of germline transcript in the class switch recombination. It has been shown that germline transcripts upstream of C_H genes aid to recruit the activation-induced cytidine deaminase (AID) to the DNA strand (68, 69). Accordingly, a similar mechanism might be applied for Ig gene rearrangements, to make the RSSs more

accessible for Rag proteins. An indirect evidence for the importance of germline transcripts in DNA recombination is that upon deletion of the promoter to prevent the germline transcription from the 5' of Dβ1 on the β locus of TCR (comparable to the μ0 transcript of the *Igh* locus, also see section 1.3.7), the rearrangement of the Dβ1 segment is destroyed, due to the diminished cleavage of RSS by Rag proteins (70). In general, these studies support a model that germline transcription of the Ig loci, as the initiation of V(D)J recombination, plays a role in regulating the accessibility of Rag to the recombining DNA sequences. In addition to the study of germline transcription according to the accessibility model, a number of chromatin modifications first identified as features of an "open" chromatin structure, correlate with V(D)J recombination accessibility at the corresponding locus. For example, over the past decades, it has been demonstrated that Ig genes accessible for recombination display chromatin modifications (histone acetylation or DNA demethylation) that are characteristics for active genes, during the early stages of B cell development (71-76) (also discussed in section 1.3.6). Further work needs to be done to elucidate whether germline transcription and its associated chromatin modifications are a cause or effect of DNA rearrangement of Ig loci.

Enhancers and promoters throughout the Ig loci play a critical role in controlling the accessibility of the gene segments on the Ig loci. They have been implicated in making the neighboring chromatin in a Rag-accessible configuration (77). The *Igh* intronic enhancer Eμ is known to be critical for V_HDJ_H recombination. Deletion of Eμ substantially impairs D-to-J_H and V_H-to-DJ_H rearrangements (78, 79). The MARs that flank the core region of Eμ are dispensiable for V_HDJ_H recombination (80), and deletion of the hs3a, hs1,2 or hs3/hs4 regions of the 3'RR on *Igh* seems not to affect V_HDJ_H recombination (81, 82) (also see section 1.9). Rearrangement at the Igκ locus was impaired but not abolished by the deletion of iEκ or the 3' Eκ alone (83, 84). But deletion of

both abrogated Igκ rearrangement (85). The controlled accessibility of Ig gene segments to Rag protein mediates DNA cleavage and recombination at only the appropriate time and place. Roles and mechanisms of these regulatory elements (enhancers, promoters, etc) in V(D)J recombination are still under active investigation.

1.3.7 Germline transcripts

Before D-to-J_H gene recombination on the *Igh* locus, germline transcription initiates from the promoter of the 3' most D gene segment (D_{Q52}) and gets spliced to the Cμ region to generate the germline transcript μ0 (39, 86), and the germline transcript Iμ initiating at Eμ also gets spliced to the Cμ region (87). Similar to μ0 and Iμ, germline V_H gene segments are transcribed in the sense direction from the V_H promoters before the onset of V_H to DJ_H gene recombination. It should be noted that these V_H promoters also initiate the transcription of the recombined V_HDJ_H exon (88, 89). The germline V_H transcription is silenced once a productive V_HDJ_H rearrangement is produced (51, 90). Introduction of a pre-rearranged μ transgene resulted in a decrease in the endogenous V_H germline transcript and a decrease in V_HDJ_H recombination on the endogenouse *Igh* locus (91). An ~ 120 kb domain which includes the D gene segments and extends to the Cμ exons was found to be hyperacetylated prior to the initiation of D-to-J_H recombination, indicating an active form of *Igh* genes for transcription (where μ0 and Iμ transcription occurs). However, germline V_H genes were not hyperacetylated at this stage but were transcriptionally active at the subsequent stage when V_H-to-DJ_H recombination occurs. This is consistent with the mechanism for the sequential recombination of D-to-J_H gene segments, followed by V_H-to-DJ_H recombination (92). It remains to be investigated whether accessibility depends on germline

transcripts, the process of germline transcription, or chromatin modification that accompany the transcription process (93).

There are also two germline transcripts described for murine Cκ on the κ light chain locus (94, 95). One of the gemline transcripts initiates 3.5 kb upstream of the 1st Jκ gene segment (Jκ1), which leads to a 8.4 kb primary transcript but produces a processed germline transcript of 1.1 kb (94, 96, 97). The other transcript initiates 50-100 bp upstream of the Jκ gene cluster, producing a 4.7 kb transcript which is also processed, resulting in a 0.8 kb processed product (98). Disruption of these transcripts by deleting the promoters led to dramatic reduction in the frequency of DNA rearrangement at the targeted κ locus (99, 100). To date, less about λ locus germline transcripts has been identified. Known are germline transcripts initiating from the 5' of the corresponding Jλ gene segments on each of the three functional JλCλ clusters and these transcripts are spliced to the Cλ segment (101, 102). Germline transcripts of the JλCλ clusters were detectable two days later than the JκCκ transcripts in vitro, and this difference was also observed in vivo: germline transcripts of κ loci were found in large pre-B cells whereas germline transcripts of λ loci were first seen at the later developmental stage of small pre-B cells. This differential activation of κ and λ loci is suggested to be one of the decisive factors for the sequential recombination of κ and λ light chain genes: κ first and then λ, and for the 10:1 ratio of κ: λ present in murine.

These murine germline transcripts are found at low abundance and there are either no or multiple start/stop sites in all three reading frames of these transcripts, suggesting that they do not encode proteins, such that they are also called "sterile" transcripts (94, 102). Despite the positive correlation between levels of germline transcripts and the efficiency of V(D)J recombination (103, 104), the definitive role of germline transcript/transcription in V(D)J recombination is still

under investigation and remains debated (105). For example, evidence for the correlation between *Igh* germline transcript and V_HDJ_H recombination includes the finding that promoter of D_{Q52} turns to be active to generate the μ0 transcript before the D-to-J_H recombination (86, 106, 107), and absence of μ0 results in complete loss of D-to-J_H gene rearrangement (79). However, impairment of Iμ germline transcription has no clear effect on V_HDJ_H recombination (108).

1.4. B cell development stages

Murine B lymphocytes develop from progenitor cells in the fetal liver and in the bone marrow (BM) after birth, through sequential rearrangements of *Igh* and *lgl* chain genes (109, 110). Successful expression of the IgH and IgL chains on the cell surface is required for B cell development (109) (Figure 1.8). The progressive stages of B cell development are well defined on the basis of patterns of *Igh* and *Igl* gene rearrangements and expression, as well as the expression of various cell surface markers (110) (Table 1-1).

Figure 1.8: Flow chart of B cell development in mice.

B cells develop from HSC to transitional B cells in the bone marrow, and then exit and enter the periphery, where they undergo further maturation to memory and Ig-secreting B cells upon activation. Stages of developing B cells are labeled under the cells with corresponding surface receptor (pre-BCR, BCR) expression. Naïve mature B cells in the germinal center may differentiate into plasma cells or memory B cells during the process of affinity maturation. Most of cells become plasmablasts, and eventually plasma cells producing Ig proteins.

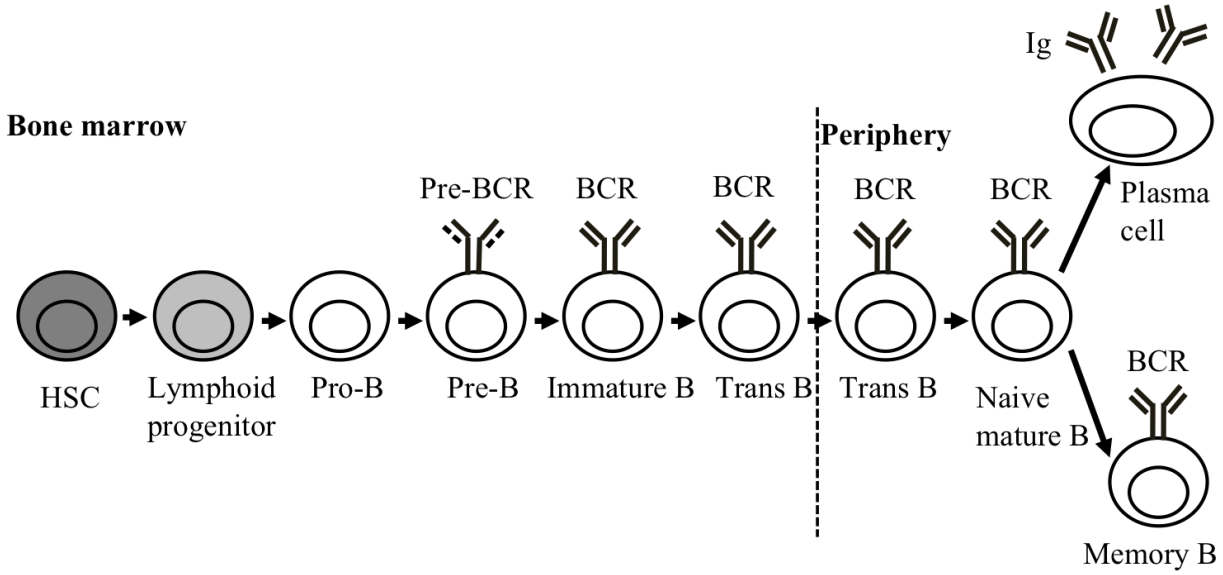


Table 1-1: Cell surface markers expressed on B cells at different stages.

The progressive stages of B cell development are well defined on the basis of expression of various cell surface molecules.

Stage	**Location**	**Cell surface markers**
Pro-B	Bone marrow	$B220^{+}CD43^{+}IgM^{-}$
Pre-B	Bone marrow	$B220^{+}CD43^{-}IgM^{-}$
Immature B	Bone marrow	$B220^{+}IgM^{+}IgD^{-}$ $CD21^{-}CD23^{-}$
Newly formed transitional 1 (NF T1)	Bone marrow	$B220^{+}CD21^{-}CD23^{-}$ $IgM^{+}IgD^{low}$
T2	Spleen	$B220^{+}IgM^{+}CD21^{low}CD23^{+}$
Mature B (FO)	Spleen	$B220^{+}IgM^{+}IgD^{+}$ ($CD21^{+}CD23^{+}$)
Marginal zone B	Spleen	$B220^{+}IgM^{+}CD21^{+}CD23^{low}$

From HSC to lymphoid progenitor, then to B lineage cells

Hematopoietic stem cells (HCS) are self-renewing and pluripotent. HSCs differentiate into IL-$7R^+$ common lymphoid progenitors (CLP) that give rise to B and T lymphocytes, as well as natural killer (NK) cells and dendritic cells (DC). B lineage cells are characterized by surface CD45R/B220 expression. The commitment to the B lineage from CLP is also marked by other processes, such as the expression of signaling molecules Igα and Igβ, surrogate light chain (SLC, including VpreB and λ5 proteins), and germline transcripts of heavy chain locus, etc. This commitment is mediated by a series of transcription factors (111, 112). For example, PU.1 protein is one of the well-known transcription factors, which controls IL-7R expression and thus controls the emergence of B lineage cells (113). The B-cell specific activator protein Pax5 not only activates B cell specific genes including CD19, BLNK, etc (114), but also represses alternative cell fates (115-118).

Pro-B cell stage

In the bone marrow, stromal cells secrete cytokines (such as IL-7) to induce TdT and RAG gene expression in lymphoid progenitor cells ($CD34^+$). These cells undergo D-to-J_H joining on the heavy chain loci (often on both alleles) and start expressing B220 ($CD45^+$) and class II MHC molecules as early pro-B cells. Subsequently, V_H segments recombine to the joined DJ_H on one of the two alleles, which completes the late pro-B cell stage.

Because of the diverse coding ends generated during the recombination, the resulting transcription unit may not be in frame. If the recombined V_HDJ_H sequence is non-productive, failing in synthesis of a heavy chain protein, V_H-to-DJ_H assembly is attempted on the second *Igh*

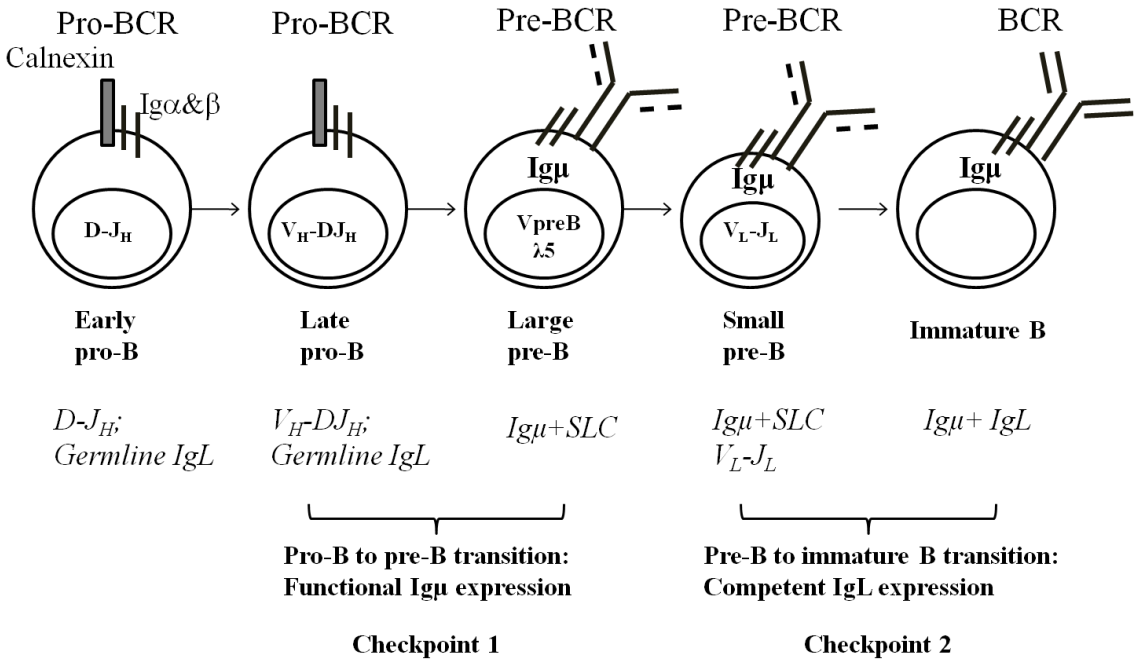
allele. Failure in these two opportunities results in cell death. B cells eventually migrate from the outer part of the bone marrow to the core as the development goes on.

Pre-B cell stage

When the recombined V_HDJ_H genes are expressed as membrane μ chains and assemble with surrogate light chain proteins (SLC, include VpreB and λ5 proteins) to form pre-B cell receptors (pre-BCR) on the cell surface, pro-B cells become pre-B cells (Figure 1.9). SLC resembles the real light chains but are invariant in all pre-B cells. The pre-BCR signals to shut down additional V_HDJ_H rearrangement, so that only one functional heavy chain is expressed, a phenomenon known as allelic exclusion (39) (see section 1.5). TdT and RAG expression is down-regulated for this "feedback-inhibition" process. Now these large pre-B cells actively divide into a clone of B cells with identical μ chains. After several rounds of proliferation, cells turn into the resting small pre-B cells and start V_L-to-J_L joining on the light chain loci by re-expressing the RAG1/2 genes. Meanwhile, SLC levels decrease and IL-7R is no longer expressed. Light chains are also expressed in the allelic excluded manner, with κ chain expression preceding λ chain expression.

Figure 1.9: Expression of B cell receptors on developing B cells in the bone marrow.

B cell receptor expression on surfaces of B cells at various differentiation stages, from pro-B cell stage through immature B cell stage in the bone marrow. Dashed lines = surrogate light chains; signaling subunit Igα/β as indicated. B cell receptors are tested at two checkpoints for cells progress to the next stage.



Immature B cell stage

Once IgL chains are successfully expressed, they replace the SLC proteins and assemble with μ chains on the surface as IgM molecules, marking the immature B cell stage. Immature B cells are sensitive to antigen binding. If bound to self antigens in the bone marrow, the cells are negatively selected and undergo apoptosis, or can be rescued by receptor editing (see section 1.6). Further rearrangements at the light chain loci replace the auto-reactive specificity with a new functional, but non-self-reactive B cell receptor, allowing further maturation of these cells (119). Immature B cells that do not bind to self antigens pass the negative selection process. They develop into transitional B cells of type 1 (T1) as a precursor to transitional B cells of type 2 (T2, the cycling immigrants from the bone marrow to the spleen).

Mature B cell stage

Transitional B cells are the linker between BM immature B and the peripheral mature B cells. B cells mature in the spleen and co-express IgM and IgD (120). B cells are now mature naive resting cells, and can be activated in the periphery by foreign antigens and differentiate into Ig-secreting plasma cells and memory B cells which respond more readily and quickly to the future exposure to the same antigens. B cells that fail to mature during development undergo apoptosis. $IgM^{+}IgD^{+}$ B cells in the bone marrow are re-circulating mature cells from the periphery (110). The Ig loci undergo further DNA modifications including CSR and SHM (Figure 1.7 and sections 1.3.5) (beyond the V(D)J recombination in pro- and pre-B cells), as the B cells mature.

There are different types of mature B cells in mice. B-1 B cells develop from fetal precursors. They are self-renewing cells mainly in the peritoneal and pleural cavities. B-1 B cells secrete

"natural" antibodies against blood-borne antigens (121). Marginal zone (MZ) B cells in mice are noncirculating, self-repleshing mature B cells in the spleen. They share with B-1 B cells the feature of self-replenishment and capacity of quick response to antigens (122), and are capable of participating in early adaptive immune responses in a T cell independent manner, similar to B-1 B cells (122). These cells localize at the border between the white and red pulp of the spleen. Follicular (FO) B cells and MZ B cells are both B-2 B cells, which develop from progenitor cells in the bone marrow, representing most of the mature recirculating B cells and reside in follicles of secondary lymphoid organs (123).

FO B cells, comprising the majority of the recirculating B cells, respond to antigens in both T-cell dependent and T-cell independent manners (123). Antigen recognition by FO B cells initiates T-cell dependent immune responses in the germinal centers of secondary lymphoid organs. B cells proliferate actively in the germinal centers with somatic hympermutation (SHM), resulting in modified antigen-specific B cells with higher affinities. Class switch recombination (CSR) is another process occurring after the activation of mature B cells via BCR, to generate antibodies of a secondary isotype, with a different effector function (see section 1.3.5, Figure 1.7).

In the current, we aim to further identify the control of B cell differentiation, especially at the pre-B to immature B cell transition.

1.5. Allelic exclusion

B cells expressing functional IgH and IgL chains are able to pair them and express an Ig molecule on the cell surface. Theoretically, there is potential for a B cell to express two distinct

functional V_HDJ_H and four productive V_LJ_L rearrangements (one on each chromosome of Igκ and Igλ loci). Nevertheless, this is not what is observed in B lymphocytes. Each mature B cell contains only one productively rearranged V_HDJ_H and one productively rearranged V_LJ_L, and expresses only a single type of IgH/IgL combination. This unique feature of B lymphocytes (also applies to T lymphocytes) is reffered to as allelic exclusion (67, 124). According to the clonal selection theory, each B lymphocyte expresses a single-specificity antigen receptor on the cell surface so that the BCR repertoire is managed at the single cell level, allowing for an effective and efficient selection of the B cells with surface receptors (cells with protective receptors maintained, cells with autoreactive receptors eliminated) and a more rapid antibody response upon antigen recognition (17).

As discussed before (section 1.3.4), rearrangement of *Igh*, *Igκ* and *Igλ* genes in developing B cells is a strictly ordered process. D and J_H gene segments are recombined first in pro-B cells on both the *Igh* loci, followed by V_H-to-DJ_H recombination on one of the two alleles. A complete V_HDJ_H exon encodes the variable region of the IgH protein. Only if the rearrangement on the first allele is non-functional (out of frame or unable to pair with SLC as a pre-B cell receptor), cells proceed to recombine the second, alternative *Igh* allele, leading to allelically excluded expression of the heavy chain. Similarly, following IgH expression, only one allele of the light chain genes is productively rearranged and expressed to assemble with the IgH protein.

A regulated model of allelic exclusion has been proposed (125), according to which, allelic exclusion is established because the initiation of V(D)J recombination of antigen receptor genes occurs on one allele at a time (67). Asynchronous allele opening for germline transcription and DNA/histone modification is thus suggested as the first step to regulate the mono-allelic

initiation of DNA recombination (124) (discussed in section 1.3.6, 1.3.7). The resultant functional receptors from the first allele inhibit V(D)J recombination on the second allele, a mechanism proposed to enforce allelic exclusion called feedback inhibition (discussed in the next paragraph). In these contexts, the regulated model for allelic exclusion includes two phases: initiation and maintainance. Mono-allelic initiation ensures allelic exclusion before the feedback signals (39). Because both *Igh* alleles are rearranged in the form of DJ_H in B lineage cells, V_H to DJ_H recombination is the allelically excluded step in *Igh* locus recombination (39).

Many studies have shown that once a functional V(D)J recombination has occurred on one allele, DNA recombination on the second allele is inhibitied, a process called "feedback inhibition" (Figure 1.10) (62, 125, 126). This "feedback" model is based on the premise that one allele recombines first and is tested for productivity and functionality (i.e. whether a recombined V_HDJ_H can be translated to an Igμ protein, and whether this Igμ protein is able to pair with SLC), before the other allele is rearranged (125). According to the feedback inhibition model, functional Ig gene expression leads to assembly of pre-BCR or BCR to signal inhibition of additional Ig gene recombination through cessation of RAG gene expression or rendering Rag proteins inaccessible to the Ig loci, or silencing the germline gene segments (67, 124). In the case of the IgH chain, productively expressed IgH chain (Igμ protein) assembles with SLC and the signaling subunit Igα/β heterodimer, to "signal" suppression of allelic recombination (e.g. shut down expression of the RAG genes) (67, 127-129). But a growing number of evidences have suggested that, in fact, SLC is not required for this "feedback inhibition". With deletion of the known SLC elements (VpreB, λ5), allelic exclusion appears to be normal (130, 131). Rather, only productive membrane Igμ expression and Igα/β signaling molecules are crucial to the maintainance of intact IgH allelic exclusion (130-133).

Assuming that ~1/3 of V(D)J recombinations are productive (in-frame), this regulated model through feedback inhibition explains the 60/40 ratio (134): the observation that 60% of the peripheral mature B cells have successful V_HDJ_H recombination on the first allele, and with DJ_H configuration on the second allele, whereas the other 40% cells have their *Igh* loci in the V_HDJ_H (x)/V_HDJ_H configuration (x= unproductive). The ratio of VκJκ/κ0 to VκJκ (x)/VκJκ cells is also around 60/40 in the periphery (κ0= germline transcript) (135), suggesting that the feedback inhibition model also applies to Ig light chain gene rearrangement.

However, there is still possibility for the presence of more than one productively rearranged IgH or IgL loci in a single, mature B cell, which is called allelic inclusion. Allelic inclusion is more often seen in Ig light chains than Ig heavy chains (136-139). Several studies reported IgL (κ or λ) double-expressing B cells at low frequency in normal mice and human or at a higher frequency in transgenic mice (140-142). It should be noted that, even when a cell makes light chains from two different *Igl* alleles, the cell often expresses only one type of Ig receptor on the cell surface. This is because only one of the light chains successfully pairs with the available heavy chain.

In summary, although actively studied during the past few years, mechanisms of allelic exclusion are still enigmatic. For example, the Ig locus transcriptional enhancers and promoters are known to be required for V(D)J recombination, however, how these *cis*-elements direct the DNA recombination process and their potential roles in allelic exclusion remain unclear (124). While we know that epigenetic marks at Ig loci (e.g. germline transcription, accessibility to Rags, active histone marks, etc) correlate with the strictly regulated monoallelic V(D)J recombination, the mechanisms of regulation at molecular levels are the next to be identified. It has been shown that all the Ig loci (*Igh, Igκ* and *Igλ*) replicate asynchronously, which is established randomly in early

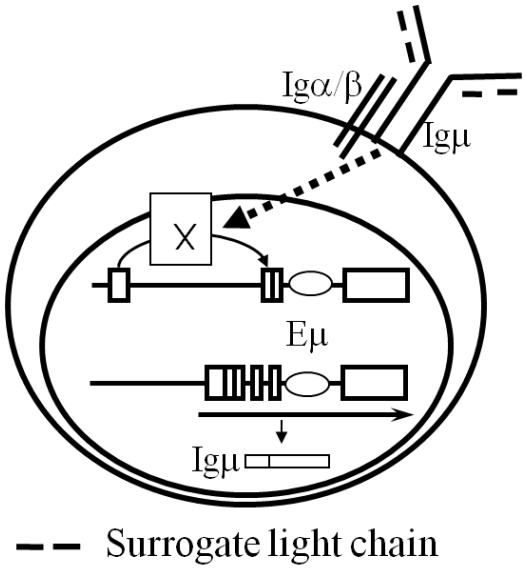
embryogenesis and maintained as an epigenetic mark (67, 143). The early-replicating *Igκ* allele is the one that is epigenetically marked as the first allele for Vκ to Jκ recombination. However, both *Igh* loci are of the DJ_H configuration in pro-B cells, suggesting an active status for V_H to DJ_H recombination at both loci. The definitive conclusions for the selection of one allele for monoallelic onset of V_H to DJ_H recombination still require expremental proofs.

Another question emerges after the finding of independence of IgH allelic exclusion on SLC. Although SLC is necessary for signaling cells to proliferate and differentate from pro-B to the pre-B cell stage, it is not required for signals that shut off further rearrangement on the second *Igh* allele. An unanswered question is what is the nature of this SLC-independent signal and how does it differ from that of the pre-BCR with SLC?

As mentioned earlier, cells can carry two productively rearranged heavy or light chain loci (V_HDJ_H/V_HDJ_H or VκJκ/VκJκ, or coexpress Igκ and Igλ chains). In some of these cells, one Ig allele encodes a μ or κ chain incapable of forming a surface expressed pre-BCR or BCR, such that allelic included Ig gene recombination display allelic exclusion at the level of surface receptor expression (132). However, in some cases, these allelic included Ig recombination results in peripheral B cells with two different IgH or IgL chains on the cell surface (more often seen in light chains) (137, 141, 142, 144, 145), suggesting that allelic exclusion prevalent in normal B cell development is not absolutely ensured nor 100% required for normal B cell development. Although such cells are rare, reasons for the escape from the stringent monoallelic initiation and feedback inhibition model for allelic exclusion need further studies.

Figure 1.10: Model for feedback inhibition of V_H-to-DJ_H recombination.

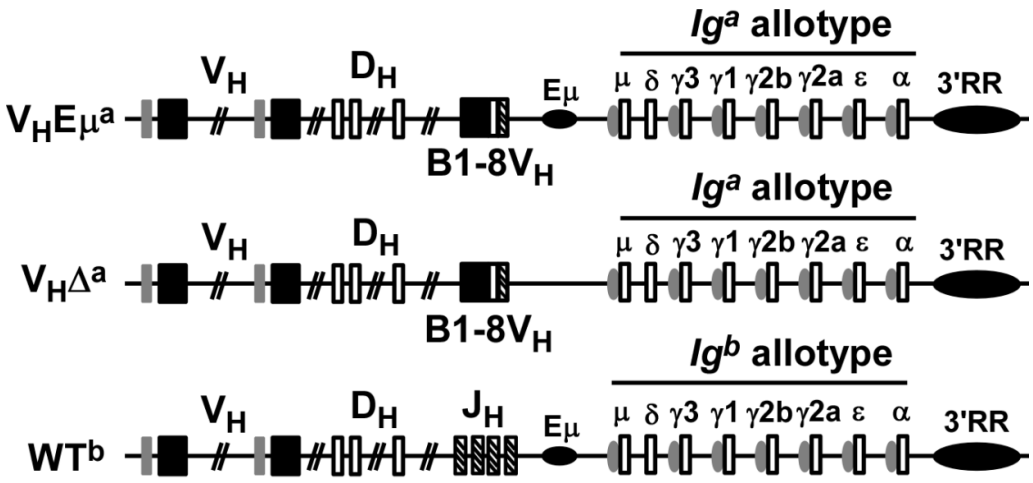
Igh locus rearrangement is ordered through the feedback regulation mechanism. Both *Igh* alleles are rearranged in pro-B cells in the form of DJ_H rearrangement, V_H to DJ_H recombination is the step for feedback regulation. Pro-B cells that have made a productive V_HDJ_H rearrangement on the first attempt do not initiate V_H to DJ_H recombination on the second allele, with a freezed DJ_H on the second allele. This is achieved through the feedback signals from the functionally expressed Igμ molecules and Igα/β signaling subunits. This model predicts that if the cells make a nonfunctional V_HDJ_H recombination on the first allele, they would rearrange V_H to DJ_H on the second allele, leading to mature B cells carrying V_HDJ_H rearrangements on both alleles (one productive and one nonproductive).



