B CELL RECEPTOR SIGNALING IN RECEPTOR EDITING AND LEUKEMIA

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LAURA BAILEY RAMSEY

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Dedication

This dissertation is dedicated to everyone who has supported me during the past five years while I attended graduate school. I love you all!

Abstract

There are many unanswered questions in the B cell field, but one of paramount importance is how B cells are tolerized to avoid autoimmunity. The majority of developing B cells are probably self-reactive, and many that make it through B cell development to the periphery show evidence of receptor editing. The question remains as to how the B cells signal to re-induce the editing machinery, whether it is a positive reactivation signal or the release of an inhibitory tonic signal. We propose that it is the release of a tonic signal through the B cell receptor that inhibits recombination when the BCR is present on the surface, but when the BCR binds self-antigen it is internalized, the inhibitory signal is abrogated, and the recombination machinery is reactivated. We tested this hypothesis using several transgenic models, including RAG2-GFP mice, HEL Ig mice, and anti-κ mice. We also used several mice deficient in BCR signaling to test this hypothesis, and found that reduced BCR signaling induces more receptor editing. When we tested mice with excessive BCR signaling or pharmacologically mimicked the BCR signal, we found a decrease in receptor editing. These experiments provide compelling evidence for the inhibitory tonic signaling hypothesis and against the activation signaling hypothesis.

Another question unanswered in the B cell field is how B cells are transformed into leukemia and lymphoma. There have been whole genome analysis studies to show that genes involved in the BCR signaling pathway are involved in many B cell malignancies, including leukemia, lymphoma and myeloma. Our studies in mice provide evidence that in combination with constitutive STAT5 activation, a loss of genes involved in B cell development (*ebf1* and *pax5*) or pre-BCR signaling (*blnk,*

PKCβ, and btk) results in leukemia. These are genes that have been shown to be deleted in B cell acute lymphoblastic leukemia (ALL). We also demonstrate an increase the phospho-STAT5 levels in adult BCR-ABL^{+} ALL patients. Herein we have developed a new mouse model for B-ALL that may be useful in testing and perfecting treatment protocols used on ALL patients.

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Chapter 2. BCR Basal Signaling Regulates Antigen-Induced Ig Light Chain Rearrangements

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I wrote much of the manuscript, re-analyzed data and prepared several of the figures, submitted the manuscript, responded to reviewers' comments and made necessary revisions. Brian Schram and Lina Tze performed experiments, analyzed data, prepared figures and assisted in writing the manuscript. Jiabin Liu, Lydia Najera, Amanda Vegoe, and Keli Hippen assisted with experiments and provided discussion related to the manuscript. Richard R. Hardy provided samples for microarray analysis. Timothy Behrens and Michael Farrar co-directed the project.

Chapter 3. Tonic BCR Signaling Represses Receptor Editing via the Ras and Calcium Signaling Pathways

This manuscript is in preparation for submission.

I performed the experiments, analyzed the data, prepared the figures, and wrote the manuscript. Amanda Vegoe helped dissect the mice and oversaw the mouse husbandry. Timothy Behrens conceptualized the project, which was directed by Michael Farrar.

Chapter 4. STAT5 Cooperates with Defects in B Cell Development to Initiate Progenitor B Cell Leukemia

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I prepared the microarray samples and analyzed the microarray data, with help from Emily Baechler and Thearith Koeuth. I prepared several figures and assisted in writing the manuscript. Mark Willette and Lynn Heltemes-Harris performed the analysis of the mice, prepared figures, and assisted in writing the manuscript. Yi Qiu and Steven Kornblau carried out the reverse phase protein array studies of human patients. E. Shannon Neeley and Nianxiang Zhang assisted Steven Kornblau and Yi Qiu with statistical interpretation of human patient data. Michael Farrar directed the project and assisted in writing the manuscript.

List of Abbreviations

Ag, antigen
ALL, acute lymphoblastic leukemia
B6, C57Bl/6 mouse strain
BCR, B cell receptor
BLNK, B cell linker
BM, bone marrow
Btk, Bruton's tyrosine kinase
CLP, common lymphoid progenitor
CML, chronic myelogenous leukemia
DAG, diacylglycerol
EBF, early B cell factor
GFP, green fluorescent protein
HA, Herbimycin A
HC, heavy chain
HEL, hen egg lysozyme
HSC, hematopoietic stem cell
i.v., intravenous
IFN, interferon
Ig, immunoglobulin
IL-7, interleukin-7
IL-7R, interleukin-7 receptor
IP3, inositol 1,4,5-triphosphate
IP4, inositol 1,3,4,5-tetrakisphosphate
IRF, interferon regulatory factor
ITAM, immunoreceptor tyrosine-based activation motif
ITIM, immunoreceptor tyrosine-based inhibitory motif
Itpkb, Inositol 1,4,5-triphosphate kinase B
Jak, Janus kinase
KI, knockin
LC, light chain
LN, lymph node
MPP, multipotent progenitor
NFκB, nuclear factor of kappa light polypeptide gene enhancer in B cells
$PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-triphosphate
$PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate
PI3K, phosphatidylinositol-3-kinase
PKC, protein kinase C
PLC, phospholipase C
PMA, 4-a-Phorbol 12-myristate 13-acetate
Pre-BCR, pre-B cell receptor
RAG, recombination activating gene
SHIP, SH2-containing inositol phosphatase
SHP-1, hematopoietic cell phosphatase, protein tyrosine phosphatase, non-receptor type 6
sIg, surface immunoglobulin

SLC, surrogate light chain
SOC, store operated calcium channel
SOCS, suppressor of cytokine signaling
STAT, Signal transducer and activator of transcription
Tg, transgenic
WT, wild type
xid, x-linked immunodeficiency

Chapter 1. Introduction

B cell development

The normal function of the immune system is to protect against attacks by invading pathogens, such as bacteria and viruses, and also abnormal cells that develop in cancer. The immune system consists of many types of cells, one of which is the lymphocyte. There are two types of lymphocytes, B and T, that are named based on the location of their development. T cells develop mostly in the thymus, while B cells develop in the bone marrow (BM). B cells are responsible for providing the antibody-mediated immune response. The immune system must have a vast repertoire of antibodies to attack any invading/abnormal cell. Through evolution the immune system has developed several processes to create a diverse repertoire, including V(D)J recombination, where gene segments are combined to create an antibody, and somatic hypermutation, where point mutations are induced to increase the antibody's affinity for antigen. Throughout these processes, B cells with antibodies to self-antigens develop and must be tolerized to avoid autoimmunity. This thesis will examine one tolerance mechanism, called receptor editing, which results from signals through the surface antibody on a B cell.

In adult vertebrates all blood cells develop from hematopoietic stem cells in the BM (Figure 1). There are several models for hematopoiesis which are not linear, but have been simplified for ease of understanding here [1]. B cells go through several distinct stages of development characterized by surface markers and rearrangement of

immunoglobulin (Ig) genes (Figure 1). Rearrangement of Ig genes is what confers upon B cells the repertoire diversity to provide protective immunity microbial invasion.

The hematopoietic stem cell (HSC) is a self-renewing cell that can differentiate into all types of blood cells. The next stage toward becoming a B cell is the multi-potent progenitor (MPP), which still has the potential to develop into erythroid, myeloid, and lymphoid lineage cells. When the interleukin 7 receptor (IL-7R) is expressed on the surface, at the common lymphoid progenitor (CLP) stage, the cells lose erythroid and myeloid lineage potential. CLPs can develop into B cells, T cells, and NK cells.

In order to become a B cell, the CLP must start expressing B220, at which point it is considered a pre-pro B cell. CD19 is the next B cell marker expressed on the surface at the early pro-B stage, when expression of RAG1/2 and the rearrangement of the heavy chain (HC) of Ig genes begins. The late pro-B stage is characterized by CD43 expression on the surface and continued HC rearrangement. The HC rearrangement creates a pre-B cell receptor (pre-BCR) that gets expressed on the surface at the pre-B stage, which sends signals to proliferate rapidly and downregulate RAG expression. The pre-BCR pairs with the signaling components, Igα and Igβ, in order to propagate signals to proliferate. After several rounds of clonal expansion, RAG is induced again, and the pre-B cell starts rearranging the light chain (LC) Ig genes, which, when paired with the HC, will be expressed on the surface of the immature B cell, with Igα and Igβ, as the B cell receptor (BCR).

When the immature B cell expresses the BCR (IgM) on the surface it is tested for self-reactivity. The BCR will later be secreted by the B cell as an antibody, so self-

reactive B cells must be deleted or rearrange their Ig genes again, in a process called receptor editing. When the BCR is not self-reactive, B cells exit the BM and migrate to secondary lymphoid organs (e.g., spleen and lymph nodes), where they continue developing through the transitional stage to the mature B cell stage, characterized by expression cell surface of IgD. At this point the mature B cell will traffic through the peripheral lymphoid tissues (spleen and lymph nodes) until it encounters the antigen for which the BCR will bind. When this occurs, the B cell engulfs the antigen, degrades it into small peptides, which are presented on the cell surface for recognition by T cells. When the T cell recognizes the antigen that the B cell is presenting, it activates the B cell to proliferate, produce more of the antibody and secrete it, as a plasma cell. The antigen then binds the invading pathogen, signaling for other cells to engulf or destroy it. When the initial response to antigen is finished, most of the B cells die, but a few of the plasma cells and memory B cells survive, so the immune system is primed for a quick response to a subsequent invasion by the same pathogen.

Transcriptional control of B cell development

There are many transcription factors necessary for B cell development. These transcription factors do not fit neatly into a linear pathway but instead are part of a complex network of cell fate regulators (Figure 2). One of the first to be expressed is Ikaros, a member of the Kruppel-like zinc finger family of transcription factors. It is necessary for Flt3 expression and B cell development. Flt3 is a cytokine receptor that propagates signals for growth and proliferation. In the absence of Ikaros, B cell development stops at the MPP stage, due, in part, to the necessity of Flt3 to become a

CLP [2]. Ikaros is also involved in the self-renewal of HSCs – without Ikaros there is a 30 – 40 fold reduction in the long-term reconstitution activity of HSCs [3]. The necessity for Ikaros can be bypassed by the expression of EBF1 [4], another transcription factor involved in B cell development.

In order for the IL-7R to be expressed, the transcription factor PU.1 has to be expressed at low levels – at high levels PU.1 induces macrophage development [5]. PU.1 is an Ets family transcription factor that is expressed in HSCs, MPPs, and all differentiating hematopoietic cells except erythroblasts, megakaryocytes, and T cells [6]. PU.1-deficient embryos die around birth, but fetal liver hematopoietic cells can be harvested and show no expression of IL-7R or Flt3 [7]. Based on these, and other studies, PU.1 has been shown to regulate cytokine-dependent proliferation, survival, and differentiation of MPPs [8].

PU.1 regulates the expression of E2A and EBF1, which are also transcription factors necessary for B cell development. They regulate many of the genes that are imperative for B cell differentiation – CD79a (Igα), CD79b (Igβ), λ5, VpreB, and RAG1/2. The E2A gene codes for two basic helix-loop-helix proteins, E12 and E47, which result from differential splicing of the mRNA. The E2A proteins bind the enhancer regions of the Ig HC locus and induce transcription necessary for HC recombination [9]. Deficiency of E2A or EBF1 results in a block at the CLP stage [10]. Early B cell factor 1 (EBF1) is expressed in B cells starting at the pro-B stage. EBF1 activates transcription of several genes and also represses transcription of genes necessary for alternative lineages [11]. E2A and EBF1 act together, often at binding sites within close proximity [12]. Mice heterozygous for both genes have a block in B

cell development at the pro-B stage and show reduced expression of many genes necessary for B cell development, including RAG1/2 [13], demonstrating how crucial these genes are for proper development of B cells.

Differentiation of CLPs to pre-proB cells depends on E2A, EBF1, and Pax5. E2A and EBF1 regulate each other and also Pax5, a transcription factor important for repressing myeloid genes and antagonizing T cell fate by downregulating the expression of Notch-1 [14]. Pax5 also induces expression of CD79a, CD19, and BLNK, important proteins for signal transduction downstream of the pre-BCR & BCR [15]. Pax5 is necessary for B cell commitment – in the absence of Pax5, pro-B cells can give rise to T cells and myeloid cells [14]. Pax5 is necessary for V to DJ HC rearrangement, and facilitates contraction of the Igh locus chromatin for recombination [16].

Transcriptional control of early B cell development is fairly well characterized, but later stages are even more complex due to the signals from many surface receptors. Many transcription factors are functionally redundant so the exact function of each is difficult to elucidate. Transcription factors involved in V(D)J recombination, IL-7R signaling, and BCR signaling will be discussed in those sections.

Interleukin-7 Receptor Signaling

Interleukin-7 (IL-7) and its receptor (IL-7R) are critical for B cell development. The IL-7R is composed of a unique α chain and a γ chain, common to many cytokines (IL-2, IL-4, IL-9, IL-15, and IL-21). In the absence of IL-7 [17], IL-7R [18], or the common γ chain [19], B cells cannot progress past the pro-B stage and have reduced numbers of CLPs. The signal through the IL-7R is propagated by phosphorylation of

JAKs & STATs (Janus kinase and Signal transducer and activator of transcription, respectively). JAK1 is associated with the IL-7Rα chain, while JAK3 is associated with the common γ chain. When the two chains are brought together by binding IL-7, JAK1/3 phosphorylate each other and the IL-7Rα chain, allowing for docking of STATs 1, 3, and 5 [20]. The JAKs then phosphorylate the STATs, which dimerize and translocate to the nucleus to activate transcription. IL-7R signaling is negatively regulated by SOCS1 (Suppressor of cytokine signaling 1) though binding of SOCS1 to JAK kinase domains and ubiquitin-mediated degradation of the signaling complex [21]. In addition to negative feedback loops, IL-7R induces a positive feedback loop through E2A and EBF, which induce expression of the *il7r* gene [22].

STAT5 is particularly important for B cell development – without both isoforms (STAT5a/b) there is a severe reduction in B cells, starting at the pro-B stage [23]. The SH2 domain of STAT5 binds to the IL-7Rα chain at tyrosine 449 when it is phosphorylated [20, 24]. STAT5 is phosphorylated by JAK1/3 on tyrosine 694 or 699 (Stat5a & Stat5b, respectively), which induces homodimerization, also via the SH2 domains and phosphotyrosines. This causes translocation to the nucleus, where the homodimers regulate anti-apoptotic genes such as bcl-2 and several caspases. Other target genes of STAT5 are cyclin D2, bcl-xL, Pax5, EBF1, and Myc [25-28]. Thus, IL-7R signaling through STAT5 is important for B cell survival and developmental progression.

V(D)J Recombination

In order to respond to any pathogen that invades the body, the immune system must have a vast repertoire of B cells. It is estimated that in humans there are 100 billion different specificities of B cells [24]. Each B cell is specific for only one antigen, however there is not a gene for each specificity. Instead, the HC of the Ig locus consists of many gene segments, which are spliced and brought together by a recombination complex, a process termed V(D)J recombination. The gene segments are termed Variable, Diversity, and Joining, based on their position in the Ig locus. Each gene segment has a recombination signal sequence (RSS) that binds the V(D)J recombinase complex. The RSS is composed of a heptamer (7 nucleotides), a spacer, which is 12 or 23 nucleotides long, and a nonamer (9 nucleotides). In order for the D gene segment to join to the J gene segment and not another D gene segment, it must follow the 12/23 rule. The D gene segments have spacers with 12 nucleotides on each side, and the V and J segments have 23 nucleotide spacers. Thus, the HC V and J segments cannot be joined together, ensuring utilization of all three gene segments. However, the LC consists of only V and J segments, but still follow the 12/23 rule. There are two LC loci, the κ and λ loci. In the κ LC locus, a 12 bp spacer flanks the V region and a 23 bp spacer flanks the J region. In order to avoid recombining the κ V gene segment to the λ J segment, the spacers are opposite in the λ locus, with the V region flanked by a 23 bp spacer and the J region flanked by a 12 bp spacer [29].

The V(D)J recombinase complex is made of the RAG (recombinase activating gene) proteins 1 & 2, DNA-dependent protein kinase, DNA ligase IV, and the Ku heterodimer (Ku70/Ku80). RAG1&2 are the only lymphoid specific components of this

complex, the others are involved in DNA repair and are ubiquitously expressed [30]. RAG1/2 initiate the DNA cleavage between RSSs and coding sequences. The recombinase complex then joins the RSSs to form a circular DNA product with the intervening DNA (Figure 4). The coding sequences are joined and occasionally additional nucleotides are added through random integration, DNA repair, or by the enzyme terminal deoxynucleotidyltransferase (Tdt). V(D)J recombination and RAG expression are tightly regulated, because aberrant recombination can lead to unwanted rearrangement, causing self-reactivity or cancer.

Only one allele of the Ig locus is rearranged at a time, a property termed allelic exclusion. Approximately half of the BCRs that are made are self-reactive, so expressing two different BCRs on the surface of a B cell would greatly increase the chance of self-reactivity [31]. Each allele of the HC is rearranged with equal frequency, so the choice is thought to be stochastic [32]. The choice of allele that is rearranged depends on epigenetic marks and positioning of the loci within the nucleus [33]. Germline transcription of the Ig genes is necessary for V(D)J recombination, presumably to open the locus for the recombination machinery. The LC consists of two loci, κ and λ, which are rearranged at different frequencies in different species. In mice, the ratio of κ to λ is approximately 20:1, however in humans it is about 2:1 [34]. This ratio correlates with the number of κ and λ V gene segments and the efficiency of rearrangements.

The HC is rearranged before the LC, with the D & J segments joining first during the early pro-B stage. The V segment is joined to the DJ rearranged gene segments at the late pro-B stage. Each rearrangement has only a 1/3 chance of putting

the coding sequences in the correct reading frame, so many of the rearrangements are non-productive. If the B cell makes a non-productive rearrangement, the HC will not be expressed on the surface and the cell will die. Once a functional HC is produced, it pairs with the surrogate LC (VpreB & λ5), and gets expressed on the surface. This is known as the pre-BCR, which sends signals to proliferate and rearrange the LC. The LC has only V and J segments, which are joined during the pre-B stage of development. If this rearrangement is functional and it pairs with the HC, then the BCR will be expressed on the surface, and rearrangement ceases.

V(D)J recombination is imperative for B and T cell development. In the absence of RAG protein, B cell development stops at the pro-B stage. Mice with RAG deficiency are very useful as chimeric hosts for analyzing donor hematopoiesis since they are unable to produce mature lymphocytes. Humans with RAG mutations are diagnosed with severe combined immunodeficiency (SCID), which is characterized by the lack of mature lymphocytes, resulting in many infections. Other forms of SCID result from mutations in the other recombinase complex proteins, indicating the importance of this process for the development of a competent immune system.

Pre-BCR Signaling

Pre-BCR signaling is necessary for B cell development, proliferation, and recombination of the LC. The pre-BCR is composed of two rearranged HCs and two surrogate LCs (heterodimers of VpreB & λ5), which associate with the signaling components Igα and Igβ. Expression of these proteins on the surface of the cell is necessary for B cell development progression beyond the pro-B stage. Mice lacking the

transmembrane region of the HC ($\mu MT^{-/-}$) or the genes encoding λ5 or VpreB have an expansion of pro-B cells and progression further in development is impaired [35-37]. The mice lacking the surrogate LC (SLC) do have some mature B cells that develop due to early LC expression and pairing with the HC on the surface to provide the necessary signal for proliferation. This does not occur in humans, however, since the lack of λ5 expression results in agammaglobulinemia and a more complete block in B cell development [38].

Not only is surface expression of the pre-BCR necessary, but signaling through the pre-BCR is crucial for B cell development as well. In mice lacking Igα or Igβ there is also a block in development at the pro-B stage, even though V(D)J recombination and HC expression are normal [39]. BLNK- and Syk-deficient mice have blockages at the pre-B stage, where they express the pre-BCR but are unable to clonally expand or induce LC recombination [40, 41]. These are two important signaling proteins that will be discussed in the BCR signaling section.

Pre-BCR signaling is necessary not only for B cell development, but also for allelic exclusion, which ensures that BCRs of a single specificity are expressed on the cell surface. The pre-BCR signal prevents the second HC allele from undergoing rearrangement by downregulation of RAG1/2 expression [42]. However, pre-BCR signaling is also important for initiating LC rearrangement and inducing expression of RAG1/2 after a few rounds of proliferation [43]. The signals through the pre-BCR that mediate RAG expression are poorly understood. RAG1/2 expression is necessary for homologous pairing of LC alleles, which is critical for LC allelic exclusion [44]. Hewitt et al. found that when homologous pairing of *Igh* or *Igl* occurs, one allele is

associated with euchromatin and is actively being transcribed and rearranged, while the other allele is associated with pericentromeric heterochromatin, which represses transcription and recombination. Pre-BCR signaling may also inhibit HC rearrangement by attenuating the IL-7R signal, which allows for histone deacetylation and inactivation of the HC V gene locus [45]. One possible mechanism for this inhibition is through interferon regulatory factors 4 and 8 (IRF4 and 8). Pre-BCR signaling induces IRF4/8 expression, which induces Ikaros and related transcription factor Aiolos. These transcription factors downregulate the pre-BCR by suppressing the SLC and inhibiting the cell cycle transition from G1 to S [46]. IRF4/8 play redundant roles, and only when both are knocked out is B cell development perturbed [47]. These double knockout B cells are blocked at the large pre-B stage, where they are hyperproliferative, suggesting they negatively regulate pre-B cell expansion.

Recently the mechanism of induction of LC recombination has been elucidated [48, 49]. FOXO1 and FOXO3a are FOX class O transcription factors that are necessary for HC recombination [50] and activation of LC recombination [48] via the expression of the RAG protein complex. These FOXO transcription factors are inhibited by PI3K signaling but induced by BLNK expression. The most recent model of LC recombination regulation postulates that the expression of the pre-BCR on the surface of the pre-B cell induces PI3K signaling through Akt that inhibits FOXO transcription factors and induces a few rounds of proliferation [51]. Then by an unknown mechanism, BLNK counteracts the PI3K signal and allows the FOXO transcription factors to induce RAG1/2 expression and promote LC recombination. The model is supported by evidence that inhibition of PI3K at the immature B cell stage results in re-

expression of RAG1/2 [48, 52]. Additionally, cultured B cells lacking the regulatory subunit of PI3K, p85α, show increased *Rag1/2* transcription and increased LC recombination [53]. Moreover, BLNK-deficient mice have a block in B cell development at the pre-B stage owing to the lack of downregulation of λ5 and impaired induction of RAG1/2 [54]. BLNK may activate LC recombination through PKCη and IRF4 [55], however, PKCη is not substantially expressed in B cells [56].

LC recombination is thought to occur in a certain time frame, with κ rearranging first, and if time allows, λ following [57, 58]. FOXO proteins initiate an apoptotic program, which may provide the fixed time frame for LC recombination [59]. To support this idea, overexpression of anti-apoptotic protein bcl-2 allows a longer lifespan and more λ^+ B cells [60]. These recent findings regarding the PI3K-Akt-FOXO pathway have greatly advanced our understanding of the control of LC recombination, but questions still about the mechanism of BLNK inhibition of this pathway.

One of the main questions remaining about pre-BCR signaling is whether or not it is induced by a ligand. Ligands that bind the pre-BCR have been identified on stromal cells [61, 62]; however, *in vitro* experiments have shown pre-B proliferation and development in the absence of BM stromal cells [63]. In the absence of ligand, aggregation of pre-BCR complexes may be enough to initiate signaling. The positively charged non-Ig region of λ5 is necessary for the initiation of pre-BCR aggregation and signaling [64], and has also been found to bind many molecules, including DNA, LPS, and galectin, one of the suggested pre-BCR ligands expressed on stromal cells [62, 65, 66]. The signals downstream of the pre-BCR are thought to be largely the same as BCR signals [61], which will be discussed in detail in the next section. The pre-BCR signal

is imperative for B cell survival, expansion, developmental progression, HC allelic exclusion, and LC recombination in B cells.

BCR Signaling

The BCR sends signals that are important for B cell development, proliferation, and survival. The BCR has no cytoplasmic domain, so the signals are propagated through Igα and Igβ (CD79a & CD79b, respectively), which have immunoreceptor tyrosine-based activation motifs (ITAMs). Antigen binding leads to BCR aggregation in lipid rafts, which are microdomains enriched for sphingolipids, cholesterol, and the tyrosine kinase Lyn [67]. The tyrosines in the ITAMs are phosphorylated primarily by Lyn, but can also be phosphorylated by other Src-family kinases, Fyn, Lck or Blk [68]. This begins a cascade of signaling propagated mainly through phosphorylation. Once the ITAMs are phosphorylated, Syk binds and is phosphorylated by another Syk molecule or a Src-family kinase also bound to Igα or Igβ. The phosphorylation of Syk results in the formation of the signalosome, consisting of BLNK, PLCγ2, Btk, PI3K, Vav, PKCβ, Carma1, as well as other proteins (Figure 3).

CD19 is also phosphorylated by Lyn, which creates binding sites for the SH2 domain of phosphatidylinositol-3-kinase (PI3K), bringing it in close proximity to the cell membrane, where it can generate phosphatidylinositol 3,4,5-triphosphate ($PI(3,4,5)P_3$) from the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$). $PI(3,4,5)P_3$ is essential for recruitment of PH-domain containing proteins, such as Btk, to the plasma membrane. Btk is important for B cell maturation and responsiveness to antigen stimulation, as demonstrated by a mutation in the PH-domain

of Btk (*xid*), which inhibits recruitment to the plasma membrane. Mice with this mutation or the absence of Btk have fewer B cells and fail to proliferate in response to IgM stimulation [69]. Humans with mutations in *btk* have a more severe disease, X-linked agammaglobulinemia, which is characterized by an almost complete absence of B cells, which are blocked at the pro-B to pre-B transition [70], indicating the importance of pre-BCR signaling to B cell survival.

There are many adaptor proteins involved in BCR signaling, which have domains that bring other important proteins together. The most proximal of these adaptors is BLNK, which, when phosphorylated by Syk, recruits Btk and PLCγ2. BLNK is essential for PLCγ2 recruitment to the plasma membrane, where it cleaves $PI(4,5)P_2$ into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) [71]. IP3 causes the mobilization of Ca^{2+} from intracellular and extracellular stores, which activates PKCβ, calmodulin, and NFAT. DAG also activates PKCβ, a kinase important for the activation of NFκB and survival [72]. The amplitude and duration of Ca^{2+} signaling direct two different pathways. The NFAT pathway is activated in response to sustained elevated Ca^{2+} signaling, while NFκB activation is dependent on the amplitude of the Ca^{2+} signal [73].

Phosphorylated BLNK binds Grb2, which binds Sos and activates the Ras/MAPK pathway [74]. Rac also gets activated by phosphorylated BLNK via binding of the guanine nucleotide exchange factor Vav, which activates MEKK, whose downstream targets are p38 MAPK and JNK [75]. Additionally, BLNK has an affect on cytoskeletal rearrangement by binding the adaptor Nck, which causes the activation of Rho, a regulator of the actin cytoskeleton [75]. Many of these pathways are

interconnected and can be activated by other means, such as Ca^{2+} activating JNK and DAG inducing the activation of Ras via RasGRP.

BCR signaling is important for B cell survival, since B cells lacking a BCR signal die and B cells with very strong BCR signals are deleted. Inducible deletion of Igβ at the immature B cell stage leads to cell death, demonstrating that the BCR signal is necessary for survival [76]. The strength of the BCR signal is regulated by several co-receptors on the B cell surface. BCR signaling is enhanced by CD19 and CD45, but attenuated by CD22, FcγRIIb and PIR-B. CD19-deficient B cells have less tyrosine phosphorylation and proliferation, owing to the inability to activate PI3K and Btk [77]. CD45 acts on the BCR signal primarily by keeping Lyn from being phosphorylated by Csk at an inhibitory residue [78]. Lyn has both positive and negative regulatory roles in BCR signaling. Lyn phosphorylates CD22 in the immunoreceptor tyrosine-based inhibitory motif (ITIM), which negatively regulates the BCR signal by recruiting SHP-1, a phosphatase that inactivates Igα, Igβ, Syk, Vav, CD19, and BLNK [79]. While there is redundancy in the positive role for Lyn (i.e. Fyn, Blk), there is no redundancy in the inhibitory role – Lyn-deficient mice develop lethal antibody-mediated glomerulonephritis owing to hyperactive B cells [80]. Lyn also phosphorylates the ITIM of FcγRIIb, which recruits the phosphatase SHIP and inhibits $PI(3,4,5)P_3$ accumulation, PI3K binding to CD19, and Ras activation [77]. PIR-B contains multiple ITIMs, which are constitutively phosphorylated and bound to SHP-1 [77]. PIR-B is more highly expressed on activated B cells than naïve B cells, suggesting that it is regulated by cell stimulation [81]. Regulation of the BCR signal is imperative for

proper B cell function – too much signaling induces autoimmunity and too little impairs B cell development.

Most of the work cited above was done on mature B cells, however there are several differences between mature and immature B cells with regards to BCR signaling. Lyn is expressed at a higher level in immature B cells, while Fyn and Fgr (other Src-family kinases) are expressed more in mature B cells [82]. PLCγ2, Btk, and BLNK are also expressed at higher levels in immature B cells, while Syk expression is decreased [83]. Inhibitory co-receptors CD22 and FcγRIIb increase with maturity as well [84], though immature B cells may not need as much inhibition because the BCR is not associated with lipid rafts as in mature B cells, so it has less accessibility to downstream signaling components [85]. Instead of inducing proliferation as in mature B cells, BCR stimulation at the immature stage induces receptor editing [86, 87], which is critical for self-tolerance. BCR signaling at the mature B cell stage induces higher expression of antiapoptotic proteins bcl-xL and A1 [88]. Bcl-2 expression is also higher in mature B cells, indicating that mature B cells are more resistant to apoptosis than immature B cells after BCR stimulation.

Similar to the pre-BCR, the question remains whether BCR stimulation is necessary for signaling at the immature B cell stage, or whether aggregation of BCR complexes is enough to trigger the BCR signal necessary for survival. When the BCR is deleted on mature B cells, they are unable to survive in the periphery [89]. Tze et al. have shown that when the BCR is deleted at the immature B cell stage, the cells "back-differentiate" and express proteins needed for LC rearrangement [52]. This suggests

that there may be a signal from the BCR that inhibits LC rearrangement, and abrogation of this signal allows expression of RAG1/2, inducing rearrangement.

The BCR signal must be carefully balanced to signal enough to keep B cells alive and avoid immunodeficiency, but not signal extensively and induce autoimmunity. Many autoimmune patients have mutations in attenuators of BCR signaling and numerous mouse models with improper BCR signaling develop autoimmune diseases. Syk-deficient mice show impaired LC allelic exclusion in fetal liver organ cultures, but the mice die *in utero* so the involvement of Syk in autoimmunity is difficult to study [90]. CD19, which facilitates BCR signaling, only needs to be increased 15 – 30% to induce autoimmunity in mice [91] and has been implicated in systemic sclerosis in humans [92]. In mouse models either deletion of Lyn or hyperactivation of Lyn results in a lethal antibody-mediated autoimmune disease [93]. Both of these mutations result in increased signaling from the BCR because Lyn has both positive and negative regulatory roles in BCR signaling. Lyn expression has been found to be lower in lupus patients than healthy controls, however these were small studies and need to be validated further [94, 95].

Hyper-signaling B cells can also be generated by mutations in inhibitory molecules, such as FcγRIIb, CD22, SHP-1, and SHIP. FcγRIIb has been implicated in autoimmunity by mouse models, as well as lupus and rheumatoid arthritis patient studies. FcγRIIb-deficient mice develop lupus-like disease [96], and genetic studies have shown significant linkage to the region containing *fcgr2* (among other genes) in lupus, rheumatoid arthritis, and diabetes patients [97-99]. CD22-deficient mice have high-affinity antibodies towards dsDNA and other self-antigens but do not develop

autoimmune disease [100]. SHP-1-deficient mice develop autoimmune disease [101, 102], but there are no clear studies that implicate SHP-1 in human autoimmune disease. Similarly, SHIP has not been implicated directly in human autoimmune disease but the knockout mice have hyperactive B cells but not autoimmunity [103]. Thus, the BCR signal is necessary for B cell developmental progression and survival, but must be tightly regulated to avoid autoimmunity.

Receptor Editing

In order to avoid autoimmunity, B cells must be tolerant to self-proteins. Tolerance is induced by clonal deletion of self-reactive cells, receptor editing of self-reactive receptors, or anergy, a non-responsiveness to antigen binding. The choice of tolerance mechanism is dependent on the BCR affinity for self-antigen. Clonal deletion generally happens to B cells that bind with high affinity to self-proteins in the BM. Receptor editing also occurs in the BM, to B cells that bind self-antigen with less affinity. If self-reactive B cells happen to escape from the BM, they can be tolerized in the periphery by anergy. Estimates are that up to 75% of BCRs generated by V(D)J recombination are self-reactive, however most of these B cells are deleted or edited in the BM [31].

Receptor editing was initially described by Nemazee [104] and Weigert [105] in 1993. They used transgenic (Tg) mouse models with a pre-rearranged self-reactive BCR to show that the self-reactive HC could pair with an edited LC in order to avoid auto-reactivity. Nemazee used an anti-MHC class I transgenic mouse to show that in addition to clonal deletion, some BM IgM^+ B cells were expressing RAG transcripts

and rearranging the LC λ locus. B cells found in the periphery expressed the Tg HC and the λ LC instead of the Tg LC and no longer bound the self-antigen. The authors concluded that instead of deleting all self-reactive B cells, some are saved by editing the LC. Weigert also came to the same conclusion but used a different Tg mouse, which had a BCR specific for dsDNA. They also found that cells in the peripheral lymphoid organs expressed the Tg HC paired with an endogenous LC rather than the Tg LC. These publications provided the initial evidence for receptor editing and provided the impetus for analyzing the mechanism of receptor editing and its involvement in autoimmune diseases.

Receptor editing most commonly occurs at the κ LC. In the mouse the κ LC rearranges before the λ LC due to an enhancer in the κ locus. Half of λ LC expressing mouse B cells show evidence of editing at the κ LC [106]. In the mouse the κ LC has approximately 140 V gene segments and only four functional J gene segments [107]. LC recombination brings together a V and a J segment, while secondary rearrangement brings together an upstream V and downstream J, resulting in excision of the primary recombination (Figure 4). Alternatively, the other κ LC allele could be rearranged, or rearrangement could occur at the λ locus. In order for rearrangement of either the HC or the LC, the locus must be 'open' to the recombinase machinery, which occurs with transcription.

The κ LC locus contains the V and J gene segments, two enhancers, two promoters and the constant region (Figure 4). Transcription is necessary for the recombinase complex to bind to the RSSs. Many transcription factors have binding sites in the enhancer regions, including NFκB, PU.1, IRF4, Pax5, and E2A [108].

These binding sites are in close proximity, so regulation of LC rearrangement may occur by transcription factors expressed at different developmental stages. When the LC is inactive at the pro-B stage, Pax5 is bound to the Eκ3' enhancer, but not at the pre-B stage. At the pre-B stage PU.1 is bound to the Eκ3' enhancer, allowing for activation of the κ locus [108]. IRF4 is induced by pre-BCR signaling, which activates the Eκ3' enhancer, induces cell cycle exit, and attenuation if IL-7R signaling [109, 110]. IRF4-deficient mice have defective receptor editing, which is more severe at the λ LC than the κ LC [111]. E2A binds both κ and λ LC loci, and loss of only one copy of E2A is enough to perturb B cell development and λ LC expression [112]. The mechanism of transcriptional regulation at the λ locus is not well understood.

Studies by Tze et al. have previously shown that receptor editing is a consequence of binding membrane bound antigen at the immature B cell stage [113]. They also showed that the level of IgM on the surface of a B cell (sIg) correlates with receptor editing [114]. After binding antigen, the receptor is downregulated and signaling through the BCR ceases [115]. Internalization of the BCR is regulated by ubiquitinylation of tyrosines in Igα and Igβ, while BCR signaling is regulated by phosphorylation of these tyrosines [115, 116]. Based on evidence that deletion of the HC at the immature B cell stage results in a global 'back differentiation' gene expression pattern [52], it is possible that after binding self-antigen at the immature B cell stage the receptor is internalized, signaling ceases, allowing expression of genes necessary for receptor editing. This possibility contradicts the generally accepted hypothesis that there is a signal through the BCR that activates receptor editing after ligation of the self-ligand to the receptor. In CD19-deficient mice, the tonic signal is

decreased due to the lack of PI3K activation, which causes increased RAG expression and stimulates receptor editing [117]. Use of a PI3K inhibitor in these same cells reduces the tonic signal, RAG expression, and level of receptor editing [118]. In addition, mice deficient in the PI3K p110δ catalytic subunit [119] or the p85α [53] regulatory subunit fail to suppress RAG expression at the immature B cell stage and induce excessive receptor editing. We propose that there is a tonic signal through the BCR that inhibits receptor editing in the absence of self-ligand, and when the ligand binds, the receptor is internalized, signaling stops, and receptor editing proceeds. Experiments in this thesis will address this important question.

