Control of B Cell Development by the Signaling Proteins Btk and Slp-65

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Control of B Cell Development by the Signaling Proteins Btk and Slp-65

Regulatie van de B cel ontwikkeling door de signaleringseiwitten Btk en Slp-65

Proefschrift

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ABBREVIATIONS

ABBREVIATIONS

AID	activation-induced deaminase
BAFF	B-cell activation factor
BCR	B cell receptor
BM	bone marrow
Btk	Bruton's tyrosine kinase
CDR	complementarity determining region
CML	chronic myelogenous leukemia
CSR	class switch recombination
DAG	diacylglycerol
DSB	double strand break
ERK	Extracellular signal-regulated kinase
Fol	follicular
GC	Germinal Center
HSC	hematopoietic stem cell
Ig	Immunoglobulin
IgHC	Immunoglobulin Heavy Chain
IgLC	Immunoglobulin Light Chain
IL-7	Interleukin-7
IL-7R	Interleukin-7 receptor
IP3	inositol triphosphate
IRF	interferon regulatory factor
ITAM	immunoreceptor tyrosine-based activation motif
MAPK	Mitogen-activated protein kinase
MHC	major histocompatibility complex
MZ	marginal zone
NHEJ	non-homologous end-joining
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP	phosphatidylinositol phosphate
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 1,4,5-trisphosphate
PIP5K	phosphatidylinositol-4-phosphate 5-kinase
PKC	protein kinase C
PLCγ2	phospholipase Cγ2
pre-BCR	precursor B cell receptor
RAG	recombination activating gene
RSS	recombination signal sequence

S-region	switch-region
SCID	severe combined immune deficiency
SH2	Src homology 2
SH3	Src homology 3
SLC	surrogate light chain
Slp-65	SH2-domain leukocyte protein of 65 kD
TCR	T-cell receptor
TDT	terminal deoxynucleotidyl transferase
TH	Tec homology region
TI	T-cell independent
TI-I	T-cell independent type I
TI-II	T-cell independent type II
TLR	Toll like receptor
TNF	Tumor necrosis factor
UNG	uracil-DNA glycosylase
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Part of this chapter was published before as a review in Seminars in Immunology: Involvement of Slp-65 and Btk in tumor suppression and malignant transformation of pre-B cells.

Semin Immunol. 2006;18:67-76.

1 PRELUDE

All organisms are continuously challenged by a variety of infectious microbial agents such as viruses, bacteria, fungi and parasites. Therefore the simplest up to the most complex organisms have developed defense mechanisms, to block assaults from hostile micro-organisms. In higher vertebrates this resulted in the development of an immune system consisting of an innate and an adaptive arm.

After penetration of the epithelia the innate arm is the first defense line that is encountered, consisting of cells equipped with *germline encoded receptors* such as Toll like receptor's (TLR's) that enable pathogen recognition on the basis of ancient molecular patterns or that detect changes in cell surface composition identifying them as virus infected. Examples of such cells are macrophages, granulocytes and natural killer cells. These cells are supported by an intricate system of soluble anti-microbial proteins such as the complement system, lysozyme and lactoferrin that also provide coverage against micro-organisms. The adaptive arm forms the second line of defense and responds in a highly specific way by virtue of *somatically rearranged receptors* to a microbial challenge, simultaneously generating immunological memory allowing swifter more vigorous responses in future challenges with the same pathogen. The adaptive immune response is carried out by two classes of lymphocytes that are generated from a common lymphoid progenitor in the bone marrow (BM). Progenitors for T cells first migrate to the thymus where they differentiate to become mature helper, cytotoxic and regulatory T cells, whereas progenitors for B cells remain in the BM and develop through several stages into mature B cells.

Aberrant lymphoid development in humans may result in various diseases such as immunodeficiency with increased vulnerability to infections, autoimmunity with immune activation to self-antigens or lymphoproliferation and malignancy.

This thesis focuses on the function of two signal transduction proteins Btk and Slp-65 in B cell development and their involvement in immunodeficiency and malignancy.

2 B CELL DEVELOPMENT

2.1 Immunoglobulins

The most important function carried out by B cells is the production of immunoglobulins (Ig), which are proteins with a vast range of specificities that can recognize antigenic structures in a highly specific way. When presented at the B cell surface these Ig are known as B-cell receptors (BCR). Upon terminal differentiation into plasma cells the B cells transform into small factories devoted to the production and secretion of Ig with a single specificity. The secreted Ig (also called antibodies) can specifically bind pathogenic structures and thereby protect the body from pathogens.

Ig are composed of 2 heavy chain proteins and 2 light chain proteins that form a Y-shaped structure that is kept together by disulfide bonds. These disulfide bonds allow great flexibility which helps in binding antigen efficiently. Both Ig heavy (IgHC) and light chains proteins (IgLC) are composed of a variable (V) region and a constant (C) region, whereby the variable region is responsible for antigen recognition and the constant region determines the effector function. The Ig are devided into 5 different categories on the basis of the IgHC C-region composition (IgM, IgD, IgG, IgA and IgE). IgM and IgG are the major immunoglobulin subclasses and are mainly responsible for clearing bacteria and viruses. IgA is mainly produced in the mucosa and defends mucosal surfaces, while IgE is thought to protect against parasites and worms, but is quite notorious due to its role in allergic disease. Furthermore, these 5 different categories may contain IgLC proteins of either the κ or the λ subtype, which does not result in any functional differences. The V region has 3 specific sites that determine immunoglobulin specificity and allow antigen recognition. These complementary determining regions 1, 2 and 3 (CDR) arise through a combination of IgHC and IgLC V regions (combinatorial diversity). The V-regions from both IgHC's and IgLC's are generated from multiple gene segments by a gene rearrangement process called Ig gene recombination (junctional diversity). Junctional and combinatorial diversity of IgHC and IgLC proteins allows the production of a wide range in specificities up to 10^{11}.

2.1.1 Immunoglobulin gene recombination

In 1890 Behring and Kitasato found that substances in serum (so-called antibodies) specifically protected vaccinated individuals from disease. The clonal selection theory that was put forward in the 1950s by Burnet explained the production of specific antibodies by proposing that the body contained many different potential antibody-producing cells that have the ability to make antibody of different specificities. Upon engagement of a cell-bound form of antibody by antigen the cell is activated and undergoes cell division, resulting in multiple clones producing the

same antibody. Since then, numerous discoveries have shed light on the cellular and molecular mechanisms that explain the generation of antigen-receptor diversity (1). This started in the 1970s-1980s with discoveries on the antigen receptor loci, their structures and rearrangements. In the 1990s the recombination activating gene 1 (RAG-1) and RAG-2 were discovered along with fundamental aspects of how they initiate V(D)J recombination (2, 3). These important discoveries provided the basis for our current understanding of V(D)J recombination (reviewed in 4, 5).

Sequential gene rearrangements are essential for the production of mature functional Ig and involve recombination of the IgHC locus and at least 1 of the 2 IgLC loci. IgHC gene rearrangement generally precedes immunoglobulin light chain (IgLC) recombination, and the IgLC κ locus rearranges before λ (reviewed in 5). The IgHC V region is created by recombination of the V, D and J gene segments to form a single exon. Recombination first starts with joining a D to a J gene segment followed by rearrangement of a V to the DJ gene segment. For the IgLC V region this process is more simple, consisting of just a V to J gene segment rearrangement.

In order to generate diversity in both IgHC and IgLC V regions the V, D and J gene segments contain multiple copies in the germline DNA. Random selection and combination of the gene segments creates great V region diversity. In addition, imprecise joining of the different gene segments with the addition of non-templated nucleotides or deletion of nucleotides adds additional variation. In a later stage of B cell development, during germinal centre reactions, diversity is amplified by the action of the enzyme activation-induced cytidine deaminase (AID) during the process of somatic hypermutation. Further diversification of the repertoire comes from combining different IgHC and IgLC V regions.

The V, D, J gene segments are flanked by recombination signal sequences (RSS), which consist of a conserved heptamer and nonamer separated by a non-conserved spacer of either 12 or 23 nucleotides in length. Gene segments of a particular type are all flanked by RSS with the same spacer length. Only gene segments that are flanked by RSS with dissimilar spacer lengths can recombine with one another, which is known as the 12/23 rule. A multimeric complex also termed the V(D)J recombinase, consisting of RAG-1, RAG-2 and the non-homologous end-joining (NHEJ) proteins DNA-dependent protein kinase (DNA-PK), Ku70, Ku80, X-ray repair cross complementing protein 4 (XRCC4), DNA ligase IV and Artemis is responsible for Ig recombination. Initially 2 RAG-1/RAG-2 complexes, also containing high-mobility group proteins, recognize and align the two RSS. Next the endonuclease activity of RAG cleaves a 12/23 pair of RSS precisely at the heptamer-RSS junction, generating coding ends that are covalently closed DNA hairpins and signal ends that are blunt and 5' phosphorylated. Signal ends are joined by DNA ligase IV to form a signal joint. Coding ends are first opened by the

nuclease activity of Artemis and then joined by DNA ligase IV to form a coding joint. Imprecision when joining the coding ends contributes considerably to diversity and includes duplication of palindromic sequences (P-nucleotide additions) and deletion or insertion of nucleotides (N-nucleotide additions by terminal deoxynucleotidyl transferase, TDT). The other enzymes that are part of the complex contribute in activating and recruiting Artemis and DNA ligase IV. Ku80 recruits DNA-PK, a protein kinase that is required to activate the nuclease activity of Artemis (6-8). XRCC4 and Cernunnos (XLF) in turn increase the activity of DNA ligase IV (9-12). Deficiency in any of these proteins blocks the formation of coding joints and results in a severe combined immune deficiency (SCID) characterized by complete arrests of T and B cell development. Recently new data showed that in addition to the classical NHEJ very rarely an alternative NHEJ pathway may contribute to end joining (13).

2.1.2 Regulation of immunoglobulin gene recombination

Somatic recombination using the RAG enzymes exposes the organism to a major danger: genomic instability. Therefore somatic recombination needs to be tightly controlled. Multiple mechanisms have evolved to ensure that the recombination machinery is only active in the right cells, at the right time and at the right place (reviewed in 4,5,14).

The first measure that preserves genomic stability regulates the recombination machinery and especially RAG-1/2 themselves. This is achieved by limiting RAG protein expression to the progenitor stages of B-cell development by regulation of transcription and by limiting RAG-2 stability. Phosphorylation by a cyclin-dependent kinase regulates RAG-2 stability in a cell cycle-dependent way and result in degradation through a ubiquitin-dependent pathway (15). Because RAG-1 remains more stable in the presence of RAG-2, degradation of RAG-2 likely influences RAG-1 protein expression as well (16).

Secondly, genomic stability is preserved by regulating substrate accessibility. The presence of RSS that meet the 12/23 rule is essential for RAG-mediated rearrangements and provides essential substrate specificity, targeting rearrangements to the Ig and T-cell receptor (TCR) loci. Similarly, this ensures that the right gene segments are joined. Still these requirements do not explain why Ig genes fully arrange only in B cells and TCR genes rearrange only in T cells. Moreover, rearrangement of both the Ig and the TCR loci is arranged in such a way that mostly only one functional allele is generated. Therefore additional mechanisms must exist to impose B-cell specific Ig gene rearrangements, T-cell specific TCR gene rearrangements and generation of only one functional allele (so-called allelic exclusion). In the mid 1980s observations that rearranging gene segments are transcribed before or coincident with their activation for rearrangement, gave the

first hint that accessibility plays a major role in determining substrate specificity (17). It was postulated that these so-called germline transcripts either helped increasing accessibility or correlated with changes in chromatin structure. Since then research has focused on showing that cis-regulatory sequences such as enhancers and promoters associated with the Ig loci are important for Ig gene recombination (18). Enhancers influence recombination and gene expression over long distances, while promoters influence recombination and gene expression more locally. The molecular basis of chromatin accessibility likely involves DNA methylation, histone modification and nuclear localization that have been shown to change in rearranging loci. The mechanism how enhancers and promoters change RSS accessibility is unknown. In gene activation promoters are able to remodel the promoter-associated nucleosome by ATP-dependent chromatin remodeling complexes. When promoters are located far away from RSS other mechanisms must come into play. An important clue to this issue came from studies of the TCR-α locus (19). In this study it was shown that rearrangement of downstream J segments strongly depended on an upstream promoter. A strong transcriptional terminator was introduced by homologous recombination after which the rearrangement frequency of downstream J segments strongly decreased. This was the first study that could demonstrate a direct link between transcription and recombination of gene segments. It was hypothesized that RNA polymerase II and its associated histone-modifying complexes play an important role in opening up the chromatin.

2.2 Early B cell development

2.2.1 B cell commitment

In adult life B cells are generated continuously through several different steps from hematopoietic stem cells (HSC) in the BM. Progressively the lineage potential of precursor cells becomes restricted through the action of multiple transcription factors (reviewed in ref. 20).

During the first step of development the HSCs loose self-renewal capacity but retain multilineage differentiation potential, differentiating into a so-called multipotent progenitor cells (MPP) (21). MPP expressing Flt3 have a lymphoid and myeloid restricted developmental potential and have limited erythromegakaryocytic potential (22). It is thought that a few of these lymphoid-primed MPP (LMPP) develop into early lymphoid progenitors (ELP) that express Rag-1 (23). From these the well-known common lymphoid progenitors (CLP) develop that are characterized by IL-7R expression, which upon transplantation can provide a rapid lymphoid-restricted (T, B, and NK) reconstitution (24). Recent data, however, suggest that CLP mainly generate B lymphocytes and NK cells (25). During the next step pro-B cells develop that represent the earliest committed B cell progenitors. In addition,

alternative models have been proposed that implicate that, despite the obvious similarities between T and B lymphocytes, these cells might not develop from a common lymphoid progenitor. Investigations by the group of Katsura, using a clonal assay which allows for T-, B- and myeloid-lineage development, indicate that commitment to T-cell and B-cell lineages occurs instead through myeloid/T and myeloid/B bipotential stages, respectively (26). The induction of the lineage-specific gene-expression program (lineage specification) and repression of alternative gene-expression programs (lineage commitment) occur gradually by the combined action of receptors and regulatory networks of transcription factors. In LMPP the specification is thought to be dependent on Flt3, as Flt3 and Flt3-ligand deficient mice have few B cell progenitors and Flt3 ligand administration influenced LMPP development.

The three transcription factors PU.1, Ikaros and E2A have been shown to be important for the development of CLP. Although the IL-7R is first expressed in CLP it is not needed for CLP development, but is essential for progression to the pro-B cell stage. B-cell specification needs expression of E2A and EBF that coordinately activate the expression of B cell specific genes such as Igα, Igβ, λ5, and VpreB1 (27-35). To finally commit the B cell progenitors to the B lymphoid lineage, E2A and EBF-1 induce Pax-5 expression (ref. 36, 37 and reviewed in ref. 38). Pax-5 expression is induced by Ebf-1 and controls multiple components of the pre-BCR and BCR and activates genes such as Igα, CD19, CD21, CD72 and Slp-65 (39, 40). Conversely, Pax-5 represses other lineages by repressing for example the M-CSF receptor and Notch1, which are required for myeloid development and T cell lineage specification, respectively (41-45). This linear hierarchical model does not fully explain the transcriptional control of early B cell development as multiple positive feedback loops are found as well, indicating that the regulatory network is much more complex than was previously appreciated.

2.2.2 Pro- and pre-B cell development

Differentiation of B lymphocytes from hematopoietic precursors in the BM is a multi-step process, involving controlled lineage- and locus-specific recombination of IgHC and IgLC gene segments, initiated by Rag-1 and Rag-2 (reviewed in ref. 46). Upon successful immunoglobulin heavy chain (IgHC) gene rearrangement, μ H chain (μHC) protein is deposited on the surface of pre-B cells, together with the non-rearranging surrogate light chain (SLC) constituents λ5 (called Ig λ-like 14.1 in human) and VpreB, in association with the Ig-α and Ig-β signal transduction subunits. This pre-BCR complex acts as a key checkpoint in B cell development to monitor the expression of a functional μHC (see for review ref. 47, 48).

