REGULATION OF VH REPLACEMENT IN HUMAN IMMATURE B CELLS BY B CELL RECEPTOR (BCR)-MEDIATED SIGNALING

By

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REGULATION OF VH REPLACEMENT IN HUMAN IMMATURE B CELLS BY B CELL RECEPTOR (BCR) – MEDIATED SIGNALING

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ABSTRACT

V_{H} replacement occurs through RAG-mediated secondary recombination to change unwanted IgH genes. In this dissertation, I focused on studying the molecular mechanism that regulates V_{H} replacement in human immature B cells.

In part I, our results show that V_{H} replacement is regulated by B cell antigen receptor (BCR) mediated signaling. Using the human EU12 µHC^{+} cells as an experimental model system, crosslinking BCR with F(ab')_{2} anti-IgM antibodies results in BCR internalization, cell proliferation arrest, and induction of V_{H} replacement. Pretreatment of human EU12 µHC^{+} cells with the protein tyrosine kinase inhibitor Genistein, Syk kinase inhibitors, and a Src kinase inhibitor blocks BCR-mediated signaling events and inhibits V_{H} replacement. Inhibition of PI3K kinase enhances V_{H} replacement, conversely, activation of PI3K by anti-CD19 antibodies inhibits BCR signaling induced V_{H} replacement. Furthermore, analyses of large numbers of IgH sequences reveal that the V_{H} replacement products are highly enriched in different autoimmune diseases and anti-viral responses.

In part II, we further dissect BCR-mediated signaling events in EU12 µHC^{+} immature B cells compare to that in human Daudi and Ramos mature B cells. EU12 µHC^{+} cells have features of human bone marrow immature B cells. After 30 minutes of
treatment with anti-IgM antibodies, almost 100% of EU12 µHC⁺ cells lost their surface BCR, in contrast, Daudi and Ramos cells only partially lost their surface BCR. After BCR internalization, restimulation of EU12 µHC⁺ cells with anti-IgM antibodies results in normal level of Erk1/2 activation, but with delayed Syk phosphorylation, reduced Ca²⁺ mobilization, and decreased FoxO1 phosphorylation. Thus, internalization of BCR on EU12 µHC⁺ immature B cells attenuates BCR signaling with selective effects on downstream signaling events, which may favor receptor editing.

Taken together, the results presented in this dissertation provide the first evidence that V_H gene replacement is regulated by BCR signaling on immature B cells. Complete internalization of BCR receptor specifically changes BCR-mediated signaling events in immature B cells.
DEDICATION

This work is dedicated to my mother and father, XIAOPIN FENG and FUSEN LIU, for their support and encouragement during all my education years. To my extended family, for their extraordinary involvement in my life and the caring and the support they give.
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Finally, I would like to thank the members of my graduate committee, Drs. Peter Burrows, Hiromi Kubagawa, John Kearney and Louis Justement, for their support, invaluable assistance, and guidance.
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<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
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<tr>
<td>BAFF</td>
<td>B cell activating factors</td>
</tr>
<tr>
<td>Bam32</td>
<td>B cell adaptor molecule of 32 kD</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
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<td>BLNK</td>
<td>B cell linker</td>
</tr>
<tr>
<td>BSAP</td>
<td>B cell specific activator protein</td>
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<tr>
<td>Btk</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>CCP</td>
<td>clathrin-coated pits</td>
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<td>CLP</td>
<td>common lymphoid progenitor</td>
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<tr>
<td>cRSS</td>
<td>ctyptic recombination signal sequences</td>
</tr>
<tr>
<td>D</td>
<td>diversity gene segment</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
<td>EBF</td>
<td>early B-cell factor</td>
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<tr>
<td>Erk</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>FL</td>
<td>Flt-3 ligand</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GC</td>
<td>germinal center</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>Igα</td>
<td>immunoglobulin α</td>
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<tr>
<td>Igβ</td>
<td>immunoglobulin β</td>
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<tr>
<td>IgH</td>
<td>immunoglobulin heavy chain</td>
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<tr>
<td>IgL</td>
<td>immunoglobulin light chain</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-7Ra</td>
<td>IL-7 receptor α</td>
</tr>
<tr>
<td>J</td>
<td>joining gene segment</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol-1, 4, 5-triphosphate</td>
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<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mIg</td>
<td>membrane immunoglobulin</td>
</tr>
<tr>
<td>MZ</td>
<td>marginal zone</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
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<tr>
<td>PH</td>
<td>pleckstrin homology</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3 - kinase</td>
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<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<td>PTK</td>
<td>protein tyrosine kinase</td>
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<tr>
<td>pTyr</td>
<td>phosphotyrosine</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RAG</td>
<td>recombination activation gene</td>
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<td>RSS</td>
<td>Recombination signal sequences</td>
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<td>SH2</td>
<td>Src homology-2</td>
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<tr>
<td>SLC</td>
<td>surrogate light chain</td>
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<tr>
<td>SOC</td>
<td>store-operated Ca^{2+} channel</td>
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<tr>
<td>Syk</td>
<td>spleen tyrosine kinase</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>Tg</td>
<td>transgenic</td>
</tr>
<tr>
<td>TNF</td>
<td>tumore necrosis factor</td>
</tr>
<tr>
<td>V</td>
<td>variable gene segment</td>
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<td>WT</td>
<td>wild type</td>
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INTRODUCTION

Overview of B Lymphocytes

*B lymphocyte origin*

Lymphocytes are divided into two major types based on their development and differentiation needs. T lymphocytes (T cells) require the thymus for maturation, whereas B lymphocytes (B cells) are independent of the thymus. The prefix ‘B’ was originated from initial studies in the chicken that antibody production requires the presence of lymphocytes in an organ called the bursa of Fabricius (Masteller et al., 1995; Glick and Whatley, 1967). Elimination of this organ by hormonal treatment gave rise to birds that were unable to produce antibodies. Later studies in mice and humans showed that B lymphocytes are generated in the fetal liver, and in the bone marrow (BM) of adults (Rolink et al., 2000; Gururajan et al., 2008).

B and T lymphocytes have similar morphology (Polliack et al., 1975) and can only be distinguished based on their cell surface molecules. During the early stage, B cells express immunoglobulins (Igs) and other glycoproteins on their surface, such as CD19, which are not found on T cells. Upon activation, B cells terminally differentiate
into plasma cells, which can produce specific antibodies to combat foreign antigens (Bewarder et al., 1996).

Development of B cells

In adults, B cells are generated from hematopoietic stem cells in the bone marrow (Rolink et al., 2001; Harris et al., 2001). Commitment of pluripotent stem cells into B lineage cells is a highly regulated series of events, namely the concerted expression of cell surface and intracellular factors, sequential rearrangement of IgH and IgL genes, and changing requirements for growth factors (Tonegawa, 1983; van Gent et al., 1996).

After commitment into the B lineage pathway, B cells develop from pro-B cells (early and late) to pre-B cells, immature B cells, and mature or naïve B cells in the bone marrow (Figure 1). The pro-B cell is the earliest B lineage cell in the bone marrow. It differs from the hematopoietic stem cell by exhibiting a B or lymphoid – restricted gene expression pattern, such as CD19 and Igα (CD79a), and by its ability to initiate rearrangement of the Ig gene locus (Gay et al., 1993; Chen et al., 1995; Cascalho et al., 1997). A pro-B cell is transformed into an early pre-B cell once the Dh and Jh gene segments are recombined at the Ig heavy chain locus while the genes for light chains still remaining in germline configuration. Future rearrangements attach one of the Vh gene segments to the DhJh segment and give rise to a new B cell stage, called the late pre-B cell, which expresses a functionally rearranged VhDhJhCμ-chain, small amounts of which can be found on the cell surface. Subsequent rearrangement of the Ig light chain
Figure 1. Early steps of B lymphocytes differentiation in bone marrow and periphery.
In bone marrow, development progresses through the pro-B, pre-B and immature B cell stages. During this differentiation, rearrangements at the immunoglobulin locus result in the generation and surface expression of the pre-BCR, which is composed of an Igμ heavy chain and surrogate light chain (VpreB and Vλ5) and followed by a mature BCR (composed of rearranged heavy and conventional κ or λ light chain genes) that is capable of binding antigen. At the immature stage, B cells undergo a selection process to prevent the development of self-reactive cells. After successfully passing through this checkpoint, cells with functional IgM on their cell surface leave the bone marrow and relocate to secondary lymphoid organ as transitional B cells. Then, they further convert into mature follicular B cells (or marginal zone B cells). Following an immune response, antigen-specific B cells develop into either plasma (antibody secreting) cells or memory B cells. Early B lymphopoiesis is controlled by a regulatory network of key transcription factors and signaling through transducers with sequential rearrangement of immunoglobulin heavy and light chain genes.
gene locus leads to the surface expression of a complete IgM molecule and to a new B cell stage, the *immature B cell*. The latter gives rise to the *mature B cell* that, as a result of an alternative splicing of the Ig heavy chain transcript, expresses both IgM and IgD at the cell surface. Mature B cells are also called naïve B cells as they did not yet encountered their cognate antigen.

Mature naïve B cells leave the bone marrow and migrate into the secondary lymphoid organs, where they either die or enter primary B cell follicles (Allman et al., 2001). A small number of antigens are T-cell independent as they can directly activate naïve B cells by binding to their antigen-specific B cell receptor (BCR). Most antigens are T-cell dependent. They can only activate naïve B cells by initiating two immune interactions: a first one between antigen presenting cells and T helper cells which leads to clonal expansion of those T helper cells with the required T cell receptor, and a second interaction between the selected and antigen primed T helper cells and mature naïve B cells (Heyzer-Williams et al., 2001). The latter one is a two way process, in which B cells present the antigen in association with MHC class II molecules to T cells and receive signals from those T cells for proliferation and differentiation. Antigen activated B cells, either with or without the interaction of antigen presenting cells and T cells, can transform into large B blasts that may differentiate into IgM producing plasma cells, which are responsible for the first line of defense against the antigen or migrate back into the primary B cell follicle in which they rapidly proliferate and differentiate into GC B cells (Kroese et al., 1990).
Cytokines that regulate early B cell development

Hematopoiesis is influenced by many different cytokines that are produced in the fetal liver and BM microenvironment. These proteins alter gene expression profiles by binding to specific receptors on hematopoietic stem cells (HSCs) and more mature progenitors. These interactions can promote proliferation and survival of receptive cells. Such signals may also induce lineage specific differentiation (Northrup and Allman, 2008).

In mouse, early B cell development is regulated mainly by two cytokines, Flt-3 ligand (FL) and IL-7. FL binds to and activates the Flt-3 tyrosine kinase, and IL-7 binds to a heterodimeric receptor composed of IL-7 receptor α-chain (IL-7Rα) and the common γ-chain (γc), which is a component of several additional cytokine receptors (Baird et al., 1999). B cell development is compromised but not completely eliminated in FL-deficient mice (Sitnicka et al., 2003). Early B cell development is largely IL-7 dependent, and IL-7null/null adults lack detectable numbers of pro-B cells (Carvalho et al., 2001). Surprisingly, the spleen of IL-7 and IL-7R-deficient adults contains readily detectable numbers of mature B cells, yet pro-B cells are not detected in BM of these mice. However, mice that lack both Flt-3 and IL-7 completely lack B-lineage cells including developing precursors (Sitnicka et al., 2003), suggesting that IL-7R and Flt-3 signaling pathways converge to regulate the growth and survival of the same progenitor cells such as common lymphoid progenitors (CLPs). CLPs are the earliest lymphoid-specific progenitor population and serve as a requisite branch point for both B and T cell development.
It has been shown that B cell development is arrested at the CLP stage in IL-7Rα<sup>null/null</sup> adults (Miller et al., 2002). This leads to experiments showing that IL-7R activity establishes a B-lineage-specific gene expression program in CLPs. The IL-7R accomplishes this mission by promoting the expression of early B-cell factor (EBF), and ectopic expression of EBF restores B cell development in IL-7 deficient animals (Dias et al., 2005; Kikuchi et al., 2008). Remarkably, the genetic targets underlying FL-mediated B cell development remain unknown. IL-7 and FL are likely to promote expression of alternative transcription factors that are critical for generating early B cell precursors.

*Transcriptional control of B cell development*

The transcriptional regulatory network that leads to specification and commitment to the B lymphocyte lineage from CLPs has been extensively studied. Gene targeting has revealed the importance of many specific transcription factors during the earliest stages of B cell commitment and differentiation *in vivo* (Figure 1). Among them, the most important players are the Ets-family transcription factor PU.1, the zinc-finger protein Ikaros, EBF, E2A, and B-cell-specific activator protein (BSAP), encoded by the Pax5 gene (Bain et al., 1994; Zhuang et al., 1994; Urbanek et al., 1994).

**PU.1 and Ikaros** PU.1, a member of the Ets family of transcription factors, is required for the generation of both lymphoid and myeloid lineages (Klemsz et al., 1990). *PU.1*-deficient mice die during early embryonic development, lack macrophages, B cells and have reduced numbers of granulocytes, natural killer (NK) and T cells (Scott et al., 1994). *PU.1*-deficient progenitor cells do not express the IL-7Rα chain on their cell
surface and therefore, they cannot respond to IL-7 signals. The IL-7Rα gene is a direct target of PU.1 as the levels of IL7Rα transcripts are reduced in CLPs of PU.1−/− mice and the promoter of the IL-7Rα gene contains a functional PU.1 binding site (DeKoter and Singh, 2000). Recent studies showed that PU.1 is not totally required for B cell development (Ye et al., 2005). Cultures of PU.1−/− progenitor cells on stromal cells in the presence of IL-3, IL-7, SCF and Flt3L allow for the outgrowth of cells that express the PU.1 target genes IL-7Rα and Ebf, but not B220 (DeKoter et al., 2002). Transduction of PU.1−/− progenitor cells with the IL-7Rα gene can partially rescue the B cell deficiency, consistent with the regulatory hierarchy of PU.1 and IL-7R. The role of Stat5 as the mediator of IL-7 signaling was confirmed by the ability of a constitutively active form of Stat 5 to rescue the B cell defect in mice with a mutation in the IL-7Rα gene. This study also showed that overexpression of Ebf results in a partial rescue of B cell development in IL-7−/− mice (Kikuchi et al., 2005). Similarly, the B cell defect of IL-7−/− CLPs was found to be overcome by the retroviral transduction of Ebf, whereas no rescue was observed with Pax5 (Dias et al., 2005).

PU.1 levels may also determine the choice between the two lineages (Klemsz et al., 1990). Bone marrow cell cultures for PU.1-deficient mice skewed toward myeloid differentiation in vitro in the presence of high levels of ectopically expressed PU.1. In contrast, lower levels of PU.1 expression favored differentiation to the B lymphocyte lineage. The dosage effect may be due to the effects of PU.1 on expression of the gene encoding the IL-7R. Low levels of PU.1 were found to be permissive for IL-7R
expression in bone marrow cultures, whereas no IL-7R expression was evident in high
PU.1 expressing cells.

Ikaros belongs to the family of zinc finger transcription factors and contributes to
HSC activity and differentiation. Initial characterization suggested that Ikaros acted as a
transcriptional activator of the lymphoid-specific terminal deoxynucleotidyl transferase
(TdT) gene and the T-cell-specific CD3δ enhancer (Georgopoulos et al., 1992; Hahm et
al., 1994). The generation of Ikaros null mice revealed marked defects in B and natural
killer (NK) cell differentiation, decreased dendritic cell (DC) and T cell populations and
to a reduced reconstitution activity of HSCs (Wang et al., 1996). Mice with a dominant-
negative mutation of Ikaros showed an additional defect that includes a complete lack of
DC and T cells. All these studies reinforce the fact that Ikaros is required for the
activation of critical lymphoid-specific genes (Wang et al., 1996). Using a novel
immunofluorescence in situ hybridization technique (Immuno-FISH), it was shown that
Ikaros associates with transcriptionally silent genes in foci containing heterochromatin.
Genes associated with a non-B lineage, such as CD4, co-localized with Ikaros, while
genes actively expressed in B cells, like CD19, did not localize to the Ikaros-
heterochromatin complexes. Genes with a stage-specific pattern of expression in B cells,
such as λ5, also associated with Ikaros when inactive, indicating that Ikaros acts as a
global negative regulator of B-lymphocyte gene expression (Brown et al., 1997). Further
investigation has defined a mechanism by which Ikaros and its related family members
mediate global repression.
The genes encoding Flk2/Flt3 and c-kit are both regulated by Ikaros. HSCs deficient for Ikaros lack the Flk2/Flt3 and c-kit receptors on their cell surface and fail to generate CLPs (Nichogiannopoulou et al., 1999). Interestingly, normal levels of Ikaros transcripts are detected in PU.1-deficient progenitors, suggesting that PU.1 and Ikaros act in distinct pathways of transcriptional control of hematopoiesis (DeKoter and Singh, 2000).

**EBF and E2A** EBF and E2A both act at the earliest pro-B cell stage and are thought to play key roles at the onset of pro-B cell differentiation. In the hematopoietic system, *Ebf* is expressed exclusively in the B cell lineage, starting from the CLP stage to mature B cells, whereas in plasma cells the expression of *Ebf* is turned off (Igarashi et al., 2002; Nagaoka et al., 2000). EBF has been initially identified via its cell-type specific activation potential of the *mb1* gene (Hagman et al., 1993). Targeted deletion of *Ebf* leads to a complete block in early B cell differentiation prior to D<sub>H</sub> to J<sub>H</sub> recombination of the *Igh* locus (Lin and Grosschedl, 1995). *Ebf*-deficient mice express normal levels of *IL-7Ra* transcripts and *Igh* germline transcripts. EBF binds to regulatory sequences of the λ5 and *VpreB* promoters and activates the expression of these genes in collaboration with the transcription factor E2A (Sigvardsson et al., 1997). EBF also collaborates with the transcription factor Runx1 in the activation of the *mb1* gene, whereby EBF acts as a “pioneer” factor that facilitates DNA demethylation and enhances chromatin accessibility in CLPs (Maier et al., 2004). Ectopic expression of *Ebf* or *E2a*, together with the recombinase-activating genes *Rag1* and *Rag2* in nonlymphoid cells is sufficient to induce D to J<sub>H</sub> recombination (Romanow et al., 2000).
Disruption of the $E2a$ gene results in a block in B cell differentiation similar to $Ebf$, prior to the onset of $D_{H1}$ to $J_{H1}$ rearrangement (Bain et al., 1994). $E2a$ encodes for two splice variants, E12 and E47, which are members of the basic helix-loop-helix (bHLH) family of transcription factors (Kreider et al., 1992). Both proteins are ubiquitously expressed and form heterodimers with cell type specific members of the bHLH family (Shen and Kadesch, 1995). In B cells, E47 is thought to be specifically dephosphorylated, leading to the formation of homodimers (Sloan et al., 1996). EBF and E2A cooperate in the regulation of components of pre-BCR, encoded by the $\lambda5$, VpreB, mb1 and B29 genes. The functional synergy of EBF and E2A has also been shown genetically. Compound heterozygous $Ebf^{+/-} \times E2a^{+/-}$ mice display a severe developmental arrest at the pro-B cell stage and have reduced expression of multiple B cell specific genes, including $\lambda5$, $Rag1$, $Rag2$, mb1 and Pax5 (O'Riordan and Grosschedl, 1999). The role of EBF in the specification of B lineage cells was confirmed by genetic bypass experiments of $PU.1$ and $E2a$ deficient mice in which retroviral transduction of $Ebf$ overcomes the block in B cell differentiation (Medina et al., 2004). Ectopic expression of $Ebf$ in HSCs leads to the abrogation of T lymphopoiesis by restricting lymphopoiesis to the B cell lineage. In addition, EBF induces apoptosis in pro-T cells by an unknown mechanism. These results suggest that EBF acts downstream of $PU.1$ and E2A. This presumed regulatory hierarchy is consistent with the presence of a $PU.1$-binding site in the first intron of the $Ebf$ gene and the presence of an E2A-binding site in the $Ebf$ promoter (Smith et al., 2002). Moreover, the expression of $Ebf$ is reduced in $E2a^{-/-}$ fetal liver hematopoietic progenitors (Seet et al., 2004). However, EBF also seems to regulate the expression of the $E2a$ gene.
(Zhuang et al., 2004), which suggests that EBF and E2A act in a feedback loop to specify
the B cell fate.