Autoimmunity is characterized by a defect in central tolerance, either receptor editing or deletion. Patients with rheumatic autoimmune diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), fail to remove autoreactive B cells in the BM and the periphery [120, 121]. In the blood of lupus patients there are more self-reactive B cells, but it is unknown whether this results from defects in editing or deletion [121]. Patients with other autoimmune diseases also show defects in receptor editing – secondary LC recombination is defective in some RA patients [120], and RAG expression is reduced in juvenile idiopathic arthritis patients [122]. Alternatively, some studies show an increase in receptor editing or RAG expression in autoimmune patients [123-125]. In the periphery, RAG is expressed in a small subset of sIg^- B cells in the germinal centers of human tonsils [126]. This phenomenon, termed receptor revision, is rare and somewhat controversial, but may generate autoantibodies in the periphery [127]. In a mouse model of lupus, the MRL/lpr strain, the B cells are defective in receptor editing, specifically, there is reduced RAG

induction and reduced downregulation of IgM and CD19 [128]. Another mouse model of autoimmune disease, the NZB mouse, has more immature B cells with low RAG expression and receptor editing [129]. The continued study of these central tolerance mechanisms in both mice and humans will aide in the development of treatments for autoimmunity, possibly providing new drug targets or increasing efficacy of current treatments.

B cell Acute Lymphoblastic Leukemia

B cell malignancies have been found at all stages of B cell development and analysis of these cells has helped define B cell developmental stages. Expansion of the lymphoid progenitor cells is known as acute lymphoblastic leukemia (ALL), and is the most common cancer diagnosis in children [130]. The disease-free survival rate for children is 80 – 90%, however, the survival rate for adults is only about 50% [130]. Nearly 80% of ALL cases involve expansion of B-lineage cells, and these cases can be classified based on the stage of B cell development. The most common type is common precursor B-ALL (nearly 80% of B-ALL cases), characterized by CD10 expression but no cytoplasmic Ig. CD10 positive cases with cytoplasmic Ig are classified as pre-B-ALL and cases with neither CD10 nor cytoplasmic Ig are classified as pro-B-ALL.

Translocations are often found in leukemic B cells and can help predict outcome. The most common translocation in children with ALL is TEL-AML1 t(12;21), which is correlated with a relatively good treatment outcome [131]. This translocation involves two transcription factors, which results in repression of normal transcription of genes necessary for HSC differentiation. In adults, the most common

translocation is BCR-ABL t(9;22), which is associated with a poor treatment outcome [131]. Here BCR stands for breakpoint cluster region, a gene with unknown function in normal cells. ABL is a tyrosine kinase, and when fused to BCR, is constitutively active, phosphorylating many targets, including STAT5, and altering signaling pathways that control proliferation, survival and self-renewal [132]. Gleevec (Imatinib), a specific inhibitor of the BCR-ABL fusion protein, can be used to treat ALL patients with this translocation. However, treated cells often develop resistant mutations in the ABL kinase domain, so new tyrosine kinase inhibitors are being developed to treat these patients [133]. Other common translocations are E2A-PBX1 and FLT3-ITD, and several have been found that involve MLL or MYC. These fusion proteins promote leukemic transformation by altering cell growth, enhancing cell survival, and blocking lymphocyte differentiation. A substantial portion of ALL tumors lack common translocations and are characterized by hyperdiploidy, with greater than 50 chromosomes. ALL is a heterogeneous disease, with many possible instigators of leukemogenesis.

Although the identification of translocations is important for diagnosis, the molecular mechanisms underlying the development of ALL have yet to be elucidated. A recent study has reported that nearly 40% of ALL patients show loss-of-function mutations in genes involved in B cell development, such as *pax5, ikzf1* (Ikaros)*,* and *ebf1* [134]. Ikaros deletions were the most common, and correlated strongly with a poor outcome for ALL patients, independent of any translocations. These patients have a 73% chance of relapse, while patients without the Ikaros mutation have a 25% chance of relapse within 5 years [135]. EBF1 correlated with poor outcome only in the high-

risk patients, and Pax5 did not correlate with poor outcome at all. The same group found mutations in JAKs in 10.7% of the ALL cases they analyzed, which were BCR-ABL negative but high-risk [136]. These whole genome array studies are helpful in defining the genetic lesions of patients without translocations, and have also been helpful in identifying factors for relapse.

The question remains whether these mutations drive the induction of leukemia or are a result of genomic instability in leukemic cells. Targeted deletion of these genes in mice shows a block in B cell development but no malignancies [2, 14, 137]. Pax5 and EBF1 each bind to the other's promoter, and B cells lacking either gene are arrested early in B cell development [137-139]. BLNK-deficient mice also show a block at the pre-B stage, but have a low incidence of pre-B-ALL (~5%) [140]. The tumor incidence increases dramatically when mice are deficient for both BLNK and Btk, indicating that the pre-BCR signal may be important for suppressing leukemia [141]. One small study has found BLNK protein missing in 16 of 34 pediatric pre-B ALL samples [142]. Thus, it appears that genes involved in B cell development play a role in the development of ALL.

The identification of a molecular mechanism that drives leukemogenesis will ultimately aide in the development of better, more specific treatments for ALL patients and the improvement of the cure rate. ALL is the second leading cause of cancer deaths among children, so even though the cure rate is 80% or higher, many children still succumb to death from it each year. The development of mouse models for ALL has been difficult since the disease is very heterogeneous. There have been a few generated with the BCR-ABL translocation and loss of tumor suppressor genes that has allowed

testing of current human treatments [143, 144]. The further development of these and other mouse models will help predict outcome to treatment and may aide in the development of new treatments. The development of a new mouse model of ALL will be discussed in Chapter 4 of this thesis.

Objectives

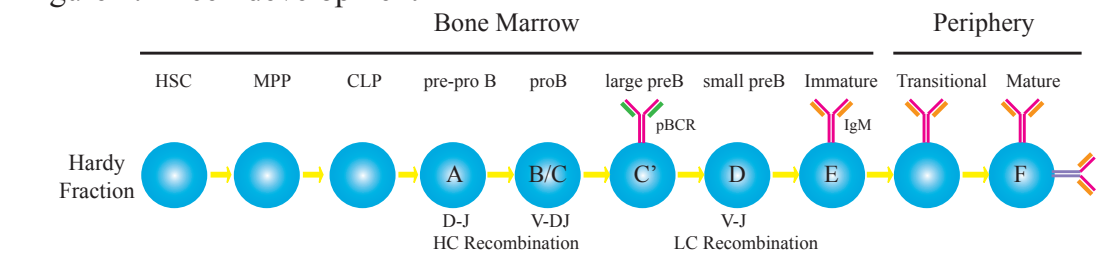
There are many unanswered questions in the B cell field, but one of paramount importance is how B cells are tolerized to avoid autoimmunity. The majority of developing B cells are probably self-reactive, and many that make it through B cell development to the periphery show evidence of receptor editing. The question remains as to how the B cells signal to re-induce the editing machinery, whether it is a positive reactivation signal or the release of an inhibitory tonic signal. We propose that it is the release of a tonic signal through the B cell receptor that inhibits recombination when the BCR is present on the surface, but when the BCR binds self-antigen it is internalized, the inhibitory signal is abrogated, and the recombination machinery is reactivated. We tested this hypothesis using several transgenic models, including RAG2-GFP mice, HEL Ig mice, and anti-κ mice. We also used several mice deficient in BCR signaling to test this hypothesis, and found that reduced BCR signaling induces more receptor editing. When we tested mice with excessive BCR signaling or pharmacologically mimicked the BCR signal, we found a decrease in receptor editing. These experiments provide compelling evidence for the inhibitory tonic signaling hypothesis and against the activation signaling hypothesis.

Another question unanswered in the B cell field is how B cells are transformed into malignant cancers. There have been whole genome analysis studies to show that genes involved in the BCR signaling pathway are involved in many B cell malignancies, from leukemia to lymphoma and myeloma. Our studies provide evidence that in combination with constitutive STAT5 activation, a loss of genes involved in B cell development or signaling results in leukemia. These are genes or proteins that have

been shown to be mutated or absent in B cell acute lymphoblastic leukemia. We also demonstrated an increase in the phospho-STAT5 levels in adult BCR-ABL^{+} ALL patients. Herein we have developed a new mouse model for B-ALL that may be useful in elucidating the origin and progression of ALL in humans.

These two important questions are tied together through BCR signaling, so when we fully understand the complex BCR signaling cascade we will have further insight into both receptor editing and B cell leukemia. We have analyzed mice with abnormal signaling to provide insight into both questions. Our results demonstrate that BCR signaling at the immature B cell stage inhibits receptor editing and pre-BCR signaling at the pre-B stage inhibits leukemia.

Figure 1. B cell development



	HSC	MPP	CLP	pre-pro B	proB	large preB	small preB	Immature	Transitional	Mature
Flt3	-	+	+	+	-	-	-	-	-	-
IL-7Rα	-	-	+	+	+	+	+/-	-	-	-
B220	-	-	-	+	+	+	+	++	++	+++
CD19	-	-	-	-	+	+	+	+	+	+
CD43	+	+	+	+	+	+	-	-	-	-
CD79a/b (Igα/β)	-	-	-	-	+	+	+	+	+	+
SLC (λ5, VpreB)	-	-	-	-	-	+	-	-	-	-
RAG1/2	-	-	-	-	+	-	+	-	-	-
CD21	-	-	-	-	-	-	-	-	+/-	+
CD22	-	-	-	-	-	-	+/-	+	+	+
CD23	-	-	-	-	-	-	-	-	+/-	+
CD24 (HSA)	+	+	+	+	+	++	++	++	+/-	-
CD25	-	-	-	-	-	+	+	-	-	-
IgM	-	-	-	-	-	+	-	+	+	+
κ/λ LC	-	-	-	-	-	-	-	+	+	+
IgD	-	-	-	-	-	-	-	-	-	+

Developmental Blockages	Ikaros PU.1	E2A EBF1 IL-7R	IL-7	RAG μMT Pax5 λ5, VpreB	Blnk Syk

Figure 1. Murine B cell development.

Shown are selected expression markers involved in characterizing B cell development. Developmental blockages of selected knockouts are shown at the bottom. Hardy Fraction refers to the naming strategy defined by Hardy et al [145]. HSC, hematopoietic stem cell. MPP, multipotent progenitor. CLP, common lymphoid progenitor. SLC, surrogate light chain. HSA, heat stable antigen. See text for details.

Figure 2. Transcription Factor Network Involved in B cell Development

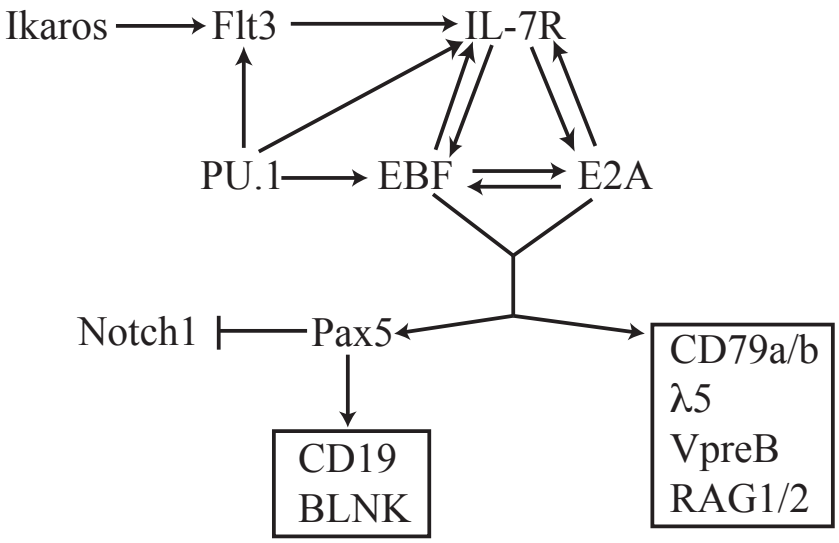


Figure 2. Transcription Factor Network Involved in B cell Development

Arrows indicate activation, perpendicular lines indicate inhibition. See text for details.

Figure 3. B cell Receptor Signaling Pathway

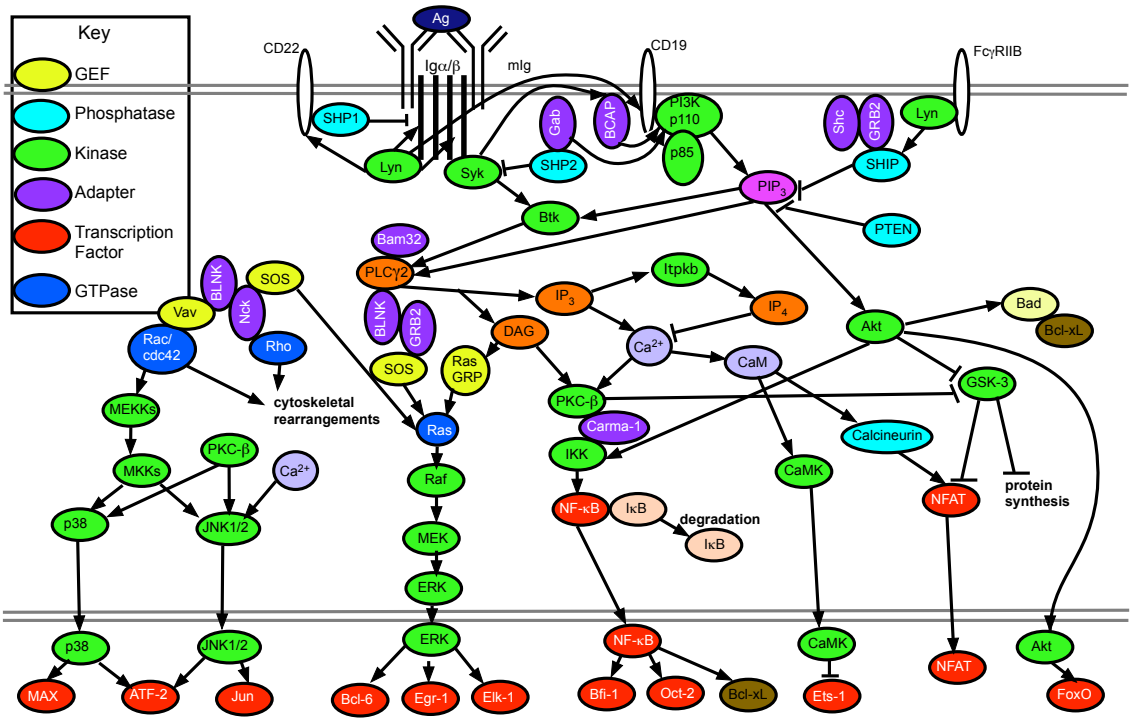


Figure 3. BCR Signaling Pathway

See key for color description. Arrows indicate activation, while perpendicular lines indicate inhibition. See text for details.

Figure 4. V(D)J Recombination & LC Receptor Editing

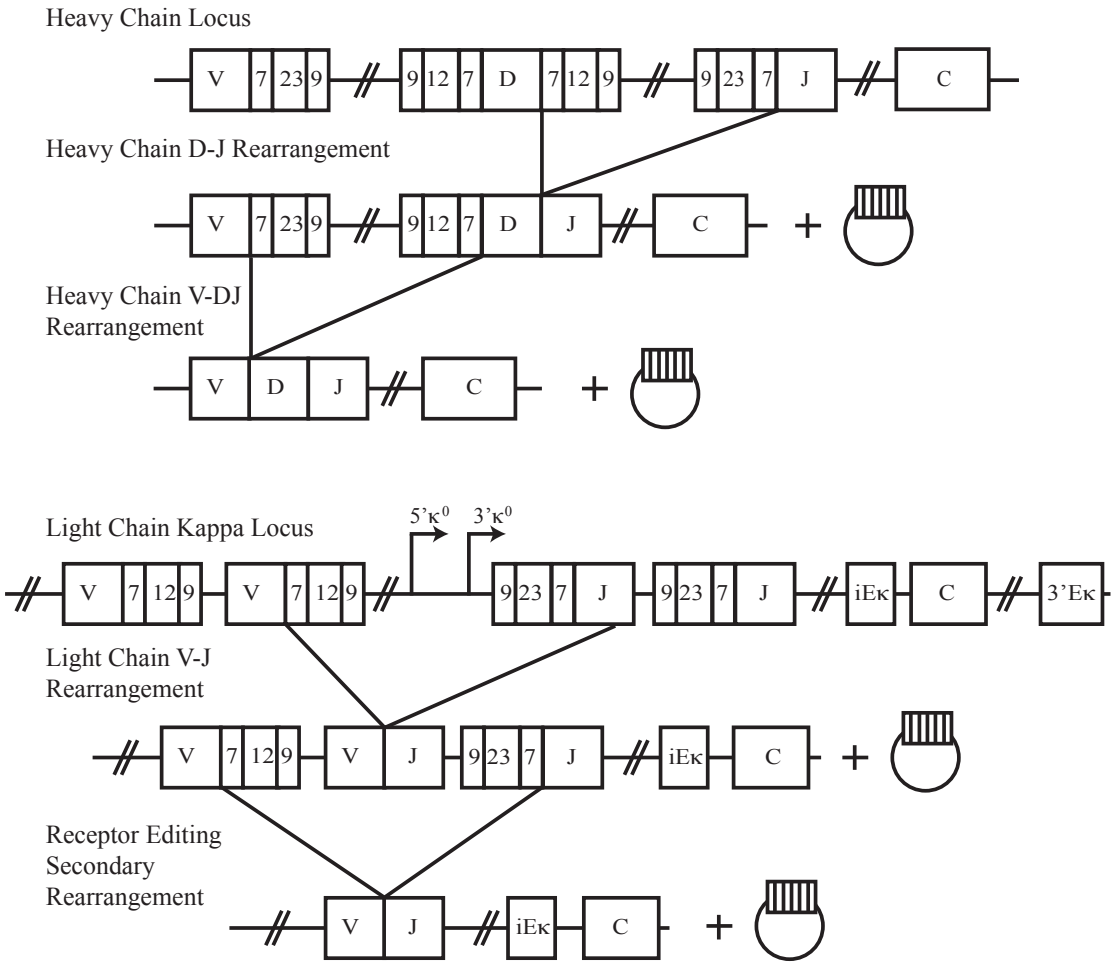


Figure 4. V(D)J Recombination & Receptor Editing

Heavy chain V(D)J recombination at the top and κ light chain recombination and receptor editing on the bottom. V, Variable gene segment. D, Diversity gene segment. J, joining gene segment. C, constant region gene segment. 7, heptamer. 9, nonamer. 12, 12 bp spacer. 23, 23bp spacer. iEκ, intronic κ enhancer. 3'Eκ, 3' κ enhancer. 5'κ^0, 5' sterile transcription start site. 3'κ^0, 3' sterile transcription start site. See text for details.

Chapter 2

BCR Basal Signaling Regulates Antigen-Induced Ig Light Chain Rearrangements

Laura B. Ramsey*[†], Brian R. Schram*[†], Lina E. Tze*[†], Jiabin Liu[†], Lydia Najera[†], Amanda L. Vegoe[†], Richard R. Hardy[‡], Keli L. Hippen[†], Michael A. Farrar[§], and Timothy W. Behrens[¶]

* Contributed equally

[†]Center for Immunology, Department of Medicine, University of Minnesota Medical School, Minneapolis, MN 55455; [‡]Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111; [§]Center for Immunology, Department of Lab Medicine & Pathology, University of Minnesota, Minneapolis, MN 55455; [¶]Immunology, Tissue Growth & Repair, Exploratory Clinical Development, Genentech, Inc., 1 DNA Way, S. San Francisco, CA 94080.

Summary

Receptor editing in the bone marrow contributes to B cell tolerance by orchestrating secondary immunoglobulin (Ig) rearrangements in self-reactive B cells. While recent evidence suggests that a majority of developing B cells may undergo receptor editing, the mechanisms that control this process are not well understood. Here, we provide evidence that editing in developing murine B cells is controlled at the level of basal B cell receptor (BCR) signaling. Immature B cells lacking the proximal BCR signaling molecule *btk* show normal expression of a recombination activating gene 2 (RAG2) – GFP BAC reporter protein following antigen incubation and receptor downregulation, despite impaired proximal BCR signaling. Incubation of immature B cells with self-antigen leads to a striking reversal in differentiation to the pro/pre-B stage of development, similar to that observed when basal BCR signaling is interrupted in immature B cells by inducible deletion of heavy chain or pharmacologic blockade of proximal BCR signaling pathways. Importantly, RAG induction, the 'back-differentiation' response to antigen, and editing in immature and pre-B cells is inhibited by a combination of phorbol ester and calcium ionophore, agents that bypass proximal signaling pathways and mimic BCR signaling. These findings support a model whereby antigen-induced receptor editing is controlled at the level of basal signaling provided by the BCR on the cell surface of developing B cells.

Introduction

Receptor editing was first described by Nemazee and Weigert [104, 105] in conventional transgenic systems where B cells altered their immunoglobulin (Ig) receptors in response to self-antigen. The design of the κ light chain (LC) locus, with variable (V) and joining (J) gene segments but no diversity (D) regions (such as those found at the heavy chain locus), allows for the editing of LCs through a series of nested deletions (or inversions in the case of certain germline Vκ genes oriented 3' to 5' compared to the bulk of the κ locus). Editing of heavy chain (HC) alleles by V_H gene replacement has also been described [146, 147], but is probably relatively uncommon compared to LC editing. A series of anti-DNA and anti-MHC Class I Ig knockin models [147-151] provided further evidence that secondary recombination at LC loci was important for maintaining tolerance to self-antigens, and suggested that editing was highly efficient [150, 151].

Immature cells exposed to self-antigen are induced to re-express RAG1 and RAG2 (or continue RAG expression from the pre-B stage) [152, 153]. Studies with mice carrying a RAG2-GFP BAC reporter transgene indicated that RAG expression is still apparent in immature B cells in the bone marrow (BM) [154, 155], and there is evidence that RAG2-GFP^{neg} IgM^{hi} immature B cells remain competent to reinitiate RAG1/2 gene transcription following receptor crosslinking by antigen [156]. The kinetics of LC replacement was examined in an experiment where the mouse Cκ constant region was replaced with human Cκ (hCκ) [157]. Heterozygous hCκ mice with a knockin anti-HEL LC, and lacking a pre-rearranged Ig HC, demonstrated the appearance of hCκ$^+$ cells with about a 2-hour delay compared to mCκ$^+$ cells. How

much of this LC replacement was due to self-reactivity vs. poor pairing of the anti-HEL LC with endogenous HCs is not clear. Similar kinetic data was observed in a knockin λ LC model [158].

As many as half, or more, of normal B cells in bone marrow (BM) may undergo some degree of editing [106, 157, 159]. Editing has also been suggested to occur in the periphery [160, 161], although there has been debate about whether this represents re-induction of RAG1/2 in mature B cells or the recruitment of immature cells to the spleen [154, 155, 161-163].

Several of the current models for receptor editing emphasize the important role for signals transduced through the crosslinked BCR in turning on, or keeping on, RAG proteins and the recombination machinery [164-166]. However, there are several studies consistent with the idea that positive signaling through the BCR may be required to turn off RAG proteins and maintain allelic exclusion. For instance, mice carrying knockin receptors for the 3-83 HC and LC show very poor allelic exclusion with a single dose of HC and LC, that is only corrected by breeding to homozygosity [167, 168]. One interpretation of these data is that a single dose of the receptor is unable to provide a sufficiently strong basal signal. B cells deficient in CD19 show strong spontaneous upregulation of RAG, loss of allelic exclusion at LC loci, and impaired positive selection [169], suggesting that the lack of positive signaling through the BCR is associated with induction of RAG and failure to progress in development. Similarly, immature B cells lacking the protein tyrosine kinase Syk show loss of LC allelic exclusion [90]. Together, these data support the hypothesis that developing B cells

require threshold signals from Ig receptors expressed on the cell surface to block further Ig gene rearrangements.

We recently reported that deletion of the BCR from immature B cells generated in IL-7 BM cultures following Cre-mediated excision of a floxed HC led to the induction of RAG expression, and new LC rearrangements [52]. Incubation of immature B cells with a tyrosine kinase inhibitor or a PI3K inhibitor led to a similar phenotype. Surprisingly, both loss of the BCR and blockade of proximal signaling pathways resulted in a global 'back-differentiation' response, where cells turned off many genes important for the mature B cell program (e.g. CD22 and Class II MHC) and turned on genes characteristic of earlier stages in B cell development (e.g. IL-7R and TdT).

These observations led us to test the hypothesis that a threshold for basal signaling may also contribute to induction of RAG genes and receptor editing in immature B cells in the HEL model system following self-antigen exposure. Our data support a model whereby basal signaling from the BCR expressed on the cell surface is required to suppress further recombination activity. In this model, self-antigen induces editing by modulating BCR surface expression and hence downregulating basal BCR signaling. In the absence of a sufficient basal BCR signal, RAG proteins are induced and allow for further rearrangements at Ig loci until a non-autoreactive receptor is produced.

Materials and Methods

Mice

RAG2-GFP BAC transgenic (Tg) [155] and human Cκ knockin mice were kindly provided by Dr. M. Nussenzweig (Rockefeller University, New York). RAG2-GFP animals were bred with MD4 anti-HEL Ig (HEL-Ig) Tg mice [170] to generate double Tg animals as described [52]. MD2/LC$^{KI/hCk}$/KLK4 membrane HEL (mHEL) Tg mice were generated by breeding mice carrying the human Cκ LC knockin allele to the animals previously described [113]. Floxed B1-8 HC knockin (B1-8f) [89] and 3-83κ LC knockin [171] mice were bred to RAG2-GFP Tg animals to generate B1-8f/3-83κ/RAG2-GFP animals. *Xid*/CbaN mice [172] were obtained from Jackson Laboratories. *Xid* females were bred with HEL-Ig/RAG2-GFP males to generate HEL-Ig/RAG2-GFP/*xid*$^{-/y}$ male mice, and *xid* males were bred with HEL-Ig/RAG2-GFP females to generate HEL-Ig/RAG2-GFP (WT-CbaN) male mice. For some experiments, control mice for *xid* experiments were HEL-Ig/RAG2-GFP mice of non-CBA background. *Lyn* deficient mice on the B6 background were obtained from Jackson Laboratories, and were bred with HEL-Ig/RAG2-GFP mice for two generations to obtain homozygous *lyn*^{null}/RAG2-GFP animals. The Hel-Ig/RAG2-GFP and B1-8f/3-83 mice were crossed onto C57Bl/6J for at least 6 generations. All mice were maintained in specific pathogen-free conditions, and were generally between 6-12 weeks of age at the time of the experiments. All experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Cell culture

Single cell suspensions of BM cells from the various mice were prepared [114], and placed into IL-7 BM culture [153, 173, 174] as previously described [52]. For secondary antigen cultures, cells were re-cultured in complete medium with HEL (Sigma, St. Louis, MO), Herbimycin A (Calbiochem, San Diego, CA), Ly290042 (Calbiochem), PP2 (Calbiochem), wortmannin (Calbiochem), PMA (4-a-Phorbol 12-myristate 13 acetate; Sigma), and/or Ionomycin (Calbiochem) for the times indicated. The $HEL^{\Delta RD}$ and $HEL^{\Delta RDGN}$ mutants, and DEL have been described previously [114, 175]. TAT-Cre mediated deletion of the floxed B1-8 HC was as described [52].

Flow cytometry and cell sorting

BM cells harvested at the end of IL-7 or secondary cultures were stained in FACS buffer (PBS, 2.5% FBS, 0.2% sodium azide) with either FITC-, PE-, CYC-, APC- or biotin-conjugated monoclonal antibodies to B220, IgM, IgM^a, IgM^b, IgD^a, CD19, CD22, CD23, CD24, CD43, CD69, CD86, integrin α4, integrin β7, PIRA/B, and IA/IE (BD Pharmingen, San Diego, CA). Staining with biotinylated antibodies was revealed by SAv-PE or SAv-APC (BD Pharmingen). In some staining conditions, annexin-V-PE and/or 7AAD (BD Pharmingen and Calbiochem) were used to exclude dead cells. For antigen culture cell sorting, cells were stained with monoclonal antibodies to IgM^a and B220 in staining buffer (1xPBS, 10% FBS) and sorted by FACSVantage (Becton Dickinson, Mountain View, CA). For purification of splenic B cells, cells were enriched using MACS separation with the B cell isolation kit and LS columns as recommended by the manufacturer (Miltenyi Biotec, Auburn, CA). Flow cytometry

analyses were performed using CellQuest (Becton Dickinson) and Flowjo (Treestar, San Carlos, CA) software.

Quantitative PCR

Genomic DNA was isolated from fresh spleen or IL-7 culture-derived cells as described [176]. Endogenous V-Jκ1 rearrangements were detected using a real time PCR assay, modified from the method previously described [176, 177]. The V-Jκ1 PCR was performed with an upstream Vκ degenerate primer: 5'-GGCTGCAG(G/C)TTCAGTGGCAGTGG(A/G)TC(A/T)-3' and a primer that annealed just downstream of Jκ1: 5'-GCCACAGACATAGACAACGGAAGAA-3' [152], with a TaqMan probe covering the Jκ constant region: 5'-TTGCCTTGGAGAGTGGCCAGAATC. All reactions were run using the following cycling conditions: 2 min at 50°C, 10 min at 95°C, then 15 sec at 95°C, and 60°C for 55 cycles. Duplicate samples were analyzed, and normalized using an 18S ribosomal genomic TaqMan probe (Applied Biosystems, Foster City CA).

Microarray gene chip analysis

Total RNA was extracted from the *in vitro* cultured cells, and biotinylated cRNA probes were synthesized and hybridized to U74Av2 probe arrays following standard Affymetrix protocols (Expression Analysis Technical Manual P/N 700218 rev. 2, Affymetrix, Santa Clara, CA), as described [52]. Three or four independent sorts were performed for each sample. All statistical analyses were performed using MS Excel (Microsoft Office X) as previously described [52]. A given transcript was considered to

be undetected if 2 out of 3 of the arrays within the group had detection p-values > 0.05, and transcripts were included for further analysis only if they were present in both control and HEL treated groups. To identify genes that were differentially expressed between control (i.e. BM 48h Ctrl, GFP Ctrl, Cre Ctrl) and experimental populations (i.e. HEL 48h, GFP HA, CreM^{lo}), we used the Student's t-test analysis with the assumptions that the sample groups followed a two-tailed distribution and had unequal variance. For visualization of the arrays, each expression value was divided by the mean of the expression values for the relevant IgM^{+} samples. These ratios were transformed into $\log_2$ space, and subjected to centered average linkage clustering using CLUSTER and visualized by TREEVIEW software [178]. The microarray data have been deposited at GEO (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE2227. The populations included are: **B6 IgM^{neg}** (B220^{+} IgM^{neg} pro- and pre-B cells from polyclonal C57Bl/6 IL-7 cultures; GEO name B6 Mneg), **Fr. D** (B220^{+} CD43^{-} IgM^{-} pre-B cells from Balb/c BM; GEO name FxD), **Fr. E** (B220^{+} IgM^{+}IgD^{-} newly formed B cells from Balb/c BM; GEO name FxE), **CreM^{lo}** (B220^{+}IgM$^{a\text{-}lo}$, B1-8f HC, 3-83 LC Mx-Cre Tg BM cultured 48h with IFN to delete the HC; GEO name Cre Mlo), **Cre Ctrl** (B220^{+}IgM^{hi}, B1-8f HC, 3-83 LC KI cultured 48h with IFN; GEO name Ctrl Mhi), **HEL 48h** (B220^{+} IgM$^{a\text{-}lo}$ GFP^{+}, HEL-Ig Tg IL-7 cultured BM incubated 48h in 1μg/mL HEL; GEO name HEL 48h), **BM 48h Ctrl** (B220^{+} IgM$^{a\text{-}hi}$ GFP^{-}, HEL-Ig Tg IL-7 cultured BM incubated 48h in media; GEO name BM 48h Ctrl), **HEL IgM^{lo}** (B220^{+} IgM$^{a\text{-}lo}$ IgD$^{a\text{-}neg}$ immature B cells from HEL-Ig Tg IL-7 BM cultures; GEO name HEL Mlo), **HEL IgM^{hi}** (B220^{+} IgM$^{a\text{-}hi}$ IgD$^{a\text{-}neg}$ immature B cells from HEL-Ig Tg IL-7 BM cultures; GEO name HEL Mhi), **GFP HA** (IgM^{+} GFP^{+}, RAG2-GFP HEL-Ig

IL-7 cultured BM incubated 24h in 400ng/mL Herbimycin A; GEO name GFPpos), and **GFP Ctrl** (IgM^+ GFP^-, RAG2-GFP HEL-Ig IL-7 cultured BM incubated 24h in media; GEO name GFPneg).

Results

Delayed kinetics of RAG2-GFP expression following antigen stimulation

Mice carrying a BCR transgene specific for hen egg lysozyme (HEL-Ig) were crossed with RAG2-GFP transgenic mice to generate HEL-Ig/RAG2-GFP double transgenic animals. The RAG2-GFP transgene allows the expression of RAG2 to be quantified at the single-cell level by flow cytometry, and RAG2-GFP expression strongly correlates with the induction of new endogenous Ig LC rearrangements in HEL-Ig B cells [52, 156]. To test the hypothesis that antigen-induced receptor editing could be a consequence of downregulation of surface IgM and subsequent loss of basal signaling rather than an 'activation' response due to acute crosslinking of the BCR, we generated *xid*/HEL-Ig/RAG2-GFP mice. *xid* (x-linked immunodeficiency) is a mutation in the pleckstrin homology domain of Bruton's tyrosine kinase (*Btk*), which impairs the recruitment of Btk to the plasma membrane and severely reduces signaling through the BCR. However, receptor internalization is not impaired in *xid* mice [179]. If positive signaling through the BCR is necessary for receptor editing, then that process should be impaired in *xid*/HEL-Ig/RAG2-GFP mice. In contrast, if removal of a basal signal is essential for receptor editing, then this process should be unperturbed or possibly enhanced in these mice.

BM from WT and *xid* HEL-Ig/RAG2-GFP transgenic (Tg) mice was cultured in IL-7 for 5 days to generate large numbers (~60-80 x 10^6) of highly purified populations of IgM^+ immature B cells (≥ 98% of WT $B220^+$ cells are $IgM^{a+}IgD^{a-}$, compared with ≥ 90% for *xid*), as previously described [114]. Cells were washed, stimulated with 1

μg/ml HEL (a concentration that saturates the HEL-Ig BCR [180]), and harvested at 3, 6 or 12 hours for flow cytometry.

Immature B cells treated with antigen undergo an initial 'activation' response as measured by global gene expression (unpublished data) and a rapid upregulation of the cell surface activation proteins CD69 and CD86, together with a downregulation of surface IgM levels over the first 12 hours of culture (Figure 1A,B). Although immature B cells do not induce CD69 and CD86 to the extent seen in mature B cells [83], they still induce robust responses that can be used to indicate the activation status of immature B cells. The decrease in surface IgM levels in response to antigen was rapid in both WT and *xid* B cells (Figure 1E). The expression of CD69 and CD86 in WT cells was approximately 70% of the 12 hour maximum after a 1.5 h pulse of antigen (not shown), and 82% of maximum after a 3 h antigen pulse. Compared to WT cells, *xid* B cells showed impaired induction of the activation antigens CD69 and CD86 at all the time points tested (Figure 1 A, B, D). Despite the strong 'activation' signal provided by antigen, there were only background numbers of RAG2-GFP positive cells through 6 hours of culture in both WT and *xid* cells, with small responses observed in both cultures at 12 hours (Figure 1C). RAG expression can be rapidly induced (within 3h) by acute pharmacologic inhibitors of BCR dependent PI3K activation (e.g. Wortmannin & Ly290042, data not shown). Therefore, the failure to see RAG2-GFP expression is not due to the fact that RAG2 induction is an inherently later response than CD69 or CD86 expression. Rather, we favor the hypothesis that prolonged downregulation of BCR basal signaling is required to induce RAG gene expression.