Characterization of patients with inherited immunodeficiency disorders and detailed genetic studies in mice demonstrated that the pre-BCR checkpoint functions as a proliferation switch that (i) controls the selective expansion of cells that produce functional μHC and (ii) redirects V(D)J recombination activity from the IgHC to the IgLC loci. Although pre-BCR ligands have been identified, it is still a matter of debate whether pre-BCR/ligand interactions are critical for cells passing through the pre-BCR checkpoint (49-52).

2.2.3 Pre-BCR signal transduction pathway

In contrast to the considerable knowledge on the signaling pathways triggered by antigen binding of the BCR in mature B cells, far less is known about those mediated by the pre-BCR. This receptor is only transiently expressed on the cell membrane and due to its constitutive activation and internalization, surface expression levels are extremely low, hampering detailed biochemical analysis of *in vivo* downstream signaling pathways. Nevertheless, signal transduction pathways were identified in cultured human pre-B cell lines, in which the pre-BCR was activated by antibodies to μHC (53), or in developmentally arrested pro-B cells from RAG-2-deficient mice, in which surface Ig-β molecules were cross-linked (54). In these experiments, pre-BCR engagement led to a rapid increase in kinase activity of the Src-family member Lyn, the accumulation of tyrosine phosphorylated immunoreceptor tyrosine-based activation motifs (ITAM) present in the Ig-α/Ig-β complex, and the formation of a lipid raft-associated calcium signaling module (Figure 1). This complex is composed of the phosphorylated tyrosine kinases Lyn, Syk, and Btk, as well as other phosphorylated proteins including Slp-65 (also called BASH or BLNK), phosphatidylinositol 3-kinase (PI3K), the GTPase-specific guanine nucleotide exchange factor Vav and phospholipase Cγ (PLCγ) (53). In this raft-associated calcium signaling module, the activity of the allosteric enzyme Syk is strongly increased by binding to the phosphorylated Ig-α or Ig-β ITAM tyrosines (55), resulting in additional ITAM phosphorylation, thereby initiating a positive feedback loop at the pre-BCR. Deficiency in Syk does not appear to affect NF-κB induction, which is in pre-B cells thought to be dependent on Src-like kinases and protein kinase C-λ (56). Rather, one of the most important substrates of Syk is the Slp-65 adapter molecule that, when phosphorylated, provides docking sites for various signaling molecules, including Grb2, Vav, Nck, Ig-α, Btk and PLCγ. Activated Btk then phosphorylates PLCγ, which leads to its full activation, resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate diacylglycerol and inositol 1,4,5-trisphosphate, both of which are second messengers required for cellular responses (Figure 1). Analyses in the mouse show that another downstream signaling cascade involves a mitogen-activated protein kinase (MAPK) pathway, the MAPK-ERK (extracellular

signal-regulated kinase) pathway, which is independent of Slp-65 and Btk (54,57). This pathway plays a major role in pre-B cell proliferation and in the mouse intersects functionally with the interleukin-7 receptor (IL-7R) pathway, which also activates the Src-family kinases Fyn and Lyn and the MAPK-ERK signaling cascade (58, 59). Although both pro-B and pre-B cells proliferate in response to IL-7, cells expressing a pre-BCR have a substantial proliferative advantage in limiting concentrations of IL-7 (60). By contrast, IL-7 does not appear to be involved in human pre-B cell proliferation. Another apparently Slp-65/Btk-independent pre-BCR signaling pathway involves PI3K and its downstream target protein kinase B (PKB, also known as Akt) (57,61,62), which promotes cell proliferation and survival.

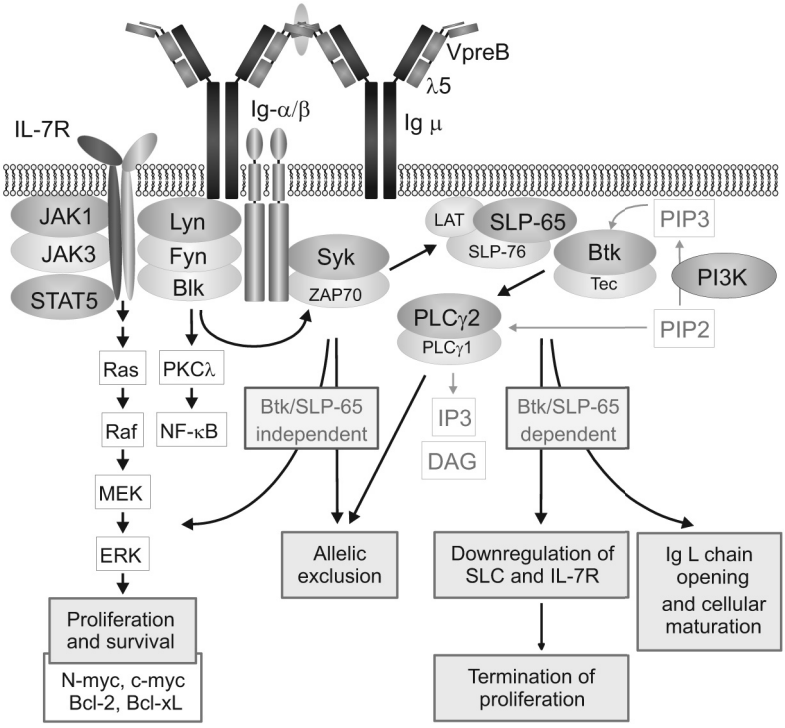

Figure 1. Model for pre-BCR signaling in the mouse, in which Btk and Slp-65 cooperate as tumor suppressors by downregulating pre-BCR and IL-7R expression.
Whereas proliferation and allelic exclusion are induced in a Slp-65/Btk independent way, a Slp-65/Btk-dependent pathway signals termination of proliferative expansion, IgLC opening and cellular maturation. Pre-BCR signaling is induced either by cell-autonomous cross-linking or through ligands expressed by stromal cells. DAG= diacylglycerol; IP3= inositol-triphosphate; JAK= Janus kinase; PIP2= phosphatidyl-inositol-4,5-biphosphate; STAT= signal transducers and activators of transcription. Other abbreviations are explained in the text.

Mice deficient for important pre-B cell receptor signaling molecules, such as Syk, Slp-65, Btk or PLCγ2 show only a partial block at the pre-B cell stage. Remarkably, detailed analyses of compound knock-out mice have indicated that homologous signaling proteins that were previously thought to be specifically expressed in other hematopoietic lineages make important contributions to pre-BCR signal transduction. As illustrated in Figure 1, these proteins include the Syk family member ZAP-70 (63), the LAT/Slp-76 adaptor molecules, which enable pre-B cell differentiation in the absence of Slp-65 (64, 65), the Tec kinase, which has the ability to partially compensate for Btk (66), and PLCγ1, which is highly expressed next to PLCγ2 in early B cell progenitors (67).

2.2.4 The pre-BCR checkpoint and pre-B cell proliferation

In agammaglobulinemia patients, mutations in the constant region of IgHC, the SLC component Ig λ-like14.1, Ig-α, Btk or Slp-65 all result in a complete or nearly complete developmental arrest at the pro- to pre-B cell transition (see for recent review ref. 68). The X-linked form of the disease, called X-linked agammaglobulinemia (XLA) or Bruton's disease, is the most frequent one and is caused by mutations in Btk (69,70). For many years, the exact location of the developmental block in XLA has been unclear, because of phenotypic heterogeneity between patients. But accurate flow cytometric analyses using an antibody specific for the SLC component VpreB demonstrated that pre-B cells from XLA patients that express the pre-BCR on the cell surface are small sized (71), indicating that Btk is required for proliferative expansion of pre-B cells in man (Figure 2).

Similarly, pre-B cell expansion is severely impaired in mice with a targeted disruption of pre-BCR components, including μHC, the SLC constituents λ5 and VpreB SLC, and Ig-α/Ig-β (72-76). In contrast, mice deficient for Slp-65 or Btk display the x-linked immunodeficiency (*Xid*) phenotype, which is mainly characterized by defects in mature peripheral B cells (77-83). In the BM, μHC-positive pre-B cells still have the capacity to progress to the large cycling stage, but show defective maturation to the next stage of small resting pre-B cells (57, 84). Btk- or Slp-65-deficient pre-B cells show an increased proliferative response to IL-7 *in vitro* (57, 84). Although Btk/Tec-double-deficient mice have an almost complete block at the $CD43^+$ pre-B cell stage, pre-B cell proliferation is intact and thus a differential ability of Tec to compensate for Btk cannot explain the phenotypic differences between XLA in human and xid in mice (66,85). A further dramatic consequence of Slp-65 deficiency is the spontaneous development of pre-B cell leukemia with high levels of the pre-BCR on the cell surface in approximately 15% of these mice at the age of 6 months (57,86). Slp-65-deficient pre-B cells cause leukemia 3-5 weeks after injection into immunodeficient RAG-2-/- / γc-/- mice. Conversely, reconstitution of

Slp-65 expression stimulated the differentiation of these cells and inhibited their potential to cause leukemia, providing strong evidence for the tumor suppressor role of Slp-65 (87).

In summary, Btk and Slp-65 appear to be essential at distinct stages in pre-B cell development in humans and mouse, since they induce pre-B cell proliferation in human and limit pre-B cell expansion in the mouse (Figure 2).

2.2.5 The pre-BCR checkpoint and pre-B cell differentiation

Next to proliferative expansion, the pre-BCR also induces termination of further rearrangement on the second IgHC allele, a phenomenon referred to as allelic exclusion. Indeed, allelic exclusion is lost in mice with a targeted disruption of the Ig μ membrane exon in which the pre-BCR cannot be deposited on the cell surface (73). Additional mouse models have provided evidence that allelic exclusion is not only dependent on correct pre-BCR assembly but also on pre-BCR signaling. Association between IgHC and the Ig-α/Ig-β heterodimers was shown to be essential for the induction of allelic exclusion (88,89). Whereas mice deficient for Syk show a partial pre-B cell block, mice deficient for both Syk and its only other known family member ZAP-70 show a complete block at the pro-B cell stage and

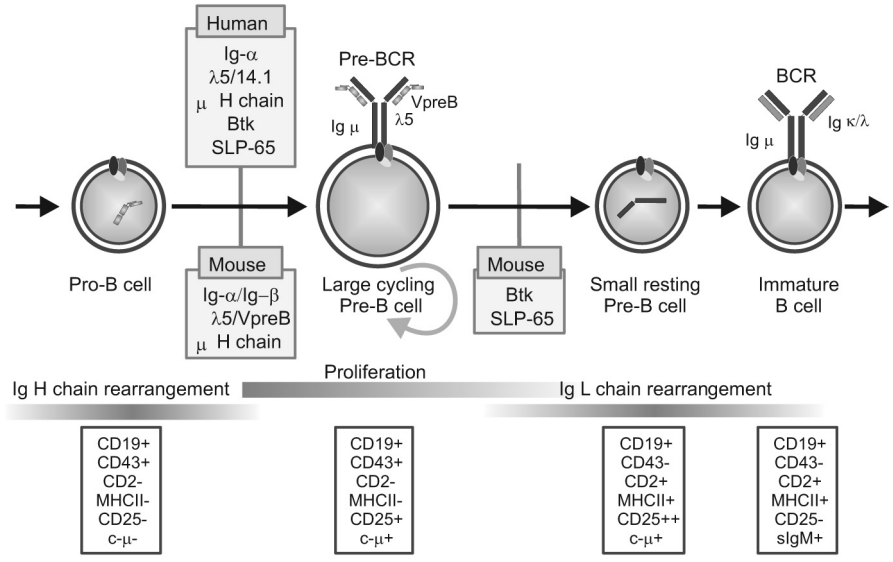

Figure 2. Stages of B lymphocyte development. Developmental blocks present either in agammaglobulinemia patients or in targeted mice with mutations in the indicated genes (shown in red). B cells are classified into four different stages, based on the cell surface marker expression and rearrangement status of the IgHC and IgLC loci (bottom). c-μ= cytoplasmic μHC; MHCII= MHC class II. Other abbreviations are explained in the text.

loss of allelic exclusion (63). Likewise, mice with reduced expression of PLCγ1 in the absence of PLCγ2 (PLCγ1$^{+/-}$PLCγ2$^{-/-}$ mice) manifest impaired IgHC allelic exclusion (67). Although activation of both PLCγ1 and PLCγ2 is dependent on Slp-65, and Slp-65-deficiency severely affects pre-BCR/BCR induced Ca^{2+} flux (67,80,81), allelic exclusion appears intact in Slp-65-deficient mice (83, 86). As both Slp-65 and LAT/Slp-76 adapter systems participate in pre-BCR-mediated calcium signaling (65) and activation of PLCγ1 and PLCγ2 (67), it is possible that allelic exclusion in the absence of Slp-65 is rescued via the LAT/Slp-76 adapters.

At the transition of large cycling into small resting pre-B cells in the mouse, Btk- or Slp-65-deficient pre-B cells show defective downregulation of SLC, the metallopeptidase BP-1, the sialoglycoprotein CD43 and the IL-7R, as well as defective upregulation of the adhesion molecule CD2, CD25 (the α-chain of the IL-2 receptor) and MHC class II (57, 84), implicating Btk- and Slp-65 in the control of cellular maturation of cytoplasmic Ig μ^+ pre-B cells.

The role of pre-BCR signaling in the induction of IgLC rearrangement is controversial (48,90). The hypothesis that pre-BCR signals play an important role is based on the observations that IgLC germline transcription and rearrangement correlate with μHC surface expression (reviewed in 90), or by expression of an activated Ras transgene in $J_H^{-/-}$ pro-B cells, which lack the ability to assemble IgHC variable regions (91). However, IgκLC transcription and rearrangement is also detectable in μHC-negative pro-B cells (92) and is increased by activation of NF-κB through lipopolysaccharide in transformed pro-B cell lines (93). Moreover, rearrangement and expression of IgLC genes can occur *in vitro* without IgHC expression (48): removal of IL-7 from cultured pro-B cells from $J_H^{-/-}$ or $\lambda5^{-/-}$ mice, which are incapable of expressing a proper pre-BCR, resulted in the apparent differentiation into IgLC expressing cells with normal frequencies and kinetics (94, 95).

The findings of reduced IgLC germline transcription and gene rearrangement in Slp-65-deficient pre-B cells (86, 96, 97) and of reduced IgLC λ usage in Btk-deficient mice (98) point to important roles for these signaling molecules in the initiation of IgLC gene rearrangement. However, they do not provide direct evidence for the involvement of these signaling proteins in the induction of IgLC gene rearrangement. A possibility remains that Btk and Slp-65 are only essential for the termination of pre-B cell proliferation, whereby cellular differentiation and IgLC gene rearrangement is subsequently initiated in a Btk/Slp-65 independent fashion.