*Pax5* B cell specific activator protein (BSAP)-Pax5 directly acts as a
transcriptional activator of several lymphoid genes such as CD19, BLNK, and Igα (Nutt
et al., 1998), through binding to their promoter or regulatory regions (Tian et al., 1997).
In addition, Pax5 acts as a transcriptional repressor of lineage-inappropriate genes,
including the M-CSF receptor gene, and thus commits the progenitor cells to the B-
lymphoid lineage and maintains that commitment by suppressing alternative cell fates
(Kee and Murre, 1998; Nutt et al., 1999; O'Riordan and Grosschedl, 1999). In line with
this fact, Pax5-deficient pro-B cells still maintain a developmental potential and can still
differentiate into NK cells, DC cells, macrophages, osteoclasts, granulocytes or even T
cells and red blood cells under appropriate conditions (Nutt et al., 1999). In addition, in
these Pax5-deficient pro-B cells, but not in wild-type (WT) pro-B cells, ectopic
expression of C/EBPα and GATA transcription factors induces a switch to the myeloid
lineage (Heavey et al., 2003; Emelyanov et al., 2002). Interaction with distinct proteins
that act as corepressors (Groucho and perhaps also TBP, Rb and PTIP) or coactivators
(DAXX) (Emelyanov et al., 2002) is regarded as the key to the differential transcriptional
activity of Pax5. Intriguingly, the transcription factor PU.1 is a direct target of the Pax5-
mediated repression and, inversely, PU.1 can inhibit Pax5 transactivation (Maitra and
Atchison, 2000). In transgenic mice, pan-hematopoietic Pax5 expression under control of
the Ikaros locus strongly promotes B cell development at the expense of T lymphopoiesis.
This is achieved by interfering with T lineage commitment and early thymocyte
development through repression of the transcription of Notch1, a key regulator of T cell specification (Souabni et al., 2002).

**NF-κB** The transcription factor nuclear factor –κB (NF-κB) is very important for adaptive immunity. It is crucial for the initial responses to pathogens, as well as for the subsequent events that lead to B cell mediated antigen-specific defense. NF-κB was originally discovered as a result of its ability to bind to a sequence in the Igκ intronic enhancers (Sen and Baltimore, 1986). NF-κB is composed of homo- or heterodimers of five rel family members: RelA (p65), RelB, c-Rel, p50 and p52 (Hayden et al., 2006).

Inactive NF-κB is sequestered in the cytoplasm bound to an inhibitory protein of the IκB family (IκBα, IκBβ or IκBε). Various signaling pathways result in the activation of a kinase that phosphorylates IκBα leading to its degradation. Once released from IκBα, NF-κB can translocate to the nucleus, bind specific DNA sequences, and regulate transcription. Importantly, one of the transcriptional targets of NF-κB is IκBα itself, leading to negative-feedback regulation of NF-κB activation (Chiao et al., 1994).

NF-κB signaling occurs through either the classical (also known as canonical) or alternative (also known as noncanonical) pathways (Bonizzi et al., 2004; Bonizzi and Karin, 2004). Whereas the classical pathway is IκB kinase β and IKKγ dependent and NF-κB activation occurs through IκB degradation (Li and Verma, 2002), the alternative pathway depends on IKKα- and NF-κB-inducing kinase (NIK) and is based on regulated processing of the p100 precursor protein (Senftleben et al., 2001). This alternative pathway is involved in NF-κB activation after stimulation by tumor necrosis factor (TNF)
superfamily members B cell activating factor (BAFF), lymphotoxin β, and CD40 ligand and is particularly important for B cell maturation and lymphoid organ formation (Hayden et al., 2006).

It has been proposed that NF-κB could be involved in the regulation of light chain gene receptor editing (Verkoczy et al., 2005). Receptor editing is stimulated in immature B cells by BCR recognition of autoantigen, which results in down-modulation of BCR from the cell surface. BCR engagement is known to activate NF-κB. From this study, it was shown that NF-κB proteins are involved in upregulating transcription of recombination-activating genes *RAG1* and *RAG2* in cells undergoing receptor editing (Verkoczy et al., 2005). A study using a fluorescent β-gal substrate and an IkBα-lacZ knockin reporter allele-defined a subpopulation of pre-B cells that contains active nuclear NF-κB and this subpopulation correlated with cells undergoing receptor editing. However, RAG expression was maintained during receptor editing process in these cells (Cadera et al., 2009). How NF-κB controls light chain editing remains to be defined.

*Transcriptional regulation of the recombination-activating genes* The first rearrangement of the immunoglobulin heavy chain loci marks the initiation of B cell differentiation. The process of recombination is highly regulated, and requires the activity of the lymphoid-specific recombination-activating genes, *Rag-1* and *Rag-2* (Oettinger et al., 1990; Schatz et al., 1989). The expression of both immature and mature antigen receptors are critical steps in normal B cell differentiation; mice lacking either of the *Rag* genes exhibit an arrest at the pro-B cell stage (Schlissel et al., 1991). Overexpression of
E47 in a pre-T cell line increases transcription of $\text{Rag-1}$, $\text{Rag-2}$ and $I\mu$, the Ig heavy chain germline transcript (Schlissel et al., 1991). Additionally, Rag-1 expression is completely abolished in the fetal liver of $E2a^{-/-}$ mice (Bain et al., 1994). The analysis of B cell differentiation in $Ebf^{+/+}E2a^{+/+}$ fetal liver indicated that the Rag genes are downstream targets of EBF and E2A (O'Riordan and Grosschedl, 1999). This result is surprising, as $\text{Rag-1}$ and $\text{Rag-2}$ are expressed in both B and T lymphocytes, whereas EBF and E2A are only expressed in B cells. However, this observation is consistent with the finding of B and T-cell specific activities in distinct regions of the $\text{Rag-1}$ and $\text{Rag-2}$ promoters. In addition, Pax5 was found to bind a region of the $\text{Rag-2}$ promoter which is important for B-cell specific activity (Schlissel et al., 1991). However, the expression of the $\text{Rag}$ genes is not diminished in $Pax5^{-/-}$ pro-B cells, suggesting that Pax5 may not be a critical initial activator (Li et al., 1993).

**Generation of a diversified Ig repertoire by V(D)J recombination**

Clonotypic antigen receptors, expressed on B lymphocytes, are critical for the diversity and specificity of humoral immune responses. It is well-established that DNA recombination of variable (V), diversity (D, only for the heavy chain) and joining (J) gene segments (V(D)J recombination) in three immunoglobulin loci (heavy chain, kappa and lambda light chains) provides the basis for formation and expression of diverse Ig receptors. Lymphocyte specific RAG1 and RAG2 proteins recognize, bind and cleave
conserved recombination signal sequences (RSS) elements, that flank V, D and J gene segments. The broken ends are subsequently joined using the ubiquitously expressed non-homologue ending joint (NHEJ) DNA double-strand break repair enzymatic machinery (Bassing et al., 2002).

The order of V(D)J recombination at the different loci is strictly regulated. Successful completion of V(D)J recombination on one heavy chain allele leads to expression of the $\mu$ polypeptide that provides a developmental checkpoint and signals for further development (Mostoslavsky et al., 2003). In addition, because V(D)J recombination involves the creation of double-stranded breaks, it must be appropriately restricted to provide genome stability and guard against loss or aberrant rearrangement of DNA. Thus, V(D)J recombination is subject to multiple stringent controls.

In B cells, V(D)J recombination begins at the immunoglobulin heavy chain locus by the joining of the DH and JH segments followed by the joining of VH-to-DJ (Bassing et al., 2002). It is the second step, joining of VH-to-DJ that is subject to the most stringent regulation. Although DHJH rearrangement can occur in progenitors not fully committed to the B cell lineage (Borghesi et al., 2004; Borghesi and Gerstein, 2004), VH-to-DJ rearrangement is limited to committed B cells at the pro-B cell stage. Following functional VH-to-DJ rearrangement and expression of a transmembrane $\mu$ protein, VH-to-DJ rearrangement is actively repressed in pre-B cells in a process termed allelic exclusion (Bassing et al., 2002). Therefore, this joining step serves as a model for developmental and lineage specific gene control, requiring mechanisms for both activation and repression. Packaging of DNA into chromatin constrains enzymatic processes, such a
recombination, repair, and transcription that use DNA as substrate. Thus, it has long been suspected that alterations in chromatin accessibility play an important role in controlling V(D)J recombination (Krangel, 2003).

During B lineage development in adult mice, RAG1 and RAG2 are expressed only in early B progenitors in the BM (Bertrand et al., 1998; Osmond, 1990) and expression ceases prior to the migration of B lineage cells to the periphery. Some studies have indicated that RAG1 and RAG2 are expressed in a subset of GC B cells of immunized mice (Han et al., 1996). The RAG positive germinal center B cells were shown to be undergoing receptor editing at the light chain locus and reactivation of the previously allelicly excluded heavy chain allele (Han et al., 1996; Han et al., 1997). However, other studies have failed to detect the re-expression of the RAG genes in the spleen except in immature B cells, presumably recent arrivals from the BM (Yu et al., 1999). Another group showed that BCR crosslinking and CD40 stimulation induces RAG expression, thus secondary rearrangement in mature B cells (Hillion et al., 2007). This study examined the mechanism controlling RAG expression in peripheral and tonsil B cells and found that following stimulation with CD40 and BCR crosslinking, IL-6 was produced, which contributes to the initiation and the extension of RAG expression, and modulates the subsequent BCR-mediated down-regulation (Hillion et al., 2007). Taken together, these results imply that mature B cells can re-express RAG and are capable of accomplishing secondary V(D)J recombination.
B cell tolerance checkpoints and mechanisms

The V(D)J recombination of immunoglobulin genes is essential to generate a diversified B cell repertoire, however, it also generates many immunoglobulin genes encoding self-reactive antibodies. The single-cell PCR analysis of B cells from healthy individuals showed that an estimated three quarters of the newly formed B cells are self-reactive, and a large proportion of those autoantibodies recognize multiple antigens. These B cells represent 55-75% of the highly diversified repertoire that is constantly generated by random Ig gene rearrangement during early B cell development in the BM (Yurasov et al., 2005; Wardemann et al., 2003). To ensure self-tolerance, autoreactive B cells producing such potentially harmful antibodies are normally efficiently removed at two checkpoints. The transition from early immature to immature B cells in the BM is considered to be a major checkpoint that eliminates a large part of the originally self-reactive cells. Once immature B cells are released from the BM, they are detected in the periphery as newly emigrant B cells (CD10⁺IgM⁺CD27⁻) that still contain a substantial number of self-reactive clones (approximately 7%). A second tolerance checkpoint occurs in their transition into the mature naïve B cell (CD10⁺IgM⁻CD27⁻) compartment. At this point, less than 5% of the peripheral naïve compartment consists of autoreactive B cells (Wardemann et al., 2003). Recently, a third checkpoint for B cell tolerance has been proposed to exist for B cell tolerance between the naïve and IgM⁺ memory compartment before B cells undergo somatic hypermutation (Tsuiji et al., 2006).
Following this ordered developmental sequence, tolerance mechanisms progressively shape the BCR repertoire and are critical to ensuring a self-tolerant B cell pool. Only 10 to 20% of the immature B cells in the bone marrow can reach to the peripheral, and 3% contribute to the mature B cell pool (Hao and Rajewsky, 2001), indicating the importance of tolerance mechanisms in restricting the development of autoreactive cells. Furthermore, one-fourth of all Abs in the human immature B cell compartment produced self-reactive Abs (Wardemann et al., 2003). This observation emphasizes that defects in one or several of these tolerance mechanisms could contribute to the development of autoimmunity.

Antibody-affinity selection seems to be the foundation of the removal of autoreactive B cells from the repertoire in the first tolerance checkpoint. First, antibodies with very high affinity for self are removed by clonal deletion in early development. Next, antigen-receptor editing by light-chain replacement occurs in many of the remaining self-reactive B cells and depends largely on the strength of BCR signaling.

B cell tolerance is less well characterized in the periphery. However, detailed analysis of BCR specificity in human peripheral B cells has demonstrated a roughly 50% reduction in self-reactivity in mature versus newly formed B cells, indicating that tolerance mechanisms are also active at this stage. In addition, deletion of self-reactive B cells occurs in vivo when self-Ag is specifically expressed in the periphery. Different from bone marrow B cells, transitional B cells in the periphery lose the capacity to edit their receptors and instead undergo deletion upon encounter with high-avidity self-Ags in vivo.
Tolerance sensitivity of immature bone marrow B cells

For B cells, the ability to recognize Ag is first manifest at the immature stage when the complete form of the BCR is expressed on the cell surface. A large body of evidences documents the sensitivity of the immature B cell to tolerance induction and negative selection. There are three known mechanisms of negative selection: receptor editing, clonal deletion, and anergy.

Receptor editing on the IgL genes Receptor editing is a molecular process that involves secondary rearrangements (mostly known at the light chain genes) that replace existing immunoglobulin molecules and generate a new antigen receptor with altered specificity. It has been estimated that 75% of human early immature B cells are self-reactive (Wardemann et al., 2003). Roughly a third of these self-reactive immature B cells are eliminated from the repertoire by receptor editing, wherein renewed immunoglobulin gene rearrangement generates a new light chain to pair with the existing immunoglobulin heavy chain in a seemingly anthropomorphic effort to generate a non-self-reactive BCR. Several studies have shown that B cells can undergo secondary heavy- or light- chain receptor editing. In the bone marrow, it is an important mechanism to maintain tolerance because it can extinguish a self-reactive specificity without having to physically eliminate a potentially autoreactive B cell.

Receptor editing was first described by Nemazee and Weigert (Tiegs et al., 1993; Gay et al., 1993) (Figure 2) in conventional BCR transgenic systems in which B cells altered the specificity of Ig receptors away from being autoreactive in response to self-Ag. A series of anti-DNA and anti-MHC class I Ig knock-in models (Chen et al., 1995; Chen
Figure 2. B cell light chain receptor editing.
During B cell development, successful recombination of the IgH and IgL genes leads to cell-surface BCR expression on immature B cells. If the BCR is bound by autoantigen, these cells will continue V(D)J recombination, resulting in receptor editing, particularly at loci that encode the IgL chain. ‘Innocuous’ (non-self-reactive) receptors promote positive selection, and these B cells are subsequently released into the periphery. Ig\textsubscript{\mu}, IgH chain with \mu constant region.

*Modified from Nemazee, D. (Nemazee, 2000)*
et al., 1997; Taki et al., 1993) provided further evidence that secondary recombination at
the L chain loci was important for maintaining tolerance to self-Ags and suggested that
the editing was highly efficient (Melamed and Nemazee, 1997; Chen et al., 1997). It was
suggested that as many as half or more of normal B cells in BM undergo some degree of
editing (Retter and Nemazee, 1998; Casellas et al., 2001).

Receptor editing is likely to be a major force in shaping the B cell antibody
repertoire (Casellas et al., 2001). However, the involvement of receptor editing in
eliminating autoreactive B cells in nontransgenic mice, which have a more variable
antigenic repertoire, remains controversial. Initial attempts to assess the frequency of
normal B cells that have undergone receptor editing depended on the determination of the
proportion of mature B cells expressing λ light chains that had also rearranged their
κ locus (Retter and Nemazee, 1998). The double “knock-in” mice studies have taken us a
step further in receptor editing research. In these animal models, one antibody κ light
chain locus carries a pre-rearranged antigen-combining region capable of undergoing
secondary rearrangements and the other locus contains a polymorphism that facilitates
detection of B cells that have undergone receptor editing. In these studies, the normal B
cell repertoire is more closely approximated than in previous studies that used pre-
rearranged heavy and / or light chains from autoreactive BCRs. The reason for this is that
the pre-rearranged antibody light chain is innocuous and is allowed to pair with
endogenous antibody heavy chains. Furthermore, B cells that have undergone secondary
rearrangements can be identified immediately by the characteristics of the antibodies that
they express. Using several different prearranged innocuous and potentially autoreactive
light chain “knock-ins”, it was determined that about 25% of developing B cells had undergone secondary light chain rearrangements. This finding suggests that receptor editing can dramatically influence the antibody repertoire (Melamed et al., 1998). By comparing developing B cells that continued to express the pre-rearranged antibody light chain (no receptor editing) with those in which receptor editing was taking place, it was found that cells undergoing receptor editing spent longer in the pre-BII stage of development than those that had not undergone receptor editing. These data argue that editing is not the result of random rearrangements at the very early pro-B cell stage followed by antigen-driven selection later in the development. Together with all the “knock-in” experiments, these finding imply that antigenic encounter promotes receptor editing at a relatively late developmental stage in the bone marrow.

In most current models of receptor editing, antigen-reactive immature B cells are envisioned to re-express RAGs, which are necessary for antibody gene rearrangement. This results in the reinitiation of light chain recombination after interaction with self-antigens (Melamed and Nemazee, 1997; Melamed et al., 1998). Paradoxically, immature B cells are extremely susceptible to BCR-induced apoptosis initiated by antigen binding (Norvell and Monroe, 1996; Norvell et al., 1995). The question of “how can immature B cells initiate receptor editing while they are simultaneously receiving an execution signal?” has been brought up and two models have been suggested to answer this question. The first model suggests that the BCR-induced response of immature B cells is developmentally regulated. Newly emerging immature B cells are specifically induced to undergo receptor editing after BCR engagement with antigen, whereas later stage immature B cells undergo apoptosis (Melamed et al., 1998). In a second model, all
immature B cells are sensitive to BCR-induced apoptosis, but survival signals provided by specialized bone marrow cells provide them with a temporary stay of execution, giving them another chance to produce a non-autoreactive BCR (Sandel and Monroe, 1999).

Taken together, the reinitiation of light chain rearrangement in immature B cells after antigenic encounter implies that BCR signaling is crucial for rendering cells competent to undergo secondary light chain rearrangements.

**Clonal deletion** The concept of clonal deletion, that lymphocytes expressing antigen receptors specific for self-antigens will be deleted during developmental, was originally proposed in 1957 (Burnet, 1976; TALMAGE, 1957). This hypothesis was first genetically tested and confirmed by analyzing the fate of immature thymocytes expressing TCRα/β antigen receptors specific for defined endogenous superantigens, which are encoded in the germline by retrovirus-like sequences (Pullen et al., 1988; Woodland et al., 1990). In contrast to ‘conventional’ antigens, which are presented to T cells as peptide fragments embedded into class I or class II MHC molecules and stimulate a small fraction of the T cell repertoire, superantigens are presented to T cells as whole polypeptides associated with class II MHC molecules and stimulate only a readily identifiable portion of the TCR repertoire (generally 2 ~ 10%). Specificity for superantigens bound to class II MHC chains is conferred to a large extent by the variable region of the TCRβ chain (Woodland et al., 1990). Using panels of TCRvβ-specific monoclonal antibodies and collections of mouse strains it was shown by several studies
that T cells that respond to endogenous superantigens are deleted during their development in the thymus (Woodland et al., 1990; Pullen et al., 1988).

Studies using BCR transgenic mice and transgenic mice expressing the cognate antigen revealed that autoreactive B lymphocytes, like T cells, can be deleted at an immature stage during development in the bone marrow or at a mature stage in the spleen or lymph nodes, provided that no T cell help is available (Goodnow, 1989; Russell et al., 1991; Nemazee and Burki, 1989).

Collectively, these studies demonstrate that signaling through antigen receptors can cause deletion of autoreactive B and T lymphocytes both during their development in the bone marrow or thymus, respectively, or at the mature stage in peripheral lymphoid organs, such as the spleen or lymph nodes. As the well established clonal deletion as B cell tolerance mechanism, we take it as a process in which clones of immature B cells are selectively deleted (negative selection) in BM.

\textit{Anergy} Anergy is a state wherein cells persist in the periphery but are unresponsive to antigen. It is responsible for silencing many self-reactive B cells (Goodnow et al., 1988; Nossal and Pike, 1980). The maintenance of anergy requires chronic binding of antigen and signal transduction. Experiments in a number of different BCR transgenic mouse models have shown that low level continuous interaction of self-reactive B cells and their cognate antigen can result in loss of responsiveness to BCR-mediated signals. These cells are prone to apoptosis, resulting in the rapid elimination of most anergic cells from the repertoire (Stevenson et al., 1989).
Unlike clonal deletion and receptor editing, anergy can occur without any previous attempts to edit away autoreactivity (Tze et al., 2005), and can presumably silence cells in which editing leaves residual low-affinity autoreactivity. Thus, the decision of which silencing mechanism is used depends upon receptor avidinity for autoantigen. Higher avidity favors editing and deletion, while low avidity interactions invoke anergy (Melchers, 2006).