In order to determine if a prolonged absence of BCR signal was necessary for RAG induction, a series of 'pulse-chase' experiments were performed. WT and *xid* B cells were incubated with antigen for periods of time before washing and returning to culture in the absence of antigen, followed by analysis at 24 and 48 hours. Despite impaired upregulation of activation antigens CD69 and CD86 due to defective BCR signaling, *xid* B cells showed RAG2-GFP responses that were comparable to those observed in control B cells (Figure 2A, B). We observed that the induction of RAG2-GFP lagged significantly behind the 'activation' response, as measured by CD69 and CD86 (Figures 1 and 2). Cells that received a short pulse of HEL (e.g. 3 hours) showed high-level induction of the activation antigen CD69, but had only modest RAG2-GFP upregulation. A prolonged incubation of immature B cells with antigen is necessary for optimal RAG2-GFP upregulation in this system, suggesting that basal BCR signals must drop below a specific threshold in order to induce RAG expression, but not CD69 or CD86. In WT immature B cells, treatment with the PI3K inhibitor Ly290042 for 3h induces RAG expression (data not shown) suggesting that when the BCR signal is completely ablated, RAG induction occurs quite rapidly. We conclude that *xid* B cells have the ability to undergo vigorous receptor editing responses following incubation with antigen, despite impaired signaling downstream of the BCR. The discordance between antigen-induced activation signals and the expression of RAG2-GFP in *xid* B cells prompted us to further analyze the signal downstream of the BCR and its role in receptor editing.

Elevated RAG2-GFP expression in *lyn*^{null} immature B cells

We next examined immature HEL-Ig/RAG2-GFP B cells deficient for the Src family kinase *lyn*, a key mediator of proximal BCR signaling. *Lyn* appears to be involved in positive regulation of BCR signaling in immature B cells by phosphorylating immunoreceptor tyrosine-based activation motifs (ITAMs) of Igα and Igβ, and *lyn* deficient immature B cells show impaired allelic exclusion and developmental progression [90]. *Lyn* deficient animals develop a lethal autoimmune disease with production of autoantibodies and fewer mature B cells in the periphery [181]. Again, we predicted that if positive signaling is necessary for RAG expression, then it should be impaired in *lyn*^{null}/HEL-Ig/RAG2-GFP mice. In contrast, if removal of a basal signal is essential, then RAG expression and receptor editing should be unperturbed or possibly enhanced in immature B cells from these mice.

We used IL-7 BM cultures to generate immature B cells from *lyn*^{null} mice. At the end of five days in culture, there were an elevated number of RAG2-GFP^{+} cells (on average, 6-fold) in *lyn*^{null}/HEL-Ig/RAG2-GFP immature B cells compared to WT B cells (Figure 2C). *Lyn*$^{+/-}$ heterozygous B cells also had increased numbers of RAG2-GFP positive cells compared to controls. Consistent with the findings in *lyn*^{null} cells, treatment of HEL-Ig/RAG2-GFP immature B cells with the Src family kinase inhibitor PP2 resulted in elevated GFP expression as compared to medium alone controls (Figure 2D). PP2 treatment resulted in higher levels of RAG2-GFP induction than were observed in *lyn*^{null} B cells, suggesting that other Src family members may partially compensate in the absence of *lyn*. Together, these findings suggest that Src family kinases contribute to the basal signal in immature B cells that suppresses RAG2-GFP.

RAG2-GFP expression correlates with IgM receptor downregulation

Tolerance has been suggested to result from endocytosis of sIg after incubation with self-antigen [182-184]. To assess the effects of antigen affinity and concentration on the kinetics of RAG2-GFP induction, we used two site-directed mutants of HEL with reduced affinity for the HEL receptor [HEL, ~10^{-9} M affinity; $HEL^{\Delta RD}$,~10^{-8} M affinity; and $HEL^{\Delta RDGN}$,~10^{-7} M], as well as duck egg lysozyme, which binds with even lower affinity (DEL, ~10^{-6} M) [114, 175]. After a 5-day IL-7 BM culture, HEL-Ig/RAG2-GFP immature B cells were incubated with HEL or the various analogues at 2.5, 0.5, or 0.1 mg/ml, and then analyzed by flow cytometry for CD69 expression at 2h, IgM expression at 24h, and RAG2-GFP responses at 48h (Figure 3A). All doses of HEL elicited CD69 activation, prolonged downregulation of IgM^a, and maximal RAG2-GFP induction. The $HEL^{\Delta RD}$ mutant induced nearly maximal upregulation of CD69 at all concentrations, while only the highest dose led to a prolonged downregulation of IgM^a and a near maximal induction of RAG2-GFP. The highest dose of $HEL^{\Delta RDGN}$ induced CD69 to relatively high levels but resulted in only a minimal RAG2-GFP response. There was no significant RAG2-GFP induction at any of the doses of DEL. These data further demonstrate the discordance between activation of immature B cells by antigen, as measured by CD69, and induction of RAG2-GFP in this system. Importantly, prolonged downregulation of surface IgM was associated with induction of RAG2-GFP, regardless of the degree of cell activation. As illustrated in Figure 3B, it appears that sIgM levels must drop below a certain threshold for efficient RAG2-GFP expression. Modest reduction in sIgM levels resulted in minimal RAG2-GFP induction. In contrast,

once sIgM levels dropped below a threshold (in these experiments an IgM MFI of ~1000) RAG2-GFP was efficiently induced. Thus, we conclude that prolonged sIgM downregulation below a specific threshold is required to induce receptor editing.

Immature B cells undergo a back-differentiation response following antigen exposure

Immature B cells that lose surface BCR expression due to Cre-mediated deletion of Ig HC show a loss of LC allelic exclusion characterized by induction of RAG genes and new LC gene rearrangements [52]. Furthermore, these cells undergo a striking reversal in development with new onset expression of pro/pre-B cell genes and extinction of many genes of the mature B cell program. A similar picture emerged when immature B cells were treated with pharmacologic agents that block proximal signaling pathways downstream of the BCR [52]. These data suggested that basal BCR signaling provides tonic signals that suppress RAG expression and holds the immature B cell genetic program in place.

We were interested in determining whether antigen-stimulated immature B cells, which undergo a prolonged downregulation of cell surface BCR expression, might show a similar 'back-differentiation' response with expression of genes indicative of an earlier stage in B cell development. Microarray analyses were used to compare gene expression profiles of HEL-Ig/RAG2-GFP double Tg immature B cells incubated with either medium alone or with 1 μg/ml HEL for 2 days. Cells were stained with antibodies against B220 and IgM^a, and untreated ($B220^+$, IgM^{a+}, $RAG2\text{-}GFP^{neg}$; Ctrl 48h) and HEL treated ($B220^+$, $IgM^{a\text{-}low}$, $RAG2\text{-}GFP^+$; HEL 48h) cells were sorted by

flow cytometry. Total RNA was isolated, converted to biotinylated cRNA probes, which were then hybridized to Affymetrix murine U74Av2 chips. Data were analyzed using Affymetrix Microarray Suite 5.0 software (see Materials and Methods).

The analysis identified 212 transcripts that were differentially expressed between medium-alone control (Ctrl 48h) and HEL-treated cells (HEL 48h) at 48 hours (see Figure 4 legend for details regarding filtering criteria). We compared the patterns of gene expression in antigen-treated cells with the data from additional arrays generated from several sorted B cell populations: pro- and pre-B cells generated from polyclonal C57Bl/6 IL-7 BM cultures (B6 IgM^{neg}); immature B cells generated from HEL-Ig Tg IL-7 BM cultures (HEL IgM^{hi}); pre-B cells, Fraction D as described by Hardy et al. [185] sorted from BM of Balb/c mice (Fr. D); and newly formed B cells, Fraction E as described by Hardy et al. [185] also sorted from BM of Balb/c mice (Fr. E) [52]. Expression values for each gene were divided by the means of IgM^{hi} cell populations (HEL IgM^{hi}, Fr. E, Ctrl 48h). These ratios were then $\log_2$ transformed, and unsupervised clustering was performed using CLUSTER, with the data visualized using TREEVIEW [178] (Figure 4). Further detailed description of the sorting schemes and array analysis is provided in the Methods section.

Previous reports have shown that RAG mRNA and protein are induced in immature B cells after BCR crosslinking [165]. Similarly, cells treated with HEL for 48 hours showed strong induction of transcripts for RAG1 and RAG2 (Table 1). In addition, a number of other interesting transcripts were upregulated, including nuclear proteins/transcription factors - Jun, Myb, Ku70, Lef-1, Ezh2, Ets2; signaling molecules - Fyn and IκBα; and cell surface proteins – IL-7R and CXCR4. Downregulated

transcripts included the nuclear protein/transcription factor: XBP-1; signaling molecules - SHP-1, Hck, Slap, PKCγ, Fes, Igα; and many cell surface molecules - CD20, CD22, CD32, PIR-A, PIR-B, Ia invariant chain, CXCR5, and integrin-α4. Strikingly, the array profile of immature B cells treated with HEL (HEL 48h) clustered together with data from pro- (B6 IgM^{neg}) and pre-B cells (Fr. D) rather than with immature B cell populations (HEL IgM^{hi}, Fr. E) (Figure 4). Many of the genes upregulated in cultures containing self-antigen are characteristically expressed in pro-B and pre-B cells, while many of the downregulated transcripts are expressed as part of the mature B cell gene program. We conclude that antigen treatment of immature B cells leads to a 'back-differentiation' of the cells, similar to that observed when immature B cells lose basal signaling due to loss of BCR.

Surface protein expression of immature B cells incubated with self-antigen

We next sought to validate the array data by investigating whether the changes in gene expression were reflected at the protein level. At time zero and after two or three days of culture with medium alone or with HEL, cells were stained with various monoclonal antibodies to cell surface markers, and analyzed by flow cytometry. Freshly isolated non-Tg spleen and BM cells from C57BL/6 mice were analyzed in parallel. The cell surface phenotype of $B220^{+}IgM^{+}$ cells immediately after expansion in IL-7 confirmed that these cells expressed many of the surface markers characteristic of *in vivo* immature B cells (Figure 5). After incubation with HEL, immature B cells showed decreased surface levels of B220, CD22, PIR-A/B and integrin-α4, and increased levels of CD43 and class II IA-IE (Figure 4B). CD23 and integrin-β7 showed

no changes in surface protein levels. In general, these protein data mirrored, and thus validated, the results obtained in the microarray analysis. Note that the populations of cells were moving together as a "unit" with all cells demonstrating the changes in surface protein levels. We have previously shown that there is no significant proliferation of immature B cells in this system after antigen treatment, and just an average 25-30% total loss of cells over the course of a two-day antigen culture [114, 156]. Together, these data rule out the possibility that the gene expression and protein changes observed are due to selective survival and/or expansion of the small numbers (<3%) of non-IgM bearing B cells typically present at the initiation of the cultures.

Antigen stimulated immature B cells show global gene expression changes consistent with a reversal in development

To define the developmental stage of antigen-incubated immature cells, we performed unsupervised hierarchical clustering of the gene expression data with data from previously described B cell populations [52]. These populations included cells derived from mice carrying a floxed B1-8 knockin HC (B1-8f), a 3-83 knockin LC, either with ($CreM^{lo}$) or without (Cre Ctrl) the interferon inducible Mx-Cre after 48 hours of IFN treatment [156], as well as immature HEL-Ig/RAG2-GFP B cells cultured for 24 hours in the absence (GFP Ctrl) or presence of 400ng/ml Herbimycin A (GFP HA) and sorted based on B220, IgM^a and RAG2-GFP expression. We also included in this analysis the following additional sorted cell populations: early IgM^{lo} immature B cells generated from HEL-Ig Tg IL-7 BM cultures (HEL IgM^{lo}), and IgM^+ immature B cells from BM of Balb/c mice (Fr. E). Expression values for each transcript were

divided by the mean expression level for all IgM^{hi} cell populations (HEL IgM^{hi}, BM 48h Ctrl, GFP Ctrl, Fr. E, and Cre Ctrl) to provide a baseline from which to compare gene expression levels. These fold-difference ratios were $\log_2$ transformed, with clustering and visualization as described above.

As shown in Figure 6A, Ag-incubated cells (HEL 48h) clustered with IL-7 culture generated pro- and pre-B cells (B6 IgM^{neg}) and normal pre-B cells (Fr. D), as well as with IgM^{neg} HC-deleted cells (Cre M^{lo}), and Herbimycin-treated HEL-Ig B cells (GFP HA). The clustering also showed that the HEL treated cells were more similar to pre-B (Fr. D) and pro-/pre-B (B6 IgM^{neg}) cells than to early immature (HEL IgM^{lo}) cells, indicating that the cells were clearly back-differentiating to an earlier B cell stage.

To further address the correlation between HEL-treated immature B cells, pre-/pro-B cells and early immature B cells, a separate clustering analysis was performed using 155 transcripts that best discriminated normal pro-/pre-B cells (B6 IgM^{neg}) from early immature B cells (HEL IgM^{lo}). Analysis of this gene list also showed that HEL-treated immature B cells BM (HEL 48h) clustered together with pre-B (Fr. D) and pro-/pre-B (B6 IgM^{neg}) cells rather than with early immature (HEL IgM^{lo}) cells (Figure 6B). We conclude that immature B cells incubated with self-antigen for 48 hours show a striking global back differentiation to an early stage of B cell development characterized by the downregulation of many mature B cell genes and the upregulation of genes normally expressed in pro-B and pre-B cells.

Suppression of RAG responses and LC rearrangements by phorbol ester and ionophore

If the loss of basal signaling from the BCR in immature B cells drives receptor editing, we hypothesized that the addition of agents that mimic BCR signals should suppress RAG expression and block the ability of antigen to induce RAG and new LC endogenous rearrangements. We tested this idea using PMA, a phorbol ester that activates protein kinase C molecules by mimicking endogenous diacylglycerol (reviewed in [186, 187]), and Ionomycin, a calcium ionophore that activates downstream calcium dependent signaling pathways [188]. Combinations of PMA and Ionomycin have long been used to initiate activation signals in lymphocytes [189, 190].

To determine the influence of PMA and Ionomycin on baseline RAG2-GFP expression in immature B cells we performed a series of dose response experiments. As single agents, and in combination, there was no evidence for strong induction of RAG2-GFP in these cells by PMA and Ionomycin (Figure 7A) across broad concentration ranges. Cell counts showed that the combination of PMA/Ionomycin was not toxic to the cells (data not shown). Quantitation of the absolute number of RAG2-GFP positive cells showed no increases with any combination of PMA and Ionomycin (data not shown). In contrast, treatment of immature B cells with HEL consistently resulted in a greater than 6-fold induction in the absolute number of GFP positive cells compared to medium alone. We conclude that treatment of immature B cells with PMA and Ionomycin does not induce RAG2-GFP expression. This observation is consistent with our hypothesis that it is the absence of basal signal that initiates RAG expression and

receptor editing, not a positive signal through the BCR which PMA and Ionomycin mimics.

The addition of PMA and Ionomycin to HEL-treated immature B cells at the initiation of the cultures blocked the upregulation of RAG2-GFP levels at 24 and 48 hours (Figure 7B). Similarly, the induction of RAG2-GFP following incubation of cells with the tyrosine kinase inhibitor Herbimycin A was also suppressed (Figure 7B). As measured by flow cytometry, PMA and Ionomycin blocked the back differentiation of cells induced by either antigen treatment or incubation with the tyrosine kinase inhibitor Herbimycin A (as well as with PI3K inhibitors – data not shown). IL-7R upregulation was blocked (not shown), and the levels of PIR-A were, in fact, higher on cells treated with antigen and PMA/Ionomycin, consistent with continued progression through development (Figure 7B). CD22 levels were similarly upregulated by PMA/Ionomycin (not shown). Surface expression of the activation marker CD86 was upregulated at 12 hours by HEL incubation (Figure 7B), and was strongly induced by the combination of PMA and Ionomycin, confirming that the cells were receiving strong 'activation' signals.

We next assayed cells cultured with HEL or HEL plus PMA/Ionomycin for new endogenous LC rearrangements using a quantitative PCR assay, and found that PMA/Ionomycin completely prevented the induction of rearrangements observed after HEL incubation for 48 hours (Figure 7C). In order to confirm these results using an additional model system, immature B cells carrying a floxed heavy chain allele and RAG2-GFP (B1-8f/3-83k/RAG2-GFP) were cultured with TAT-Cre [191] for 2 days with or without PMA and Ionomycin (Figure 7D). The upregulation of RAG2-GFP in

cells undergoing Cre-mediated deletion of the BCR was also blocked by the combination of PMA and Ionomycin. We conclude that PMA and Ionomycin, presumably by activating protein kinase C and calcium-dependent pathways, block RAG induction and the onset of new endogenous LC rearrangements in these systems.

PMA/Ionomycin suppress LC receptor editing in pre-B cells

Finally, we tested the influence of PMA and Ionomycin on pre-B cell receptor editing to self-antigen in a newly developed *in vivo* model system [113]. In this model, mice that carry an anti-HEL HC transgene, an anti-HEL LC knockin allele, and a membrane-bound HEL transgene (mHEL) have an expanded pre-B cell compartment and show vigorous LC editing responses that lead to the accumulation of high numbers of non-HEL binding (edited) B cells in the periphery [113]. For the experiments described here, a human Cκ knockin allele [157] was introduced into these mice (generating $HC^{Tg/+}/LC^{KI/hCk}$/mHEL animals), so that we could monitor the frequency of editing to the alternate LC allele by staining cells for hCκ.

We sorted editing pre-B cells from the BM of these mice ($B220^{+}CD43^{neg}IgM^{neg}$) to high purity (>98%) (Figure 8A,B), and then cultured the cells *in vitro* with the survival factor BAFF alone or with BAFF plus PMA/Ionomycin for 18 h. Over this short culture period, there were no significant differences in overall cell numbers as determined by live cell gating (Figure 8C) and trypan blue staining. Importantly, pre-B cells incubated with PMA/Ionomycin generated increased numbers of immature B cells that bound HEL in a sandwich assay, and ~5-fold reduced numbers of hCκ edited cells

(Figure 8C). Thus, PMA/Ionomycin suppresses ongoing LC receptor editing responses in pre-B cells in this system.

Discussion

Despite many elegant studies demonstrating that receptor editing is an efficient mechanism to modify the antigen specificity of BCRs expressed on developing B cells, the mechanisms that guide and control the process remain poorly understood. The data reported here suggest that an important signal for receptor editing in response to self-antigen is the absence or reduction of basal signaling from surface BCRs on developing B cells. In light of these findings, current models that describe the regulation of receptor editing may need to be revised.

The induction of RAG1 and RAG2 mRNA and protein in response to BCR crosslinking in immature B cells is well established [164], and previous reports have emphasized the role of positive signaling through the BCR following exposure to self-antigen as a key stimulus for editing. One aspect of the 'positive signaling' model that remains unexplained, however, is the slow kinetics of RAG induction observed when IL-7 BM culture-derived immature B cells are incubated with specific antigen [152]. As shown in Figure 1, despite a rapid 'activation' response following BCR stimulation, as measured by CD69 and CD86 induction, RAG2-GFP positive cells were present at low levels before 24 hours, and then peaked at 48 hours. Cells stimulated with antigen for short pulses (~e.g. 3 h) showed significant activation responses, however did not undergo strong induction of RAG2-GFP. Similarly, in time-course microarray studies, we have observed that RAG induction does not occur in parallel with the generalized

activation response that follows antigen incubation (peaking around 6 h), but instead parallels the 'back-differentiation' response (which begins around 24 h) culminating in an induction of the pro/pre-B genetic program and a relative extinction of the mature B cell program. Together with our observations that B cells lacking the proximal signaling molecule *btk* show normal RAG2-GFP induction following antigen incubation despite impaired activation responses, and that lyn^{null} immature B cells show elevated basal RAG2-GFP expression, these data support the hypothesis that a major driving factor for RAG induction by antigen in this model system is a reduction in basal or tonic BCR signaling due to downregulated levels of surface BCR. Moreover, we propose that basal signaling must drop below a certain threshold in order to turn off RAG expression, and the lyn^{null} immature B cells are below this threshold, while the *xid* immature B cells are not.

This idea is further supported by our finding that the gene expression profile of cells stimulated with antigen is highly similar to that of cells which have lost BCR expression following Cre-mediated deletion of heavy chain, or following treatment with the protein tyrosine kinase inhibitor Herbimycin A. In each case, these cells turn on scores of genes characteristic of pro- and pre-B cells, and turn down or off many other genes that comprise the mature B cell program. The parallels observed between the three experimental models suggest that similar mechanisms may be at work.

One prediction from our 'reduced signaling' model for receptor editing is that editing should be inhibited if cells are provided with surrogates for positive signaling. This prediction was borne out by the PMA/Ionomycin data, where it was shown that PMA/Ionomycin blocked the induction of RAG2-GFP^{+} cells following treatment with

antigen or a tyrosine kinase signaling inhibitor, or in response to loss of the BCR through Cre-mediated deletion. Furthermore, in a novel transgenic model system where all pre-B cells are undergoing editing to membrane self-antigen, PMA/Ionomycin treated cells showed reduced levels of ongoing LC editing (Figure 7). This experiment was particularly informative, as it more closely mimics true *in vivo* receptor editing, and suggests that positive signaling suppresses editing in pre-B cells. PMA/Ionomycin also effectively suppressed RAG mRNA in Jurkat T cell signaling mutants with elevated basal RAG2 levels [192]. Interestingly, either PMA alone or Ionomycin alone did not suppress RAG as efficiently as the combination (unpublished observations), suggesting that a complex downstream signal may be required for efficient RAG suppression and then positive selection of immature B cells into the mature pool. Our observation that cells treated with PMA/Ionomycin expressed higher levels of surface proteins such as PIR-A and CD22 than cells not stimulated, is consistent with the idea that signaling induces positive selection of immature B cells to the mature cell stage. That the same signal required for RAG suppression might also be used to stimulate positive selection is not surprising, and would mirror the situation observed in T cell positive selection [193-195]. A potential caveat we cannot exclude is that the signals generated by PMA and Ionomycin may interfere with a positive tolerogenic signal set by differential BCR activation of downstream signaling pathways such as those involving ERK, JNK, NFAT, and NFκB. We feel that this is unlikely as PMA and Ionomycin treatment also blocked RAG2-GFP induction following Tat-Cre mediated receptor deletion or Herbimycin A treatment. Neither of these latter situations is subject to the above caveat as these situations involve inhibition of all downstream BCR-dependent signals. Thus,

the simplest explanation of the PMA/Ionomycin results is that they simply mimic basal BCR signaling and thereby prevent RAG2-GFP induction.

Based on these data, we propose the following model for the regulation of LC receptor editing, where basal signaling through the BCR in early immature B cells is required to suppress RAG gene expression and then signal for positive selection of cells. Developing B cells with a successful HC rearrangement at the pro-B stage express HC in concert with invariant surrogate LCs VpreB and λ5, undergo several rounds of clonal expansion and then enter the resting, pre-B stage [32]. Prolonged signaling through the pre-BCR (perhaps involving the NFκB pathway [196] induces κ germline transcription and activation of RAG transcription and translation. LC gene rearrangements then initiate, generally at κ, and continue until a functional LC is produced that can pair with HC, move through the secretory pathway and be expressed as a functional BCR on the cell membrane.

In this model, a critical step in the pre-B to immature-B transition is the accumulation of sufficient Ig on the cell surface that is competent to produce tonic BCR signals. Of note, the levels of IgM expressed by immature B cells is very high, perhaps higher than at any other stage of B cell development, and we suggest that this high level expression is required to produce an adequate basal signal. Furthermore, immature B cells have little or no expression of many inhibitory receptors, such as CD22 and PIR-A, which are present on mature B cells. This may facilitate positive signaling through the BCR at the immature B cell stage.

Cells bearing Ig receptors with high levels of self-reactivity likely never display significant levels of surface Ig (i.e., they are endocytosed immediately after cell surface expression) and LC alleles continue to rearrange, generally on the activated allele initially [33]. For receptors with low-level self-reactivity - insufficient to signal for receptor downregulation - the increased signaling through the receptor may "assist" the basal signal, and result in a termination of RAG and progression in development. LCs that assemble poorly or that fail to pair with heavy chain in a way that promotes adequate basal signaling would be edited, again because a sufficient basal BCR signal is not delivered. Thus, in this model, the signal for receptor editing in response to self-antigen is not primarily driven by cross-linking of surface BCRs, but rather by the absence of a sufficient basal signal due to prolonged antigen-induced BCR downregulation.

It seems likely that the level of signaling a B cell receives at this critical step in development, either basal or 'assisted' by self-antigen, may be important for further differentiation steps towards the various alternate fates of B cells – anergy, or the follicular, marginal zone, or B-1 pathways. We suggest that occasionally cells will express high levels of BCR, and only later during the immature stage encounter strongly self-reactive antigens [156]. These cells might receive a strong initial signal from self-Ag, but the primary consequence of that signal would be a downmodulation of surface Ig. The subsequent prolonged loss of basal signal would result in the re-initiation of RAG1/2 and the 'back-differentiation' program, thereby providing an opportunity to revise the receptor by editing. Importantly, this model does not rule out the possibility that signaling through the receptor, perhaps under certain co-stimulation circumstances,

may drive the induction of RAG. We believe that our proposed model is consistent with the data available, and provides a number of testable hypotheses for future work.

What is the nature of the basal signal driving the cessation of RAG expression at the pre-B/immature B cell transition? It is apparent from the work of Monroe and colleagues that the minimal cellular machinery required to drive B cell development is the Ig α/β heterodimer [197]. We hypothesize that the basal signal is transmitted through the following series of interacting proteins: the BCR, Igα/β, *syk*, *lyn*, *btk*, and *PI3K*. Knockouts of *syk* [90], *lyn* [198], *PI3K p85α* [53], *PI3K p110δ* [119], and *btk* (Figure 1), and inhibition of PI3K with inhibitors [52], all result in a phenotype compatible with a role for basal signaling in suppressing RAG induction. Of interest, a recent report showed that a high percentage of B cells that "squeak" through development in human X-linked agammaglobulinemia, a disorder characterized by mutations in *btk*, have a peripheral B cell repertoire enriched for autoimmune BCRs [199]. The authors propose that inefficient signaling through the BCR may favor the development of autoreactive B cells, which is consistent with the model proposed here. Further downstream molecules conveying the basal signal may include PLCγ and PKCβ; inhibitor studies and further genetic dissection will be required to explore the role of these molecules. Recently Verkoczy et al. showed that PLCγ2 played a significant role in down-regulating RAG expression and B cell positive selection [53].

These findings have significant implications for our understanding of allelic exclusion, whereby each B cell expresses a single BCR [32]. In general, the mechanisms that initiate and maintain allelic exclusion are not well understood. HC allelic exclusion requires the expression of a functional membrane-bound HC protein,

since mice lacking the Cμ transmembrane domain show a complete block in B cell development at the pro-B stage, and B cells fail to establish HC allelic exclusion [200]. HC allelic exclusion also requires the Ig receptor-associated signaling proteins Igα and Igβ [201-203]. Less is known about the signaling requirements for LC allelic exclusion, where the situation is complex due to the presence of two κ and two λ alleles and the potential for multiple rearrangements at each locus.

There is evidence that accessibility of chromatin to the V(D)J recombination machinery is important for controlling Ig (and TCR) rearrangements (reviewed in [204]). Factors contributing to accessibility include the initiation of germline transcripts, remodeling of histones, and the methylation status of individual alleles. Two basic models have been proposed to account for allelic exclusion: regulated and stochastic. In the regulated model, a functional rearrangement on one allele provides a signal that prevents, through feedback inhibition, further rearrangements on the other allele [34, 205]. In the stochastic model, the probability and efficiency of V(D)J rearrangement is sufficiently low that allelic exclusion is obtained by 'default'.

While experimental data examining rearrangement status in single cells seems to favor the 'regulated' model [206], a study from Schlissel and colleagues using a knockin GFP allele into Jκ1 provides support for a stochastic model to explain the initiation of LC allelic exclusion [33]. A key finding was that only ~5% of pre-B cells showed sterile κ transcripts, and expression was mono-allelic. Furthermore, culturing of these GFP^+ sterile κ^+ pre-B cells led to preferential rearrangement of the GFP allele. The authors propose that the low frequency of cells bearing sterile transcripts for κ in the pre-B compartment may reflect competition for limiting numbers of transcription

factors that can drive sterile κ transcription. As noted [206], these data are also consistent with a regulated model, where pre-B cells activate sterile κ transcription only for a brief time and in a developmentally regulated fashion. In this model, cells with germline transcripts either undergo functional rearrangement and developmental progression or are arrested in the compartment, perhaps to undergo a second round of locus activation at a later time. The issues of initiation and, then, maintenance of allelic exclusion are central to the concept of receptor editing, where allelic exclusion is maintained in the setting of the potential for multiple rearrangements at a locus. The data presented here are consistent with the regulated model.

In summary, we have now shown that three distinct perturbations of immature B cells – deletion of the BCR, treatment with proximal signaling inhibitors, and antigen treatment – result in a highly similar 'back-differentiation' of cells to an earlier stage in B cell development. The plasticity exhibited by these cells is remarkable, and suggests that the genetic program of B cells may be particularly flexible. This idea is further supported by the work of Busslinger and colleagues, who have shown that Pax5 is important for the maintenance of the B cell program [207]. Early B cells that lose Pax5 show the ability to back-differentiate into stem cells and re-program for the T lineage. Mature B cells with induced deletions of Pax5, 'lose their place' in development, and back-differentiate to the pro-B stage. Among the target genes for Pax5 are the Iga and Igb signaling molecules, and we speculate that impaired transcription of these, and perhaps other, crucial signaling proteins may lead to a loss of basal BCR signaling and the developmental regression observed. Because mature B cells are poised to differentiate, following appropriate signaling, into highly efficient Ig-secreting plasma

cells, the developmental plasticity observed in B cells may reflect a ‘ready-state’ at the level of chromatin remodeling or other epigenetic alterations that allow for rapid genetic reprogramming.

Acknowledgements

We thank M. Nussenzweig for providing RAG2-GFP and human Cκ knockin mice; K. Rajewsky for B1-8f/3-83k mice, and TAT-Cre; F. Batliwalla, P. Gregersen, W. Ortmann, E. Baechler, A. Becker and J. Plumb-Smith for assistance with the microarray experiments; D. Corcoran, H. Jecklin, A. Illes and D. Fods for assistance in genotyping and animal husbandry; J. Peller for assistance in cell sorting; and L. Heltemes-Harris, K. Murphy and M. Schlissel for discussion and suggestions.

Figure 1.

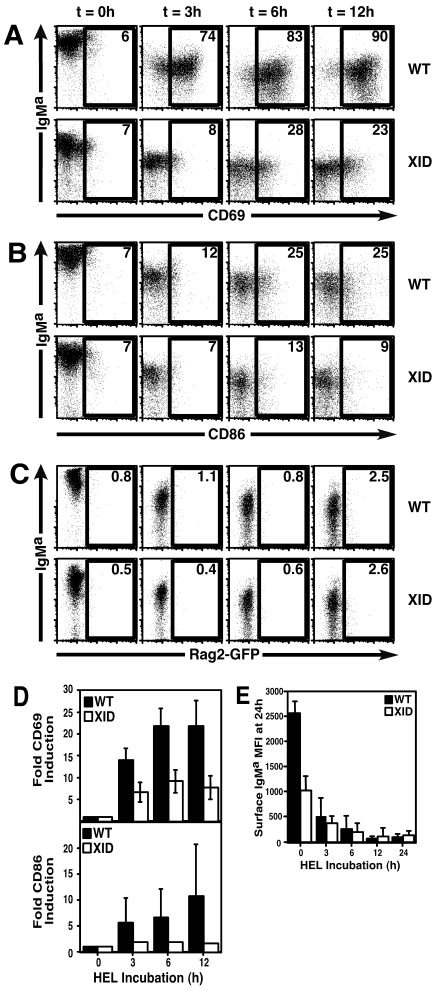

t = 0h
t = 3h
t = 6h
t = 12h
A
6
74
83
90
WT
7
8
28
23
XID
IgMa
CD69
B
7
12
25
25
WT
7
7
13
9
XID
IgMa
CD86
C
0.8
1.1
0.8
2.5
WT
0.5
0.4
0.6
2.6
XID
IgMa
Rag2-GFP
D
WT
XID
Fold CD69 Induction
Fold CD86 Induction
HEL Incubation (h)
E
WT
XID
Surface IgMa MFI at 24h
HEL Incubation (h)

Figure 1. ***Xid* immature B cells show impaired antigen-induced activation responses, but normal IgM downregulation.** Bone marrow from HEL-Ig/RAG2-GFP (WT) or *xid*/HEL-Ig/RAG2-GFP (XID) mice was expanded in IL-7 for 5 days. Immature B cells ($B220^{+}$, IgM^{hi}, IgD^{neg}) were then washed, and stimulated with 1 μg/ml HEL and assayed at 0, 3, 6 and 12 hours. Flow cytometry plots show the kinetics of surface IgM^{a} downregulation and the induction of (A) CD69, (B) CD86 and (C) RAG2-GFP in $B220^{+}$ gated populations. Numbers indicate the percentage of B220 gated cells positive for each marker. Summary data demonstrating (D) the kinetics of activation as determined by CD69 and CD86 fold induction over media condition, and (E) the downregulation of surface IgM in WT and XID immature B cells (n=4 experiments, mean ± SD).

Figure 2.

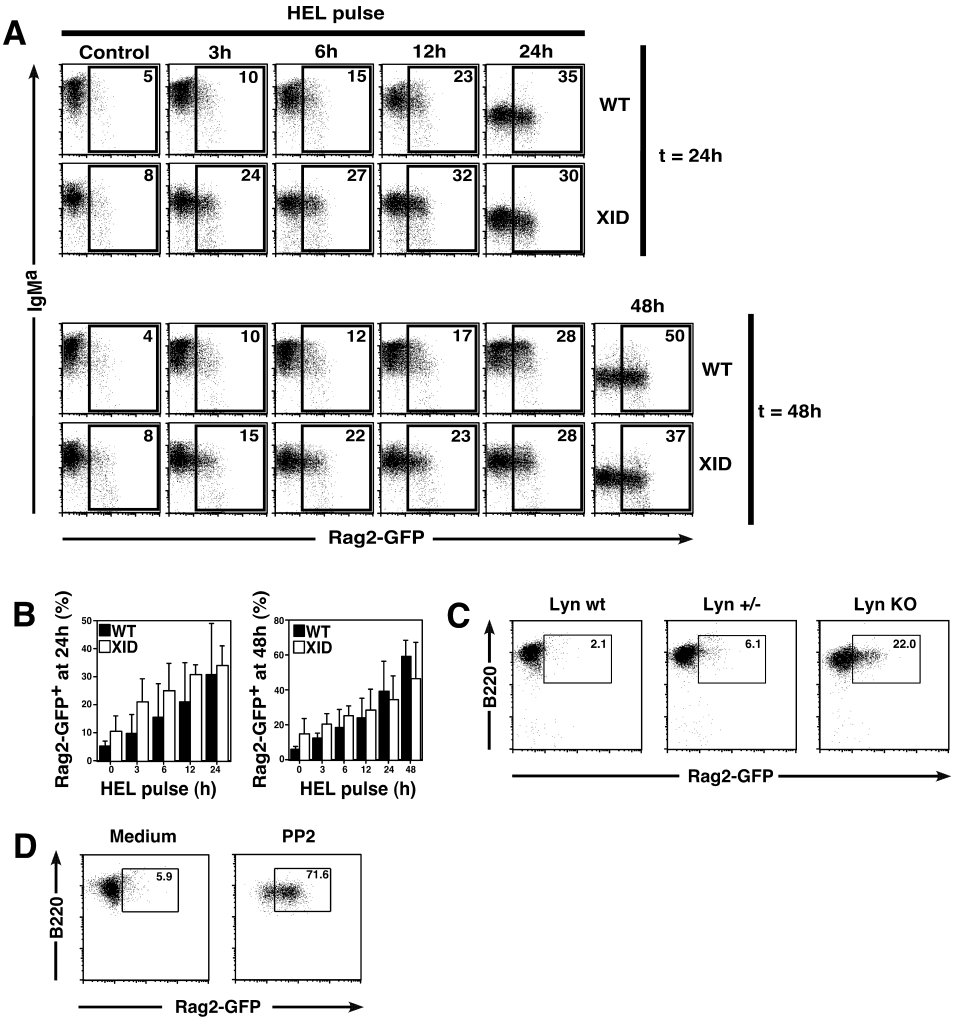

A
HEL pulse
Control
3h
6h
12h
24h
5
10
15
23
35
WT
8
24
27
32
30
XID
t = 24h
IgMa
48h
4
10
12
17
28
50
WT
8
15
22
23
28
37
XID
t = 48h
Rag2-GFP
B
Rag2-GFP+ at 24h (%)
WT
XID
HEL pulse (h)
Rag2-GFP+ at 48h (%)
WT
XID
HEL pulse (h)
C
Lyn wt
Lyn +/-
Lyn KO
2.1
6.1
22.0
B220
Rag2-GFP
D
Medium
PP2
5.9
71.6
B220
Rag2-GFP

Figure 2. RAG2-GFP responses in *xid* and *lyn*^{null} immature B cells. (A) Wildtype HEL-Ig/RAG2-GFP (WT) and *xid*/HEL-Ig/RAG2-GFP immature B cells (XID) were pulsed with 1 μg/ml HEL for 3, 6, 12 or 24h, and then assayed at 24 or 48 hours for expression of surface IgM and RAG2-GFP. Data represent B220^{+} gated cell populations. (B) Summary data for percent RAG-2-GFP^{+} cells at 24 and 48h of cells following treatment with HEL for the indicated times (n=4 experiments, mean ± SD). (C) *Lyn*-deficient immature B cells show elevated resting RAG2-GFP levels. Bone marrow from WT, *lyn*$^{+/-}$ and *lyn*^{null} HEL-Ig/RAG2-GFP Tg immature B cells was expanded in IL-7 for 5 days and then analyzed by flow cytometry. Representative of >4 animals of each genotype. (D) HEL-Ig/RAG2-GFP immature B cells were cultured for 48h in medium alone or with the Src-family kinase inhibitor PP2 (10 mM), and RAG2-GFP was measured by flow cytometry.