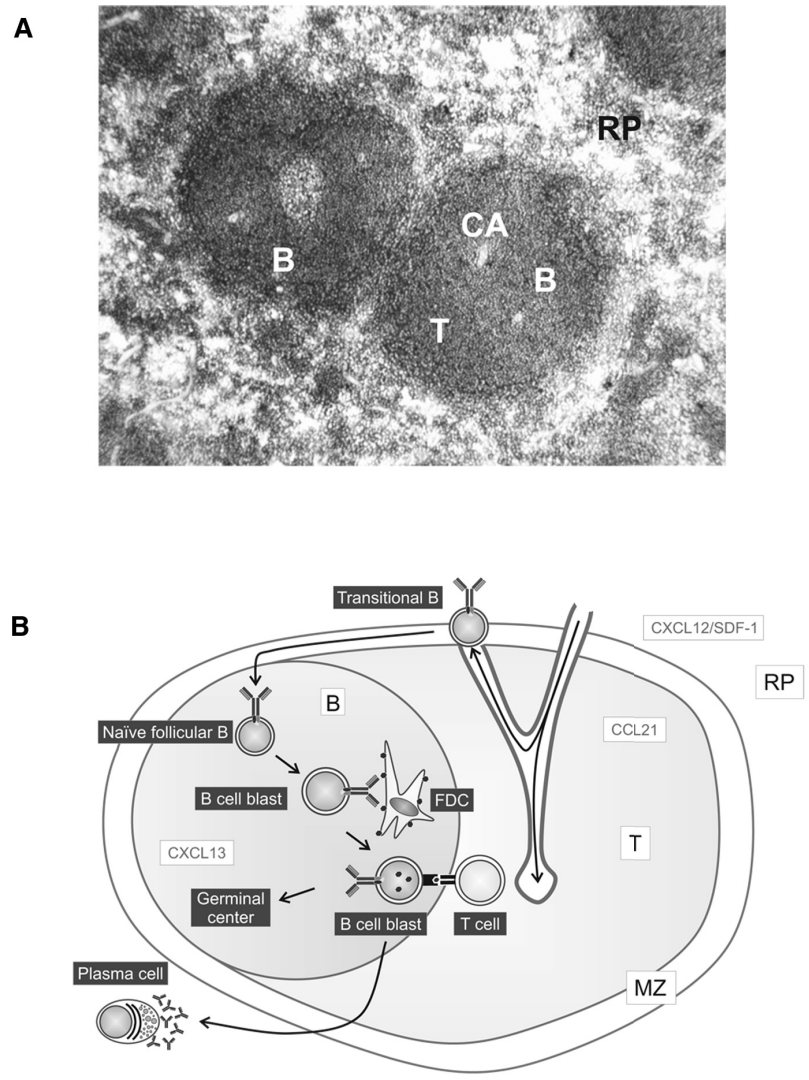

Figure 3. Splenic architecture and B cell migration.
A,B) The spleen is composed of white pulp and red pulp (RP). The white pulp contains lymphocytes and consists of a central arteriole (CA) with a surrounding T-cell area (T). Surrounding the central T cell area, B cell follicles (B) can be found. The marginal sinus marks the boundary between the follicular B cell area and the marginal zone (MZ). B) Upon arrival in the spleen the transitional B cells increase sensitivity to CXCL13 which guides them into the splenic follicle where they can mature into naïve follicular B cells that scan the B cell follicle for antigens bound to follicular dentritic cells (FDC). After antigen recognition the B cells become B cell blasts and upregulate CCR7, which initiates migration to the B-T cell area boundary where they receive T cell help and can either directly differentiate into plasma cells or take part in a germinal center reaction. Plasma cells upregulate CXCR4 that allows them to migrate to the red pulp.

2.3 Late B cell development

2.3.1 Immature and transitional B cell stages

After successful rearrangement of the IgHC and IgLC gene segments, these are jointly expressed on the B cell surface as the BCR. Expression of the BCR acts as the second key checkpoint in B cell development and monitors the production of a functional IgLC and prevents the formation of autoreactive B cells.

Young adult mice are estimated to produce about $2x10^7$ immature B cells per day (99). Of these $2\text{-}3x10^6$ cells were shown to reach the spleen daily (100). Therefore only 10-20% of the immature B cells produced in the BM enters the spleen. From these new splenic immigrants only 50% will enter the pool of long-lived mature B cells, finally resulting in an immature B cell success rate of only 5-10% (101). Immature B cells entering the spleen can undergo further maturation to so-called transitional B cells. The T1 stage is $AA4.1^+$ $CD21^-$ $CD23^-$ and is followed by the T2 stage $AA4.1^+$ $CD21^-$ $CD23^+$ after which cells can develop into mature B cells (Figure 3A) (102-104). Recently the group of Cambier showed that the previously described intermediate T3 stage actually represents an anergic B cell population (104). Transitional B cells are short-lived and upon BCR cross-linking are unable to proliferate and unable to generate an immune response. Subsequently some B cells are being selected into the marginal zone (MZ) B cell subset, while most compete for entry into the splenic B cell follicles where they will become follicular (Fol) B cells.

2.3.2 The BCR checkpoint

The fact that 90% of the immature B cells goes into apoptosis is thought to be the resultant of two processes: positive and negative selection. First, expression of the BCR checks for correct assembly and pairing of the IgHC and IgLC components. Several lines of evidence suggest that low-level basal signaling from the BCR is required for maturation of immature B cells and maintenance of mature B cells. Most convincing was the case when the BCR genes were conditionally deleted in mature B cells which resulted in depletion of all peripheral B cells, showing that low-level basal signaling from the BCR was essential for surivival of peripheral B cells (105).

Secondly, B cells are subjected to a BCR-dependent negative selection process, that evolved to ensure the efficient removal of autoreactive B cells, and prevents autoimmune disease. Here BCR signaling determines whether a cell can live or is instead tolerized by central or peripheral negative selection mechanisms. The negative selection mechanisms active in the BM operate at the immature and the later transitional stages of B cell development. Receptor editing and anergy induction are the most frequent approaches taken to induce tolerance, whereas

clonal deletion only takes place when receptor editing fails to edit away high-affinity for self (106,107). It is thought that self-antigen valency (i.e. the number of antigenic determinants) and abundance together with receptor affinity determine which negative selection process is triggered (106).

In the periphery limiting amounts of B-cell activation factor (BAFF), highly selective T cell help and follicular exclusion of autoreactive B cells all contribute to maintaining tolerance. BAFF is a member from the tumor necrosis factor (TNF) family and is a key survival factor during B-cell maturation. Excessive BAFF production corrupts B-cell tolerance and leads to autoimmunity (108). Recently, additional negative selection checkpoints have been described governing plasma cell differentiation and maintenance (109,110).

2.3.3 B cell subsets

In the mouse, three functionally and developmentally different mature B cell subsets cooperate to provide immune protection (111,112). Because Fol and MZ B cells have a common path of development in the adult, they are also referred to as B-2 B cells. Follicular B cells (IgMlow IgDhigh CD23+ CD21int CD5-) are found in the B cell follicles of the secondary lymphoid organs, and form the major B cell population (Figure 3A,B). They are the main players in the humoral adaptive immune response that is triggered by antigen exposure and activated T helper cells. The adaptive humoral immune response involves Ig class switching, somatic hypermutation and differentiation into plasma or memory B cells. In contrast to Fol B cells, MZ B cells (IgMhigh IgDlow CD23- CD21+) do not reside in B cell follicles but rather at the margin of the white pulp in the spleen (Figure 3A, B). Here they are appropriately situated to quickly catch and react to blood borne pathogens in a T cell independent way. Recently it was shown that MZ B cells were not confined to the MZ but continuously traveled between the marginal zone and follicular areas (113). Migration to the follicle is dependent on the chemokine receptor CXCR5, whereas return to the marginal zone required the sphingosine 1-phosphate receptors S1P(1) and S1P(3). Marginal zone-follicle shuttling of marginal zone B cells provides an efficient mechanism for blood antigen capture and delivery to follicular dendritic cells. MZ B cells can also function in T cell dependent reactions (112). The third B cell subset, the B-1 cells, forms a small but important population in the mouse that can be subdivided into B-1a and B-1b B cells. The B-1a B cells (IgMhigh IgDlow CD23- CD43+ CD5+) have been studied best. During adult life they can be found in the peritoneal and pleural cavities, spleen and gut where they form a self-renewing population. B-1a B cells are the major source of the 'natural antibodies' that constitutes most of the serum IgM in mice. These weakly autoreactive innate-like Ig are thought to be involved in the early stages of infection and may have housekeeping functions such as clearance of senescent cells.

Recent studies revealed a distinct function for B1b lymphocytes, which closely resemble memory B cells in a number of aspects. In contrast to the development of conventional B cell memory, which requires the formation of germinal centers and T cells, the development of B1b cell-mediated long-lasting antibody responses occurs independent of T cell help. Specific recognition of T-cell independent (TI) antigens by B1b cells and the highly protective antibody responses mounted by them clearly indicate a crucial role for this subset of B cells (114-116).

From birth on, Fol and MZ B cells are produced from hematopoietic stem cells in the BM. After successful IgHC and IgLC rearrangements, immature B cells migrate to the spleen where they become transitional B cells. Here some B cells are being selected into the MZ B cell subset, while most compete for entry into the splenic B cell follicles where they will become Fol B cells. In contrast, B-1a B cells are primarily generated from progenitors in the fetal liver and neonatal BM and are most likely maintained by lifelong self-renewal.

The choice of differentiation into either of the three B cell subsets is strongly influenced by signals originating from the BCR. Evidence for this came from multiple studies using BCR signaling compromised / enhanced mice or BCR transgenic mice, that resulted in loss or specific generation of certain B cell subsets (117,118). Moreover it has been shown that analogous to T cell development, also in B cell development, positive selection based on low-level BCR reactivity to self-antigens is important for the development of the B-1 and MZ B cell populations (119, 120).

It has long been speculated what the exact role of the BCR was during positive selection. Mechanistically, BCR-mediated positive selection into one of the B-cell subsets, might be based on either the BCR signal strength (a matter of signal quantity), the BCR specificity (a matter of signal quality) or a combination of both (121,122). Here BCR signal quality may include BCR specificity with variable engagement of BCR signaling pathways, but may also include activation of Ig co-receptors such as CD19, CD21, CD22 and CD72. Two groups independently reported that BCR signaling quantity rather than BCR signal quality was the major determining factor in cell subset differentiation decisions (121,123). They used transgenic mice expressing the Epstein Barr virus encoded protein, LMP2A, which mimics a constitutive-active-BCR. Transgenic LMP2A expression allowed the generation of BCR negative 'B cells', and therefore resulted in a model where BCR signaling quantity could be evaluated independently of BCR signaling quality. High LMP2A expression levels induced B-1 B cell development whereas intermediate expression levels resulted in Fol and MZ B cell development (121). Therefore BCR signal quantity rather than quality seems to be decisive for B cell fate decisions.

2.3.4 The humoral response

Antibodies provide three mechanisms to clear pathogens: neutralization, opsonization and activation of the complement cascade. Specificity of the humoral immune response to pathogens is achieved by demanding two different signals for B cell activation. The first signal is delivered by the BCR after binding antigen. The second signal is often provided by helper T cells that recognize fragments of degraded antigen, presented as peptides bound to MHC II molecules on the B-cell surface. Because this system requires BCR mediated endocytosis, degradation, presentation on MHC II molecules and specific recognition by helper T cells, this phenomenon was called 'linked recognition'. Additionally, this system requires separate activation of T cells by activated antigen presenting cells. CD40L on the activated T cell surface activates the B cells driving the resting B cell into cell cycle. Proliferation is further enhanced by IL-4 that is secreted in a polar fashion by the T-helper 2 cells. In contrast to the T cell dependent activation, B cells can also be activated in a TI fashion. TI type I (TI-I) responses require two signals: one coming from cross-linked BCR recognizing repetitive antigenic structures and another provided by a co-receptor such as CD21 or innate receptors including TLR4 and TLR9 (124). TI type II (TI-II) reactions are critically dependent on intact BCR signaling pathways, because mice deficient for BCR signaling proteins such as Btk and Slp-65 show defective responses. TI-II antigens have highly repetitive structures that efficiently cross-link BCR, resulting in swift IgM antibody production (125). TI-II responses are essential for defense against bacteria such as *Streptococcus Pneumoniae* and *Haemophilus Influenzae type B* that commonly escape triggering of the immune system by hiding protein structures under their polysaccharide capsule. Because children do not develop fully effective responses to carbohydrate antigens until approximately five years of age, they are at increased risk for developing severe infections with these bacteria (126).

Recent developments, including the generation of different knock-out models and the application of two-photon fluorescence microscopy have greatly increased our knowledge on the dynamics of humoral immune responses (reviewed in 127, 128). Before activation, recirculating naïve B cells enter secondary lymphoid organs from the blood to scan for their specific antigens (Figure 3). Within the lymphoid organs the B cells migrate towards the B cell follicles in a CXCR5-dependent fashion. Its ligand CXCL13 is expressed by follicular stromal cells. Within the follicles the B cells rapidly scan the area and follicular dendritic cells for antigen. Upon encounter of the specific antigen, B cells swiftly upregulate CCR7 transcription and surface expression while sustaining CXCR5 expression. Upregulation of CCR7 subsequently results in directed migration of antigen-engaged B cells towards the T cell zone. Encounter with activated helper T cells in the B–T boundary region

subsequently results in B cell activation, as was described above. Both the B and T lymphocytes now proliferate forming a primary focus. After a number of cell cycles some of the B lymphocytes differentiate into antibody secreting cells also called plasma cells providing early protection by secretion of specific antibody.

A few B cells from the primary focus migrate into a primary lymphoid follicle where they continue to proliferate and ultimately form a germinal center. Germinal centers are classically separated in a dark zone and a light zone, and contain follicular dendritic cells, T cells and B cells. The first model of germinal center function was proposed in 1994 by MacLennan (129). Since then new data have confirmed but also challenged several features. One of the most important changes is that new data stress the importance of T cell help in germinal center (GC) B cell selection (130). Preferentially in the dark zone the GC B cells prepare for mitoses, undergo extensive somatic hypermutation and perform class switch recombination. During class switch recombination (CSR) the IgHC isotype is switched from IgM to either IgG, IgA, or IgE through the action of activation-induced cytidine deaminase (AID) at so-called switch (S) regions.

AID has been shown to deaminate cytosine to uracil in single-stranded but not double-stranded DNA whereby processing of these uracils results in hypermutation in variable and switch regions, and DNA double strand breaks (DSB) which are important for CSR. CSR is thought to be targeted to specific S regions by selective transcriptional activation of germline transcripts. AID and uracil-DNA glycosylase (UNG)-dependent DSB are processed through a cascade of events mediated by base excision repair (BER), mismatch repair, NHEJ, and error-prone polymerases that facilitate coupling of S regions (131).

Several hours after entering the G1 phase of the cell cycle, some of the cells loose CXCR4 expression and CXCL12 responsiveness, which allows them to migrate to the light zone in response to CXCL13 (reviewed in 128). In the light zone the cells likely rapidly scan the follicular dendritic cell network for antigens and receive prosurvival BCR signals. Depending on the BCR affinity the centroblasts will be able to present few or lots of peptide-MHCII complexes on the cell surface. As T cells can only polarize to a single cell at a time, only GC B cells with the highest number of antigen peptide-MHCII complexes will be able to interact stably with a helper T cell. Cells exiting the germinal center rapidly differentiate into long-lived plasma cells or memory B cells.

2.3.5 *Transcriptional control of plasma cell and memory B cell differentiation*

Commitment to the memory B cell fate or plasma cell fate is thought to occur in the light zone of the germinal center. After antigen encounter B cells differentiate into plasmablasts, plasma cells or memory B cells (Figure 2). Plasmablasts are short-lived and found in extrafollicular foci in peripheral lymphoid organs. Long-lived plasma cells are mainly noncycling, found preferentially in the BM and mainly generated from germinal centers (132). In the last few years a lot has been learned about the transcriptional control of memory versus plasma cell development. Strikingly, the gene-regulatory network seems to be dominated by transcriptional repression. Pax-5, Mitf, Bach2 and Bcl-6 that are expressed in activated B cells mainly repress differentiation. In mature B cells Pax-5 represses non-B cell lineage programs but also genes such as Prdm1 (the gene encoding Blimp-1), Xbp-1 en Ig J chain. Expression of Bcl-6 and Bach2 in memory B cells in their turn suppress plasma cell development by repressing Blimp-1 whereas the transcription factor Mitf represses Irf-4.

Although it is unclear how these repressive elements are silenced, it has been shown that differentiation and high Ig secretion require IRF-4, XBP-1 and Blimp-1 (133). Blimp-1 is thought to be the master regulator of plasma cell differentiation because it is able to induce plasma cell differentiation in transfected B cell lines and its B-cell specific deletion prevents development of plasma cells *in vivo* (133). Furthermore, Blimp-1 was shown to suppress Pax-5 activity, thereby initiating loss of B cell identity whereas alternative differentiation into memory B cells was blocked by repressing the memory B cell specific transcription factor Bcl-6. The secretory phenotype of plasma cells was shown to be dependent on XBP-1, which was thought to be a downstream target of Blimp-1 (134). More recently IRF-4 has been added to this transcriptional scheme. IRF-4 is expressed in immature B cells, absent in GC B cells (centroblasts) and reexpressed in cells exiting the germinal center (centrocytes) and plasma cells. It was shown that IRF-4 is essential for plasma cell differentiation and CSR (135, 136).