Models of Ig-transgenic mice expressing lower-avidity BCRs support this point of view. In the anergic state, immature B cells retain the short life expectancy of other immature and naïve B cells, i.e., a half-life of 2 to 4 days. Hence, they will quickly disappear from the system. One difference between anergic and naïve B cells is that anergic B cells are expected to be engaged by the presence of an autoantigen, whereas naïve B cells do not react with autoantigens present in bone marrow. In fact, it has been shown that continuous presence of autoantigens is required to keep anergic B cells in an anergic state (Gauld et al., 2005). Removal from the autoantigenic influence or inhibition of this influence by soluble autohaptens, which are unable to crosslink the BCR converts anergic B cells to naïve B cells. With the short half-life of 2-4 days, the pool of $2 \times 10^7$ immature B cells, half of which are edited, supplies the spleen with $5 \times 10^6$ immature B cells per day in mouse. These cells enter a further differentiation pathway via transitional B cells. T1 appears to precede T2 and this might be considered to be the immediate precursor of naïve mature B cells in the spleen. B cell activating factor (BAFF) helps B cells to form the B cell-rich regions, follicles, in spleen. In 2006, Merrell et al. (Merrell et al., 2006) identified the T3 transitional compartment of B cells in the spleen as a major compartment of anergic B cells. They have shown that these cells are kept in their anergic
state by autoantigens present in the spleen. A collection of anergic and non-anergic Ig-transgenic mouse strains was used to define the phenotype of an anergic B cells. The results indicate that at least one third of all immature B cells in the spleen are anergic T3 type cells. Their antigen binding repertoires appear to be shifted toward the recognition of autoantigens. In summary, this study has suggested that continuous presence of the splenic autoantigens is required to maintain the anergic state (Merrell et al., 2006).

In total, All together, there are several features associated with anergy, attenuated BCR signaling, reduced membrane immunoglobulin (mIgM) expression, altered migration and localization, an inability to interact productively with helper T cells and reduced lifespan. These mechanisms act in concert to limit the ability of anergic cells to participate in immune responses (Merrell et al., 2006). Chronic BCR stimulation by antigen promotes the activity of negative feedback loop and this is rapidly extinguished upon autoantigen withdrawal. This circuitry governs receptor signaling and promotes B-cell death through parallel molecular pathways.

\[ V_H \text{ replacement} \]

The concept of \( V_H \) replacement

Most of previous studies on receptor editing have been focused on the IgL genes. However, editing of autoAb-producing B cells also involve the H chain (Radic and Zouali, 1996). Developing autoreactive B cells may edit their Ag receptor specificity by
the process of $V_H$ replacement. In contrast to the relative easy access of secondary light chain VJ rearrangement, the secondary rearrangement of an upstream $V_H$ gene to a preformed $V_HD_HJ_H$ gene appeared theoretically difficult, since all the intervening $D_H$ gene segments were deleted during the primary $V_H$ to $D_HJ_H$ rearrangement, which leaves no 12-bp RSS to recombine with the 23-bp RSS flanked $V_H$ genes (Radic and Zouali, 1996; King and Monroe, 2001). However, functional IgH genes have been found to be generated in mouse pre-B cells lines carrying nonfunctional IgH rearrangements. Comparison of the functional V(D)J joints with the previous non-functional V(D)J joints in these cases indicated that the secondary recombination could be mediated through the use of a cRSS site embedded within the third framework region of the rearranged $V_H$ gene, a rearrangement process named $V_H$ replacement (Kleinfield et al., 1986; Reth et al., 1986; Covey et al., 1990) (Figure 3). This $V_H$ replacement mechanism is quite intriguing, because the cRSS motif is found in 40 out of 44 human functional $V_H$ genes and in almost two thirds of mouse $V_H$ genes (Zhang et al., 2004). Therefore, it provides a unique RAG-mediated recombination mechanism to change preformed IgH genes.

$V_H$ replacement in mice

$V_H$ replacement was first identified in A-MuLV-transformed murine pre-B (Reth et al., 1986) and B lymphoma cell lines (Kleinfield and Weigert, 1989). Studies of the biological functions of $V_H$ replacement have been performed in several mouse models to further establish that if $V_H$ replacement occurs in vivo. A preformed VDJ$_H$ transgene which was inserted into the $J_H$ region of the IgH locus has been particularly instructive in
Figure 3. Model of serial $V_H$ replacement mediated by cryptic recombination signal sequence (RSS).
With each round of $V_H$ replacement, the previous rearranged $V_HDJ_H$ gene is replaced by a new $V_H$ gene, leaving behind a footprint in the CDR3 region of the new immunoglobulin (Ig) $H$ gene.
examining the heavy chain editing process (Bertrand et al., 1998). These models allow
the study of the inserted VDJH in a close to natural state.

The first occurrence of \( V_H \) replacement was tested in knock-in mice carrying a
rearranged IgH V(D)J joint at the \( J_H \) locus encoding anti-DNA antibodies (Chen et al.,
1995). \( V_H \) replacement was demonstrated in these mice by tracking the loss of the
inserted VDJH using anti-idiotypic antibodies, followed by DNA sequencing of the \( V_H \)
gene in the idiotype negative cells (Gay et al., 1993; Chen et al., 1995). In these mice, the
artificially inserted \( V_H \)DJH genes could be efficiently deleted by three types of
recombination events: \( V_H \) to \( V_H \)DJH, \( D_H \) to \( V_H \)DJH, and \( V_H \) to \( D_H \) to \( V_H \)DJH (Chen et al.,
1995; Chen et al., 1997). Only the \( V_H \) to \( V_H \)DJH recombination mediated by the cRSS can
occur in normal mice. The other two are artifacts of the targeted IgH locus, in which an
intact \( D_H \) region is located upstream of the rearranged \( V_H \)DJH gene. In these mice, the \( V_H \)
replacement employed to delete the anti-DNA specificity appeared to occur at the pre-B
to immature B transitional stage in the BM (Chen et al., 1995; Zhang et al., 2004),
presumably after encounter with self-antigen. Taken together, these results suggested that
the heavy chain receptor editing could eliminate self-reactive antibodies.

Evidence of \( V_H \) replacement occurrence has also been observed in other murine
models. The generation of a knock-in mouse carrying a self-reactive heavy-chain gene
could only be achieved after disruption of the cRSS site (Hertz and Nemazee, 1997),
suggesting that \( V_H \) replacement could occur in an efficient way. \( V_H \) replacement also
appears to occur frequently in a quasi-monoclonal (QM) mouse expressing a knock-in
IgH V(D)J region encoding an anti-NP (4-hyroxy-3-nitrophenyl acetyl) antibody.
(Cascalho et al., 1997). In this mouse model, all the genes for the heavy chain were either deleted or inactivated, and only three of the light chain genes were remained, and a gene construct for just one heavy chain was inserted (Williams, 1996). All available Ig gene rearrangements in these mice produced antibodies capable of binding only to the NP happen. However, by the time the mice reached maturity, about 20% of peripheral B cells and up to 50% of the peritoneal B cells were found to have lost the knock-in $V_H$ gene and NP reactivity, presumably because of secondary IgH gene rearrangement (Cascalho et al., 1997). Sequence analyses of Ig genes from the QM mouse confirmed $V_H$ to $V_HDJ_H$ rearrangement, the canonical $V_H$ gene replacement mechanism previously described \textit{in vitro}. The ability of the QM mouse to mount a protective anti-viral response using antibodies generated through $V_H$ replacement also suggested that $V_H$ replacement can contribute to repertoire diversification (Lopez-Macias et al., 1999) and can produce monoclonal antibodies against different immunogens (Golub et al., 2001). Using a ligation-mediated PCR (LM-PCR) assay, another group has identified $V_QM$ double-stranded DNA breaks indicative of $V_H$ replacement intermediates from bone marrow in the absence of self antigen, suggesting that $V_H$ replacement is another mechanism contributing to the generation of Ig diversity during B cell development and it occurs as a natural process (Lopez-Macias et al., 1999).

The occurrence of $V_H$ replacement has been well documented in knock-in mice carrying different IgH genes within the IgH locus. RAG mediated double-stranded DNA (ds-DNA) breaks at the VH-cRSS border have been used as the indicator for ongoing $V_H$ replacement, which can be detected by LM-PCR. The production of excision circles
generated at the same time can be detected by excision circle PCR. However, it has also been reported that it is difficult to detect $V_{H}$ replacement in pro-B, pre-B and immature B cells from normal mouse BM (Watson et al., 2006). In this study, 3 out of 48 LM-PCR reactions have been identified as special ds-DNA breaks at the expected cRSS sites in BM immature B cells. In addition, analysis of mouse IgH sequences from this study identified only one IgH gene as a potential $V_{H}$ replacement product (Watson et al., 2006). Another report showed that $V_{H}$ replacement occurs in normal mouse pro-B cells, but are undetectable in pre-B and immature B cells (Davila et al., 2007). $V_{H}$ replacement occurs efficiently in homozygous mice carrying non-functional IgH genes on both IgH alleles, but it is not efficient enough to compete with the wild-type IgH allele. In knock-in mice, pro-B cells carrying non-functional rearrangements on both IgH alleles are blocked from further development, while in normal mice these cells might be eliminated before having the chance to undergo secondary recombination (Zhang, 2007).

Serial $V_{H}$ replacement events in the EU12 cell line

The molecular basis and biological function of $V_{H}$ replacement in humans was not studied for a long time because there was no suitable experimental model system. The recent studies in our lab began with the demonstration that a unique cell line, EU12 (Wang et al., 2003), which was established from a childhood leukemia patient, undergoes continuous pro-B cell to pre-B and B cell differentiation in vitro and thus proved to be a good model to study human $V_{H}$ replacement. In EU12 cells, $RAG1$, $RAG2$, and terminal deoxynucleotidyl transferase ($TdT$) gene expression can be detected at the pro-B stage. $TdT$ expression is terminated at the pre-B stage while $RAG1$ expression is reduced at the
immature B cell stage. Analysis of the BCR repertoire in different subpopulation indicated limited intraclonal diversification of $V_H$ and $V_L$ gene usage in the EU12 cell line (Wang et al., 2003). All the IgH sequences were found to include the same $D_H$ 3-10 $J_H$4 joint, but with different $V_H$ genes, including $V_H$2-5, $V_H$3-7, $V_H$3-11 and $V_H$1-8 genes. Analyses of the $V_H$-$D_H$ junction regions in the EU12 IgH sequences suggested that this cell line was undergoing serial $V_H$ replacement through the use of the cRSS site (Wang et al., 2003). Double-stranded DNA breaks were detected at the $V_H$2-5 cRSS in EU12 cells using the LM-PCR assay. In addition, different $V_H$ replacement excision circles generated through predicted serial $V_H$ replacement events could be detected using seminested primer sets (Zhang et al., 2004). Based on all these observations, we conclude that there is ongoing $V_H$ replacement in the EU12 cell line (Figure 3).

**Ongoing $V_H$ replacement in human bone marrow immature B cells**

In humans, 40 out of 44 functional $V_H$ germline genes contain heptameric cRSS motifs (5’-TACTGTG-3’) and potentially could participate in $V_H$ replacement recombination (Radic and Zouali, 1996). The first evidence for the natural occurrence of $V_H$ replacement in humans was provided by the detection of ds breaks at the cRSS border in BM immature B cells but not in pro-B, pre-B, naïve B or memory B cells (Zhang et al., 2003) (Figure 4). In theory, $V_H$ replacement should also occur in pro-B cells with nonproductive $V_HDJ_H$ rearrangements or in pre-B cells expressing IgH chains that do not pair with the surrogate or conventional light chains. However, LM-PCR assays failed to detect ds DNA breaks at the cRSS borders in pro or pre-B cells. Studies using human bone marrow samples have indicated that the number of immature B cells and their
Figure 4. $V_H$ replacement during B lineage cell development.
The potential stages at which $V_H$ replacement occurs to rescue pro-B cells containing non-functional IgH genes, and to edit preformed IgH genes at the bone marrow immature stage or in the newly emigrated immature B cells in the periphery are indicated.
differentiation stage are critical for the successful detection of ds DNA breaks at the cRSS borders. The estimated number for $V_H$ replacement detection in purified immature B cells used per LM-PCR experiment is ~300. Reduction of the cell number can cause difficulty in detecting cRSS ds DNA breaks (Zhang, 2007).

Immature B cells are generated throughout human life in the BM, they migrate into the circulation as they mature (Nunez et al., 1996). During inflammatory responses, pro, pre, and immature B cells can migrate to secondary lymphoid organs, such as the spleen and and lymph nodes in response to the intriguing signals. These cells may express RAG genes and have the ability to editing their light chain genes (Nussenzweig, 1998). The occurrence of $V_H$ replacement in immature B cells is consistent with the abilities of these cells to express RAG genes and to change their IgL genes. Thus the immature B cell stage is a very delicate stage for editing both IgH and IgL genes (Zhang, 2007).

**Regulation of $V_H$ replacement**

With the detection of ds breaks, $V_H$ replacement is suggested to be a receptor editing process which might be regulated in a similar fashion as light chain editing. It has been shown that crosslinking of the BCR on immature B cells induces RAG expression and the secondary light chain gene rearrangement (Hertz and Nemazee, 1997; King and Monroe, 2000). It remains to be investigated whether the same signaling stimulation will induce $V_H$ replacement. Another important issue concerns the accessibility of the IgH locus for recombination. During primary recombination, IgH recombination preceeds
light chain recombination, and the ordered secondary recombination process is assumed to be regulated by differential locus accessibility. It is unlikely that immature B cells would make both IgH and IgL loci accessible to allow light chain receptor editing and V_H replacement occur at the same time (Nussenzweig et al., 1987).

V_H replacement in EU12 cells preferentially use a neighboring upstream V_H gene to replace the previously rearranged V_H gene (Mansilla-Soto and Cortes, 2003; Zhang et al., 2003). Neighboring upstream V_H genes may be more accessible to the RAG proteins because of their proximity to the V_H promoter and the E_M enhancer. In addition, V_H replacement may also be influenced by signaling events that affect cell survival (Sandel and Monroe, 1999). Stimulation provided by the bone marrow environment to protect immature B cells from dying could theoretically be an important determinant for V_H replacement in the same fashion as for light chain gene editing. Abnormal signaling events blocking an apoptotic pathway could also have an enhancing effect on V_H replacement. Understanding the molecular mechanisms that regulate V_H replacement has important significance.

**Biological consequences of V_H replacement for the human B cell repertoire**

V_H replacement could provide a mechanism to edit unwanted IgH genes, either ones that are non-functional or ones that encode self-reactive antibodies.

Based on current knowledge, continuous V_H replacement not only rescues pro-B cells carrying a nonfunctional V_HD_HJ_H but also generates intraclonal diversity. More importantly, with each round of V_H replacement, the resulting IgH gene renews the entire
VH coding region except for a short stretch of 3’ nucleotides from the replaced VH gene that is retained in the VH-DH joint. This residual sequence, which is also known as the ‘VH replacement footprint’, can serve as a marker that can be used to search for potential VH replacement products in primary B cells. The Ig heavy and light chains each contain three hypervariable (complementarity determining regions or CDR) segments and four conserved frame work regions. The CDRs are the most diverse regions of the antibody molecule. CDR3 is in direct contact with antigen and is the most variable portion of the Ig molecule. The VH replacement footprints preferentially contribute charged amino acids into the CDR3 regions. This appears to be a natural feature of VH replacement in that the 3’-ends of all human and mouse VH germline genes primarily encode charged amino acids, regardless of their reading frames. The biological significance of the charged amino acids deposited into the CDR3 regions by VH replacement remains unclear; however, these residues likely contribute to antigen-binding specificity and affinity. Furthermore, highly charged amino acids are frequently seen in autoreactive antibodies. Arg residues are often present within the IgH CDR3 regions of anti-DNA antibodies (Radic et al., 1993). Thus, VH replacement may also have the potential to generate autoreactive antibodies. On the other hand, through IgH gene sequences analyses derived from B lineage cells of normal humans of different ages, we could conclude that potential VH replacement products are present in roughly 5% of the analyzed IgH genes (Zhang, 2007; Zhang et al., 2003). This suggests that VH replacement products contribute significantly to the diversification of the human B cells repertoire.

The significant contribution of VH replacement to the normal B cell repertoire together with the fact that this process preferentially deposits charged amino acids into
the CDR3 region seems contradictory. It is certain that $V_H$ replacement renews the entire $V_H$ coding region, and the addition of charged amino acids definitely could alter the specificity of the antibody. This could also help to improve the affinity of antigen-binding toward charged antigens, such as the negatively charged bacteria inner walls. This speculation fits the idea that $V_H$ replacement may facilitate protection against bacteria or viral infection.

**B cell receptor and BCR signaling**

B cell development is an ordered process that allows for the sequential expression and assembly of the B cell antigen receptor (BCR). BCR controls the fate of B lymphocytes. Additionally, the avidity and the context in which antigen is encountered determine both cell fate and differentiation in the periphery. Thus, BCR signaling can either positively select and maintain immunocompetent B cells or lead to silencing or even elimination of those cells that respond to autoantigens (Wang and Clark, 2003) (Figure 5).

**Pre-BCR Signaling**

The pre-B cell receptor (pre-BCR), composed of immunoglobulin $\mu$ heavy chains, the monomorphic, non-rearranging light chain-like proteins, VpreB and $\lambda 5$, and the Ig$\alpha$/Ig$\beta$ signal-transducing heterodimer on pre-B cells (Karasuyama et al., 1990; Kudo and
Figure 5. Downstream B cell receptor mediated signaling pathways.
After binding to Ag, the Igα and Igβ cytoplasmic tails are phosphorylated on the ITAM tyrosines by Src-family tyrosine kinases (SFTKs) and Syk. Syk then activates multiple signaling pathways, including: calcium flux, PKC activation, Erk pathway, and JNK activation. NF-κB can also be activated by SFTKs. Modified from Wang et al (Wang and Clark, 2003)
Melchers, 1987; Tsubata and Reth, 1990; Burrows et al., 2002), provides signals for proliferation and maturation of developing pre-B cells. The production of a functional pre-BCR is the first checkpoint during B cell development (Kitamura et al., 1991; Mundt et al., 2001). Deleting any component of the pre-BCR leads to a pro-B cell block in development. This developmental arrest implies that signaling occurs through the pre-BCR. This idea is further supported by a block at the pro-B cell stage in mice that lack expression of the BCR-associated tyrosine kinase Syk or the signaling adaptor protein BLNK(SLP65) and in humans lacking Igα, λ5, BLNK, or the protein kinase Btk (Wang and Clark, 2003). These genetic studies demonstrate a requirement for the early forms of the BCR and its signaling capacity in order for proper B cell development to proceed.

The pre-BCR monitors the formation of a functional µHC. Signaling initiated via the pre-BCR promotes cellular proliferation and RAG1 and RAG2 downregulation to interrupt the Ig using a transgenic mice model expressing SLC throughout B cell development, it was recently shown by Hendriks’ group that silencing of SLC genes is not essential for the limitation of pre-B cell proliferation, but is rather required for the prevention of constitutive activation of B cells (van Loo et al., 2007). Successful Ig LC gene rearrangement leads to complete BCR expression, and is followed by additional checkpoints when the resulting immature B cells progress into the mature B cell stage (Sabbattini and Dillon, 2005; Burrows et al., 2002).
Initiation of BCR signaling

The BCR was first identified in 1970 (Raff et al., 1970), a multimeric complex consisting of an antigen-recognition structure, the membrane-bound Ig (mIg), associated non-convalently with a heterodimer of Igα (CD79a) and Igβ (CD79b). Except for IgG, the cytoplasmic tails of the five types of mIg are very short and all lack any known signaling capacity (Martin and Goodnow, 2002). Igα/β functions as the BCR signaling subunit and couples the receptor to intracellular signal transducer elements (Williams et al., 1994). Only the completely assembled BCR is able to reach the cell surfaces and transduces signals into the cell (Figure 5).