Figure 3.

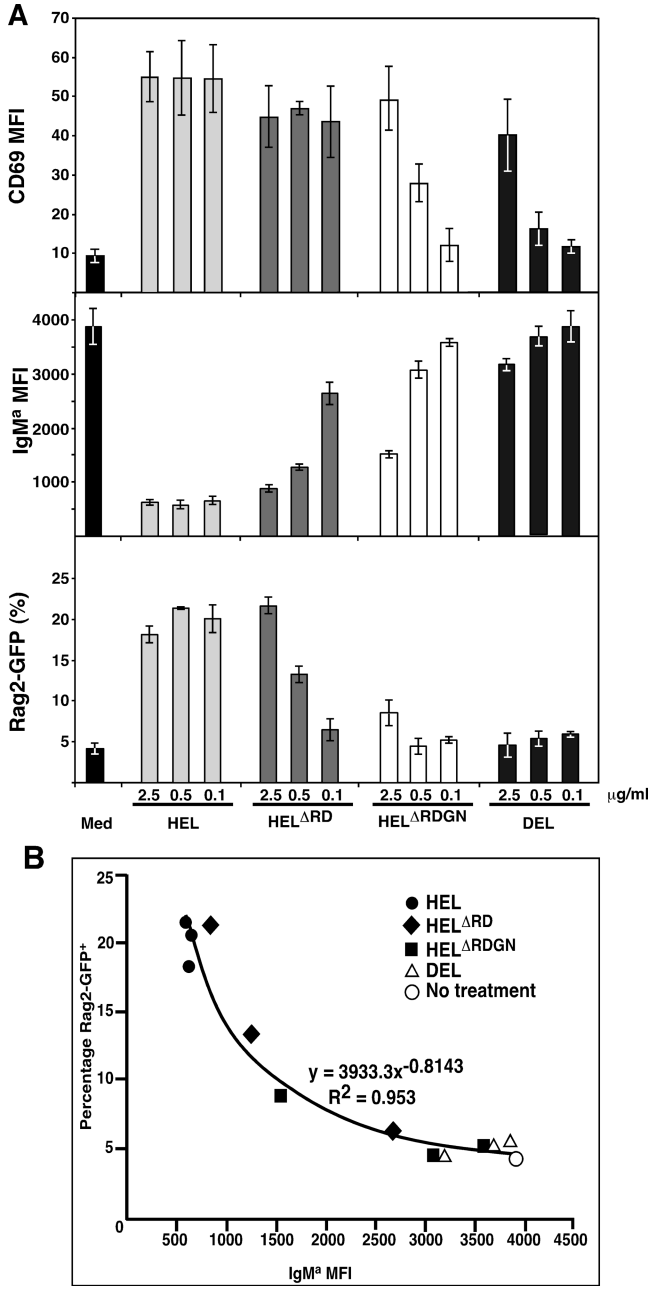

A
CD69 MFI
IgMa MFI
Rag2-GFP (%)
2.5 0.5 0.1
μg/ml
Med
HEL
HELΔRD
HELΔRDGN
DEL
B
Percentage Rag2-GFP+
IgMa MFI
HEL
HELΔRD
HELΔRDGN
DEL
No treatment
y = 3933.3x-0.8143
R2 = 0.953

Figure 3. Relationship between RAG2-GFP expression and surface IgM. HEL-Ig/RAG2-GFP Tg immature B cells were cultured *in vitro* with HEL, $HEL^{\Delta RD}$, $HEL^{\Delta RDGN}$, or DEL, at 2.5, 0.5, or 0.1 mg/ml. Aliquots of cells were then washed and analyzed by flow cytometry for CD69 at 2h, IgM at 24h, and RAG2-GFP at 48h. (A) Error bars represent range of two similar experiments. (B) Average IgM^a MFI versus average percentage $RAG2\text{-}GFP^+$.

Figure 4.

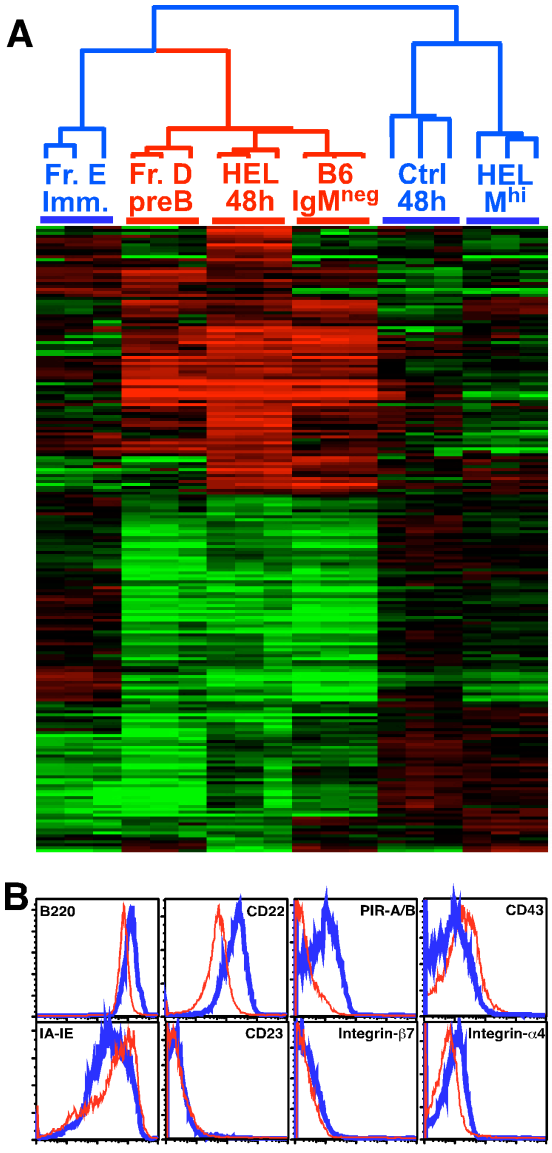

A
Fr. E Imm.
Fr. D preB
HEL 48h
B6 IgM^neg
Ctrl 48h
HEL M^hi
B
B220
CD22
PIR-A/B
CD43
IA-IE
CD23
Integrin-β7
Integrin-α4

Figure 4. Back-differentiation of antigen-stimulated immature B cells. (A) Immature B cells from HEL-Ig/RAG2-GFP Tg IL-7 BM cultures were stimulated with either medium alone or with 1 μg/ml HEL for 2 days. Cells were then stained with antibodies to IgM^a and B220 and sorted by FACS. RNA was isolated from medium alone treated cells ($B220^+$, IgM^{a+}, GFP^{neg}; **Ctrl 48h**) and HEL treated cells ($B220^+$, IgM^{a-lo}, GFP^+; **HEL 48h**), followed by cRNA synthesis and hybridization to Affymetrix U74A GeneChips. Microarray analysis identified 212 transcripts that were differentially expressed in HEL stimulated cells by the following criteria: Student's t-test < 0.05, average expression value difference ≥ 200 and average fold change ≥ 2.0. Other cell populations used for this analysis included: sorted IgM^{neg} pro/pre-B cells from IL-7 cultured BM of non-Tg mice ($B220^+$, IgMneg, IgD^{neg}; **B6 IgM^{neg}**), IgM^{hi} cells from IL-7 BM cultures of HEL-Ig Tg mice (IgM^{hi}, IgD^{neg}; **HEL IgM^{hi}**), pre-B ($B220^+$, $CD43^{neg}$, IgM^{neg}; **Fr. D preB**) and newly formed B cells ($B220^+$, IgM^{hi}, IgD^{neg}; **Fr. E imm.**) from BM of Balb/c mice. Individual expression values were divided by the mean expression level all IgM^{hi} populations (HEL IgM^{hi}, Fr. E imm. and Ctrl 48h), and the ratios were transformed into $\log_2$ space. Red represents an increase in expression, and green represents a decrease in expression relative to the mean of control IgM^{hi} populations. Each column represents a single array from an independent sorting experiment. (B) HEL-Ig/RAG2-GFP immature B cells were incubated with medium alone (blue) or HEL (red) for 2 days. Cells were then stained with antibodies to B220, IgM, and either CD22, PIR-A/B, CD43, IA-IE, CD23, integrin-β7, or integrin-α4, and analyzed by flow cytometry. Data represent $B220^+IgM^+$ gated cell populations.

Figure 5.

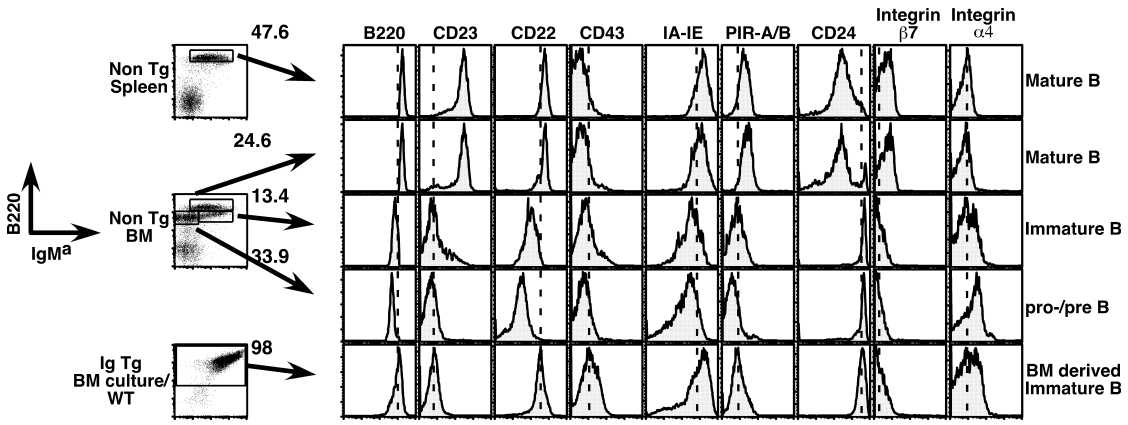

47.6
Non Tg
Spleen
24.6
13.4
Non Tg
BM
33.9
B220
IgMa
98
Ig Tg
BM culture/
WT
B220
CD23
CD22
CD43
IA-IE
PIR-A/B
CD24
Integrin β7
Integrin α4
Mature B
Mature B
Immature B
pro-/pre B
BM derived
Immature B

Figure 5. Expression of immature B cell markers following IL-7 bone marrow culture. Shown are flow cytometric analyses of HEL-Ig/RAG2-GFP cells that were expanded in IL-7 culture for 5 days and then stained with monoclonal antibodies to B220, IgM, CD23, CD22, CD24, PIR-A/B, IA-IE, integrin-α4 and integrin-β7. Freshly isolated splenic and BM cells from non-Ig Tg mice were simultaneously stained with the same antibodies for comparison. Plots shown are cells gated by size, IgM and B220.

Figure 6.

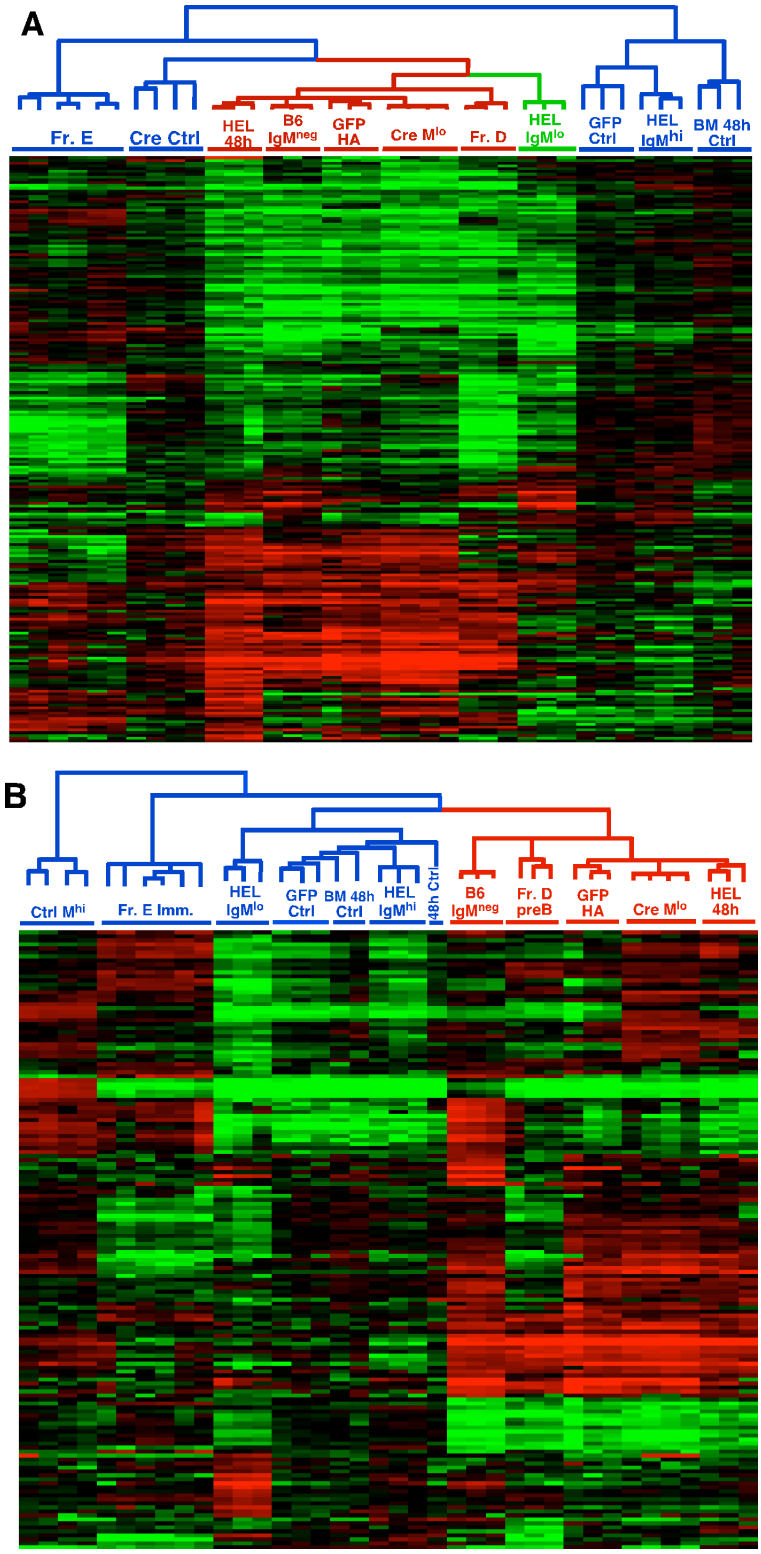

A
Fr. E
Cre Ctrl
HEL 48h
B6 IgM^neg
GFP HA
Cre M^lo
Fr. D
HEL IgM^lo
GFP Ctrl
HEL IgM^hi
BM 48h Ctrl
B
Ctrl M^hi
Fr. E Imm.
HEL IgM^lo
GFP Ctrl
BM 48h Ctrl
HEL IgM^hi
48h Ctrl
B6 IgM^neg
Fr. D preB
GFP HA
Cre M^lo
HEL 48h

Figure 6. Antigen-treated immature B cells show similar gene expression profiles as cells that have lost the BCR or have been treated with a tyrosine kinase inhibitor. (A) The data shown in Figure 4 was clustered with additional array data from several cell populations: B1-8f/3-83κ/Mx-Cre immature B cells treated with 1000 U/ml IFNαβ that have undergone surface IgM deletion (**Cre M^{lo}**) and similarly treated IFNαβ treated control B1-8f/3-83κ cells (**Cre Ctrl**); HEL-Ig/RAG2-GFP immature B cells treated with 400ng/ml Herbimycin A (**GFP HA**) and DMSO control (**GFP Ctrl**); early immature B cells from HEL-Ig Tg IL-7 BM culture (IgM^{lo}, IgD^{neg}; **HEL IgM^{lo}**) and from BM of Balb/c mice (B220^{+}, IgM^{lo}, IgD^{neg}; **Fr. E**). Clustering was performed as described for Figure 4, except that expression values from each array were divided by the mean of HEL IgM^{hi}, Fr. E imm, BM 48h Ctrl, GFP Ctrl and Cre Ctrl. (B) Clustering was performed using the 155 transcripts that best discriminate between pro/pre-B cells (B6 IgM^{neg}) and early immature B cells (HEL IgM^{lo}), dividing expression values by the mean of HEL IgM^{hi}, Fr. E imm, BM 48h Ctrl, GFP Ctrl and Cre Ctrl.

Figure 7.

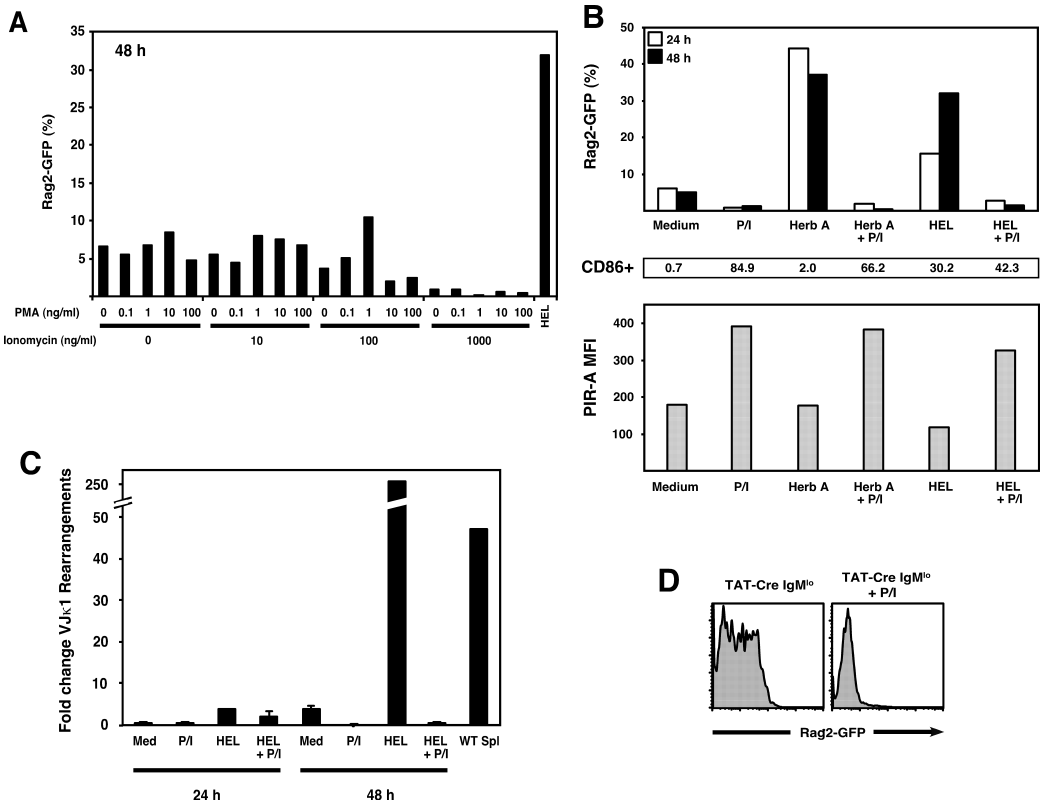

A
48 h
Rag2-GFP (%)
PMA (ng/ml)
Ionomycin (ng/ml)
HEL
B
24 h
48 h
Rag2-GFP (%)
Medium
P/I
Herb A
Herb A + P/I
HEL
HEL + P/I
CD86+
0.7
84.9
2.0
66.2
30.2
42.3
PIR-A MFI
C
Fold change VJκ1 Rearrangements
Med
P/I
HEL
HEL + P/I
WT Spl
24 h
48 h
D
TAT-Cre IgMlo
TAT-Cre IgMlo + P/I
Rag2-GFP

Figure 7. Suppression of RAG2-GFP and Ig rearrangements in immature B cells by PMA and Ionomycin. (A) HEL-Ig/RAG2-GFP Tg immature B cells were incubated with medium alone or with PMA and Ionomycin in various combinations for 48 hours, and then analyzed for RAG2-GFP expression by flow cytometry. Cells were gated by forward and side scatter, and by B220. (B) HEL-Ig/RAG2-GFP immature B cells were cultured in medium alone, with 1 μg/ml HEL or 300 ng/ml Herbimycin A in the presence or absence of PMA (10 ng/ml) and Ionomycin (60 ng/ml) for 2 days. Cells were analyzed by flow cytometry for CD86 expression at 12 hours (%), and RAG2-GFP (%) and PIR-A levels at 48h. The data are representative of 4 experiments. (C) HEL-Ig/RAG2-GFP immature B cells were cultured in medium alone or with 1 μg/ml HEL in the presence or absence of PMA/Ionomycin. Genomic DNA was isolated at 24 or 48 hours and analyzed for new endogenous VJκ1 rearrangements using a TaqMan real-time quantitative PCR assay. Results are shown as fold change in VJκ1 rearrangements, compared to day 0 levels. (D) Immature B cells carrying a floxed heavy chain allele and RAG2-GFP (B1-8f/3-83k/RAG2-GFP) were cultured with TAT-Cre [191] for 2 days with or without PMA (10 ng/ml) and Ionomycin (60 ng/ml). Shown are flow cytometry plots of RAG2-GFP expression in live lymphocyte gated $B220^{+}/IgM^{low}$ cells that had lost surface BCR as a result of Cre-induced heavy chain deletion. The data are representative of 4 experiments.

Figure 8.

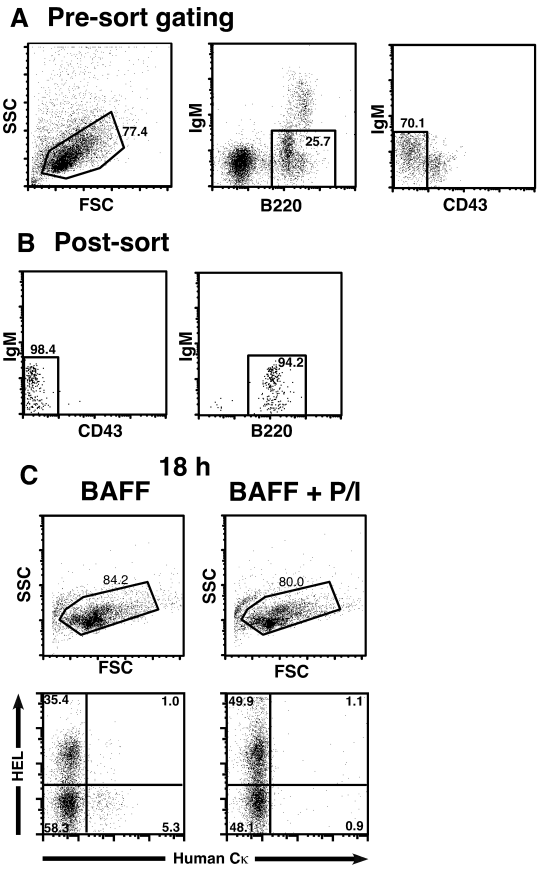

A Pre-sort gating
SSC
FSC
77.4
IgM
B220
25.7
IgM
CD43
70.1
B Post-sort
IgM
CD43
98.4
IgM
B220
94.2
C
18 h
BAFF
BAFF + P/I
SSC
FSC
84.2
SSC
FSC
80.0
HEL
35.4
1.0
58.2
5.3
49.9
1.1
48.1
0.9
Human Cκ

Figure 8. Pre-B cell LC editing to membrane self-antigen is inhibited by PMA/Ionomycin. (A) Bone marrow from $HC^{Tg/+}/LC^{KI/hCk}$/mHEL mice was harvested and stained with CD43, B220 and IgM antibodies. Panels demonstrate the gating strategy used to sort the population of $B220^{+}IgM^{neg}CD43^{neg}$ pre-B cells. 25.7% of the cells in the noted FSC/SSC gate were $B220^{+}IgM^{neg}$. 70.1% of the $B220^{+}IgM^{neg}$ cells (gate in middle panel) were $CD43^{neg}$. (B) Post-sort analysis demonstrating the purity of the pre-B cell population. (C) Pre-B cells were then cultured in the presence of 200 ng/ml BAFF alone (BAFF), or BAFF plus 10 ng/ml PMA and 60 ng/ml Ionomycin (BAFF+P/I) for 18 hours. Cells were then analyzed by flow cytometry to quantitate editing to non-HEL binding (lower quadrants) and hCκ (lower right quadrant) cells. Upper panels show the FSC/SSC gate. The cell populations shown in the lower panels are gated for $B220^{+}$ cells. Representative of 3 independent sorts and experiments.

Table I. *Selected differentially expressed transcripts in Ag-treated immature B cells*

GenBank Accession No.	Transcript	Mean Expression Value[a]		Fold Difference[b]	Nominal *p* Value[c]
		Ctrl 48h	HEL 48h	HEL 48h/Ctrl 48h	
V(D)J recombination					
M38700	Ku70	1,730	8,729	5.0	0.0002
M29475	Rag-1	740	18,307	24.7	0.0064
M64796	Rag-2	140	4,211	30.1	0.0040
Cell surface molecules					
M62541	CD20	69,274	10,883	−6.4	0.0182
U35330	H2-DMb1	2,005	329	−6.1	0.0200
U96684	PIR-A3	5,105	1,065	−4.8	0.0315
AF038149	PIR-B	2,081	574	−3.6	0.0309
U35323	H2-DMa	18,499	5,477	−3.4	0.0271
X71788	CXCR5	1,932	700	−2.8	0.0030
M23158	B220	6,926	2,572	−2.7	0.0483
M31312	FcγRIIb	7,493	2,784	−2.7	0.0295
X13450	Igα	82,226	34,113	−2.4	0.0464
X00496	Invariant chain	113,345	50,790	−2.2	0.0082
X53176	Integrin α_4	1,239	566	−2.2	0.0009
L02844	CD22	7,012	3,301	−2.1	0.0148
L23636	Flt-3L	1,095	549	−2.0	0.0430
Z80112	CXCR4	4,268	9,027	2.1	0.0004
M29697	IL7-R	1,860	6,157	3.3	0.0245
Intracellular signaling effectors					
J03023	Hck	22,712	2,351	−9.7	0.0146
X12616	Fes	1,084	208	−5.2	0.0028
M68902	SHP-1	15,165	4,079	−3.7	0.0043
L28035	PKCγ	4,009	1,097	−3.7	0.0335
U29056	Slap	5,764	1,759	−3.3	0.0119
X15373	IP3R	2,093	680	−3.1	0.0295
AI843864	PIP5K-2a	3,064	1,477	−2.1	0.0065
AI842940	PLCγ2	15,024	5,965	−2.5	0.0168
M27266	Fyn	1,072	2,496	2.3	0.0493
AI642048	IκBα	3,120	7,880	2.5	0.0001
L41495	Pim-2	2,961	6,804	2.3	0.0431
Nuclear molecules/transcription regulators					
AW123880	Xbp1	3,802	1,948	−2.0	0.0074
L25674	Nr2f6	1,108	2,322	2.1	0.0145
AA762325	Nkx6–2	829	2,272	2.7	0.0118
D16503	Lef-1	600	1,652	2.8	0.0441
M12848	Myb	14,008	42,315	3.0	0.0032
X12761	Jun	405	1,233	3.0	0.0268
J04103	Ets2	256	1,052	4.1	0.0034
U52951	Ezh2	2,201	9,442	4.3	0.0014
X89749	Tgif	2,063	9,549	4.6	0.0028
U47543	Nab2	355	1,923	5.4	0.0031

Table 1. Selected differentially expressed transcripts in Ag-treated immature B cells. [a]Mean expression values of selected transcripts from the 212 total transcripts identified as significantly differentially expressed between immature B cells untreated (Ctrl-48h) or treated with HEL (HEL-48h) for 48h. Data represent the mean of three individual sorts for each cell population. [b]Fold change of mean expression values from HEL-treated and untreated immature B cells. Positive values denote upregulation and negative values denote downregulation. [c]Student's *t* test calculated assuming two-tailed distribution with unequal variance.

Chapter 3

Tonic BCR Signaling Represses Receptor Editing via the Ras and Calcium Signaling Pathways

Laura B. Ramsey[†], Amanda L. Vegoe[†], Timothy W. Behrens[¶], Michael A. Farrar[†]

[†]Center for Immunology, Department of Medicine, University of Minnesota Medical School, Minneapolis, MN 55455; [¶]Immunology, Tissue Growth & Repair, Exploratory Clinical Development, Genentech, Inc., 1 DNA Way, S. San Francisco, CA 94080

Summary

Receptor editing is a tolerance mechanism that inhibits self-reactive B cells from reaching the periphery and causing autoimmunity. It occurs through secondary replacement of light chain immunoglobulin genes or switching from the κ locus to the λ locus. It is estimated that nearly half of the B cells that make it to the periphery have gone through receptor editing, which makes this process very important for the development of a normal immune system [106]. While many of the proteins involved in receptor editing have been identified, the mechanism controlling receptor editing remains elusive. A recent set of experiments suggested that tonic signaling through the BCR and PI3K represses RAG expression at the immature B cell stage, and that receptor editing occurs in the absence of tonic signals [52, 86]. These findings prompted us to test BCR signaling mutants, that both increase and decrease signaling, in a recently developed model system of receptor editing, the anti-κ transgenic mouse [208]. These mice express a pseudo-antibody transgene that binds the κ LC, rendering all κ-expressing B cells self-reactive and inducing expression of the λ LC. We have used these mice as hosts for bone marrow chimeras with BCR signaling mutants as donors. We first tested NFκB1- and CARMA-deficient cells as donors in these chimeras, and saw no significant differences in editing compared to WT. This suggests that either NFκB1 is not involved in tonic BCR signaling that inhibits receptor editing, or that there is functional redundancy in the NFκB pathway. Importantly, we found that gain-of-function signaling mutants Itpkb$^{-/-}$ and Raf-CAAX transgenics showed less editing than WT in these chimeras, supporting the hypothesis that tonic BCR signaling inhibits receptor editing through PI3K, which is upstream of both the

Ras/Raf/MEK/ERK pathway at Ca^{2+} signaling, which are affected in these mutants.

Introduction

Maintenance of B cell tolerance is critical for avoiding autoimmunity. The three processes that tolerize B cells to self-proteins are clonal deletion, functional inactivation (anergy) and receptor editing. These processes may involve different forms of antigen (e.g., soluble versus membrane-bound) or different binding affinities for the antigen receptor. Evidence suggests that as many as 75% of the B cell receptors (BCRs) generated during development are self-reactive [31] but these cells are usually tolerized and autoimmunity is avoided. Clonal deletion usually occurs when the antigen affinity for the BCR is high at the immature B cell stage, while anergy occurs in the periphery. It is estimated that up to 50% of B cells that make it out of the bone marrow (BM) have gone through receptor editing, which makes this process crucial for normal immune system function [106].

Receptor editing occurs at the immature B cell stage in the BM, usually via excision of a rearranged light chain (LC) gene and rearrangement of a new LC. The LC loci contain variable (V) and joining (J) gene segments that are rearranged by the RAG protein complex at the pre-B stage of development. Earlier, at the pro-B stage, the heavy chain (HC) is rearranged, which consists of variable, diversity and joining segments. The structure of the LC loci makes sequential replacements possible, so when one LC is part of a self-reactive BCR, it can be rearranged, avoiding cell death through receptor editing. An upstream V gene segment is rearranged with a downstream J segment, excising the previously rearranged gene.

Receptor editing was first identified by Nemazee [104] and Weigert [105], each using a different transgenic BCR system. Since then, many proteins involved in the mechanism of receptor editing have been elucidated but the signals triggering receptor editing are still elusive. The choice of tolerance mechanism may be driven by BCR signaling strength – with very strong signals inducing clonal deletion, moderate signals inducing receptor editing, and low signals inducing anergy. When the receptor binds antigen it is internalized, and signaling ceases [115]. We have found that the surface expression of IgM negatively correlates with RAG2-GFP induction, indicating that there is a basal signal from the BCR that inhibits receptor editing [86]. We have shown previously that the basal BCR signal through PI3K is necessary for maintaining the developmental stage and preventing receptor editing [52, 86]. The basal signal through the BCR is necessary for survival and progressing through development, however if the BCR signal is too strong, the cells will die by clonal deletion.

Recently, a new system has been developed by the Nemazee lab to analyze receptor editing in a polyclonal system [208]. This system involves a pseudo-antibody transgene that is ubiquitously expressed and binds to the κ LC, rendering all κ-expressing B cells self-reactive. This system involves both clonal deletion (cell numbers are about half of WT) and receptor editing (all B cells express the λ LC). We are using these anti-κ mice as hosts for bone marrow chimeras, where the hosts are lethally irradiated and donor BM is injected intravenously to expand and populate the lymphoid organs. In this way, we are able to test the editing efficiency of the donor BM without necessitating a complex mouse breeding scheme. The original description of the anti-κ chimeras showed that editing was decreased to half the level of WT when

RAG heterozygous donor BM was tested in this chimera system [208]. They also tested bcl-2 transgenic BM in the chimera system and found enhanced λ LC expression due to the lengthened lifespan of the B cells with the anti-apoptotic bcl-2 transgene. Thus, we believe this is a great model system for analyzing receptor editing in a polyclonal repertoire.

To test the hypothesis that BCR signaling inhibits receptor editing, we have used the anti-κ chimera system with donors carrying various signaling mutations – Itpkb$^{-/-}$, Raf-CAAX, NFkB1$^{-/-}$, and CARMA1$^{-/-}$. Our group has previously shown that basal BCR signaling inhibits receptor editing *in vitro* [86], however, we wanted to extend these studies with *in vivo* analysis using a polyclonal repertoire of B cells. If the hypothesis is true, we would expect BCR signaling mutations that result in less signaling to induce more editing, while BCR signaling mutations that result in more signaling would result in less editing.

The mutants we have chosen with increased BCR signaling are Itpkb$^{-/-}$ and Raf-CAAX. Inositol 1,4,5-triphosphate kinase B (Itpkb) plays an inhibitory role in BCR signaling [209, 210]. Itpkb phosphorylates the 3' position of the inositol ring to convert inositol 1,4,5-triphosphate (IP3) into inositol 1,3,4,5-tetrakisphosphate (IP4). After BCR stimulation, PLCγ2 produces diacylglycerol (DAG) and IP3. IP3 then binds to its receptors on the endoplasmic reticulum, allowing the release of Ca^{2+} stored there, which also induces store-operated calcium (SOC) channels in the plasma membrane to open and allow more Ca^{2+} influx and activation of NFκB, JNK and NFAT pathways [211]. The Cooke group postulates that self-reactive BCRs activate Itpkb through PLCγ and Ca^{2+}, which induces production of IP4, dampening SOC channel activity, and reducing

the Ca^{2+} concentration. This allows receptor editing to occur, saving B cells that would otherwise be clonally deleted [209]. The Itpkb-deficient mice also show increased Bim expression, a pro-apoptotic member of the bcl-2 family, indicating that Itpkb may regulate survival in B cells [209, 212]. The mice generated by Cooke's group show no defect in B cell development [210], although another Itpkb-deficient mouse shows decreased numbers of all B cell subsets in the BM starting at the pro-B stage [212]. We have chosen to analyze the mice generated by Cooke's group because the immature B cells from the BM of these animals have increased Ca^{2+} activity after BCR stimulation [209]. We hypothesize that this increase in BCR signaling will result in a decrease in receptor editing in our anti-κ chimera system due to enhanced inhibition of recombination machinery.