This current study attempts to address the mechanism for the maintainance of Ig heavy chain allelic exclusion at the pre-B to immature B cell transition, by heavy chain locus intronic enhancer Eμ. A recent study on a mouse line that is heterozygous for a pre-assembled heavy chain variable region (V_H) gene (B1-8V_H) but lacked Eμ ($V_H\Delta^a/WT^b$, Figure 1.11), showed a pronounced defect in allelic exclusion in both the immature and mature B cell pools (146). Approximately 20% splenic B cells in these heterozygous mice expressed Igμ from both $V_H\Delta^a$ and WT^b *Igh* alleles as heavy chain "double-producers". These IgH-"double-producers" were absent in the matched mouse line with the same B1-8V_H knock-in gene but retained Eμ ($V_HE\mu^a/WT^b$, Figure 1.11). This defect was not due to an inefficient feedback inhibition of *Igh* gene assembly in the $V_H\Delta^a/WT^b$ pre-B cells. In fact, both the $V_H\Delta^a$ and $V_HE\mu^a$ alleles strongly inhibited V_H-to-DJ_H assembly on the WT^b allele. But neither allele operated this feedback inhibition completely (i.e. neither allele was successful in preventing V_HDJ_H rearrangement on the second allele in every B lineage cell). Rather, there were rare but equal levels of precursor cells that circumvented this inhibition and expressed both the IgH alleles in the pre-B cell populations of both mouse strains. Nevertheless, these gave rise to IgH-double-producing immature B cells only in the $V_H\Delta^a/WT^b$ mice but not in the $V_HE\mu^a/WT^b$ mice (146). Accordingly, we postulate that it takes some time for assembly of the pre-BCR subsequent to heavy chain variable region gene assembly. During this process of assembly, V_H to DJ_H gene rearrangement takes place on the second allele in a subset of pre-B cells. Therefore, we proposed that there is a second "check-point" for heavy chain allelic exclusion operating after and distinct from the feedback inhibition, which occurs at the pre-B to immature B cell transition and is dependent upon the intronic enhancer Eμ (see section 1.9.1) (146).

Figure 1.11: Diagram of wild-type (WT^b) and B1-8V_H knock-in loci with and without Eμ (designated $V_HE\mu^a$ and $V_H\Delta^a$, respectively).

Exons are shown as boxes, enhancers are indicated as horizontal black ovals (Eμ; 3' regulatory region = 3'RR), "switch" sequences (sites of class switch recombination) upstream of the constant region genes are indicated as vertical ovals, constant regions are labeled (e.g. μ, δ, etc.). B1-8V_H is a pre-assembled V_H gene that is inserted in place of the joining gene (J_H) region. As indicated, the constant region genes in the knock-in loci are of the Ig^a allotype while they are of the Ig^b allotype in the WT locus. The WT^b allele in the heterozygotes is in the germline configuration, but if functionally rearranged, it would produce an Igμ^b chain distinguishable from that produced by the knock-in loci (Igμ^a).



1.6. Positive and negative selection of B cells

Developing B lymphocytes produce a highly diverse Ig/BCR repertoire essential for the defense against foreign pathogens. This diversity is achieved through random V(D)J gene recombination and IgH/IgL assembly. These processes simultaneously lead to the generation of B cells with null or self-reactive receptors that need to be removed from the repertoire by positive or negative selection, respectively. Positive selection is the process that has a minimum signaling requirement through the antigen receptors for the cell to survive and differentiate to the next stage (147). The BCR signals dictate the likelihood of nascent B cells to continuously differentiate and completely mature. B cells with adequate BCR signaling pass the positive selection process, so that a robust and protective mature B cell pool is established and maintained (147).

During the process of maturation in the bone marrow and periphery, B cells are also tested for interaction with self-antigens, which is known as negative selection, to avoid production of B cells that may cause autoimmune diseases. B cells expressing receptors that bind to self antigens are negatively selected at the immature B cell stage. These selection processes also occur in the secondary lymphoid tissues to further insure a protective but non-autoreactive antibody repertoire. Currently well known negative selection mechanisms include clonal deletion, receptor editing, and anergy (148). Within the $IgM^{+}IgD^{-}$ immature B cell compartment, if the maturing B cells strongly interact with multivalent self antigens in the bone marrow, they undergo apoptosis and are clonally deleted. Those that recognize and bind to soluble self antigens are not killed, and instead, they move to the periphery and become anergic expressing low levels of IgM. Others that do not react to self antigens migrate to the periphery, expressing normal levels of IgM and

IgD, which are mature naïve B cells that might fight against their cognate antigens. Auto-reactive B cells may avoid apoptosis or anergy by modifying their receptor specificities, through secondary somatic Ig gene recombination to express new IgH and IgL combinations which replace the original specificity and become non-autoreactive. This is the mechanism called receptor editing, rescuing self-specific B cells to establish central tolerance (discussed in section 1.8).

1.7. B cell receptor signaling

To achieve a diverse functionally competent, but non-autoimmune antibody repertoire, B cells undergo sequential gene expression and membrane assembly of receptors (pro-BCR, pre-BCR and BCR) to initiate the intracellular signaling pathways, which set thresholds for several selection checkpoints as B cells progress to the next stage. These signaling complexes all consist of an Igα/β heterodimer, carrying an immunoreceptor tyrosine-based activation motif (ITAM) in their intracellular domain. B cell antigen receptor mediated signaling is required throughout B cell development, peripheral maturation and repertoire selection (149).

1.7.1 Pre-BCR signals

The Igα/β signaling heterodimers associate with protein calnexin on the plasma membrane of pro-B cells. It has been proposed that this complex constitutes a pro-B-cell receptor that elicits intracellular differentiation signals for V_HDJ_H gene assembly (150). Igμ is the product of the successfully recombined heavy chain gene (IgH). It assembles with the surrogate light chain (SLC) proteins (λ5 and VpreB), and then with the signaling subunit Igα/Igβ to form a pre-B cell antigen receptor complex (pre-BCR) on the pre-B cell membrane (151-153). The ability of Igμ

chain to pair with the SLC is a quality control parameter to assess the μ chain for successive B cell differentiation. This includes clonal expansion of $Ig\mu^+$ pre-B cells and allelic excluded expression of heavy chain loci. Monitored by the pre-BCR signaling, pre-B cells subsequently recombine their light chain genes and further differentiate to the immature B cell stage with surface IgM expression.

Two checkpoints directed by pre-BCR have been defined during B cell development in the bone morrow. The 1st one is for a functional heavy chain for B cells to progress to the pre-B cell stage, and the 2nd one ensures a competent light chain for pre-B cells to differentiate into immature B cells (Figure 1.9) (154). Through activation and recruitment of a series of kinases, pre-BCRs (similar to BCR, discussed below) triggers a phosphorylation cascade to signal the developmental progression of pro- and pre-B cells, and hence the cells' ability to progress to the immature B cell stage is tested at these two checkpoints.

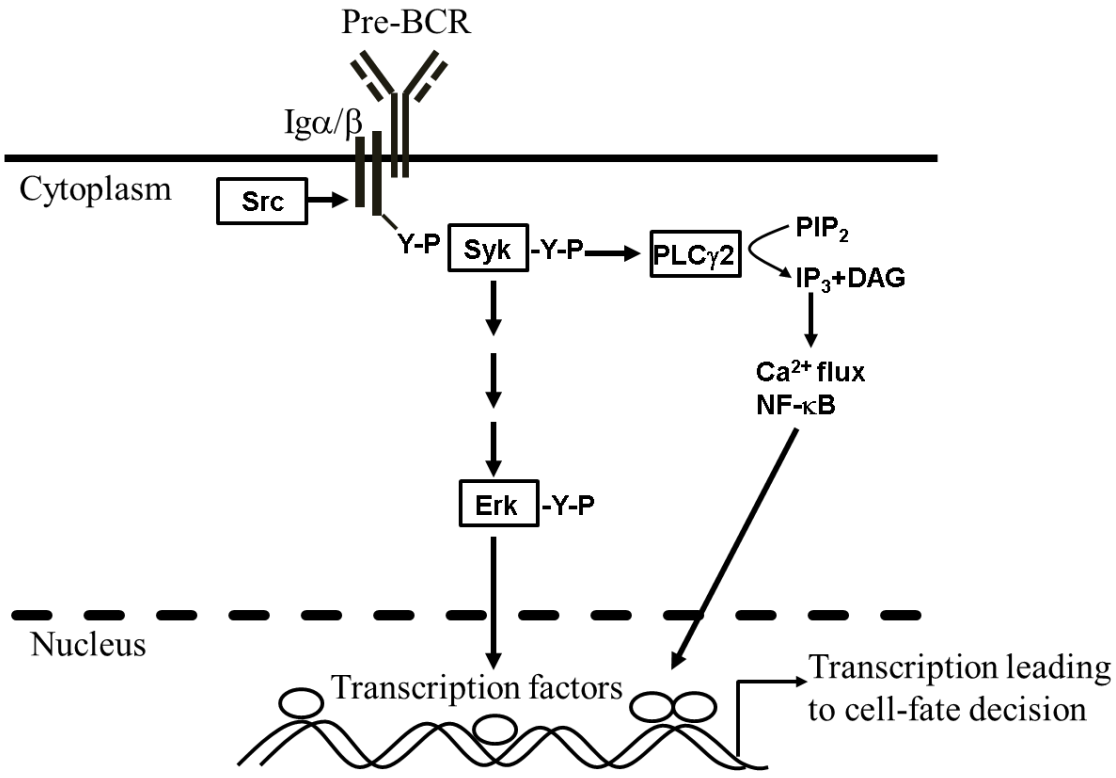
1.7.2 BCR signals

Rearranged immunoglobulin light chains (IgL) replace SLCs and form B cell receptors (BCRs), which define the immature B cell stage (149, 154, 155). To avoid autoimmunity, newly-emerging B cells with self-reactive BCRs are subject to negative selection. Cross-linking the BCRs with self-antigen initiates cell signals that lead to cell death, anergy or light-chain "editing" which replaces the autoreactive BCR with an innocuous one (139, 156, 157) (discussed in section 1.6). On the other hand, tonic (or basal) signaling through the pre-BCR and BCR, independent of ligand-binding, is needed to select B cells with functional receptors (positive selection), promoting B cell maturation (158-160).

Pre-BCR and BCR send out tonic signals in the form of tyrosine phosphorylation. Their signaling capacities are essential for the continuing B cell survival and maturation. Different status of protein tyrosine phosphorylation evokes differential cellular responses and determines the cell fates (149, 161) (Figure 1.12).

Figure 1.12: Pre-BCR mediated signaling model.

The pre-BCR is composed of membrane Igμ molecules, SLC, and associated Igα/β heterodimers. Activation of pre-BCR induces coordinated activation of various signaling cascades. Central to this involves phosphorylation of ITAMs (immunoreceptor tyrosine-based activation motifs) in the cytoplasmic tails of Igα/β scaffold proteins, mediated by Src-family protein tyrosine kinases. Phosphorylated ITAMs recruit and activate Syk tyrosine kinase, which then activates multiple signaling pathways including: Erk pathway, PLCγ2 pathway, etc. These pre-BCR-dependent pathways collectively regulate the transcriptional events to direct B cell fate decisions.



BCR signaling pathways start from receptors composed of both a signaling (Igα/Igβ heterodimer) and an antigen-binding (V_H/V_L of an Ig molecule) subunit. Antigen-mediated cross-linking of the antigen-binding moiety on Ig molecules is one way to initiate signal transduction into the cytoplasm of the cell, by way of the long cytoplasmic tails of the Igα/Igβ chains which associate with the Ig molecules. Pre-BCR signaling, however, does not always require exogenous ligands. Whether ligand-dependent or ligand-independent, activation of B cells through pre-BCR/BCR requires coordination of many different signal transduction pathways instead of any single transducer. One of the final outcomes of receptor signaling is stimulated or inhibited (or up- or down-regulated) transcription of the target genes, which, in turn, controls the intracellular events (such as DNA recombination) and the cellular activities (such as differentiation, proliferation). Defective signal transduction may have severe effects on the development of B cells and the immune system (149, 158-164).

Ligand-independent activation of pre-BCR/BCR (i.e. tonic signaling) involves phosphorylation of tyrosine residues on ITAMs within the cytoplasmic domains of Igα/β cluster by Src-family protein tyrosine kinases (PTKs)(160) (Figure 1.12). These Src-family PTKs are associated with the receptors, including Lyn, Blk and Fyn in B cells. Phosphorylated ITAMs then provide docking sites for other PTKs to form a receptor signaling complex which triggers further downstream signaling events (165, 166). These include recruitment and activation of Syk, which is a vital PTK for B cell development (167). Activated Syk binding to the ITAM then activates a series of signaling proteins such as the adaptor protein BLNK (B-cell-linker protein), Btk (Bruton's tyrosine kinase), etc (168). These initial signaling events provide a scaffold for the assembly of essential elements of the signaling complex. Syk phosphorylated BLNK is joined by Btk, and Btk activates phospholipase C γ2 (PLCγ2) to induce calcium flux and protein kinase C

(PKC) activation (169). It binds to Btk and PLCγ2 to mediate the activation of PLCγ2. BLNK is also necessary for activation of other signaling molecules such as Grb2, etc. Grb2 activates the extracellular signal-regulated kinase (ERK) pathway, which participates in the processes of cell expansion and differentiation (161, 170). BLNK is required for pre-BCR-mediated B cell development and BCR-mediated B cell activation (171-173). Src-family tyrosine kinases themselves activate nuclear factor-κB (NF-κB). Reciprocally, serine/threonine kinases negatively regulate ITAM activity by serine/threonine phosphorylation (149, 174). These tyrosine kinases may also be counterbalanced by protein tyrosine phosphatases (PTPs) (175). Therefore, the signaling quality and/or quantity through pre-BCR and BCR depends upon the balance between the activation of the signaling molecules through PTKs and the down-regulation through PTPs (176, 177).

Alterations of BCR signaling components such as Syk or Btk, lead to abnormal B cell development. Reduced BCR expression results in diminished tonic BCR signaling, which significantly impairs immature B cell differentiation to mature B cells (178). Mutation studies of elements that participate in signal transduction from the BCR (Igα, CD45, and Btk), indicate that BCR signaling is indispensible for a diverse and protective B cell repertoire. These together suggest that pre-BCR and BCR signaling is qualitatively and quantitatively regulated in developing cells.

1.8. Receptor editing

Ig genes undergo V(D)J recombination to express a functional antigen receptor, and this is known as the primary somatic gene rearrangement. Receptor editing is the ongoing Ig gene rearrangement in cells that already have an antigen receptor, a process also known as secondary

rearrangement, which may occur on the same chromosome as the primary rearrangement or on the alternative one. Secondary rearrangement not only rescues cells expressing a nonproductive primary V(D)J gene, but also alters the autoreactive specificity of antigen receptors on self-reactive B cells, rescuing them from apoptosis (19, 179-181). About 20-50% of peripheral naïve B cells have undergone receptor editing (182). Therefore, receptor editing acts as an important mechanism for self-tolerance. It also increases the chances for B cells to survive and therefore the efficiency of the production of a robust antibody repertoire.

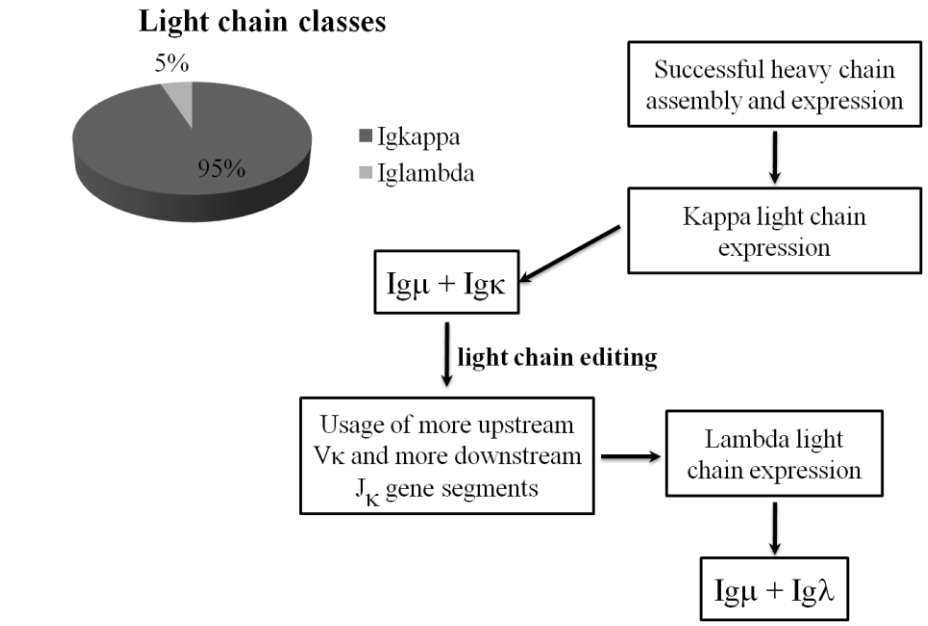
B cell receptor editing involves the same recombination machinery as the primary V(D)J rearrangement (e.g. Rag1 and Rag2 proteins). Receptor editing occurs mainly at the Ig light chain locus (also termed light chain editing), through Vκ-Jκ or Vλ-Jλ secondary rearrangements (179, 182-184). Vκ (Vλ) gene segments can rearrange to available Jκ (Jλ) gene segments by the 12/23 rule. However, for the *Igh* locus, V_H to DJ_H rearrangement deletes all the remaining D gene segments, which are required for V_HDJ_H recombination on the *Igh* loci. Even though V_H to V_HDJ_H recombination occurs by a conserved heptamer sequence in some recombined V_H gene segments (a process called V_H replacement) (185, 186), the frequency of V_H replacement is very much lower than light chain editing. For this reason, light chain editing is referred as a common process to modify BCR specificity. During the process of light chain editing, the rearranged VκJκ gene is replaced by a more upstream Vκ gene segment and a more downstream Jκ gene segment. Failing after multiple attempts on κ allele rearrangement, B cells start to express the λ allele (Figure 1.13). Autoimmune models have shown defective receptor editing, suggesting the importance of this process to prevent autoimmunity (187, 188). It has been noted that light chain editing may not delete the initial autoreactive IgL. Instead, light chain allelically- or isotypically-

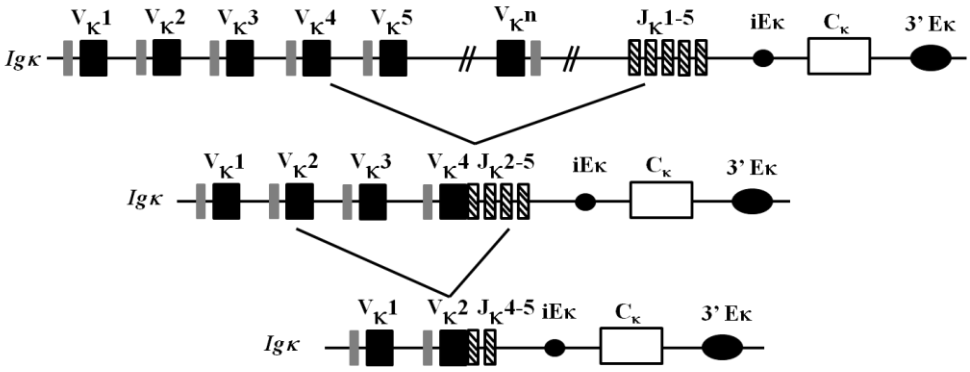
included B cells survive and mature, which may result in re-expression of the autoreactive specificity at the mature, Ig-secreting plasma B cell stage (179).

Figure 1.13: Model for light chain editing.

Upper panel: Diagram for the process of light chain editing during the maturation of B cells. B cells can avoid apoptosis by modifying the light chain Vκ and Jκ genes so as to express a different specificity. If a cell fails to express a functional but non-autoreactive Igκ, it starts to express the lambda light chain. 95% of normal peripheral B cells express the kappa light chain.

Lower panel: Secondary kappa light chain recombination. During the process of light chain editing, the rearranged VκJκ gene is replaced by a more upstream Vκ gene segment and a more downstream Jκ gene segment. All V, J_κ and C_κ gene segments are in germline configurations but not shown to scale. Dark filled boxes = V_κ coding segments; hatched boxes = J_κ coding segments; open boxes = C_κ coding segment. A leader (L) sequence upstream of each V_κ gene segment is shown as a gray box. Not all V_κ genes are shown. Slash marks indicate DNA segments not shown.


Light chain classes
5%
95%
Igkappa
Iglambda
Successful heavy chain assembly and expression
Kappa light chain expression
Igμ + Igκ
light chain editing
Usage of more upstream Vκ and more downstream J$_κ$ gene segments
Lambda light chain expression
Igμ + Igλ


Igκ
V$_κ$1 V$_κ$2 V$_κ$3 V$_κ$4 V$_κ$5 V$_κ$n J$_κ$1-5 iEκ C$_κ$ 3' Eκ
Igκ
V$_κ$1 V$_κ$2 V$_κ$3 V$_κ$4 J$_κ$2-5 iEκ C$_κ$ 3' Eκ
Igκ
V$_κ$1 V$_κ$2 J$_κ$4-5 iEκ C$_κ$ 3' Eκ

1.9. Regulators of *Igh* gene expression

Several *cis*-regulatory elements are identified throughout the *Igh* and *Igl* loci (Figure 1.2, 1.3). On the *Igh* locus, enhancers are located between the J_H and Cμ region and at the 3' end, Eμ and 3'RR respectively. There are promoters at 5' of V_H, D and C_H genes. These *cis*-acting regulators collectively govern Ig gene expression. V(D)J rearrangement is also strictly controlled in a stage and cell-type specific manner by these *cis*-acting regulators, presumably by the accessibility control mechanism as discussed in section 1.3.6. In addition, these *cis*-elements also control the process of CSR and SHM. Rearrangements of the heavy and light chain gene segments generate recombined V(D)J genes coding for IgH and IgL chains. As a consequence of the DNA deletion during this process, *cis*-regulating elements (like promoters and enhancers) are brought together for function. RNA polymerase DNA binds to promoters to initiate mRNA synthesis. Enhancers enhance transcription levels of genes, often by binding *trans*-regulating factors (transcription factors) and bringing these to the promoter region to enhance transcription (189, 190). In addition to the "classic" function attributed to enhancers (to enhance gene transcription), these DNA enhancer sequences appear to have many other functions, as well (discussed below). The regulatory functions of these immunoglobulin enhancers are therefore essential to Ig expression as well as B cell development, and are not yet fully understood.

1.9.1 Heavy chain intronic enhancer Eμ

Eμ is a *cis*-acting transcriptional enhancer lying in the intron region between the J_H and Cμ coding sequences of the *Igh* locus (Figure 1.2). It is a 1 kb fragment consisting of a core region (cEμ, 220 bp with enhancer activity) and two nuclear matrix attachment regions (MARs, facilitating cEμ activity) flanking it (191-193). Eμ has been shown to be essential for

transcription of *Igh* genes in early stage B cells of transgenic animals (192-194). Eμ has also been shown to be required for efficient heavy chain variable region (V_H) gene assembly (78, 103, 195). Targeted deletion of this enhancer region results in reduced D-to-J_H, and V_H-to-DJ_H recombination (78-80, 196). It is the cEμ, but not MARs, that is essential for promoting V_HDJ_H recombination (78-80, 195, 196).

A number of studies have shown that Eμ is active at the pro-B cell stage to enhance V_H gene assembly (85, 197). Replacement of iEκ (the intronic enhancer of *Igκ* locus, Figure 1.3) with Eμ leads to *Igκ* rearrangement in pro-B cells and absence of *Igκ* rearrangement in pre-B cells, suggesting Eμ's specificity of controlling V_HDJ_H recombination at the pro-B cell stage. Further investigation is warranted for the timing and mechanism of Eμ's activity and function in Ig expression and B cell development.

In previous studies, we circumvented the need for Eμ in V_H gene assembly to study its functions after this process. This was done using an Eμ-deficient *Igh*^{a} allele with a pre-assembled heavy chain variable region gene B1-8V_H knocked into the endogenous *Igh* locus ($V_H\Delta^a$, Figure 1.11). In the absence of Eμ, cells with a pre-assembled V_H gene progress to the pre-B cell stage and rearrange the light chain genes, and then eventually mature and populate the periphery. The feedback-inhibition of V_H-to-DJ_H joining on the second *Igh* locus occurs at the same frequency/efficiency as in Eμ-intact pre-B cells (as discussed in section 1.5, Figure 1.10). However, IgH allelic exclusion is compromised in the emerging immature B cells, suggesting a two-step process involved in allelic exclusion and Eμ's function at the second "check-point" of this central mechanism to ensure B cell single specificity (146). In other words, Eμ serves as a mediator of the second "check-point" for allelic exclusion after feedback inhibition. In addition,

as a transcriptional enhancer, Eμ drives heavy chain gene expression in a cell-type specific manner in lymphoid cells (198-200). Deletion of Eμ resulted in half of the wild-type level of Igμ expression in pre-B cells (146). We have proposed that Eμ's role in positive selection at the pre-B to immature B cell transition is likely transcription-level dependent (146).

Studies on Eμ's role in somatic hypermutation (SHM) and class switch recombination (CSR) were first carried out also in the $V_H\Delta^a$ model. Both SHM and CSR took place on the $V_H\Delta^a$ allele, however, at a decreased rate. This decrease was transcription-independent. Instead, Eμ's function in these two important processes for Ig modification is to assist recruitment of the SHM/CSR machinery to the *Igh* loci in *cis* (201).

In general, a model of Eμ's roles in regulating B cell development is that it promotes heavy chain variable region gene assembly, and then enhances the transcription of the newly assembled IgH gene, most importantly at the pre-B cell stage in the bone marrow. At the mature B cell stage in the periphery, Eμ serves to recruit the necessary machinery to the *Igh* locus and regulate its modification.

1.9.2 3' Regulatory Region (3'RR) of heavy chain locus

There is a series of *cis*-elements located downstream of the Cα region, the 3' regulatory region (3'RR), consisting of DNase I hypersensitive sites. Lying away from the rearranged V_H genes, the 3'RR enhances the IgH promoter activity over a long distance. These enhancers don't influence V_H gene rearrangement, but control class switch recombination (CSR) at later stages of B cell development (202, 203). It has been suggested that the enhancer reactivity of 3'RR activates transcription from promoters flanking the S regions of C_H gene segments, and therefore,

regulates CSR (204, 205). In addition, 3'RR is proved to sustain high-level IgH gene transcription in Ig-secreting cells, even in the absence of Eμ (205-210). Loss of the 3'RR in Ig-secreting cells had a dramatic effect on Ig transcription even when Eμ remained on the affected allele (211, 212). These two findings led to the hypothesis that the role of Eμ in promoting IgH transcription could be supplanted by the 3'RR at the plasma-cell stage. Later studies have shown that the 3'RR reaches its highest activity and starts regulating Ig gene expression at immature and mature B cell stages, to ensure high levels of IgH expression starting from the immature B cell stage (213-215). Human c-*myc* gene expression, driven by hs3a-hs1,2-hs3b-hs4 (the four enhancers at the DNase I hypersensitive sites on murine *Igh* locus, Figure 1.2), were at low levels in pro-B and pre-B cells, but high levels in immature and mature B cells (214), supporting the notion that 3'RR has no or weak enhancer activity at the pro-B and pre-B cell stages. For this reason, Eμ is essential for IgH transcription in these early stage B cells. On the other hand, since Igμ is expressed at a much lower level in pro-/pre-B cells than in immature and mature B cells, 3'RR might have a stronger enhancer activity than Eμ does, and is most active in late-stage B cells.

1.10. Disorders of immune system

1.10.1 Immunodeficiency

The integrity of the immune system is essential for the body to defend against infections. If the immune system is missing or even compromised, then the host will be highly susceptible to pathogens, causing immunodeficiency diseases which are often fatal. Defects in immune system may occur in any component during any developmental stage. For example, there is humoral immune deficiency caused by defective B cells, plasma cells or antibodies; T cell deficiency

resulting in compromised cellular and humoral immune responses, and complement deficiency. Immunodeficiency may also result from reduced numbers of granulocytes, or absence of spleen function. The immunodeficiency can be inherited or acquired. Primary immunodeficiency is congenital, making the body highly susceptible to infections from infancy onward. Secondary immunodeficiency is acquired because of various immunosuppressive agents, such as drug treatment, malnutrition, or infection. Disturbed or inadequate development of the B cells therefore renders the host liable to immunodeficiency. Elucidation of the regulation mechanisms of B cell development is vitally important.

1.10.2 Autoantibodies and their role in autoimmunity

Failure to distinguish self-constituents from non-self allows an immune response against the body's own cells, and this aberrant reaction is autoimmunity. B and T lymphocytes may express receptors with specificities to self-antigens during development. Self-tolerance is achieved through negative selection processes (apoptosis, receptor editing or anergy) that prevent the outgrowth of self-reactive lymphocytes. Abnormal regulation of these selection processes, or disruption by external factors may result in loss of self-tolerance. Autoantibodies are hallmarks for the humoral responses inappropriately directed against self-components, which can mount effective destruction against self-determinants. The first evidence of autoimmue disease due to continuous self-specific antigen and antibody interaction, was the identification of autoantibodies in glomerulonephritis patients (216). Systemic lupus erythematosus is another autoimmune disease in which the immune system attacks the hosts' cells and tissues, resulting in inflammation and tissue damage. It is characterized by antibodies to dsDNA and/or nuclear proteins in the hosts (217). Although the correlation between autoantibodies and systemic

autoimmune disease is widely known, the contribution of autoantibodies to the autoimmune disease, as well as the defective regulatory processes that account for the pathogenesis remain to be elucidated. For example, subtle alterations in the signaling pathways that influence BCR responses to self-antigens may predispose an individual to autoantibody production and possibly autoimmune disease. Most of the currently known human autoimmune diseases result from both genetic factors and environmental effects, both of which collectively dysregulate the B cell functionality and thereby confer susceptibility to autoimmune diseases (218). In order to achieve a diverse population of B cells capable of producing high-affinity antibodies with no pathological autoreactivity, the development of B cell needs to be precisely regulated to avoid self-reactivity. Study of the negative selection and the regulatory mechanisms of autoreactive B cells is therefore, challenging and of great importance. New insight into abnormal survival of autoreactive B cells and the pathogenesis of autoimmunity is demanded.