Many efforts have been spent on determining how BCR aggregation induces phosphorylation of the ITAM tyrosines. The resting BCR is associated with Src family tyrosine kinases, such as Blk, Lyn, and Fyn, which become activated following receptor ligation (Burkhardt et al., 1991). The spleen tyrosine kinase (Syk) can also be found in the resting receptor complex. The association of these tyrosine kinases with the receptor is mediated by unique tyrosine-independent motifs embedded within the cytosolic tail of Igα (Papavasiliou et al., 1995).

Engagement of BCR with Ag or anti-receptor Ab mediates clustering of associated Src family kinase molecules leading to phosphorylation of ITAM tyrosines (Yamanashi et al., 1991; Clark et al., 1992). This leads to recruitment of additional Src family kinases as well as Syk which, upon recruitment, becomes tyrosyl phosphorylated and activated (Kurosaki and Tsukada, 2000; Kurosaki et al., 1995). Syk phosphorylates the unique B cell linker protein, BLNK/SLP-65, which is essential for the activation of
Bruton’s tyrosine kinase (Btk) and phospholipase C (PLC) γ2 (Fu et al., 1998; Ishiai et al., 1999). Phosphorylation of Syk can also signal propagation through multiple pathways, including PI(3)K pathway and the classical NF-κB1 pathway. Subsequent tyrosyl phosphorylation of CD19 leads to PI(3)K recruitment to the membrane and generation of phosphatidylinositol (PtdIns)-3,4,5P3 from PtdIns(4,5)P2 (Hippen et al., 1997; Buhl and Cambier, 1999). In the presence of inositol 5-phosphatases, e.g., SHIP, phosphatidylinositol 3-kinase activation should also lead to the generation of PtdIns(3,4)P2. The pleckstrin homology (PH) domain-containing signaling intermediaries Btk and PLCγ translocate to the plasma membrane where they bind PtdIns(3,4,5)P3, facilitating PLCγ-mediated hydrolysis of phosphoinositides, generation of inositol-1,4,5-trisphosphate (Ins(1,4,5)P3 and mobilization of calcium (Li et al., 1996).

BCR and Ag engagement can also initiate signaling cascades that lead to activation of the Ras-MAPK pathway, phosphatidylinositol-3-kinase, and phospholipase Cγ (PLCγ) (Fu et al., 1998; Ishiai et al., 1999). The BCR triggering ultimately induces gene expression patterns that can promote cell activation, apoptosis, or anergy, depending upon the balance of enhancing and inhibitory influences that vary according to the stage in B cell differentiation (Healy and Goodnow, 1998).

Signaling through the BCR is a complex process that has been predominately studied in mature or activated B cell lines. However, it is clear that BCR signaling is responsible for a wide range of distinct physiological responses through the entire period of B cell development, indicating that discrete signaling pathway subsets are
developmentally coordinated as cells mature. Until now, the regulatory mechanism of BCR signaling in immature B cells still remains unclear.

**Signaling through the immature B cell receptor**

The recombination of conventional Igκ or Igλ light chain genes is required for the development of immature B cells (Zou et al., 2003). Truncation of the cytoplasmic tail of Igα causes a dramatic decrease in the numbers of immature B cells, indicating the importance of BCR signaling at this stage (Torres et al., ), while a similar truncation in Igβ has no effect on the production of immature B cells, demonstrating that the functions of Igβ are redundant of Igα during the pro-B to pre-B transition. Moreover, truncation of the Igα cytosolic tail inhibits the generation of immature B cells to a greater degree than pre-B cells. This suggests that as B cell progenitors progress through development, successively higher signaling capacity is required (Wang and Clark, 2003).

Although receptor editing occurs in the immature B cells, it is still not completely clear that how the BCR signaling initiates editing. One possibility is that receptor editing is triggered by the absence of basal BCR signaling that occurs following ligand-induced downregulation of the BCR (Tze et al., 2005). From this point of view, the lack of BCR signaling would allow immature B cells to back-differentiate to an earlier stage in which RAG and other recombinase machinery genes are expressed. This conclusion is based on the observation that conditional deletion of the BCR in immature B cells initiates new light chain rearrangements and the re-expression of pre-B cell markers (Tze et al., 2005; Tze et al., 2003). Alternatively, editing may result from a BCR-dependent transient arrest
during development at the pre-B/immature B transition state (Casellas et al., 2001), a stage at which RAG is still expressed. Anergy, another mechanism of immature B cell tolerance and the outcome of low affinity receptor crosslinking, results in the activation of Erk signaling pathways and translocation of nuclear factor of activated T cells (NFAT) to the nucleus without the activation of NF-κB (Healy et al., 1997). This suggests that anergy may arise from the activation of a subset of the signaling pathways activated by the BCR. On the other hand, it is also possible that anergy involves specific signaling effectors not requisite for normal cell activation, for example it is known that B cells deficient in protein kinase Cγ become activated in response to anergizing ligands.

The BCR mediates distinct cell fate decisions at the immature and mature B cell stages. It has been proposed that B cell fate is determined by the balance between survival and death signals initiated through the BCR while other co-stimulatory signals might be involved subsequently. Under conditions when BCR expression at the cell surface generates a basal (tonic) signal, it might be sufficient for immature B cell survival (Bureau et al., 2002). When some immature B cells have BCRs with high avidity for self-antigen, a strong BCR signal is generated, thus leading to the death fate for these cells, while in mature B cells, basal BCR signaling acts as a maintenance signal for cell survival (Lam et al., 1997).

Regulation of PTKs

The sequential activation of three different types of PTKs, Lyn, Syk, and Btk, is required to regulate downstream effectors in BCR signaling.
Lyn plays a central role in maintaining the balance between positive and negative B cell signaling pathways, in regulating B cell tolerance and the development of autoimmunity (Zouali and Sarmay, 2004). Analysis of signaling pathways in LYN−/− mice showed that regulation of BCR signaling is a complicated process in which the activating role of Lyn is mediated by the phosphorylation tyrosine-based activation motifs (ITAMs) of Igα/β, and CD19, and the subsequent recruitment of signaling enzymes such as Syk, PLCγ2, and PI3Ks. These positive effects are balanced by the negative regulatory role of Lyn in B cells potentially through two pathways. First, Lyn is responsible for phosphorylating tyrosine residues of the negative BCR coreceptor FcγRIIb. This action may account for the enhanced BCR signaling observed in LYN−/− B cells. Second, Lyn was shown to negatively regulate B cells by opposing the effect of Syk on BCR-mediated activation of Akt/PKB. Deregulation of Akt/PKB correlates with the BCR-mediated hyperresponsiveness of LYN−/− B cells (Cornall et al., 1998).

The key role of Lyn in establishing and maintaining peripheral tolerance also comes from studies of the consequences of sustained activation of Lyn in vivo using a targeted “gain-of-function” mutation (Hibbs et al., 2002). LYN+/+ mice express a constitutively activated form of Lyn. This experimental design allows study of the consequences of constitutive engagement of both stimulatory and inhibitory signaling pathways. Phenotypically, these mice have reduced conventional B-2 cells, downregulation of surface IgM and costimulatory molecules, and elevated numbers of B-1a cells. Increased Ca2+ flux in response to BCR stimulation and intensified positive signaling were observed in vitro. Surprisingly, these LYN+/+ mice develop autoreactive
antibodies, suggesting that enhanced positive signaling can override constitutive negative signaling (Nishizumi et al., 1995).

Syk is a central signaling element for many ITAM-containing receptors. Syk was originally identified at the protein level as a 40 kDa proteolytic fragment containing the catalytic domain that was isolated from bovine thymus based on its ability to phosphorylate a synthetic peptide substrate (Geahlen and Harrison, 1986). A kinase of the same size was isolated from porcine spleen (Kobayashi et al., 1990). Antibodies against this active fragment identified the full-length enzyme as a 72 kDa protein, a size confirmed when the cDNA was sequenced (Zioncheck et al., 1988). Syk has a tandem pair of Src homology 2 (SH2) domains separated by a 60-amino acid linker (linker A). A C-terminal catalytic domain is connected to the tandem SH2 domains by a stretch of 106-amino acids (linker B) that contains multiple sites of phosphorylation (Livak and Schatz, 1997). A role for Syk in cellular signaling was first identified in B lymphocytes (Hutchcroft et al., 1991), but Syk is expressed in many cell types including most cells of the hematopoietic system, and at lower levels in some epithelial cells, fibroblasts, hepatocytes, vascular smooth muscle cells, endothelial cells and neuronal cells (Yanagi et al., 2001).

Upon BCR activation, the phosphorylation of the two tyrosines within the Igα/β ITAM leads to the physical recruitment of Syk to the site of the clustered receptor in an interaction mediated by its tandem pair of SH2 domains (Ottinger et al., 1998). The binding of Syk to the phosphorylated ITAMs of clustered BCR complexes leads to its activation. In vitro studies indicate that the simple binding to Syk in solution to a dually
phosphorylated peptide with the sequence of an ITAM is sufficient to fully activate the kinase (Cheng et al., 1995). An analysis of Syk’s structure by electron microscopy suggests an autoinhibited conformation comparable to that of Zap-70 indicating that Syk is likely to be regulated and activated in an analogous fashion (Tsang et al., 2008). Consequently, the binding of Syk to its reaction product, a dually phosphorylated ITAM, activates the enzyme and generates a positive feedback loop to promote the phosphorylation of additional ITAM tyrosines to recruit even more Syk molecules to the clustered BCR complexes (Rolli et al., 2002).

Syk can catalyze the phosphorylation of ITAM tyrosines and at least one non-ITAM tyrosine (Y204) on Ig-α when recruited to the BCR. These residues lie within predicted AP-2 binding sites and the conversion of all three to non-hydrophobic residues severely impairs receptor internalization. If the activity of Syk is then inhibited, the Syk-receptor complex rapidly dissociates and the receptor is internalized most likely due to its rapid dephosphorylation by protein tyrosine phosphatases. Thus phosphorylated receptors that bind and activate Syk are retained at the cell surface while clustered, but nonphosphorylated receptors are internalized (Hou et al., 2006). The persistence of Syk-BCR complexes at the plasma membrane likely plays an important role in determining the length of time that Syk remains active following the initial engagement of the receptor. The prolonged activation of Syk is required for some receptor-stimulated events such as the activation of the NFAT transcription factor, which requires Syk to remain active for more than one hour following receptor ligation (Oh et al., 2007). The binding
of Syk to the adaptor protein BLNK/SLP65 at the plasma membrane also provides a mechanism for its prolonged activation (Kulathu et al., 2008).

Once activated, Syk catalyzes the phosphorylation of multiple protein substrates that are important for transducing the antigen receptor interaction into the appropriate physiological response. The consequences of a proteins phosphorylation by Syk vary depending on the nature of the substrate and the site that is modified. For a subset of Syk’s substrates, the addition of a phosphate group induces conformational changes that lead to alterations in the intrinsic activity of the phosphorylated protein. The phosphorylation on tyrosine of PLCγ2, Btk, hematopoietic progenitor kinase-1 (HPK1) and the Vav1 guanine nucleotide-exchange factor leads to their activation (Rodriguez et al., 2001; Kurosaki and Tsukada, 2000). For many substrates, phosphorylation on tyrosines instead promotes protein-protein associations by generating docking sites that are recognized by proteins that have SH2 domains or other phosphotyrosine-binding motifs. In fact, it has been observed that Syk preferentially phosphorylates tyrosines within motifs that can then be recognized by group I SH2 domains. Thus, the phosphorylation of many Syk substrates including BLNK/SLP-65, LAB/NTAL/LAT2, 3BP2, BCAP, BANK and GCET generates scaffolds for the assembly of larger signaling complexes (Kurosaki, 2002). For example, the phosphorylation of BLNK/SLP65, a major Syk substrate in B cells, creates docking sites that bind Btk and PLC-γ to generate a protein complex that regulates the mobilization of calcium.
In summary, multiple factors act in concert to influence the activity of Syk in order to regulate the quality and quantity of the signal that is sent from the BCR, which ultimately determines the physiological outcome of receptor engagement.

CD19

An important step in BCR signaling is the phosphorylation of CD19. The CD19 coreceptor can induce positive signals that enhance B cell responses. CD19 physically associates with the BCR through intracellular and extracellular motifs (Pesando et al., 1989) and has also been shown to facilitate pre-BCR signaling (Krop et al., 1996). Previous work showed that signals from BCR and CD19 are critical for LC isotype (κ versus λ) exclusion. The absence of CD19 results in an inability to downregulate RAG genes and there is disproportionate development of B cells expressing λ. LC develop. The signaling pathway that regulates RAG gene expression and leads to LC allelic exclusion and receptor editing still remains unclear. However, RAG gene expression is enhanced by the PI3K inhibitor wortmannin (Verkoczy et al., 2005) suggesting a downstream effector role of PI3K for both BCR and CD19. Furthermore, in CD19−/− mouse B cells, reduced PI3K activation was demonstrated by a decrease in BCR-mediated Akt phosphorylation. Activation of PI3K appears to be essential for CD19 signaling function. When an altered form of CD19 that lacks critical tyrosines required for PI3K binding was expressed in CD19−/− mice, the knockout phenotype was not rescued (Wang et al., 2002), however CD19 lacking other tyrosines, including those involved in recruitment of Src family
kinases and Vav, could restore the function. This clearly shows the direct interaction between CD19 and PI3K.

**BCR signaling through the Phosphoinositide 3-kinases (PI3K) pathway**

The PI3Ks are a family of enzymes that regulate diverse biological functions in every cell type by generating lipid second messengers (Donahue and Fruman, 2004). Gene-targeting studies have revealed that PI3K subunits are correlated with B cell signaling. Three class IA PI3K catalytic subunits, encoded by the p110α, β or δ genes, are all expressed in B cells. The function of class I PI3Ks is to convert phophatidylinositol-4,5-bisphosphate (PtdInsP₂) to phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns(3,4,5)P₃) at the inner leaflet of the plasma membrane. PtdInsP₃ acts as a binding site for numerous intracellular enzymes that contain pleckstrin-homology domains (PH domains) with selectivity for this lipid. The first demonstration of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ production in murine B cells activated via the BCR was shown using chromatographic methods (DeFranco, 1994). Subsequently, it was also shown that pharmacological inhibitors of PI3K could block proliferation of primary B cells in human (agaard-Tillery and Jelinek, 1996). Also, downstream of PI3K the serine/threonine kinase AKT/PKB plays an important role in B cell proliferation, growth, survival, and metabolism (Koyasu, 2003).

In B cells, PI3K is activated within seconds of antigen-receptor interaction. There is strong evidence to show that Syk activity is required for PI3K activation. Deletion of Syk in the chicken B cell line DT40 impairs BCR-stimulated production of...
PtdIns(3,4,5)P$_3$ (Brunati et al., 1995). Once Syk is activated and followed by phosphorylation of the co-receptor CD19 and B cell PI3K adaptor (BCAP), providing binding sites for PI3Ks. BCR ligation leads to the recruitment by CD19 of PI3K via its p85$\alpha$ regulatory subunit, the generation of lipid products such as PIP$_3$, the attendant recruitment to the plasma membrane of PH domain-containing proteins such as PLC$_{\gamma}2$ and cytoplasmic kinases, such as Btk and Akt.

BCR-stimulated Akt phosphorylation was nearly abolished in p85$\alpha$- and p110$\delta$-deficient B cells, which indicates that they are directly acting downstream of the BCR.

During BCR-Ag interaction, a major pathway initiating from PI3K is mediated by Bruton’s tyrosine kinase (Btk) (Bolland et al., 1998). Btk is a Tec family kinase that signals through the adaptor protein B cell linker protein (BLNK) and phospholipase C (PLC)$_{\gamma}2$ to mediated BCR-induced Ca$^{2+}$ flux and activation of protein kinase C (PKC)$\beta$ (Mecklenbrauker et al., 2002). (Details about calcium signaling will be discussed later)

**The Forkhead box family and BCR signaling**

The Forkhead Box, Subgroup O (Foxo) family is an important group of Akt substrates (Burgering and Kops, 2002). Foxo1, Foxo3a, Foxo4 and Foxo6, has been identified from yeast to humans. Foxo factors increase transcription of many target genes that are involved in cell cycle arrest and the promotion of apoptosis and metabolism. It has been shown that BCR crosslinking leads to PI3K-Akt-dependent phosphorylation of Foxo1 and subsequent nuclear exclusion (Stahl et al., 2002). Overexpression of Foxo1 or
Foxo3a protein in activated primary B cells leads to cell cycle arrest and apoptosis (Yusuf et al., 2004).

Two recent studies of the regulation of BCR editing suggest that autoreactive signaling via BCRs causes the nuclear accumulation of the Foxo family transcription factors, which can directly activate transcription of \( \text{Rag1} \) and \( \text{Rag2} \) to promote receptor editing (Amin and Schlissel, 2008; Herzog et al., 2008). These findings suggest a molecular link between autoreactive BCRs and secondary rearrangements that provide stimulation that facilitates B cell tolerance.

At the immature B cell stage, expression of an innocuous (non-autoreactive) BCR generates ligand-independent (‘tonic’) intracellular signals that downregulate \( \text{Rag1}/\text{Rag2} \) expression and promote survival and further differentiation (Waskow et al., 2008). On the other hand, cell surface expression of an autoreactive BCR can also lead to antigen-BCR interaction, internalization of antigen-BCR complexes and activation of intracellular signals that upregulate \( \text{Rag1}/\text{Rag2} \), and inhibit differentiation and initiate secondary \( V_\kappa \) rearrangements that replace the primary \( \kappa \) light chain rearrangement (Waskow et al., 2008). Both tonic and antigen-induced signaling requires the PI3K-Akt kinases and the SLP-65 adaptor protein (Verkoczy et al., 2005). One group has found that signaling through PI(3)K and Akt negatively regulates Ig \( \kappa \)-chain rearrangement in a SLP-65-deficient pre-B cell line. Akt-mediated phosphorylation of Foxo proteins inhibits their transcriptional activity by promoting their cytoplasmic localization and degradation (Herzog et al., 2008). Another group found that tonic BCR signaling in immature B cells suppresses \( \text{Rag1}/\text{Rag2} \) expression through Akt-mediated phosphorylation and
inactivation of Foxo1, whereas binding of antigen to an autoreactive BCR leads to antigen-BCR internalization and blockage of this regulation (Amin and Schlissel, 2008). Based on this study, it was proposed that Foxo1 drives $\text{Rag1/Rag2}$ transcription by directly binding to sequences in the $\text{Rag1/Rag2}$ locus.

Taken together, these findings indicate that in developing B lymphocytes, Foxo proteins function downstream of the BCR signaling pathway as a molecular mediator that regulates $\text{Rag1/Rag2}$ transcription and thus BCR editing. Innocuous BCRs expressed on immature B cells signal through PI(3)K-Akt to phosphorylate and inactivate Foxo transcription factors, suppressing $\text{Rag1/Rag2}$ expression, whereas antigen engagement of autoreactive BCRs expressed on immature B cells initiates SLP-65 mediated signals that quench BCR-mediated tonic activation of PI(3)K-Akt and Foxo phosphorylation, which promotes nuclear accumulation of Foxo proteins to directly activate $\text{Rag1/Rag2}$ transcription (Rowh and Bassing, 2008).

$\text{Ca}^{2+}$ transport and signaling in B lymphocytes

Calcium (Ca$^{2+}$) is a second messenger with an important role in almost all cell types. In T cell, B cells, mast cells, and many other cell types, Ca$^{2+}$ signals are involved in numerous fundamental cellular processes and regulation of cell fate determination including proliferation, differentiation, apoptosis, and gene transcription (Lewis, 2007). After the engagement of immunoreceptors such as the BCR, TCR, and the Fc receptors on mast cells and NK cells, the intracellular concentration of calcium ions is increased. The concerted Ca$^{2+}$ mobilization initially from intracellular stores and subsequently from extracellular resources is essential to transmit signals from various surface receptors,
leading to specific cellular responses (Berridge et al., 2003). Depending on the developmental stage of the B cell, engagement of the BCR can deliver Ca\textsuperscript{2+} - regulated signals with diversified biological outcomes, i.e. clonal expansion, activation, differentiation, or cell apoptosis (Niiro and Clark, 2003). During the absence of antigen, the BCR on peripheral B cells provides a tonic maintenance or survival signal that also requires the presence of Ca\textsuperscript{2+} mobilizing proteins (Reth et al., 1986).