Recent data shed new light on this transcriptional picture. Rag-1 deficient mice that were reconstituted with Prdm1-deficient/ GFP knock-in fetal liver cells were analyzed for plasma cell differentiation (137). Although these mice lacked a plasma cell compartment, pre-plasmablasts were detected that generated substantial amounts of all Ig isotypes. Pre-plasmablasts lacked detectable amounts of Blimp-1 and IRF-4 but expressed XBP-1. Therefore plasma cell differentiation is initiated independently of Blimp-1 but requires Blimp-1 for full commitment to the plasma cell fate.

3 B CELLS IN DISEASE

3.1 Immunodeficiency

Loss of effective immunity against infectious agents can have dramatic impact on health, resulting in recurring or even lasting infections and wasting. Diseases characterized by T cell defects are most severe and can be complicated by life threatening infections from early age on, while B cell defects generally result in a more benign course. In this section defects in pre-BCR and BCR signaling caused by Btk and Slp-65 deficiency will be discussed.

3.1.1 X-linked agammaglobulinemia or Bruton's disease

The first description of X-linked agammaglobulinemia (XLA) dates back from 1952 when Bruton reported on an eight-year-old boy with recurrent bacterial sepsis and absence of serum immunoglobulins (138). Reasoning that the lack of Ig might be the cause of the frequent infections found in this boy he started administering subcutaneous gammaglobulin on a monthly basis, which resulted in a dramatic improvement in the boys condition. When more cases were discovered it was noticed that most patients were male and that the disease was inherited in an X-linked pattern (139). Later on, in 1978, it was noted that patients with XLA virtually lack peripheral B cells and that B-cell precursors found in the BM are unable to replicate normally (140). Furthermore it was shown that the defect in XLA was B cell intrinsic as only B cells from females carrying the XLA mutation had a non-random pattern of X-inactivation (141). Finally in 1993 a European group and an American group simultaneously discovered the defective gene in XLA, Bruton's tyrosine kinase (Btk) (69,70). In the same period Btk was also shown to be defective in the well known murine immunodeficiency model Xid (142,143). In the decade that followed it became clear that approximately 15% of agammaglobulinemia cases was not caused by Btk deficiency (144). Mutations in the constant region of IgHC, the SLC component Ig λ-like14.1, Ig-α, Ig-β or Slp-65 all resulted in a complete or nearly complete developmental arrest at the pro- to pre-B cell transition and an agammaglobulinemia phenotype (68,145-151).

Btk is a member of the Tec family of cytoplasmic tyrosine kinases and is encoded in 19 exons at Xq22 in man. All Tec kinase family members except Txk/Rlk contain an amino-terminal pleckstrin homology domain that localizes the protein to the membrane by binding to phophatidylinositol lipids. The PH domain is followed by three protein interaction domains: Tec homology region (TH) and the Src homology 3 (SH3) and SH2 domain (Figure 4). The catalytic SH1 domain is located on the C terminal end. Btk is expressed at all B cell differentiation stages with the exception of plasma cells (152,153). Furthermore, it is expressed in myeloid cells and platelets.

Btk has been reported to function under a large variety of receptors such as the BCR, Fcε receptor, TLR4, Epo receptor, CD38, CCL5, TLR2, IL-10R and IL-6R.

Slp-65 like its close relative in T cells (Slp-76) is a linker protein. It is encoded in 17 exons on the long arm of chromosome 10. The amino-terminal region of the protein is rich in tyrosines and tyrosine motifs of the YXXP motif that are found in multiple tyrosin-phosphorylated adaptor proteins such as Slp-76, Dok and Cbl. Furthermore, it contains a highly conserved leucine zipper that is responsible for recruitment of Slp-65 to the plasma membrane. The mid-portion of the molecule contains a proline-rich region that has efficient SH3-domain binding properties. Finally the C-terminus consists of an SH2-domain that allows interaction with other tyrosine phosphorylated proteins. Slp-65 is expressed in B cells and myeloid cells and up till now has been mainly reported to function under the BCR. Recently it was shown that CD303 on plasmacytoid dendritic cell signals via a BCR-like signalosome involving Syk, Slp-65 and PLCγ2 (154).

XLA is a recessive fully penetrant disorder that is X-linked. Therefore most patients are male, although rarely female patients have been reported. There is no clear relation between the specific mutation in Btk and the severity of disease. Likely the phenotype of XLA patients is modified by other genetic factors and environmental factors. XLA is one of the most common primary immunodeficiencies with an incidence of 1/190,000 male births in the USA (155). The clinical course is highly variable between patients with XLA. Most are diagnosed before the age of 5 years and only a few are recognized at a later age (155,156). The majority of patients is diagnosed after hospitalization for a severe infection. Infections in children within the first year of life include pyoderma gangrenosum, perirectal abscess, cellulitis, impetigo, pseudomonas or staphylococcal sepsis and neutropenia. Patients diagnosed between 13 and 40 months of age (approximately 50%) mainly present with respiratory symptoms such as recurrent otitis, sinusitis, pneumonia, arthritis, meningitis, diarrhea and conjunctivitis. In addition to frequent bacterial infections, patients with XLA also show increased susceptibility to enteroviral infections such as echo, coxsackie and polio virus that can induce a dermatomyositis-like syndrome or fatal chronic encephalitis (157-161). Furthermore, patients have more frequent infections with giardia.

Autosomal agammaglobulinemia caused by defects in genes such as the constant region of IgHC, the SLC component Ig λ-like14.1, Ig-α, Ig-β or Slp-65 all result in a phenotype similar to that found in XLA patients. However, these patients do seem to be diagnosed younger and to suffer from a higher incidence of severe complications (162).

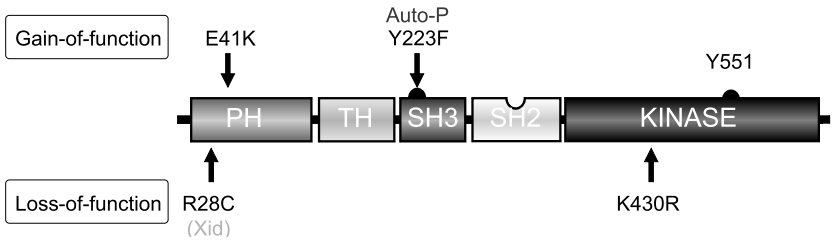

Figure 4. Linear model of Bruton's tyrosine kinase.
Shown are the 5 different domains (explained in the text). The positions of the gain-of-function mutations E41K and Y223F are indicated by arrows pointing downward. The position of loss-of-function mutations such as R28C and K430R are shown by arrows pointing upward. Furthermore the major phosphorylation site Y551 is shown.

Current therapy includes monthly administration of intravenous immunoglobulins and aggressive use of antibiotics. This therapeutic regimen has markedly improved the outcome of patients with XLA and resulted in a death rate of 1%/year during a 4.5 year follow-up, which is similar to that found in normal persons (155). Nevertheless it is difficult to tell whether the patients treated with the current therapy will have normal life expectancy, as most of the patients that were followed were relatively young and may still develop long-term complications reducing life expectancy.

3.1.2 X-linked immunodeficiency and Btk and Slp-65 deficiency

The well-characterized mouse model X-linked immunodeficiency (Xid) is caused by an R28C missense mutation resulting in replacement of a conserved arginine residue by a cysteine residue (Figure 4). Therefore R28C-Btk is unable to bind the plasma membrane with its PH domain and transmit BCR signals. Nevertheless Xid mice have a mild phenotype compared to XLA patients, characterized by a mild arrest of B cell development at the large pre-B cell stage, reduction of approximately 50% of the normal number of B cells in the periphery, more immature B cells in the spleen (IgM^{high}/IgD^{low}), a lack of B-1a cells, shortened survival of MZ B cells and low serum IgM and IgG3 levels (163). Furthermore Xid mice responded poorly to TI-II antigens.

Like Xid mice, Slp-65-deficient mice have a less profound arrest of B cell development than is found in humans. B cell development was affected at the pre-B cell stage resulting in increased proportions of large-pre B cells and failure to efficiently progress to the small pre-B cell stage (80,82). Additionally the mice had more immature B cells in the spleen, fewer mature $IgM^{lo}IgD^{hi}$ B cells and had a 5-40% reduction in the number of B cells. Furthermore they lacked B-1a B cells, showed low serum IgM and IgG3 serum titers and demonstrated poor TI-II

responses. Moreover, mutant B cells had reduced Ca^{2+} responses to BCR cross-linking and proliferated poorly upon CD40 ligation or LPS stimulation.

Initially the mild Xid phenotype was attributed to the 'weakly pathogenic' R28C mutation. Soon, however, it was discovered that R28 mutations were also found in patients with classical XLA (164). Moreover mice deficient for Btk had a phenotype that was no different from that found in Xid mice (78,79,165).

Careful studies of B cell development in Btk- and Slp-65-deficient humans and mice, and various pre-BCR and cytokine receptor deficient mice, provided important clues to the origin of the differences found between Btk-deficient mice and men. In mice progression to the large pre-B cell stage and proliferation are dependent on signals emanating from both the pre-BCR and the IL-7R, as mice deficient for Igα, Igβ, Syk, IL-7, IL-7Rα chain, IL-7Rγ chain or JAK3 have an arrest of B cell development at the pro-B to pre-B cell transition and fail to undergo pre-B cell expansion (47,166-170). In XLA and autosomal forms of agammaglobulinemia, B cells are also arrested at a late pro-B cell stage and are unable to progress to the large pre-B cell stage and enter cell cycle (71). In contrast, patients with severe combined immunodeficiency (SCID) caused by disruption of the IL-7Rα chain, IL-7Rγ chain or JAK3 show normal B cell development (171-174). Therefore pro-B cell to large pre-B cell progression in mice seems to be highly dependent on both IL-7R and pre-BCR signaling, while human pro-B cell to large pre-B cell differentiation strongly depends on pre-BCR signaling only. Although pre-BCR signaling is essential for developmental progression and initiation of proliferation in mice, B cell development in Xid mice is mildly arrested at the large pre-B cell stage where the cells efficiently enter cell cycle but stop proliferation less efficiently (84). A possible explanation for the discrepancy between mice and men is that other Tec family members compensate for the Btk deficiency in mice. Supporting this notion, it was shown that Tec and Itk could rescue the Ca^{2+} mobilization defect in XLA patients (175). Strangely however, most human B cells contain Tec, which essentially is activated by the same triggers that activate Btk (176). Moreover, as described before, mice double-deficient for Btk and Tec displayed increased proliferation at the large pre-B cell stage but defective termination of proliferation and differentiation (66). Similar results were obtained for Slp-65 and PLCγ2 and their respective family members Slp-76 and PLCγ1 (64). Therefore Btk and Slp-65 have different roles in pre-BCR signaling in humans than in mice with respect to initiation of proliferation. Taken together, these data indicate that both IL-7R signaling and redundancy of pre-BCR signaling components contribute to a milder phenotype of Btk or Slp-65 deficency in mice.

Mechanistically, the arrest of differentiation found in XLA may be a direct consequence of a failure to enter the cell cycle. Alternatively, failure to efficiently

initiate differentiation and IgLC rearrangements in the absence of adequate pre-BCR signaling is likely to also contribute. This issue is still a matter of heavy debate and has therefore been studied by us in detail in mouse models (Chapter 2).

3.1.3 Constitutive active Btk mutants

Soon after the identification of Btk, a Btk mutant was shown to induce transformation of fibroblasts (177,178). The constitutive active E41K Btk mutant was able to transform NIH 3T3 fibroblasts in a soft-agar culture system (177,178). This mutation, which was identified using a retroviral random mutagenesis scheme (177,178), is located in the pleckstrin homology (PH) domain and is associated with increased Btk tyrosine phosphorylation, in particular of the regulatory Y551 residue (Figure 4).

Btk activation is initiated by transphosphorylation at position Y551 by Lyn or Syk kinase, which promotes the catalytic activity of Btk and subsequently results in its autophosphorylation at position Y223 in the SH3 domain. Btk activation upon (pre-)BCR activation is dependent on its membrane association through interactions of the PH domain with PIP3, which is a second messenger synthesized from PIP2 by PI3K. In contrast, E41K-Btk manifests increased membrane localization in quiescent cells, independent of PI3K activity, probably resulting from increased affinity for PIP2 (179). Transforming activity is kinase-domain dependent and is potentiated by introduction of a second mutation at position Y223 (177,178). However, Y223 phosphorylation does not appear to be essential for *in vivo* Btk function in the mouse (180). Transfection of E41K-Btk also enabled IL-5 independent growth of the mouse Y16 pro-B cell line, although much less efficient than v-Abl (177). Experiments involving specific targeting of Btk to the membrane using various fusion proteins showed that membrane association of Btk is essential and sufficient for its activation (181).

Also in B cells, E41K-Btk has constitutively active signaling properties as expression of E41K-Btk in Ramos B cells resulted in increased sustained cytoplasmic Ca^{2+} upon BCR cross-linking (175). Furthermore, transgenic expression of E41K-Btk in mice induced the expression of the early activation antigen CD69 on splenic B cells and enhanced their blast formation in culture and promoted IgM+ plasma cell differentiation *in vivo* (182,183). Finally, expression of E41K-Btk in early B cell differentiation resulted in an almost complete arrest at the immature B cell stage in the BM, which represents the first stage at which autoreactive B cells are susceptible to apoptosis (183). These data show that Btk is a key element of BCR signaling that strongly determines B cell responsiveness and that aberrancies can result in a large variety of diseases ranging from non-responsive immunodeficient states to hyperactive B cells.

3.2 Acute lymphoblastic leukemia

ALL is the most common form of childhood malignancy, whereby the majority of cases arise from B cells arrested at the precursor-B cell stage. The molecular events that arrest pre-B cell differentiation in leukemic pre-B cells are not known in detail, although extensive molecular characterization have revealed chromosomal translocations giving rise to various oncogenic fusion genes in a significant fraction of B-lineage ALL (Table 1) (184).

Table 1

B-ALL	85% of the pediatric ALL cases are of B-lineage origin. 80% can be further classified by cytogenetics and genetic translocations.	
Translocation	**Frequency**	**Characteristics**
BCR-ABL t(9;22)(q34;q11)	3%	also called Philadephia chromosome. Unfavourable prognosis.
E2A-PBX1 t(1;19)(q23;p13.3)	5%	might not affect prognosis
MLL-AF4 t(4;11)(q21;q23)	5% overall 85% younger than 1 year	although most often the AF4 gene on chromosome 4 is the fusion partner, about 40 different partners have been shown to fuse with MLL; any MLL rearrangement is considered a high-risk feature in ALL
TEL-AML1 t(12;21)(p13;q22)	22%	relatively favourable prognosis
Hyperdiploidy	25%	relatively favourable prognosis

Adapted from (185)

3.2.1 Basic concepts of acute lymphoblastic leukemia

Clinical symptoms and signs in children with leukemia result from infiltration of mainly BM but also other organs such as liver, spleen, testes, lymph nodes and the central nervous system. In most cases the symptoms develop over several weeks with malaise, pallor, abnormal bruising, hepatosplenomegaly, lymphadenopathy and bone pain as most common presenting symptoms.

Diagnosis is made using careful examination of blood and BM by morphological, cytogenetic and immunological methods. On the basis of morphology and immunological phenotyping, ALL is further subdivided into T-ALL (15% of cases) and B-ALL (85% of cases). Prognosis is dependent on cytogenetic features, involvement of central nervous system, involvement of testes and initial response to therapy whereby minimal residual disease monitoring plays a major role.

Current therapies use combination chemotherapy, intrathecal chemo-therapy and, in high risk groups allogeneic BM transplantation to cure patients from leukemia. New developments include antibody based therapy, small-kinase

inhibitors (imatinib) and better risk stratification according to pharmacogenetic data. Pharmacogenomics will be of great value for improving leukemia treatment (that is, reducing toxicity and increasing efficacy) by facilitating optimal treatment selection and appropriate dose individualization thereby reducing side affects and mortality (185). With current treatment regimens overall 5-year survival is around 85%.