1.11. Aims of study

To make a mature B cell population that produces a highly diverse antibody repertoire, which recognizes numerous foreign antigens but lacks anti-self reactivity, B cell development is regulated through intracellular signals propagated by B cell receptors (149, 156, 161, 162). Disruption of any element of the pre-BCR or BCR (the SLC, membrane Igμ, Igα or Igβ) or of the downstream signaling pathways of these receptors blocks B cell development, indicating that not only membrane expression but also the signaling capacity of these receptors are critical for the development and maintenance of the mature B cell population (161, 163, 164).

Several different experimental models have suggested that the ligand-independent, constitutive (tonic or basal) signaling strength of the pre-BCR and BCR is directly proportional to their

membrane densities. Reduced receptor expression translates into diminished tonic signaling, impairing B cell maturation and skewing the development of B cell subsets (178, 219-223). In the current study, we explore the hypothesis that one role of the transcriptional enhancer Eμ is to ensure sufficient Igμ protein levels in pre-B and nascent immature B cells, and thus sufficient BCR density, to support positive selection into the immature B cell compartment. The following aims are to further characterize the defects associated with the absence of Eμ in the $V_H\Delta^a$ model, identify the changes in BCR signals, and test the effects of "allelic inclusion" on antibody production. Understanding the nature and extent of these defects (in development, BCR repertoire and BCR signaling) caused by expression of an Eμ-deficient allele, will provide us with new insights into the mechanisms regulating B cell development.

Aim 1. Study the effects of Eμ-deficiency on early B lymphocyte development after V_HDJ_H recombination.

In previous studies, we circumvented the need for Eμ in V_H assembly to study its functions after this process. To do this, we created an Eμ-deficient *Igh*^{a} allele with a pre-assembled heavy chain variable region gene B1-8V_H knocked into the endogenous locus ($V_H\Delta^a$, Figure 1.11). We found that, in pre-B cells, this allele was expressed at half the level of an identical but Eμ-intact allele ($V_HE\mu^a$), resulting in ~½ normal cytoplasmic Igμ levels (146). We propose that this reduction in Igμ expression causes a decrease in the strength of signals emanating from newly emerging BCRs, which should result in signals insufficient for positive selection and thereby an inefficient transition from pre-B to immature B cell stage.

1a. To test this hypothesis, we will look at the efficiency with which pre-B cells differentiate into immature B cells. Our previous study suggests that this transition should be impaired if we analyze the bone marrow B cell subpopulations in mice heterozygous or hemizygous for the $V_H\Delta^a$ allele, with comparison to the corresponding $V_HE\mu^a$ mice. Steady-state levels of pre-B and immature B cell subsets from the bone marrow of mice carrying the $V_H\Delta^a$ allele will be examined.

1b. To completely study the effects of Eμ-deficiency on B lineage cell development, we will also take note of B cell subsets other than pre-B and immature B cell stages, including transitional stage B cells in the bone marrow and mature B cells in the spleen. In this way, the rate at which cells differentiate from one compartment to the next will be described.

Aim 2. Test the hypothesis that Eμ ensures Igμ levels and thereby amplifies BCR signal strength for the positive selection of pre-B cells into immature B cell stage.

In this aim, we seek to test the relationship between Eμ deletion and pre-BCR/BCR signal strength. The differences between pre-BCR/BCR signals that can and cannot support early B cell differentiation will be examined.

2a. We will compare the signal strengths of pre-BCRs dependent solely on μ expressed from the $V_H\Delta^a$ and $V_HE\mu^a$ alleles, respectively. Since the Igμ level in pre-B cells of $V_H\Delta^a$ mice is ~½ that of $V_HE\mu^a$ mice, we would expect impaired B1-8 H-chain pre-BCR signals in the absence of Eμ. We will test this hypothesis by measuring phosphorylation of downstream targets in the pre-B cells.

2b. Tonic BCR-signals in $V_H\Delta^a$ and $V_HE\mu^a$ immature B cells will also be compared, to explore the relationship between Eμ deficiency and BCR signal strength.

Aim 3. Test the contribution of light chain editing (i.e. light chain gene sequence) in the development of B cells expressing the Eμ-deficient allele.

We have evidence that the Eμ-deficient allele is posing problems at the pre-B to immature B cell transition from our analysis of mature, splenic B cells that expressed Igμ from only the Eμ-deficient IgH allele ($V_H\Delta^a$ single-producers). These cells have undergone extensive light-chain editing, a process that takes place in pre-B cells transitioning to the immature B cell stage (146, 179). We suggest this is a sign of weak BCR signals (low Igμ) insufficient for a prompt "turn-off" of the recombination machinery (the recombination-activating genes RAG-1 and RAG-2) and for progression to the immature B cell stage.

3a. We postulate that the extensive receptor editing, in response of $V_H\Delta^a$-B cells to reduced BCR signals, serves to create an IgH/IgL combination with superior signaling properties to drive the pre-B to immature B cell transition in the absence of Eμ, through formation of a BCR with higher stability and/or a specificity that can ratchet up the signals above the threshold required for the pre-B to immature B cell transition. As a test of this hypothesis, we ask whether providing the Eμ-deficient B cells with a pre-assembled Vκ knock-in gene (we use 3-83Vκ gene for the current study) would affect the rate of pre-B to immature B cell transition, to assess the contribution made by light chain editing (i.e. light chain sequence) to overcome the low μ levels.

3b. We found a breach of allelic exclusion in $V_H\Delta^a/WT^b$ mice, with >20% splenic B cells expressing two IgH alleles as "double-producers", which were not found in $V_HE\mu^a/WT^b$ mice.

Determining whether the Vκ knock-in light chain affects the outgrowth of these double-producers should also provide insight into the cell selection processes that explain the mechanism of allelic exclusion. Our hypothesis is that the BCR signal is the determining factor in whether the second "check-point" for allelic exclusion is breached (previously described as an Eμ-dependent "check-point" at the pre-B to immature B cell transition)

Besides bi-allelic expression of the IgH chains, receptor editing is another means by which the Eμ-deficient B cell development could be rescued. So the level of light chain editing (downstream Jκ usage and/or λ chain expression) will be analyzed in the $V_H\Delta^a$/3-83κ mice.

Aim 4. Test the long-held notion that allelic "inclusion" supports the development of autoreactive B cells.

An early prediction made by Burnet, when describing his theory of clonal immature B cell selection, was that cells with receptors of more than one specificity would be at risk of co-expressing both protective and autoreactive receptors. We will test cells expressing both the $V_H\Delta^a$ and the wild type IgH alleles (double-producers), for their secretion of autoreactive antibodies.

4a. Preliminary anti-nuclear antibody (ANA) assay of sera from $V_H\Delta^a$/WT^b mice indicated a propensity of autoreactivity in the animals expressing double-producers. Matched strains of mice will be compared for the sera reactivity to certain auto-antigens (e.g. dsDNA).

4b. Double-producers and single-producers from $V_H\Delta^a/WT^b$ mice will be directly analyzed for autoantibody (anti-dsDNA antibody) secretion in vitro, to determine the origin of the autoreactivity.

The results of these studies, in large part, have now been published (Peng, C. and L. A. Eckhardt. 2013. Role of the *Igh* Intronic Enhancer Eμ in Clonal Selection at the Pre-B to Immature B Cell Transition. *The Journal of Immunology* 191: 4399-4411).

Chapter 2: Materials and Methods

2.1. Mice

All mice in this study were maintained under pathogen-free conditions in the Animal Facility at Hunter College, City University of New York. All protocols were reviewed and approved by Hunter College Institutional Animal Care and Use Committee.

2.1.1. B1-8V_H knock-in mice

The heavy chain variable region gene knock-in mice $V_HE\mu^a/V_HE\mu^a$, with a pre-assembled V_HDJ_H gene segment (B1-8V_H) on both the endogenous *Igh* loci, were originally described as B1-8i mice, and supplied by Dr. K. Rajewsky's lab (CBR Institute for Biomedical Research, Boston, MA) (144). The B1-8V_H gene represents a recombined Ig variable region derived from a 4-hydroxy-3 nitrophenylacetyl binding antibody (B1-8). The B1-8V_H "knock-in" gene replaces the endogenous D_{Q52} and the J_H gene segments on the *Igh* locus. There is a silent point mutation in codon 92 of the B1-8V_H gene, which inactivates the internal heptameric RSS, preventing endogenous V_H replacement such that the stability of this gene segment is increased.

The $V_H\Delta^a/V_H\Delta^a$ mouse line carries the same B1-8V_H knock-in gene but with the intronic enhancer Eμ removed from both the *Igh* loci (146). Both $V_HE\mu^a$ and $V_H\Delta^a$ alleles in this study encode a allotype Ig proteins. $V_HE\mu^a/V_HE\mu^a$ and $V_H\Delta^a/V_H\Delta^a$ homozygous mouse strains were crossed to C57BL/6J mice (described as WT^b/WT^b mice, The Jackson Laboratory, Bar Harbor, ME, stock#000664) for 10 to 13 generations to make congenic $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ heterozygous offspring (Figure 1.11). The IgH allele of C57BL/6J mice (WT^b) encodes b

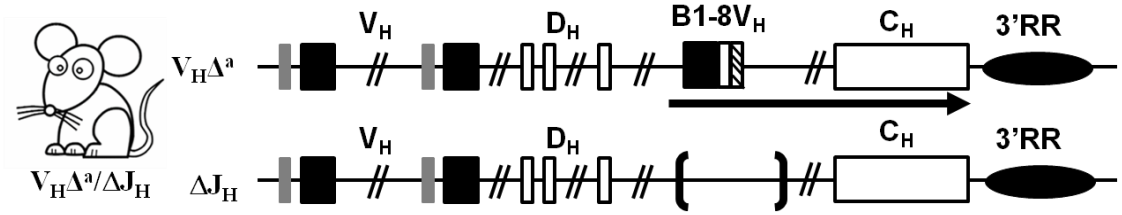
allotype Ig proteins. In some cases, the heterozygous mice were intercrossed and the offspring were analyzed to further eliminate the variations of the genetic background.

2.1.2. ΔJ_H mice

The $\Delta J_H/\Delta J_H$ mice lack both Eμ and the J_H gene segments on the *Igh* locus, precluding V_HDJ_H recombination (224) (The Jackson Laboratory, B6.129P2-Igh-J^{tm1Cgn}/J; stock#002438). These mice are not able to produce antibodies and hence no functional B cells. They were mated to $V_HE\mu^a/V_HE\mu^a$ and $V_H\Delta^a/V_H\Delta^a$ mice to generate $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ hemizygous offspring (Figure 2.1). B cell development in these mice depends entirely on the B1-8V_H knock-in allele, with or without Eμ ($V_HE\mu^a$ or $V_H\Delta^a$ allele, respectively).

Figure 2.1: Diagram of *Igh* loci of hemizygous B1-8V_H knock-in mice, $V_H\Delta^a/\Delta J_H$.

B1-8V_H is a pre-assembled V_HDJ_H gene that is knocked into the *Igh* locus, replacing the D_{Q52} and the J_H gene segments. Shown in picture is the $V_H\Delta^a$ allele, with the intronic enhancer Eμ removed from the locus. Eμ and J_H genes are deleted from the *Igh* loci of $\Delta J_H/\Delta J_H$ mice. Shown in the bracket are genes deleted on the ΔJ_H locus, prohibiting V_HDJ_H recombination and therefore Ig expression from this allele.



2.1.3. RAG1$^{-/-}$ mice

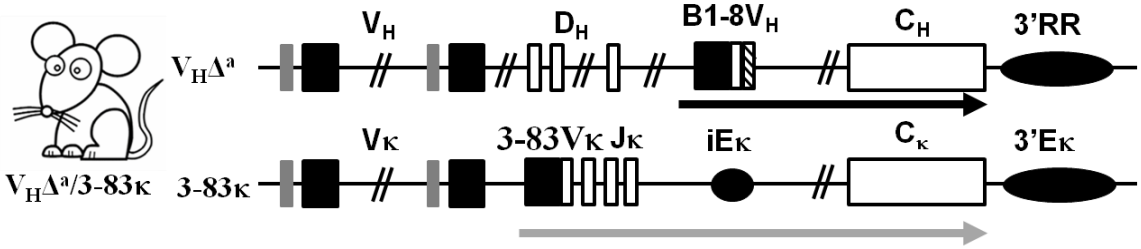
RAG1 genes were deleted on both chromosomes of RAG1$^{-/-}$ mice (225) (The Jackson Laboratory, B6.129S7 RAG1 <TM1MOM>/J; stock#002216). They are immunodeficient as they lack both mature B and T lymphocytes, due to the inability to assemble the BCR and TCR variable region genes. $V_HE\mu^a/V_HE\mu^a$ and $V_H\Delta^a/V_H\Delta^a$ mice were mated with RAG1$^{-/-}$ mice on the C57BL/6J background, resulting in $V_HE\mu^a/WT^b$ RAG1$^{-/-}$ and $V_H\Delta^a/WT^b$ RAG1$^{-/-}$ progeny. B cell development in these progeny stalls at the pre-B cell stage because the cells cannot undergo light chain gene rearrangements without Rag1 protein.

2.1.4. 3-83Vκ knock-in mice

The light chain variable region (V_L) gene knock-in mice, 3-83κ/3-83κ, with the pre-assembled 3-83Vκ knock-in gene on both the kappa light chain loci, replacing the endogenous Jκ1 and Jκ2 gene segments, were supplied by Dr. R. Pelanda (Integrated Department of Immunology, National Jewish Health, Denver, CO). A previous study showed that the B1-8V_H/3-83Vκ combination yields a B cell receptor (BCR) with innocuous specificity (226) (Figure 2.2).

Figure 2.2: Diagram of *Igh* and *Igκ* loci of B1-8V_H and 3-83Vκ double knock-in mice, $V_H\Delta^a$/3-83κ.

One heavy chain locus with the pre-assembled B1-8V_H gene is shown on top: $V_H\Delta^a$, encoding Igμ^a proteins; Shown below it is one light chain locus with the pre-assembled V_L gene: 3-83Vκ, encoding 3-83 Igκ proteins.



2.2. Preparation of DNA

2.2.1. Isolation of genomic DNA

Each mouse tail piece at about 0.5 cm was incubated in 500 μl tail lysis buffer (100 mM Tris-HCl, 5 mM EDTA, 200 mM NaCl and 0.2% SDS) with 20 μl proteinase K (Sigma-Aldrich Cat.#P6556, 10 mg/ml in dH_2O stored at -20 ºC) at 56 ºC overnight. Then the genomic DNA from the tails was isolated by phenol/chloroform extraction using the Phase Lock Gel™ (PLG, 5 Primer Cat.#2302830), according to the manufacturer's protocol. Extracted DNA was dissolved in TE (10 mM Tris pH 7.6, 1 mM EDTA) buffer at 37 ºC for 2 hours and stored at 4 ºC for further analysis.

2.2.2. Purification of calf thymus double-stranded DNA (dsDNA)

250 mg of calf thymus DNA (Sigma-Aldrich, Cat.#D1501-1G) was added to 40 ml TE buffer. The solution was stirred on the rocking platform for 2-3 days for the DNA to dissolve. DNA solution was split to 10 ml aliquots and fragmented by sonication in a beaker with ice for 1.5 minutes. Equal volumes of TE equilibrated 1:1 phenol/SEVAG (phenol: chloroform: isoamyl alcohol = 25: 24: 1) solution (10 ml) were added to each aliquot (10 ml), and mixed by inversion. The mixtures were centrifuged at 14000 rpm for 5 minutes and DNA was in the upper layer. Phenol/SEVAG extraction was repeated using the upper layer DNA solution. 1/10 (1 ml) the volume of the original solution (10 ml) of 3 M NaOAc was then added and mixed with the DNA solution by inversion. And then equal volume (10 ml) of 100% ethanol was added and mixed by inversion. After 5 minutes of centrifugation at 1400 rpm, the DNA was in the precipitate and the supernatant discarded. 70% ethanol was used to wash the DNA pellet. Dried DNA pellets were

dissolved in the original volume (10 ml) of TE buffer, and 0.01% sodium azide was added. DNA segments were tested by agar gel electrophoresis and stored at 4 ˚C. DNA solution was filtered through a 0.45 μm filter before use, and dsDNA came through the filter whereas the ssDNA was bound to the filter.

2.3. Genotyping mice

$V_HE\mu^a$ and $V_H\Delta^a$ alleles were detected by polymerase chain reaction (PCR) using the genomic tail DNAs, as were wild-type (germline configuration) *Igh* (WT^b), *Igκ*, 3-83κ, wild-type RAG1, and RAG1-deletion loci (described below). Mice sera were analyzed by ELISA for IgM^a and IgM^b as one indication of the genotype of the *Igh* loci ($V_HE\mu^a$ or$V_H\Delta^a$ versus WT^b, see section 2.7).

2.4. Polymerase Chain Reaction (PCR)

Genotyping PCRs were performed with HotStar Plus Taq polymerase (Qiagen, Cat.#203646) according to the manufacturer's protocol. The following primer pairs were used to amplify the tail-tip genomic DNA:

Table 2-1: Primers used to amplify genomic DNA for genotyping.

[a, b]The Jκ1 and Jκ2 region is missing on the 3-83Vκ knock-in allele.

[c, d, e]RAG1 gene primers:

http://jaxmice.jax.org/protocolsdb/f?p=116:2:4183119992152012::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:329,002216

Primer	Locus	Sequence
Primer number 2 (146)	Within the 3' homology region of B1-8$V_H\Delta$[a]; nucl. 136031-136054, GenBank AC_073553	5'-CAGAGGGAGTTCACACAGAGCATG-3'
Primer number 4 (146)	Immediately 5' of the leader exon of B1-8V_H; nucl. 312629-312655, GenBank BN_000872	5'-TCTTTACAGTTACTGAGCACACAGGAC-3'
Primer E3HR (201)	Within the 3' homology region of targeting vector to generate B1-8i mice (144); complementary to nucl. 134588-134613, GenBank AC_073553	5'-CTCCACCAACACCATCACACAGATTC-3'
JH4f (146)	Within J_{H4}; nucl. 134365-134389, GenBank AC_073553	5'-CTATGGACTACTGGGGTCAAGGAAC-3'
VDJ junction primer (146)	The unique V_HDJ_H junction sequence of B1-8V_H	5'-CGCAAGATACGATTACTACGG-3'
Wild-type Igκ (forward	Between Jκ1 and Jκ2 of the WT κ locus; nucl. 39919879-	5'-GATTCTGGCACTCTCCAAGG-3'

primer)	39919898,GenBank NT_039353	
Wild-type Igκ (reverse primer)	Between Jκ2 and Jκ3 of the WT κ locus; complementary to nucl.39920274-39920293,GenBank NT_039353	5'-CCAACCTCTTGTGGGACAGT-3'
3-83Vκ knock-in (forward primer)[a]	Upstream of 3-83Vκ coding sequences; within the 3-83Vκ insertion, which replaces Jκ1-Jκ2 on the WT κ locus (227)	5'-GCGGCCGCACACTATATTTTCCTCCTTC-3'
3-83Vκ knock-in (reverse primer)[b]	3'end of 3-83Vκ coding sequences; within the 3-83Vκ insertion, which replaces Jκ1-Jκ2 on the WT κ locus (227)	5'-GTCGACAACACACAACAAAGAACAAC-3'
WT-RAG1-F[c]	RAG1 forward primer; nucl. 42522471-42522490, GenBank NT_039207	5'-GAGGTTCCGCTACGACTCTG–3'
WT-RAG1-R[d]	Common reverse primer for RAG1 and RAG1-deletion mutant; complementary to nucl. 42522016-4252235, GenBank NT_039207	5'-CCGGACAAGTTTTTCATCGT–3'
dRAG1-F[e]	Forward primer for RAG1-deletion mutant	5'-TGGATGTGGAATGTGTGCGAG–3'

Primers number 4 and E3HR generate an 834 bp product from only the $V_HE\mu^a$ allele, but not other IgH alleles. Primer number 2 and VDJ junction primer are specific for the $V_H\Delta^a$ allele and generate a 387 bp product. JH4f and primer E3HR generate a 249 bp product from only the wild type *Igh* (WT^b) locus. Wild-type Igκ primers anneal to wild type kappa locus and generate a 415 bp product. 3-83Vκ knock-in gene primers anneal to 3-83Vκ gene and generate a 1.1 kb product. Primers WT-RAG1-F and WT-RAG1-R generate a 474 bp product from the wild type RAG1 locus, whereas WT-RAG1-R and dRAG1-F generate a 530 bp product from the RAG1 deficient mutant. All primer pairs are specific for their designated genes, and don't generate a detectable product from other gene loci under the reaction conditions.

2.5. Gel electrophoresis in agarose gels

Agarose gel electrophoresis was used for the detection of DNA products of the PCR. 1% agarose was dissolved in the electrophoresis buffer TAE (Tris/Acetate/EDTA). DNA molecules are negatively charged in the TAE buffer because of the ionized phosphate residues. Once the electric field is provided, DNA fragments move to the positive electrode at different rates. The smaller the fragment is, the faster it moves in the gel. In most cases of this study, the gel electrophoresis was performed at 110 V. DNA molecules in the gel were stained with ethidium bromide (EtBr), which intercalates to DNA and fluoresces under the UV light. By comparing to DNA markers, the sizes of the DNA products were estimated.

2.6. Real-time reverse transcriptase (RT) PCR

To quantify RAG2 and germlineIgκ transcripts in pre-B cells by the quantitative real time RT-PCR, bone marrow B cells were first enriched by positive selection for $B220^+$ cells using B220

MicroBeads kit (Miltenyi Biotech). Total RNA was extracted by RNeasy mini-prep kit (Qiagen, Cat.#74104) following the manufacturer's protocol, and the RNA products were treated with RNase free DNase (Qiagen, Cat.#79254) to remove genomic DNA. RNA solutions were adjusted to 100 μg/ml in RNase-free water.

One-step, real-time RT-dependent PCRs were performed with the isolated RNAs, using the QuantiFast SYBR Green RT-PCR kit (Qiagen, Cat.#204154). Primers for κ germline transcripts (0.8 kb (228); GenBank NT_039353) were 5′-CAGTGAGGAGGGTTTTTGTACAGCCAGACAG-3′ (forward, immediately upstream of Jκ1; nucl. 39919702-39919732) and 5′-CTCATTCCTGTTGAAGCTCTTGACAATGGG-3′ (reverse, within Cκ; complementary to nucl. 39923911-39923940); Primers for detecting RAG2 mRNA were from Qiagen (QuantiTect Primer Assay, QT00253414). Samples were normalized using primers for mRNA from the house keeping gene *hgprt1* (hypoxanthine/guanine phosphoribosyl transferase; QuantiTect Primer Assay QT00166768). PCR reactions were performed on the 7500 Real-Time PCR System (Applied Biosystems) at the biology department of Hunter College, CUNY. Data were analyzed with the software supplied by Applied Biosystems (User bulletin #2). Reverse-transcriptase negative controls were included to exclude the possibility of genomic DNA contamination.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

Antibodies expressed from the V_H knock-in (IgM^a) and/or wild-type (IgM^b) IgH alleles were detected and distinguished in the sandwich-type ELISA, using allotype-specific antibodies. Biotinylated mouse anti-mouse IgM^a antibody (BD Biosciences, Cat.#553515) and biotinylated mouse anti-mouse IgM^b antibody (BD Biosciences, Cat.#553519) were used to detect a and b

allotype IgM, respectively. Blood samples were collected from $V_HE\mu^a/V_HE\mu^a$, $V_H\Delta^a/V_H\Delta^a$; $V_HE\mu^a/WT^b$, $V_H\Delta^a/WT^b$; $V_HE\mu^a/\Delta J_H$, $V_H\Delta^a/\Delta J_H$, and C57BL/6J mice. Sera samples were prepared by spinning blood samples at 12,000 rpm for 5 min and stored at 4 ˚C with 0.1% NaN_3 in PBS.

2.7.1. Quantification of immunoglobulin

For IgM^a and IgM^b proteins: For both qualitative and quantitative ELISAs, 96-well immunoplates (NuncMaxisorp, Thermo Scientific) were coated with rat anti-mouse IgM antibody (BD Biosciences, Cat.#553405) at 4 ˚C overnight. Plates were then blocked with blocking buffer (3% bovine serum albumin, BSA; 0.1% NaN_3 in phosphate-buffered saline, PBS) at room temperature (RT) for one hour. To quantify IgM^a and/or IgM^b, mouse sera or culture supernatants were diluted and each dilution was analyzed in duplicate. After applying mouse sera or culture supernatants and one hour incubation at RT, plates were washed 3 times with washing buffer (1x PBS, 0.05% Tween-20). 100 μl per well 1 μg/ml biotinylated anti-allotype antibodies were then added to detect a or b allotype IgM. After one hour incubation at RT, plates were washed 3 times and 100 μl 1:1000 diluted streptavidin-horse radish peroxidase (SA-HRP; BD Biosciences, Cat.#554058) was added to each well to detect the anti-allotype antibodies. After one hour incubation at RT, plates were washed 3 times and ABTS (Southern Biotech, Cat.#0401-01) was applied as the substrate for HRP. Plates were read at 405 nm, using the PowerWave HT microplate spectrophotometer (BioTek, Winooski, VT), and data were analyzed by the software Gen5 (BioTek). A titration curve for each assay was generated with purified mouse immunoglobulin of the relevant Ig allotype. OD405 of all tested samples was in the linear range of the assay.

2.7.2. Test for anti-dsDNA antibodies

For anti-dsDNA antibody: Detection of IgM^a or IgM^b anti-dsDNA antibodies by ELISA was performed as previously described by others (229). Briefly, calf thymus DNA (Sigma-Aldrich, Cat.#D1501) was sonicated and purified by phenol extraction, and then was diluted to 100-150 μg/ml in PBS and filtered through a 0.45 μm microcellulose filter (Millipore, Bedford, MA) to remove ssDNA. 100 μl dsDNA solution was added to each well of the Immulon-2 HB 96-well plate (Dynatech, Chantilly, VA). Plates were dried for 48 hours at 37 ˚C and blocked with 3% BSA in PBS (pH 7.4) for one hour at room temperature. Culture supernatant or diluted sera samples from mice of various genotypes were added and incubated for two hours at 37 ˚C. Wells were then washed with 0.05% Tween-20 in PBS. Binding of IgM^a molecules to dsDNA was revealed with biotinylated mouse anti-mouse IgM^a antibody and streptavidin-HRP at 405 nm. A purified mouse monoclonal IgM^a anti-dsDNA antibody, kindly provided by Dr. L. Spatz laboratory (Department of Microbiology and Immunology, Sophie Davis School of Biomedical Education, City College of New York), was also serially diluted by two-folds beginning with a concentration of 0.5 μg/ml, and was used to generate a standard curve. OD405 of all samples analyzed was in the linear range of the standard curve. The relative levels of IgM^a or IgM^b anti-dsDNA antibodies were normalized to IgM^a or IgM^b protein levels (total amount of IgM^a or IgM^b in each serum sample was quantified by ELISA as described above). Consistency of all ELISA results was confirmed by 3 repeated assays of each sample.

2.8. B cell isolation and in vitro cell culture

Single-cell suspensions were prepared from the bone marrow and spleen in cold RPMI-1640 media (Mediatech, Inc. Cat.#17-104-CI) containing 5% fetal bovine serum (FBS, Thermo Fisher

Scientific, Cat.#SH30070.03). Erythrocytes were lysed by ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.4). In some cases, B lineage cells were isolated by magnetic cell sorting. Bone marrow B lineage cells were positively selected with the MACS B220 MicroBeads kit (Miltenyi Biotech, 130-049-501), and splenic B cells were enriched by negative selection of CD43-expressing cells with the CD43 microbeads (B cell isolation kit, mouse, Miltenyi Biotech, 130-090-862). For some experiments, a FACSVantage (BD Biosciences) was used to identify and separate IgM^{a+b+} and IgM^{a+b-} cells before placing these separate populations into culture (described below).

For lipopolysaccharide (LPS)-stimulated cultures, splenic B cells isolated from 10-week-old mice were cultured at 37 ºC, 5% CO_2, for 7 days starting at a density of 5 x 10^5 cells/ml in RPMI full media (RMPI-1640, supplemented with 20% FBS; 50 μM β-mercaptoethanol, Life Technologies, Cat.#21985-023; 2 mM L-glutamine, Life Technologies, Cat.#25030-081; 100 U/ml penicillin, 100 μg/ml streptomycin, Life Technologies, Cat.#15140-122; 100 μM non-essential amino acids, Life Technologies, Cat.#11140-050) with 25 μg/ml LPS (*Escherichia coli*, serotype 055:B5; Sigma-Aldrich, Cat.#L6511). Cell culture supernatants were collected and assayed by ELISA for the presence of IgM^a or IgM^b antibodies to dsDNA and for the quantification of total IgM^a or IgM^b antibodies.