Several biochemical and genetic studies have identified BLNK as a critical upstream regulator of BCR signaling mediated Ca\textsuperscript{2+} mobilization (Jumaa et al., 1999). BLNK is a multidomain adapter protein. Two signaling features of BLNK are particularly important for the initiation of Ca\textsuperscript{2+} mobilization. First, tyrosine-phosphorylated BLNK recruits the key enzymes for Ca\textsuperscript{2+} elevation, Btk and PLC-\(\gamma\)2 into a complex via the enzyme’s SH2 domains (Fu et al., 1998). This complex allows phosphorylation of Btk, following the activation of PLC-\(\gamma\)2. Secondly, BLNK targets the Ca\textsuperscript{2+} initiation complex to the inner plasma membrane layer, providing PLC-\(\gamma\)2 with access to its substrate PIP\textsubscript{2} (Kohler et al., 2005). Activated PLC-\(\gamma\)2 at the plasma membrane hydrolyzes PIP\textsubscript{2} into DAG and soluble IP\textsubscript{3}. When B cells are stimulated, DAG recruits and activates several signal effectors like PKC-\(\beta\) and Ras guanine nucleotide release protein 3, which couple BCR activation to the classical NK-\(\kappa\)B and MAPK pathways separately. IP\textsubscript{3} has at least three receptors which are ubiquitously expressed ligand-gated channels of the ER membrane (Kurosaki et al., 2000).

The phases of Ca\textsuperscript{2+} mobilization have different profiles in each of the primary B-cell subsets. Immature transitional B cells in the spleen are new immigrants from the
bone marrow. These immature B cells that do not react with self-antigens become resistant to BCR-induced apoptosis and eventually develop into mature B cells (Su et al., 1999). Flow cytometry experiments have shown that BCR ligation triggers a distinct biphastic Ca\textsuperscript{2+} flux in both immature and mature B cells, which may contribute to the different biological responses of these cells to antigen encounter. Moreover, silencing of autoreactive B cells and maintenance of their unresponsiveness to BCR activation is associated with unusually high basal levels of intracellular Ca\textsuperscript{2+} (Gauld et al., 2005).

B cells have many ways to adjust the Ca\textsuperscript{2+} response proximal and distal to the BCR. Among them, protein tyrosine phosphatases act as negative regulators because of their critical role in the initiation of Ca\textsuperscript{2+} mobilization (Veillette et al., 2002). It has also been shown that BCR and the CD19 coreceptor working together strongly enhance the responses and lower the antigen threshold for B cell activation. Phosphorylation of the cytosolic adaptor protein Bam32 after BCR ligation augments Ca\textsuperscript{2+} mobilization also promotes endocytic BCR internalization (Niiro and Clark, 2003).

Collectively, BCR-induced Ca\textsuperscript{2+} signaling depends on the precise coordination of BCR-specific and ubiquitously expressed effector proteins. Both cytosolic and transmembrane adaptor proteins work together as signal organizers to ensure the activation of Ca\textsuperscript{2+} - sensitive downstream pathways, which ultimately determine appropriate cellular responses, including elimination of autoreactive B cells or proliferation and differentiation of immunocompetent B cells into antibody-secreting plasma cells.
Interaction of BCR with cognate antigen (Ag) initiates B cell mediated immune responses, followed by BCR-mediated internalization, processing and presentation of Ag-derived peptides to CD4⁺ T cells by major histocompatibility complex (MHC) class II molecules. Two important events are rapidly initiated when Ag engage to the BCR: a phosphorylation cascade that results in the production of secondary signaling intermediaries (Gold et al., 1991; Braun et al., 1979) and the internalization of Ag-BCR complexes. Both are required for normal humoral immune responses. Once Ag is bound, one of the earliest events during Ag processing in B cells is the internalization of the BCR. The precise mechanisms regulating BCR internalization remain exclusive, although a number of molecular mechanisms that could potentially play a role have been proposed.

Studies using anti-BCR antibodies (Ab) have documented that BCR signaling is required for BCR endocytosis and lipid rafts have been indicated as platforms for BCR function (Stoddart et al., 2000). The molecular mechanism connecting BCR signaling to endocytosis is defined as only when clathrin is associated with rafts and is tyrosine phosphorylated following BCR crosslinking that BCR internalization occurs. Therefore, lipid rafts spatially organize signaling cascades with clathrin to regulate BCR internalization (Salamero et al., 1995). Gene targeting has shown that the Igβ ITAM tyrosines modulate ligand-induced signaling by regulating BCR internalization. Although the roles of these ITAMs in BCR internalization is still controversial (Patterson et al., 2006), activation of PTKs is known to be required for BCR internalization. Other prior
work also suggests that Src kinase-mediated BCR signaling plays a critical role in BCR internalization (Pure and Tardelli, 1992).

Notably, a recent study (Putnam et al., 2003) using a well characterized murine cell line expressing a BCR specific for the hapten PC demonstrated that BCR-mediated internalization of cognate Ag occurs mostly through a lipid raft-independent mechanism. The results show that Src kinases and Syk-mediated BCR signaling are not essential for BCR-mediated Ag internalization. Moreover, different ligands could elicit distinctive internalization pathways, as shown by comparing anti-Ig Ab and Ag as BCR ligands. Compared to Ag-BCR internalization, the Ab-BCR complex is more dependent on signaling and highly relies on cholesterol and the actin cytoskeleton (Caballero et al., 2006). Using anti-IgM antibody as a ligand in a Lyn-knockout chicken B cell line provided evidence for Lyn-mediated BCR signaling induced BCR internalization (Ma et al., 2001). These results confirm that the nature of the ligands could be an important variable when analyzing BCR internalization, especially the relationship between signaling and endocytosis. Overall, this study suggests that the roles of early signaling and the internalization of BCR-ligands are variable depending on the interaction of the ligand and the BCR.

Meanwhile, another group (Hou et al., 2006) proposed that BCR signaling and internalization are actually mutually exclusive events. Their study provides direct evidence that once a BCR engages antigen it is either phosphorylated, retained on cell surface or it is internalized. This was demonstrated by using both biochemical and confocal microscopic techniques. The mutation experiment of Igα ITAM tyrosines
showed that multiple tyrosines within Ig\(\alpha\) are required for BCR internalization. An *in vitro* study using splenic B cells from BLNK\(^{-/-}\) mice also showed that phosphorylated BCRs are not internalized but retained on cell surface. More interestingly, only a small fraction of surface Ig\(\alpha\) was phosphorylated following BCR engagement. A mathematical model of the observed exclusive relationship between receptor internalization and phosphorylation has also been proposed. Using this method we have learned that the observed competition between receptor phosphorylation and internalization enhances signaling responses to low avidity ligands.

Another important player in BCR internalization is the B lymphocyte adaptor molecule of 32 kDa (Bam32). Upon BCR ligation, Bam32 is recruited to the plasma membrane where it associates with BCR complexes and redistributes and is internalized with BCRs (Dowler et al., 1999). BCR crosslinking induces colocalization of Bam32 with lipid rafts, clathrin, and actin filaments. Usage of a Src family PTKs inhibitor blocked both BCR-induced tyrosine phosphorylation of Bam32 and BCR internalization. Moreover, both BCR internalization and BCR-induced actin polymerization are impaired in Bam32\(^{-/-}\) cells. In summary, Bam32 functions downstream of Src family PTKs to regulate BCR internalization *via* an actin-dependent mechanism (Niiro et al., 2004).

The study presented in this dissertation explores the molecular regulation mechanism of V\(\text{H}\) gene replacement in human immature B cells and the biological significance of V\(\text{H}\) replacement in autoimmune diseases and anti-viral responses. The effects of BCR-internalization in BCR-mediated downstream signaling events were also analysed.
REGULATION OF VH REPLACEMENT IN HUMAN IMMATURE B CELLS BY B CELL RECEPTOR (BCR)-MEDIATED SIGNALING

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Abstract

V_{H} replacement provides a unique RAG-mediated recombination mechanism to edit unwanted IgH genes in mouse and human. Here, using the human EU12 µHC^{+} cells as an experimental model system, we show that crosslinking of the B cell antigen receptor (BCR) results in BCR internalization, cell proliferation arrest, and the induction of V_{H} replacement, as measured by elevated levels of double stranded DNA breaks at the V_{H1}, V_{H3} cryptic RSS borders and increased release of V_{H} replacement excision circles. Pretreatment of EU12 µHC^{+} cells with different protein tyrosine kinase inhibitors, Genistein, Syk II, Syk III, or PP1, blocks BCR-mediated signaling events and prevents BCR stimulation induced V_{H} replacement. Interestingly, BCR signaling mediated induction of V_{H} replacement can be further enhanced by pretreatment with PI3K inhibitors, but clocked by CD19 co-stimulation. Moreover, ongoing V_{H} replacement can be detected in the newly emigrated immature B cells from peripheral blood of healthy donors and tonsillar samples, which can be further enhanced by BCR stimulation. Sequence analyses show that the frequencies of V_{H} replacement products are significantly elevated in IgH genes derived from autoimmune diseases and in IgH genes encoding anti-viral antibodies, indicating a previously unrealized biological significance of V_{H} replacement in human diseases.
Introduction

The variable region exons of immunoglobulin (Ig) genes are assembled in developing B lineage cells by somatic DNA recombination processes to join the previously separated variable (V), diversity (D) (for heavy chain only), and joining (J) gene segments (Tonegawa, 1983; Rajewsky, 1996; Bassing et al., 2002). This random V(D)J recombination process mediated by the recombination activating gene products (RAG1 and RAG2) and is essential for the generation of a diversified antibody repertoire (Schatz et al., 1989; Oettinger, 1992). However, it also produces a large number of non functional Ig genes or Ig genes encoding autoreactive antibodies. These unwanted Ig genes can be edited by RAG-mediated secondary recombination, a process known as receptor editing (Tiegs et al., 1993; Gay et al., 1993). In addition, B cells expressing self-reactive B cell receptor (BCR) will be eliminated by clonal deletion or anergy to establish central tolerance (Nemazee and Weigert, 2000; Zhang, 2007; Wardemann et al., 2003).

Most of the previous works on receptor editing primarily focused on the Ig light chain genes. The organizations of the κ and λ light chain gene loci allow continuous secondary recombination by joining an upstream Vκ or Vλ gene with a downstream Jκ or Jλ gene, respectively, until there are no available VL or JL genes or the recombinases become inactive (Nemazee and Hogquist, 2003; Retter and Nemazee, 1998; Zhang, 2007). Light chain gene editing occurs frequently in mouse models. Using an engineered mouse with one κ allele marked with the human Cκ region, it was estimated that about 25% of the peripheral B cells have edited their κ chain genes (Wardemann et al., 2003). Upon stimulation through the BCR, up to 70% of murine immature B cells altered their
light chain genes (Melamed et al., 1998). Light chain gene editing is confined at the bone marrow early immature B cell stage, followed by strict negative selection (Sandel and Monroe, 1999).

Recent studies also suggest that antigen stimulation of immature B cells initiates signaling through BLNK, thereby promotes Foxo1 nuclear accumulation and RAG induction to facilitate receptor editing (Herzog et al., 2008). Conversely, mice deficient for Foxo1 have reduced levels of Rag expression and light chain recombination (Cadera et al., 2009).

V_{H} replacement occurs through RAG-mediated secondary recombination between a cryptic recombination signal sequence (cRSS) embedded within the frame work 3 region of a previously rearranged V_{H} gene and the 23bp recombination signal sequence (RSS) of an upstream V_{H} gene. The concept of V_{H} replacement was originally proposed to explain how murine pre-B leukemia cells are able to generate functional IgH genes from non-functional IgH rearrangements (Kleinfeld et al., 1986). Later experiments using different knock-in mouse models demonstrated that V_{H} replacement is used to edit IgH genes encoding anti-DNA antibodies (Chen et al., 1995); to change the knocked-in IgH gene encoding a monoclonal anti-NP antibody (Casellas et al., 2001); and to rescue B cell development in mice carrying two non-functional IgH rearrangements (Lutz et al., 2006; Koralov et al., 2006). Despite of these observations, evidence for V_{H} replacement in normal mice is still hard to find (Watson et al., 2006; Davila et al., 2007).

Ongoing V_{H} replacement has been observed in the developing human B lineage leukemia cell line EU12 and in human bone marrow immature B cells (Zhang et al.,
Purified RAG1 and RAG2 core proteins can bind to and cleave DNA substrates containing the cRSS motifs, providing the molecular basis that $V_H$ replacement occurs through RAG-mediated recombination. Sequence analyses for the presence of $V_H$ replacement footprints within D-J junctions of existing IgH genes have estimated that $V_H$ replacement products contribute to up to 5% of the primary human antibody repertoire (Zhang, 2007).

It is not clear how $V_H$ replacement is regulated. The studies of $V_H$ replacement in mouse models carrying knock-in IgH transgene encoding anti-DNA antibodies or IgH gene encoding anti-NP antibodies suggested that $V_H$ replacement could be induced upon antigen interaction with cell surface receptor (Chen et al., 1995; Cascalho et al., 1997). The occurrence stage of $V_H$ replacement in human bone marrow immature B cells also suggests that $V_H$ replacement might be regulated by BCR-mediated signaling (Zhang et al., 2003). Stimulation through the BCR leads to a wide range of biological responses, which depend on the developmental stage of the B cell and the properties of the antigen (Cambier et al., 1994; Goodnow et al., 1995). In a recent transgenic mouse study, change of BCR expression levels delayed B cell development and induced light chain receptor editing (Liu et al., 2009). We predict that engagement of the BCR on human immature B cells induces $V_H$ replacement. EU12 $\mu$HC$^+$ cells have many features as human bone marrow immature cells (Wang et al., 2003). Using EU12 $\mu$HC$^+$ cells as an experimental model system, we demonstrate that crosslinking BCR induces $V_H$ replacement and further dissect the responsible signaling events for regulation of $V_H$ replacement. Moreover, we find that ongoing $V_H$ replacement occurs in the newly emigrated immature B cells in the peripheral blood of healthy donors and in human tonsil, which is also
controlled by BCR-mediated signaling. Taken together, these results indicate that $V_H$
replacement is regulated by BCR-mediated signaling in human immature B cells.
Results

Crosslinking of BCR on EU12 μHC+ cells induces BCR internalization and inhibits cell proliferation

Our previous studies showed that V_{H1} replacement occurs spontaneously in human EU12 cells under normal tissue culture conditions (Zhang et al., 2003). The μHC+ cells in EU12 culture are phenotypically similar to human bone marrow immature B cells, which are IgM+CD10+CD24^{high}. As an initial step to test the BCR signaling capacity, we performed FACS analyses to determine if crosslinking of BCR induces Ca^{++} influx in the EU12 μHC+ cells. EU12 μHC+ cells were pre-loaded with Fluo-3 dye and stimulated with goat F(ab')_2 anti-IgM antibodies. Crosslinking of BCR results in elevated levels of intracellular Ca^{++}, which can be observed within 10 seconds after the addition of anti-IgM antibodies (Figure 1A). These results confirm that the BCRs on the EU12 μHC+ cells are capable of transducing signals.

When the parental EU12 cells are treated with the goat F(ab')_2 anti-IgM antibodies, the μHC+ subpopulation is diminished from the parental culture (Figure 1B, top panel). The loss of the surface IgM staining is not due to anti-IgM antibody masking the epitope, because anti-Igλ antibodies also failed to detect the μHC+ cells (Figure 1B, bottom panel). To determine if the disappearance of the EU12 μHC+ cells is due to cell death, we purified the EU12 μHC+ cells and treated them with the same amount of goat F(ab')_2 anti-IgM antibodies (2 μg/ml). After three days, the majority of the EU12 μHC+ cells are still viable, but lost their surface μHC and Igλ expression (Figure 1C).
Detailed analyses of the cell surface BCR expression showed that treatment of EU12 μHC⁺ cells with anti-IgM antibodies quickly induced BCR internalization, which could be observed as soon as after 1 minute of stimulation. After 30 minutes of BCR crosslinking, almost all of the EU12 μHC⁺ are negative for surface BCR expression (Figure 1D). The anti-IgM antibody mediated complete internalization of BCR on the EU12 μHC⁺ cells differs from that of mature B lineage cells, such as Daudi and Ramos, in which only a fraction of BCRs is internalized with the same treatment condition.

Cell cycle analysis further showed that crosslinking BCR on the EU12 μHC⁺ cells did not induce significant cell death. After two days of stimulation, only 20% of the cells under the anti-IgM antibody treatment were apoptotic as compared to 10% of the apoptotic cells in the control cultures (Figure 1E). Nevertheless, crosslinking of BCR on the EU12 μHC⁺ cells inhibits cell proliferation, which could be seen after two days of anti-IgM antibody treatment. On day 3, the EU12 μHC⁺ cells in the anti-IgM treatment experiment began to die (Figure 1F).

*Crosslinking of BCR induces V_h replacement in the EU12 μHC⁺ cells*

Next, we test whether crosslinking of BCR affects V_h replacement in the EU12 μHC⁺ cells. EU12 μHC⁺ cells (1 × 10⁶ cells/ml) were cultured without or with goat F(ab’)₂ anti-IgM antibodies (2 μg/ml) for three days. Genomic DNA was prepared and subjected to semi-quantitative ligation mediated (LM)-PCR to monitor double-stranded (DS) DNA breaks at the V_h₃ cRSS boarders as an indication of V_h replacement (Figure 2A). Crosslinking of BCR resulted in an accumulation of DS DNA breaks at the V_h₃ cRSS sites, indicating the induction of V_h replacement (Figure 2B). DNA sequence
analyses of the LM-PCR products confirm that the DS DNA breaks occur at the VH3-7 cRSS sites (data not shown). As we showed previously, the majority of the EU12 µHC+ cells express IgH genes with different VH3-7DJH4 rearrangements (Wang et al., 2003). Therefore the induced VH replacement is targeting the functionally expressed VH3-7 genes. Indeed, the results of two independent experiments showed that crosslinking of BCR on the EU12 µHC+ cells induced an accumulation of VH1 to VH3 replacement excision circles, in which the upstream VH1-8 gene has replaced the preexisting VH3-7 rearrangement (Figure 2C).

To accurately measure the relative levels of DS DNA breaks at the VH cRSS borders, we developed a real time LM-PCR assay following published protocols (Curry et al., 2005) (Figure 2D). First, we test the feasibility of this modified assay. Ligation of genomic DNA samples with biotin-labeled DNA linkers enriched the double-stranded DNA breaks almost 70 fold at the VH1 cRSS, and nearly 30 fold at the VH3 cRSS borders comparing to controls with no ligase added (Figure 2E). In our treated samples, crosslinking of BCR on the EU12 µHC+ cells resulted in a 14 to 15 fold induction of DS DNA breaks at the VH1 or VH3 cRSS sites as measured by the real time LM-PCR (Figure 2F). These results are consistent with our semi-quantitative LM-PCR studies and confirm that crosslinking of BCR induces VH replacement in the EU12 µHC+ cells.

*Blocking BCR-mediated signaling events prevents anti-IgM antibody induced VH replacement in the EU12 µHC+ cells*

Signaling from the BCR is mediated through a cascade of protein tyrosine phosphorylation events, which can be blocked by different protein kinase inhibitors.
Pretreatment of EU12 µHC⁺ cells with a general protein tyrosine kinase inhibitor, Genistein, prevents cellular protein tyrosine phosphorylation triggered by BCR stimulation. Specifically, it reduces BCR signaling induced phosphorylation of Erk, Akt, and Foxo1 (Figure 3A). Convincingly, treatment of EU12 µHC⁺ cells with Genistein ablated anti-IgM antibody induced V₄ replacement, as measured by LM-PCR detecting DS DNA breaks at the V₄ cRSS borders (Figure 3A). The reduction of DS DNA breaks at the V₄ cRSS site is not due to Genistein-mediated cell toxicity, because treatment of EU12 µHC⁺ cells with Genistein for 24 hours did not result in a considerable change in cell viability.

Similarly, pretreatment of EU12 µHC⁺ cells with Syk kinase inhibitors, Syk II and Syk III, or Src kinase inhibitor, PP1, blocks BCR signaling induces global protein tyrosine phosphorylation and prevents anti-IgM antibody induced V₄ replacement in the EU12 µHC⁺ cells (Figure 3A). Pretreatment of EU12 µHC⁺ cells with Syk II, Syk III, or PP1 all inhibits BCR signaling induced activation of the Akt pathway and prevents Foxo1 phosphorylation. However, the three drugs have different effects on the MAP kinase pathway. Pretreatment of cells with Syk II inhibits Erk phosphorylation, but the use of Syk III induces Erk phosphorylation in both controls and anti-IgM antibody treated EU12 µHC⁺ cells.