The other increased signaling mutant we have chosen to analyze in the anti-κ chimera system is Raf-CAAX. These transgenic mice have a human Raf gene with the CAAX motif of K-Ras, which causes localization of Raf to the plasma membrane and constitutive activation [213]. Normally, BCR stimulation activates Ras, which recruits Raf to the plasma membrane, where it phosphorylates MEK, which phosphorylates ERK, allowing for activation of transcription factors necessary for proliferation and survival, such as c-Myc and Elk-1. The Raf-CAAX transgene is under the control of the lck proximal promoter and Eμ enhancer for expression in B and T lineages, beginning before the pro-B cell stage. This Raf-CAAX mouse has an increased number of pro-B cells and fewer immature and mature B cells [25]. The activation of MEK is increased 3-fold in BM B cells, to a level similar to BCR stimulation of mature B cells [214]. This signal mimics pre-BCR signaling and is able to push RAG2-deficient B

cells through the pro-B stage into the pre-B stage of development [214]. Raf-CAAX mice have normal V to DJ joining on the HC, demonstrating that the Ras/Raf/MEK/ERK pathway is not inhibiting V(D)J recombination. We propose that the increase in signal is enough to inhibit receptor editing at the immature B cell stage. We will test this by using the Raf-CAAX BM as donor BM in the anti-κ chimera system.

It has been suggested that NFκB is required for Igλ expression and rearrangement [58, 215], so we chose NFκB1- and CARMA1-deficient mice as our reduced BCR signaling donors in the anti-κ chimera system. There are two signaling cascades that activate NFκB. The classical pathway of activation downstream of the BCR involves PKCβ activation of the CARMA1/Bcl10/Malt1 complex, followed by IKK (IκB kinase) phosphorylation and degradation of inhibitor of NFκB (IκB), which leads to the activation of the heterodimer RelA/NFκB1. The alternative pathway of activation, which is activated by BAFF in immature B cells, involves the phosphorylation and proteolytic processing of NFκB2 and leads to the activation of the RelB/NFκB2 heterodimer. Activation of each heterodimer allows translocation to the nucleus, where they bind NFκB DNA sites and activate gene transcription.

There are many important downstream targets of NFκB, including RAG [196]. The NFκB1-deficient mice have normal B cell development and expression of the κ and λ LCs [216], while NFκB1/2 double knockouts have impaired development at the immature B cell stage, and development is completely blocked in the spleen at the transitional stage, indicating they may not have enough basal signaling to progress

through development [217]. HC Tg NFκB1-deficient mice show elevated RAG expression and LC rearrangements in cultured BM cells, suggesting NFκB1 could have an inhibitory role in regulating RAG expression [196]. CARMA1-deficient mice have been generated by several groups [196, 218-221]. They all show no defects in B cell development in the BM but have defective activation upon BCR stimulation, i.e. no proliferation, activation of NFκB or JNK. To our knowledge, LC rearrangement and receptor editing have not been analyzed in these mice. We anticipate that reduced signaling from these mice would result in an increase in receptor editing, if indeed there is an inhibitory signal repressing RAG via NFκB1.

Materials & Methods

Mice

Anti-κ mice with the CD45.1 congenic marker were kindly provided by Dr. M. Weigert (University of Chicago) and have been described previously [208]. CD45.1 mice were obtained from Jackson Laboratories (Stock number 002014) and were bred to the anti-κ mice to maintain the congenic marker. Itpkb-deficient mouse bones [210] were kindly provided by Dr. M. Cooke (Novartis, Basel, Switzerland). Raf-CAAX mice have been described previously [213]. NFκB1-deficient mice on a B6 background were obtained from Jackson Laboratories (Stock number 006097). CARMA1-deficient mice [218] were generously provided by Dr. D. Littman (New York University). All mice were maintained in specific pathogen-free conditions, and were generally between 6-12 weeks of age at the time of the experiments. All experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Bone Marrow Chimeras

All recipient mice were congenically marked with the CD45.1 allele, while donors carried the CD45.2 allele. Recipients were either anti-κ transgenic or littermate controls. Recipients received 900 rads of γ irradiation from a Cs source on the morning of the transfer. 0.2 to 16 million cells were transferred i.v. per recipient. After 6 weeks the recipients were killed and bone marrow, spleen and lymph nodes were analyzed by flow cytometry. Chimera sample sizes: C56Bl/6 into anti-κ: 4, C56Bl/6 into CD45.1: 4, $Itpkb^{-/-}$ into anti-κ: 7, $Itpkb^{-/-}$ into CD45.1: 4, Raf-CAAX into anti-κ: 6, Raf-CAAX into

CD45.1: 7, NFκB1$^{-/-}$ into anti-κ: 4, NFκB1$^{-/-}$ into CD45.1: 9, CARMA1$^{-/-}$ into anti-κ: 7, CARMA1$^{-/-}$ into CD45.1: 5.

Flow cytometry

Single cell suspensions were prepared from lymphoid tissues by mashing with a plunger over a 70μm filter and washing with FACS buffer (PBS, 2.5% FBS, 0.2% sodium azide). Erythrocytes were eliminated from bone marrow and spleen samples by treatment with ammonium chloride. Cells were stained in FACS buffer with APC-Igλ (BioLegend) or FITC-Igλ (Becton Dickinson) and PE- Igκ (clone 187.1, Southern Biotech), Pacific blue-B220 (eBioscience), and Alexa Fluor® 700-B220 (eBioscience). Stained cells were analyzed on an LSRII flow cytometer (Becton Dickinson) and analyzed using the FlowJo software (Treestar). Cells were gated on forward and side scatter to avoid contamination of dead cells or debris.

Results

Anti-κ mice [208] were used as hosts for bone marrow chimeras. The anti-κ transgene forces editing away from surface expression of the κ LC to the λ LC. In order to test whether the BCR signal regulates editing through an inhibitory signal or an activation signal, several BCR signaling mutants were tested as BM donors in anti-κ chimeras. First, WT C57Bl/6 BM was used to replicate previously published data [208] and provide a baseline for comparison. Twelve million WT cells were injected i.v. into lethally irradiated CD45.1 anti-κ transgenic mice or CD45.1 littermates. After six weeks the lymphoid tissues were harvested and analyzed by flow cytometry. The WT cells edited efficiently away from κ expression in the anti-κ hosts, while showing normal κ and λ expression in the CD45.1 hosts (Figure. 1).

Itpkb-deficient mice have defective receptor editing in anti-κ chimeras

To test the hypothesis that the BCR signal inhibits editing we tested BCR signaling mutants that signal excessively, Itpkb-deficient mice [210] and Raf-CAAX transgenic mice [213]. Inositol 1,4,5-triphosphate kinase B (Itpkb) converts inositol 1,4,5-triphosphate (IP3) into inositol 1,3,4,5-tetrakisphosphate (IP4) upon BCR stimulation. IP3 activates calcium signaling, so the conversion of IP3 to IP4 is a negative feedback loop that dampens the calcium signal after BCR stimulation. In the absence of Itpkb, the calcium signal is enhanced and lasts longer due to greater SOC channel activity [210]. Mice lacking Itpkb have a defect in T cell development but normal B cell development in the BM. However, all peripheral subsets of B cells are reduced and they are unresponsive to BCR stimulation.

When Itpkb-deficient BM was used to make BM chimeras with anti-κ hosts, we observed a defect in receptor editing. At the immature B cell stage there were nearly twice as many residual κ-expressing cells and significantly fewer λ-expressing cells (44% of WT, p = 0.008) (Figure 1). Therefore, the editing ratio of λ to κ was significantly lower than the WT editing ratio (39% of WT, p = 0.02) (Figure 2A). This defect was also observed in the periphery in the spleen and LN, but not in the $B220^{hi}$ mature recirculating B cells in the BM. When Itpkb-deficient BM was used to make BM chimeras with CD45.1 hosts, there was no difference in the percentage of κ- or λ-expressing cells (p = 0.60 and p = 0.12, respectively), nor the editing ratio in the immature or mature B cell subsets in the BM (p = 0.65 and p = 0.62, respectively). However, the editing ratio was significantly higher in the spleen (1.9-fold, p = 0.014) and LN (1.4-fold, p = 0.023). This may be due to a difference in the response to the BCR signal in the periphery versus the BM. Itpkb-deficient cells show defects in editing only in the presence of an overwhelming stimulus to edit, not in a normal environment, presumably because the defect in BCR signaling is only evident after BCR stimulation, which is significant in the anti-κ hosts and not the CD45.1 hosts.

The Ras/Raf/MEK/ERK pathway inhibits receptor editing

Raf-CAAX BM was used as donors for BM chimeras in order to test the Ras signaling pathway downstream of the BCR. Raf-CAAX mice have an expansion of pro-B, pre-B and immature B cells in the BM [213]. These mice have a transgene that includes the human c-Raf-1 gene and the farnesylation signal of K-Ras that localizes Raf to the membrane, mimicking Ras-mediated activation of Raf [222]. This provides

for constitutive activation of Raf and its downstream effectors, such as MEK, ERK, and Ets-family transcription factors. When the BM from Raf-CAAX mice was used as donors for anti-κ chimeras, there was a defect in editing similar to the Itpkb-deficient chimeras. They have a 2.5-fold increase in percentage of residual κ-expressing cells and 2.4-fold fewer λ-expressing cells ($p = 0.0023$) in the immature BM compartment (Figure 1). The editing ratio is 4.9-fold lower at the immature B cell stage ($p = 0.005$), but also in the spleen (6-fold, $p = 0.0014$) and LN (4-fold, $p = 0.052$)(Figure 2). When Raf-CAAX BM was used to make chimeras with CD45.1 hosts, there were lower percentages of κ- and λ-expressing cells in the BM (57% and 37% of WT, respectively), probably due to the expansion of pro-B cells (Figure 1). The editing ratio in these chimeras was 69% lower than WT in the immature B cells in the BM ($p = 0.0095$), but significantly higher in all mature B cell tissues (average 1.6-fold increase). Since the Ras pathway is constitutively active in all of these cells, these results suggest that BCR signaling inhibits editing at the immature B cell stage but may enhance editing in the periphery.

NFkB1-deficient cells edit as well as WT

If the hypothesis that there is a signal downstream of the BCR that inhibits receptor editing were true, we would expect that B cells with a defective BCR signal would edit more than WT. In order to test this we used NFκB1- and CARMA1-deficient cells as donors in our chimeras. Removing many of the BCR signaling proteins results in a defect in B cell development since the pre-BCR and BCR signals are necessary for progression through developmental stages. We chose to use NFκB1-

and CARMA1-deficient cells because they have normal B cell development but still show defects in B cell activation in response to BCR stimulation [216].

When NFκB1-deficient BM was used in anti-κ chimeras we saw no difference in expression of κ or λ LCs in the BM ($p > 0.4$ for each, Figure 1). We may not have seen the expected increase in editing due to the near perfection of editing of WT cells in the anti-κ chimeras. The spleen and LN showed reduced editing ratios (7.6-fold and 5.7-fold, respectively), owing to the lack of activation in NFκB1-deficient cells (Figure 2). In CD45.1 chimeras, expression of κ and λ LCs were both reduced 1.9-fold ($p < 0.001$), but the editing ratio was nearly identical to WT ($p = 0.94$). The editing ratio is slightly elevated in the spleen (36% higher than WT, $p = 0.0012$) but not in the LN or mature recirculating B cells in the BM. These results are consistent with previous reports on NFkB1-deficient mice having normal κ and λ LC expression in the periphery [216].

CARMA1-deficient cells have normal receptor editing

CARMA1-deficient cells have a phenotype nearly identical to NFκB1-deficient cells due to their inability to activate NFκB [218]. In our chimeras they show virtually the same results as the NFκB1-deficient cells. They have no defect in editing in the immature or mature BM subsets in either the anti-κ chimeras or the CD45.1 chimeras (Figure 2). They also show a 1.9-fold increase in the editing ratio in the spleens of CD45.1 chimeras, but it is slightly decreased in the LN (77% of WT) in these same chimeras. In contrast to the NFκB1-deficient cells, they have no decrease in the editing ratio in the spleen or LN of anti-κ chimeras. The reason for this discrepancy could be

the activation of NFκB via other means (e.g. the alternative pathway). The CARMA1-deficient cells are unresponsive to BCR stimulation but are still able to activate NFκB via TNF receptors [218].

Discussion

These data show that an increase in BCR signaling impairs receptor editing at the immature B cell stage. In the presence of the anti-κ transgene there is an overwhelming stimulus to edit away from κ, and cells with an increased BCR signal do not edit as well, as shown by a lower editing ratio in both the Itpkb-deficient cells and the Raf-CAAX transgenic cells. In the absence of the anti-κ transgene there is less of a stimulus to edit. The Itpkb-deficient cells have normal development without BCR stimulation, and show no difference from WT in chimeras without the anti-κ transgene. The Raf-CAAX transgenic cells also show a defect in editing in the CD45.1 chimeras, presumably due to the signal through Ras/Raf/MEK/ERK that is suppressing editing. This fits our model well, showing an increase in BCR signaling inhibits editing, rather than inducing receptor editing, as the current model would suggest.

Our model (Figure 3) postulates that BCR signals inhibit receptor editing through the Ras/Raf/MEK/ERK pathway and Ca^{2+} signaling. These two pathways are both downstream of PI3K activation, which we have previously shown inhibits RAG2-GFP expression *in vitro* [52]. Ca^{2+} signaling is impaired in B cells lacking PI3K subunits p85α or p110δ, which also show increased RAG expression and LC editing [53, 119]. ERK phosphorylation is also blocked by PI3K inhibitors, indicating the Ras pathway is downstream of PI3K [223, 224]. CD19 is necessary for maximal PI3K activation, and genetic ablation of CD19 results in an increase in RAG expression and receptor editing [117]. CD19 needs only be increased 15 – 30% in mice to cause autoimmunity, decreased expression of sIgM, and enhanced BCR signaling [91]. These

mice would be interesting to test in our anti-κ chimera system to validate the results presented here.

The lack of an increase in editing in the NFκB1- and CARMA1-deficient cells may indicate that the system is already editing as well as possible, or may suggest that NFκB is not involved in the suppression of receptor editing. Verkoczy et al. have recently shown an increase in RAG expression and LC rearrangements in HC Tg NFκB1-deficient cultured BM B cells [196]. The HC transgene accelerates B cell development, which may account for the difference from our results with a polyclonal repertoire.

Another possibility for the lack of increase in editing in our system is that there is redundancy in the repression of editing, and the alternative pathway is activated, so NFκB2 is substituting for NFκB1. When both NFκB1 and NFκB2 are ablated, B cell development is impaired [217] and λ LC expression is decreased [58], but not when just NFκB1 is knocked out [216]. Derudder et al also found that NFkB signaling is not necessary for receptor editing, but suggested that NFκB activation prolongs the editing time frame that may be necessary for λ LC expression [58]. They found that anti-κ mice lacking IKKγ, and hence the classical NFκB pathway, produce fewer IgM^+ cells, and assumed that these were λ LC-expressing cells, but did not actually show λ LC expression on them. They suggest that the decreased percentage of λ^+ B cells in mice lacking both NFκB activation pathways is due to the lack of survival protein Pim2. They were able to rescue this defect with bcl-2 expression, implying that it is the expression of pro-survival proteins that allows λ LC recombination to occur at a later

time point than κ LC recombination, similar to what was found for bcl-xL, another bcl-2 family member [177]. This concept does not discount our model that the BCR signal inhibits receptor editing, but lends no support either.

Others have suggested that there are two distinct signaling pathways downstream of the BCR, the tonic signal involving PI3K and an activation signal involving Btk and BLNK [51]. Herzog et al suggest that PI3K functions to activate Akt/PKB, which inhibits the FOXO proteins, which normally promote Rag transcription and LC recombination. We have shown here that PI3K may act through Ras & Ca^{2+} signaling to repress LC recombination in multiple ways. Herzog et al also suggest that Btk and BLNK inhibit the PI3K pathway through an unknown mechanism, which allows for FOXO activation, induction of RAG expression, and LC recombination. If this were occurring through the PKCβ/CARMA1/NFκB pathway, we would have expected to see a decrease in editing in our anti-κ chimeras, but we saw normal editing in the BM. This implies that either this pathway is not involved in the signal downstream of Btk/BLNK that activates LC recombination, or there is redundancy in the pathway and other molecules (possibly including NFkB2) are compensating for the lack of NFκB1 activation.

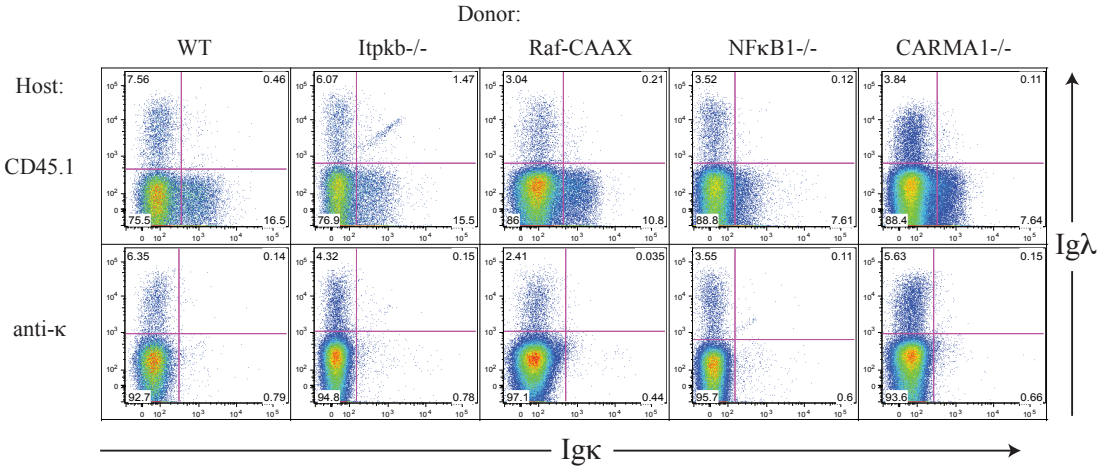
In the periphery, it seems that the increase in BCR signaling results in increased editing, not decreased as in the BM. The Itpkb and Raf-CAAX mutants both show a significantly higher percentage of λ-expressing cells and evidence of receptor editing in the spleen and lymph nodes (Figure 2). This may be due to a difference in the downstream effectors expressed at the different stages of B cell development. In the periphery, stimulation through the BCR is meant to expand the B cells and induce an

immune response. In contrast, in the BM, stimulation through the BCR is indicative of auto-reactivity and induces editing. RAG2 expression is induced in immature but not mature B cells after BCR stimulation and calcium signaling [83]. This lends support to our model where BCR binding of self-antigen at the immature stage induces downregulation of sIg and BCR signaling, allowing RAG expression and receptor editing.

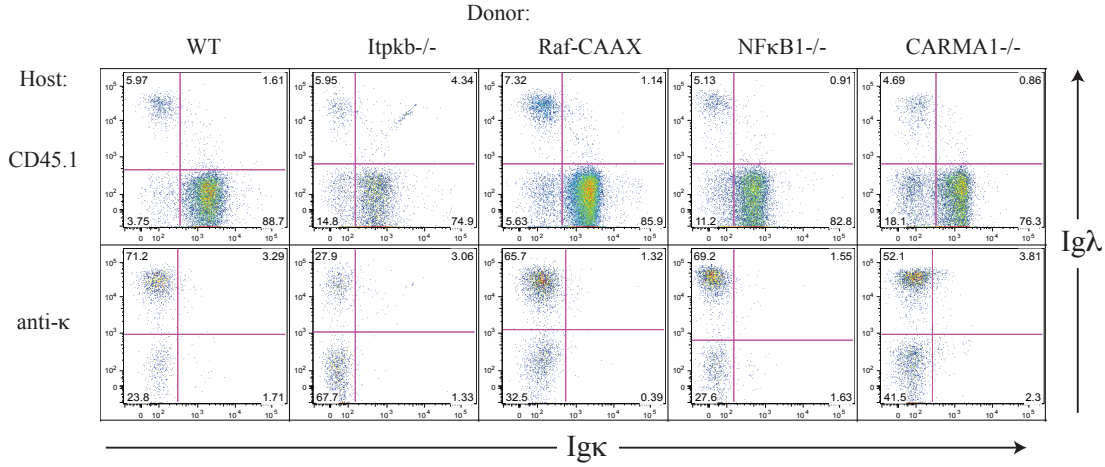
The exact mechanism of inhibition of receptor editing through tonic signaling remains elusive, but we have shown here that it could be through activation of the Ras and Ca^{2+} pathways. Future studies will need to be done to elucidate the effect that ERK and Ca^{2+} have on receptor editing. Other mutations could be tested in this system, like a constitutively active ERK mutant, to ensure that the inhibition of editing that we see in the Raf-CAAX mutants is definitely occurring through ERK. Recently, a constitutively active Akt mutant has been generated [225], which would also be interesting to test in this system, since recent reports have shown that Akt inhibits LC recombination through FOXO proteins [48]. The BCR signalosome is a complex network of interactions that is hard to parse out with knockouts, but the anti-κ system reported herein is able to analyze the effect of single molecules on receptor editing.

Figure 1. Light chain expression in BM chimeras

A. Bone Marrow (B220^{int})



B. Bone Marrow (B220^{hi})



C. Bone Marrow (B220^{int})

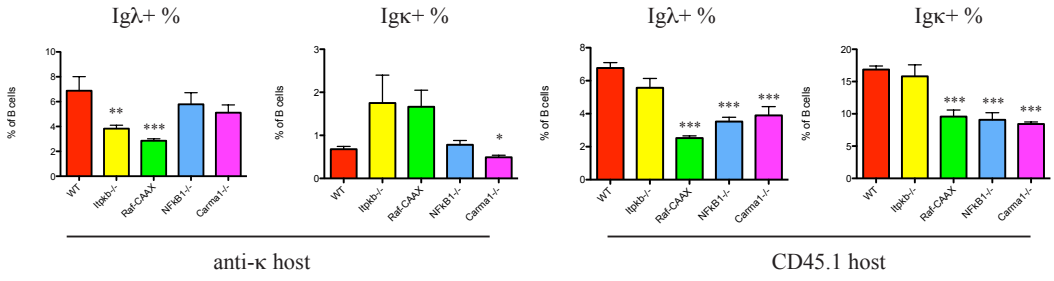


Figure 1. Light chain expression in BM chimeras.

Flow cytometry of the BM was performed, with a forward/side scatter gate for lymphocytes, then a $CD45.2^{+}$ $B220^{int}$ (A & C) or $B220^{hi}$ (B) gate was applied. A & B: The x-axis shows Igκ expression, while the y-axis shows Igλ expression. C: The percentages of Igκ and Igλ-expressing cells are plotted with standard error shown as error bars. Percentages in anti-κ hosts are on the left, while percentages in CD45.1 hosts are on the right. Asterisks indicate two-tailed student's t-test values, * - <0.05, ** - <0.01, *** - <0.005.

Figure 2. Editing ratio in BM chimeras

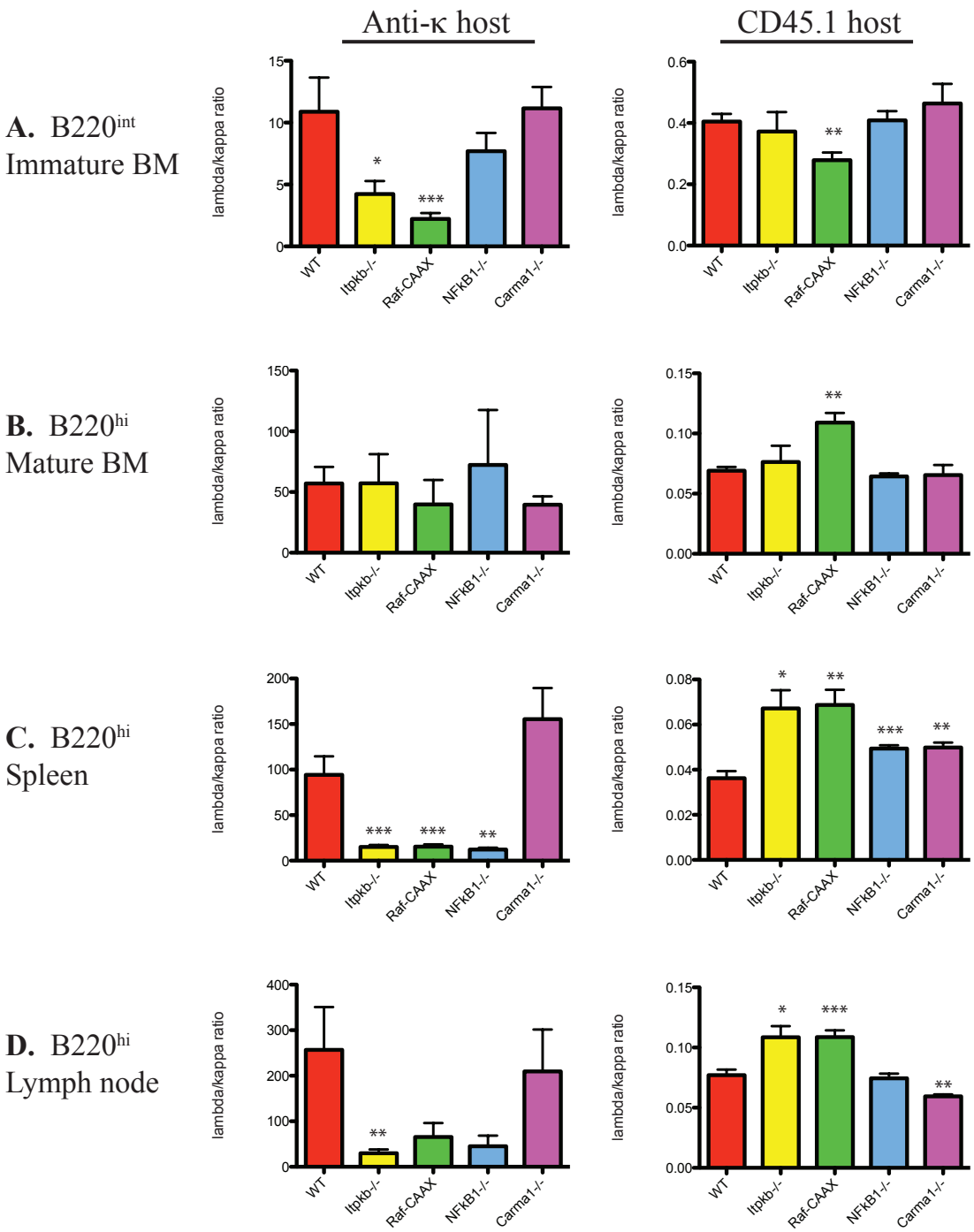


Figure 2. Editing Ratio in BM Chimeras.

Flow cytometry of BM, spleen, and lymph nodes was performed, with a forward/side scatter gate for lymphocytes, then CD45.2 and B220 gates applied. The percentage of λ-expressing cells was divided by the percentage of κ-expressing cells to obtain the editing ratio, plotted with standard error shown as error bars. Asterisks indicate two-tailed student's t-test values, * - <0.05, ** - <0.01, *** - <0.005. The left column shows the ratio in anti-κ chimeras, while the right column shows the ratio in CD45.1 chimeras. $B220^{int}$ cells from the BM are shown in A, while mature recirculating $B220^{hi}$ cells from the BM are shown in B. $B220^{hi}$ cells from the spleen and lymph node are shown in C & D, respectively.

Figure 3. Model of Receptor Editing Inhibition by BCR Signals

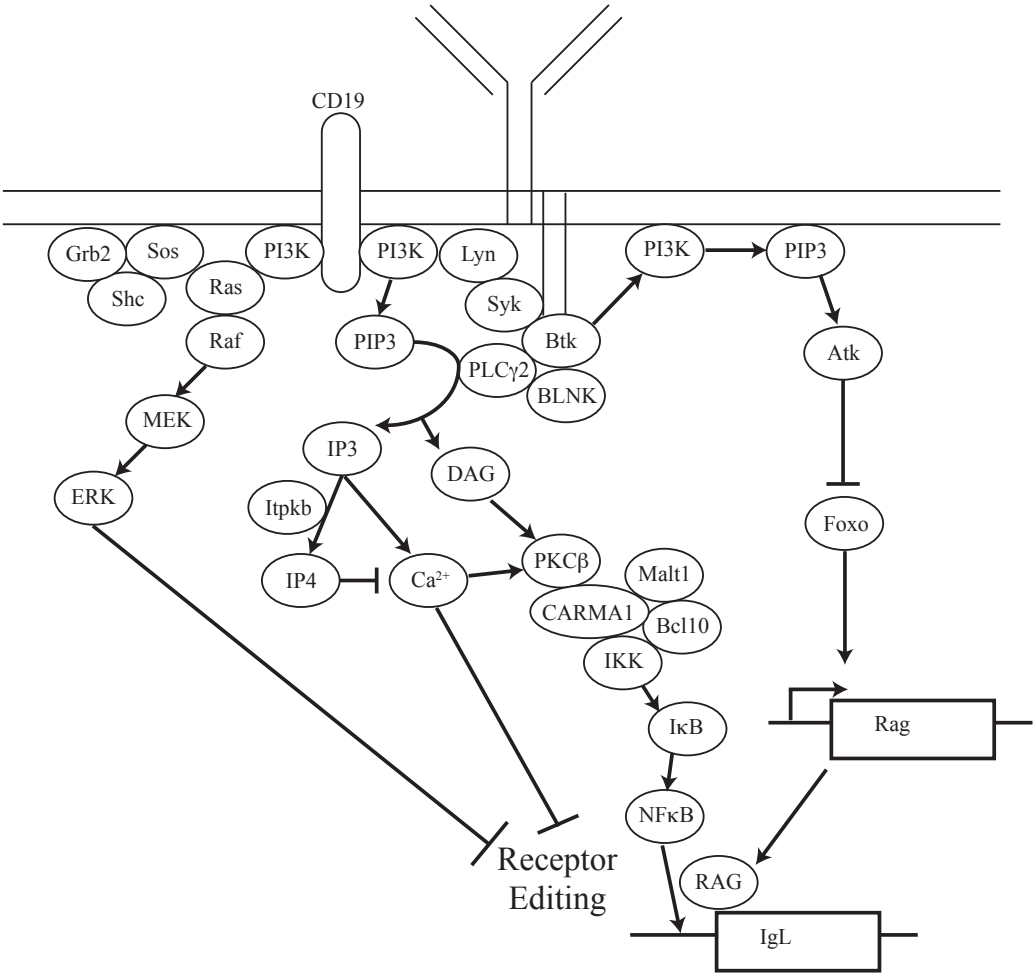


Figure 3. Model of Receptor Editing Inhibition by BCR Signals

The complex pathway of BCR signaling is not linear, but is simplified here for understanding. PI3K inhibits receptor editing through Ras activation and Ca^{2+} signaling. PI3K has also been shown to inhibit Rag transcription through Akt inactivation of FOXO proteins. Additionally, a pathway through Btk and BLNK has been suggested to inhibit the PI3K/Akt/FOXO pathway, enhancing LC recombination.

Chapter 4

STAT5 Cooperates with Defects in B Cell Development to Initiate Progenitor B Cell Leukemia

Laura B. Ramsey[1*], Lynn M. Heltemes-Harris[1*], Mark J.L. Willette[1*], Yi Hua Qiu[2], E. Shannon Neeley[3], Nianxiang Zhang[4], Thearith Koeuth[5], Emily C. Baechler[5], Steven M. Kornblau[2], and Michael A. Farrar[1]

*Contributed equally to the results presented here

[1]Department of Laboratory Medicine and Pathology, Center for Immunology, The Cancer Center, University of Minnesota, 312 Church St. SE, Minneapolis, MN 55455; [2]Department of Stem Cell Transplantation and Cellular Therapy, Section of Molecular Hematology and Therapy, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 448, Houston, TX 77030-4009; [3]Department of Statistics, Brigham Young University, 223 TMCB, Provo, UT 84602; [4]Bioinformatics and Comp. Biology, MD Anderson Cancer Center, Houston, TX 77030; [5]Department of Medicine, Center for Immunology, University of Minnesota, 312 Church St. SE, Minneapolis, MN 55455

Summary

Acute lymphoblastic leukemia (ALL) is characterized by chromosomal aberrations including translocations, duplications, and mutations. Recent studies have reported that 40% of human ALL samples harbor loss-of-function mutations in genes required for B cell development such as *pax5*, *ebf1* and *ikaros* [134]. However, since ALL is characterized by a fair degree of genomic instability, and since mice deficient in *pax5*, *ebf1* or *ikaros*, do not develop progenitor B cell leukemia, it remains unclear whether these mutations in human ALL are simply passengers in the transformation process or whether they drive the initial transformation. To address this question, we developed a mouse model in which STAT5 activation cooperates with defects in genes involved in pro-B (*ebf1*, *pax5*) or pre-B cell (*blnk*, *btk*, *PKCβ*) differentiation to initiate highly penetrant aggressive ALL. Likewise, we found that ~35% of adult human ALL specimens had elevated phospho-STAT5 levels. Importantly, increased STAT5 phosphorylation in BCR-ABL$^+$ ALL correlated with a significantly poorer outcome to Imatinib treatment. Our results demonstrate that mutations in genes critical to B cell development cooperate with activated STAT5 to initiate ALL and that STAT5 activation has important implications for treatment of patients.

Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease characterized by an expansion of lymphocytes in the blood. Eighty percent of cases involve expansion of B cells, while 20% of cases are T cell ALL. Although the cure rate is above 80%, ALL is still the second leading cause of cancer death among children. The cure rate of adults that develop ALL is merely 40%. Many ALL cases involve translocations, including t(12;21) TEL-AML1, which is more common in children, and t(9;22) BCR-ABL, which is more common in adults. BCR-ABL is a fusion protein of the breakpoint cluster region and ABL kinase, rendering the kinase constitutively active, altering signaling pathways that control proliferation and survival. This translocation is associated with a particularly poor outcome, but is now treated with an ABL-specific kinase inhibitor, Imatinib, which has greatly improved survival. The BCR-ABL translocation is also found in chronic myelogenous leukemia (CML), and mouse models have shown that BCR-ABL expression in hematopoietic stem cells (HSCs) is enough to induce CML-like disease, but additional genetic alterations are required for development of ALL-like disease [143, 226].

Recently, many gene expression studies have been performed to identify genetic abnormalities that may cooperate with the BCR-ABL translocation to induce ALL [134, 227-229]. In one study, 40% of patients had loss-of-function mutations in genes regulating B cell development, with *ikaros*, *pax5*, and *ebf1* among the most common [134]. Deletion of *ikaros* was particularly associated with BCR-ABL$^+$ ALL, with 83.7% of patient samples harboring deletions [230]. Deletion of *pax5* occurred in 51% of BCR-ABL ALL cases, 95% of which also had *ikaros* deletions.

Ikaros, Pax5, and EBF are transcription factors important for normal B cell development [137, 138, 231]. Mice deficient in any of these transcription factors have severely impaired B cell development, however they do not develop leukemia. This caused us to wonder if these mutations are actually the driving mutations behind leukemogenesis or just passengers in the process, mutated due to the inherent genomic instability of leukemic cells. Mice lacking the adaptor protein BLNK develop ALL at a low rate (~5%), and also have defects in B cell development [140, 232]. When mice lack BLNK and Btk, they develop leukemia at a much higher rate (75%) [141], indicating that pre-BCR signaling may have a role in preventing leukemogenic transformation.

We observed a five-fold expansion of pro-B and pre-B cells in mice with a constitutively active STAT5b transgene, *STAT5b-CA*, and a 1 – 2% incidence of ALL-like disease [28, 233]. Herein we have analyzed these leukemic mice further, and also crossed the *STAT5b-CA* transgenic mice with mice lacking pre-BCR signaling components (BLNK, Btk, PKCβ) or B cell development transcription factors (Pax5 and EBF1). All of these crosses developed highly penetrant leukemia, with either a pro-B or pre-B phenotype, indicating the importance of these genes in driving leukemogenesis.