2.9. Flow cytometry

2.9.1. Cell analysis

Single cells were stained for surface and intracellular molecules by standard procedures. In general, 1x10^6 cells were incubated with the corresponding monoclonal antibodies in FACS

staining buffer (1x PBS, 5.6 mM glucose, 0.1% BSA, and 0.1% NaN_3), at 4 °C for 20 min. Monoclonal antibodies from BD Biosciences included: fluorescein-isothiocyanate (FITC) conjugated anti-mouse CD3ε (BD553061), anti-mouse IgM (BD553408), anti-mouse IgM^a (BD553516), anti-mouse Igκ (BD550003), anti-mouse CD23 (BD553138); phycoerythrin (PE) conjugated anti-mouse IgM^b (BD553521), anti-mouse CD43 (BD553271), anti-mouse CD21 (BD552957); allophycocyanin (APC) conjugated anti-mouse B220 (BD553092). Biotin-conjugated antibodies were revealed with streptavidin-PE (BD554061). Monoclonal antibodies from Southern Biotech included: PE conjugated anti-mouse Igλ (Cat.#1175-09), and biotin conjugated anti-mouse IgD (Cat.#1120-08). Cells were washed in FACS washing buffer (1x PBS, 5.6 mM glucose, and 0.1% NaN3) after staining. Dead cells were excluded from analysis by propidium iodide staining.

For cytoplasmic Igμ, Igκ and Igλ staining, bone marrow cells were isolated and incubated with an antibody to B220 (and/or antibodies to other cell surface markers, as appropriate for the experiment to identify the pre-B, and/or immature B cell subsets). Cells were then fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences, Cat.#554714) buffer at RT for 20 min. Intracellular Igμ was stained with FITC conjugated anti-mouse IgM (BD553437), and intracellular Igκ and Igλ was revealed by antibodies described above.

Phosphoflow staining: To detect the phosophorylation status of intracellular signaling molecules, freshly isolated single-cell suspensions ($1 \times 10^6 \sim 5 \times 10^6$ bone marrow cells) were surface stained with anti-B220-APC (in some cases also anti-sIgM-PE, BD553409 or anti-sIgM-FITC, BD553408), and then washed with PBS and resuspended in pre-warmed (37°C) RPMI 1640 medium (up to 5×10^6 cells/ml), incubated for 5 minutes. Sodium pervanadate (10 μl per 1 ml of

cell suspension) was then added immediately into the cell suspension to a final concentration of 60 μM for 5-minute incubation at 37 °C.

Sodium pervanadate inhibits intracellular tyrosine phosphatases, which results in accumulation of tyrosine phosphorylation (and/or hyperphosphorylation of tyrosine kinases). It has been used to analyze the relative amount of tyrosine-phosphorylated protein, which is an indicator of the activity of the signaling pathways (158, 159, 230, 231). Sodium pervanadate was prepared by mixing 1 ml of 20 mM sodium orthovanadate (Na_3VO_4, pH 10.0, diluted in water) and 330 μl of 30% H_2O_2 at room temperature for 5 minutes, to yield a 6 mM pervanadate solution with the remaining H_2O_2, and was used at a final dilution of 1:100 (or diluted to 100 μM in RPMI to make a 2x solution).

Cells were then washed with PBS, fixed and permeabilized with Cytofix/Cytoperm buffer and incubated with anti-phosphotyrosine antibody (BD558008), anti-pErk (BD612592), anti-pSyk (BD560081) or one of the corresponding isotype-matched, control antibodies with irrelevant specificity, at 4 °C for 30 min. For phosphorylated Syk: the mouse anti-Syk antibody (I120-772) is specific for the mouse Syk phospholytation site tyrosine 342 (pY342). For phosphorylated Erk: the mouse anti-Erk1/2 monoclonal antibody (20A) recognizes the phosphorylated threonine 203 and tyrosine 205 (pT203/pY205) residues in murine Erk1, and the T183/Y185 in murine Erk2. For phosphorylated total tyrosine: the mouse anti-phosphotyrosine antibody (PY20) detects all phosphorylated tyrosine residues.

For all flow-cytometry experiments, cell analysis was on a FACSCalibur (BD Biosciences), data were acquired with CellQuest (FACS instruments), and data were further analyzed with FlowJo

software (Tree Star, Inc.). Lymphocytes were gated according to side and forward scatter, and further gates were applied as described in individual experiments.

2.9.2. Cell sorting

Cell sorting of IgM^{a+b+} double-positive, and IgM^{a+b-} single-positive splenic cells was done with the FACSVantage^{TM} (BD Biosciences). Cells were first enriched using the MACS B cell negative magnetic selection kit (described above), and then surface stained with anti-B220-APC, anti-IgM^{a}-FITC and anti-IgM^{b}-PE for sorting by flow cytometry.

2.10. Statistical analysis

Statistical tests for differences among the mouse lines were performed with GraphPad Prism 5 software. Student's unpaired two-tailed *t*-test was used to assess the statistical significance of the differences. Differences with a p-value of less than 0.05 were considered significant.

Chapter 3: Results

The results of these studies, in large part, have now been published (Peng, C. and L. A. Eckhardt. 2013. Role of the *Igh* Intronic Enhancer Eμ in Clonal Selection at the Pre-B to Immature B Cell Transition. *The Journal of Immunology* 191: 4399-4411).

3.1. Eμ-deficient ($V_H\Delta^a$) B cells have impaired pre-B to immature B cell transition

— *Immature B cell compartment is diminished in the bone marrow of Eμ-deficient mice*

We previously described two strains of mice in which a pre-assembled heavy chain variable region gene (B1-8V_H) had been inserted into the endogenous *Igh*^{a} locus, either leaving the intronic enhancer Eμ intact ($V_HE\mu^a$), or deleting it ($V_H\Delta^a$) (described in chapter 2, Figure 1.11) (146). Mice heterozygous for the Eμ-deficient allele ($V_H\Delta^a/WT^b$) showed a striking defect in allelic exclusion. This was not due to a defect in feedback inhibition of DNA rearrangement on the wild-type allele (this occurred normally, as in the $V_HE\mu^a/WT^b$ mice), but instead reflected a defect at a second "check-point" that operates at the pre-B to immature B cell transition (described in chapter 1) (146). Further evidence that the Eμ-deficient allele was posing problems at this transition came from an analysis of mature, splenic B cells that expressed Igμ from only the Eμ-deficient allele. These cells had undergone unusually high levels of light-chain editing, a process that takes place in pre-B cells transitioning to the immature B cell stage (146). This finding, together with the fact that the Eμ-deficient allele produced Igμ at ½ normal levels in pre-B cells, led us to propose that absence of Eμ, in an otherwise functional IgH gene, results in emerging BCRs that cannot achieve the density required to signal "positive selection" into the immature B cell compartment.

To further test this hypothesis in the current study, we compared B cell subsets in the bone marrow of B1-8V_H knock-in heterozygous mice that retained ($V_HE\mu^a/WT^b$) and lacked Eμ ($V_H\Delta^a/WT^b$). Because of the B1-8V_H knock-in heavy chain gene, B cells largely bypass the pro-B cell stage (with no need for V_HDJ_H assembly), rapidly progressing to the pre-B cell stage for light chain rearrangement. Antibodies to cell surface markers including anti-B220, anti-IgM and anti-IgD were used to identify pro- and pre-B cells ($B220^+IgM^-IgD^-$), immature B cells ($B220^+IgM^+IgD^-$), and mature, recirculating B cells ($B220^+IgM^+IgD^+$). Wild-type C57BL/6J (WT^b/WT^b) mice were analyzed as a control, whose $B220^+IgM^-IgD^-$ subset includes not only pre-B cells, but also pro-B cells undergoing heavy chain variable region (V_H) gene recombination. As shown in Figure 3.1A, the immature B cell compartment in $V_H\Delta^a/WT^b$ mice (12.18% of the total $B220^+$ BM B cells) was ½ the size of that in $V_HE\mu^a/WT^b$ (24.80%) and WT^b/WT^b (22.63%) mice. Although the majority (~90%, (146)) of immature B cells in the $V_H\Delta^a/WT^b$ mice express only the $V_H\Delta^a$ allele as Ig heavy chain (IgH) "single-producers", there is still possibility of developmental "rescue" by co-expressing the WT^b allele as IgH-"double-producers", which may compensate for the loss of Eμ on one of the IgH alleles and drive these ~10% cells through the pre-B to immature B cell transition at a normal efficiency. We therefore next examined the B cell subsets in the "hemizygous" mice where B cell development is entirely dependent upon the B1-8V_H knock-in loci. This was done by making the $V_HE\mu^a$ and $V_H\Delta^a$ alleles heterozygous with an allele that lacked the J_H gene segments (ΔJ_H; no V_H gene assembly possible). As shown in Figure 3.1B, the immature B cell compartment in $V_H\Delta^a/\Delta J_H$ mice was also ½ that of the $V_HE\mu^a/\Delta J_H$ mice (13.51% versus 23.54%). Data from multiple mice of these and the $V_HE\mu^a/WT^b$, $V_H\Delta^a/WT^b$ and WT^b/WT^b genotypes were quantified in the bar graphs of Figure 3.1C. In all cases, the ratio of Eμ-deficient pro-/pre-B to immature B cells was about

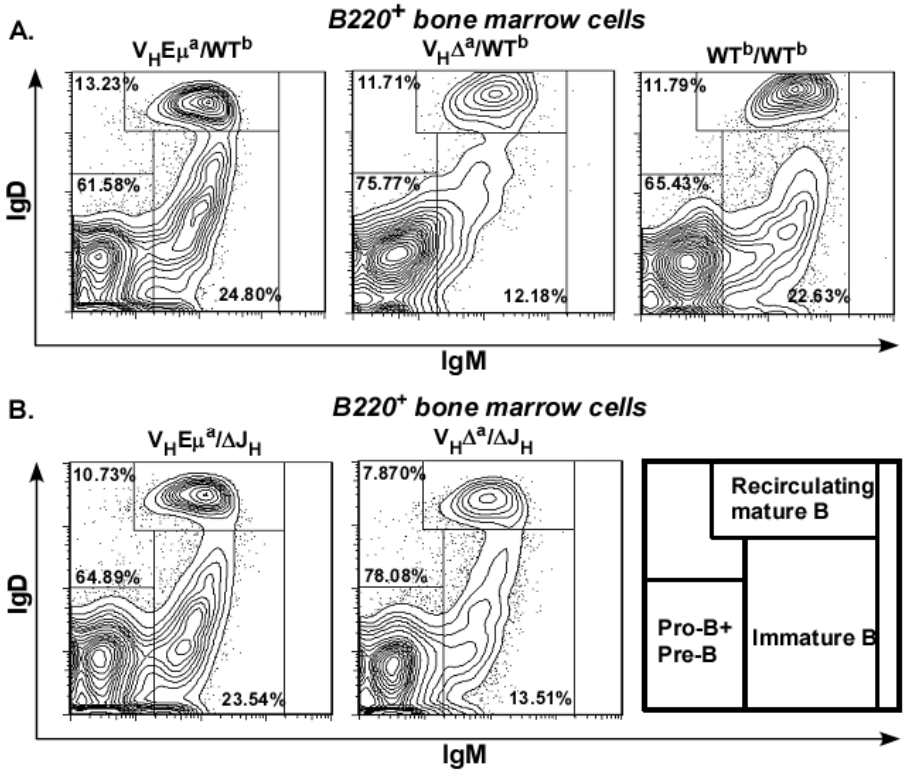
twice that of Eμ-intact ones, demonstrating that presence of the $V_H\Delta^a$ allele resulted in a less efficient transition from the pre-B to immature B cell stage.

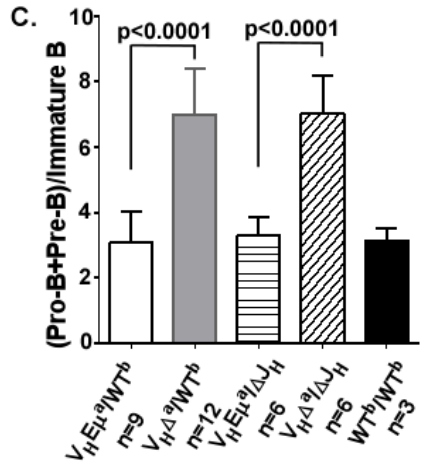
Figure 3.1: Immature B cell compartment is diminished in $V_H\Delta^a/WT^b$ and $V_H\Delta^a/\Delta J_H$ mice.

A. Representative flow-cytometry plots of bone marrow cells gated for $B220^+$ (B-lineage cells) and analyzed for IgD and IgM surface expression: mature B cells (IgD^+IgM^+), immature B cells (IgD^-IgM^+), pro-B+pre-B cells (IgD^-IgM^-). Gates and percentage cells in gates are indicated (% calculated relative to total $B220^+$ cells). $V_H\Delta^a/WT^b$ = heterozygous for the wild-type (Ig^b) allele and the Eμ-deficient allele with B1-8V_H knock-in; $V_HE\mu^a/WT^b$ differs from $V_H\Delta^a/WT^b$ only by virtue of the presence of Eμ on the B1-8V_H knock-in allele. Both of the knock-in lines are on the C57BL/6J background; WT^b/WT^b = normal C57BL/6J mouse as control.

B. Representative flow-cytometry plots of bone marrow B-lineage cells from hemizygous V_H knock-in mice ($V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$). Since the allele lacking the J_H region (ΔJ_H) cannot assemble a V_H gene, the "knock-in" allele is effectively "hemizygous". Analyses as in A.

C. Quantification of flow-cytometry data from the indicated numbers (n=) of mice of each genotype. Genotypes explained in A and B above. Ratios of (pro-B + pre-B) to immature B cells are compared (thereby leaving aside comparisons of re-circulating, mature B cells). Animals were age-matched (3-4 months). Error bars indicate Standard Deviation (SD); p-values for differences in means were determined by two-tailed *t*-test obtained (GraphPad Prism 5).


A.
B220+ bone marrow cells
VHEμa/WTb
VHΔa/WTb
WTb/WTb
13.23%
61.58%
24.80%
11.71%
75.77%
12.18%
11.79%
65.43%
22.63%
IgD
IgM
B.
B220+ bone marrow cells
VHEμa/ΔJH
VHΔa/ΔJH
10.73%
64.89%
23.54%
7.870%
78.08%
13.51%
Recirculating mature B
Pro-B+ Pre-B
Immature B
IgD
IgM


C.
(Pro-B+Pre-B)/Immature B
p<0.0001
p<0.0001
VHEμa/WTb n=9
VHΔa/WTb n=12
VHEμa/ΔJH n=6
VHΔa/ΔJH n=6
WTb/WTb n=3

To rule out the possibility that the observed increase in the ratio of pre-B to immature B cells was due to an abnormal expansion of pre-B cells, and since broader changes in cell numbers in the bone marrow of these animals might be missed by the above analyses, B-lineage cell counts were collected from each bone marrow subpopulation. The results were consistent with those described above: the only difference among the various genotypes was a reduction of immature B cell numbers in the Eμ-deficient mice (both the $V_H\Delta^a/WT^b$ and $V_H\Delta^a/\Delta J_H$ animals) (Table 3-1). We conclude that in the bone marrow of Eμ-deficient mice, there was no extra pre-B cell accumulation, but immature B cells did not emerge in normal numbers.

Table 3-1: Cell numbers in the bone marrow of mice carrying the B1-8V_H knock-in allele (with and without Eμ).

Bone marrow cells from the indicated genotypes were stained for B cell surface markers B220, IgM and IgD (n=number animals analyzed). Cell numbers for the indicated subpopulations were calculated from percentages obtained in flow-cytometry, multiplied by the total cells recovered (20000 cells collected and analyzed for each animal). Lymphocytes were identified by conventional forward and side-scatter gates; live cells among these were further identified by Propidium Iodide (PI) stain-exclusion; B-lineage cell subpopulations were identified as described both in the text and in Figure legend 3.1. Cell numbers are provided ± SD. n = number of animals analyzed. p-values were calculated by two-tailed student's *t*-test using GraphPad Prism 5. p>0.05: no significant difference. p<0.05: significant difference (italicized and bold in table).

	$V_H\Delta^a/WT^b$ n=4	$V_HE\mu^a/WT^b$ n=4		$V_H\Delta^a/\Delta J_H$ n=6	$V_HE\mu^a/\Delta J_H$ n=6	
Lymphocytes	3970±293	4020±246	p=0.89988	4690±194	4486±183	p=0.46253
Live cells	3859±300	3883±249	p=0.95261	4563±190	4355±175	p=0.43753
B220+ cells	573±51	720±46	p=0.07036	1216±166	1225±48	p=0.60969
(Pro+Pre-B) cells	341±61	377±41	p=0.61772	983±130	778±28	p=0.15489
Immature B cells	39±5	115±19	***p=0.01406***	142±23	234±16	***p=0.00859***
Mature B cells	191±21	224±34	p=0.48124	88±14	108±11	p=0.27722
Ratio of (pro/pre-B) to immature B	8.709±0.795	3.598±0.418	***p=0.00025***	7.215±0.451	3.395±0.216	***p<0.0001***

3.2. Eμ-deficient ($V_H\Delta^a$) B cells develop normally after the immature B cell stage

— *Absence of Eμ does not adversely affect cell development after the immature B cell stage*

Immature B cells differentiate to "transitional" B cells, and the first in a phenotypically-distinguishable series of such cells is the transitional B cells of type 1 (T1 B cell) in the bone marrow. The cell surface markers including B220, CD21, CD23, IgM, and IgD were stained and analyzed to compare the newly formed T1 (NF T1, $B220^+IgM^+IgD^{low}$) B cell compartments in the bone marrow of $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice. Among IgM^+ B cells in the bone marrow, the $CD21^-CD23^-$ cells constitute the combined pool of immature and NF T1 B cells. As shown in Figure 3.2A and 3.2B, this combined pool in $V_H\Delta^a/\Delta J_H$ mice was ½ the size of that in $V_HE\mu^a/\Delta J_H$ mice (22.43% versus 42.16%), consistent with the diminished immature B cell compartment in these animals. However, the ratios of immature to NF T1 B cells (distinguished by levels of IgM and IgD expression; immature, $B220^+IgM^+IgD^-$; NF T1, $B220^+IgM^+IgD^{low}$ (232-235)) were indistinguishable in these two mouse strains (3.72±0.18 versus 3.41±0.18; Figure 3.2C, D). This demonstrates that although the size of NF T1 B cell compartment was reduced in mice expressing the Eμ-deficient allele, the reduction was directly related to the smaller number of immature B cell "precursors" from which they arose. There was no evidence that the efficiency of NF T1 cell development was itself compromised by the deletion of Eμ.

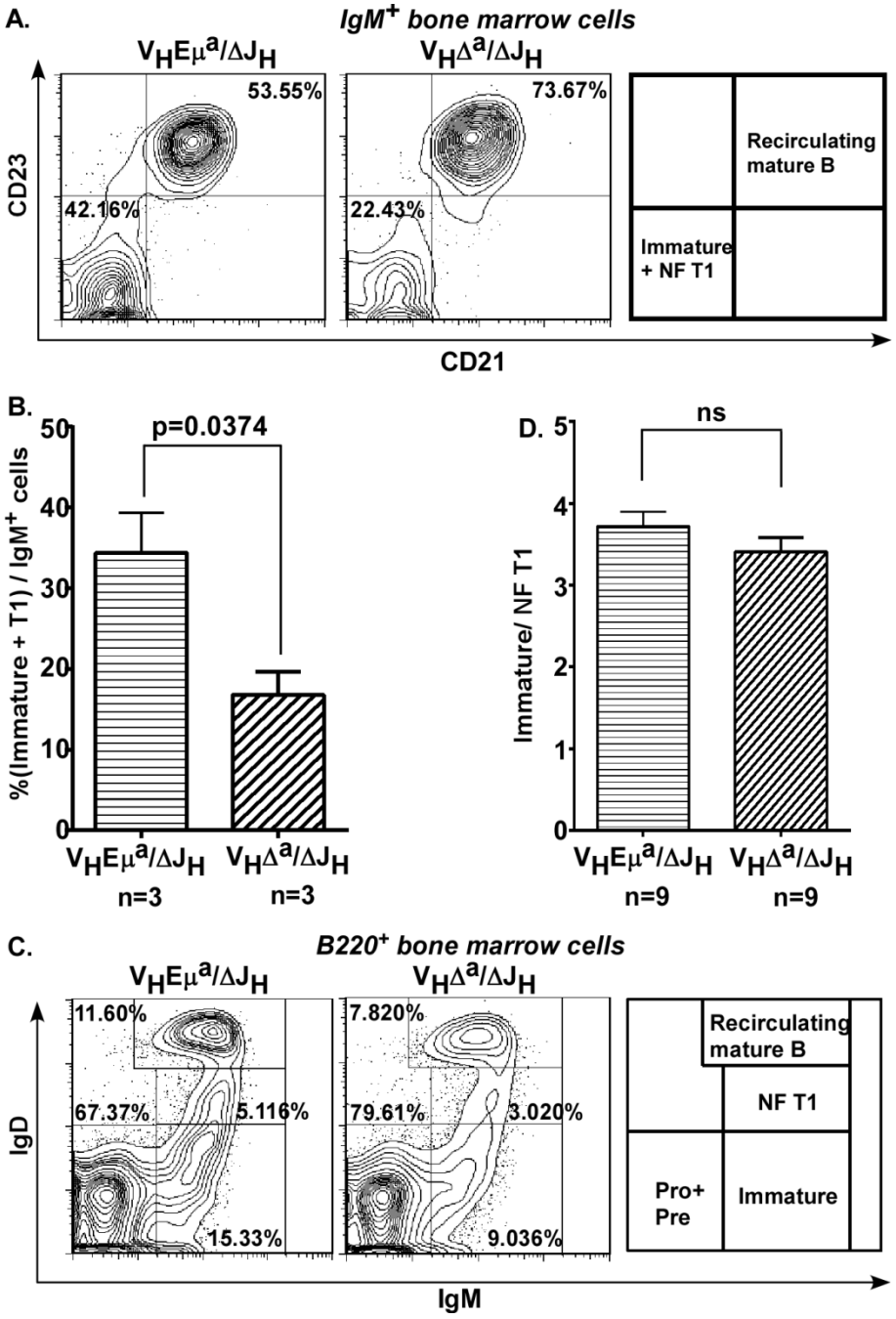
Figure 3.2: Immature and transitional (T1) B cells are found at similar ratios in $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice.

A. Representative flow-cytometry plots of bone marrow cells gated for IgM^+, and analyzed for CD23 and CD21 surface expression. Quadrants for recirculating, mature B cells ($CD21^+CD23^+$) and for a mixture of immature B and NF T1 (newly-formed T1) B cells ($CD21^-CD23^-$) are indicated. Percentage cells are calculated relative to total IgM^+ cells.

B. Bar graphs comparing immature+NF T1 B cell pools in $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice (gated as shown in A). Three (n=3) age-matched animals (2-3 months) of each genotype were analyzed. Error bars show SD. p-value calculated by a two-tailed student's *t*-test.

C. Representative flow-cytometry plots of bone marrow cells gated for $B220^+$ and analyzed for IgM and IgD expression. Gates are shown for immature B cells (IgM^+IgD^-), NF T1 B cells (IgM^+IgD^{low}), and recirculating, mature B cells (IgM^+IgD^{high}). Percentage cells are calculated relative to total $B220^+$ cells.

D. Ratios of IgD^-/IgD^{low} (immature/NF T1) B cells in the bone marrow of $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice. Subsets gated as in C. Numbers of mice analyzed are indicated (n=); mice were 2-3 months of age. Error bars show SD. ns = non-significant (calculated by two-tailed student's *t*-test).


A.
IgM+ bone marrow cells
VHEμa/ΔJH
VHΔa/ΔJH
53.55%
73.67%
42.16%
22.43%
CD23
CD21
Recirculating mature B
Immature + NF T1
B.
p=0.0374
%(Immature + T1) / IgM+ cells
50
40
30
20
10
0
VHEμa/ΔJH
VHΔa/ΔJH
n=3
n=3
D.
ns
Immature/ NF T1
5
4
3
2
1
0
VHEμa/ΔJH
VHΔa/ΔJH
n=9
n=9
C.
B220+ bone marrow cells
VHEμa/ΔJH
VHΔa/ΔJH
11.60%
7.820%
67.37%
5.116%
79.61%
3.020%
15.33%
9.036%
IgD
IgM
Recirculating mature B
NF T1
Pro+ Pre
Immature

Eμ-deficient B cell development appears to be normal after the immature B stage, even with a restricted generation of immature B cells. Consistent with our previous study, normal numbers of B cells were found in adult spleen of $V_H\Delta^a/WT^b$ mice (Table 3-2, Figure 3.3, (146)). The proportion of B cells (~50% of total live lymphocytes) in the spleen of $V_H\Delta^a/WT^b$ mice wasn't different from that of $V_HE\mu^a/WT^b$ mice. Study of chimeras reconstituted with normal and B-cell deficient bone marrow cells have shown that the continuous pre-B cell input is necessary for maintaining a physiologically normal peripheral B cell compartment, but the number of peripheral B cells is independent of pre-B cell numbers (236), indicating that the peripheral B cell compartment reaches its homeostasis autonomously. Although the turnover of naive B cells in the spleen is high because they are short-lived and there is continuous influx of bone marrow immature B precursors, recent studies have shown that these naive mature B cells, like naive T cells and natural killer cells (237-240), undergo a homeostatic proliferation independent of antigen in the spleen to expand this cell pool (241, 242). And then the antigen-dependent proliferation in the follicles largely increase the size of mature B cell compartment by producing memory and plasma cells, reaching a physiological homeostasis in the spleen (243, 244).

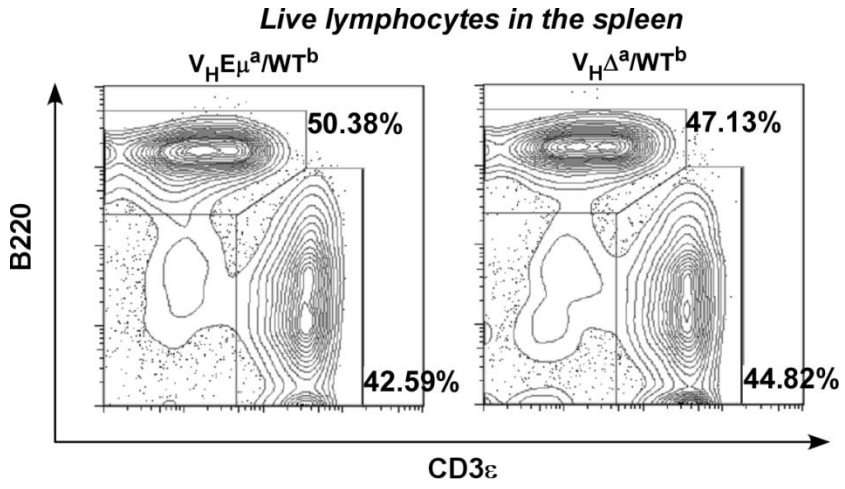
Table 3-2: Cell numbers in the spleen of mice carrying the B1-8V_H knock-in allele (with and without Eμ).

Cells from the spleens of different heavy chain modified mice with and without Eμ were recovered and stained for B cell surface markers (B220) and T cell surface marker (CD3ε). Cell number for lymphocyte subpopulations were calculated based on total cell count (20000 cells collected for each animal) and the percentage of total cells comprising each subpopulation determined by flow cytometry. Dead cells were excluded by PI dye staining. p-values were calculated by two-tailed student's *t*-test. $p>0.05$: no significant difference; $0.01<p<0.05$: significant difference; $0.001<p<0.01$: very significant difference; $p<0.001$: extremely significant difference. n = number of animals analyzed.

	$V_H\Delta^a/WT^b$ n=2	$V_HE\mu^a/WT^b$ n=3		$V_H\Delta^a/\Delta J_H$ n=3	$V_HE\mu^a/\Delta J_H$ n=3	
Lymphocytes	3850 ± 10	4547 ± 293	p=0.16292	4427 ± 29	4547 ± 293	p=0.70457
Live cells	3725 ± 8	4257 ± 325	p=0.29356	4054 ± 115	4257 ± 325	p=0.58586
B220+ B cells	1674 ± 121	1926 ± 411	p=0.47886	1691 ± 334	1926 ± 411	p=0.48663
CD3+ T cells	1636 ± 52	1870 ± 187	p=0.19789	1543 ± 266	1870 ± 187	p=0.15670

Figure 3.3: Normal percentage of B cells in the spleen of $V_H\Delta^a/WT^b$ mice.