3.2.2 The BCR-ABL1 fusion protein

A translocation between chromosomes 9 and 22, which is known as the Philadelphia chromosome, is found in a minor fraction of ALL patients but in most chronic myelogenous leukemia (CML) patients (Figure 5). The translocation involves a fusion of the breakpoint cluster region (BCR) with the proto-oncogene ABL1, leading to the expression of the chimeric fusion protein BCR-ABL1 (reviewed in ref. 186). In contrast to ABL1, which codes for the tightly regulated tyrosine kinase c-Abl that is involved in cell growth and survival, DNA-damage responses and actin dynamics, BCR-ABL1 is aberrantly localized in cells, forms oligomers, and is constitutively activated. BCR-ABL1 drives malignant transformation of pre-B cells and activates multiple signaling molecules and pathways, including those involved in cell survival and proliferation (Stat5, Ras, Raf, MAPK, ERK, PI3K/Akt, NF-κB, c-Myc, Bcl-XL, Bcl-2, Cyclin D2, and p27Kip), signaling (Lyn, Vav) and DNA repair (BRCA1, DNA-PK, Rad51, p53). As a result, BCR-ABL1 ALL cells manifest autonomous survival and proliferation and bypass selection for functional expression of a pre-BCR on the cell surface. Accordingly, molecules needed for transduction of survival signals through the pre-BCR are silenced. The majority of BCR-ABL1+ ALL carry nonproductively rearranged IgHC due to VH gene segment replacement (187). Those BCR-ABL1+ ALL cells that harbor a functional IgHC gene were found to be unresponsive to pre-BCR engagement (187). BCR-ABL1 kinase activity is linked to the expression of a truncated isoform of Slp-65, which may contribute to the compromised pre-BCR signaling. Inhibition of BCR-ABL1 kinase activity by the specific inhibitor STI-571 (Imatinib mesylate, Gleevec) reconstituted selection of pre-B ALL cells expressing the pre-BCR, corrected the expression of Slp-65, and restored the capacity of the surviving cells to differentiate into IgM+ immature B cells (187). Interestingly, defective Slp-65 expression may potentially cause the initiation of the secondary, mostly nonproductive Ig VH replacements, which would be consistent with the finding that interruption of basal IgM signaling in immature B cells, e.g. with the tyrosine kinase inhibitor herbimycin A or the PI3K inhibitor wortmannin, led to a strong induction of RAG expression (188).

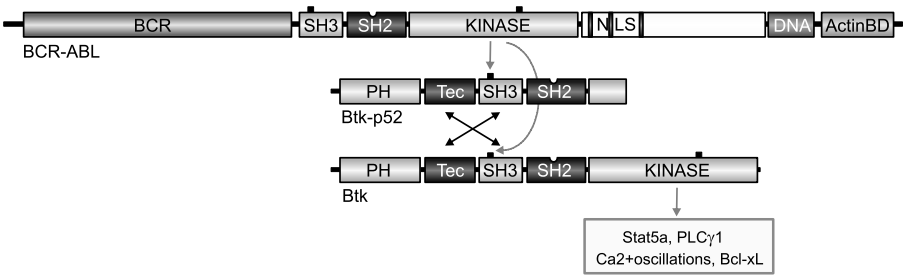

Figure 5. Model for BCR-ABL1-dependent activation of Btk in ALL.
Truncated Btk-p52 acts as a linker, enabling BCR-ABL1 to phosphorylate full length Btk at position Y223 in the SH3 domain, which initiates the indicated downstream survival signals. Full length and Btk-p52 interact through association of proline-rich regions in the Tec-homology domain and the SH3 domain. PH= pleckstrin-homology domain, SH2,3= Src-homology domain-2 and -3; NLS= nuclear localization signal; DNA= DNA-binding domain; ActinBD= actin binding domain; other abbreviations are explained in the text.

Specific pharmacological inhibitors have been instrumental for the identification of downstream targets that critically contribute to ABL-mediated leukemogenesis. Global PI3K inhibitors, such as wortmannin and LY294002, block both myeloid and lymphoid transformation, showing that activation of PI3K is one of the essential ABL signaling mechanisms (see ref. 189 for a comprehensive review). Using the Src-kinase inhibitor CGP76030, which prolonged survival of mice with B-lineage ALL, the Src-family kinases Lyn, Hck and Fgr were identified as essential targets of BCR-ABL1, specifically in lymphoid transformation. Lyn is also essential for survival of primary BCR-ABL1+ CML blast cells, as ablation of the Lyn kinase by short interfering RNA targeting induced apoptosis of these cells (190). Likewise, inhibition of Btk by the inhibitor LFM-A13 induced apoptosis in BCR-ABL1+ leukemia cells to a similar extent as inhibition of the BCR-ABL1 kinase activity itself. This finding, however, does not necessarily implicate Btk as an important target of BCR-ABL1 (see below), because the inhibitor LFM-A13 is not completely Btk-specific and inhibits Janus kinase 2 (JAK2) signaling as well (191).

3.2.3 Defective splicing of Slp-65 transcripts

Analysis of a panel of childhood pre-B ALL samples revealed that 16 out of 34 had either complete loss or drastic reduction of Slp-65 expression (87). No genomic mutations in the Slp-65 locus were present, but Slp-65 transcripts contained alternative exons that introduced premature stop codons. Although in one study it was concluded that Slp-65 deficiency is a rare event in pre-B cell ALL (192), defective Slp-65 expression was found in a significant fraction of pre-B ALL and mature B cell lymphoma (ref. 187 and M. Müschen, pers. comm.). In BCR-ABL1+ pre-B ALL cells, expression of truncated isoforms of Slp-65 was linked to BCR-ABL1 kinase activity (187).

Cancer-specific defects in mRNA splicing in the absence of genomic mutations are not unique for the Slp-65 gene, but have also been identified for various other signaling molecules (reviewed in ref. 193). At the moment, the relation between Slp-65 deficiency and other characteristics in ALL, such as IgHC expression or the presence of additional genetic abnormalities such as translocations is not clear. This may be due to the heterogeneity of additional mutations involved in the etiology of the disease, but may also point to a secondary role of defective Slp-65 expression in cellular transformation. In either case, the absence of Slp-65 at the pre-B cell checkpoint may contribute to the oncogenic transformation. One hypothesis is that defective Slp-65 expression allows aberrant V(D)J recombinase expression in proliferating pre-B cells (87), which would be supported by analyses of the IgHC VH regions in Slp-65-deficient pre-B ALL, indicating continued IgHC rearrangement and VH replacement (187). Consistent with this possibility, reconstitution of Slp-65 expression in Slp-65-deficient leukemia and lymphoma cells resulted in downregulation of RAG-1/-2 expression and prevented IgHC rearrangement and VH replacement (M. Müschen, pers. comm.). Taken together, these observations indicate that loss of Slp-65 expression and the accompanying pre-B cell differentiation arrest may be an important primary cause of pre-B ALL. However, at the moment it is not clear whether loss of Slp-65 is a common leukemogenic event.

3.2.4 Truncated BTK splice variants

In man, Btk signaling is not only critical for the induction of proliferation of pre-B cells (Figure 2), but also for cell survival, as it induces Bcl-xL expression and inhibits the pro-apoptotic effects of Fas ligation in mature B cells (194,195). Therefore, it was hypothesized that malignant transformation of pre-B cells could be dependent on Btk signaling. In accordance with the anti-apoptotic function of Btk, treatment of B-lineage ALL cell lines with the rationally designed specific Btk-inhibitor leflunomide metabolite analog LFM-A13, which binds to the catalytic site within the kinase domain, indeed enhanced the sensitivity of these cells to ceramide- or vincristine-induced apoptosis (196). These findings suggested that Btk might be constitutively activated in human B cell leukemia. Molecular analyses showed that the majority of ALL samples contained aberrant Btk splice variants, mostly encoding Btk proteins with a truncated kinase domain (197). Although in mice Btk function during B cell development is partially independent of its catalytic activity (84), this does not appear to be the case in man: to date ~110 unique missense mutations in the kinase domain have been identified, all leading to XLA phenotypes (198). Thus, truncated kinase-inactive Btk proteins were not expected to be functionally signaling for pre-B cell proliferation in man, and may even have a dominant negative effect (199). Taken together, these findings were in apparent conflict, as the observed anti-

apoptotic effects of Btk in ALL were not consistent with the presence of aberrant Btk splice variants encoding non-functional kinase-inactive Btk. However, they could be explained by assuming that the Btk splice variants do have a function in supporting cellular proliferation or survival. In agreement with this hypothesis, Btk ALL cells containing dominant-negative Btk isoforms exhibited a selective advantage after γ-irradiation (199). Although this may suggest that the Btk splice variants protect from radiation-induced apoptosis, it is also possible that basically the absence of functional Btk leads to resistance to γ-irradiation induced apoptosis (200).

Intriguingly, a novel function of truncated kinase-inactive Btk was identified in BCR-ABL1+ pre-B-ALL. BCR-ABL1 induces aberrant splicing of Btk, resulting in the presence of Btk-p52 (lack of exon 15, loss of reading frame) and Btk-p62 (in frame deletions of exons 15 and 16) isoforms. Although c-Abl can phosphorylate Btk at position Y223 in the SH3 domain, BCR-ABL1+ is unable to physically interact with full-length Btk (201). However, in BCR-ABL1+ pre-B-ALL, the BCR-ABL1 fusion protein utilizes the expression of the truncated splice variant Btk-p52 as a linker molecule to constitutively phosphorylate full-length Btk and Btk-p52 at Y223 (202) (Figure 5). In this complex full length Btk and Btk-p52 can bind to each other as a result of stable intermolecular interactions between the proline rich region within the Tec domain of one molecule and the SH3 domain of a second molecule (203). As a result, activated Btk provides signals that otherwise would be transmitted by the pre-BCR, such as activation of PLCγ1 and induction of Bcl-xL (202) (Figure 5). Activation of Btk and PLCγ1 is independent of Slp-65 expression, which is defective in BCR-ABL1+ ALL (187). The presence of BCR-AB1L, full-length Btk, Btk-p52 and PLCγ1 is also required for autonomous Ca^{2+} oscillations in these ALL cells (202), as well as for phosphorylation and nuclear translocation of Stat5, which was previously shown to be a direct substrate of Btk (204). In this context, there is a remarkable parallel with another Tec-family member, Etk/Bmx, which was previously shown to be a critical mediator of Src-induced cell transformation and STAT3 activation in liver cells (205).

4 AIM OF THE THESIS

In recent years, the signaling cascades that are involved in pre-BCR and BCR signalling have been resolved in considerable biochemical detail. Far less is known about the exact role of signaling proteins in the *in vivo* context of pre-BCR and BCR-mediated processes such as survival, developmental progression, selection, activation and tolerance. These downstream effects of pre-BCR and BCR-signaling are likely dependent on the developmental stage, environmental cues and the pool

of signaling proteins and their cooperation, present at a certain timepoint. Therefore, we set out to study how the two signal transduction proteins Btk and Slp-65 impact on B cell development and maturation. Moreover, we wanted to study the molecular mechanisms by which various defects in Btk and Slp-65 result in the development of immunodeficiency and malignancy. To this end, we studied several Btk and Slp-65 loss-of-function or gain-of-function mouse models in detail.

In Chapter 2, we addressed the question of the role of Btk in pre-B cells in the initiation of IgLC rearrangement and developmental progression. The findings of the pre-B cell arrest in Btk-deficient or Slp-65-deficient agammaglobulinemia patients, together with reduced IgLC germline transcription and gene rearrangement in Slp-65-deficient pre-B cells and reduced λ IgLC usage in Btk-deficient mice pointed to important roles for these signaling molecules in the initiation of IgLC gene rearrangement. However, they do not provide direct evidence for the involvement of these signaling proteins in the induction of IgLC recombination. The possibility remains that Btk and Slp-65 are only essential for the termination of pre-B cell proliferation, whereby cellular differentiation and IgLC gene rearrangement is subsequently initiated in a Btk/Slp-65 independent fashion. In Chapter 2, we show that Btk is able to signal for developmental progression at the pre-B cell stage independent of proliferation. It is therefore likely that the developmental defect in XLA patients also reflects failure to initiate rearrangement of the IgLC loci.

The defects in B cell development and B cell function in Btk-deficient and Slp-65-deficient mice are comparable. In particular, the phenotypes of these two mice at the pre-B cell stage, including defective termination of IL-7 driven proliferation, impaired upregulation of developmentally regulated markers such as CD2, CD25 and MHC class II and particularly the impaired downregulation of SLC expression are remarkably similar. Therefore, it was surprising that Slp-65 acts as a tumor suppressor at the pre-B cell checkpoint, whereas Btk-deficient mice do not develop any pre-B cell malignancies. To identify a possible role of Btk as a tumor suppressor, Btk-deficient mice were crossed onto the Slp-65-deficient background and mice were followed for the development of malignancy. Chapter 3 shows that Btk and Slp-65 cooperate as tumor suppressors and that low-level transgenic expression of a constitutive active Btk mutant prevents tumor formation in Slp-65/Btk double mutant mice.

Previously, it was shown that Btk partly functions independent of its catalytic activity, as transgenic expression of a kinase-inactive Btk mutant partially restored pre-B cell and B cell defects in Btk-deficient mice. Therefore, the mechanism by which Btk performs its tumor suppressor activity might either involve Btk kinase activity or rather its adaptor function. To investigate this in more detail we crossed Btk/Slp-65 double-deficient mice to the kinase-inactive Btk mutant mouse and

showed that Btk represses tumor formation in a kinase-independent way (Chapter 4).

In Chapter 5 we hypothesize that deregulated V(D)J recombination contributes to oncogenesis of Slp-65-deficient pre-B cells. This was suggested by several findings, such as high-level expression of Rag1/ Rag2 and ongoing IgLC rearrangements in Slp-65-deficient pre-B leukemias. Crosses of Slp-65-deficient mice onto the p53-deficient background helped us to assess the effect of unrepaired DNA damage. Furthermore, we crossed Btk/Slp-65 double-deficient mice with transgenic mice expressing a pre-rearranged IgHC. Such a transgene is thought to reduces the duration of the the pro-B cell stage in which V(D)J recombination occurs. Our results indicate that deregulated V(D)J recombination activity may contribute to malignant transformation of Slp-65 pre-B cells.

Although the phenotype of Btk-deficiency can be explained on the basis of its role in pre-BCR and BCR signaling, numerous other receptor signaling pathways have been shown to contain/use Btk as well. In Chapter 6 we investigate involvement of Btk in responsiveness to chemokines. We show that Btk mediates chemokine-controlled migration and *in vivo* homing.

In Chapter 7, the General discussion of the experimental work of this thesis, the data obtained are discussed in the context of available literature. In this chapter also suggestions are done for future studies relevant for the field of B cell development.

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CHAPTER 2

Bruton's tyrosine kinase and Slp-65 regulate pre-B cell differentiation and the induction of immunoglobulin light chain gene rearrangement

Bruton's tyrosine kinase and Slp-65 regulate pre-B cell differentiation and the induction of immunoglobulin light chain gene rearrangement

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SUMMARY

Bruton's tyrosine kinase (Btk) and the Slp-65 adapter protein transmit pre-B cell receptor signals that are essential for efficient developmental progression of large cycling into small resting pre-B cells. We show that Btk- and Slp-65-deficient pre-B cells have a specific defect in immunoglobulin λ light chain germline transcription. In Btk/Slp-65 double deficient pre-B cells both κ and λ germline transcripts are severely reduced. Although these observations point to an important role of Btk and Slp-65 in the initiation of light chain gene rearrangement, a possibility remained that these signaling molecules are only required for termination of pre-B cell proliferation or for pre-B cell survival, whereby differentiation and light chain rearrangement is subsequently initiated in a Btk/Slp-65 independent fashion. Because transgenic expression of the anti-apoptotic protein Bcl-2 did not rescue the developmental arrest of Btk/Slp-65 double deficient pre-B cells, we conclude that defective light chain opening in Btk/Slp-65-deficient small resting pre-B cells is not due to their reduced survival. Next, we analyzed transgenic mice expressing the constitutively active Btk mutant E41K. Expression of E41K-Btk in immunoglobulin heavy chain negative pro-B cells induced (i) surface marker changes that signify cellular differentiation, including downregulation of surrogate light chain and upregulation of CD2, CD25 and MHC class II, and (ii) premature rearrangement and expression of κ and λ light chains. These findings demonstrate that Btk and Slp-65 transmit signals that induce cellular maturation and immunoglobulin light chain rearrangement, independent of their role in termination of pre-B cell expansion.