We next investigated which downstream factor might be responsible for BCR signaling induced V₄ replacement in the EU12 µHC⁺ cells. We specifically analyzed the effects of the PI3K pathways using PI3K kinase inhibitor LY294002. Interestingly, blocking PI3K promotes V₄ replacement (Figure 3B). To further explore the effects of
PI3K activation on $V_H$ replacement, we treated EU12 $\mu$HC$^+$ cells with anti-CD19 monoclonal antibodies. Treatment of EU12 $\mu$HC$^+$ cells with anti-CD19 antibodies completely inhibits BCR stimulation induced $V_H$ replacement (Figure 3C).

Taken together, these results confirm that $V_H$ replacement in the EU12 $\mu$HC$^+$ cells is induced by BCR-mediated signaling. Activation of PI3K pathway seems negatively regulates $V_H$ replacement in the EU12 $\mu$HC$^+$ cells.

$V_H$ replacement occurs in the newly emigrated immature B cells from peripheral blood and is regulated by BCR stimulation

Our previous studies showed that $V_H$ replacement occurs in human bone marrow immature B cells (Zhang et al., 2003). A small fraction of IgM$^-$CD27$^+$CD10$^+$ B cells in the peripheral blood has similar features as their bone marrow immature B cell counterparts in which these cells re-express RAG1/2 genes and are capable of editing their light chain genes. To determine if $V_H$ replacement occurs in peripheral immature B cells, we purified newly emigrated immature B cells (IgM$^+$CD27$^+$CD10$^+$) and naïve mature B cells (IgM$^+$CD27$^-$CD10$^+$) from peripheral blood of healthy donors (Figure 4A). With nested primer PCR, we are able to detect LM-PCR products corresponding to the DS DNA breaks at the $V_{H3}$ cRSS borders in the newly emigrated immature B cells but not in the naïve mature B cells of six analyzed healthy donors (Figure 4B). DNA sequences of the LM-PCR products confirm that these DS DNA breaks occurred at $V_{H3-9}$, $V_{H3-11}$ or $V_{H3-13}$ cRSS sites (Figure 4C). These results show that $V_H$ replacement occurs naturally in newly emigrated immature B cells in the peripheral blood of healthy donors.
Next, we tested if BCR signaling affects $V_H$ replacement in primary immature B cells. Purified newly emigrated immature B cells (3000 cells) were seeded in 100 µl media in 96 well plate without or with goat F(\text{ab}'\text{)}_2\text{ anti-IgM antibodies (2 µg/ml). Naïve mature B cells were used as controls. After 24 hours, genomic DNA was purified and analyzed by LM-PCR to detect DS DNA breaks at the $V_{H3}$ cRSS borders. Anti-IgM antibody treatment induced $V_{H3}$ cRSS DS DNA breaks in the primary immature B cells but not in the naïve mature B cells (Figure 4D). Thus, $V_H$ replacement in primary immature B cells can be enhanced through BCR stimulation.

$V_H$ replacement occurs in tonsillar immature B cells

Human tonsil contains a large number of B lineage cells. Among them, a subpopulation of CD24$^{\text{high}}$IgM$^+$ B cells also has similar features as the bone marrow immature B cells (Sims et al., 2005) (Figure 5A). Using LM-PCR approach, we were able to detect DS DNA breaks at the $V_{H1}$ cRSS and the $V_{H3}$ cRSS sites in tonsillar immature B cells (CD24$^{\text{high}}$IgM$^+$), but not in the CD24$^{\text{low}}$IgM$^+$ mature B cells (Figure 5B). DNA sequence analysis of the LM-PCR products confirms that $V_H$ replacement occurs at the $V_{H3-9}$, $V_{H3-23}$, and $V_{H3-64}$ cRSS sites (Figure 5C). Furthermore, treatment of tonsillar immature B cells with anti-IgM antibodies also induces DS DNA breaks at the $V_{H3}$ cRSS sites (Figure 5D), indicating that $V_H$ replacement in tonsillar immature B cells can be further enhanced by BCR stimulation, same as that in the newly emigrated immature B cells in the peripheral blood.
Elevated frequencies of $V_H$ replacement products in IgH genes derived from autoimmune diseases and in IgH genes encoding anti-viral antibodies

Our previous analyses of IgH genes derived from healthy donors revealed that $V_H$ replacement products contribute to the diversification of human antibody repertoire (Zhang et al., 2003). To explore the biological significance of $V_H$ replacement, we analyzed 14,358 IgH sequences from the IMGT database. The overall frequency of $V_H$ replacement products in these IgH sequences is 10%, which is significantly higher than what has been found in healthy donors. We speculate that a large number of IgH sequences deposited at the IMGT database is derived from diseased subjects. Indeed, detailed analyses show that the frequencies of $V_H$ replacement products are significantly elevated in IgH genes derived from different autoimmune subjects, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and Sjögren’s syndrome (Figure 6A). Interestingly, the frequencies of $V_H$ replacement products are also significantly elevated in IgH genes derived from patients with chronic viral infection, such as EBV infection, or IgH genes encoding anti-viral antibodies, such as anti-CMV antibodies, anti-hepatitis C E2 antigen antibodies, anti-HIV antibodies, and anti-hepatitis B surface antigen antibodies (Figure 6B). In contrast, the frequency of $V_H$ replacement products is very low in a group of IgH genes encoding anti-streptococcus pneumonia antibodies (Figure 6C). These results reveal a previously unrealized contribution of $V_H$ replacement to autoimmune diseases and anti-viral response in humans.
Discussion

Receptor editing provides an important mechanism to change unwanted Ig genes through RAG-mediated secondary rearrangements. It has been well documented that murine immature B cells have the capacity to re-induce RAG gene expression and to edit Ig light chain genes upon BCR stimulation (Nemazee, 2000). Our previous studies showed that $V_H$ replacement occurs in human bone marrow immature B cells, which should be considered as part of the receptor editing process to change unwanted IgH genes. Here, we show that $V_H$ replacement in human immature B cells is regulated by BCR-mediated signaling events.

Human EU12 cells undergo spontaneous differentiation from pro-B cell to the pre-B cell and to immature B cell stages (Wang et al., 2003). The $\mu$HC$^+$ subpopulation of EU12 cells has similar features as human bone marrow immature B cells. Using the EU12 $\mu$HC$^+$ cells as an experimental model system, we found that crosslinking of BCR induces BCR internalization and cell proliferation arrest. The lacking of significant induction of apoptosis upon BCR crosslinking could be due to the immortalization nature of this leukemia cell line. As expected, BCR crosslinking strongly induces RAG-mediated double stranded DNA breaks at the $V_{H3}$ cRSS boarders and the release of $V_{H1}$ to $V_{H3}$ replacement excision circles. Analyses of the DNA sequences of the LM-PCR products showed that the $V_{H3}$ cRSS breaks targeted the $V_{H3-7}$ genes, which is one of the dominant $V_H$ genes expressed in the EU12 $\mu$HC$^+$ culture. Thus, crosslinking of BCR induces $V_H$ replacement targeting the functionally expressed IgH genes in the EU12 $\mu$HC$^+$ cells.
Crosslinking of BCR induces a cascade of protein tyrosine phosphorylation events (Gazumyan et al., 2006; Gold and DeFranco, 1994). Using different protein tyrosine kinase inhibitors, we further dissected BCR-mediated signaling pathways that might be responsible for the induction of $V_H$ replacement in the EU12 $\mu$HC$^+$ cells. Upon BCR engagement, Syk kinase is quickly recruited to the membrane proximity of BCR, which initiates the phosphorylation of other signaling components (Tsang et al., 2008). Indeed, blocking Syk kinase activity by global protein tyrosine kinase inhibitor Genistein or by specific Syk kinase inhibitors, Syk II or Syk III, prevents anti-IgM antibody induced $V_H$ replacement in the EU12 $\mu$HC$^+$ cells. Moreover, pre-treatment of EU12 $\mu$HC$^+$ cells with Src kinase inhibitor, PP1, also prevents anti-IgM antibody induced $V_H$ replacement. These results provide the first evidence that $V_H$ replacement in the EU12 $\mu$HC$^+$ cells is regulated by BCR-mediated signaling. Comparison of the different effects of these inhibitors on BCR-mediated signaling events provide additional information for downstream BCR signaling pathways that might be responsible for the induction of $V_H$ replacement. For example, Genistein and Syk II treatments inhibit BCR-induced Erk phosphorylation, while Syk III treatment strongly induces Erk phosphorylation. These results suggest that activation of Erk kinase alone is not able to induce $V_H$ replacement. On the other hand, recent studies in murine pre B cells showed that elevated expression of FOXO1 can induce Rag gene expression and thus induces Igκ gene recombination (Amin and Schlissel, 2008). In our studies, crosslinking of BCR induces Akt and FOXO1 phosphorylation, which can be effectively blocked by Genistein, SykII, SykIII and PP1 treatments. FOXO1 has been suggested to be involved in light chain receptor editing, however it is still yet to determine if FOXO1 is involved in $V_H$ replacement regulation in
the EU12 μHC+ cells. Interestingly, blocking of PI3K activation enhances BCR induced V_H replacement, conversely, activation of PI3K through crosslinking of CD19 inhibits BCR induced V_H replacement. These results are consistent with the observations of CD19 function in light chain editing and indicate that activation of the PI3K negatively regulates V_H replacement.

We have previously shown that V_H replacement occurs in human bone marrow immature B cells. A subset of newly emigrated immature B cells in peripheral blood has similar features as bone marrow immature B cells. Using LM-PCR approach, we were able to detect ds DNA breaks at the V_H3 cRSS boarders in the newly emigrated immature B cells from all the analyzed healthy donors. Similarly, double stranded DNA breaks corresponding to the V_H3 cRSS sites can be detected in tonsillar immature B cells. These results are consistent with our previous observation that V_H replacement naturally occurs in primary immature B cells. Moreover, the ongoing V_H replacement in primary immature B cells can be further enhanced by anti-IgM antibody stimulation. Thus, we conclude that immature B cells have the capacities to change both Ig heavy and light chain genes upon BCR stimulation.

The successful detection of RAG-mediated double stranded DNA breaks at the V_H3 cRSS sites in our study is slightly different from previously reported studies analyzing V_H3 cRSS DS DNA breaks in murine B cell subsets. From our experiences, detection of double-stranded DNA breaks at the cRSS boarders by LM-PCR is dependent on sufficient number of immature B cells and the complete digestion of nuclear proteins with proteinase. It should also be mentioned that the level of RAG protein expression in
the EU12 μHC⁺ cells is not changed upon BCR stimulation, which might be an abnormal feature of the EU12 leukemia cell line.

Having evidence that V₉ replacement occurs in the peripheral immature B cells, we further explored the potential contribution of V₉ replacement in human diseases. Due to the location of the cRSS motif, V₉ replacement recombination could leave a stretch of nucleotides from the previously rearranged V₉ gene into the newly formed IgH gene at the D-J junction. Although it is hard to be experimentally proven, the existence of such V₉ replacement “footprints” provides the only clue for the occurrence of V₉ replacement in human IgH repertoire. Using this method, we have estimated that V₉ replacement products contribute to about 5% of the normal B cell repertoire. Here, analyses of 14,358 IgH sequences from the IMGT database revealed that the frequencies of V₉ replacement products are significantly higher in IgH genes derived from two large categories, namely, autoimmune diseases and anti-viral responses. We speculate that prolonged exposure of immature B cells to antigens, either self antigens or viral antigens, might induce V₉ replacement and the enrichment of V₉ replacement products in autoimmune diseases or chronic viral infection, suggest a potentially significant contribution of V₉ replacement to generate autoreactive or anti-viral antibodies.

Taken together, these results showed that V₉ replacement in human immature B cells is regulated by BCR-mediated signaling events and the elevated frequencies of V₉ replacement products in autoimmune diseases and anti-viral responses suggested a previously unrecognized contribution of V₉ replacement to human diseases.
Experimental Procedures

Antibodies and Reagents

Phycoerythrin (PE) conjugated anti-CD34 antibodies (Pool) were purchased from IMMUNOTECH (France); Cy5-conjugated AffiniPure Donkey Anti-Human IgM F(ab’)2 Fragments were purchased from Jackson ImmunoResearch (West Grove, PA); Fluorescein (FITC) conjugated goat F(ab’)2 anti-Human lambda (λ chain specific) antibodies, Goat anti-Human IgM F(ab’)2 (µ chain specific, LE/AF) were purchased from Southern Biotech (Birmingham, AL); Anti-CD10-FITC, anti-CD27-PE, and anti-CD24-PE were from Becton Dickinson PharMingen (San Diego, CA);

Cell culture and treatment

The human B lineage EU12 cells and the EU12 µHC+ subpopulation were maintained in RPMI medium with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, 50 µM β-mercaptoethanol and were routinely monitored for cell surface µHC, Igλ, and CD34 expression by FACS. The EU12 µHC+ cells were purified from the EU12 parental cells by FACS sorting on a MoFlow cell sorter (Cytomation, Fort Collin, CO). For anti-IgM antibody treatment, 1× 10^6 µHC+ cells were seeded in 1 ml complete RPMI1640 media alone or supplemented with Goat F(ab’)2 anti-human IgM antibodies at 2 µg/ml for various time period. After treatment, cells were washed once with 1X PBS, collected and subjected to FACS, LM-PCR, excision circle PCR, real time LM-PCR, or Western blot analyses. When specified, 10 × 10^6 µHC+ EU12 cells were pretreated with 50 µg/ml genistein (Sigma-Aldrich), 10 µM Syk
inhibitor II (2-(2-Aminoethylamino)-4-(3-trifluoromethylanilino)-pyrimidine-5-carboxamide, Dihydrochloride, Dihydrate; 574712; Calbiochem), 10 µM Syk inhibitor III (3,4-Methylenedioxy-β-nitrostyrene; 574713; Calbiochem), 10 µM PP1 (EI275-0001; Biomol International), 10 µM LY294002 (PI3 Kinase inhibitor), 10 µM Akt VIII and 10 µM MEK1/2 U0126 for 30 min prior to anti-IgM antibody stimulation. For mock treatment, the equivalent amount of dimethyl sulfoxide (DMSO) was used.

**Ca++ influx Assays**

Ca++ influx assays were performed using the Fluo-3AM fluorescence dye following the manufacture’s suggestion. Briefly, EU12 µHC+ cells were washed once with RPMI media with 1% FBS once before resuspension at a concentration of 10^6 cells/ml and incubation with Fluo-3AM (1mM) /Pluronic® F-127 at 1:1 ratio (Molecular Probes, Invitrogen) in the dark at 37°C for 30 min. After incubation, the fluorochrome-labeled cells were analyzed on a C6 Flow Cytometer (Accuri Cytometers Inc., MI). Various dosage of goat F(ab’)2 anti-IgM antibodies (2 µg, 5 µg and 10 µg/ml) were added directly into each sample tube at 30 sec after collecting events. FACS data were analyzed by using the CFow software package (Accuri Cytometers Inc., MI)

**Cell cycle analysis**

EU12 µHC+ Cells (1 × 10^6/ml) were treated as described above. After stimulation, cells were pelleted and resuspended in 1 ml of ice-cold 70% ethanol and fixed for 1 h at 4°C. Cells were then washed twice with cold PBS and treated with RNase (final concentration 0.5 µg/ml) for 30 min at 37°C. DNA was stained with PI (10µg/ml, Sigma)
for 30 min at 37°C in dark. DNA content was analyzed on a FACS Calibur. FACS results were analyzed by the Win MDI2.8 software (Stanford University, CA).

Detection of RAG-mediated double stranded DNA breaks at the VH cRSS sites by LM-PCR and real time LM-PCR

To investigate if crosslinking BCR affects V_{H} replacement, 1 × 10^6 purified EU12 µHC^+ cells were incubated with goat F(ab')2 anti-IgM (2 µg/mL) for 24 hr. Genomic DNA was isolated using standard proteinase K digestion followed by phenol/chloroform extraction method. For LM-PCR, 1 µg DNA sample was ligated with 20 pmol of the re-annealed double-stranded (ds) DNA linker at 16°C. Equal volume of stopping solution was added to terminate the ligation reaction at 95°C for 15 min. To detect ds breaks at V_{H3} cRSS site, serial 5 fold diluted treated and control ligation samples were used as templates in two rounds of semi quantitative LM-PCR. For the first round, 5 µl of total ligation samples was used as template and amplified with sense primer V_{H3} together with an antisense primer LinkcRSS1. For the second round PCR, 2 µl of the first round PCR products was used as template and amplified with an internal V_{H3n} sense primer and the linkeRSS2 antisense primer. The conditions for both LM-PCR reactions are performed as described (Zhang et al., 2003). Second round PCR products (10 µl) were separated on 2% agarose gel and visualized after ethidium bromide-staining. Positive PCR products were subcloned into PCRII vector and sequenced to verify the location of double-stranded DNA breaks.

Real-time LM-PCR assay was performed essentially as described (Curry et al., 2005). Double-stranded (ds) DNA linkers were labeled with biotin-dCTP by Klenow fill-
in reaction. Genomic DNA from treated or control EU12 µHC+ cells was ligated to biotin labeled DS linker overnight at 14°C and a mock ligation with no T4 ligase was used as a negative control. The ligation samples were subjected to BamHI / HindIII (5 units per sample) digestion at 37°C for 2 hr. Biotin-tagged DNA was enriched by using Streptavidin conjugated magnetic beads (Promega). After washing twice with 1 ml of 1X TE buffer (pH 8.0), the enriched DNA samples were eluted by incubation at 75°C for 15 min. After centrifugation at 13,000g for 10 min, the supernatant was subjected to real time PCR analyses using the V_{H1} and V_{H3} sense primers, which are derived from the V_{H} framework 2 regions, and the LinkeRSS2 primers. Real time PCR was performed on an ABI PRISM® 7900HT Sequence Detection System using the SYBR GREEN PCR Master Mix (Applied Biosystems) with cycling conditions suggested by the manufacturers (Applied Biosystems). The values obtained for double-stranded DNA breaks were normalized to unligated GAPDH Genomic DNA.

Detection of V_{H} replacement excision circles

V_{H} replacement excision circle PCR assay was performed as previously described (Zhang et al., 2003). Briefly, cellular DNA was extracted from 1 × 10^6 EU12 µHC+ cells untreated or treated with Goat F(ab’)2 anti-IgM antibodies (2 µg/ml) separately. Two rounds of PCR amplification with seminested primer sets were performed. The second round PCR products (10 µl) were separated on 2% agarose gel electrophoresis and visualized by EtBr staining. The primer sequences are listed in Supplementary Table 1.
The effects of preincubation with BCR signaling inhibitors followed by cross-linking BCR signaling on $V_H$ replacement were investigated as following description. Prior to 30 min incubation with different inhibitors followed by 5 min stimulation with Goat F(ab’)$_2$ anti-IgM (2 µg/ml), $10 \times 10^6$ cell aliquots were washed twice with cold PBS (Gibco) and incubated for 2 hr in 1 × GIBCO Opti-MEM I Reduced-Serum Medium (Invitrogen). Western blotting was performed as described (Ehrhardt et al., 1999). Briefly, cells were harvested, washed with cold PBS once and spun down before lysed by lysis buffer containing 1% Nonidet P-40, 50 mM Tris.HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, and the addition of 1 tablet of protease inhibitor, PMSF (40 µg/ml), and phosphatase inhibitor Na$_3$VO$_4$ (0.2 mM), Na$_2$MoO$_4$ (1 mM), and β-glycero-phosphate (5 mM). Whole cell lysate proteins were quantitated by using the BCA protein assay reagent (Thermo fisher Scientific). Collected lysates were boiled at 95°C for 5 min in 5X loading buffer. Equal amount of proteins were resolved by 10% SDS / PAGE (Bio Rad) before transfer to nitrocellulose membranes (Amersham Biosciences), then were probed with rabbit anti-phospho-Syk (#2170; Cell signaling Technology), anti-phospho-Akt (Ser 473) (#9916), anti-Akt (pan) (#4691), anti-phospho-PI3K p85(Tyr458)/p55(Tyr199) (#4228), anti-PI3 Kinase p85 (19H8) (#4257), anti-phospho-FoxO1 (Ser356) (#9461), anti-FoxO1 (C29H4) (#2880), anti-phospho-p38 MAP Kinase (Thr180/Tyr182) (#9211), anti-p38 MAP Kinase (#9212), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#9101), anti-p44/42 MAPK (Erk1/2) (#9102), anti-phospho-SAPK/JNK (Thr183/Tyr185) (#9251), anti-SAPK/JNK (#9252) antibodies all from Cell signaling Technology (Beverley, MA) or mouse anti-Syk (6D784; sc-73088; Santa Cruz Biotechnology). Monoclonal antibody to
β-actin (AC-15; Sigma) and monoclonal antibody to phosphorylated tyrosine (4G10; upstate) were also used. AlphaView Imaging software (Alpha Innotech) was used for gel densitometry analysis. The above experiments were also performed in parallel with collected genomic DNA subjected to LM-PCR, excision circle PCR and real time LM-PCR assays.