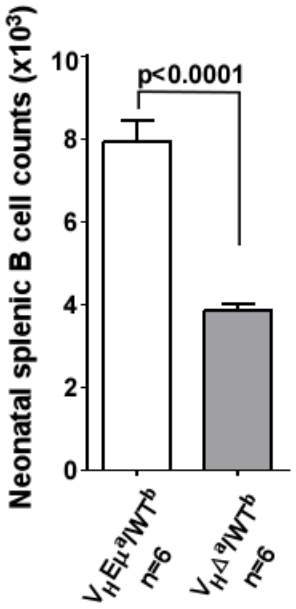
Splenic lymphocytes form different heavy chain modified mice with and without Eμ were recovered and stained for B cell surface markers (B220) and T cell surface marker (CD3ε). Percentages of lymphocyte subpopulations are calculated relative to the total live lymphocytes. Shown is a representative histogram of 3 individual experiments.



For this reason, it was not surprising to find normal B cell numbers in spleens of $V_H\Delta^a/WT^b$ mice, despite the immature B cell deficiency evident in the bone marrow. On the other hand, B cells in the spleens of neonatal mice are essentially immature B cells, whereas mature B cells are absent throughout the 1st week of life (245). During this neonatal period (1st week of life), the newborn actively develop their immune system to reach a hematopoietic homeostasis with mature B and T lymphocytes, under the cooperation of the infant spleen, bone marrow and liver (246, 247). Neonatal splenic B cell counts were collected in $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice. As shown in Figure 3.4, in the $V_H\Delta^a/WT^b$ mice, new-born splenic B cells (analyzed on the day of birth) were half the number found in $V_HE\mu^a/WT^b$ newborns. The early wave of immigrants to spleen, therefore, is reduced in size in the Eμ-deficient mice. We suggest that as the splenic B cells eventually mature, the mature B cell compartment reaches its normal size over time by homeostatic and antigen-induced proliferation (241), and then remains at this normal level once built up, masking the aberrant immature B cell subsets.

Figure 3.4: Neonatal spleens of $V_H\Delta^a/WT^b$ mice have fewer B cells.

Splenic B cell counts of the indicated genotypes. Newborn mice were analyzed at birth (day 1). n=number of spleens analyzed. Bar graph shows numbers of $B220^+$ cells per 10^5 total splenic cells. Error bars indicate Standard Deviation (SD); p-value determined by two-tailed *t*-test (GraphPad Prism 5).

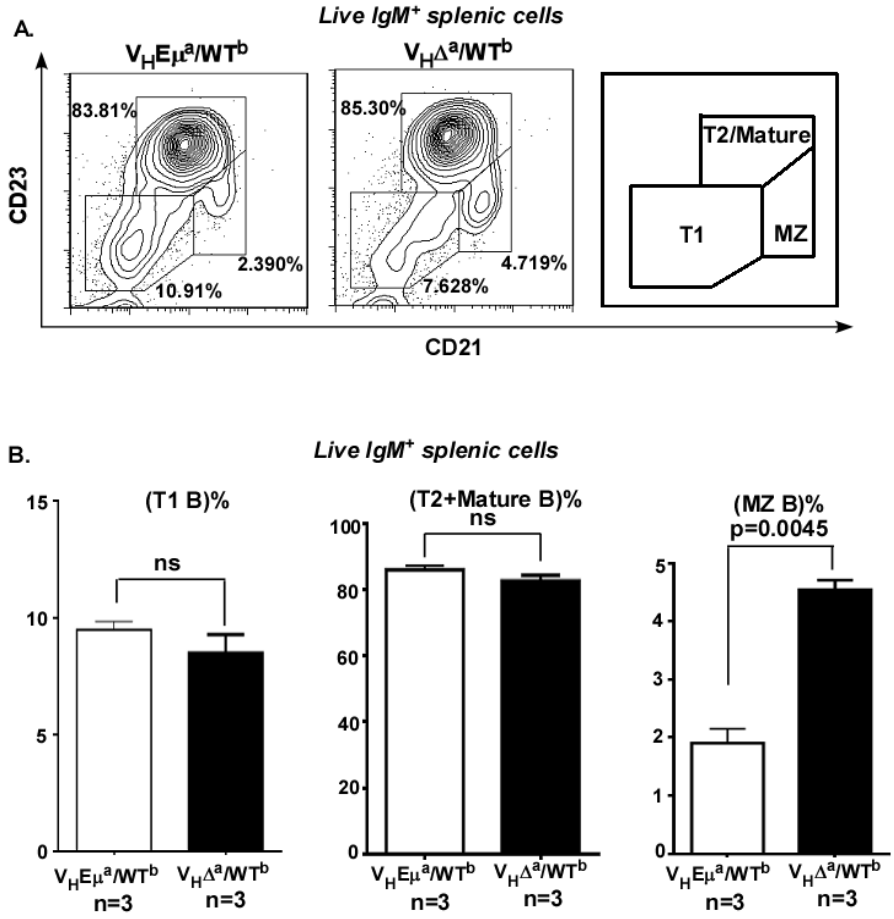


We also analyzed B cell subsets in the spleens of adult $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice. As shown in Figure 3.5, steady-state B cell numbers were the same for both T1 ($IgM^+CD21^-CD23^-$) and T2+mature ($IgM^+CD21^+CD23^+$) B cell compartments in the two strains. In our previous studies, we found that in the $V_H\Delta^a/WT^b$ mice, IgH-double-producers were enriched in the marginal zone B cells of the spleen (146). Consistent with those earlier findings, we again noticed that the marginal zone B cell population ($IgM^+CD21^+CD23^{low}$) was at a higher frequency in$V_H\Delta^a/WT^b$ mice than in $V_HE\mu^a/WT^b$ mice (4.719% versus 2.390%, percentages relative to total IgM^+ splenic lymphocytes) (Figure 3.5).

Figure 3.5: B cell subsets in the spleen of V_H knock-in mice with and without Eμ.

A. Flow cytometry profiles of IgM^+ splenic B lymphocytes, and analyzed for CD21 and CD23 expression. T1 B cells ($CD21^-CD23^-$), T2+mature B cells ($CD21^+CD23^+$), marginal zone B cells ($CD21^+CD23^{low}$). Gates and percentage cells in gates are indicated (% calculated relative to total $B220^+$ cells). Data shown are representative of at least 3 age-matched animals (3-4 months) of each genotype.

B. Percentages of T1 B, T2+mature B, and MZ B cells in the spleen of indicated genotypes (calculated relative to IgM^+ cells). n= number of animals analyzed. p-value determined by two-tailed student *t*-test. Error bars show SD.



In conclusion, when Eμ is missing, bone marrow B-cell development is partially impaired at the pre-B to immature B cell transition, so that the immature B cell compartment does not accumulate to a normal size. B cell populations at other stages including the earlier pre-B and the later transitional and mature B cell stages, however, are not affected directly by Eμ-deletion.

3.3. Eμ-deficient B1-8V_H heavy chain ($V_H\Delta^a$) is expressed at a lower level in pre-B cells

As a *cis*-control transcriptional enhancer, one of Eμ's important functions is to enhance Ig heavy chain expression after V_H gene assembly. For this reason, it is worthwhile to examine the Igμ expression level in pre-B cells from Eμ-deficient mice. To do so, $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice were bred onto the $RAG1^{-/-}$ background. In the absence of Rag1 protein, B cell development in $RAG1^{-/-}$ mice with wild type Ig loci (WT^b/WT^b $RAG1^{-/-}$) is arrested at the pro-B cell stage, which can be identified by surface CD43 (leukosialin) expression in combination with the cell size analysis by FACS (89). In $V_HE\mu^a/WT^b$ $RAG1^{-/-}$ and $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ mice, however, the B1-8V_H knock-in heavy chain gene restores the arrested $RAG1^{-/-}$ pro-B cells to the small pre-B cell stage. $B220^+$ BM cells from these V_H knock-in, $RAG1^{-/-}$ mice are smaller in size (Figure 3.6A) and express lower levels of CD43 than the comparable $B220^+$ BM cells from the WT^b/WT^b $RAG1^{-/-}$ mice (Figure 3.6B), supporting the idea that BM B cells of these V_H knock-in mice ($V_HE\mu^a/WT^b$ $RAG1^{-/-}$and $V_H\Delta^a/WT^b$ $RAG1^{-/-}$) have progressed to but are stalled at the pre-B cell stage. Bone marrow cells from $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ and $V_HE\mu^a/WT^b$ $RAG1^{-/-}$ mice were isolated and assayed by flow cytometry to measure cytoplasmic Igμ protein levels. As previously reported, the progression from pro-B, through large pre-B, to small pre-B was not adversely affected by Eμ deletion (Figure 3.6A, B and (146)), but Igμ expression was reduced in the pre-B cells of $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ mice to ½ the levels seen in pre-B cells of $V_HE\mu^a/WT^b$ $RAG1^{-/-}$ mice

(Figure 3.6C, D and (146)). B-lineage cells in the bone marrow of the $V_HE\mu^a/WT^b$ $RAG1^{-/-}$ and $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ mice, therefore, differ only with respect to the levels of cytoplasmic Igμ available for pre-BCR assembly.

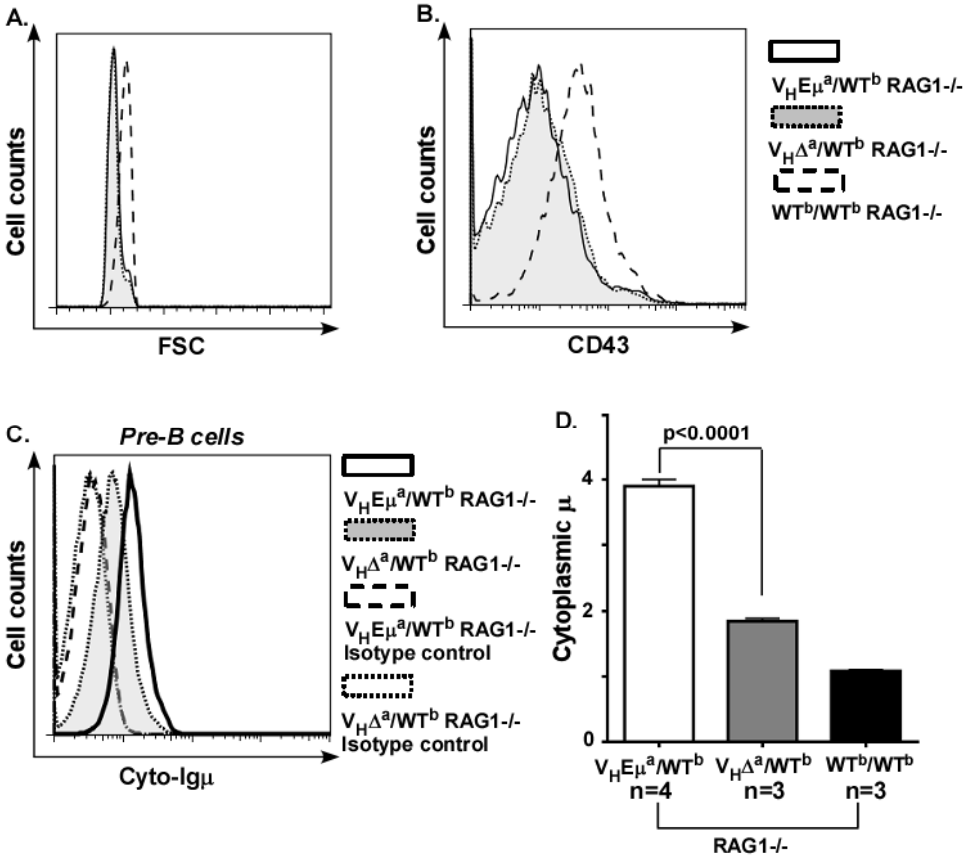
Figure 3.6: Reduced cytoplasmic Igμ expression in pre-B cells of $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ mice.

A. Forward scatter plot showing sizes of WT pro-B cells versus B1-8V_H knock-in pre-B cells. $B220^+$ lymphocytes from bone marrow of RAG1-deficient $V_HE\mu^a/WT^b$, $V_H\Delta^a/WT^b$ and WT^b/WT^b mice were analyzed. Genotypes as indicated in B.

B. Flow cytometry plot of CD43 expression of WT pro-B cells (WT^b/WT^b $RAG1^{-/-}$) versus B1-8V_H knock-in pre-B cells ($V_HE\mu^a/WT^b$ $RAG1^{-/-}$ and $V_H\Delta^a/WT^b$ $RAG1^{-/-}$).

C. Representative histogram of $B220^+$ bone marrow cells stained for intracellular Igμ and analyzed by flow cytometry (genotypes indicated in legend). Isotype control: cells treated with an irrelevant antibody of the same isotype as that of the monoclonal antibody to mouse Igμ.

D. Bar graphs quantifying mean fluorescence of cytoplasmic Igμ in pre-B cells analyzed as shown in A. Error bars show SD; p-values calculated by GraphPad Prism 5.


A.
Cell counts
FSC
B.
Cell counts
CD43
$V_HE\mu^a/WT^b$ RAG1-/-
$V_H\Delta^a/WT^b$ RAG1-/-
WT^b/WT^b RAG1-/-
C.
Pre-B cells
Cell counts
Cyto-Igμ
$V_HE\mu^a/WT^b$ RAG1-/-
$V_H\Delta^a/WT^b$ RAG1-/-
$V_HE\mu^a/WT^b$ RAG1-/- Isotype control
$V_H\Delta^a/WT^b$ RAG1-/- Isotype control
D.
p<0.0001
Cytoplasmic μ
0
2
4
$V_HE\mu^a/WT^b$ n=4
$V_H\Delta^a/WT^b$ n=3
WT^b/WT^b n=3
RAG1-/-

3.4. Small pre-B cells of Eμ-deficient ($V_H\Delta^a$) mice have reduced B cell receptor signaling

Light chain gene assembly after Igμ expression, with the consequent formation of a signaling-competent B cell receptor, is essential for the transition from the pre-B to immature B cell stage. Having confirmed that this transition was compromised in $V_H\Delta^a/WT^b$ and $V_H\Delta^a/\Delta J_H$ mice, we asked whether the defect could be explained by a decrease in the signaling strength of newly-emerging BCRs. In pre-B cells, pre-BCR signals dominate over newly-emerging BCRs because light chain gene assembly is ongoing and most pre-B cells lack a functionally assembled IgL gene. While our interest was in the signals of newly-emerging B1-8 H-chain BCRs, we reasoned that any change in their signal due to the observed reduction of Igμ in $V_H\Delta^a/WT^b$ pre-B cells would similarly affect B1-8 H-chain pre-BCR signals in these cells. We therefore focused on B1-8 H-chain pre-BCR signals in $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice since their receptor structures were identical, which is B1-8 Igμ + SLC in both. However, the amount of Igμ available for pre-BCR assembly differed significantly. To directly compare pre-BCR signals in the small, non-proliferating pre-B cell population poised for Ig light chain gene assembly and expression, we tested the $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice on the $RAG1^{-/-}$ background, where there is no light-chain gene assembly, thereby restricting signals to those mediated by the pre-BCR.

Membrane expression of Igμ in the form of a pre-BCR or BCR, induces intracellular signals as a series of tyrosine phosphorylation (ligand-independent or antigen-induced), which controls B cell differentiation and selection. The pre-BCR and BCR initiated signaling process depends critically on the ITAM region on the cytosolic tail of the Igα/β molecules (154, 158, 160, 164, 167, 248). After phosphorylation of tyrosine residues on the ITAMs, the protein tyrosine kinase (PTK) Syk (spleen tyrosine kinase) is recruited to the phospho-ITAMs and activated, which turns

on various signaling pathways for further signal transduction (175, 249). Syk is known to be a protein kinase involved in mediating multiple signaling events, such as phospholipase C γ2 (PLCγ2) activation and calcium mobilization (149, 250-252). Deletion of Syk led to developmental block at the pro-B to pre-B cell transition, which is a hallmark of defective pre-BCR signaling (253-255). Syk-deficient cells with a transgenic BCR failed to maintain the mature B cell pool, suggesting the critical role of Syk in BCR signaling (252). Extracellular signal-regulated kinase (Erk) is a protein kinase downstream of protein tyrosine kinase Syk (256). It is a key participant in pre-BCR and BCR mediated tonic signaling pathway which regulates the activities of a series of transcription factors and thereby their target genes (170, 178). B lymphocytes in mice deficient in both Erk1 and Erk2 were defective in pre-BCR-dependent transcriptional events, showing impaired cell expansion and a block at the pro-B to pre-B transition (170). Since Syk and Erk are essential for both pre-BCR and BCR signaling, it is conceivable that the cytoplasmic phosphorylation of total tyrosine, Syk and Erk depends on and is directly proportional to B cell receptor signal strength.

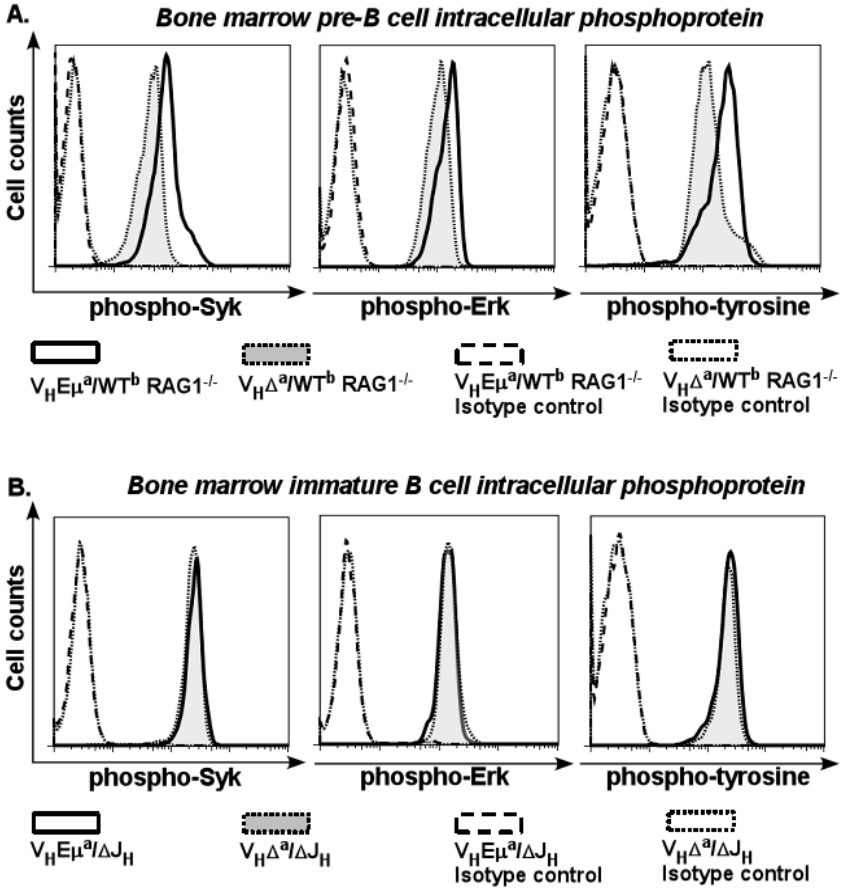
Bone marrow B cells from $V_HE\mu^a/WT^b$ $RAG1^{-/-}$ and $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ mice were treated with sodium pervanadate, a protein-tyrosine phosphatase (PTP) inhibitor, in order to analyze tonic signals mediated by the pre-BCR (158, 165). The effect of sodium pervanadate in signaling studies is to block the activity of intracellular PTPs which counterbalance signals generated by the receptors, so that the weak or transient signals can be stabilized and revealed (158, 231, 257, 258). The pervanadate-treated cells were then permeabilized and incubated with antibodies specific for phosphorylated Erk (pErk), for phosphorylated Syk (pSyk), and for phospho-tyrosine residues, in general (pTyr). As shown in Figure 3.7A, mean levels of these phosphorylated

proteins (pSyk, pErk, and of global tyrosine-phosphorylated proteins) were lower, in every case, in $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ pre-B cells.

Figure 3.7: Pre-BCR signals are attenuated in the small pre-B cells of $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ mice.

A. Histograms of pre-B cells stained with antibodies to the phosphorylated (activated) forms of the indicated proteins. $B220^+$ cells from bone marrow of $V_HE\mu^a/WT^b$ $RAG1^{-/-}$ and $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ mice were treated with the phosphatase inhibitor pervanadate and then fixed and permeablized before incubation with antibodies. pErk = phosphorylated form of "extracellular signal-regulated kinase"; pSyk = phosphorylated form of "spleen tyrosine kinase"; pTyr = phosphorylated tyrosine residues on any/all cell proteins. Controls are pre-B cells stained with isotype-matched antibodies (no specificity for mouse proteins). Histograms shown are representative of 3 independent experiments with 2 month-old mice of the indicated genotypes.

B. Histograms of immature B cells stained with antibodies to the phosphorylated (activated) forms of the indicated proteins as discussed in A. $B220^{low}sIgM^+$ bone marrow cells from bone marrow of $V_H\Delta^a/\Delta J_H$ and $V_HE\mu^a/\Delta J_H$ mice were analyzed. Histograms shown are representative of 3 independent experiments with 2.5 month-old mice of the indicated genotypes.


A.
Bone marrow pre-B cell intracellular phosphoprotein
Cell counts
phospho-Syk
phospho-Erk
phospho-tyrosine
VHEμa/WTb RAG1-/-
VHΔa/WTb RAG1-/-
VHEμa/WTb RAG1-/- Isotype control
VHΔa/WTb RAG1-/- Isotype control
B.
Bone marrow immature B cell intracellular phosphoprotein
Cell counts
phospho-Syk
phospho-Erk
phospho-tyrosine
VHEμa/ΔJH
VHΔa/ΔJH
VHEμa/ΔJH Isotype control
VHΔa/ΔJH Isotype control

3.5. Eμ-deficient B1-8V_H heavy chain ($V_H\Delta^a$) is expressed at the normal level in immature and mature B cells

We and others have suggested that once cells reach the immature B cell stage, Eμ is no longer required for normal levels of Igμ mRNA and protein. This is because the 3' regulatory region (3'RR, Figure 1.2) becomes active and can efficiently enhance Igμ expression at this stage (213, 214, 259). To test this hypothesis, we compared Igμ protein levels in pre-B, immature and mature B cells of $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice**.** In these mice, B cell development and BCR expression is entirely dependent upon the $V_HE\mu^a$ and $V_H\Delta^a$ alleles, respectively. Bone marrow cells were stained for surface B220 and IgM (sIgM). Cells were then fixed, permeablized and incubated with antibodies to cytoplasmic Igμ. Immature B cells were identified as $B220^{low}sIgM^+$. As shown in Figure 3.8A, surface IgM levels varied over a broad range in these immature B cells from these mice, but the overall profiles overlapped when Eμ was and was not present on the knock-in allele ($V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ cells, respectively). Similarly, as has been reported before for $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice (146), splenic B cells from $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice expressed identical levels of surface IgM (Figure 3.8B). Cytoplasmic Igμ in the immature bone marrow cells were of no difference between these two genotypes (Figure 3.8D, F). This contrasts with what was seen in pre-B cells ($B220^+sIgM^-$, no surface IgM) from the same animals. As in the pre-B cells from $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ mice (Figure 3.6C, D), cytoplasmic Igμ levels were reduced in pre-B cells from $V_H\Delta^a/\Delta J_H$ to almost ½ that in pre-B cells from $V_HE\mu^a/\Delta J_H$mice (Figure 3.8C, F). Reduced cytoplasmic Igμ in pre-B cells and normal cytoplasmic Igμ levels in immature/mature BM B cells were also evident when the entire population of $B220^+$ bone marrow cells were similarly analyzed (Figure 3.8E). Note that the peaks corresponding to cells

with high levels of cytoplasmic Igμ (immature/mature B cells) were juxtaposed for $B220^{+}$ of both genotypes, but the peaks for cells with comparatively low cytoplasmic Igμ (pre-B cells) were displaced from one another, with the peak for$V_H\Delta^a/\Delta J_H$ pre-B cells lying left (lower cytoplasmic Igμ) of that for$V_HE\mu^a/\Delta J_H$ pre-B cells. In summary, the Eμ-deficient allele produced significantly less Igμ protein in pre-B cells than did the same gene with Eμ present, but this deficiency no longer existed in immature/mature BM B cells, nor in splenic B cells.

Figure 3.8: Igμ is not reduced in immature B cells expressing an Eμ-deficient allele.

A. Representative histogram of surface IgM-staining on immature B cells of $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice. Immature B cells identified and gated as $B220^{low}sIgM^+$. Isotype control = cells incubated with an irrelevant fluorescent antibody with the same isotype as the monoclonal antibody to mouse Igμ. Representative of 4 independent experiments.

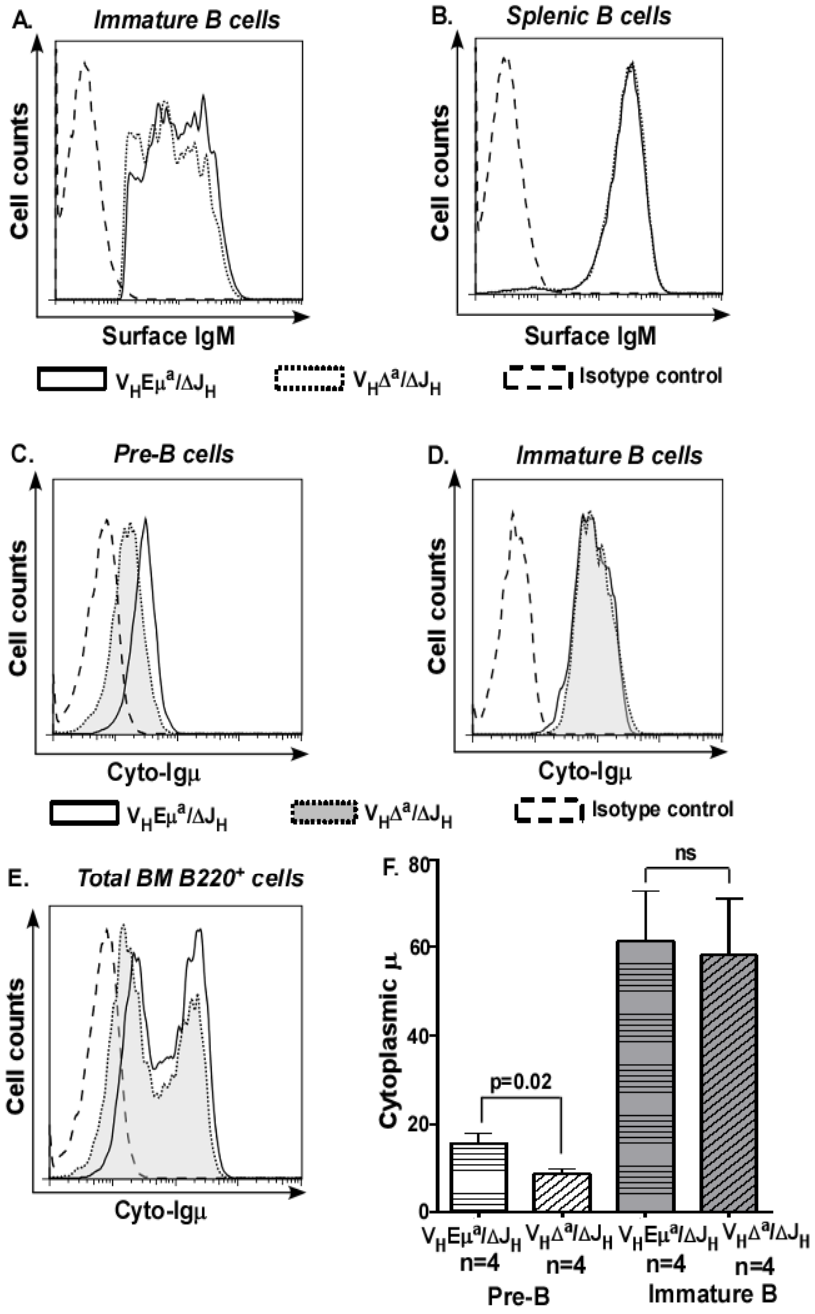
B. Representative histogram of surface IgM-staining on splenic B cells of $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice. $B220^+$ splenic cells were analyzed for surface IgM expression. Representative of 4 independent experiments.

C. Representative histogram of cytoplasmic Igμ-staining in pre-B cells from $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice. Bone marrow cells gated for $B220^+sIgM^-$ (pre-B cells). Shown is Representative of 4 independent experiments.

D. Representative histogram of cytoplasmic Igμ-staining in immature B cells of $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice. Bone marrow cells gated for $B220^{low}sIgM^+$ (immature B cells). Representative of 4 independent experiments.

E. Representative histogram of cytoplasmic Igμ-staining in bone marrow $B220^+$ cells from $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice. Peaks on the left correspond to cytoplasmic Igμ in pre-B cells; peaks on right correspond to cytoplasmic Igμ in immature and mature B cells. Representative of 4 independent experiments.

F. Bar graph quantifying cytoplasmic Igμ in pre-B and in immature B cells from bone marrow of $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice. Mice of each genotype (3 months old) were analyzed in four individual experiments. Error bars show SD. p-value calculated by two-tailed student's *t*-test. ns = non-significant.