Materials and Methods

Mice

All mice have been previously described [28, 137, 179, 207, 234, 235]; the University of Minnesota IACUC approved all animal experiments. Tumors were identified by visual examination and palpation; mice were euthanized upon tumor identification. Spleen, lymph nodes, bone marrow and peripheral blood were isolated from tumor-bearing mice and used for further experiments. $B220^{+}CD19^{+}$ lymph node cells were isolated using magnetic bead separation (Miltenyi Biotech) and used to isolate RNA, DNA and protein. Kaplan-Meyer survival curves were created using Prism software. Survival of *STAT5b-CA x xid, STAT5b-CA x PKCβ*$^{-/-}$*, STAT5b-CA x blnk*$^{+/-}$*, STAT5b-CA x rag2*$^{-/-}$*, STAT5b-CA x ebf1*$^{+/-}$ *and STAT5b-CA x pax5*$^{+/-}$ was compared to *STAT5b-CA*-negative littermate controls; all mice were monitored for one year or more. Deaths were indicative of tumor development.

Flow Cytometry

Single cell suspensions were prepared from the above tissues and stained with the following antibodies: α-IgM (Jackson ImmunoResearch), α-IgD (11-26), α-BP-1 (FG35.4), PE α-CD4 (L3T4), Pacific Blue α-CD4 (GK1.5, BioLegend), α-CD8 (53-6.7), α-CD19 (1D3), α-CD24 (M1/69), α-CD25 (PC61.5), α-CD43 (S7), α-CD45R (RA3-6B2), α-CD117 (2B8), α-CD127 (A7R34), α-CD135 (A2F10), α-AA4.1, α-GITR (DTA-1), α-pre-BCR (SL165; BD Biosciences) and α-GR-1 (RB6-8C5). All antibodies were obtained from eBioscience unless otherwise indicated. SA-PerCP-Cy5.5 (eBioscience) was used to detect biotinylated antibodies. Cells were assayed on

a LSRII flow cytometer (BD Biosciences); data was analyzed using FlowJo software (Treestar).

V(D)J Recombination

DNA recombination of the *Igh* locus was detected by PCR as described [4]. Briefly, DNA was purified from $B220^{+}CD19^{+}$ lymph node cells using High Pure PCR Template Preparation Kit (Roche). A serial dilution (1, 10 and 100 ng) of DNA was subjected to PCR using primers specific for $V_HJ558\text{-}DJ_H$, $V_HQ52\text{-}DJ_H$ and $V_H7183\text{-}DJ_H$ rearrangements or actin (control). Southern blot analysis was performed using a digoxigenin-labeled $D_HFL16\text{-}J_H4$ probe (generously provided by Dr. Harinder Singh) or an actin probe and detected using chemiluminescent anti-digoxigenin (Roche).

Western Blot

Whole cell lysates were prepared from 2 x 10^7 B cells in RIPA buffer with HALT protease and phosphatase inhibitors (Pierce). Protein concentrations were determined with the BCA protein assay (Pierce). Forty μgs of each sample were loaded into each well, separated by gel electrophoresis and transferred to a polyvinylidene difluoride membrane using an iBlot transfer system (Invitrogen). Blots were probed with rabbit anti-Myc (Cell Signaling). Bound antibodies were detected by Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen) and images were acquired on an Odyssey Infrared Detection System (Licor).

RT-PCR

Complementary DNA was synthesized from 200 ng of total RNA using Superscript III reverse transcriptase (Invitrogen) and a random hexamer primer in a total volume of 20 μl. One μl of cDNA template was used in a 20 μl reaction. Primers for *cyclin D2* and *hprt* have been previously described [25]. Reactions using Platinum quantitative PCR SuperMix-UDG with ROX were set up according to the instructions provided by the manufacturer (Invitrogen) and carried out on a 7000 Sequence Detection PCR System (Applied Biosystems). Amplification conditions were as follows: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Normalized values were calculated as described [236].

Microarray

Total RNA was extracted from either sorted pre-B control cells (C57Bl/6, *STAT5b-CA* or *xid*) or $B220^{+}CD19^{+}$ leukemic cells from lymph nodes of tumor-bearing mice using an RNeasy kit (Qiagen). cRNA probes were synthesized and hybridized to Mouse 430 2.0 arrays following Affymetrix protocols and statistical analyses were performed as previously described [52]. Clustering and visualization of data was performed using CLUSTER and TREEVIEW software [178]. Sample Sizes: C57Bl/6 pre-B = 5, *STAT5b-CA* pre-B = 4, *xid* pre-B = 3, *STAT5b-CA x ebf1*$^{+/-}$ = 5, *STAT5b-CA x rag2*$^{-/-}$ = 4, *STAT5b-CA* spontaneous = 6, *STAT5b-CA x xid* = 7, *STAT5b-CA x blnk*$^{+/-}$ = 5, *STAT5b-CA x PKCβ*$^{-/-}$ = 4.

Cooperation Response Synergy Score

Significantly different genes were defined by having an absolute fold change greater than 1.5, an absolute difference in expression levels of 200, a p-value of less than 0.05 and a false discovery rate of less than 0.1, comparing the average of B6 pre-B to *STAT5b-CA x xid* for each probe. Probes with no annotation were removed and probes of the same gene were averaged to provide one expression level per gene. A synergy score was calculated by comparing the average of *STAT5b-CA* pre-B (a), the average of *xid* pre-B (b), and the average of the *STAT5b-CA x xid* (c), using the following formula: $(a+b)/c \leq 0.9$ for genes overexpressed in the tumors, and $c/a + c/b \leq 0.9$ for genes underexpressed in the tumors [237]. This resulted in a list of 417 genes. GO biological processes were used to categorize each gene.

Reverse Phase Protein Assays (phospho-STAT5)

129 ALL samples and 10 $CD34^+$ control samples were collected and analyzed using reverse phase protein arrays as previously described [238]. These samples were collected by the University of Texas M.D. Anderson Leukemia sample bank under the IRB approved protocol Lab01-473 and utilized under protocol Lab05-0654.

Data Analysis

Data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). Canonical pathways analysis identified the pathways from the Ingenuity Pathways Analysis library that were most significant to the data set.

Genes from the data set that met the absolute fold change cutoff of at least 1.5 (versus C57Bl/6 pre-B), absolute difference of at least 200, FDR [239] of less than 0.05, and were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base, were considered for the analysis. The significance of the association between the data set and the canonical pathway was measured by Fischer's exact test; the $\log_2$(p-value) is plotted on the x-axis.

Results

The transcription factor STAT5 plays a critical role downstream of the IL-7R in initiating pro-B cell differentiation [25] and inhibiting premature pre-B cell differentiation [44]. Alterations in STAT5 expression have not been reported in previous microarray screens of ALL, thereby raising questions about the importance of STAT5 in ALL [134, 229]. However, STAT5 has been suggested to play a role in BCR-ABL and TEL-JAK2-dependent transformation of murine B cells [240, 241], although the relative importance of this pathway in human leukemia remains to be determined. To address these questions, we analyzed transgenic mice expressing a constitutively active form of STAT5 (STAT5b-CA) throughout B and T cell development [28].

Approximately 1-2% of *STAT5b-CA* mice develop progenitor B cell leukemia [28, 233], characterized by grossly enlarged spleen and lymph nodes (Figure 1a) and elevated numbers of B cell progenitors in the blood and bone marrow (data not shown). These cells typically express the IL-7R but minimal levels of IgM; most leukemic cells also express pre-BCR, suggesting that they represent pre-B cell leukemia (Figure 1b). These leukemic cells appear to be clonal or possibly oligoclonal in origin as demonstrated by an analysis of V(D)J rearrangement patterns (Figure 1c).

To further characterize the leukemia that spontaneously arose in *STAT5b-CA* mice, we compared gene expression profiles from *STAT5b-CA* leukemic cells to sorted C57Bl/6 pre-B cells (Figure 1d). Some of the most significant changes involved well-known oncogenes such as *c-myc* (up 9-fold, Figure 2a) and *ccnd2* (up 6-13-fold, Figure 2b). Intriguingly, the asparagine synethetase gene (*asns*), a major target in

chemotherapy for ALL, was also upregulated in four out of six *STAT5b-CA* leukemias (up ~9-fold, Figure 1d). Finally, a number of genes involved in B cell development (*ebf1*, *ikaros*) and pre-BCR signaling (*syk*, *blk*) appeared to be downregulated in multiple *STAT5b-CA* spontaneous leukemic samples. These results suggest that the combination of STAT5 activation coupled with downregulation of genes in B cell differentiation and signaling plays a critical role in driving pro-B or pre-B cell transformation.

Based on the above findings, we tested whether crossing *STAT5b-CA* mice to mice lacking genes important for pre-BCR signaling, including the adaptor *blnk*, and the downstream kinases *btk* and *PKCβ*, would result in increased incidence of leukemia. Importantly, $blnk^{+/-}$, *xid* (*btk*-defective mice) and $PKC\beta^{-/-}$ control mice never developed leukemia (Figure 3). In contrast, >75% of *STAT5b-CA* x $blnk^{+/-}$, *STAT5b-CA* x *xid* and *STAT5b-CA* x $PKC\beta^{-/-}$ mice developed ALL between 6-42 weeks after birth (Figure 3). Tumors from these mice resembled the "spontaneous" *STAT5b-CA* tumors as they were IL-$7R^{+}$, $CD19^{+}$ and pre-BCR^{+} but expressed reduced levels of B220; consistent with a pre-B cell-like phenotype, these leukemic cells lacked CD43 and c-kit (Figure 3 and 4). Thus, STAT5 activation combined with defects in pre-BCR signaling results in highly penetrant pre-B ALL.

The transcription factors EBF1 and Pax5 regulate expression of many components of pre-BCR signaling, including *CD79a* and *blnk* [242]. To examine the role of these two genes in leukemia, we created *STAT5b-CA* x $ebf1^{+/-}$ and *STAT5b-CA* x $pax5^{+/-}$ mice. Loss of both alleles of *pax5* in mature B cells results in back differentiation and development of progenitor B cell leukemia with a median age of

onset of ~9 months [243]. However, *ebf1*$^{+/-}$ and *pax5*$^{+/-}$ mice never develop leukemia [137, 207] (Figure 3). In contrast, loss of one allele of either *pax5* or *ebf1* in combination with constitutively active STAT5b resulted in rapid 100% induction of leukemia (median onset 50 and 109 days, respectively; Figure 3). The majority of these leukemias have not completely lost *pax5* and/or *ebf1* expression as demonstrated by the presence of *CD19* and *CD79a*, respectively (Figures 4 and 5). Thus loss of only a single allele of *pax5* or *ebf1* is sufficient to promote transformation. Finally, to determine if the primary mechanism responsible for transformation is due to a block in early B cell differentiation, we generated *STAT5b-CA x rag2*$^{-/-}$ mice, which cannot progress beyond the late pro-B cell stage. Surprisingly, these mice also came down with leukemia. However, penetrance was lower (~60%) and the median age of tumor onset was significantly later in these mice (208 days; Figure 3). This latter finding suggests that while inhibiting B cell differentiation is important, other effects also contribute to transformation.

To more fully characterize the leukemia generated in our mouse models, we performed microarray analysis to compare changes in mRNA expression in C57Bl/6, *STAT5b-CA* non-transformed and *xid* pre-B cells, to those in leukemic cells from *STAT5b-CA x blnk*$^{+/-}$, *STAT5b-CA x xid*, *STAT5b-CA x PKCβ*$^{-/-}$, *STAT5b-CA x ebf1*$^{+/-}$ and *STAT5b-CA x rag2*$^{-/-}$ mice. These studies confirmed our earlier observation that these tumors showed downregulation of genes involved in B cell development and pre-BCR signaling. Interestingly, hierarchical clustering of the microarray data suggested that there were two distinct subsets of leukemia in our dataset (Figure 6). The first set was comprised primarily of leukemic cells derived from *STAT5b-CA x ebf1*$^{+/-}$ and

STAT5b-CA x rag2$^{-/-}$ mice, while the second set was comprised largely of leukemic cells from *STAT5b-CA x blnk*$^{+/-}$, *STAT5b-CA x xid* and *STAT5b-CA x PKCβ*$^{-/-}$ mice. Spontaneous *STAT5b-CA* tumors could be found in each subset. These subsets also can be identified based on phenotypic analysis using cell surface markers such as CD25, c-kit, BP-1 and pre-BCR (Figures 3 and 4).

There were clear similarities between these distinct subsets (referred to hereafter as EBF/Rag and BLNK/PKCβ subsets) including upregulation of potential oncogenes such as *c-myc*, *ccnd2* and *r-ras2* (Table I). However, there were also notable differences between the two subtypes. First, Ingenuity Pathway Analysis indicated that the EBF/Rag subset, but not the BLNK/PKCβ subset, was characterized by an upregulation of genes involved in multiple metabolic pathways (Figure 7). Second, the BLNK/PKCβ subset, but not the EBF/Rag subset, showed a clear decrease in genes involved in BCR/pre-BCR signaling (Figures 7c and 8). Finally, while both subsets showed perturbations in NFκB signaling, NFκB1 expression was clearly elevated in the EBF/Rag subset when compared to the BLNK/PKCβ subset (up 2.8 fold, $p < 0.0001$). This is consistent with the possibility that downregulation of the BLNK/Btk/PKCβ pathway results in decreased NFκB1 activation and hence inhibits pre-B to immature B cell differentiation. NFκB2 activation was also noted in both subsets (data not shown), although the underlying mechanism likely differs. In the EBF/Rag subset we observed upregulation of multiple members of the TNF receptor superfamily including *tnfrsf18* and *tnfrsf1b* (Figure 9). Likewise, RANKL (*tnfsf11*) was expressed at very high levels in the EBF/Rag subset but not in the BLNK/PKCβ subset or C57Bl/6 pre-B cells (Figure 9). Thus, aberrant expression of TNF receptor family members or their ligands

may play an important role in driving NFκB2 (and NFκB1) activation in the EBF/Rag subset of tumors. In the BLNK/PKCβ leukemias we noted decreased expression of *traf3* and *cyld* (Figure 10*)*. The TRAF3/CYLD signaling module negatively regulates NFκB2 signaling. Reduced expression of these signaling components has been shown to underlie NFκB2 activation in multiple myeloma cells [244, 245] and may allow for heightened NFκB2 signaling in the BLNK/PKCβ subset of tumors. Thus, STAT5 activation coupled with at least a partial loss of function in genes that make up either a network of transcription factors governing B cell development, or the pre-BCR signaling pathway, leads to two distinct forms of highly penetrant leukemia.

Malignant cell transformation often requires the cooperation of two or more oncogenic mutations, which can synergize to alter downstream signaling pathways. To determine whether *STAT5b-CA* and *xid* were cooperating to synergistically activate specific signaling pathways, we calculated the synergy score and identified cooperation response genes [237]. This was done by comparing gene expression patterns in B6 WT pre-B cells, *STAT5b-CA* non-leukemic pre-B cells, *xid* pre-B cells, and *STAT5b-CA x xid* leukemic cells (see Materials & Methods). 417 cooperation response genes (CRGs) were identified, 286 that were increased, among them: *asns, socs2, socs3, cish, rras, myc, ccnd2, zap70, tcrg,* and *vegfa*. There are multiple SOCS family members upregulated, indicating these cells are trying to attenuate cytokine and growth hormone signaling. R-ras and Myc are other known oncogenes that may be cooperating to transform these cells. Zap70 and TCRγ expression indicate these cells are losing B cell specificity, which is common in B-ALL. Among the 131 downregulated CRGs: *foxp1, bach2, igl, nfkbid* (IκBδ)*, il2ra* (CD25)*, and dntt* (Tdt). Among these, Foxp1, Igl and

Tdt are all involved in LC recombination, indicating the tumor cells are at an earlier stage than the sorted pre-B cells. The lower CD25 expression also supports this idea. Bach2 is a tumor suppressor that is downregulated by BCR-ABL and associated with deletions in ALL patients [246]. Decreased expression of IκB may allow for constitutive activation of NFκB, which is associated with many types of cancer, including ALL [247, 248]. Many of the CRGs are involved in metabolism & transport (31%), which could increase the biosynthesis for rapid cell division, and signal transduction (19%), which could free the cells from external regulatory cues (Figure 11).

To determine whether STAT5 also plays a critical role in human ALL, we examined ALL patient samples by a newly described reverse phase proteomics approach [238]. Consistent with previous microarray studies [229] we found no increase in total STAT5 protein in human ALL (Figure 12). In contrast, >35% of ALL patient samples (n=129) exhibited clearly elevated levels of phosphorylated STAT5 when compared to normal adult control samples (n=10)(Figure 13a). The level of phospho-STAT5 varied significantly between ALL cytogenetic subsets with the highest expression observed in BCR-ABL$^+$ ALL (Figure 14). Historically, the overall survival of BCR-ABL$^+$ patients is quite poor, although the recent introduction of Imatinib as a therapy for such patients has improved survival [249]. Intriguingly, we found that phospho-STAT5 status prior to treatment with Imatinib predicted the response to subsequent Imatinib therapy with higher levels of phospho-STAT5 correlating with dramatically poorer overall survival (Figure 13b).

Discussion

Herein we describe a new mouse model that closely mimics human ALL. These findings suggest that STAT5 acts as a central hub that is critical for the induction of both pro-B and pre-B cell leukemia. STAT5 drives transformation by cooperating with loss-of-function mutations that cluster in two distinct pathways, one involving EBF1 and Pax5, which are critical for pro-B differentiation, and the second involving pre-BCR signaling, which is required for pre-B differentiation. Recently, Nakayama et al published similar findings regarding *STAT5b-CA x Blnk*$^{+/-}$ mice [233]. The authors found that BLNK inhibits JAK3 and induces apoptosis in the presence of the STAT5b-CA transgene, thereby suppressing leukemogenesis. This provides a model for the pre-BCR signaling pathway in preventing leukemia.

We have shown here that BLNK, Btk, PKCβ, EBF1 and Pax5 are all involved in the development of pre-B ALL. When combined with constitutive STAT5 signaling, which mimics IL-7R signaling, we find that nearly all of these mice develop leukemia. We conclude that these genes are not merely passengers in leukemogenesis, but drivers of transformation. Cells deficient for pre-BCR signaling proteins are blocked in B cell development at the pre-B stage, where they are proliferating rapidly in response to IL-7 [233]. When IL-7R signaling is enhanced with the STAT5b-CA transgene, the cells are unable to induce apoptosis via BLNK and leukemia results. Cells deficient in EBF1 or Pax5 have earlier blocks in development, but still proliferate in response to IL-7. We have shown here that lack of only a single allele of either of these genes is sufficient to promote transformation in conjunction with STAT5b-CA. The leukemic cells from these mice are blocked at the pro-B stage, when RAG proteins are expressed. Excessive

proliferation and aberrant RAG expression could lead to translocations similar to those seen in ALL patients.

Typically human B-ALL is modeled in mice by xenografts of patient cells [250] or expression of a BCR-ABL retrovirus [251]. Although these methods are effective at analyzing certain leukemic cells, we feel that our novel intact mouse model is a simpler and more physiologically relevant system. The BCR-ABL retroviral mouse model is widely used, but only 3% of children and 25% of adult ALL cases harbor this translocation [131]. Our model is more physiologically relevant because 40% of pediatric ALL cases harbor mutations in B cell development genes, such as *pax5* and *ebf1*. We have shown these mutations induce ALL when STAT5 is activated, which was found in 35% of adult ALL cases (Figure 13). This mouse model could easily be used to optimize current treatments or test new pharmacologic reagents.

We have demonstrated that STAT5 activation is increased in 35% of BCR-ABL$^+$ ALL patients, and those with lower STAT5 activation respond better to Imatinib therapy (Figure 4). These findings have important implications for human health, as we have demonstrated that a relatively simple procedure involving analysis of STAT5 phosphorylation allows one to predict responsiveness of BCR-ABL$^+$ ALL to Imatinib treatment. Thus, strategies to inhibit STAT5 activation may be useful for treating subsets of BCR-ABL$^+$ adult patients, as well as a newly described class of BCR-ABL-like ALL, which has a poor prognosis and develops with high frequency in pediatric patients [227]. Many patients develop resistance to Imatinib after several rounds of treatment [233]. STAT5 has been shown to be activated by BCR-ABL in retrovirally-

transformed cells [252, 253]. Thus, STAT5 inhibitors could potentially be useful in treating BCR-ABL^{+} Imatinib-resistant patients.

Acknowledgements

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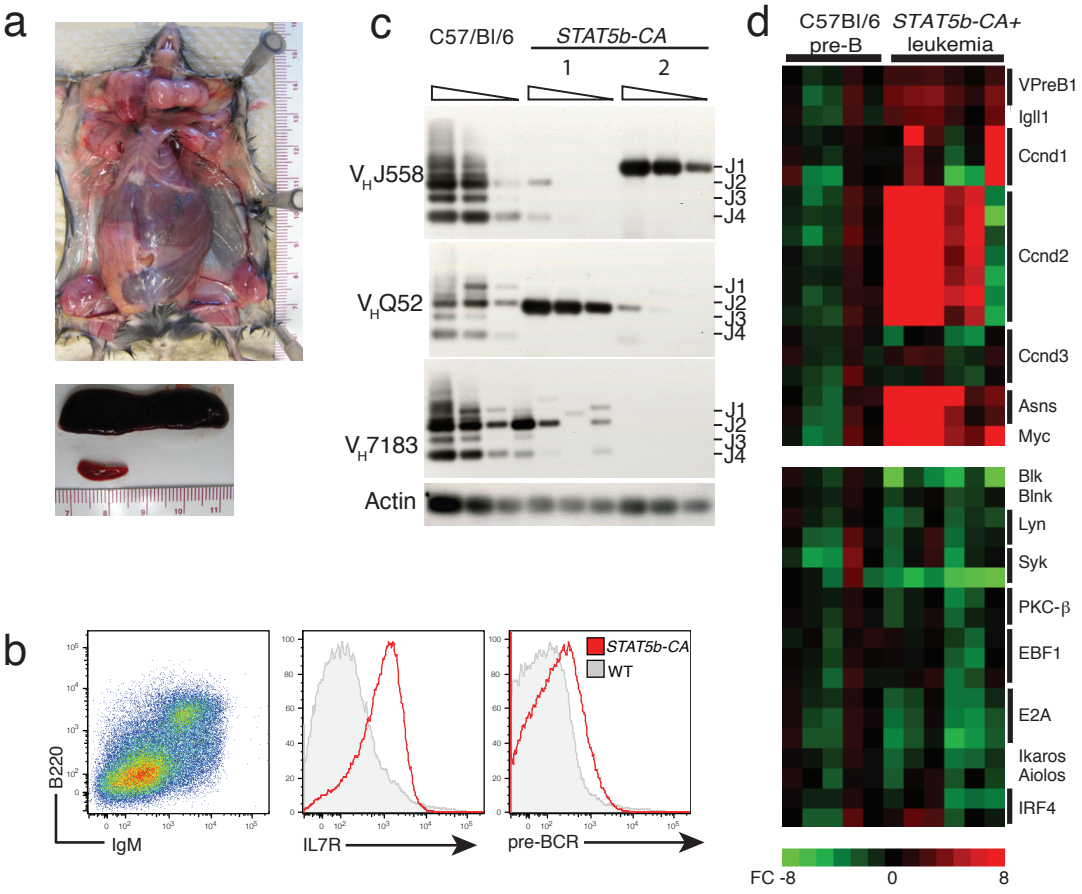
Figure 1


a
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C57/Bl/6
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d
C57Bl/6 pre-B
STAT5b-CA+ leukemia
VPreB1
Igll1
Ccnd1
Ccnd2
Ccnd3
Asns
Myc
Blk
Blnk
Lyn
Syk
PKC-β
EBF1
E2A
Ikaros
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IRF4
FC -8
0
8
b
B220
IgM
IL7R
pre-BCR
STAT5b-CA
WT

Figure 1. Spontaneous tumors in *STAT5b-CA* mice. **(a)** Top panel. Photo of a *STAT5b-CA* mouse that developed ALL-like leukemia. Bottom panel. Spleen from a leukemic *STAT5b-CA* mouse compared to wild-type C57Bl/6 control mouse. **(b)** Flow cytometric analysis of B220, IgM, IL-7R and pre-BCR expression on lymph node cells from *STAT5b-CA* leukemic and C57Bl/6 mice. $B220^{hi}IgM^{hi}$ cells represent remaining non-transformed B cells. Grey histograms represent staining for these markers on mature $B220^{+}$ splenic B cells from C57Bl/6 mice. **(c)** PCR analysis of *Igh* rearrangements in genomic DNA from *STAT5b-CA* tumor mice. DNA was analyzed using primers that detect $V_HJ558\text{-}DJ_H$, $V_HQ52\text{-}DJ_H$ and $V_H7183\text{-}DJ_H$ rearrangements and assessed by Southern blot. Wedges above lanes indicate a 10X serial dilution. PCR with *actin*-specific primers was used as a loading control. **(d)** Microarray analysis of *STAT5b-CA* tumors. Representative genes involved in B cell signaling and differentiation, cell cycle and potential genes involved in human ALL are shown. The color gradient indicates a fold-change from -8 to +8. C57Bl/6 pre-B cells were FACS sorted from BM as $CD19^{+}$, $B220^{+}$, $CD43^{-}$ and IgM^{-}; n = 5. STAT5b-CA+ leukemic cells were column purified from enlarged lymph nodes with CD19 and B220 beads; n = 6.

Figure 2. Cyclin D2 and myc expression in *STAT5b-CA* ALL

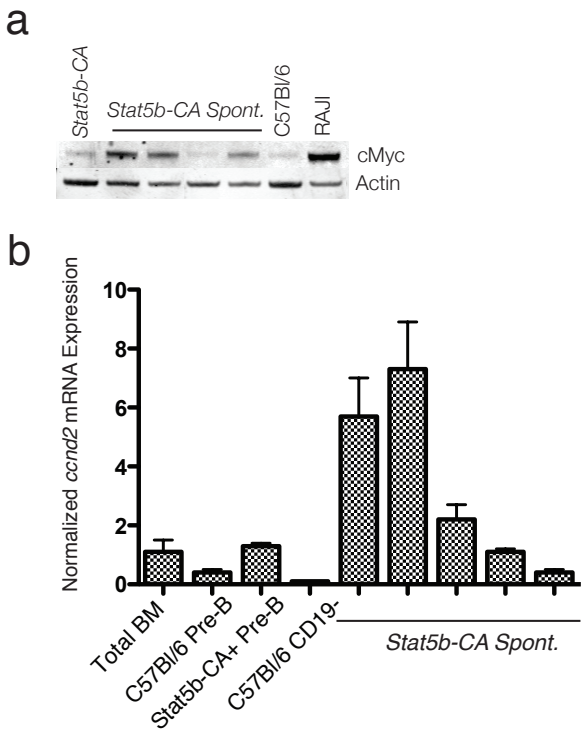


Figure 2. Cyclin D2 and Myc expression in *STAT5b-CA* ALL. **(A)** Western blotting for c-Myc protein in splenic B cells from non-leukemic *STAT5b-CA* mice (lane 1), leukemic cells from *STAT5b-CA* mice (lanes 2 to 5), splenic cells from C57Bl/6 control mice (lane 6), and RAJI cells (lane 7). **(B)** *Cyclin D2* (*ccnd2*) mRNA expression in *STAT5b-CA* leukemic cells was quantitated using TaqMan probes. Results were normalized to *hprt* expression.

Figure 3

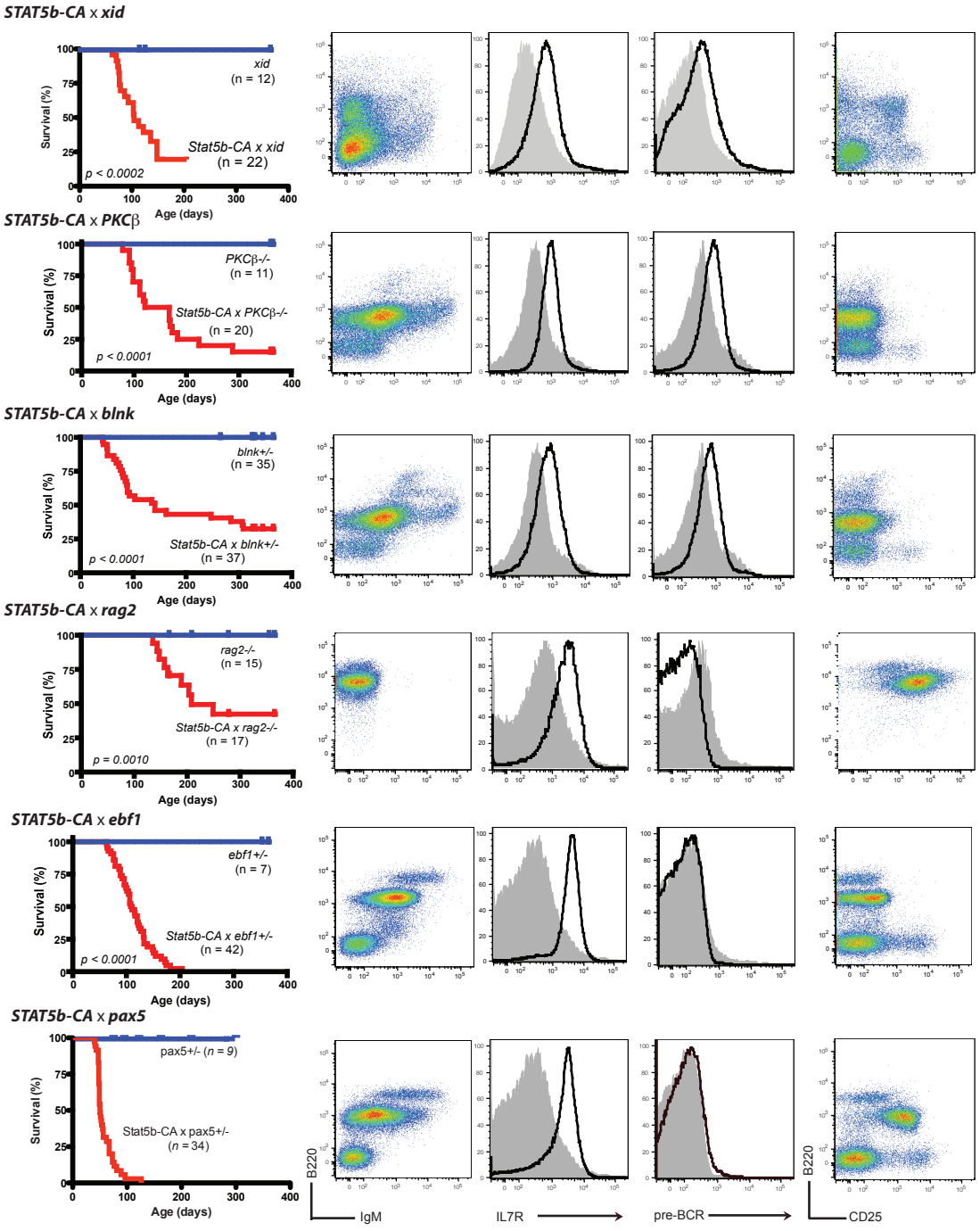

STAT5b-CA x xid
Survival (%)
xid
(n = 12)
Stat5b-CA x xid
(n = 22)
p < 0.0002
Age (days)
STAT5b-CA x PKCβ
PKCβ-/-
(n = 11)
Stat5b-CA x PKCβ-/-
(n = 20)
p < 0.0001
STAT5b-CA x blnk
blnk+/-
(n = 35)
Stat5b-CA x blnk+/-
(n = 37)
p < 0.0001
STAT5b-CA x rag2
rag2-/-
(n = 15)
Stat5b-CA x rag2-/-
(n = 17)
p = 0.0010
STAT5b-CA x ebf1
ebf1+/-
(n = 7)
Stat5b-CA x ebf1+/-
(n = 42)
p < 0.0001
STAT5b-CA x pax5
pax5+/- (n = 9)
Stat5b-CA x pax5+/-
(n = 34)
B220
IgM
IL7R
pre-BCR
B220
CD25

Figure 3. *STAT5b-CA* causes a significant increase in tumor incidence in all mouse crosses. Left. Kaplan-Meyer survival analysis of mice of the indicated genotype. The number (n) of mice analyzed is shown. Right. Representative flow cytometric analysis of B220, IgM, IL-7R, pre-BCR and CD25 expression on lymph node cells from leukemic mice listed above. Grey histograms represent staining for these markers on mature $B220^{+}$ B cells from the lymph nodes of C57Bl/6 mice.

Figure 4. Flow cytometric analysis of leukemic mice

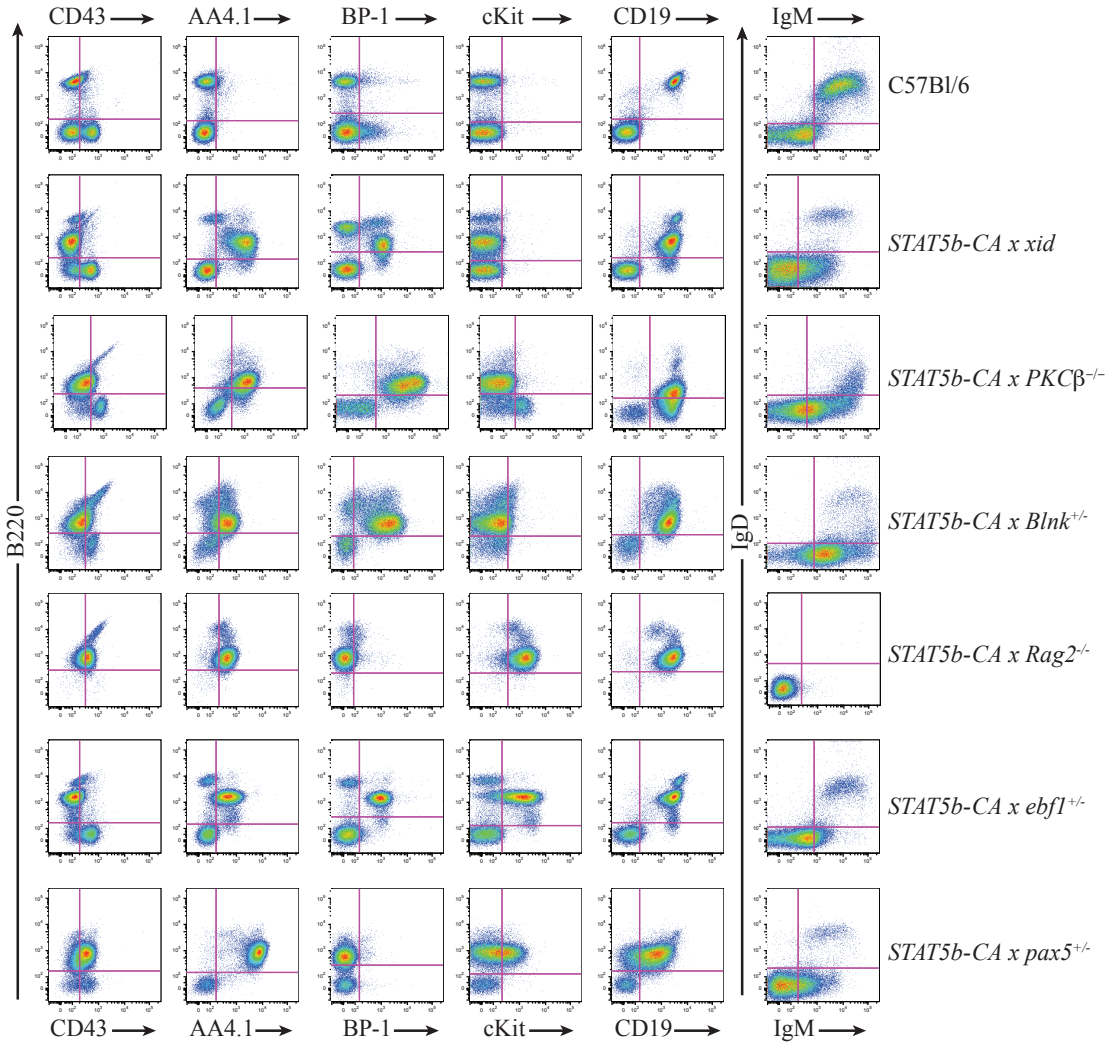


Figure 4. Flow cytometric analysis of lymph node cells from *STAT5b-CA x xid, STAT5b-CA x PKCβ*$^{-/-}$*, STAT5b-CA x blnk*$^{+/-}$*, STAT5b-CA x rag2*$^{-/-}$*, STAT5b-CA x ebf1*$^{+/-}$ *and STAT5b-CA x pax5*$^{+/-}$ tumor mice. Representative flow cytometric analysis of B220, CD43, AA4.1, BP-1, c-Kit, CD19, IgM and IgD expression on lymph node cells is shown. Doublets were gated out and a lymphocyte gate was set based on side and forward scatter properties. All gates shown are based on wild type lymph node cells isolated from control C57Bl/6 mice.