INTRODUCTION

During B cell development, Ig gene rearrangement is ordered such that the Ig H chain locus generally rearranges before the Ig κ and λ L chain loci (Reviewed in Ref. (1, 2). Productive V(D)J recombination of the Ig H chain gene results in surface deposition of the precursor-B cell receptor (pre-BCR), which consists of μ H chain, the non-rearranging VpreB and λ5 surrogate light chain (SLC) proteins and the Ig-α/CD79a and Ig-β/CD79b signaling components. The pre-BCR is a key checkpoint in B cell development to monitor the assembly of a functional Ig H chain and to terminate further H chain rearrangements, thus ensuring that only one functional Ig H chain is synthesized, a phenomenon referred to as allelic exclusion. Pre-BCR expression induces proliferative expansion of cytoplasmic H chain positive pre-B cells and their progression into small resting pre-B cells in which Ig L chain rearrangement occurs (1, 2).

In mice deficient for components of pre-BCR complex, including Ig-α, Ig-β, SLC, or the downstream tyrosine kinase Syk, μ H chain positive pre-B cells are unable to proliferate (Reviewed in Ref. (3). Specifically, the non-Ig-like unique tail of λ5 was shown to be essential for the activation of downstream signal transduction pathways (4). In contrast, disruption of Bruton's tyrosine kinase (Btk) or the adapter protein SH2 domain-containing leukocyte-specific phosphoprotein of 65 kDa (Slp-65, also known as BASH or BLNK) showed that these pre-BCR signaling components are crucially involved in the termination of IL-7 driven expansion of large cycling pre-B cells (5, 6). Slp-65-deficient mice spontaneously develop pre-B cell tumors expressing high levels of pre-BCR on the cell surface (6, 7). Although Btk-deficient mice do not develop tumors, Btk cooperates with Slp-65 as a tumor suppressor, since the incidence of pre-B cell leukemia is significantly higher in Slp-65/Btk double deficient mice, when compared with Slp-65 single deficient mice (8). Using transgenic (Tg) mice expressing the kinase-inactive Btk mutant K430R, we recently showed that Btk exerts its tumor suppressor function independent of its kinase activity (9).

During the transition of large cycling into small resting pre-B cells in the mouse, Btk- or Slp-65-deficient cells show defective downregulation of SLC, the metallopeptidase BP-1 and the sialoglycoprotein CD43, as well as defective upregulation of the adhesion molecule CD2, the IL-2 receptor CD25 and MHC class II (5, 8). Btk-deficient cells also manifest a specific ~3 hours developmental delay within the small pre-B cell compartment. Thus, it appears that in addition to their function in the termination of IL-7 driven proliferation, Btk and Slp-65 are also involved in cellular maturation of cytoplasmic Ig μ^+ pre-B cells.

The role of pre-BCR signaling in the induction of pre-B cell differentiation and Ig L chain rearrangement is controversial (2, 4). The hypothesis that pre-BCR signals are responsible for the redirection of V(D)J recombination activity from the Ig H chain to the L chain loci is based on the observations that Ig H chain surface expression correlates with transcription of unrearranged Ig L chain gene segments. This so-called germline transcription has been implicated in regulation of accessibility of Ig loci to the V(D)J recombinase, because it precedes or coincides with V(D)J recombination (See for review: (2). Second, in the absence of pre-BCR function, e.g. in mice with targeted disruption of Ig H chain, Ig-α, Ig-β, SLC components or Syk, Ig κ locus rearrangement is diminished. Third, expression of an activated Ras transgene induces L chain rearrangement in $J_H^{-/-}$ pro-B cells, which lack the ability to assemble Ig H chain variable regions (10). Fourth, the findings of reduced Ig L chain germline transcription and gene rearrangement in Slp-65-deficient pre-B cells (7, 11) and of reduced λ L chain usage in Btk-deficient mice (12, 13) points to important roles for these signaling molecules in the initiation of Ig L chain rearrangement. Nevertheless, evidence for a direct involvement of pre-BCR signaling in the induction of L chain V(D)J recombination is lacking. On the contrary, it has also been reported that rearrangement and expression of Ig L chain genes can occur without Ig H chain expression. Ig κ transcription and rearrangement is detectable in Ig μ^- pro-B cells (14) and is increased by activation of NF-κB by lipopolysaccharide in transformed pro-B cell lines (15). Removal of IL-7 from cultured pro-B cells from $J_H^{-/-}$ or $\lambda5^{-/-}$ mice, which are incapable of expressing a proper pre-BCR, resulted in the apparent differentiation into cells that transcribe and rearrange Ig L chain loci (16). Moreover, the observations that SLC- and Ig H chain-deficient pre-B cells rearrange Ig L chain at normal frequencies and with normal kinetics, argue for a model in which the initiation of Ig L chain rearrangement is independent of pre-BCR expression (4, 17).

As deficiency for Btk or Slp-65 affects IL-7 responsiveness, cell surface marker expression and Ig L chain rearrangement in pre-B cells, it is possible that Btk and Slp-65 are directly involved in all three processes. Alternatively, Btk and Slp-65 signaling may only be required for the termination of IL-7 driven pre-B cell proliferation and thus the differentiation of large cycling to small resting pre-B cells. The subsequent cell surface marker changes and Ig L chain rearrangements may then be initiated independent of Btk and Slp-65 signaling. In a third model, Btk and Slp-65 also do not regulate pre-B cell differentiation or the induction of Ig L chain locus activation, but they only support survival of small resting pre-B cells.

To distinguish between these possibilities, in the current report we investigated the effects of Btk and Slp-65 deficiency on transcriptional activation of Ig L chain loci in pre-B cells. By crossing Btk/Slp-65 double deficient mice onto

a Bcl-2 Tg background (18), we determined whether providing an extended time window for Ig L chain rearrangement rescued their severe arrest at the pre-B to immature B transition. Finally, we studied Tg mice that express the constitutively active Btk mutant E41K-Btk under the control of the CD19 promoter region (8, 19-21). The CD19 promoter region targets expression of transgenes to all stages of B cell development, including pro-B cells (21). Therefore, the E41K-Btk and E41K-Y223F-Btk mice enabled us to investigate whether Btk signaling has the capacity to prematurely induce cell surface marker modulations and Ig L chain rearrangement in pro-B cells, thus independent of the developmental progression of large cycling into small resting pre-B cells.

MATERIALS AND METHODS

Mice and Genotyping

Mice deficient for Btk (22), RAG-1 (23) or μMT (24) and *E_μ-2-22* Bcl-2 Tg mice (18) were on the C57BL/6 background; Slp-65$^-$ mice (25) were on the Balb/c background. The VH81X-μ Tg mouse strain (26) was a generous gift from J.F. Kearney. E41K-Btk and E41K-Y223F-Btk Tg mice have been described previously (8, 21). The different composite genotypes were on a mixed background and in single experiments littermates were compared. All mice were bred and maintained at the Erasmus MC animal care facility under specific pathogen free conditions. Mice were analyzed at the age of 8-16 weeks.

For mouse genotyping, tail DNA was analyzed by Southern blotting of BamHI digests using a partial human Btk cDNA probe (bp 133-1153), as described previously (21, 22). Alternatively, the presence of Btk transgenes, endogenous mouse Btk WT or Btk KO alleles were evaluated by PCR (8, 27). PCR assays were also used to determine the genotypes for Slp-65 (8), RAG-1 and μMT mice, using standard primers (www.jax.org), and to determine the presence of the *E_μ-2-22* Bcl-2 (12) or VH81X-μ transgenes (forward primer specific for framework I: 5'-CGCGCGGCCGCGTGGAGTCTGGGGGAGGCT-3' and reverse primer specific for the transgene CDR3 region: 5'-CCCAGACATCGAAGTACCAGCTACTACCATG-3' (26).

Flow cytometric analyses

Preparations of single-cell suspensions, flow cytometry procedures and monoclonal antibodies have been described previously (5, 22). For intracellular staining of cytoplasmic proteins, cells were first stained for cell surface markers and subsequently fixed in 2% paraformaldehyde and permeabilized using 0.5% saponin (21). The anti-

SLC hybridoma LM34 ((28) was kindly provided by A. Rolink, University of Basel, Switzerland). Events (1-5 x 10^5) were scored using a FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences, Mountain View, CA).

IL-7 driven BM cultures

Cultures of (pre-)B cells from total BM in the presence of 100 U IL-7 (Sigma), and quantification of IL-7 dependent proliferative responses of total BM cells have been described previously (5).

RNA isolation and analysis

Total BM cell suspensions were depleted of erythrocytes by standard ammonium chloride lysis. For mice that were on the μMT background, cell suspensions were enriched for B-lineage cells by magnetic sorting using anti-B220-coated microbeads (Miltenyi Biotec). Ig L chain-negative pre-B and Ig L chain-positive immature B cell fractions were obtained from IL-7 driven BM cultures. Cells were labeled with biotinylated anti-Igκ (187.1) and anti-Igλ (R26-46) antibodies, incubated with streptavidin-coated microbeads (Miltenyi Biotec), and subjected to cell separation by AutoMACS (Miltenyi Biotec).

Total RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep system (Sigma). Two μg total RNA was used as a template for double stranded cDNA synthesis using reverse transcriptase (Superscript II; Invitrogen) and random hexamer primers. Samples were diluted serially threefold before amplification using PCR primers specific for the germline Ig L chain transcripts $\lambda1^0$, $\lambda2^0$, $\lambda3^0$, Vλ1/2, $\kappa^0$0.8 and $\kappa^0$1.1, functional Vλ1Cλ1 (29, 30) RAG-1, RAG-2 (31) and β-actin. PCR products were separated by standard agarose electrophoresis and visualized by ethidium bromide staining.

RESULTS

Reduced germline λ L chain transcription in the absence of Btk

To investigate the effect of Btk signaling on transcriptional activation of Ig L chain loci, we evaluated Ig L chain germline transcription in pre-B and B cells from wild-type and Btk-deficient mice. Total bone marrow (BM) cell suspensions were cultured in the presence of IL-7 for 5 days and subsequently without IL-7 for 2 days. Removal of IL-7 strongly induces pre-B cells to exit from the cell cycle and to initiate stepwise activation of Ig κ and λ L chain gene rearrangement (17, 29, 30). We purified surface L chain negative pre-B cell and surface L chain positive B cell fractions (Figure 1A), and used RT-PCR to assay expression of germline κ and λ transcripts.

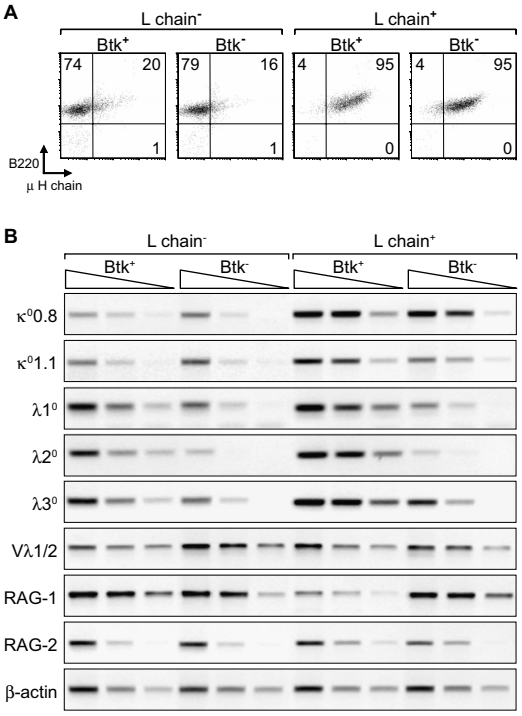

Figure 1. Reduced germline λ L chain transcription in Btk- pre-B and immature B cells.
(A) Flow cytometric characterization of sorted surface Ig- pre-B cell and surface Ig+ immature B cell fractions from IL-7 driven BM cultures. (B) Detection of Ig L chain germline and RAG-1/2 transcripts. Ig κ+λ L chain negative and positive fractions were obtained by magnetic sorting. Total RNA from these cell fractions was reverse transcribed, diluted serially threefold, and used as a template for amplification of the transcripts indicated at the left. Amplification of a β-actin (bottom) was performed as a control. Products were fractionated by gel electrophoresis and detected with ethidium bromide.

As shown in Figure 1B, we found that Btk+ and Btk– pre-B cells expressed comparable levels of κo0.8 and κo1.1 germline transcripts, which are initiated in different regions 5' of Jκ and spliced to the C_κ region (29). In contrast, the expression levels of germline λ transcripts were significantly reduced in Btk– pre-B cells, as compared with Btk+ pre-B cells. In particular the levels of λ1o and λ2o transcripts, which initiate 5' of the $J_\lambda 1$ and $J_\lambda 2$ region and are spliced to $C_\lambda 1$ and $C_\lambda 2$, respectively (29), were reduced in Btk-deficient pre-B cells (Figure 1B). Interestingly, this effect of Btk appeared to be specific for the germline transcripts of the λ J-C cluster, because transcripts of the Vλregion were present at similar levels in Btk+ and Btk- pre-B cells (Figure 1B). Although successful V_L-to-J_L rearrangement results in loss of germline transcription on productive alleles, transcription of unrearranged L chain alleles continues in mature B cells (32). We therefore also analyzed surface Ig+ B cells and observed a

specific reduction in the expression of λ1° and λ2° transcripts in Btk-deficient B cells, while Vλ, κ°0.8 and κ°1.1 transcripts were present at normal levels (Figure 1B).

Developmental progression of large to small pre-B cells is also accompanied by the upregulation of transcription of the recombination activating genes RAG-1 and RAG-2 (1). We observed similar levels of RAG-1/2 transcripts in Btk^+ and Btk^- pre-B cells (Figure 1B), indicating that Btk is not involved in the initiation of RAG transcription in pre-B cells. Interestingly, RAG-1 expression was downregulated in Btk^+ Ig^+ B cells, but not in Btk-deficient Ig^+ B cells (Figure 1B), suggesting that termination of RAG-1 expression upon productive L chain recombination is Btk-dependent.

Taken together, the finding of a specific reduction in the levels of germline λ1° and λ2° transcripts in Btk-deficient (pre-)B cell fractions indicates that Btk signals are needed for the activation of the Ig λ L chain locus for recombination by opening of the λ J-C clusters.

Synergistic roles of Btk and Slp-65 in the induction of L chain rearrangement

The analysis of germline transcription as a marker for L chain locus accessibility in vivo is complicated by the fact that ongoing V_L-to-J_L rearrangement will destroy its germline configuration and thereby result in loss of germline transcription. Therefore, we bred Btk and Slp-65 single and double mutant mice to RAG-1 deficient mice that carry the Ig H chain transgene V_H81X (26). In this background, B cell progenitors progress to the pre-B cell stage because of the presence of the pre-rearranged Ig H chain, which ensures pre-BCR expression and cellular proliferation (33). However, the inactivation of RAG-1 precludes any L chain gene rearrangement and cells are arrested at the small pre-B cell stage (Figure 2A).

Detailed flow cytometric analyses of the four groups of mice (WT, Btk-deficient, Slp-65-deficient and Btk/Slp-65 double deficient mice) on the RAG-$1^{-/-}$ V_H81X Tg background revealed that the pre-B cells exhibited elevated expression of CD43 and SLC and reduced expression of CD2, CD25 and MHC Class II, whereby the defects were most pronounced in the Btk/Slp-65 double mutant (Figure 2B). These findings show that the previously reported aberrant phenotypes of Btk and Slp-65 single or double deficient pre-B cells, which reflect their impaired cellular maturation (5, 8), were preserved on the RAG-$1^{-/-}$ V_H81X Tg background. We additionally found that downregulation of IL-7R and c-kit expression on the surface of pre-B cells was impaired in the absence of Btk and Slp-65 (Figure 2B).