A.
Immature B cells
Cell counts
Surface IgM
B.
Splenic B cells
Cell counts
Surface IgM
$V_HE\mu^a/\Delta J_H$
$V_H\Delta^a/\Delta J_H$
Isotype control
C.
Pre-B cells
Cell counts
Cyto-Igμ
D.
Immature B cells
Cell counts
Cyto-Igμ
$V_HE\mu^a/\Delta J_H$
$V_H\Delta^a/\Delta J_H$
Isotype control
E.
Total BM B220+ cells
Cell counts
Cyto-Igμ
F.
Cytoplasmic μ
80
60
40
20
0
p=0.02
ns
$V_HE\mu^a/\Delta J_H$ n=4
$V_H\Delta^a/\Delta J_H$ n=4
$V_HE\mu^a/\Delta J_H$ n=4
$V_H\Delta^a/\Delta J_H$ n=4
Pre-B
Immature B

3.6. Immature B cells of Eμ-deficient ($V_H\Delta^a$) mice have normal B cell receptor signaling

The BCR signals in immature B cells represent only those that have successfully passed the tests of both positive and negative selections, with sufficient and appropriate signaling properties. We asked whether the return to equivalent levels of intracellular Igμ and of surface IgM would lead to equivalent tonic signaling. $B220^{low}sIgM^+$ immature B cells from the $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice were analyzed for signaling in the same manner as described for pre-B cells. As shown in Figure 3.7B, flow-cytometry plots of the phosphorylated, active forms of the relevant kinases and overall phosphorylated tyrosine levels were indistinguishable for the immature B cells from these two mouse strains. This was in striking contrast to the signals from pre-B cells in the V_H knock-in mouse strains with and without Eμ (Figure 3.7A). The difference in signaling detected within the pre-B cells was not found within the immature B cells, consistent with the reduced Igμ levels in pre-B cells but same IgM expression in immature B cells with and without Eμ.

3.7. Onset of κ-light chain gene transcription and rearrangement is normal in pre-B cells expressing the Eμ-deficient heavy chain ($V_H\Delta^a$)

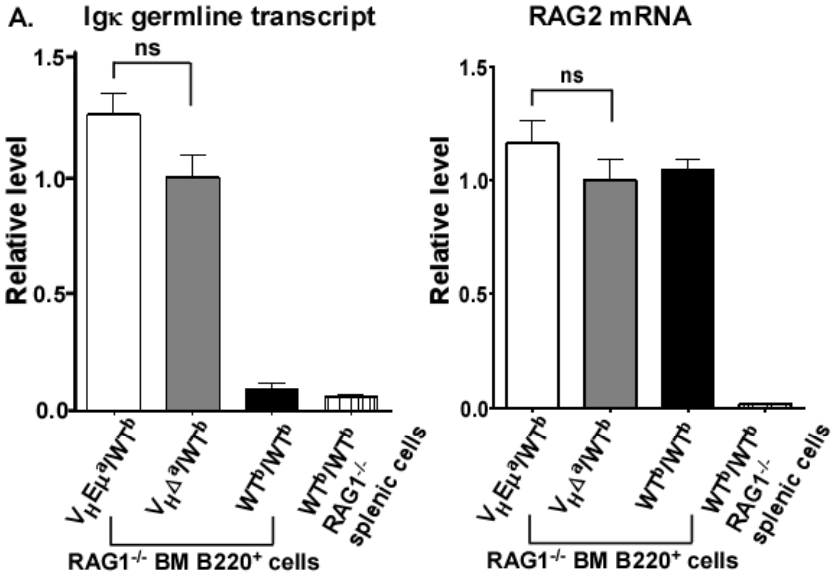
There could be a smaller immature B cell compartment if light-chain gene rearrangement is not activated normally in Igμ-expressing pre-B cells. Since the onset of light chain gene rearrangement is dependent upon pre-BCR signaling (161), and we have found that B1-8 H-chain pre-BCR signals were reduced in pre-B cells of $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ mice (Figure 3.7A), we asked whether the reduced signals had resulted in a defect in the activation of light-chain gene assembly, thereby leading to less efficient population of the immature B cell pool. To answer this, we examined two hallmarks of Igκ locus activation: Igκ germ line gene transcription

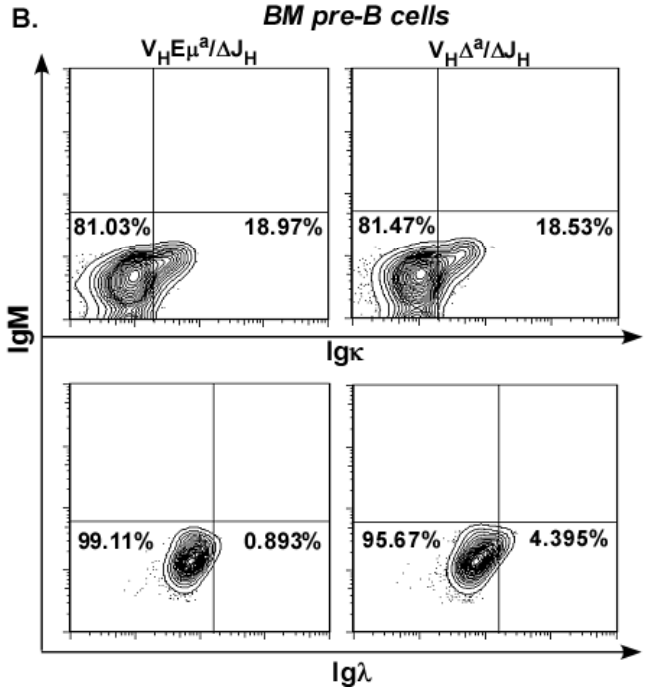
(94) and recombination-activating gene 2 (RAG2) expression. Pre-B cells from $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ and $V_HE\mu^a/WT^b$ $RAG1^{-/-}$ mice were isolated, and germline transcripts from the kappa light chain (Igκ) locus and RAG2 mRNA quantified by reverse transcriptase-dependent, quantitative (real-time) polymerase chain reaction (PCR). As shown in Figure 3.9A, Igκ germ line transcripts and RAG2 transcripts (both essential for subsequent Vκ-to-Jκ recombination) were found at comparable levels in the pre-B cells of $V_HE\mu^a/WT^b$ $RAG1^{-/-}$ and $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ mice. That the Igκ germ line transcripts were dependent upon pre-BCR signals was supported by the fact that $B220^+$ bone marrow cells from $RAG1^{-/-}$ mice without the V_H knock-in (and therefore unable to make a pre-BCR; WT^b/WT^b $RAG1^{-/-}$ in Figure 3.9A, left panel) were devoid of these transcripts. The same cells from WT^b/WT^b $RAG1^{-/-}$ mice were positive for RAG2 transcripts (Figure 3.9A, right panel), since these are pro-B cells poised to undergo D-to-J_H and V_H-to-DJ_H recombination but unable to do so because of the absence of RAG1. These results suggested that the onset of light-chain gene rearrangement was not disturbed in $V_H\Delta^a/WT^b$ pre-B cells, despite the lower B1-8 H-chain pre-BCR signals in these cells.

Figure 3.9: RAG2 and germline kappa light chain (Igκ) gene transcription is normal in pre-B cells of mice expressing the Eμ-deficient allele.

A. Bar graphs comparing RAG2 and germline Igκ transcript levels in $B220^{+}$ lymphocytes from bone marrow of RAG1-deficient $V_H\Delta^a/WT^b$, $V_HE\mu^a/WT^b$ and WT^b/WT^b mice. Germline Igκ transcripts (left panel) and RAG2 mRNA (right panel) levels were determined by quantitative RT-PCR. Igκ and RAG2 mRNA of $V_H\Delta^a/WT^b$ $RAG1^{-/-}$ pre-B cells are set as 1.0. Pro-B cells of WT^b/WT^b, $RAG1^{-/-}$ littermates served as a negative control for Igκ germline transcripts (absence of pre-BCR precludes onset of Igκ transcription) and as a positive control for RAG2 expression (absence of IgH gene assembly sustains expression of the RAG1 and RAG2 genes). mRNAs from WT^b/WT^b, $RAG1^{-/-}$ splenic cells (no B cells) were included as negative controls for both analyses. All analyses were normalized to *hgprt1* mRNA to exclude variations of input RNA templates. Five age-matched animals (2 months) of each genotype were analyzed. Statistical significance (ns: not significant) was determined by two-tailed student's *t*-test.

B. Representative flow-cytometry plots of $B220^{+}sIgM^{-}$ bone marrow lymphocytes (pre-B cells), stained for cytoplasmic Igκ (upper panels) or for cytoplasmic Igλ (lower panels). % positive cells are indicated in the lower right quadrant of each plot. Data are representative of analyses of three age-matched mice (2 months) of each genotype.


A.
Igκ germline transcript
RAG2 mRNA
ns
ns
Relative level
1.5
1.0
0.5
0.0
$V_HE\mu^a/WT^b$
$V_H\Delta^a/WT^b$
WT^b/WT^b
WT^b/WT^b RAG1$^{-/-}$ splenic cells
RAG1$^{-/-}$ BM B220$^+$ cells


B.
BM pre-B cells
$V_HE\mu^a/\Delta J_H$
$V_H\Delta^a/\Delta J_H$
81.03%
18.97%
81.47%
18.53%
IgM
Igκ
99.11%
0.893%
95.67%
4.395%
Igλ

In mice that are not deficient for RAG1, Igκ germline transcription and RAG1/RAG2 expression are followed by Vκ gene assembly and subsequent Igκ protein expression. We compared percent pre-B cells expressing cytoplasmic Igκ in the pre-B cells of $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice. The percentages of pre-B cells expressing Igκ were similar in these two mouse strains (18.97% versus 18.53%, Figure 3.9B upper panels). In short, germline Igκ transcription, RAG2 transcription, and cytoplasmic Igκ protein expression in pre-B cells were unaffected by the deletion of Eμ, again suggesting a normal onset of Igκ rearrangement in the pre-B cells of $V_H\Delta^a/\Delta J_H$ mice. We conclude that pre-BCR signaling, while reduced in pre-B cells expressing an Eμ-deficient IgH gene, remains sufficient to induce normal levels of light chain gene assembly. The smaller immature B cell compartment in these animals, therefore, results from a deficiency operating after this process.

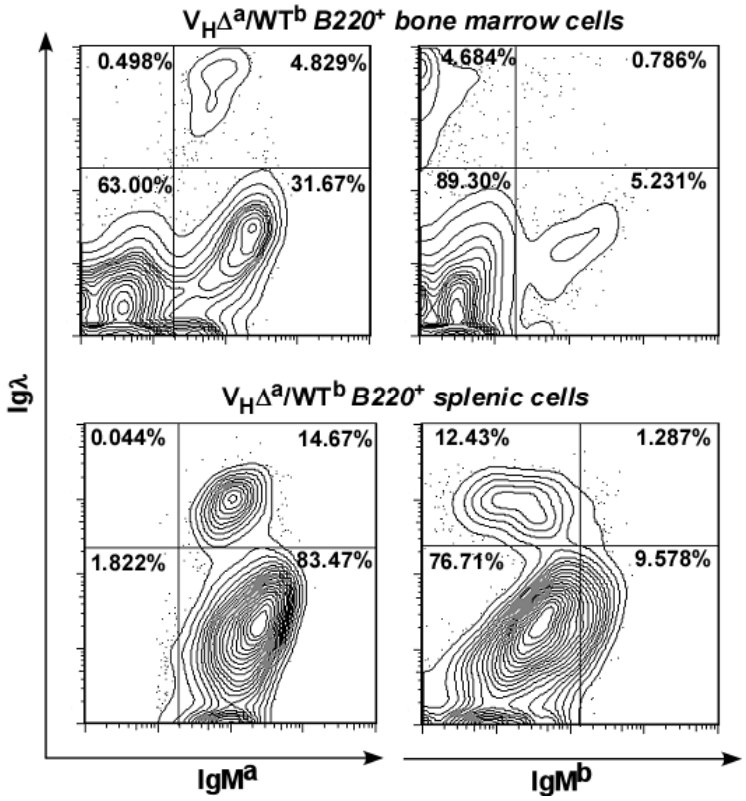
3.8. Eμ-deficient ($V_H\Delta^a$) B cells have undergone increased light chain editing

Vλ assembly and Igλ protein expression can occur following Igκ usage and is generally regarded as evidence of light chain editing (to rescue cells that have not been able to assemble a functional Igκ gene or that express an Igμ/κ BCR with autoreactive properties). We compared percent pre-B cells expressing cytoplasmic Igλ in the pre-B cells of $V_HE\mu^a/\Delta J_H$ and $V_H\Delta^a/\Delta J_H$ mice. There was a detectable subpopulation of pre-B cells expressing cytoplasmic Igλ only in the $V_H\Delta^a/\Delta J_H$ mice (4.395%, Figure 3.9B lower panels). This finding is consistent with our previous study showing increased light-chain editing in splenic B cells from these mice (146). As discussed in more detail later, we believe this light-chain editing resulted not from expression of autoreactive BCRs but from the inability of many newly-formed BCRs to reach the signaling threshold for the pre-B to immature B cell transition.

In addition, in $V_H\Delta^a/WT^b$ heterozygous mice, "single-producers" express their BCRs entirely dependent upon Igμ from an Eμ-deficient allele. These single-producers from the immature and splenic B cell compartments included more lambda chain producers than their "double-producer" counterparts. As shown in Figure 3.10, in the bone marrow (upper panel) and spleen (lower panel) of $V_H\Delta^a/WT^b$ mice, surface IgM^a+ cells (left panels) include a subpopulation that express the lambda light chain. But when cells express IgM^b as double-producers (which must also co-express IgM^a from the pre-assembled V_H gene, right panels), they do not express Igλ, suggesting that there is no excessive light chain editing in these IgH-double-producers. This is consistent with the evidence of splenic B cells having undergone unusually high levels of light-chain editing in $V_H\Delta^a/\Delta J_H$ mice (B cells in these mice are all IgH-single-producers) (146).

Figure 3.10: Increased light chain editing in Eμ-deficient $V_H\Delta^a/WT^b$ B cells.

Cell surface staining of Igλ and IgM^a/IgM^b on bone marrow and splenic B cells in $V_H\Delta^a/WT^b$mice. $B220^+$ live lymphocytes gated and shown. Most cells expressing both heavy chain alleles are not expressing λ chains, whereas ΔEμ-single producers are more likely to express the λ light chain.



3.9. A pre-assembled light chain gene (3-83Vκ) partially rescues allelic exclusion and pre-B to immature B cell transition in mice expressing the $V_H\Delta^a$ heavy chain

As described previously and shown in this study, mice heterozygous for a functional, but Eμ-deficient IgH allele ($V_H\Delta^a/WT^b$ mice) exhibited a profound defect in allelic exclusion, with approximately 20% splenic B cells expressing both the mutant heavy chain and an assembled WT^bheavy chain ("double-producers", (146)). Immature and splenic B cells whose BCRs are entirely dependent upon Igμ from an Eμ-deficient allele (as in $V_H\Delta^a/\Delta J_H$ mice or "single-producers" in $V_H\Delta^a/WT^b$ mice) showed evidence of having undergone unusually high levels of light-chain editing (Figure 3.10 and (146)). We have suggested that both phenotypes are due to a decrease in Igμ which results in diminished B1-8V_H-BCR signals often insufficient to reach the threshold for positive selection. To circumvent this problem, cells that have only the Eμ-deficient allele available for expression (as "single-producers") continue to edit their light chain until they generate a BCR with superior signaling ability, overcoming the defects of low receptor density. Double-producers, on the other hand, assemble and express the second IgH^b allele (that retains Eμ), thereby reversing their deficiency in Igμ levels, giving these cells a selective advantage.

To further test the hypothesis that light chain editing in single-producers is functionally related to the transition of pre-B cells to the immature B cell stage, and to the outgrowth of double-producers in $V_H\Delta^a/WT^b$ mice, we asked whether limiting cells to a single light chain sequence would not only affect the pre-B to immature B cell transition of single-producers but also the relative advantage afforded to double-producers in the same animals. $V_H\Delta^a/WT^b$ mice were bred to a strain that carries a functional 3-83Vκ knock-in gene (a pre-assembled kappa light chain variable region gene) on the *Igκ* locus. B cell subpopulations were compared in the resulting

$V_H\Delta^a/WT^b$ 3-83κ/Igκ mice versus the $V_H\Delta^a/WT^b$ mice. As shown in Figure 3.11 and Figure 3.12, the relative size of the immature B cell pool was greatly increased in the presence of the 3-83Vκ knock-in gene (Figure 3.11A left panel, Figure 3.11C and Figure 3.12). Importantly, this increase was accompanied by fewer double-producers in both the immature and splenic B cell compartments (Figure 3.11A middle and right panels and Figure 3.11B). Igλ-producing cells also reduced in number by the 3-83Vκ knock-in light chain (from 11.3% to 4.7% splenic B cells, Figure 3.13). We interpret these results as evidence that the B1-8V_H/3-83Vκ combination results in a BCR with stronger signaling properties than the average BCR in normal wild-type cells expressing a wide repertoire of light chain genes. This IgH/IgL combination largely compensates for the lower than normal levels of Igμ because of Eμ-deletion. The result is a more rapid progression of single-producers from the pre-B to the immature B cell stage (after little or no light chain editing), and also a reduction in the selective advantage of double-producers over single-producers in these animals.

Figure 3.11: 3-83Vκ knock-in gene reduces the incidence of double-producers in $V_H\Delta^a/WT^b$ mice.

A. *Left panels:* Flow cytometry profiles of bone marrow cells gated for lymphocytes, and analyzed for B220 and IgM expression. $B220^{low}IgM^+$ = immature B cells (% immature B cells indicated). *Middle panels:* Flow cytometry profiles of bone marrow immature B cells as gated on the left, analyzed for IgM^a and IgM^b expression (% double producers within immature B cell population indicated). *Right panels:* Flow cytometry profile of $B220^+$ splenic cells (B cells), analyzed for IgM^a and IgM^b expression. (% double producers within splenic B cells indicated). Data shown are representative of at least 4 age-matched animals (3-4 months) of each genotype.

B. Data of double-producers as analyzed in A. n= number of animals analyzed. p-value determined by two-tailed student *t*-test. Error bars show SD.

C. Ratios of pro/pre-B to immature B cells in the bone marrows of indicated genotypes (calculated as in Figure 3.1). n= number of animals analyzed. p-value determined by two-tailed student *t*-test. Error bars show SD.

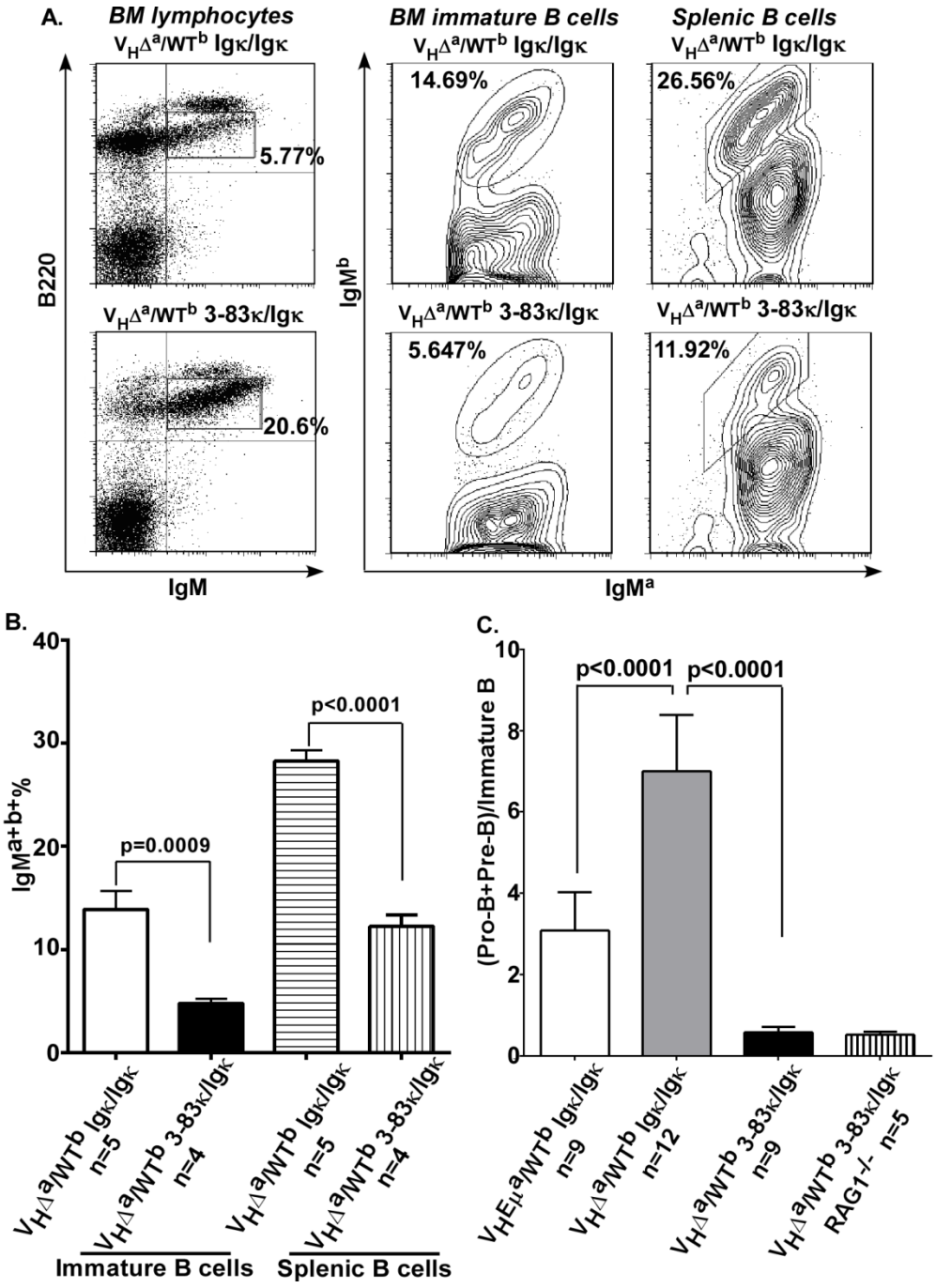

A.
BM lymphocytes
BM immature B cells
Splenic B cells
VHΔa/WTb Igκ/Igκ
VHΔa/WTb 3-83κ/Igκ
5.77%
20.6%
14.69%
26.56%
5.647%
11.92%
B220
IgM
IgMb
IgMa
B.
IgMa+b+%
p=0.0009
p<0.0001
VHΔa/WTb Igκ/Igκ n=5
VHΔa/WTb 3-83κ/Igκ n=4
VHΔa/WTb Igκ/Igκ n=5
VHΔa/WTb 3-83κ/Igκ n=4
Immature B cells
Splenic B cells
C.
(Pro-B+Pre-B)/Immature B
p<0.0001
p<0.0001
VHEμa/WTb Igκ/Igκ n=9
VHΔa/WTb Igκ/Igκ n=12
VHΔa/WTb 3-83κ/Igκ n=9
VHΔa/WTb 3-83κ/Igκ RAG1-/- n=5

Figure 3.12: 3-83Vκ knock-in gene increases the efficiency of pre-B to immature B cell transition in Eμ-deficient mice.

A. Flow cytometry profiles of $B220^+$ bone marrow lymphocytes, and analyzed for IgM and IgD expression. Immature B cells (IgD^-IgM^+), pro-B+pre-B cells (IgD^-IgM^-). Gates and percentage cells in gates are indicated (% calculated relative to total $B220^+$ cells). Data shown are representative of at least 3 age-matched animals (3-4 months) of each genotype.

B. Ratios of pro-B+pre-B to immature B cells in the bone marrows of indicated genotypes (calculated as in Figure 3.1). n= number of animals analyzed. p-value determined by two-tailed student *t*-test. Error bars show SD.

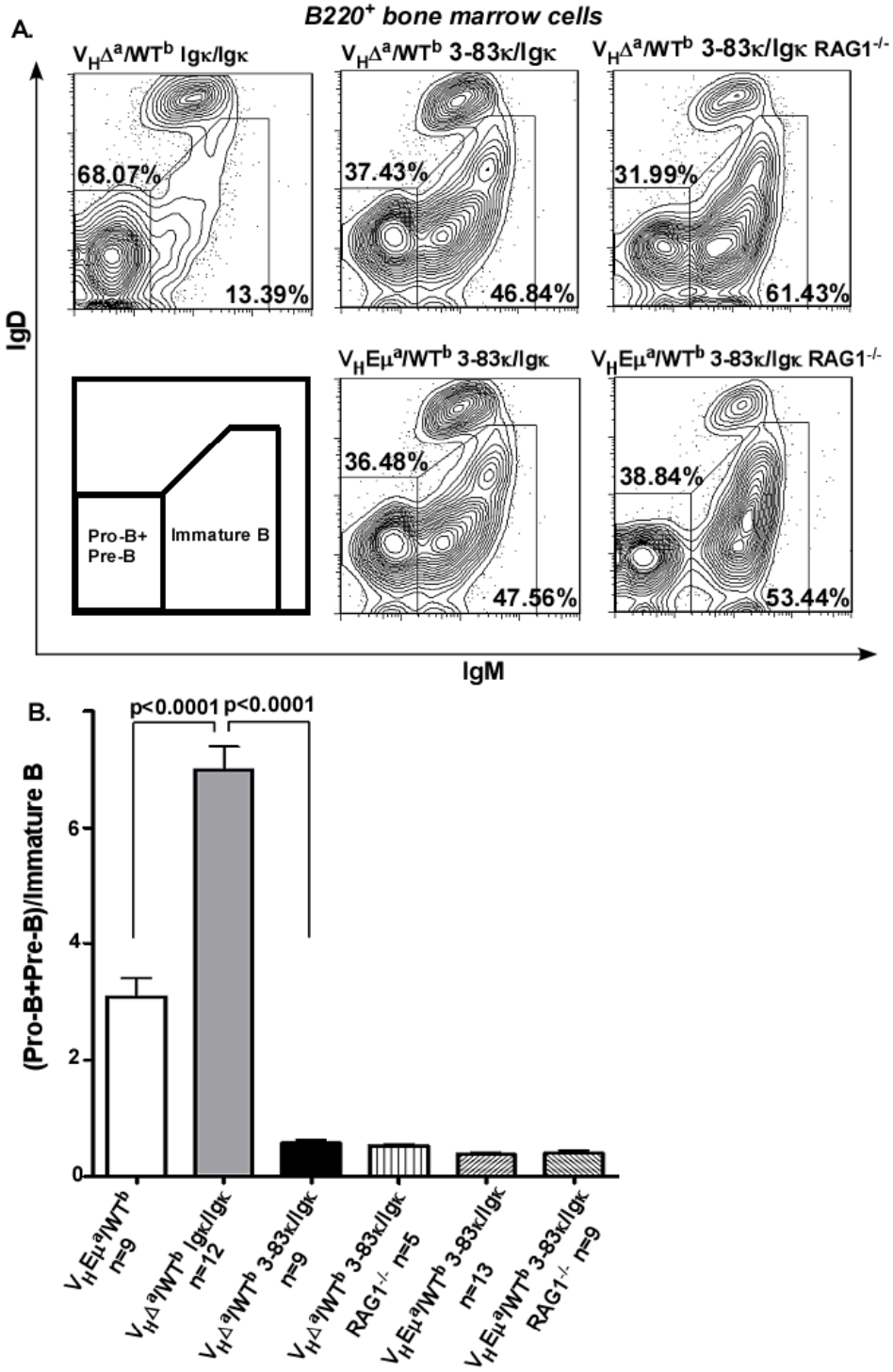
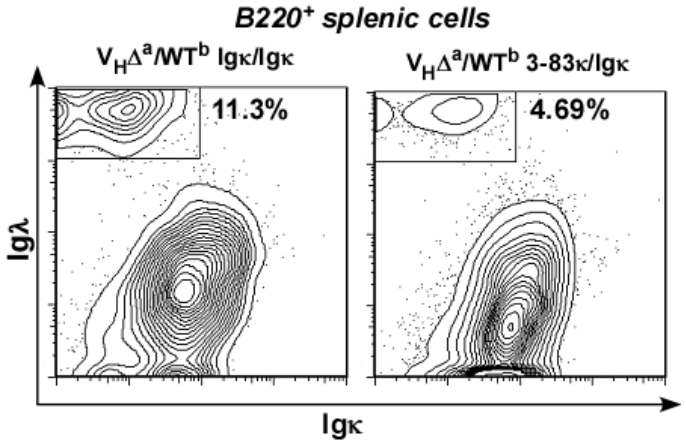

A.
B220+ bone marrow cells
VHΔa/WTb Igκ/Igκ
VHΔa/WTb 3-83κ/Igκ
VHΔa/WTb 3-83κ/Igκ RAG1-/-
68.07%
13.39%
37.43%
46.84%
31.99%
61.43%
VHEμa/WTb 3-83κ/Igκ
VHEμa/WTb 3-83κ/Igκ RAG1-/-
36.48%
47.56%
38.84%
53.44%
Pro-B+ Pre-B
Immature B
IgD
IgM
B.
p<0.0001
p<0.0001
(Pro-B+Pre-B)/Immature B
0
2
4
6
VHEμa/WTb n=9
VHΔa/WTb Igκ/Igκ n=12
VHΔa/WTb 3-83κ/Igκ n=9
VHΔa/WTb 3-83κ/Igκ RAG1-/- n=5
VHEμa/WTb 3-83κ/Igκ n=13
VHEμa/WTb 3-83κ/Igκ RAG1-/- n=9

Figure 3.13: **Decrease in Igλ-producers in mice expressing both the Eμ-deficient IgH allele and a 3-83Vκ knock-in Igκ allele.**

Flow cytometry profiles of $B220^+$ splenic lymphocytes (B cells) analyzed for Igκ and Igλ expression. % Igκ and Igλ expressing cells calculated relative to total $B220^+$ cells. Genotypes indicated above the plots. Plots shown are representative of 3 independent experiments.