Figure 5. CD79a Expression

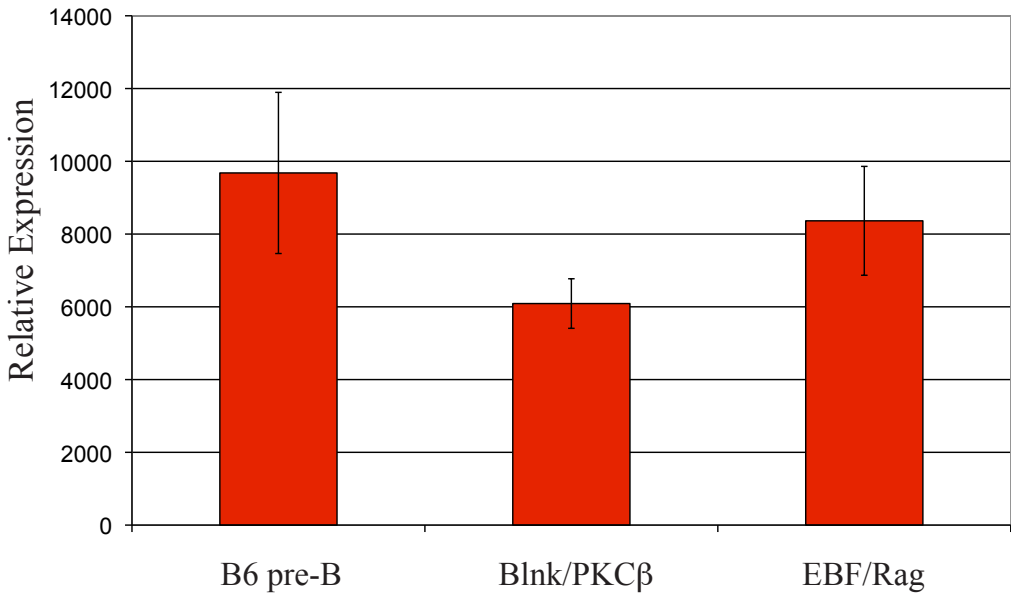


Figure 5. *CD79a* expression. Relative microarray gene expression levels of *CD79a* in C57Bl/6 pre-B cells, BLNK/PKCβ tumors and EBF/Rag tumors. Two-tailed student's t-test shows no significant difference in expression for *CD79a* in EBF/Rag or BLNK/PKCβ leukemic cells relative to control pre-B cells, with p-values of 0.18 (BLNK/PKCβ) and 0.64 (EBF/Rag).

Figure 6. Microarray analysis of tumor samples

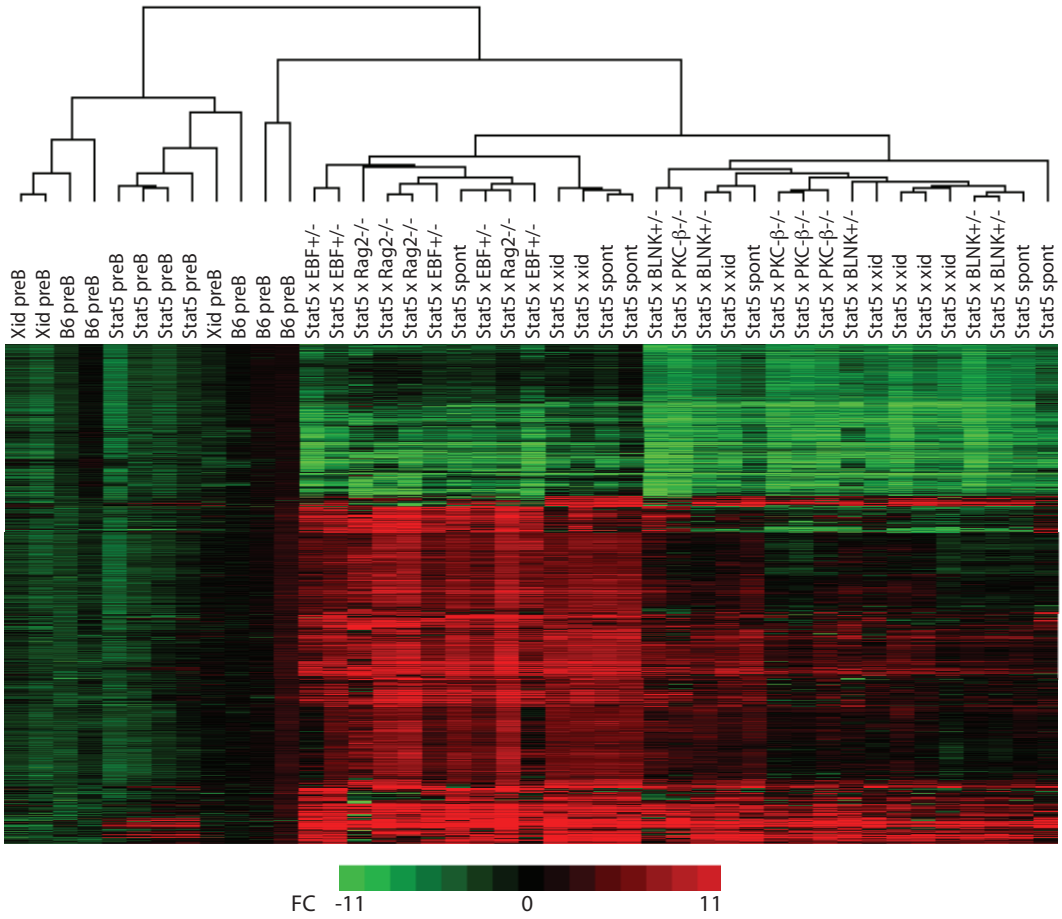


Figure 6. Microarray analysis of tumor samples from indicated mice. The average of the tumor samples was compared to the average of the C57Bl/6 pre-B samples for every probe set. Identification of genes with an absolute fold change of 1.5, absolute difference of 200, and a False Discovery Rate (FDR) [239] of less than 0.05 generated a gene list of 2091 genes. The color gradient indicates fold changes from -11 to +11. See sample sizes in Materials and Methods.

Figure 7a. Canonical pathways differentially regulated in the Blnk/PKCβ subset

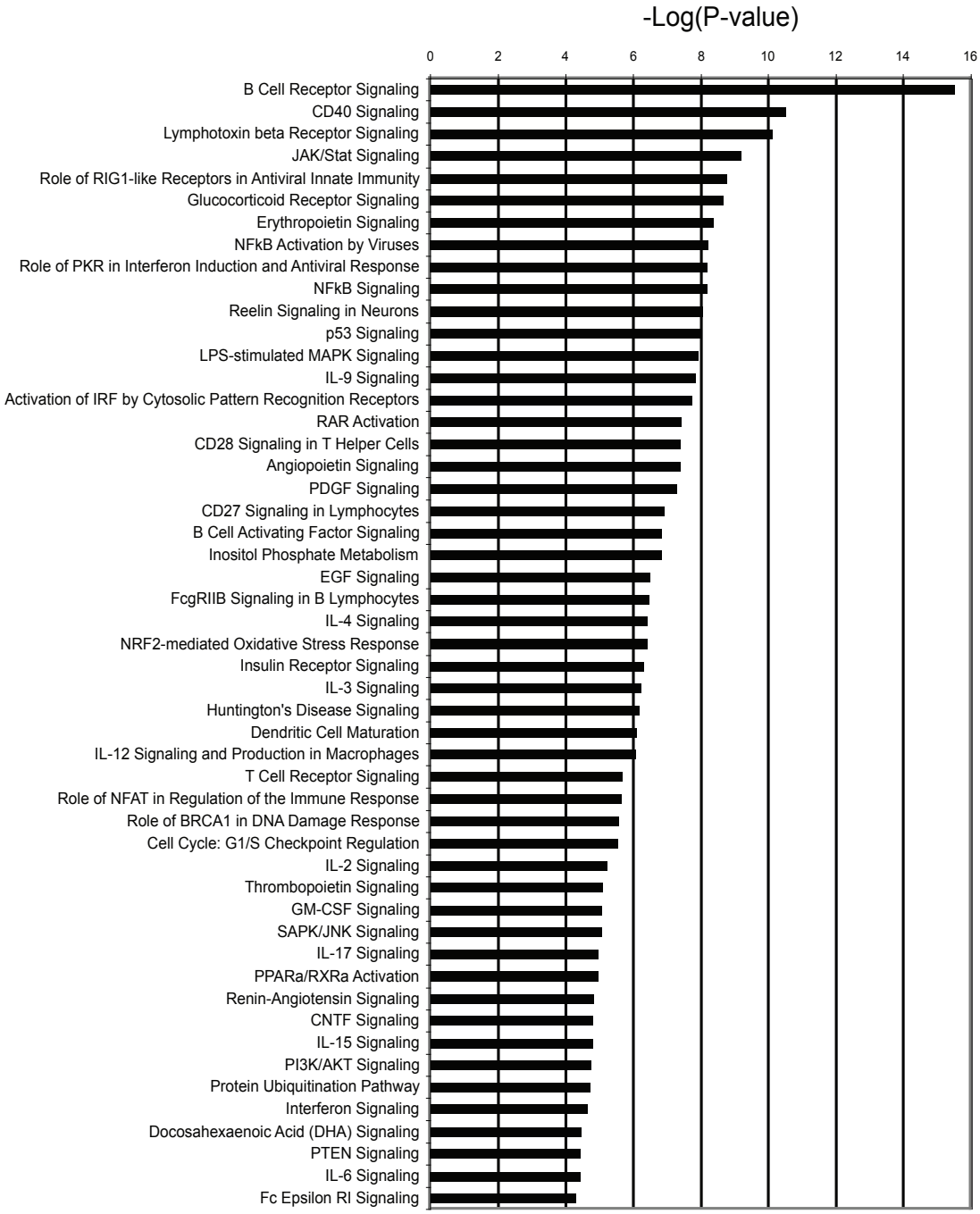


Figure 7b. Canonical pathways differentially regulated in the EBF/Rag subset

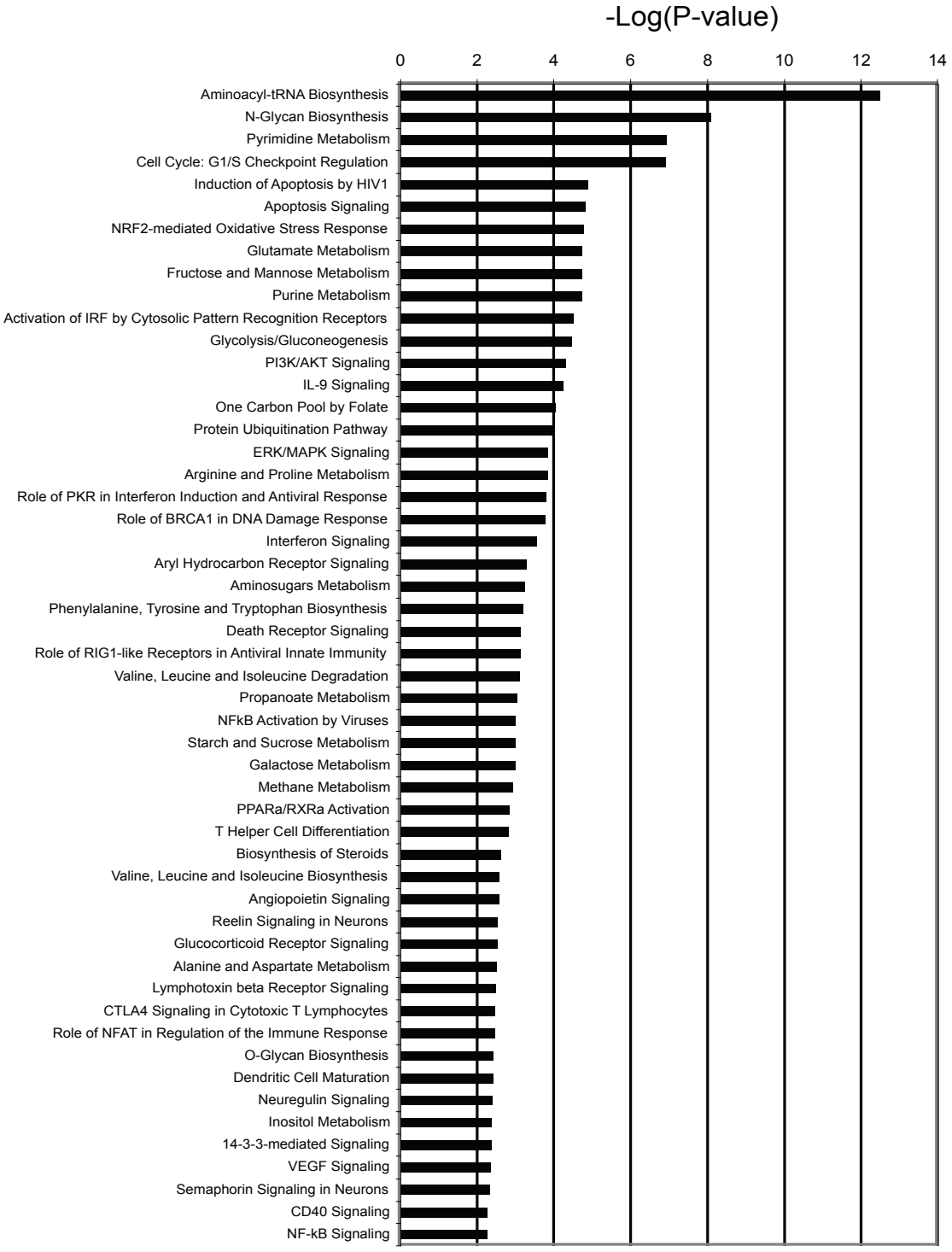


Figure 7c. Categorization of Differentially Regulated Genes

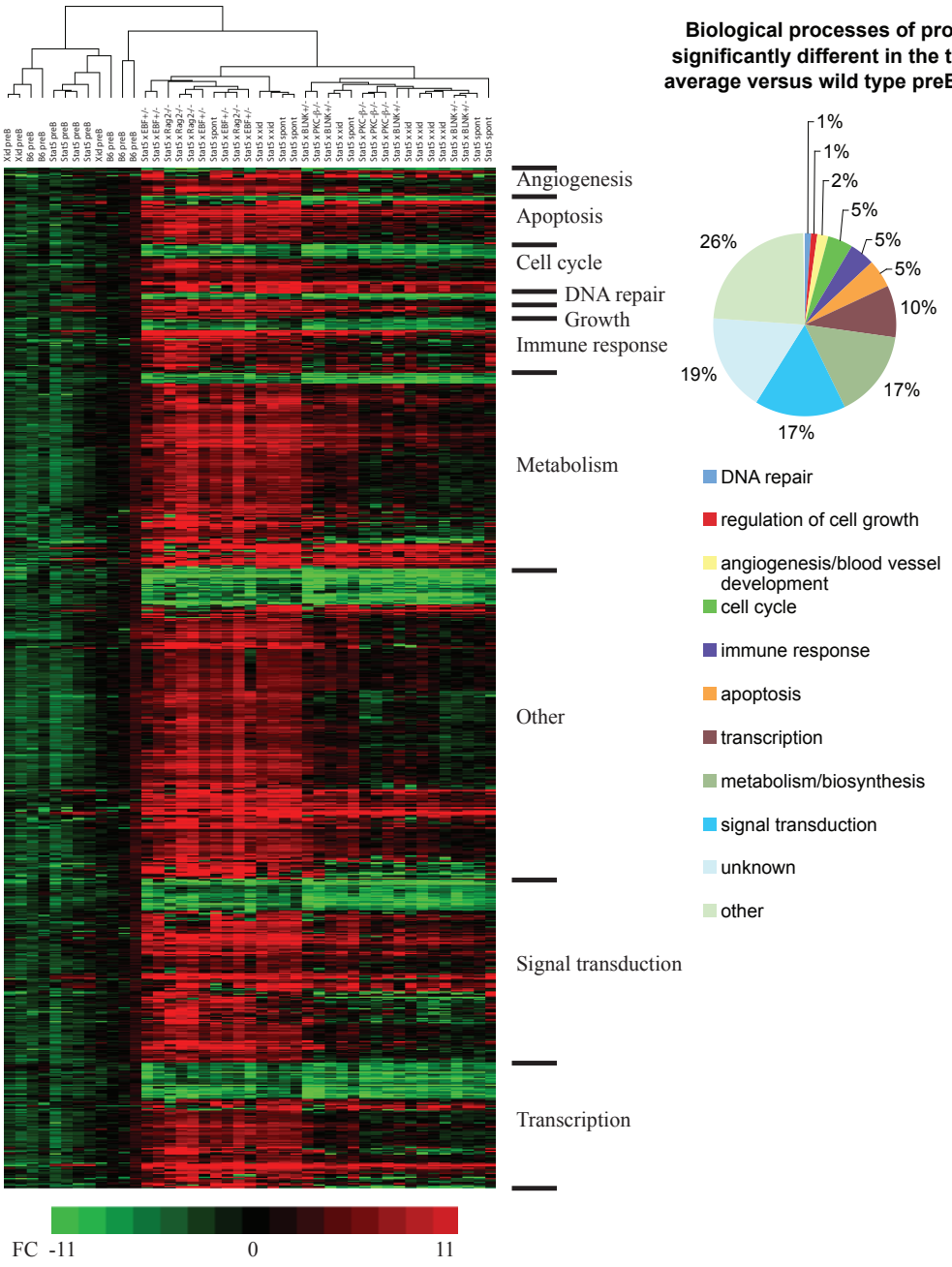


Figure 7. Canonical Pathways differentially regulated in tumor samples. Data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). The top 50 pathways are shown here for **(A)** the BLNK/PKCβ subset and **(B)** the Ebf/Rag subset, relative to C57Bl/6 pre-B cells. The significance of the association between the data set and the canonical pathway was measured by Fischer's exact test; the $\log_2$ p-value is plotted on the x-axis. **(C)** Categorization of differentially regulated genes in the tumors. Microarrays were carried out and analyzed as described in methods. The 1000 most significant genes, ranked by FDR, 811 of which had GO (Gene Ontology) biological process terms, were sorted into general categories. The probes in each category were clustered using CLUSTER and TREEVIEW (see methods summary), while the arrays were fixed in the same order as Figure 6. The percentages of probes in each category are shown in the pie chart. The color gradient shows a fold-change from -11 to +11.

Figure 8a. B Cell Receptor Signaling in the Blnk/PKCβ Subset

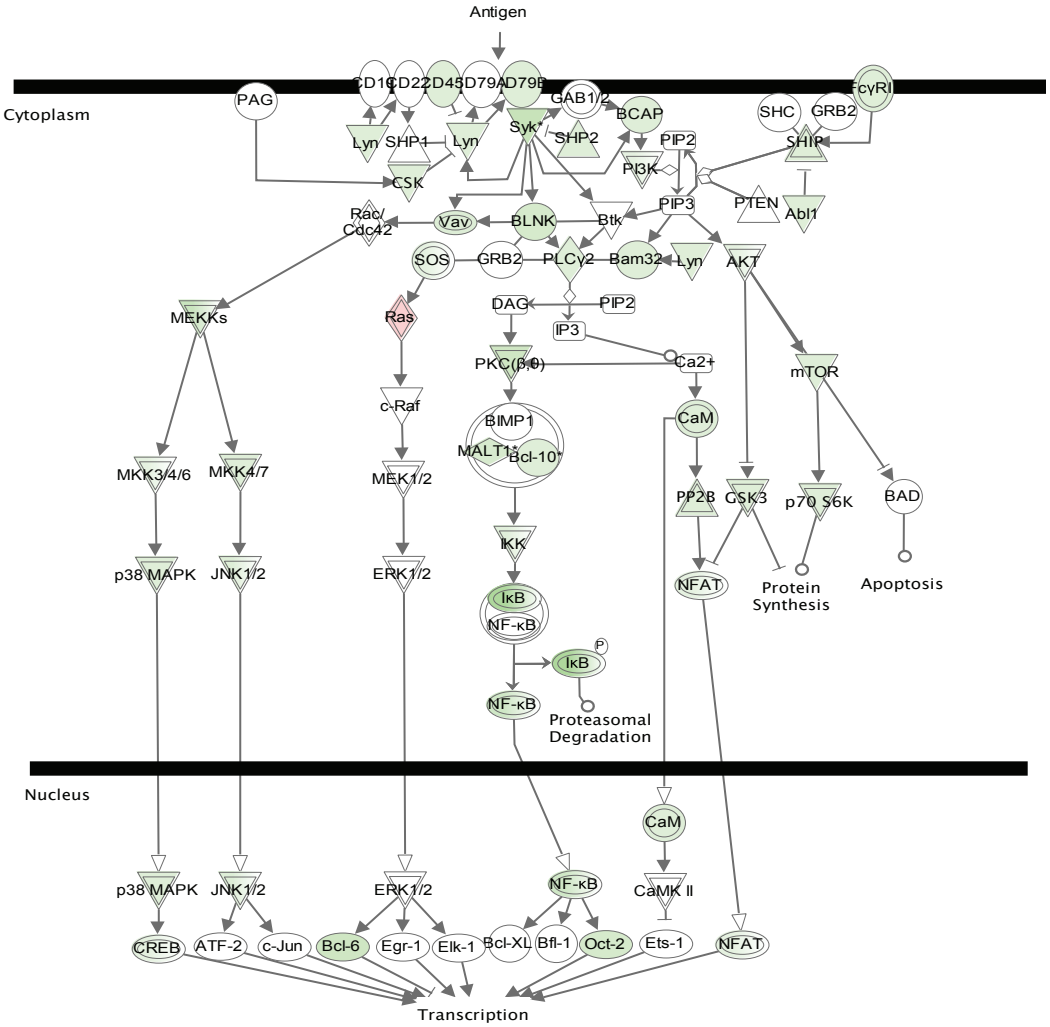


Figure 8b. B cell Receptor Signaling in the EBF/Rag subset

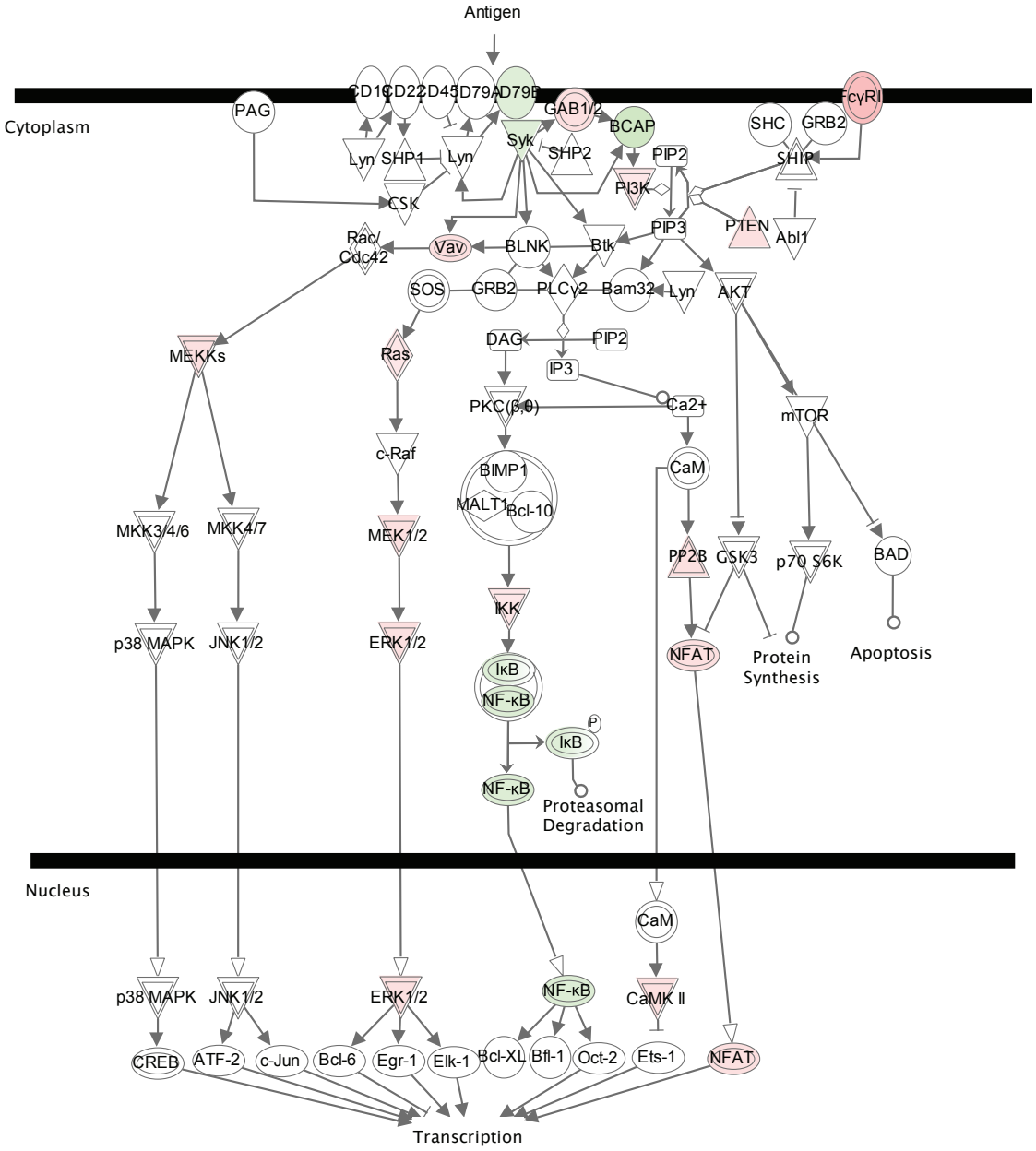
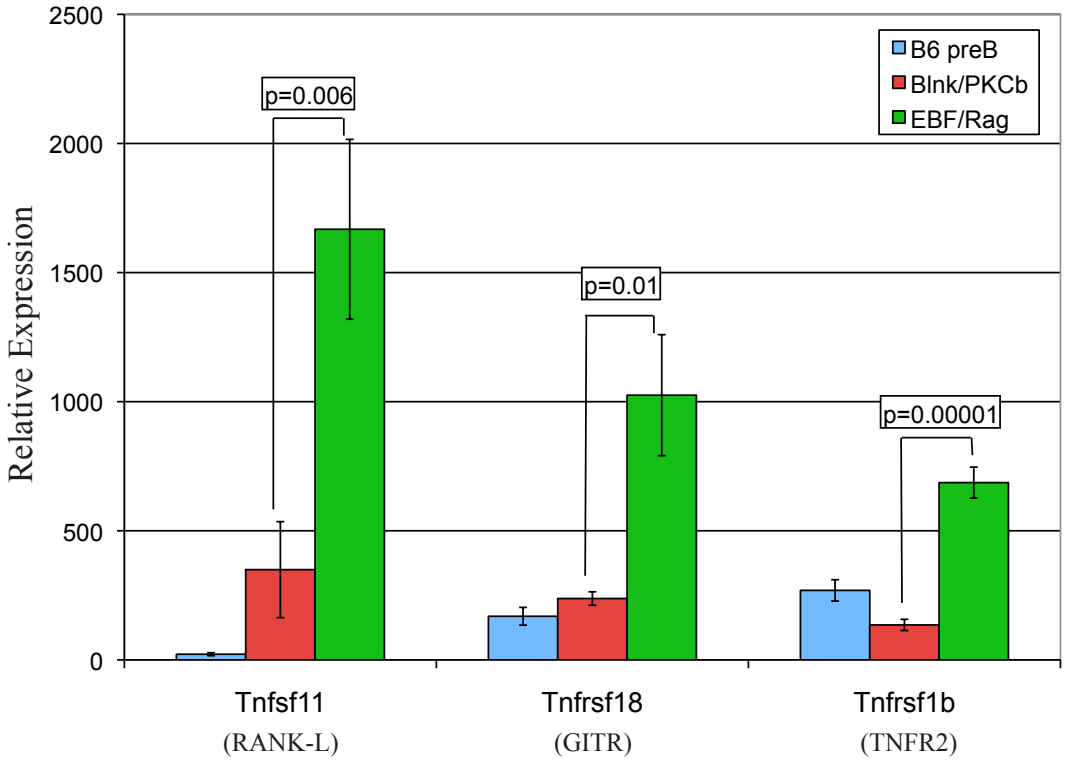


Figure 8. Expression of BCR signaling pathway genes.

Data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). The BCR signaling pathway is shown, overlaid with color density based on the fold change (green is down, red is up) in the specified tumor sample average versus the C57Bl/6 pre-B sample average. **(A)** BLNK/PKCβ subset. **(B)** EBF/Rag subset.

Figure 9. TNFR Expression

A



B

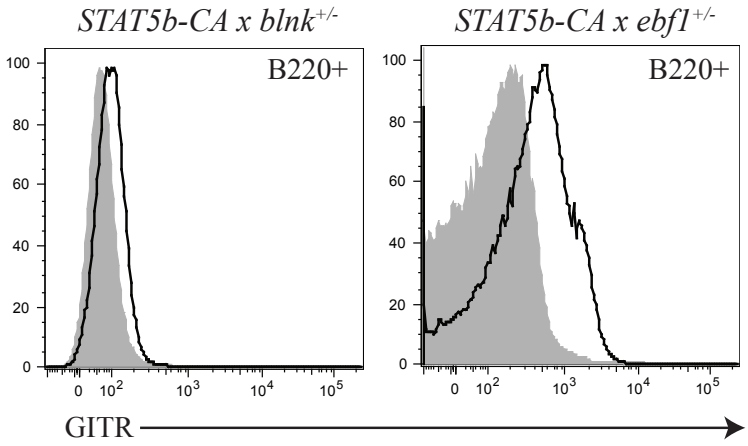


Figure 9. Expression of TNF/TNFR superfamily genes. **(A)** Relative microarray gene expression of *tnfsf11* (RANK-L), *tnfrsr18* (GITR) and *tnfrsf1b* (TNFR2) are shown. Error bars represent standard error. P-values were determined by two-tailed student's t-tests. **(B)** Flow cytometry showing increased GITR expression on *STAT5b-CA x ebf1*$^{+/-}$ versus *STAT5b-CA x blnk*$^{+/-}$ and C57Bl/6 pre-B cells. Grey histograms represent staining for these markers on mature B220^{+} splenic B cells from C57Bl/6 mice.

Figure 10. Regulators of NFκB2 signaling

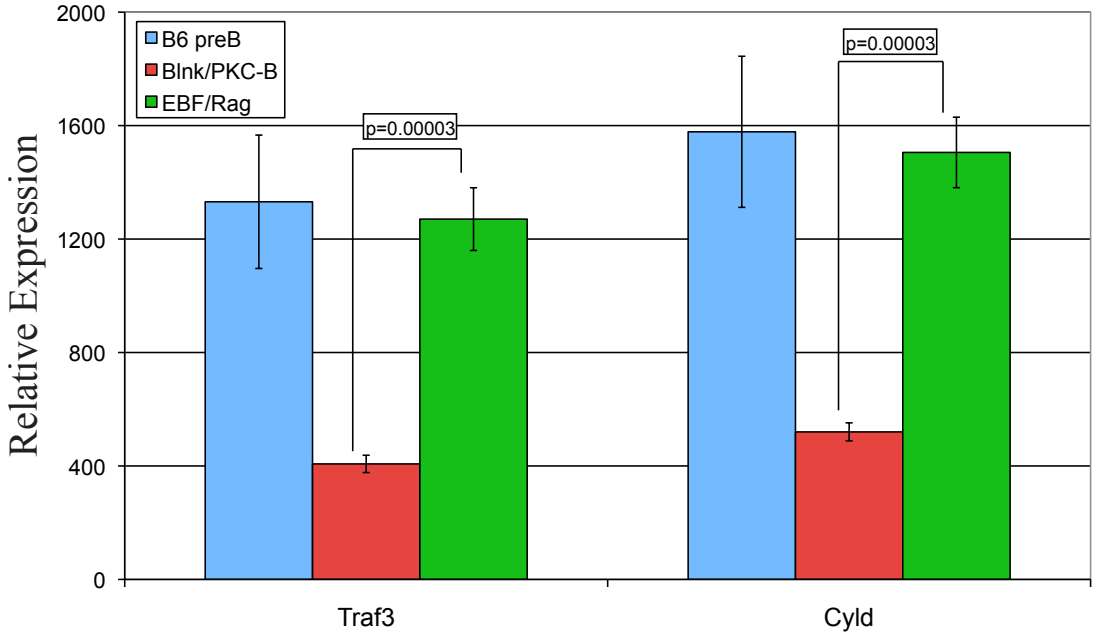
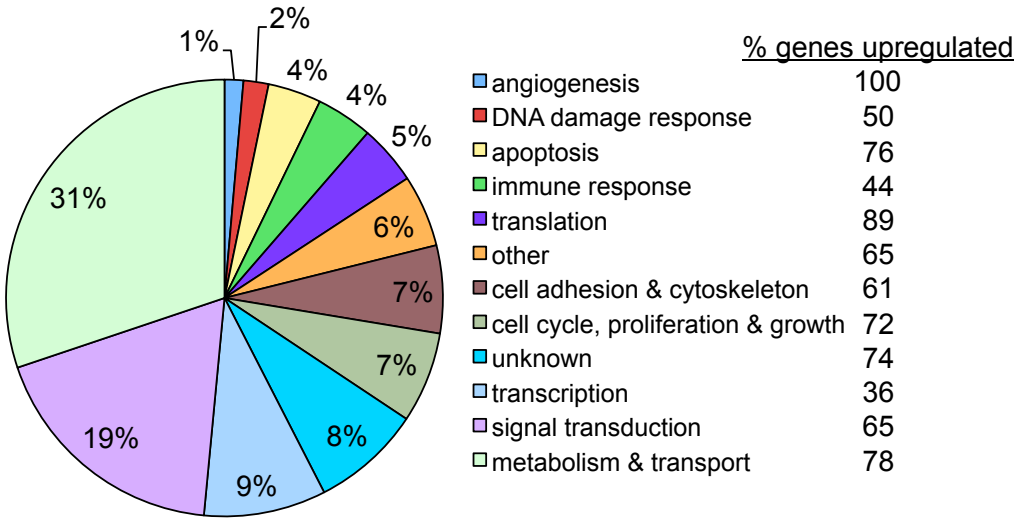


Figure 10. Regulators of NFκB2 signaling. Relative microarray gene expression of *traf3* and *cyld*. Error bars represent standard error. P-values were determined by two-tailed student's t-tests. None of the EBF/Rag values are significantly different than the C57Bl/6 pre-B values ($p>0.1$).

Figure 11

A. *STAT5b-CA x xid* Cooperation Response Genes by GO category



B. Heat map of *STAT5b-CA x xid* Cooperation Response Genes

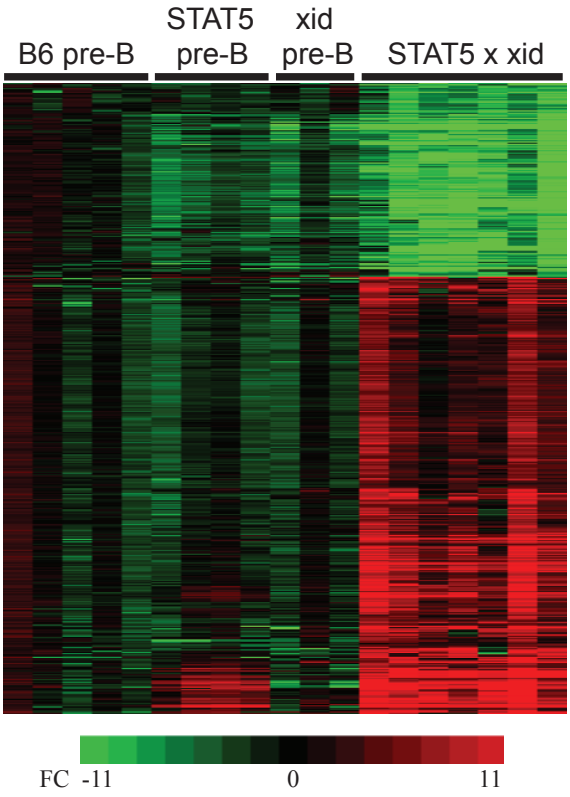


Figure 11. Cooperation response genes in *STAT5b-CA x xid* tumors. **(A)** CRGs were categorized by Gene Ontology (GO) category. Pie chart shows all 417 differentially regulated genes. Right column shows percentage of each category that is upregulated. **(B)** Heat map of 417 differentially regulated CRGs. Green indicates downregulation, red indicates upregulation based on B6 pre-B average. The color gradient shows a fold change from -11 to +11.