From the four groups of mice, BM cell suspensions were analyzed for the expression of Ig κ and λ L chain germline transcripts. In RT-PCR experiments, we observed a specific reduction in the expression of λ1° and λ2° transcripts in the absence of either Btk or Slp-65. Expression levels of λ3° were not significantly

affected by the absence of Btk or Slp-65 (Figure 3). We detected a modest reduction in the expression levels of the κ°0.8 and κ°1.1 germline transcripts in Slp-65 deficient pre-B cells, when compared to wild-type or Btk-deficient mice (Figure 3). In contrast, in the Btk/Slp-65 double deficient pre-B cells the levels of both κ and λ germline transcripts were severely reduced.

The observed differences between the four groups of mice did not appear to result from differences in the ratio between large cycling and small resting pre-B cells, as the forward scatter profiles of the pre-B cell populations in these mice were comparable (Figure 2B). Consistent with this, similar differences in surface marker expression profiles and Ig κ and λ germline transcription between the four groups of RAG-1$^{-/-}$ V_H81X Tg mice were observed in *in vitro* IL-7 driven BM cultures, in which developmental progression from large cycling into small resting pre-B cells was induced by IL-7 withdrawal (data not shown).

The finding that in the absence of either Btk or Slp-65 λ germline transcription is reduced while κ germline transcription is not dramatically affected indicates differential regulation of κ and λ locus activation. Therefore, either (i) the opening of the λ L chain J-C clusters is essentially dependent on Btk- and Slp-65-mediated signaling pathways, or alternatively, (ii) in the absence of these signaling proteins development does not efficiently progress to a stage critical for λ activation.

Defective Ig L chain opening in Btk/Slp-65-deficient pre-B cells is not due to their reduced survival

Interestingly, the Btk/Slp-65 dependency of κ and λ germline transcription parallels the order of transcriptional activation of the L chain loci (29, 30): the κ locus and the λ V cluster, which are only marginally affected by the absence of Btk or Slp-65, open early in B cell development, and the λ J-C cluster, which is severely affected by the absence of one of the two signaling molecules, opens late in B cell development. Thus, our findings can also be explained by a model in which Btk and Slp-65 signals are mainly essential for the survival of small resting pre-B cells that initiate Ig L chain rearrangement. The almost complete arrest of B cell development in Btk/Slp-65 double deficient mice at the pre-B cell stage (8, 34), and the absence of germline L chain transcripts, would in this model reflect the short life span of Btk/Slp-65 double deficient pre-B cells.

To investigate this issue, we determined whether enforced expression of the anti-apoptotic Bcl-2 gene, which is thought to provide an extended time window per cell for Ig L chain rearrangement (35), is able to rescue the severe block in B cell development in Btk/Slp-65 double deficient mice. We crossed Btk/Slp-65 double deficient mice with Eμ-Bcl-2 Tg mice (18) and investigated the size of the

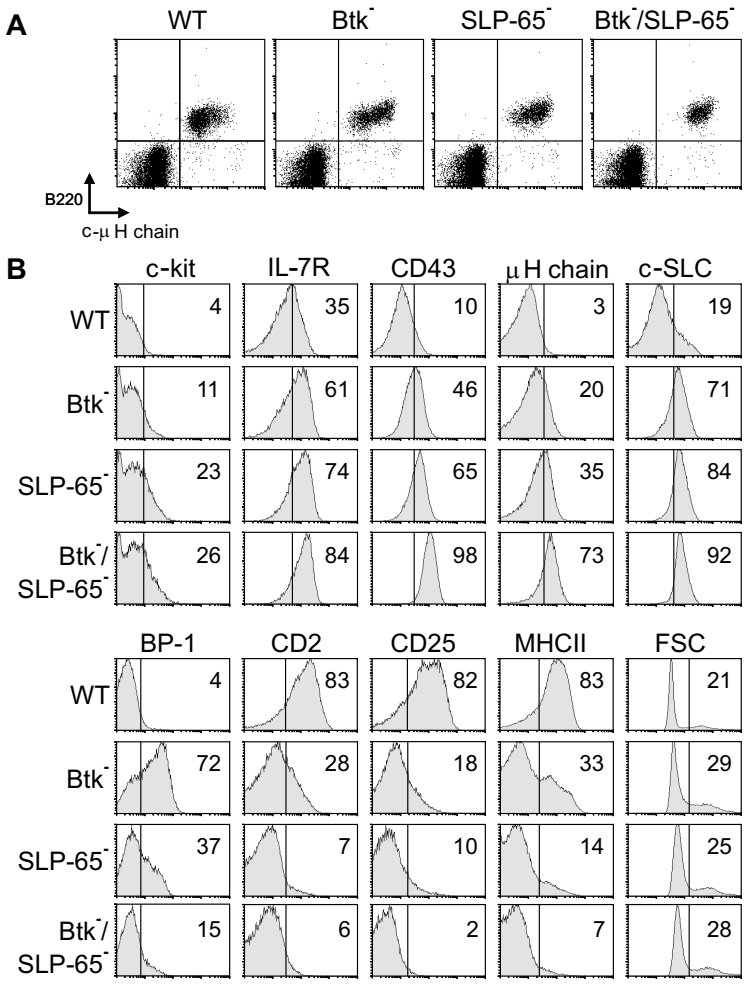

Figure 2. Defective pre-B cell maturation in Btk and Slp-65 deficient pre-B cells.
(A) Flow cytometric characterization of the B cell population from the indicated mice on a RAG-1$^{-/-}$ VH81X Tg background. BM lymphoid cells were gated on the basis of forward and side scatter characteristics. The expression profile of B220 versus cytoplasmic μ H chain is plotted. (B) Expression of markers that are normally downregulated or induced during pre-B cell differentiation. B220^{+}cy-μ^{+} pre-B cells were gated and the expression data of the indicated markers and forward scatter (FSC) are shown as histograms. Numbers indicate the percentage of positive cells. Plots are representative for four mice of each genotype.

immature B cell population by flow cytometry. We found that in the presence of the Bcl-2 transgene, the size of the immature B cell population was still severely reduced (Figure 4). Moreover, analysis of cytoplasmic μ H chain-positive pre-B cells indicated that increasing survival of Btk/Slp-65 double deficient pre-B cells did not rescue the defective downregulation of SLC expression in these cells (Figure 4).

Likewise, the Bcl-2 transgene failed to correct the modulation of the CD43, CD2 and CD25 surface markers or the (not shown). We verified that Tg Bcl-2 diminished apoptosis in Btk/Slp-65-deficient pre-B cells as effective as in wild-type pre-B cells, by analysis of cell survival of cultured BM pre-B cells upon IL-7 withdrawl (Figure 4B).

Collectively, these findings demonstrate that protection of Btk/Slp-65 double deficient pre-B cells from apoptosis does not result in the termination of SLC expression or the initiation of L chain rearrangements. Therefore, we conclude that defective Ig L chain opening in Btk- and Slp-65-deficient pre-B cells is not due to reduced survival.

E41K-Btk induces cell surface marker changes in μ- pro-B cells

Next, we aimed to investigate whether Btk signaling has the capacity to induce cell surface marker modulation and Ig L chain transcription and rearrangements, independent of the development progression of large to small pre-B cells. We employed Tg mice expressing the constitutively active E41K-Btk mutant under the control of the CD19 promoter region, which were crossed onto the Btk-deficient background.

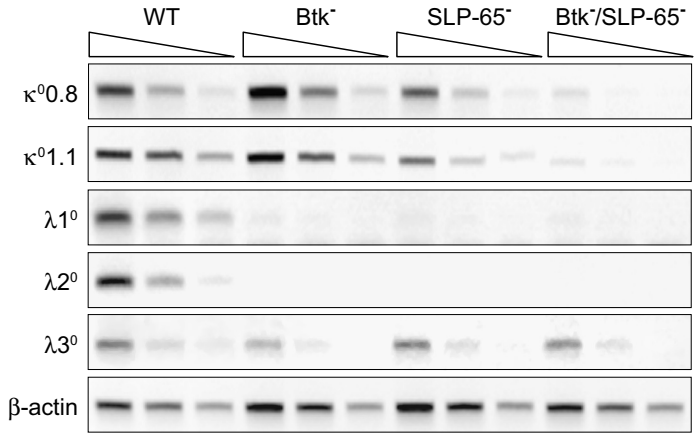

Figure 3. Defective germline transcription of Ig L chain loci in Btk and Slp-65 deficient pre-B cells. Expression of the indicated Ig L chain germline transcripts was analyzed by RT-PCR. Total RNA from BM fractions of the indicated four groups of mice was reverse transcribed, diluted serially threefold, and used as a template for amplification of the transcripts indicated at the left. Amplification of β-actin (bottom) was performed as a control. Products were fractionated by gel electrophoresis and detected with ethidium bromide. Data shown are representative for 3 mice examined in each group.

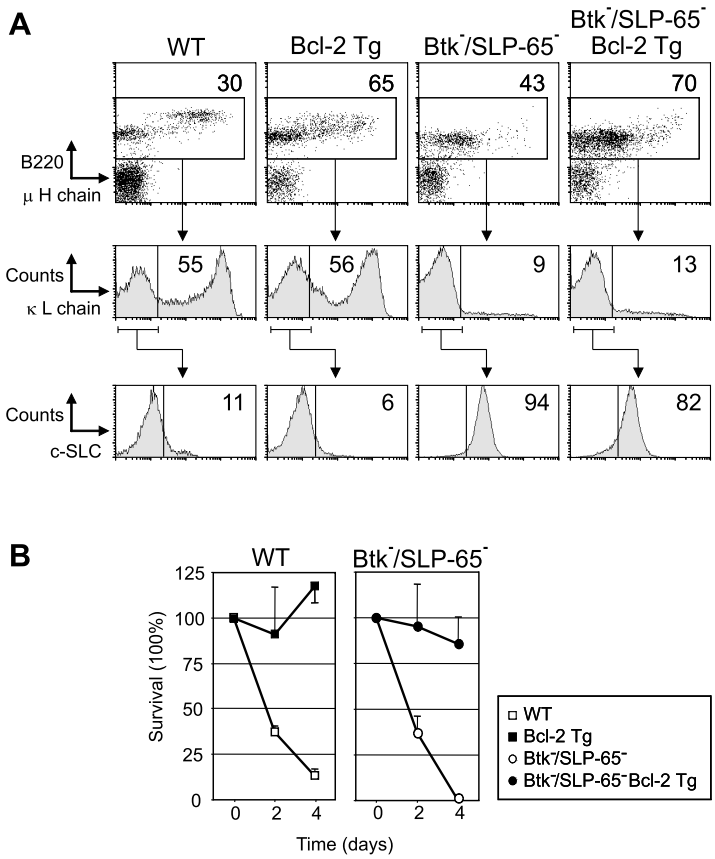

Figure 4. Bcl-2 expression does not rescue the pre-B cell arrest in Btk/Slp-65 double deficient mice.
(A) Flow cytometric analysis of BM lymphoid cells from the indicated mouse groups. Expression profiles of B220 and μ H chain are displayed as dot plots. Total $B220^+$ B-lineage fractions were gated and analyzed for the expression of κ L chain on the cell surface. Next, pro/pre-B cell fractions ($B220^+$, κ^-) were analyzed for cytoplasmic expression of SLC. Data are shown as histograms, whereby numbers indicate the percentage of positive cells. Plots are representative for 3-8 mice of each genotype. (B) *In vitro* survival after IL-7 withdrawal of BM cultures from the indicated mouse genotypes.

Although expression of transgenic E41K-Btk resulted in a dose-dependent deletion of B cells at the transition of IgM^{low} to IgM^{high} $B220^{low}$ immature B cell stage in the BM (8, 21), it nevertheless completely corrected the impaired modulation of pre-B cell surface markers in Btk-deficient mice (Figure 5A). In vitro BM culture experiments showed that expression of E41K-Btk increased IL-7 responsiveness of pro-B cells and reduced IL-7 responsiveness of pre-B cells (Figure 5). E41K-Btk did not increase IL-7Rα chain transcription or surface expression in pro-B cells (data

not shown), and therefore it appears that this effect of E41K-Btk is based on the functional intersection of pre-BCR and IL-7R signaling pathways that induce pre-B cell proliferation (36).

To examine the capacity E41K-Btk to mimic pre-BCR activation and consequently signal for premature cellular maturation of cytoplasmic μ^- pro-B cells *in vivo*, we characterized the $CD19^+B220^{low}$ cytoplasmic μ^- pro-B cell fractions in the BM from Btk^+, Btk^- and E41K-Btk Tg mice (Figure 6A). In Btk^+ and Btk^- mice, almost all $CD19^+B220^+$ cytoplasmic μ^- pro-B cells contained SLC. In contrast, in E41K-Btk Tg mice ~40% of the pro-B cells did not express detectable levels of SLC in the cytoplasm. When compared with Btk^+ or Btk^- mice, the pro-B cell fraction from E41K-Btk Tg mice contained a significantly larger subpopulation of cells expressing CD2, CD25 and MHC class II. In all groups of mice, pro-B cells were largely $CD43^+$. Also E41K-Y223F-Btk pro-B cells manifested reduced expression of SLC and significant induction of the pre-B cell markers CD2, CD25 and MHC class II (data not shown).

To exclude the possibility that the analyses of pro-B cells are confounded by Ig μ H chain positive pre-B cells that have subsequently lost or downregulated Ig μ H chain expression, we crossed Btk^+, Btk^-, and E41K-Btk Tg mice onto the μMT background (24). In μMT mice, cells are arrested at the pro-B cell stage, as the membrane exon of the Ig H chain μ constant region is disrupted and therefore Ig μ H chain cannot be expressed on the cell surface. Also on the μMT background, we found that expression of E41K-Btk resulted in downregulation of SLC and the induction of CD2, CD25 and MHC class II in cytoplasmic μ H chain negative pro-B cells (Figure 6B).

The phenotypic marker profiles induced by E41K-Btk and E41K-Y223F-Btk in pro-B cells reflected the phenotypic changes that are normally associated with developmental progression of large cycling into small resting pre-B cells. Therefore, we conclude that E41K-Btk expression in pro-B cells mimics pre-BCR signaling, and induces cell surface marker changes in these cells, even in the absence of functional Ig μ H chain proteins.

E41K-Btk induces L chain rearrangement in μ- pro-B cells

Next, we investigated the effect of Tg E41K-Btk expression on Ig L chain rearrangement in pro-B cells, both on the wild-type background and on the μMT background. Whereas in wild-type mice <5% of $CD19^+B220^{low}$ cytoplasmic μ H chain negative pro-B cells express detectable levels of Ig κ L chain proteins in their cytoplasm, this proportion was significantly increased to ~10-15% of pro-B cells in E41K-Btk Tg mice (Figure 6AB). In the total population of E41K-Btk μMT $CD19^+B220^+$ pro-B cells approximately equal proportions of cells exclusively expressed Ig H or Ig L chains, whereas the fraction of H and L chain double positive pro-B cells was low (Figure 6C). This finding indicates that in E41K-Btk μMT pro-B cells the majority of functional κ L chain rearrangements are performed in the

absence of a productive H chain rearrangement. As shown in Figure 6D, Ig κ L chain expression was mainly found in those pro-B cells in which expression of pre-B cell specific markers was induced. Moreover, the fractions of pro-B cells in which SLC expression was downregulated and CD2, CD25 or MHC class II were induced were largely overlapping (Figure 6D).