*Purification of primary immature B cells from human PBMC and tonsils*

All study protocols involving human samples were approved by the institutional review board (IRB) at University of Alabama at Birmingham (UAB) and University of Nebraska Medical Center (UNMC). Peripheral blood samples (10 - 20 ml) were obtained from healthy donors with informed consent. Mononuclear cells (PBMCs) were isolated using density gradient centrifugation with Lymphocyte Separation Medium (Mediatech, Herndon, VA).

The newly emigrant immature B cells as CD19⁺ IgM⁺CD27⁻CD10⁺ cells and mature naïve B cells as CD19⁺ IgM⁺CD27⁻CD10⁻ were purified by FACS sorting on Dako Cytomation MoFlo (Fort Collins, CO). Post-sort purity check was applied to the newly sorted B-cell subpopulations on FACScalibur (BD Bioscience).

Roughly same number of sorted human immature and mature B cells was seeded in 24 well plate with medium alone or addition of anti-IgM antibodies (2 µg/ml). Cells were collected and genomic DNA was subjected to LM-PCR as described above after 24 hr. Selected positive LM-PCR products were sent to sequence to confirm the location of
the double-stranded DNA breaks. This experiment has been done more than 6 times (6 donors).

Tonsillar lymphocytes were prepared by tissue mincing and filtration through a 70-mm wire mesh and density gradient centrifugation. Tonsillar Immature B cells (IgM⁺CD24⁺) cells and mature B cells (IgM⁺CD24⁻) were purified on a Moflo Sorter. Sorted cells purity was checked by FACS calibur (BD).

Roughly same number of sorted tonsillar immature and naïve mature B cells was seeded into 24 well plates with 100 µl medium alone or presence of anti-IgM antibodies (2 µg/ml). After 24 hr, cells were collected and genomic DNA was then subjected to LM-PCR assay. This experiment has been done for more than 3 times.
Acknowledgments

The authors would like to thank Drs. Larry Gartland (UAB), Marion Spells (UAB) for helping with cell sorting and FACS analysis. ZZ is supported in part by NIH grants AI074948, AI076475, and AI073174.


Figure 1, Liu
Figure 1. EU12 μHC\(^+\) cells are sensitive to BCR crosslinking. (A) Crosslinking BCR induces Ca\(^{2+}\) influx in EU12 μHC\(^+\) cells. Cells were loaded with 1 μM Fluo-3 AM dye and with 0, 2, or 10 μg/ml goat F(ab')\(_2\) anti-IgM antibodies. (B) Crosslinking BCR with anti-IgM antibodies (2 μg/ml) eliminates μHC\(^+\) cells in the EU12 culture. (C) Treatment of purified EU12 μHC\(^+\) cells with anti-IgM antibodies induces internalization of BCR. (D) Crosslinking BCR induces quick BCR internalization. EU12 μHC\(^+\) cells were stimulated at 37°C with goat F(ab')\(_2\) anti-human IgM (2 μg/ml) for 0 min, 1 min, 5 min, 30 min, 1 h and 4 h. Cell surface BCR expression was monitored by FACS. (E) BCR crosslinking induces cell cycle arrest in EU12 μHC\(^+\) cells. EU12 μHC\(^+\) cells (1 × 10\(^6\)/ml) were cultured for the indicated time period in the presence of goat F(ab')\(_2\) anti-IgM antibodies (2 μg/ml) and DNA content was analyzed by FACS. The hypodiploid peak, indicative of apoptotic cells, was analyzed using Win MDI2.8 software and showed as the percentage of total cells. (F) Live cell number counts after anti-IgM antibody treatment. EU12 μHC\(^+\) Cells (1 × 10\(^6\)) were cultured with goat F(ab')\(_2\) anti-IgM antibodies (2 μg/ml) for 3 days. Results represented are mean value with standard deviation from three independently performed experiments.
Figure 2, Liu

A

B
Control Anti-IgM

C
Control Anti-IgM Control Anti-IgM

D

E

F

Relative level
Ligation - + - +
V_{H1} V_{H3}

Fold induction
Anti-IgM - + - +
V_{H1} V_{H3}
Figure 2. BCR crosslinking induces V_H replacement in the EU12 µHC^+ cells. (A) Diagram shows the V_H replacement process in EU12 cells. Double-stranded DNA breaks and excision circles can be used as indication of the V_H replacement occurrence. (B) LM-PCR detection of RAG-mediated double-stranded DNA breaks at V_H3 cRSS site after treatment without or with goat F(ab’)2 anti-IgM antibodies (2 µg/ml). Serial 5 fold diluted DNA templates were used. (C) Nested primer PCR detection of V_H3 to V_H1 excision circles. The CD19 promoter region was amplified by PCR as a control for DNA input. All PCR products were sequenced with V_H specific primers. These experiments have been repeated for more than 3 times. (D) Diagram shows using biotin labeled double-stranded DNA linkers to enrich double stranded DNA breaks. (E) Detection of enriched double-stranded DNA breaks at V_H1 and V_H3 cRSS sites with biotin labeled double-stranded DNA linker using real-time LM-PCR approach. The + or – symbols indicate the presence or absence of T4 DNA ligase in the ligation reaction, respectively. (F) Treatment of EU12 µHC^+ cells with anti-IgM antibodies induces double-stranded DNA breaks at the V_H1 and V_H3 cRSS sites. Untreated and treated EU12 µHC^+ cell genomic DNA samples were ligated to biotin-labeled ds linkers as indicated in the diagram (D) and subjected to real time LM-PCR assays. Results shown are mean values from triplicated reactions.
Figure 3, Liu

A

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LM-PCR

- V₁₅₅ cRSS
- CD19
Figure 3, Liu

B

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- Anti-IgM
- p-Syk
- Syk
- p-Erk
- Erk
- p-PI3K
- p85
- p-Akt
- Akt
- p-FoxO1
- FoxO1
- β-actin

C

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- Anti-IgM
- V_H3 cRSS
- CD19

LM-PCR

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Figure 3. Treatment of EU12 cells with protein tyrosine kinase inhibitors blocks BCR signaling and inhibits anti-IgM antibody induced V_{H} replacement in EU12 μHC^{+} cells. For Western blot, EU12 μHC^{+} cells (10 x 10^6/ml) were stimulated with goat F(ab’)_2 anti-IgM antibodies (2 μg/ml) for 3 min in the presence of medium alone, DMSO, Genestein (5 μM), Syk II (5 μM) or Syk III (5 μM), PP1 (5 μM) and LY294002 (5 μM). Cells were lysed and analyzed by SDS-PAGE and Immunoblot. Blots were probed with specific antibodies for the indicated BCR signaling components. β-actin serves as a loading control (A, B top). For LM-PCR analysis, EU12 μHC^{+} cells were treated under the same condition as described above for 24 h. Genomic DNA was isolated and used as template for LM-PCR analysis. The CD19 genomic DNA was amplified as a loading control. Results are representatives of at least three independent experiments.
Figure 4, Liu

A

B

C

D

VH3-9 ATACTGTGGAATTCTAGATCTCCTAGATACTGTGGAATTCTAGATCTCCTAGA
VH3-11 TGAGAGCCGAGGACACGGCCGTGATTACTGTGGAATTCTAGATCTCCAAGC
VH3-13 TGAGAGCCGAGGACATGGCTGTGATTACTGTGGAATTCTAGATCTCCTAAG

n i n i n i n

Anti-IgM

VH3 cRSS

GAPDH
Figure 4. Occurrence and regulation of $V_H$ replacement in the newly emigrated human immature B cells. (A) Identification and purification of newly emigrated immature B cells (i, IgM⁺CD27⁻CD10⁺) and mature naïve B cells (m, IgM⁺CD27⁻CD10⁻) from peripheral blood of health donors. (B) Detection of double-stranded DNA breaks at the $V_H3$ cRSS sites by LM-PCR in the newly emigrated immature B cells. (C) DNA sequences of the LM-PCR products confirmed that the double-stranded DNA breaks occur at $V_H3$-9, $V_H3$-11 or $V_H3$-13 cRSS sites. (D) Crosslinking BCR induces $V_H$ replacement in human newly emigrated immature B cells but not in mature naïve B cells. Genomic DNA was extracted and ligated with double-stranded DNA linker overnight. The ligation product was being used as template for ligation-mediated PCR assay. In both studies, two rounds of LM-PCR were performed (total 60 cycles). GAPDH housekeeping gene was used as the control for cell-sorting DNA yield (40 cycles). i, immature B cells, n, naïve mature B cells. These experiments have been repeated for more than 6 times.
Figure 5, Liu

A

B

C

VH3-9  TGAGAGCTGAGGACACGGCTTTGTCTATCTGTGAATTCAAGATCTCTTAAG
VH3-23  CAGCATTGGATCTGCGTGGGACACCTACTGTGAATTCAAGATCTCTTAAG
VH3-64  TGAGAGCTGAGGACATGGCTGTATCTGTGAATTCAAGATCTCTTAAG

D

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Anti-IgM

VH3 cRSS

GAPDH
Figure 5. BCR crosslinking induces $V_H$ replacement in tonsillar immature B cells.

(A) Purification of tonsillar B cell subpopulations by FACS. Tonsillar Immature B cells (i, CD24$^{hi}$IgM$^+$) and (m, CD24$^+$ IgM$^+$) mature naïve B cells. (B) LM-PCR detection of double-stranded DNA breaks as indication of $V_H$ replacement events at both $V_{H1}$ and $V_{H3}$ cRSS sites. (C) After BCR cross-linking, $V_H$ replacement is being induced in tonsillar immature B cells but not in mature B cells. (D) Sequence analysis of the LM-PCR products confirmed the occurrence and induction of double-stranded DNA breaks at the $V_{H3}$ cRSS sites. The boxes indicate cRSS heptamers. Linker primer sequences are underlined. Representative results from three independently performed experiments are shown.
Figure 6, Liu

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Figure 6. Potential biological significance of $V_H$ replacement in autoimmune diseases or anti-viral responses. Frequencies of $V_H$ replacement products in IgH sequences derived from autoimmune diseases (A), in IgH genes encoding anti-viral antibodies or derived from viral infections (B), and in IgH genes encoding anti-Streptococcus pneumonia antibodies (C). The frequencies of $V_H$ replacement products in different subcategories of IgH sequences were compared with that in the normal peripheral B cells. Statistic significance was determined by two tailed Chi-square test with Yates’ correction. (*) indicates $P < 0.05$ and (**) indicated $P < 0.0001$. 
INTERNALIZATION OF BCR IN EU12 μHC⁺ IMMATURE B CELLS
SPECIFICALLY CHANGES DOWNSTREAM SIGNALING EVENTS

JING LIU, KATHLEEN MARRAN-NICHOL, KAIHONG SU, AND ZHIXIN ZHANG
Abstract

Engagement of B-cell antigen receptors (BCRs) on immature B cells or mature B cells leads to completely different cell fate decisions. The underlying mechanism remains unclear. Here we show that crosslinking BCR on EU12 μHC⁺ immature B cells results in complete receptor internalization. After BCR internalized, restimulation of these cells showed impaired Ca²⁺ flux, delayed Syk phosphorylation, and decreased FoxO1 phosphorylation, which are totally different from that in mature B cells. However, sustained phosphorylation level and reactivation of Erk are still comparable with that in the mature B cells. Taken together, these results indicate that BCR internalization specifically changed downstream signaling events, thus might contribute to the different signaling pathways in human immature B cells and mature B cells, which may favor receptor editing in immature B cells.
Introduction

The development of B lymphocytes from hematopoietic progenitor cells in the bone marrow (BM) to mature, recirculating B cells can be divided into distinct stages according to the expression of specific marker proteins and the rearrangement status of the IgH and L chain gene loci (Burrows and Cooper, 1990; King and Monroe, 2000). Immature B cells are the first to express the complete form of the B-cell antigen receptors (BCRs) and to recognize and respond to specific antigens (Tze et al., 2005). BCR expressed on immature and mature B cells composed of Ag-binding membrane Ig (mIg) noncovalently associated with a disulfide-linked heterodimer, Igα/Igβ (Campbell and Cambier, 1990).

Most of our current understandings of BCR-mediated signaling events are based on analyses of BCR on mature B cells. Receptor crosslinking elicits several biologic responses, including cell activation, proliferation, differentiation, anergy, and cell death. One of the early signals to initiate these divergent responses is the immediate phosphorylation of the tyrosine residues in the two immunoreceptors tyrosine-based activation motifs (ITAMs) in Igα and Igβ by the protein tyrosine kinases (PTKs) Lyn and Syk (Beavitt et al., 2005; Sada et al., 2001). The Src family kinase Lyn predominantly phosphorylates the first ITAM tyrosine, whereas Syk phosphorylates and binds to both ITAM tyrosines (Kulathu et al., 2008). The expression of Syk is required for normal B cell development. Mice carrying a deletion of the Syk gene are embryonic lethal, as expression of Syk is required for maintenance of vascular integrity (Turner et al., 1995). The adaptor protein SH2 domain-containing leukocyte protein of 65 kDa (BLNK)
is then recruited to the BCR complex, where it is phosphorylated by Syk (Fu et al., 1998). Tyrosine phosphorylated BLNK serves as an adaptor to organize a signaling complex by recruiting Btk, Vav, PLCγ2, Grab2 and Rac1 (Kurosaki and Tsukada, 2000). Formation of this “signalosome” is important for fully activation of Ca^{2+} influx, and the PI3K, MAPK, and NF-kB pathways (Kurosaki et al., 1995; Yanagi et al., 2001).

Although BCRs expressed on immature B cells and mature B cells share all the same components, BCR engagement induces totally opposite outcomes in the immature B cells versus mature B cells (Norvell et al., 1995). In mature B cells, BCR signaling induces proliferation, affinity maturation, and class-switch recombination during T cell-dependent responses and ultimately drives the differentiation of B cells into memory cells and antibody-secreting plasma cells (Heyzer-Williams and Heyzer-Williams, 2005). In contrast, BCR stimulation in immature B cells leads to receptor editing, clonal deletion, or anergy (Norvell et al., 1995).

It is still not clear how the same antigen receptors induce completely opposite outcomes in different stages of B cells. Human EU12 μHC^{+} cells have features of BM immature B cells (Wang et al., 2003). Our previous studies have shown that ongoing V_{H} replacement occurs in the EU12 μHC^{+} cells, which can be induced by crosslinking BCR (Liu et al., separate manuscript). Interestingly, treatment of EU12 μHC^{+} cells with F(ab’)_2 anti-IgM antibodies results in complete internalization of surface BCR, which is in sharp contrast to that observed in Daudi and Ramos mature B cells. Detailed analyses showed that BCR internalization specifically changes BCR mediated downstream
signaling events in EU12 $\mu$HC$^+$ immature B cells, which might favor the decision of receptor editing rather than cell proliferation.
Results

Crosslinking BCR induces different level of BCR internalization in human EU12 µHC⁺ immature B cells versus in Daudi and Ramos mature B cells

As an initial experiment to compare the different signaling events triggered by BCR engagement in immature versus mature B cells, EU12 µHC⁺, Daudi, and Ramos cells were stimulated with goat F(ab’)_2 anti-IgM antibodies (2 µg/ml) for different time periods and analyzed for cell surface BCR expression by FACS. Treatment with anti-IgM antibodies caused a very quick BCR internalization in the EU12 µHC⁺ cells, which can be seen as soon as after 1 min of addition of anti-IgM antibodies (Figure 1A). After 30 min, almost all the cells lost their surface BCRs. In contrast, treatment of Daudi and Ramos cells with the same concentrations of antibodies only resulted in internalization of a fraction of BCRs on each cell, as seen by the reduction of surface BCR expression level. After 4 h, about 96% of Daudi cells and 78% of Ramos cells still express surface IgM (Figure 1A, B). To further verify this finding, the three cell lines were treated without or with Cy5 labeled F(ab’)_2 anti-IgM antibodies (2 µg/ml) for 30 min, and cell surface BCR expression was visualized under immunofluorescence microscopy. In untreated cells, all the BCRs are on the cell surface. After treatment, all the Cy5 labeled antibodies are internalized with no surface staining in the EU12 µHC⁺ cells. On the other hand, for both mature Daudi and Ramos cells, a large fraction of Cy5 labeled anti-IgM antibodies are still remained on the cell surface (Figure 1B). These results show that the EU12 µHC⁺ immature B cells are highly sensitive to internalize their cell surface BCRs upon BCR crosslinking.
To determine the effects of BCR internalization on subsequent BCR-mediated signaling events, we compared EU12 µHC⁺, Daudi, and Ramos cells for their capabilities to evoke calcium influx upon restimulation through BCR ligation before and after BCR internalization. Direct stimulation of the three cell lines with goat F(ab’)₂ anti-IgM antibodies (10 µg/ml or 20 µg/ml) induced Ca²⁺ influx (Figure 2B) in a dose-dependent manner. In the second set of experiments, all the three cell lines were first treated with goat F(ab’)₂ anti-IgM antibodies (2 µg/ml) for 30 min, washed off the excessive antibodies, recovered in complete growth medium for 4 h at 37°C (as illustrated in Figure 2A), and restimulated with goat F(ab’)₂ anti-IgM antibodies (10 µg/ml or 20 µg/ml). Upon restimulation, EU12 µHC⁺ cells showed dramatically reduced Ca²⁺ influx compared to that during direct stimulation. Ca²⁺ influx in Daudi and Ramos cells upon restimulation is also reduced (Figure 2B). These results reveal that internalization of BCRs on all three cell lines affect the quantities of BCR-mediated signaling events by reduction of Ca²⁺ influx.

To further determine if BCR internalization affects BCR-mediated downstream signaling events, we performed Western blot analyses to determine the phosphorylation status of different signaling molecules. As described before, all the three cell lines were either directly treated with goat F(ab’)₂ anti-IgM antibodies (10 µg/ml) or pretreated with...
goat F(ab’)2 anti-IgM antibodies (2 µg/ml) for 30 min, washed, recovered, and then restimulated at 2 h and 4 h with goat F(ab’)2 anti-IgM antibodies (10 µg/ml) for three minutes. With direct stimulation, EU12 µHC+ cells exhibited similar activation of downstream signaling patterns compared to that in mature Daudi and Ramos cells, except BLNK phosphorylation (Figure 3B). For restimulation, global protein phosphorylation in the EU12 µHC+ cells is almost unchanged with anti-IgM antibody treatment (10 µg/ml) at 2 h and 4 h time points. More specifically, restimulation of EU12 µHC+ cells did not induce phosphorylation of Syk and FoxO1 at 3 min. Interestingly, restimulation of EU12 µHC+ cells still induced similar level of Erk protein phosphorylation. Thus, internalization of BCR in the EU12 µHC+ cells not only attenuates BCR-mediated signaling events, but also changes the downstream signaling events with normal phosphorylation of Erk kinase, but not Syk or FoxO1.