Figure 12. Total STAT5 Protein Expression

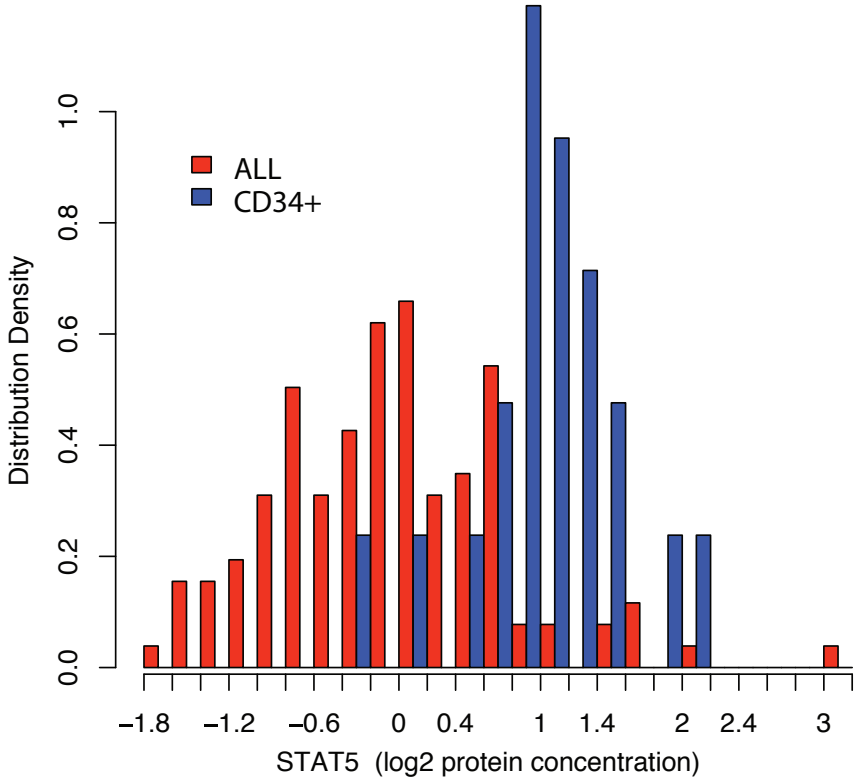


Figure 12. Total STAT5 protein is not increased in ALL. The distribution of total STAT5 protein expression in leukemia cells from 129 ALL patients (red bars) and 10 $CD34^{+}$ progenitor cell controls (blue bars) are shown. Total STAT5 protein levels in leukemic cells were decreased relative to control cells.

Figure 13 STAT5 activation in human ALL

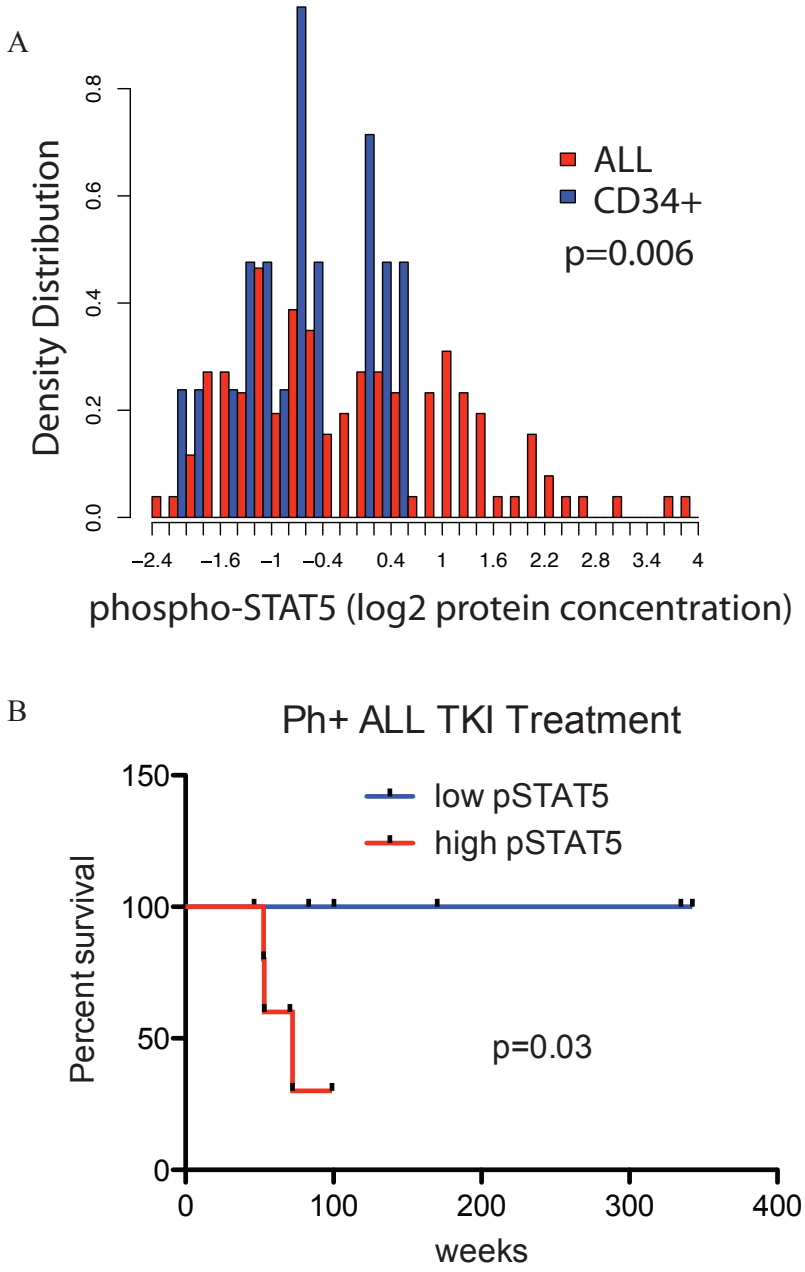


Figure 13. (a) STAT5 activation in human ALL. Shown is the distribution of STAT5 phosphorylation levels (p-Tyr-694/699) in 129 newly diagnosed ALL samples (red bars) and controls ($CD34^+$ progenitor cells; blue bars show data from 10 controls printed in replicate). **(b)** Overall survival of $BCR\text{-}ABL^+$ patients treated with Imatinib. $BCR\text{-}ABL^+$ patients were separated into two equal groups representing higher (red line, n=5) and lower (blue line, n=6) levels of phospho-STAT5. All of these patients were subsequently treated with combination chemotherapy including a tyrosine kinase inhibitor (TKI) Imatinib and overall survival was determined. Patients with higher phospho-STAT levels did significantly worse than patients with lower phospho-STAT5 (p=0.03, Log Rank (mantle-cox) test).

Figure 14. STAT5 phosphorylation in different cytogenetic subsets of ALL

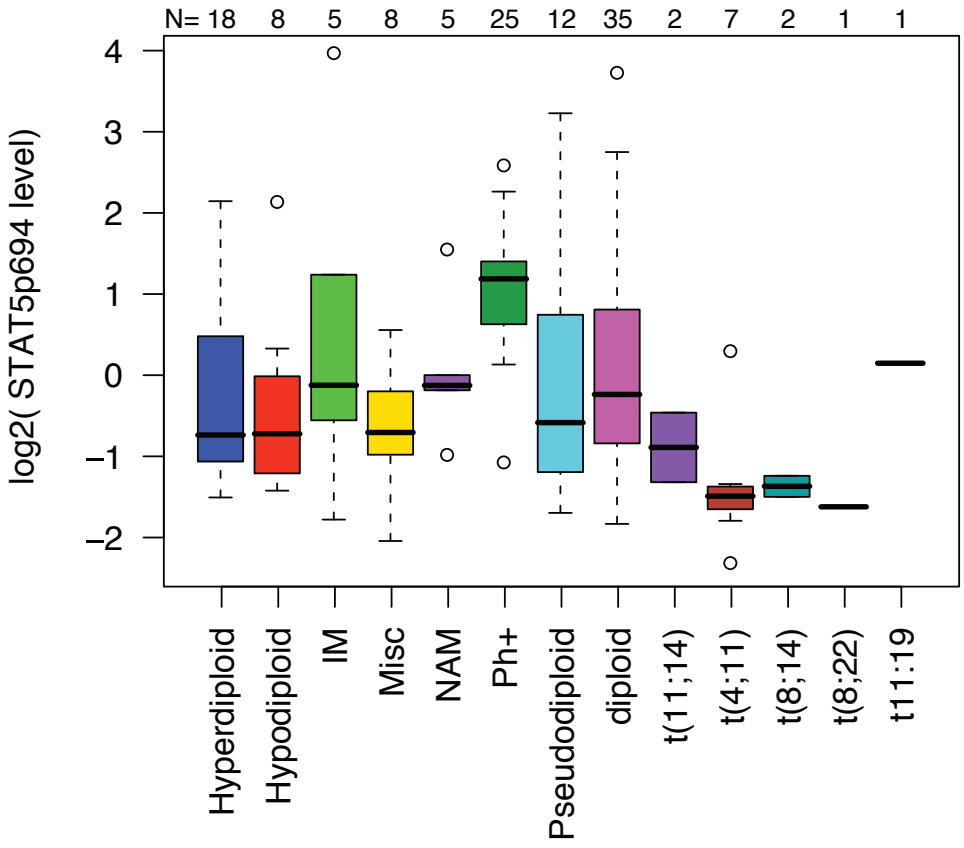


Figure 14. Distinct STAT5 phosphorylation in different cytogenetic subsets of ALL. Shown are the relative levels of STAT5 phosphorylation on tyrosine 694/699 from ALL in different cytogenetic subsets including hyperdiploid, hypodiploid, immature B cell (IM), miscellaneous (Misc), no analyzable metaphases (NAM), BCR-ABL$^+$ (Ph$^+$), pseudodiploid, diploid, t(11;14), t(4;11), t(8;22) and t(11;19). Solid black bars represent the median while solid boxes represent 25-75% range; dashed lines give ± 2 S.D. and open circles represent outliers. The number of samples for each subset is listed above the plot. Some subsets, including most notably Ph$^+$ ALL, are characterized by high STAT5 activation.

Table Ia. Top 50 genes sorted by fold change

Probe set	Gene Title	Gene Symbol	B6 avg	Stat5 avg	Xid avg	Stat5 BLNK	Stat5 PKC-B	Stat5 spont	Stat5 xid	Stat5 Rag	Stat5 EBF	tumor ave	FC	p value	FDR
1436115_at	gene model 266, (NCBI)	Gm266	3	51	5	351	633	293	561	73	166	358	140.30	4.7E-07	3.4E-05
1416325_at	cysteine-rich secretory protein 1	Crisp1	23	24	12	1890	992	774	1559	8847	306	2125	93.76	0.01248	0.03580
1429759_at	ribosomal protein S6 kinase polypeptide 6	Rps6ka6	3	2	1	247	2	830	411	3	1	294	93.00	0.00440	0.02043
1422188_s_at	T-cell receptor gamma, variable 3	Tcrg-V3	36	376	25	1899	2465	1948	1119	8910	4621	3149	88.65	7.7E-05	0.00123
1416049_at	glycine decarboxylase	Gldc	17	114	5	1101	2383	1116	1281	1029	847	1260	74.83	2.8E-06	0.00011
1415857_at	embigin	Emb	28	67	67	1507	1063	1242	979	5109	2526	1909	68.19	3.7E-07	2.8E-05
1454997_at	methionine sulfoxide reductase B3	Msrb3	5	3	4	100	10	471	18	420	718	283	51.90	0.01631	0.04081
1448110_at	sema domain, immunoglobulin domain (Ig), transmembrane dom	Sema4a	16	52	16	250	238	286	373	2566	1199	735	46.49	0.00027	0.00297
1450521_a_at	T-cell receptor gamma, variable 4	Tcrg-V4	44	227	22	1036	1100	1339	960	5854	2938	2014	45.39	5.0E-05	0.00089
1451944_a_at	tumor necrosis factor (ligand) superfamily, member 11	Tnfsf11	5	8	2	58	191	68	33	645	456	211	43.60	0.00120	0.00887
1449470_at	distal-less homeobox 1	Dlx1	5	11	9	4	44	99	10	371	827	209	42.27	0.00503	0.02208
1415978_at	tubulin, beta 3	Tubb3	8	30	8	373	244	153	352	434	147	281	35.69	3.1E-08	7.6E-06
1426851_a_at	nephroblastoma overexpressed gene	Nov	10	53	5	136	1018	123	91	834	400	370	35.53	0.00157	0.01076
1415800_at	gap junction protein, alpha 1	Gja1	9	9	6	118	69	203	158	19	1371	326	34.35	0.02309	0.04797
1426852_x_at	nephroblastoma overexpressed gene	Nov	12	93	5	188	932	128	113	988	454	402	33.80	0.00113	0.00850
1419083_at	tumor necrosis factor (ligand) superfamily, member 11	Tnfsf11	22	13	7	183	558	233	112	2040	1370	656	30.05	0.00055	0.00507
1427029_at	HtrA serine peptidase 3	Htra3	19	75	11	300	776	266	295	1658	222	516	26.96	0.01244	0.03576
1421816_at	similar to Glutathione reductase, mitochondrial precursor (GR) (	LOC630729	25	26	15	295	432	883	688	883	781	669	26.62	1.2E-07	1.7E-05
1449065_at	acyl-CoA thioesterase 1 /// similar to acyl-CoA thioesterase	Acot1	30	17	18	667	690	899	1004	60	893	749	24.70	1.7E-05	0.00042
1436127_at	corticotropin releasing hormone binding protein	Crhbp	129	1495	176	3206	1240	4242	4229	590	3717	3129	24.19	2.0E-08	5.5E-06
1438934_x_at	sema domain, immunoglobulin domain (Ig), transmembrane dom	Sema4a	53	165	60	661	677	653	792	3669	1468	1210	22.83	1.7E-05	0.00042
1416576_at	suppressor of cytokine signaling 3	Socs3	13	60	22	145	123	207	228	634	553	302	22.38	0.00021	0.00248
1420353_at	lymphotoxin A	Lta	13	24	8	64	179	143	90	771	604	278	22.20	0.00139	0.00984
1460214_at	Purkinje cell protein 4	Pcp4	38	50	52	67	457	2650	954	215	69	837	22.19	0.00984	0.03154
1419292_at	HtrA serine peptidase 3	Htra3	16	67	10	169	222	408	187	1075	163	342	21.92	0.01541	0.03974
1422189_x_at	T-cell receptor gamma, variable 4	Tcrg-V4	109	316	50	1176	1163	1754	1236	6219	3355	2302	21.05	2.6E-05	0.00054
1455056_at	LIM domain only 7	Lmo7	65	222	16	405	440	1432	946	4384	762	1301	20.02	0.00231	0.01389
1421375_a_at	S100 calcium binding protein A6 (calcyclin)	S100a6	74	57	70	1474	970	1023	864	2530	2147	1429	19.35	2.6E-06	0.00011
1424226_at	RIKEN cDNA 9030617O03 gene	9030617O03Rik	23	21	10	165	88	559	290	727	778	431	19.13	1.9E-06	8.7E-05
1418471_at	placental growth factor	Pgf	19	100	16	123	205	188	200	297	1086	341	17.91	0.00095	0.00754
1437992_x_at	gap junction protein, alpha 1	Gja1	24	19	5	183	103	249	226	15	1750	426	17.90	0.02436	0.04943
1448276_at	tetraspanin 4	Tspan4	17	12	18	104	107	224	102	952	477	296	17.49	0.00014	0.00190
1422102_a_at	signal transducer and activator of transcription 5B	Stat5b	314	6273	126	3681	4599	6392	5538	7195	5431	5479	17.46	2.2E-16	9.4E-13
1447792_x_at	G protein-coupled receptor 174	Gpr174	28	20	21	94	175	296	75	1607	947	472	16.62	0.00134	0.00960
1420805_at	myosin light chain 2, precursor lymphocyte-specific	Mylc2pl	329	892	389	4952	3855	1576	3927	12563	7576	5331	16.22	4.0E-07	3.0E-05
1437137_at	RIKEN cDNA 6430550H21 gene	6430550H21Rik	34	71	7	292	430	449	426	507	1049	520	15.41	7.6E-06	0.00024
1436590_at	protein phosphatase 1, regulatory (inhibitor) subunit 3B	Ppp1r3b	32	20	68	139	154	145	172	1755	926	485	15.33	0.00292	0.01635
1418764_a_at	bisphosphate 3'-nucleotidase 1	Bpnt1	159	849	49	1532	2351	3170	2028	2287	2975	2397	15.05	8.3E-13	1.2E-09
1456212_x_at	suppressor of cytokine signaling 3	Socs3	38	93	38	184	164	419	313	1462	988	551	14.47	0.00107	0.00818
1449991_at	CD244 natural killer cell receptor 2B4	Cd244	48	41	43	614	641	329	356	1618	994	695	14.39	1.6E-05	0.00042
1420603_s_at	retinoic acid early transcript 1, alpha /// retinoic acid early transcr	Raet1a /// Raet1b	24	41	12	354	243	461	529	134	175	343	14.25	2.3E-07	2.3E-05
1422398_at	histone cluster 1, H1e	Hist1h1e	282	182	113	16	28	33	12	12	18	20	-14.18	0.02164	0.04642
1457729_at	Transcribed locus	---	238	69	120	11	4	16	14	35	20	16	-14.55	0.01001	0.03186
1433643_at	calcium channel, voltage-dependent, alpha2/delta subunit 1	Cacna2d1	651	658	169	21	9	114	39	15	18	40	-16.22	0.00026	0.00291
1437054_x_at	protamine 1	Prm1	493	233	137	30	12	21	15	7	89	29	-16.88	0.00235	0.01395
1439379_x_at	protamine 1	Prm1	597	236	133	39	9	19	8	2	113	31	-18.96	0.00191	0.01225
1449472_at	G-protein coupled receptor 12	Gpr12	792	317	519	13	17	83	30	55	45	41	-19.18	0.00196	0.01247
1430357_at	H3 histone, family 3B	H3f3b	2085	1480	520	104	105	121	66	58	39	82	-25.29	0.00705	0.02688
1454752_at	similar to RNA binding motif protein 24 /// RNA binding motif p	Rbm24	1272	518	424	23	27	63	54	91	43	50	-25.42	0.00302	0.01664
1452730_at	ribosomal protein S4, Y-linked 2	Rps4y2	366	142	109	3	3	6	4	9	3	5	-78.58	0.00301	0.01665

Table Ib. Top 50 genes sorted by p-value

Probe set	Gene Title	Gene Symbol	B6 avg	Stat5 avg	Xid avg	Stat5 PKC-B	Stat5 EBF	Stat5 spont	Stat5 xid	Stat5 Rag	Stat5 BLNK	tumor ave	FC	p value	FDR
1422102_a_at	signal transducer and activator of transcription 5B	Stat5b	314	6273	126	4599	5431	6392	5538	7195	3681	5479	17.46	2.2E-16	9.4E-13
1449211_at	bisphosphate 3'-nucleotidase 1	Bpnt1	586	2186	212	4091	5412	6098	3996	5243	3357	4701	8.02	1.7E-13	3.6E-10
1418764_a_at	bisphosphate 3'-nucleotidase 1	Bpnt1	159	849	49	2351	2975	3170	2028	2287	1532	2397	15.05	8.3E-13	1.2E-09
1434380_at	guanylate binding protein 6	Gbp6	531	2122	606	5438	8503	4417	4542	7744	5595	5855	11.03	1.3E-12	1.3E-09
1452324_at	plasmacytoma variant translocation 1	Pvt1	27	18	23	219	307	406	249	521	246	320	11.82	4.8E-11	4.0E-08
1449109_at	suppressor of cytokine signaling 2	Socs2	770	2044	1019	5413	4925	6325	5996	8480	3769	5773	7.50	2.4E-10	1.7E-07
1428785_at	angiomotin-like 1	Amotl1	186	311	102	826	1044	1081	1180	802	1042	1022	5.51	4.8E-10	2.9E-07
1437918_at	RIKEN cDNA 4930539E08 gene	4930539E08Rik	150	148	128	357	477	429	380	548	384	425	2.83	1.4E-09	7.2E-07
1426631_at	pseudouridylate synthase 7 homolog (S. cerevisiae) /// hypothetic	Pus7	149	121	105	573	1399	1048	955	1901	594	1059	7.10	4.1E-09	1.9E-06
1424942_a_at	myelocytomatosis oncogene	Myc	344	278	258	2046	4271	3220	2721	6560	2220	3395	9.88	7.1E-09	3.0E-06
1436443_a_at	KDEL (Lys-Asp-Glu-Leu) containing 1	Kdelc1	337	331	321	1102	1633	1738	1359	2329	1217	1546	4.59	7.8E-09	3.0E-06
1448560_at	BH3 interacting domain death agonist	Bid	28	54	35	176	332	240	225	393	181	253	9.04	1.0E-08	3.5E-06
1436194_at	PRELI domain containing 2	Prelid2	102	129	87	1676	484	643	1015	1064	657	891	8.72	1.3E-08	4.1E-06
1417398_at	related RAS viral (r-ras) oncogene homolog 2	Rras2	824	922	660	1837	3986	4700	3994	5537	2408	3794	4.60	1.4E-08	4.2E-06
1436127_at	corticotropin releasing hormone binding protein	Crhbp	129	1495	176	1240	3717	4242	4229	590	3206	3129	24.19	2.0E-08	5.5E-06
1439496_at	Stonin 1	Ston1	121	242	88	344	1860	1401	1214	2986	863	1414	11.65	2.2E-08	5.7E-06
1415978_at	tubulin, beta 3	Tubb3	8	30	8	244	147	153	352	434	373	281	35.69	3.1E-08	7.6E-06
1428667_at	monoamine oxidase A	Maoa	209	248	138	509	685	878	883	1677	637	864	4.14	4.1E-08	9.6E-06
1451345_at	methylthioadenosine phosphorylase	Mtap	212	224	163	977	1711	1741	1154	2496	899	1467	6.92	4.7E-08	1.0E-05
1434288_at	basic, immunoglobulin-like variable motif containing	Bivm	87	82	64	151	411	385	324	668	264	362	4.17	4.8E-08	1.0E-05
1449623_at	thioredoxin reductase 3	Txnrd3	270	189	305	1082	2038	2489	1862	1220	1011	1691	6.26	5.1E-08	1.0E-05
1455956_x_at	cyclin D2	Ccnd2	300	1027	415	1963	1290	3905	3677	5006	2046	3023	10.09	6.6E-08	1.3E-05
1423868_at	thioredoxin reductase 3	Txnrd3	122	143	133	687	1264	1583	1192	628	599	1046	8.60	7.7E-08	1.4E-05
1418507_s_at	suppressor of cytokine signaling 2	Socs2	909	1397	995	4439	5033	6231	5563	9673	2786	5544	6.10	8.1E-08	1.4E-05
1448539_a_at	aspartoacylase (aminoacylase) 3	Acy3	25	19	54	201	218	308	257	179	228	239	9.57	1.0E-07	1.7E-05
1435465_at	kelch repeat and BTB (POZ) domain containing 11	Kbtbd11	284	285	207	1260	2631	3061	3103	5580	2657	3028	10.67	1.1E-07	1.7E-05
1447683_x_at	methyltransferase-like 1	Mettl1	48	36	41	313	289	319	211	603	249	314	6.49	1.1E-07	1.8E-05
1418406_at	phosphodiesterase 8A	Pde8a	149	151	97	551	1148	513	959	1537	863	910	6.11	1.2E-07	1.8E-05
1421816_at	similar to Glutathione reductase, mitochondrial precursor (GR) ((	LOC630729	25	26	15	432	781	883	688	883	295	669	26.62	1.2E-07	1.7E-05
1460192_at	oxysterol binding protein-like 1A	Osbpl1a	143	279	104	691	824	1008	808	1752	678	935	6.55	1.2E-07	1.7E-05
1423869_s_at	thioredoxin reductase 3	Txnrd3	269	205	238	896	1750	2147	1751	993	889	1480	5.50	1.2E-07	1.7E-05
1428550_at	YdjC homolog (bacterial)	Ydjc	198	135	164	678	866	997	836	2032	788	998	5.03	1.3E-07	1.7E-05
1434745_at	cyclin D2	Ccnd2	775	1522	734	2773	3101	5331	5117	7828	2329	4431	5.72	1.3E-07	1.6E-05
1424505_at	required for meiotic nuclear division 1 homolog (S. cerevisiae)	Rmnd1	370	693	221	2107	905	1470	1864	937	1738	1524	4.12	1.3E-07	1.6E-05
1425156_at	guanylate binding protein 6	Gbp6	161	400	181	825	2369	858	857	1962	881	1243	7.72	1.3E-07	1.6E-05
1416022_at	fatty acid binding protein 5, epidermal	Fabp5	459	358	325	2425	1686	2475	2876	3542	1943	2484	5.41	1.3E-07	1.6E-05
1419410_at	basic leucine zipper transcription factor, ATF-like	Batf	143	131	138	515	661	482	379	1034	318	537	3.74	1.6E-07	1.8E-05
1416122_at	cyclin D2	Ccnd2	884	2069	937	3450	2685	6458	6340	8744	3352	5229	5.91	1.7E-07	1.8E-05
1421498_a_at	RIKEN cDNA 2010204K13 gene	2010204K13Rik	73	38	54	414	456	552	377	1195	348	529	7.23	1.8E-07	2.0E-05
1434766_at	Mus musculus, clone IMAGE:1512359, mRNA	---	41	76	32	250	400	472	365	53	302	326	7.96	2.0E-07	2.1E-05
1435292_at	TBC1 domain family, member 4	Tbc1d4	77	113	72	312	515	712	735	1408	384	671	8.74	2.1E-07	2.1E-05
1420603_s_at	retinoic acid early transcript 1, alpha /// retinoic acid early transcr	Raet1a /// Raet1b	24	41	12	243	175	461	529	134	354	343	14.25	2.3E-07	2.3E-05
1452280_at	FERM, RhoGEF (Arhgef) and pleckstrin domain protein 1 (chon	Farp1	40	33	24	57	292	331	213	456	174	254	6.29	2.4E-07	2.3E-05
1420570_x_at	T-cell leukemia/lymphoma 1B, 3	Tcl1b3	935	812	955	2318	2950	2382	2169	3680	2580	2617	2.80	2.5E-07	2.4E-05
1441945_s_at	abhydrolase domain containing 14A	Abhd14a	290	198	213	513	790	1001	732	1610	763	883	3.04	2.7E-07	2.5E-05
1416329_at	cytoplasmic FMR1 interacting protein 1	Cyfip1	453	428	403	685	3335	2304	1649	3062	1036	2007	4.43	2.7E-07	2.5E-05
1450986_at	nucleolar protein 5	Nol5	390	451	413	1284	1725	1747	1524	2713	1539	1724	4.42	2.7E-07	2.4E-05
1419515_at	FYVE, RhoGEF and PH domain containing 2	Fgd2	185	246	139	639	1138	722	669	1603	416	831	4.49	3.0E-07	2.6E-05
1434301_at	RIKEN cDNA D330050I23 gene	D330050I23Rik	335	263	212	733	814	1152	883	934	739	888	2.65	3.1E-07	2.7E-05
1455247_at	angiomotin-like 1	Amotl1	79	131	43	573	812	968	974	756	658	816	10.38	3.2E-07	2.7E-05

Table I. The top 50 probe sets by fold change (Ia) or p-value (Ib) are listed here. The gene list was generated by an absolute fold change between the average of all tumor samples and the C57Bl/6 pre-B sample average of at least 1.5, an absolute difference in expression levels of at least 200 and a FDR of less than 0.05, resulting in 2091 probe sets.

Chapter 5. Discussion

Receptor editing is a major mechanism of B cell tolerance, evidenced by the fact that as many as 50% of B cells that escape the BM show evidence of editing [106, 157, 159]. Many of the proteins involved the mechanism of receptor editing have been elucidated, but the control of the mechanism remains unknown. We have shown evidence here that there is a basal signal that regulates receptor editing (Chapters 2 & 3). This basal signal is important for maintaining the developmental stage and for survival of immature B cells. When the basal signal is abrogated, through deletion of the HC or pharmacologic inhibition, the cells back-differentiate to an earlier stage of B cell development [52]. This thesis provides evidence that the basal signal involves PI3K and its downstream effector pathways through Ras and Ca^{2+}.

If an activation signal induces RAG expression and receptor editing, as conventional wisdom suggests, we would expect pharmacologic mimicry of the BCR signal to induce RAG expression. In fact, we observed the opposite. We used PMA and Ionomycin to mimic the DAG and Ca^{2+}, but instead of inducing RAG expression they actually suppressed it beyond background levels (Chapter 2). They also inhibited LC rearrangements and induced expression of maturation markers, indicating that they were progressing through development instead of editing. This supported our previous findings that a basal signal inhibits RAG expression instead of activating it [52].

In order to analyze the kinetics of receptor editing, we used RAG2-GFP Tg cells so we could visualize RAG expression and receptor editing. We incubated HEL

Tg/RAG2-GFP immature B cells with HEL antigen and found a correlation between the downregulation of sIg and the induction of RAG2-GFP, which did not correlate with the activation response indicated by CD69 upregulation. When we used short pulses of HEL we saw activation but no editing. Additionally, when we used mutants of HEL that bound the receptor with less avidity, we found that induction of RAG2-GFP correlates with the level of sIg. The mutants were able to activate the cells almost as well as HEL, but they were not able to induce editing as well.

To examine the molecules involved in the basal signal we used *xid* mice, lacking functional Btk, and *lyn* knockout mice. The *xid* mice showed impaired activation responses but had normal RAG2-GFP expression when incubated with antigen, indicating that receptor editing is not impaired in these mice. Alternatively, the lyn^{null} mice had increased RAG2-GFP expression, indicating Lyn is involved in the basal signal that inhibits receptor editing. Furthermore, when we incubated immature B cells with the Src-family kinase inhibitor PP2, we saw an even greater induction of RAG2-GFP than in the lyn^{null} mice, indicating that Lyn is not the only Src-family kinase involved in basal signaling through the BCR.

To further characterize cells that are editing in the presence of self-antigen, we performed microarrays on antigen treated, Herbimycin A treated, and HC-deleted immature B cells and compared them to untreated pro-B, pre-B, and immature B cells. Tze et al. had previously demonstrated that HC deletion at the immature B cell stage induces back-differentiation to an earlier stage in B cell development [52]. Our analysis of antigen-treated cells showed a similar phenotype. Again, when the tyrosine kinase inhibitor Herbimycin A was used to block BCR signaling in immature B cells we saw

back-differentiation. This indicates that the basal BCR signal is indeed inhibiting receptor editing and promoting maturation. When the basal signal is lost, RAG expression is induced and receptor editing occurs.

To better understand which proteins regulate receptor editing we utilized a newly developed mouse model, the anti-κ mouse. These mice have a ubiquitously expressed transgene that encodes a pseudo-antibody reactive with the κ LC, which causes editing and expression of the λ LC on all B cells [208]. We used these mice as hosts for BM chimeras where we injected donor BM from mice with defects in BCR signaling and compared it to WT donor cells (Chapter 3). To test the hypothesis that BCR signaling inhibits receptor editing we used cells with excessive signaling, Itpkb$^{-/-}$ and Raf-CAAX Tg, and cells with impaired signaling, NFκB1$^{-/-}$ and CARMA1$^{-/-}$. We found impaired editing in the immature B cell compartment of the cells that had excessive signaling, but found no difference from WT in cells with impaired NFκB signaling. Therefore, the signal inhibiting RAG expression and receptor editing may be redundant or may not involve NFκB. The inhibition of editing in cells with hyperactive signaling implies that the basal signal does, in fact, inhibit receptor editing not only *in vitro* as shown in Chapter 2, but also *in vivo*.

The basal signal inhibiting RAG likely involves PI3K signaling. When wortmannin or LY294002 were used to inhibit PI3K in immature B cells, RAG expression was induced [53]. Additionally, mice lacking PI3K subunits p85α [53] or p110δ [119] have increased basal RAG expression in immature B cells. PI3K is upstream of both the Ras and Ca^{2+} pathways, among others. We tested the Ras pathway with the Raf-CAAX donor cells, which have a B/T cell-specific transgene that allows

for constitutive activation of Raf [213]. This results in higher levels of phospho-ERK, which is downstream of Raf via MEK phosphorylation. We also tested the Ca^{2+} pathway with Itpkb-deficient cells. Itpkb is a kinase that inhibits IP3 activity, therefore reducing Ca^{2+} signaling downstream of the BCR. Itpkb-deficient cells have higher Ca^{2+} signaling after BCR stimulation [209, 210]. Both Raf-CAAX and Itpkb deficiency result in increased editing in the anti-κ chimera system, indicating they are repressing RAG more than WT. We propose that the basal signal through PI3K inhibits RAG expression and receptor editing through activation of the Ras/Raf/MEK/ERK pathway as well as the Ca^{2+} pathway.

This is in contrast to the widely accepted model that BCR signaling induces receptor editing, however, we feel that we have sufficient evidence to reject that model. As we have shown here, the basal signal from the BCR is important for repressing receptor editing, and the activation response does not correlate well with RAG expression and receptor editing. We propose there is a threshold of signaling that must be reached to inhibit receptor editing, which is not reached in Lyn-, or PI3K-deficient cells. Autoimmune patients with defects in BCR signaling may also not reach this threshold, which would explain the excessive receptor editing in some patients [123-125]. If we can further characterize the molecular pathway that regulates receptor editing, we may be able to stop excessive receptor editing in patients and ameliorate their disease.

BCR signaling must be tightly regulated to avoid autoimmunity and immunodeficiency, but may also be involved in the development of leukemia. In the absence of pre-BCR signaling, B cell development is impaired at the pre-B stage,

during a clonal expansion phase of rapid proliferation. This rapid proliferation, in conjunction with a transforming mutation, can result in cancer, as in the case in B-ALL. B-ALL is a heterogeneous disease that is often characterized by translocations, however the molecular mechanisms underlying the development of the disease remain unknown. Mullighan et al. found that 40% of B-ALL patients have defects in genes involved in B cell development [134]. We have shown here that when coupled with STAT5 activation, deficiency in genes involved in B cell development (*ebf1* and *pax5*) or genes involved in pre-BCR signaling (*blnk*, *PKCβ*, and *btk*) induce leukemia (Chapter 4).

A low percentage of mice with *STAT5b-CA* alone develop leukemia, however when crossed with mice lacking one copy of *ebf1* or *pax5*, all the mice develop tumors. Alone, $ebf1^{+/-}$ or $pax5^{+/-}$ mice do not develop leukemia, but when crossed with mice expressing constitutively active STAT5, they show complete penetrance of leukemia, indicating that these mutations could contribute to leukemogenesis in ALL patients. Mice lacking certain pre-BCR signaling proteins have a developmental blockage at the pre-B stage, where they are rapidly proliferating in response to IL-7. When IL-7R signaling is mimicked with constitutive STAT5 activation, and the cells are blocked at this stage of development they also develop leukemia, indicating it may be a developmental blockage or the lack of pre-BCR signaling that enhances leukemogenesis.

We have developed a new mouse model for ALL that could be useful for pharmacologic studies in the future. We found that approximately 35% of adult ALL patients have elevated phospho-STAT5 levels, and those with higher levels are more resistant to treatment with Imatinib. Future treatments may be aimed at inhibiting

STAT5 activity if these results can be replicated and validated elsewhere. Our mouse model could be used to study pharmacologic inhibitors of STAT5 or IL-7R signaling for use in ALL treatment. These studies provide new insight into the molecular mechanism of development of ALL in a murine model, and the model may yield new leads for understanding the origin and progression of human ALL.

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Phone: 301–634–7197 • Fax: 301–634–7829
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Laura Ramsey
University of Minnesota
6-230 NHH 312 Church St SE
Minneapolis, MN 55455
612-625-3608
Email: robin444@umn.edu

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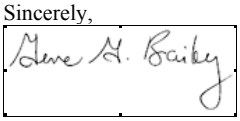

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