3.10. In $V_H\Delta^a/WT^b$ mice, allelically "included" B cells (expressing two different IgHs) bear a higher probability of auto-reactivity

A relationship between the expression of two different BCRs and autoreactivity has long been conjectured as an evolutionary explanation for the development of "allelic exclusion" in B lymphocytes. Having developed a mouse strain with a profound defect in allelic exclusion, we asked whether the double-producers from these animals were at increased risk of expressing autoreactive receptors.

In a preliminary screen by ANA (anti-nuclear antibody) test for autoimmune antibodies, 8/13 (61%) sera from the $V_H\Delta^a/WT^b$ animals showed moderate to high levels of reactivity to the nuclei, while 3/20 (15%) sera from the $V_HE\mu^a/WT^b$ animals showed the comparable reactivity (data not shown). To extend these analyses, we examined sera from mice of these and additional genotypes for anti-dsDNA reactivity by enzyme-linked immunosorbant assay (ELISA). Included in these analyses were two mouse strains that carried the $V_H\Delta^a$ allele but were unable to generate double-producers, either because they were homozygous for the $V_H\Delta^a$ allele ($V_H\Delta^a/V_H\Delta^a$) or because they lacked a functional second heavy chain allele ($V_H\Delta^a/\Delta J_H$). To directly compare IgM expressed from the B1-8V_H knock-in allele, which is present in all genotypes with and without Eμ, we used an anti-IgM^a antibody to detect the anti-dsDNA antibodies in these mouse sera. As shown in Figure 3.14A, the IgM^a in sera from $V_H\Delta^a/WT^b$ animals contained significantly higher anti-dsDNA reactivity than did IgM^a from any of the other genotypes ($V_HE\mu^a/WT^b$, $V_H\Delta^a/V_H\Delta^a$, $V_H\Delta^a/\Delta J_H$ or $V_HE\mu^a/\Delta J_H$).

To test more directly for anti-dsDNA BCRs among B cell double-producers in the spleen, we isolated and cultured $B220^{+}$ spleen cells from $V_H\Delta^a/WT^b$ mice (contain ~20% double-producers) and $V_HE\mu^a/WT^b$ mice (no double-producers) with LPS for 7 days. As shown in Figure 3.14B left panel, these two cultures produced comparable amounts of IgM^a, rising well above the background by Day 3. However, culture supernatants from the $V_H\Delta^a/WT^b$ culture showed a striking rise in IgM^a antibodies reactive to dsDNA over time, while this did not occur in the $V_HE\mu^a/WT^b$ cell cultures (Figure 3.14B right panel).

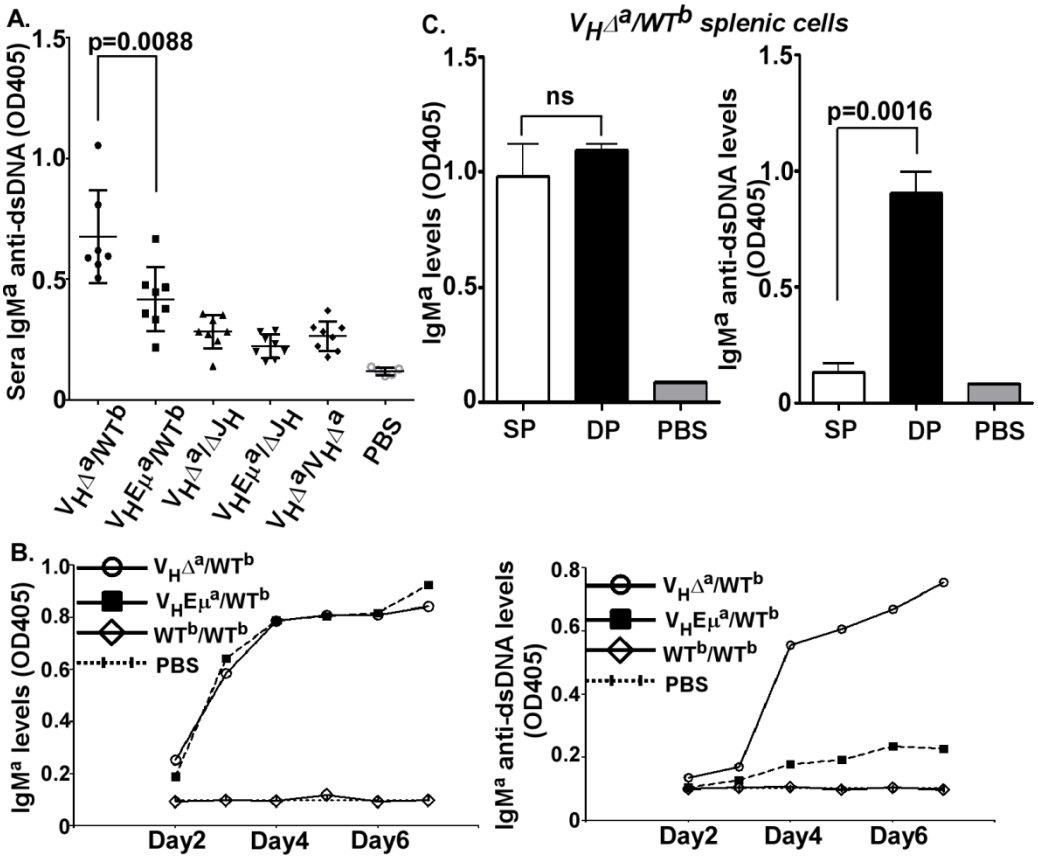
To prove whether this IgM^a anti-DNA antibody (expressed from the $V_H\Delta^a$ IgH gene) was being produced by the single-producers and/or by the double-producers in the $V_H\Delta^a/WT^b$ animals, we sorted $V_H\Delta^a/WT^b$ splenic B cells into these two subpopulations (IgM^{a+b-} single-producers, and IgM^{a+b+} double-producers) and cultured them separately with LPS. Culture supernatants were analyzed on day 7, for their anti-dsDNA antibody levels. As shown in Figure 3.14C left panel, again IgM^a secretion overall was the same in both cultures. However, only the double-producers secreted IgM^a anti-dsDNA antibody (Figure 3.14C right panel). We concluded therefore, that anti-dsDNA BCRs are more prevalent on the double-producers of $V_H\Delta^a/WT^b$ mice than on single-producers in the same mice or in $V_HE\mu^a/WT^b$ mice. Their anti-dsDNA reactivity is associated with IgM^a protein expressed from the $V_H\Delta^a$ knock-in IgH. As discussed below, we interpret this as evidence that the BCRs formed with $Ig\mu^a$ from the Eμ-deficient allele are not only of insufficient density to signal positive selection but also are unable to signal negative selection.

Figure 3.14: Anti-dsDNA reactivity from B1-8V_H allele of B cells expressing two different receptors in $V_H\Delta^a/WT^b$ mice.

A. IgM^a (B1-8V_H) anti-dsDNA proteins in sera from mice of the indicated genotypes. Y-axis = (OD405 of IgM^a anti-dsDNA)/(OD405 of total IgM^a) in sera. This ratio of the standard IgM^a anti-dsDNA monoclonal antibody is set at 1.0 and not shown in figure. Means and standard deviations are shown as horizontal lines with vertical brackets Dots correspond to sera from individual animals analyzed at 5-6 months of age. PBS was used as a negative control.

B. IgM^a (B1-8V_H) anti-dsDNA in supernatants of LPS-stimulated cultures, and harvested at the indicated times. Both total IgM^a proteins (left panel) and IgM^a anti-dsDNA antibodies (right panel) in splenic B cell cultures were compared, from $V_H\Delta^a/WT^b$, $V_HE\mu^a/WT^b$ and WT^b/WT^b mice. PBS was used as negative a control. Data shown is representative of 3 individual experiments.

C. IgM^a anti-dsDNA antibody secretion by double-producers. $V_H\Delta^a$ single-producers (SP, IgM^{a+b-}) and $V_H\Delta^a/WT^b$ double-producers (DP, IgM^{a+b+}) from spleens of $V_H\Delta^a/WT^b$ mice were sorted, cultured in LPS and culture supernatants harvested at day 7. Total IgM^a proteins produced by SP and DP (left panel); IgM^a anti-dsDNA antibodies produced by SP and DP (right panel). PBS was used as anegative control. Bar graph is a statistical analysis of 3 individual experiments. p-values were calculated by two-tailed student's *t*-test. ns= non-significant.


A.
p=0.0088
Sera IgMa anti-dsDNA (OD405)
1.5
1.0
0.5
0
VHΔa/WTb
VHEμa/WTb
VHΔa/ΔJH
VHEμa/ΔJH
VHΔa/VHΔa
PBS
C.
VHΔa/WTb splenic cells
ns
p=0.0016
IgMa levels (OD405)
IgMa anti-dsDNA levels (OD405)
SP
DP
PBS
B.
IgMa levels (OD405)
VHΔa/WTb
VHEμa/WTb
WTb/WTb
PBS
Day2
Day4
Day6
IgMa anti-dsDNA levels (OD405)

As we have previously shown, $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice differ profoundly with respect to allelic exclusion (146). The double-producers among the immature and splenic B cell subsets, characteristic of $V_H\Delta^a/WT^b$ mice, are absent in $V_HE\mu^a/WT^b$ mice. However, there are double-producers in $V_HE\mu^a/WT^b$ mice, which are restricted to the peritoneal cavity of these mice (146). As a consequence of these cells, IgM^b is present in the sera of $V_HE\mu^a/WT^b$ mice at about the same level as in $V_H\Delta^a/WT^b$ mice. We used this as a means for asking whether the absence of Eμ (resulting in lower Igμ and hence BCR density) was responsible for the increased frequency of anti-dsDNA antibodies, or alternatively, any BCR on double-producers (even when both BCRs are encoded by Eμ-intact alleles) would have a greater likelihood of autoreactivity. We found no difference in serum IgM^b anti-dsDNA level in $V_HE\mu^a/WT^b$ versus $V_H\Delta^a/WT^b$ versus C57BL/6J (WT^b/WT^b) mice (Figure 3.15A), although the IgM^b-BCR showed some background levels of anti-dsDNA reactivity both in sera (Figure 3.15A). Splenic B cells from $V_HE\mu^a/WT^b$, $V_H\Delta^a/WT^b$ and C57BL/6J mice were cultured in vitro and analyzed for secretion of anti-dsDNA proteins with IgM^b allotype. There is very low level (close to background PBS control) of IgM^b in the culture supernatant from $V_HE\mu^a/WT^b$ splenic B cells since few, if any, detectable splenic B cells making IgM^b in these mice. Double-producers from $V_H\Delta^a/WT^b$ mice secrete IgMb proteins, which showed reactivity to dsDNA (Figure 3.15C). Not only $V_H\Delta^a/WT^b$, but also C57BL/6J splenic B cells secreted IgM^b and these proteins showed anti-dsDNA reactivity as well, consistent with the IgM^b-anti-dsDNA detected in sera. There is reasonable concordance between the levels of IgM^b-anti-dsDNA levels and IgM^b protein levels in the cell cultures of $V_H\Delta^a/WT^b$ and C57BL/6J splenic B cells: the more IgM^b expressed, the higher anti-dsDNA activity detected (Figure 3.15B). This demonstrates that IgM^b secreted by $V_H\Delta^a/WT^b$ B cells are not more likely to be autoreactive. We suggest, accordingly, there is no alteration in the selection of BCRs

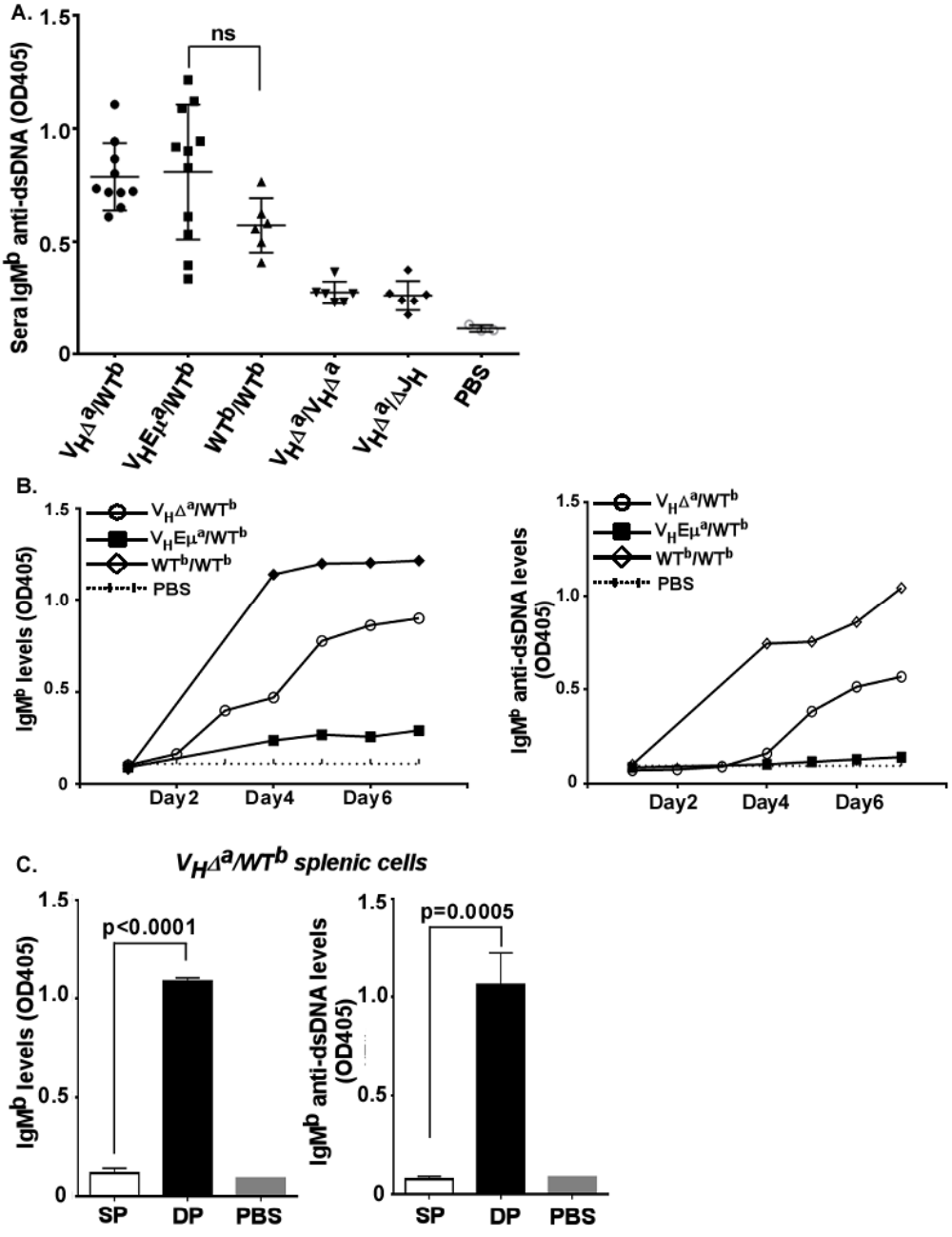
containing Igμ from the WT^b allele that retains Eμ. Only BCRs comprised of $Ig\mu^a$ from the Eμ-deficient allele showed evidence of defective negative selection in double-producers.

Figure 3.15: Anti-dsDNA reactivity from WT^b allele of B cells expressing two different receptors in $V_H\Delta^a/WT^b$ mice.

A. IgM^b (WT^b) anti-dsDNA proteins in sera from mice of the indicated genotypes. Y-axis = (OD405 of IgM^b anti-dsDNA)/(OD405 of total IgM^b) in sera. Means and standard deviations are shown as horizontal lines with vertical brackets. Dots correspond to sera from individual animals analyzed at 5-6 months of age. PBS was used as a negative control.

B. IgM^b (WT^b) anti-dsDNA in supernatants of LPS-stimulated cultures, and harvested at the indicated times. Both total IgM^b proteins (left panel) and IgM^b anti-dsDNA antibodies (right panel) in splenic B cell cultures were compared, from $V_H\Delta^a/WT^b$, $V_HE\mu^a/WT^b$ and WT^b/WT^b mice. PBS was used as a negative control. Data shown is representative of 3 individual experiments.

C. IgM^b anti-dsDNA antibody secretion by double-producers. $V_H\Delta^a$ single-producers (SP, IgM^{a+b-}) and $V_H\Delta^a/WT^b$ double-producers (DP, IgM^{a+b+}) from spleens of $V_H\Delta^a/WT^b$ mice were sorted, cultured in LPS and culture supernatants harvested at day 7. Total IgM^b proteins produced by SP and DP (left panel); IgM^b anti-dsDNA antibodies produced by SP and DP (right panel). PBS was used as a negative control. Bar graph is a statistical analysis of 3 individual experiments. p-values were calculated by two-tailed student's *t*-test. ns= non-significant.


A.
Sera IgM^b anti-dsDNA (OD405)
ns
$V_H\Delta^a/WT^b$
$V_HE\mu^a/WT^b$
WT^b/WT^b
$V_H\Delta^a/V_H\Delta^a$
$V_H\Delta^a/\Delta J_H$
PBS
B.
IgM^b levels (OD405)
IgM^b anti-dsDNA levels (OD405)
Day2
Day4
Day6
C.
$V_H\Delta^a/WT^b$ splenic cells
p<0.0001
p=0.0005
SP
DP
PBS

Chapter 4: Discussion

4.1. B cell development and B cell receptor signaling of Eμ-deficient mice

4.1.1. Deletion of Eμ results in an impaired pre-B to immature B cell transition, leaving other developmental stages unaffected

4.1.2. Deletion of Eμ reduces Igμ expression, resulting in suboptimal receptor signaling in pre-B cells but not in immature B cells

In both mice and humans, pre-B to immature B cell transition in the bone marrow is an important checkpoint at which an emerging BCR is tested for its functionality and autoreactivity (162, 260, 261). Cells expressing anti-self receptors are arrested at this transition and subsequently clonally deleted (180, 262-265). Signals propagated by these auto-reactive receptors can also reactivate the recombination machinery (RAG genes) to induce receptor editing of IgH or IgL chain genes in pre-B cells. As a result, non-self-reactive receptors replace the original autoreactive specificity and rescue the B cell differentiation into the immature B cell stage (226, 266-269). On the other hand, cells expressing non-autoreactive receptors continue to differentiate by signals sent out from the pre-BCR and BCR in a ligand-independent and/or ligand-dependent manner (158, 162). In the present study, we provide evidence that nascent BCR signals must reach a signaling threshold for the positive selection of the pre-B cells into the immature B cell stage and that the BCR's ability to reach this threshold is a function of both receptor structure (IgH/IgL combination) and abundance. Insufficient nascent BCR signals result in sustained light chain rearrangements in pre-B cells for a better IgH/IgL combination. The role of the *Igh* intronic enhancer, Eμ, at this transition, is to ensure sufficient expression of the IgH gene (and hence

enough abundance of BCR), so that most IgH/IgL combinations form BCRs reaching the signaling threshold for positive selection.

In the absence of Eμ, Igμ was expressed at half of the normal level in pre-B cells, which was correlated with discernibly lower levels of tyrosine phosphorylation for the total cytoplasmic proteins, as well as for the pre-BCR and BCR signaling molecules Syk and Erk. We interpret this as evidence for a decrease in pre-BCR and BCR receptor signaling strength in these pre-B cells. We conclude therefore, the low Igμ levels translate into low pre-BCR and BCR levels that in turn lead to tonic signals of reduced average signal strength. Correlated with the lower Igμ and lower receptor signals, we found the immature B cell compartment to be less efficiently populated in these Eμ-deficient mice, such that at the steady state, it was half the size of that in the genetically matched mouse strain with Eμ intact and with twice the amount of Igμ in pre-B cells. In our Eμ-deficient model, the decreased Igμ in pre-B cells, therefore, compromises the positive signal sent by a newly-formed BCR which is required for the transition from the pre-B to immature B cell stage. As a result, pre-B cells expressing both the Eμ-deficient and the wild-type *Igh* alleles are at a selective advantage because their tonic signals through the emerging BCRs could reach the threshold for survival.

This is consistent with a previous study which showed that BCR signals are critical to maintaining immature B cells at the immature B cell stage (222). Ablation of tonic BCR signals in immature B cells through inducible gene deletion of surface Ig or chemical inhibition of tyrosine kinases, resulted in back-differentiation of the immature B cells into the pre-B cell stage and recommencement of light chain editing (222). The current study provides further and more direct evidence that BCR signaling plays a dominant role for B cell differentiation, especially at

the pre-B to immature B cell transition. We further demonstrate that there is a signaling threshold for pre-B cells to differentiate into immature B cells. Reduced BCR signals, not solely loss of all BCR signals as caused by BCR ablation, leads to defective transitioning.

While others have described the effects of perturbing Igμ levels and/or BCR signals in genetically-modificed mice (e.g. (178, 222, 270-273), a unique feature of the current experimental system is that Igμ expression is not irreversibly depressed throughout B cell development in $V_H\Delta^a$ mice. Instead, Igμ expression returns to wild-type levels in Eμ-deficient immature B cells and in their descendants ((146) and Figure 3.8). As we and others have shown, the transcription of IgH and hence Igμ levels, are controlled by not only the *Igh* intronic enhancer Eμ but also a downstream regulatory region at the 3' end of the *Igh* locus (3'RR) (215, 274, 275). As shown here, Igμ levels in pre-B cells depend in large part upon the transcriptional enhancer Eμ, whereas earlier studies have shown that the 3'RR sustains high-level IgH gene transcription in Ig-secreting cells, even in the absence of Eμ (206-210). Reciprocal loss of the 3'RR in Ig-secreting cells led to a dramatic decrease in Ig transcription (206, 211, 212). These findings suggested that Eμ's role in promoting IgH transcription could be supplanted by the 3' IgH enhancers at the plasma-cell stage. Later studies have shown that the 3'RR becomes active as early as the immature B cell stages (213-215). Taking all these observations together, we suggest accordingly that 3'RR up-regulates Igμ expression at immature and mature B cell stages to the normal level, and Igμ expression becomes independent of Eμ when cells reach the immature B cell stage. As we show in immature B cells, this return to wild-type Igμ level correlated with a return to normal levels of BCR-signals (as measured by phosphorylation of tyrosine residues, active forms of Syk and Erk) and resumption of normal B cell development

past this stage (Figure 3.2, 3.3, 3.7, 3.8). The levels of phosphorylated signaling molecules in pre-B and immature B cells are in agreement with the relative Igμ levels in these cell populations, confirming our hypothesis that as a critical constituent of pre-BCR and BCR, Igμ levels correspond to the receptor levels and therefore the tonic signaling strengths of BCRs in developing B cells.

More recently, a hypomorphic μHC model caused by reporter gene (GFP: green fluorescence protein) insertion into the intron between Eμ and J_H gene segments, showed also reduced Igμ expression but from all IgH genes formed (by V_HDJ_H recombination) on the affected allele. This effect (variable) on Igμ transcription was at all B cell developmental stages. In this model, BM B-cell development was partially impaired at all stages and affected cells had increased receptor editing; and the distribution of B cell subsets was skewed (B-1a, B-1b, marginal zone, and B-2 B cells). In addition, with lower Igμ level, there was reduced level of BAFF receptors on splenic B cells and impaired antibody responses, suggesting decreased BCR signaling (271). This study is supportive to our notion that reduced Igμ expression results in reduced pre-BCR and BCR levels and suboptimal signals, which perturb the pre-B to immature B cell transition and trigger ongoing light chain editing (see section 4.2).

4.2. Receptor editing in Eμ-deficient B cells

4.2.1. Cells expressing only the $V_H\Delta^a$ allele undergo extensive receptor editing

4.2.2. A pre-assembled 3-83Vκ light chain gene rescues the pre-B to immature B cell transition during $V_H\Delta^a$ B cell development

4.2.3. A pre-assembled 3-83Vκ light chain gene partially reduces the incidence of IgH double-producers in $V_H\Delta^a/WT^b$ mice

Importantly, the current study shows that the significance of the enhancer Eμ is not limited to its roles in IgH gene assembly and transcription. It serves another important function in ensuring the development of a broad B cell receptor repertoire among emerging B cells. This means absence of Eμ affects not only emerging B cell numbers (size of immature B cell compartment, as discussed above), but also the emerging B cell repertoire (discussed below).

In cells expressing low levels of Igμ, formation of a functional IgL gene is often insufficient to signal differentiation to the immature B cell stage because of the low BCR levels and signaling. In such cells, even when they have successfully expressed BCRs, light chain gene rearrangements do not cease (as demonstrated by the hallmarks of light chain "editing"), because these cells cannot efficiently "sense" the presence of BCRs through the under-expressed signals. There would be insufficient RAG down-regulation, resulting in ongoing light chain recombination or developmental arrest. We postulate that this increased light chain editing also reflects a more stringent requirement for the BCR potency due to the reduced μ levels at the pre-B cell stage, which drives the cell to create an IgH/IgL combination with better signaling properties. Evidences for this are 1) reduced immature B cell compartment in Eμ-deficient mice; and 2) increased light-chain editing suggested by Igκ gene structures (more usage of the most downstream Jκ gene segment) and by Igλ gene expression (more Igλ-producing cells) in immature B cells expressing an Eμ-deficient IgH gene. The expected outcome would be a reduction in the BCR repertoire because of the more stringent requirements for Ig light chain structure. In the presence of Eμ and of higher lgμ proteins, however, more IgH and IgL

combinations meet the necessary threshold more frequently, allowing for an expanded (and likely more protective) repertoire of B cell receptors.

We show further evidence that IgL sequence and structure affect B cell development in Eμ-deficient mice with low Igμ at the pre-B cell stage. Provided with a pre-assembled 3-83Vκ knock-in IgL gene, $V_H\Delta^a/WT^b$ mice had an increased size of immature B cell compartment, so that the ratio of (pro-B+pre-B)/immature B cells reduced from an average of 7 to <1. We suggest therefore, the B1-8V_H and 3-83Vκ pair exemplifies an IgH and IgL combination which signals better than others, so that even when Igμ level is low, the basal level of signaling by this receptor is sufficient to support the pre-B to immature B cell transition. We selected to use the 3-83Vκ knock-in gene because it had already been shown that B1-8H-chain and 3-83κ light chain form a functional BCR that can support B cell development (226). But we didn't know whether this combination was able to circumvent the defect caused by low Igμ levels due to Eμ-deficiency. Had this BCR (B1-8H/3-83κ) been of lower-than-threshold signaling properties, we might have found a more pronounced decrease of immature B cell compartment. This was not what we found, however. A relative decrease in size of the (pro-B+pre-B) cell compartment and increase in size of the immature B cell compartment are commonly found in IgH/IgL knock-in mice as long as the BCR is not autoreactive (e.g. (226)). What was remarkable in the B1-8V_H and 3-83Vκ double knock-in mice, however, was the effect on heavy chain allelic exclusion: double-producers significantly reduced in numbers among both immature and splenic B cell compartments in $V_H\Delta^a/WT^b$ 3-83κ/Igκ mice, as compared to $V_H\Delta^a/WT^b$ Igκ/Igκ mice.

As described earlier, one of the striking phenotypes in mice heterozygous for the Eμ-deficient allele ($V_H\Delta^a/WT^b$), is the defect in allelic exclusion (146). Approximately 20% splenic B cells

expressed Igμ from both the $V_H\Delta^a$ knock-in and the wild-type IgH chains ("double-producers"). A fundamental requirement for the clonal selection theory of B lymphocytes is that each B cell expresses an antigen receptor of a single specificity, so that individual B cell clones are effectively and efficiently selected for their functionality and against their auto-reactivity during development, and also quickly produce antibodies upon exposure to their cognate antigen (17). The core mechanism responsible for allelic exclusion involves feedback inhibition of V_H to DJ_H joining on the second allele after successful V_HDJ_H recombination has taken place on the first allele. We have previously shown that this process occurs normally, in both $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice. Both the $V_H\Delta^a$ and $V_HE\mu^a$ alleles strongly inhibit V_H to DJ_H assembly on the WT^b allele, but neither fully blocks the generation of pre-B cells with a second functional V_H gene on the WT^b allele. These rare precursors to double-producers are present in the pre-B cell populations of both $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice (146). When Eμ remains on the V_H knock-in allele, however, immature and mature B cells never emerge from these rare precursors, revealing a second, Eμ-dependent, "check-point" for IgH allelic exclusion at the pre-B to immature B cell transition. We suggest that this, in fact, is another manifestation of the inability of the $V_H\Delta^a$ allele to produce Igμ, and therefore BCR, at levels sufficient for positive selection. "Double-producers" in $V_H\Delta^a/WT^b$ mice are not eliminated at this check-point because the functionally assembled IgH gene on the second allele, which retains Eμ, allows for BCR levels sufficient for positive selection. We assume that this puts double-producers at a selective advantage over single-producers (no Eμ) at the pre-B to immature B cell transition in $V_H\Delta^a/WT^b$ mice due to the lower Igμ expression from only the $V_H\Delta^a$ allele.