Delayed Syk activation and sustained Erk phosphorylation after BCR internalization

In mature B cells, ligation of BCR with an antigen will activate Syk. Fully activated Syk phosphorylates BLNK, thereby leading to formation of BCR signalosome to full activate downstream signaling pathways including the PI3K, the mitogen-activated protein kinase (MAPK), and the NF-κB pathways (Rolli et al., 2002). However, after internalization of BCR in EU12 µHC+ cells, restimulation with anti-IgM F(ab’)2 antibodies (10 µg/ml) can not fully activate Syk after 3 min. However, the same treatment can still induce CD19 and Erk phosphorylation. To further determine Syk activation status in EU12 µHC+ cells during restimulation, we compared syk phosphorylation at different time points after either direct stimulation or restimulation.
For direct stimulation, Syk phosphorylation can be detected as soon as 1 min after engaging BCR and reached to peak level of phosphorylation at 3 min and quickly diminished after 10 min. However, for restimulated cells, Syk phosphorylation is clearly delayed, which can be barely detected at 3 min, and lasted all the way till 15 or 30 min after restimulation (Figure 4). There are not dramatic changes for CD19 and Erk phosphorylation between direct stimulation versus restimulation. It should be pointed out that Erk phosphorylation is sustained after directed stimulation, even in cells completely lost their cell surface BCR (Figure 4, lanes 7 and 8). These results suggest that internalization of BCR alters downstream signaling events in the EU12 µHC⁺ cells.

*Distribution of phosphorylated Syk and Erk in stimulated EU12 µHC⁺ cells*

Next, we performed immunofluorescence studies to visualize phosphorylated Syk or Erk proteins in three cell lines upon direct stimulation or restimulation. In directly stimulated EU12 µHC⁺, Daudi, or Ramos cells, crosslinking BCR with Cy5 labeled F(ab’)₂ anti-IgM antibodies (10 µg/ml) induced Syk and Erk phosphorylation (Figure 5A, B, left panels). In restimulated EU12 µHC⁺ cells, Syk phosphorylation is diminished. Interestingly, phosphorylated Erk can be detected in the EU12 µHC⁺ cells even 4 h after the first wave of stimulation, and can be further enhanced by restimulation with anti-IgM antibodies (Figure 5A, B, right panels). CD19 phosphorylation is also decreased in pretreated cells upon restimulation compare to directly treated cells (Figure 5C). These results are consistent with our Western blot results indicating that internalization of BCR in the EU12 µHC⁺ cells specifically changed BCR-mediated signaling events during restimulation.
Discussion

BCRs expressed on immature B cells and mature B cells have the same components, however, upon stimulation, they transduce different signals, which result in different outcomes (Norvell et al., 1995). For immature B cells, engagement of BCR induces receptor editing, clonal deletion, or anergy; for mature B cells, stimulation of BCR induces cell activation and proliferation. Understanding how the same BCRs can transduce different signals is a long standing question in immunology research.

We are currently using the EU12 μHC+ cells as an experimental model system to study the molecular regulation of V_H replacement. The EU12 μHC+ cells are phenotypically similar to bone marrow immature B cells (Zhang et al., 2003). We noticed that the EU12 μHC+ cells are highly sensitive to BCR crosslinking to internalize their cell surface BCRs (Liu, unpublished observations). Treatment of EU12 μHC+ cells with low concentration of goat F(ab')2 anti-IgM antibodies (2 μg/ml), internalization of BCR can be detected as soon as after 1 min of treatment. After 30 min, almost all the EU12 μHC+ cells lost their cell surface IgM expression and become μHC-. Such complete BCR internalization is totally different from that in other mature B lineage cells, such as Daudi or Ramos. With the same treatment, majority of the Daudi or Ramos cells remain μHC+, although the levels of cell surface IgM expression are reduced.

Internalization of cell surface BCR clearly attenuates the downstream signaling events. As expected, restimulation of EU12 μHC+ cells after internalization of cell surface IgM showed dramatic reduction of BCR induced Ca^{2+} influx. With the same
treatment, Ca^{2+} influx in Daudi or Ramos cells upon restimulation is also attenuated. Such results are easy to interpret, because reduction of the cell surface BCR expression attenuates BCR-mediated signaling events.

Direct comparison of the anti-IgM antibodies induced protein tyrosine phosphorylation patterns in the three different cells lines after BCR internalization showed significant differences. After BCR internalization, EU12 μHC⁺ cells failed to induce global protein tyrosine phosphorylation upon restimulation with higher concentration of anti-IgM antibodies at 2 h and 4 h time points. However, in Daudi or Ramos cells, the patterns of anti-IgM antibody induced protein tyrosine phosphorylation are similar during both direct stimulation and restimulation experiments. More specifically, after internalization of BCR, restimulation of EU12 μHC⁺ cells results in delayed Syk phosphorylation and almost diminished FoxO1 phosphorylation. Such changes are consistent with the low level of cell surface BCR expression on the EU12 cells. However, anti-IgM antibody mediated activation of Erk kinase in the EU12 μHC⁺ cells seems not to be affected by BCR internalization. In fact, sustained Erk phosphorylation can be detected in the EU12 μHC⁺ cells even after they lost almost all of their cell surface BCRs. Restimulation of EU12 μHC⁺ cells after BCR internalization can still mount a similar wave of Erk phosphorylation. These results demonstrate that internalization of BCR in the EU12 μHC⁺ cells specifically changes BCR-mediated downstream signaling events. Such changes might be due to the differential activation thresholds of individual signaling factors. For the Erk kinase, the activation threshold might be very low. With minimal level of cell surface BCR stimulation, Erk kinase can be phosphorylated to activate the downstream signaling events. However, for Syk kinase,
after internalization of majority of the cell surface BCRs, restimulation can no longer fully activate Syk. Given the important roles of Syk kinase in BCR mediated early signaling events in mature B cells, delayed and attenuated Syk activation might affect the formation of the signalosome to fully activate the downstream PI3K and NF-κB pathways (Figure 6). Indeed, we observed diminished FoxO1 phosphorylation upon restimulation of EU12 µHC+ cells as an indication for altered PI3K downstream signaling pathways. In a separate study, we found that BCR crosslinking only induces very weak NF-κB activation in the EU12 µHC+ cells compared to that in Ramos cells (Hong, unpublished observations). More importantly, a recent study has showed direct evidence that Erk activation is involved with light chain receptor editing using a specific inhibitor of MEK1/2 that blocks Erk1/2 (Mazari et al., 2007). Corresponding to the sustained activation of Erk kinase in treated EU12 µHC+ cells, we postulate that Erk activation upon BCR crosslinking might play a role in V_{H} replacement regulation.

Among the major BCR-mediated signaling pathways, activation of the PI3K and NF-κB pathways is very important for cell survival and proliferation (Figure 6). Here, our results demonstrate that internalization of BCR in the EU12 µHC+ cells specifically attenuates BCR-mediated activation of the PI3K and NF-κB activation, but does not alter BCR-mediated activation of Erk kinase. We propose that such changes in the EU12 µHC+ immature B cells after BCR internalization will lead to cell proliferation and the induction of V_{H} replacement.
Material and Methods

Cell lines and treatment

The EU12 µHC⁺ cells are purified from the parental EU12 cells that established from a child with replapsed B-cell precursor ALL (Zhou et al., 1995; Zhou et al., 1998; Wang et al., 2003). Ramos and Daudi are B cell lymphomas obtained by tissue culture adaptation of Burkitt lymphomas (Klein et al., 1975; PULVERTAFT, 1965; Klein et al., 1968; Kerr et al., 1991). In all experiments, cell lines were cultured in RPMI 1640 medium, supplemented with 100 units/ml penicillin/streptomycin, 10% heat-inactivated FBS, 50 µM β-mercaptoethanol, and 2 mM L-glutamine. Cells were maintained at densities of 0.1 to 1 × 10⁶/ml. For direct stimulation, all cells are stimulated with goat F(ab’)₂ anti-IgM antibodies (10 µg/ml) at room temperature. For restimulation experiments, cells were pretreated with goat F(ab’)₂ anti-Human IgM antibodies at 2 µg/ml for 30 min, washed, recovered in complete growth medium for 4 h and then restimulated with 10 or 20 mg/ml goat F(ab’)₂ anti-IgM antibodies. Ca²⁺ influx, western blot, and immunofluoresence staining were all performed under these two conditions.

Antibodies and Reagents

Immunofluorescence assays used the following monoclonal antibodies (mAbs): Cy5-labeled AffiniPure Donkey Anti-Human IgM F(ab’)2 fragment were purchased from Jackson ImmunoResearch (West Grove, PA); Goat F(ab’)₂ anti-Human IgM (µ chain specific, LE/AF) were purchased from Southern Biotech (Birmingham, AL); Goat Anti-Rabbit Alexa 488 were bought from Invitrogen. Phospho-Syk, Phospho-Erk, Phospho-
CD19 antibodies were purchased from Cell signaling. Anti-goat serum was kindly provided by Dr. Kaihong Su.

**Cell-surface staining and flow cytometry analysis**

Cell surface staining was performed with various mAbs after blocking with PBS + 2% FBS. After cell surface staining and washing, flow cytometric analysis was performed with live cell gating by Accurri FACS machine using CFLOW Plus software.

**Ca^{2+} influx Assays**

Intracellular Ca^{2+} levels were monitored by FACS analyses using the Fluo-3AM fluorescence dye. Briefly, EU12 µHC^{+} cells were preloaded with 1µM Fluo-3AM with pluronic-F127 at 1:1 ratio (molecular Probes, Invitrogen) for 30 min at 37°C in the dark. Cells are washed and resuspended in RPMI media containing 1% FBS. Cells were maintained in the dark at room temperature before use. Samples were analyzed on an Accuri FACS cytometer by using FL1 vs time to establish baseline fluorescence for unstimulated cells. Different stimuli were added at 30 sec after analyzing on the cytometer and continued for a total length of 10 min. FACS data were analyzed by using the CFlow software package (Accuri Cytometers Inc., MI)

**Antibody-mediated receptor internalization of B cells**

EU12 µHC^{+}, Daudi, or Ramos cells were used in these experiments. Approximately 1× 10^6 cells were washed once with cold FACS buffer, followed by
incubation with monoclonal goat F(ab’)2 anti-IgM antibodies (2 µg/ml) (Southern Biotech, AL) at 37°C. Cells were collected at different time points for FACs analysis.

Immunofluorescence Analysis

Different cells were directly stimulated or restimulated as described before. After restimulation, cells were fixed with 3.7% formaldehyde at room temperature for 10 min in the dark, followed by incubation with 0.1% Triton X-100 for 5 min on ice. Untreated cells were used for both negative and secondary antibody staining controls. Cells were washed twice with 1 x PBS and blocked with PBS + 1% goat serum at room temperature for an hour. Primary antibody for phospho-Syk, phosphor-Erk and phosphor-CD19 (1:200 dilution) in PBS + 2% BSA were added separately to cells and incubated at the room temperature for an hour in the dark. Excessive primary antibodies were removed by washing. Cells were then resuspended in PBS + 2% BSA containing goat anti Rabbit-Alxa488 (Invitrogen) secondary antibodies (1:1000 dilution) for an hour. The samples were then washed intensively in cold PBS for three times, 5 min each. Cells were then mounted on glass slides and visualized on an Olympus X81 fluorescence microscope and photographed at ×60 magnification under oil. All image data was analyzed using Slidebook Imaging software.

Western blotting

For global tyrosine phosphorylation, EU12 µHC+, Daudi and Ramos cells (10 × 10⁶/ml) were either directly treated with anti-IgM F(ab’)2 antibodies (10 µg/ml) for 3 min or cells were pretreated with anti-IgM F(ab’)2 antibodies (2 µg/ml) for 30 min, washed,
recovered in complete RPMI medium for 4 h and then restimulate with anti-IgM F(ab’)2 antibodies (10 µg/ml) for 3 min. Cells were washed once with 1 x PBS, whole cell lysates were collected using the 1% TNT buffer (Stadanlick et al., 2008) and the phosphorylation status of the BCR signaling molecules CD19, Lyn, Syk, BLNK, Erk, Akt and FoxO1 were analyzed with phosphospecific antibodies (Cell signaling). Reduced samples were separated by 10% SDS-PAGE and immunoblotted.
Acknowledgements

The authors would like to thank Wanqin Xie for helping with the immunofluorescence staining, Sang Yong Hong for helpful discussion. ZZ is supported in part by NIH grants AI074948, AI076475, and AI073174.
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Figure 1

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Figure 1. Anti-IgM antibody induces BCR internalization in EU12 µHC+ immature B cells and in mature Daudi or Ramos B cells. (A) EU12 µHC+, Daudi, and Ramos cells were treated with goat F(ab’)2 anti-IgM antibodies (2 µg/ml). The cells were stained with the Cy5 labeled anti-IgM antibodies at different time points and subjected to flow cytometric analysis. For the restimulation experiments, EU12 µHC+, Daudi and Ramos cells were pretreated with goat F(ab’)2 anti-IgM antibodies (2 µg/ml) for 30 min, recovered in medium for 4 h and subjected to FACS analysis. The percentages of cell surface IgM expression are indicated. (B) Immunofluorescence analysis. For untreated cells, EU12 µHC+, Daudi or Ramos cells were fixed and stained with Cy5 labeled anti-IgM antibodies. For anti-IgM antibody treated cells, EU12 µHC+, Daudi and Ramos cells treated with Cy5 labeled anti-IgM (2 µg/ml) for 30 min. Cells were then fixed and visualized on glass slides by fluorescence microscopy.
Figure 2

A

Direct Stimulation  Wash  Restimulation

0 h  0.5 h  4 hr  Time

B

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[Diagram showing FLuo3 vs Time (s) for different conditions and treatments]
Figure 2. BCR internalization reduces Ca$^{2+}$ influx upon restimulation. (A) Schematic representation of different stimulation protocols for EU12 μHC$^+$, Daudi, and Ramos cells with goat F(ab’)$_2$ anti-IgM antibodies. (B) EU12 μHC$^+$, Daudi and Ramos cells were prepared as outlined in (A). EU12 μHC$^+$, Daudi and Ramos cells were either directly treated with goat F(ab’)$_2$ anti-IgM antibodies (10 µg/ml or 20 µg/ml) or pretreated with goat F(ab’)$_2$ anti-IgM antibodies (2 µg/ml) for 30 min, washed, recovered for 4 h, then restimulated with F(ab’)$_2$ anti-IgM antibodies (10 µg/ml or 20 µg/ml). Baseline fluorescence was established with the mock experiments without addition of stimulatory antibodies. Arrows indicates the time point when antibodies were added. Results shown are representative of three independent experiments.
Figure 3

A

Direct Stimulation     Wash     Restimulation     Restimulation
0 h     0.5 h     2 hr     4 hr     Time

B

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Anti-IgM

p-Tyr

p-Syk
Syk
p-BLNK
BLNK
p-CD19
CD19
p-Lyn
Lyn
p-Erk
Erk
p-Akt
Akt
p-FoxO1/3a
FoxO1
β-actin
Figure 3. Internalization of BCR changes downstream signaling events upon restimulation. (A) Schematic representation of different treatment for EU12 µHC⁺, Daudi, or Ramos cells with F(ab’)₂ anti-IgM antibodies. (B) EU12 µHC⁺, Daudi and Ramos cells were stimulated follow the indicated scheme with F(ab’)₂ anti-IgM antibodies and cell lysates were prepared. BCR-mediated downstream signaling events were monitored by western blot analyses using 4G10 (p-Tyr), phosphorylated CD19 (p-CD19), phosphorylated Lyn (p-Lyn), phosphorylated BLNK (p-BLNK), phosphorylated Erk (p-Erk), phosphorylated Akt (p-Akt) or phosphorylated FoxO1/3a (p-Foxo1/3a). Membranes were stripped and re-probed with antibodies against CD19, Lyn, Syk, BLNK, Erk, Akt, FoxO1 and β-actin as controls.
Figure 4

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127
Figure 4. Internalization of BCR results in delayed activation of Syk, and sustained activation of Erk1/2 in EU12 μHC⁺ cells upon restimulation. EU12 μHC⁺ cells were directly stimulated with goat F(ab’)_2 anti-IgM antibodies (10 μg/ml) or pretreated with F(ab’)_2 anti-IgM antibodies (2 μg/ml) for 30 min, washed, recovered and restimulated upon F(ab’)_2 anti-IgM antibodies (10 μg/ml). Cells were collected at 0, 1, 3, 10, 15 and 30 min and analyzed for CD19, Syk, and Erk phosphorylation. The membranes were stripped and blotted with anti-CD19, anti-Syk, anti-Erk, or anti-β-actin antibodies as controls.
Figure 5

A

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![Image](image13)

B

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![Image](image17)

C

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![Image](image21)
Figure 5. Internalization of BCR changes downstream signaling events upon restimulation. EU12 µHC+, Daudi, and Ramos cells were directly stimulated with Cy5 labeled anti-IgM antibodies (10 µg/ml) for 3 min or pretreated with F(ab’)2 anti-IgM (2µg/ml) for 30 min, washed, recovered for 4 h and restimulated with Cy5 labeled anti-IgM antibodies (10 µg/ml) for 3 min. Fixed and permeablized cells were analyzed by immunofluorescence microscopy for phosphorylated Syk (A), Erk1/2 (B) and CD19 (C). Scale bar, 10 µm. Data shown are representative of > 150 cells analyzed.
Figure 6
Figure 6. Proposed mechanisms by which BCR induces proliferation in mature B cells versus receptor editing in immature B cells. In mature B cells, BCR engagement by foreign antigen leads to phosphorylation of CD19 and tyrosine kinases such as Syk, Lyn. Subsequently, phosphorylation of BLNK will lead to signalosome formation. These will lead to fully activation of the PI3K, MAPK, and NF-κB pathways and promote cell proliferation. In immature B cells, crosslinking BCR induces BCR internalization. BCR signaling is less efficient and impaired with delayed Syk activation, but sustained Erk activation. Insufficient activation of PI3K leads to accumulation of unphosphorylated FoxO1 in the nucleus. These changes may favor receptor editing mechanism.
CONCLUSION

$V_H$ replacement refers to RAG product mediated secondary recombination between a previously rearranged $V_H$ gene and an upstream unrearranged $V_H$ gene. In human, ongoing $V_H$ replacement was originally found in a B cell leukemia EU12 cell line and in human bone marrow immature B cells. As a mechanism to edit unwanted IgH genes, $V_H$ replacement renews the entire $V_H$ coding region and extends the immunoglobulin heavy chain CDR3 regions preferentially with charged amino acids and contributes to about 5% of the human B cell repertoire. However, how $V_H$ replacement is regulated remained unknown.

In the first part of this dissertation, I investigated how $V_H$ replacement is regulated in human immature B cells. EU12 $\mu$HC$^+$ cells have many features of human bone marrow immature B cells. Using the EU12 $\mu$HC$^+$ cells as an experimental model system, we showed that crosslinking BCR results in receptor internalization, cell proliferation arrest, and induction of $V_H$ replacement, as determined by detection of double-stranded DNA breaks and $V_H$ replacement excision circles. Pretreatment of human EU12 $\mu$HC$^+$ cells with specific protein tyrosine kinase inhibitors, such as Genistein, SykII, SykIII, or PP1, blocks BCR-mediated signaling events and inhibits anti-IgM antibody induced $V_H$ replacement. In addition, pretreatment of EU12 $\mu$HC$^+$ cells with PI3K inhibitor enhances $V_H$ replacement, while activation of the PI3K pathway by crosslinking CD19 inhibits $V_H$ replacement. Taken together, these results indicates that the PI3K pathway negatively regulates $V_H$ replacement process. Moreover, ongoing $V_H$ replacement can be detected in the newly emigrated immature B cells from peripheral of healthy donors and human tonsillar samples, which can be further enhanced by BCR stimulation. In summary, our results show that $V_H$ replacement in EU12 $\mu$HC$^+$ cells is regulated by BCR mediated
signaling events and that the PI3K pathway plays a negative role in the induction of VH replacement.

In the second part of the dissertation, I further compared BCR-mediated signaling events in immature EU12 µHC⁺ cells versus that in mature Daudi and Ramos. As expected, loss of immature B cell surface BCRs attenuated BCR-mediated Syk, Lyn activation and calcium mobilization upon restimulation. Moreover, time dependent assays showed delayed activation of Syk kinase in EU12 µHC⁺ cells. BCR signaling mediated phosphorylation of FoxO1 is also diminished. However, BCR signaling mediated Erk activation is almost unchanged. These results showed that BCR internalization in EU12 µHC⁺ cells not only completely changes the BCR signaling quantity, but also leads to qualitative changes to favor receptor editing in immature B cells.

Taken together, the results presented in this dissertation provided the first evidence that VH gene replacement is a BCR signaling mediated process. The rapid internalization of BCR on immature B cells specifically changes the downstream signaling patterns, which may direct immature B cells for receptor editing and induction of VH replacement.


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