Consistent with this hypothesis, the current study shows that genetic manipulations that augment the pre-B to immature B cell transition in single producers from $V_H\Delta^a/WT^b$ mice (using the pre-assembled 3-83Vκ gene), simultaneously augment allelic exclusion (i.e. reduce the outgrowth of cells with BCRs of two different specificities as double-producers). Double-producers are at a selective advantage at the pre-B to immature B cell transition in $V_H\Delta^a/WT^b$ mice only because of the signaling defect caused by low Igμ levels in single-producers. In the $V_H\Delta^a/WT^b$ 3-83κ/Igκ mice, this signaling defect is greatly reversed because of a potent BCR comprised of B1-8V_H and 3-83Vκ. All these observations support our hypothesis that BCR signal is a determining factor for cells to traverse the pre-B to immature B cell stage, and in the selection "check-point" for allelic exclusion.

4.3. Pre-BCR dependent B cell progression is normal in Eμ-deficient B cells

While these lower Igμ levels strongly affect the phenotype of emerging B cells, which includes a more limited repertoire and the persistence of double-producers, our data suggest that they have no clear effect on the outcomes of pre-BCR signaling in our Eμ-deficient, B1-8V_H knock-in model. We have found that the processes preceding the pre-B to immature B cell transition are much less dependent upon Igμ levels than is the development and selection of immature B cells.

As we have shown previously, the 1st "check-point" in allelic exclusion (i.e. the feedback inhibition of V_H-to-DJ_H rearrangement on the 2nd *Igh* locus), while mediated by membrane Igμ and the signaling molecules Igα and Igβ (67, 124, 132, 272, 273, 276), is fully operational in B1-8V_H knock-in animals and is unaffected by Eμ deletion (146).

The pre-BCR (composed of Igμ, SLC, Igα and Igβ) induces differentiation of the pro-B to the pre-B cell stage, proliferative expansion of large pre-B cells, differentiation of large pre-B to small pre-B cells, and subsequent down-regulation of SLC expression as well as onset of Ig light chain gene rearrangements in non-dividing small pre-B cells (132, 161, 272, 273, 277-279). In our Eμ-deficient, B1-8V_H knock-in model, none of these pre-BCR-associated processes was adversely affected. Pro-B and pre-B cell differentiation appeared normal in the absence of Eμ. In both $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ mice, all cells carry a productively rearranged B1-8V_H heavy chain, and as a result, they largely bypass the pro-B cell stage and progress directly to the small pre-B stage for light chain assembly. There is no obstruction at the pro-B to pre-B cell transition, and instead, pro-B cell compartments were of no discernible differencce in size in the two strains (both reduced relative to wild-type, Figure 3.6A, 3.6B), suggesting an equivalent response to pre-BCR signals. We found equivalent numbers of pre-B cells in the $V_H\Delta^a/WT^b$ versus $V_HE\mu^a/WT^b$ mice, as well, suggesting that the pre-BCR-mediated proliferative expansion and differentiation were also taking place normally in the absence of Eμ.

A caveat to this interpretation is that deficient pre-B cell expansion might be masked by these cells' inefficient, BCR-mediated exit into the immature B cell compartment. Otherwise, pre-B cell numbers might have been expected to rise because of this inefficient exit. Alternatively, pre-BCR-mediated proliferation may be normal, and instead, apoptosis of BCR-signal-deficient cells balances the slow exit.

In any case, in small, non-dividing pre-B cells, re-expression of RAG-1 and RAG-2 genes and Igκ locus germ line transcription (as a prelude to Igκ gene assembly) (161, 279), both being

processes mediated by pre-BCR signals, occurred at equivalent levels whether Eμ was present or absent on the expressed Igμ allele (Figure 3.9).

Taken together, all of these findings support the conclusion that the signaling threshold for pre-BCR-mediated processes is lower that that of BCR-mediated ones. As a result, pre-BCR signaled events are not affected by the absence of Eμ as long as a functional V_H gene (such as B1-8V_H) has already been assembled. The pre-BCR density and therefore signal strength reaches the signaling "threshold" in $V_H\Delta^a$ mice, so that the development of precursor B cells until small pre-B stage is not affected. Rather, after V_H gene assembly, Eμ's importance is next manifested only at the pre-B to immature B cell transition where BCR (not pre-BCR) signaling is required and tested.

4.4. Comparison with previous studies: strengths of the $V_H\Delta^a$ model

As mentioned earlier, this study extends and refines the earlier studies by BCR ablation, providing evidence of a signaling threshold at the pre-B to immature B cell transition. While loss of BCR and/or BCR signaling results in defective cell differentiation, a reduction of pre-BCR/BCR signaling strength would also cause deficiencies.

Others have shown that truncation of the Igα cytoplasmic tail causes significant decrease of pre-B and immature B cell numbers, supporting the importance of receptor signaling (280). Notably, however, the inhibition of immature B cells generation was greater than was the effect on pre-B cell generation, suggesting that higher signaling capacities are required as B cells progress through development (149). This is in agreement with our finding that pTyr, pSyk and pErk levels increase progressively from the pro-B to pre-B, and then to the immature B cell stage (data

not shown). Taken together, we suggest that pre-BCR signals are not as "density" dependent as are the BCR signals.

Previous models for study on receptor signaling involved either ablations of BCR genes that led to cell death or involved mutants that affected BCR levels or signaling throughout B cell development, not allowing for a focus on BCRs' roles at a specific transition point. For example, in the hypomorphic μHC model, the transcriptional depression was sustained throughout development. Igμ expression was reduced to 1/10 of the wild-type level, which resulted in developmental deficiencies at all stages during B cell maturation. Therefore, it was difficult to assign a mutant phenotype conclusively to perturbation of any one particular selection process (i.e. it was difficult to assign a specific role for Igμ level at specific points of transition). In comparison, our Eμ-deficient V_H knock-in model provides us with a system where Igμ expression is less severely impaired (to only half the wild-type level), and this impairment occurs only in pre-B cells. Consequently, the reduction of BCR levels is limited to the pre-B to immature B cell transition, allowing for a focus on BCR signaling at this particular and functionally important juncture, when B cell receptors are tested for their signaling competence and autoreactivity. This experimental system also allows us to identify the specific stage at which Eμ's functions (and its effects on Igμ expression) are critical.

Moreover, another advantage of this experimental system is that we can directly attribute effects to Igμ mRNA and protein levels, without the confounding effects of differences in nucleic acid/amino acid sequence, since all comparisons among cell stages in $V_H\Delta^a$ mice and between $V_HE\mu^a$ and $V_H\Delta^a$ mice are of cells expressing the same recombined IgH gene with identical V_H and IgH promoter. The only difference is the presence or the absence of the intronic enhancer,

and therefore emerging B cells differ only in the Igμ (mRNA and protein) level at the pre-B to immature B cell transition. This relatively small reduction in Igμ expression at this transition yields large effects on the B cell and antibody repertoire, both reducing the breadth and allowing for development of B cells with two different receptors.

4.5. Eμ-deficient $V_H\Delta^a/WT^b$ heterozygous mice are prone to B cell autoimmunity

Finally, as we pointed out in the results, allelic exclusion has long been conjectured to have evolved as a means for ensuring the clonal selection of each B cell, either positively (selected for expansion, when receptor binds an invading pathogen) or negatively (selected for elimination, when receptor binds a host product) depending on the specificity of the BCR it displays (18). The separate selection of individual B cells is an efficient means for assuring that protective receptors are maintained and autoreactive receptors are eliminated (18). If B cells carry more than one receptor, then antigen-induced expansion of such allelic included cells would result in simultaneous production of the protective and autoreactive antibodies. In support of this notion, several studies have identified the presence of allelic included expression of either IgH or IgL in mouse and human B cells carrying autoreactive specificity (137, 138, 145, 281-287). These studies with Ig transgenic or Ig 'knock-in" mice have demonstrated that, co-expression of self-reactive and non–self-reactive receptors enables developing B cells to escape negative selection and differentiate into mature lymphocytes.Interpretations include: 1) the receptor with high avidity to self-antigen is concealed in the cytoplasm and is not detectable on the cell surface, so that the B cell co-expresses a second receptor with an innocuous specificity for maturation. Only when the innocuous BCR is stimulated is the second, autoreactive antibody revealed through secretion from the resulting plasma cell (Ig-secreting) cell (137, 145); 2) allelic- or isotype-

included co-expression of two light chains (express two Igκ alleles, or Igκ and Igλ chains) results in competition for assembly with the available IgH chain, diluting the surface density of both BCRs and therefore making neither sufficiently "visible" for negative selection (137, 138, 282, 284). However, the mechanisms for the survival of these B cells with dual-receptors have not yet been thoroughly elucidated.

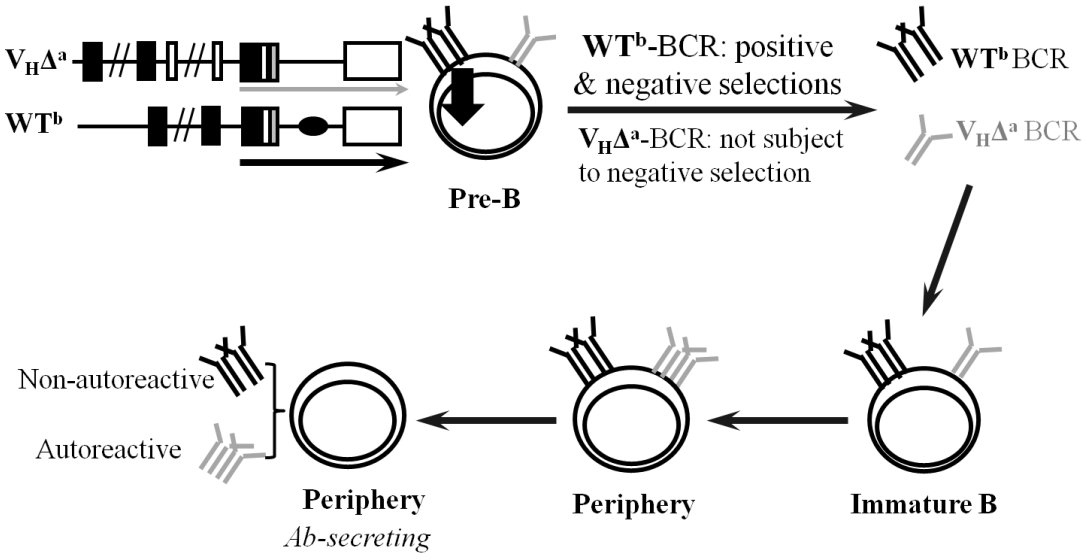
While allelic "inclusion" constitutes a risk-factor for autoimmunity, several studies have shown that "double-producers" are not, *a priori*, autoreactive (141, 288). Studies by Makdasi and Eilat (2013) provided evidence that allelic-included B cells in diseased autoimmune mice (systemic lupus erythematosus, SLE) were generally not autoreactive. Reciprocally, B cells with DNA-specific BCRs were no more likely to be allelically included (double-producers) than were non-autoreactive B cells (288). The authors suggested that light chain allelic exclusion is merely a byproduct of receptor editing, but not a mechanism for autoreactive receptors to be ignored for negative selection. Otherwise, allelically included B cells should retain both autoreactive and non-autoreactive receptors (288). This conclusion is reminiscent of another previous study, which showed that allelic inclusion does not necessarily result in autoreactive B cells (141). We suggest that allelic inclusion should not be viewed as an absolute marker for autoimmunity: while some double-producers will produce both autoreactive and innocuous BCRs, others will emerge with two innocuous (protective) ones. Allelic inclusion, therefore, constitutes a risk-factor for autoimmunity, not a singular cause for it.

In the current study, the double-producers in high numbers in $V_H\Delta^a/WT^b$ mice (but not in $V_HE\mu^a/WT^b$ mice) provide an example of how "allelic-included" cells can contribute to the risk for autoimmunity. We find that these "double-producers" ($V_H\Delta^a$+WT^b) include a subpopulation

with autoreactive BCRs expressed from the Eμ-deficient ($V_H\Delta^a$) allele. We suggest that the difference in the expression level of Igμ from two different IgH alleles explains the cells' ability to transit from pre-B to immature B cells, unchecked for the specificity of the light chain plus μ-heavy chain from the $V_H\Delta^a$ allele. As we have shown in mice dependent solely upon expression of the $V_H\Delta^a$ allele, this Eμ-deficient allele drives expression of a BCR often insufficient for positive selection. We suggest that actually this makes it also impervious to negative selection at the pre-B to immature B cell transition. When a 2nd IgH allele with Eμ is expressed, the resultant double-producer will be selected on the basis of this 2nd "wild-type" heavy chain, for both positive and negative selection. Given that a newly formed B cell receptor (IgH/IgL combination) is autoreactive at a frequency as high as 75% (260, 289), these double-producers will sometimes prove autoreactive because of the inability to sense and test the autoreactive properties of BCRs formed by B1-8 H-chain (from the $V_H\Delta^a$ allele, at half of the wild-type leve) plus light chain at the pre-B to immature B cell transition. For this reason, protective and auto-reactive receptors will arise on single cells in emerging immature B cells (Figure 4.1). As the "double-producers" mature and differentiate into plasma cells, the tested IgM^b molecules and the untested IgM^a molecules are both secreted. Consistent with that hypothesis, we found that only in the $V_H\Delta^a/WT^b$ mice (not in $V_HE\mu^a/WT^b$ mice expressing the same B1-8 H chain), and only in double-producers from $V_H\Delta^a/WT^b$ mice, were there anti-dsDNA antibodies associated with the B1-8 H chain ($Ig\mu^a$; Figure 3.14).

Figure 4.1: Model for auto-reactive $V_H\Delta^a/WT^b$ double-producers.

In $V_H\Delta^a/WT^b$ mice, newly emerging immature B cells may express both the knock-in and the wild-type (WT) *Igh* alleles in order to pass positive selection at the pre-B to immature B cell transition. These cells express the Eμ-deficient allele ($V_H\Delta^a$) at half the level of the WT-*Igh* (WT^b). The $V_H\Delta^a$-BCR is "invisible" for both positive and negative selection due to its lower density. Instead, cells are positively and negatively selected only by the WT^b-BCR. Therefore, the resultant Ig-secreting cells will produce both non-autoreactive antibodies (from the WT^b-BCR) and autoreactive antibodies (from the $V_H\Delta^a$-BCR) once activated.



In general, although allelic-inclusion does not always involve an auto-reactive receptor, we predict that when it does, cells with one self-reactive receptor will escape clonal deletion, allowing cells to differentiate into autoantibody secreting plasma cells and pose a threat of autoimmunity. It should be noted, however, in our Eμ-deficient V_H-knock-in mice, except for the increased IgM anti-dsDNA and anti-nuclear antigen reactivity in sera, we have found no evidence of autoimmune disease in these animals up to 6 months of age. Assays for sera IgG^a-anti-dsDNA antibodies were negative, suggesting that the cells secreting IgM^a-anti-dsDNA antibodies were not commonly undergoing class-switching (in germinal centers) and forming IgG-secreting plasmacytes. No proteinuria was detected and blood urea nitrogen levels in these mice were indistinguishable from age-matched wild-type animals (data not shown), suggesting that there was no development of glomerulonephritis or autoimmune disease in our model.

Nevertheless, the difference in the development of IgM^a-anti-dsDNA antibodies in the matched mouse strains ($V_H\Delta^a/WT^b$ versus $V_HE\mu^a/WT^b$) and in double- versus single-producers clearly shows that BCR selection is profoundly affected by the presence of a second BCR with alternative specificity. Presumably, if within an autoimmune mouse background, the double-producers (expressing one Eμ-deficient V_H-knock-in allele) would increase the likelihood of autoimmune disorders.

4.6. It is the Eμ-deficient V_H knock-in allele ($V_H\Delta^a$), not the WT^b allele, that predispose $V_H\Delta^a/WT^b$ heterozygous mice to B cell autoimmunity

As noted above, it has been suggested that double-producers might give rise to auto-antibodies because neither of two alternative BCRs is present at high enough levels to make it subject to negative selection. In our model, however, one BCR (IgM^a) is presumably present at half the

level of the other (IgM^b). We focused on IgM^a anti-dsDNA antibodies since our question was whether the antigen-specificity of the B1-8 H chain BCRs was assessed differently in single-producers (where this BCR drives positive selection and thus, would be expected to be subject to negative selection, as well) versus double-producers (where we predict that this BCR is neither driving positive selection nor is subject to negative selection). We have shown that the IgM^a BCR is behaving differently (with respect to selection) when expressed on single-producers versus double-producers.

It might be asked whether there is a reciprocal effect on the IgM^b BCR even though it is expressed at twice the levels of the IgM^a BCR at the critical pre-B to immature B cell transition. Serum IgM^b from the $V_H\Delta^a/WT^b$ and $V_HE\mu^a/WT^b$ animals proved informative in this regard. We were able to look at IgM^b anti-dsDNA antibodies in sera of both $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice, since $V_HE\mu^a/WT^b$ mice produce IgM^b at similar levels as $V_H\Delta^a/WT^b$ mice, presumably deriving from the double-producers found among peritoneal B cells of $V_HE\mu^a/WT^b$ mice (which were not found in the immature B and splenic B cell compartments in these mice) (146). Anti-dsDNA antibodies are a hallmark of lupus erythematosus in humans, but such antibodies, particularly IgM anti-dsDNA, are also found in healthy people. Similarly, we found detectable amounts of IgM^b anti-dsDNA antibodies in C57BL/6J mice. It should be noted that there was anti-dsDNA reactivity associated with these IgM^b proteins in sera from both $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice and at the same frequency (and both had levels equivalent to normal age matched C57BL/6J mice), suggesting that a subset of BCRs with anti-dsDNA IgM^b encoded by the WT^b IgH locus was retained in the repertoire as a result of antibody diversification, probably due to their mild anti-dsDNA affinity allowing the cells to survive negative selection.

IgM^b BCRs in both $V_HE\mu^a/WT^b$ and $V_H\Delta^a/WT^b$ mice represent a broad range of V_H and V_L combinations, and the $Ig\mu^b$ chain is produced from an allele that retains Eμ, so we would expect these BCRs to be subject to both positive and negative selection whether expressed in single or double-producers entering the pre-B to immature B cell transition. Consistent with that expectation, there was no difference in serum IgM^b anti-dsDNA level in $V_HE\mu^a/WT^b$ (IgM^b from double-producers) versus $V_H\Delta^a/WT^b$ (IgM^b from double-producers) versus C57BL/6J (IgM^b produced from single-producers) mice.

4.7. Summary

In summary, our observations, together with those previously published, provide compelling evidence to support a model in which the constitutive signaling strength of newly emerging BCR must reach a threshold in order to promote the pre-B to immature B cell transition. The IgH intronic enhancer Eμ's important function is to amplify IgH transcription at the pre-B cell stage, such that in combination with most IgH promoters, signaling through a functional BCR reaches this threshold.

Loss of Eμ from a productive IgH allele has profound effects on B cell development and Ig repertoire. This results in under-expressed receptor density in pre-B cells, attenuates the receptor signaling strength in these cells and yields notable effects on the emerging immature B cells. It impairs cell differentiation efficiency, reduces the breadth of receptor repertoire, and may also lead to a selection process allowing survival of B cells with two different receptors which increases the frequency of autoreactive cells. Eμ's important function in allelic exclusion is to insure enough levels of Igμ expression to preclude the need for expression of a second allele, and

to insure that both BCRs made by the rare double-producer precursors are subject to negative selection. This study, in essence, supports the assumption underlying the "clonal selection" theory, that B cells express single-specificity receptors to prevent simultaneous activation of protective and autoreactive antibody responses.

A broader implication is that any elements compromising IgH levels (Igμ and therefore pre-BCR and BCR signals) at this critical transition from pre-B to immature B cell stage during B cell development, such as certain weak V_H promoters, genetic polymorphisms in enhancer sequence and strength, polymorphisms in transcription factors required for enhancer or promoter function, will affect the efficiency of cell selection at this important transition stage. A population of B cells with multiple receptors will arise because the "weak-signaling" IgH allele functions in neither positive nor negative selection, providing a higher potential for autoreactivity. Concurrently, such "weak" alleles because of insufficient Igμ will force the use of a restricted repertoire of light chains in emerging single-producers because of the need to overcome the impaired signaling properties and rescue positive selection, compromising the development of a robust and protective antibody repertoire. All these suggest a mechanism by which individuals may be predisposed to more restricted versus broader Ig repertoires and/or to autoimmunity.

Chapter 5: Future Directions

Using the B1-8V_H knock-in model, we are able to further describe the defects of B cell development and the antibody repertoire caused by expression of an Eμ-deficient IgH gene, which deepens our understanding of the nature and mechanism of B cell development regulation. There remain some questions that are worthy of further study.

5.1. Are the B cell development defects (impaired pre-B to immature B cell transition, breach in allelic exclusion, and light chain editing toward a more restricted receptor repertoire, etc) in $V_H\Delta^a/WT^b$ mice a direct result of low Igμ levels?

As proved in the previous and current studies, Eμ's role is to increase Igμ levels in pre-B cells and thereby to ensure that the signals from newly-formed BCRs are sufficient for the pre-B to immature B cell transition and allelic excluded expression of Ig heavy chains. A future experiment worthy of consideration is to more directly test the hypothesis that reduced Igμ is the sole cause for the impairment in pre-B to immature B cell transition and allelic exclusion. If confirmed, the underlying hypothesis is that Eμ is not alone in affecting the antibody repertoire of emerging B cells, since numerous factors (such as V_H promoter strength, polymorphisms in enhancer sequence and strength, transcription factors for enhancer or promoter activity) also contribute to the expression level of newly assembled IgH gene.

RNA interference technology could be used to knock down Igμ mRNA expression from the $V_HE\mu^a$ allele, to an extent equal to that of the $V_H\Delta^a$ allele. The prediction is that the knock-down of Igμ through RNAi to ½ the WT level in $V_HE\mu^a$ B cells will result in a diminished immature B cell repertoire, emergence of double-producers in the immature and splenic B cell populations,

and higher light chain editing in single-producers. To accomplish this goal, the conditional RNAi technology described by Miething's lab could be used (290). Mice carrying the inducible shRNA (short hairpin RNA, specific for Igμ from the $V_HE\mu^a$ allele) could be mated to carry the $V_HE\mu^a$ allele for this experiment.

Alternatively, if factors (e.g. the sequence of Eμ, interaction of Eμ with other *cis*- or *trans*-acting factors, etc) other than the reduced Igμ level were the causes for the development defects in $V_H\Delta^a/WT^b$ mice (impaired pre-B to immature B cell transition, breach in allelic exclusion, and light chain editing toward a more restricted receptor repertoire, etc), then knock-down of Igμ through RNAi should not result in these defects (or not to the same extent).

5.2. Can the B cell development defects (impaired pre-B to immature B cell transition, breach in allelic exclusion, and light chain editing toward a more restricted receptor repertoire, etc) in $V_H\Delta^a/WT^b$ mice be amplified by decreased BCR signals, or rescued by augmented BCR signals?

We inferred that the reduced Igμ level led to a lower BCR density, which compromised the cells' ability to produce sufficient receptor signals for positive selection of pre-B cells into immature B cell stage. It is informative if we test B cell development in $V_H\Delta^a/WT^b$ mice by controlling the levels of BCR signals through manipulations of the downstream signaling pathway. The hypothesis is that the defect in allelic exclusion, increase in light chain editing, as well as the diminished immature B cell compartment could be reversed by augmented BCR signals, or amplified by decreased BCR signals.

$V_H\Delta^a/WT^b$ mice could be mated to mice which carry mutations that increase ($Lyn^{-/-}$) or decrease ($Btk^{-/-}$) the BCR signals. Lyn is one of the Src-family tyrosine kinases. Its inhibitory activity has been proved by an increase in BCR signaling in immature B cells in the bone marrow of $Lyn^{-/-}$ mice (Jackson Laboratories B6.129S4-Lyn^{tm1Sor}/J, stock#002515) (291, 292). In the double-mutant $V_H\Delta^a/WT^b$ $Lyn^{-/-}$ mice, BCR signals at the pre-B to immature B cell transition should be increased by relief from the inhibition mediated by Lyn protein. Deletion of the gene for Bruton's tyrosine kinase ($Btk^{-/-}$, Jackson Laboratories B6.CBA-Btk<xid>/AllmJ, stock#009361)) reduces BCR signals. Double-mutant $V_H\Delta^a/WT^b$ $Btk^{-/-}$ mice should have decreased BCR signals, leading to more double-producers and further increases in light-chain editing. $V_HE\mu^a/WT^b$ $Btk^{-/-}$ mice would be expected to generate double-producers in ithe immature and splenic B cell population.

5.3. What are the attributes of light chains that can support the pre-B to immature B cell transition when Igμ levels are low?

It has been shown that $V_H\Delta^a$-single-producers in $V_H\Delta^a/WT^b$ mice undergo higher light chain editing than their ($V_H\Delta^a$+WT^b)-double-producer counterpart or cells from $V_HE\mu^a/WT^b$ mice. Provided with a pre-assembled light chain gene 3-83Vκ, $V_H\Delta^a$-single-producers pass positive selection at the pre-B to immature B cell transition more efficiently. Our prediction is that some IgH/IgL combinations elevate signals to direct pre-B cells into the immature B cell pool through either their more stable structure or because of their specificity. In other words, some IgH/IgL combinations have better than average signaling properties because they are more efficient to assemble with each other, or are more stable on the cell surface.

Other IgH/IgL combinations may signal more efficiently because of their antigen-specificity. According to the notion that autoreactive receptors lead to greater signals which result in apoptosis, we suggest that through moderate anti-self specificity, some IgH/IgL combinations might be able to signal positive selection in the absence of Eμ, when tonic signals are otherwise below the necessary threshold.

To test these hypotheses, we could ask what is the difference between light chains that can and cannot support the pre-B to immature B cell transition? The BCR repertoire would be more self-reactive in $V_H\Delta^a/\Delta J_H$ mice than in $V_HE\mu^a/\Delta J_H$ mice, or V_Ls with better signaling properties are used more frequently in $V_H\Delta^a/\Delta J_H$ mice than in $V_HE\mu^a/\Delta J_H$ mice. Immature and splenic B cells from mice of both genotypes could be isolated, and the light chain gene sequences cloned by PCR, sequenced and compared. Self-reactivity and signaling capacities of these light chains could then be tested in cell lines and in an animal model. The prediction is that the light chain repertoire in $V_HE\mu^a/\Delta J_H$ mice is broader than in $V_H\Delta^a/\Delta J_H$ mice, and the limited BCR repertoire of $V_H\Delta^a/\Delta J_H$ mice might display more self-reactivity or stronger signaling properties. The emerging immature B cells are of particular interest. The mature B cells in the spleen are subject to additional selection processes, but could also be looked at to see whether there is any unexpected consequence with a restricted immature B cell repertoire from which they arise.

5.4. To what extent does Eμ contribute to autoreactivity?

While the $V_H\Delta^a/WT^b$ model is a very useful system to study Eμ's functions in regulating B cell differentiation, it also allows for the study of naturally-occurring double-producers with regard to their autoimmune risk.

To gain an appreciation of what proportion of allelically-included B cells carry autoreactive receptors in this model, we could work with Dr. Betty Diamond's lab (The Feinstein Institute for Medical Research, North Shore-Long Island Jewish Health Care System, Manhasset, NY), to mark cells expressing anti-DNA BCRs and thereby directly enumerate B cells with BCRs specific for DNA. The Diamond lab has identified a peptide that serves as a surrogate for DNA and that is biotin-labeled (293). When this peptide binds to cells with anti-DNA specificity, the cells can be detected by fluorescently-labeled avidin through flow cytometry. By comparing single-producers and double-producers from immature and splenic $V_H\Delta^a/WT^b$ mice, with $V_HE\mu^a/WT^b$ mice as controls, we expect to see a higher number of DNA-reactive cells among $V_H\Delta^a/WT^b$-double-producers than among $V_H\Delta^a$-single-producers, and we should be able to assess the overall frequency of anti-DNA cells among these double-producers.

In addition, more anti-self specificities could be tested in sera and cells from the $V_H\Delta^a/WT^b$ mice, besides anti-dsDNA specificity. Of particular interest would be anti-self specificities not found in WT^b/WT^b mice (i.e. WT^b-IgH plus IgL with this specificity is completely negatively selected, and only the $V_H\Delta^a$-IgH plus IgL with such specificity has a chance to pass negative selection). Then we could more precisely test the model for the emergence of autoreactive receptors in $V_H\Delta^a/WT^b$-double-producers.

Moreover, if we put the $V_H\Delta^a$ allele within an autoimmune mouse background, such as MRL/lpr, NZB/NZW or NOD, these autoimmune disease prone models will be more informative if we study and characterize their pathology. We predict that these models, incombination with the Eμ-deficient V_H knock-in, will develop more severe symptoms that resemble human SLE or type 1

diabetes, including organ malfunction (glomerulitis: proteinuria, elevated BUN, kidney histopathology; insulitis: pancreas-specific auto-antibodies, etc) and mortality.

Chapter 6: References

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