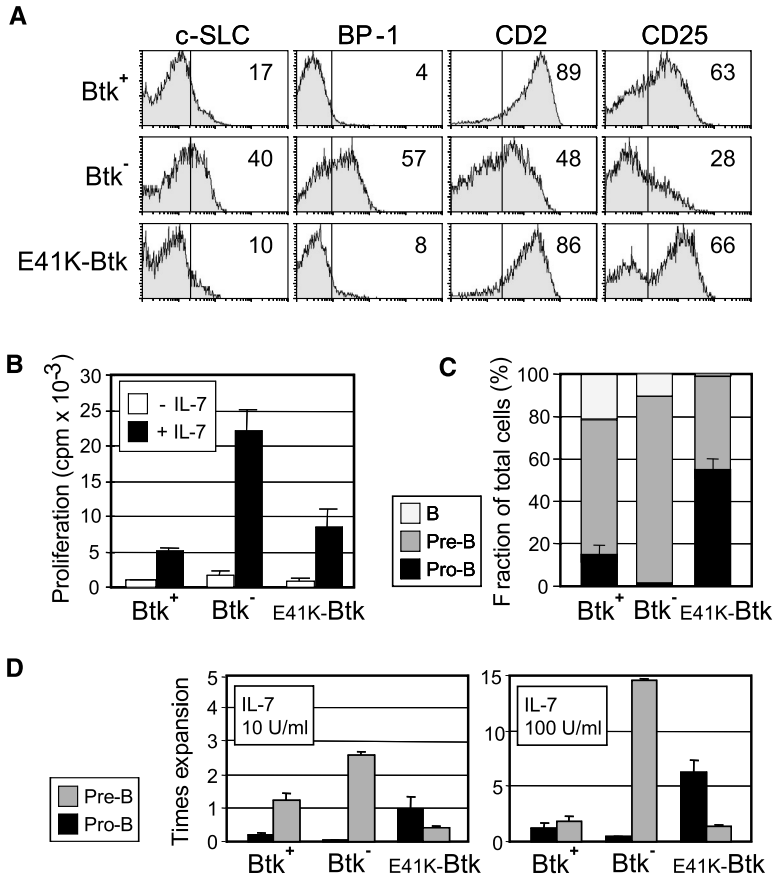

Figure 5. The effect of E41K-Btk expression on pre-B cells.
(A) E41K-Btk expression corrects the impaired modulation of cell surface markers in Btk-deficient pre-B cells. Cytoplasmic SLC and CD2 expression was investigated in pre-B cell fractions ($B220^+IgM^-$, c-μ H $chain^+$) whereas BP-1 and CD25 were analyzed in total pro-B/pre-B cell fractions ($B220^+IgM^-$) from the indicated mice. Data shown are representative for 6-11 animals per group. (B) E41K-Btk expression corrects increased IL-7 responsiveness of Btk-deficient B-lineage cells. Analysis of proliferative responses to IL-7 of total BM fractions from the indicated mice, as determined by [^{3}H]-thymidine incorporation after 5 days of culture in the presence or absence of 100 U/ml IL-7. Error bars are SEM from 3-7 mice per group. (C) Increased proportions of pro-B cells in BM cultures from Btk^{Act} Tg mice. The distribution profile over the indicated B-lineage subpopulations of IL-7 driven total BM cultures of the indicated mice after 5 days of culture in the presence of 100 U/ml IL-7. Error bars are the SEM values for pro-B cells from 3-7 mice per group. (D) Constitutively active Btk supports expansion of pro-B cells in IL-7 driven BM cultures. The bars indicate the relative expansion of $c\text{-}\mu^-$ pro-B and $c\text{-}\mu^+$ pre-B cells during culture with 10 or 100 U/ml IL-7 at day 5, as compared with the pro-B and pre-B cell numbers at the start of the culture, which were set to one.

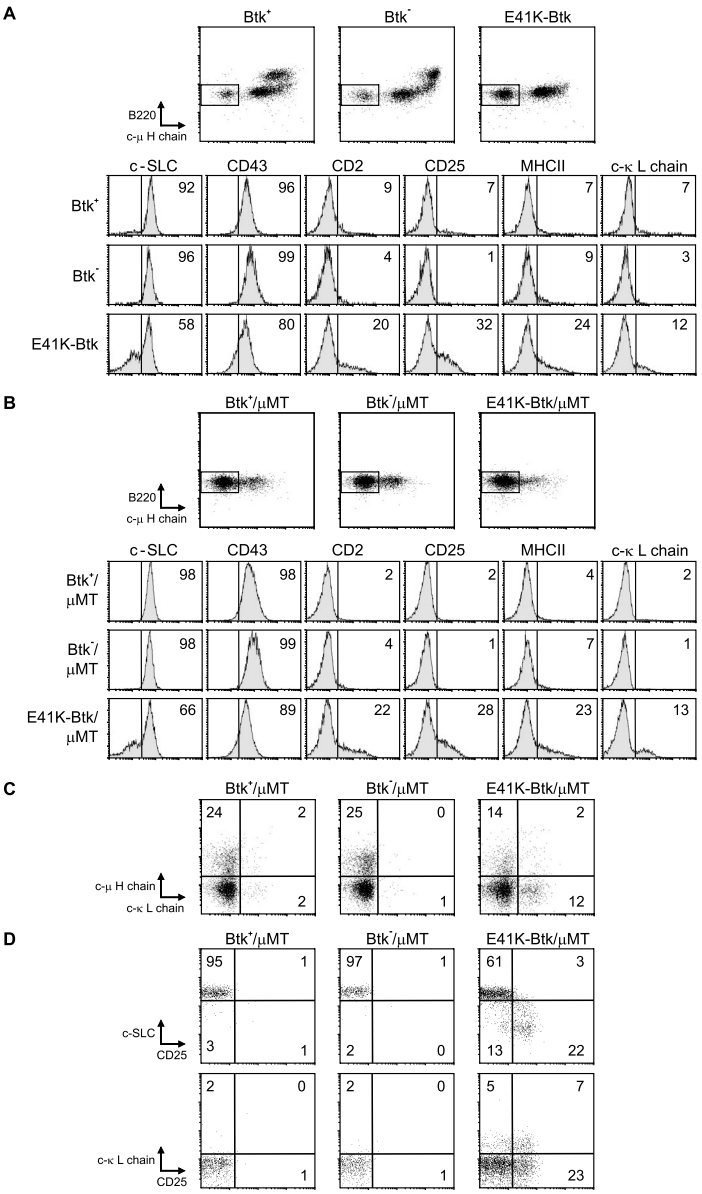

Figure 6, E41K-Btk induces phenotypic changes in cytoplasmic μ⁻ pro-B cells.
Flow cytometric expression profiles of the indicated markers in pro-B cells of Btk⁺, Btk⁻ and E41K-Btk mice on a wild type (A) or a μMT background (B, C, D). Flow cytometric analysis of surface B220 and cytoplasmic μ H chain expression on total CD19⁺ B-lineage cells in the BM (A, B, upper parts). CD19⁺B220⁺cμ⁻ pro-B cells were gated and analyzed for the expression of cytoplasmic SLC, surface CD43, CD2, CD25 and MHC class II, and cytoplasmic κ L chain (A, B, lower parts). The results are displayed as filled histograms in which the percentages of positive cells are indicated. (C) Expression profiles of cytoplasmic μ H chain and κ L chain in B220⁺CD19⁺ BM fractions of the indicated mice. (D) CD19⁺B220⁺cμ⁻ pro-B cells from the indicated mice were gated and analyzed for the expression of CD25, SLC and κ L chain. Data shown are representative of 4-8 animals examined within each group.

Using similar flow cytometric techniques we were unable to detect Ig λ L chain protein in E41K-Btk pro-B cells. However, when we performed RT-PCR analyses using primers specific for Vλ1 and Cλ1, rearranged λ L chain transcripts were readily detectable in purified E41K-Btk μMT pro-B cell fractions (Figure 7A). The levels of the Vλ1Cλ1 transcripts in E41K-Btk μMT pro-B cells were increased, when compared with control Btk$^+$ or Btk$^-$ μMT pro-B cell fractions. Consistent with the induction of L chain rearrangement by the E41K-Btk transgene, increased levels of κ^o0.8, κ^o1.1, $\lambda1^o$, and $\lambda2^o$ germline transcripts were detected in E41K-Btk μMT pro-B cells (Figure 7B).

Taken together, the finding of germline transcription and recombination of Ig L chain gene segments in E41K-Btk pro-B cells, which do not express functional μ H chain proteins, demonstrates that E41K-Btk has the capacity to transmit signals that induce Ig κ and λ L chain rearrangement.

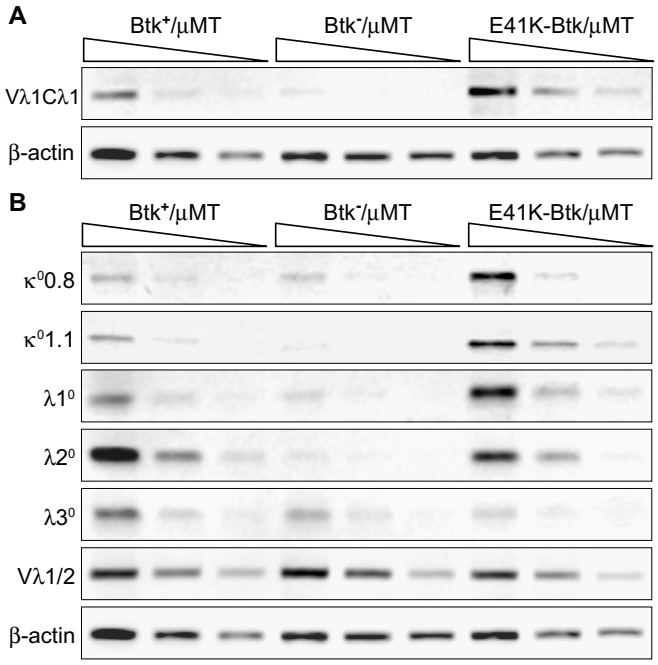

Figure 7. E41K-Btk induces L chain germline transcription and gene rearrangement in pro-B cells. RT-PCR analysis for the detection of λ L chain transcripts from rearranged Vλ1-Cλ1 segments (A) and κ and λ L chain germline transcripts (B) in Btk$^+$, Btk$^-$ and E41K-Btk mice on a μMT background. Total RNA from BM fraction, enriched for B220$^+$ cells, was reverse transcribed, diluted serially threefold, and used as a template for amplification of the indicated transcripts. Amplification of β-actin (bottom) was performed as a control. Products were fractionated by gel electrophoresis and detected with ethidium bromide. Data shown are representative for 3 mice examined in each group.

Cooperation of IL-7 withdrawal and pre-BCR signaling

Pre-B cell receptor signaling mediates selective response to IL-7 at the pro-B to pre-B cell transition via an ERK/MAP kinase-dependent pathway (36). It has however been reported that removal of IL-7 induces Ig L chain rearrangement in cultured pro-B cells, even in the absence of functional Ig H chain protein (16). To investigate cooperative effects of pre-BCR signaling and IL-7 withdrawal, we cultured total BM cells from Btk$^+$, Btk$^-$ and E41K-Btk μMT mice in the presence of IL-7 for 5 days and subsequently without IL-7 for 2 days. Consistent with reported findings (16), we observed that the Btk$^+$ μMT pro-B cell cultures contained cytoplasmic κ L chain-positive cells (~20%, Figure 8). However, the expression of Ig κ L chain was partially dependent on Btk signaling, because in Btk$^-$ μMT pro-B cell cultures the proportions of cytoplasmic κ L chain-positive cells were consistently lower (~10%, Figure 8). Likewise, the levels of germline $\lambda^0$1 and $\lambda^0$2 transcripts were severely decreased in Btk$^-$ μMT pro-B cell cultures, when compared to Btk$^+$ μMT pro-B cell cultures (data not shown). In E41K-Btk μMT pro-B cell cultures the proportions of Ig κ$^+$ cells were similar to those found in Btk$^+$ μMT cultures, and levels of $\lambda^0$1 and $\lambda^0$2 transcripts were slightly increased (Figure 8 and data not shown).

In these IL-7 driven pro-B cell cultures, Btk activity had a more dramatic effect on the expression of the developmentally regulated markers SLC, CD2 and CD25 (Figure 8). In E41K-Btk μMT pro-B cell cultures, SLC was expressed in only ~16% of cells, as compared with ~42% and ~91% in Btk$^+$ and Btk$^-$ μMT pro-B cell cultures, respectively. Likewise, CD2 and CD25 expression was induced in ~70% of the cells in E41K-Btk μMT pro-B cell cultures, whereas expression of these markers was very low in Btk$^-$ μMT pro-B cells (Figure 8).

In summary, these findings indicate that in pro-B cell cultures removal of IL-7 and Btk signaling have cooperative effects on initiation of L chain rearrangement, but modulation of SLC, CD2 and CD25 expression is essentially dependent on Btk signaling.

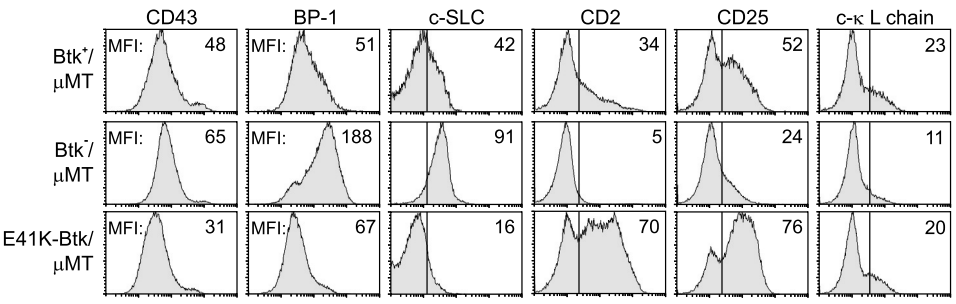

Figure 8. Cooperative effects of pre-BCR signaling and IL-7 withdrawal.
Flow cytometric expression profiles of the indicated markers in pro-B cells of Btk$^+$, Btk$^-$ and E41K-Btk mice on μMT background that were cultured in the presence of IL-7 for 5 days and for 2 additional days without IL-7. CD19$^+$B220$^+$ pro-B cells were gated and analyzed for the expression of the indicated markers. Data shown are representative of 4-8 animals examined within each group.

DISCUSSION

The pre-BCR checkpoint monitors functional Ig H chain rearrangement and triggers clonal expansion and developmental progression of Ig μ^+ pre-B cells (3). Hereby the downstream signaling components Slp-65 and Btk serve as feedback inhibitors that limit pre-B cell proliferation. Mice deficient for Slp-65 or Btk show a partial block at the large cycling pre-B cell stage, while an almost complete arrest is observed in Slp-65/Btk double-mutant mice (6-8, 11-13, 25, 27, 34, 37). These findings suggest that Btk and Slp-65 are involved in cellular maturation of pre-B cells and in the initiation of Ig L chain recombination, but evidence for their direct involvement in these processes is lacking. A possibility remains that Btk and Slp-65 are only required to terminate pre-B cell expansion and to advance pre-B cells to a stage at which Ig L chain rearrangement may be initiated in a Btk/Slp-65 independent fashion. Moreover, defective initiation of L chain rearrangements in Btk/Slp-65-deficient pre-B cells may also be explained by an essential role of these molecules in the survival of small resting pre-B cells.

In this report, we show (i) that protection of Btk/Slp-65 double deficient pre-B cells from apoptosis did not enhance modulation of pre-B cell surface marker expression or L chain rearrangement, and (ii) that premature expression of the E41K-Btk mutant E41K induced the pre-B cell developmental program in pro-B cells, including germline transcription and productive rearrangement of Ig L chain and modulation of cell surface markers. Therefore, we conclude that activated Btk does not only act to terminate proliferation or to increase survival of pre-B cells, but has the intrinsic capacity to signal for cellular maturation and the initiation of L chain rearrangement. It is highly unlikely that E41K-Btk signals for cellular maturation and Ig L chain recombination by inducing cell cycle arrest in pro-B cells, because expression of E41K-Btk increased IL-7 responsiveness in pro-B cells (Figure 5). Although we observed cooperation of IL-7 withdrawal and pre-BCR signaling *in vitro* (Figure 8), modulation of SLC, CD2 and CD25 expression was essentially dependent on Btk signaling and was not induced by IL-7 withdrawal.

The E41K gain-of-function mutation in the pleckstrin homology (PH) domain of Btk induces transformation of 3T3 fibroblasts and this capacity is augmented by mutation of Y223, the main autophosphorylation site of Btk (19, 20). The E41K and E41K-Y223F mutants are associated with enhanced Btk membrane localization, activation and calcium signaling in cultured cell lines (19, 20, 38, 39). Several lines of evidence show that these mutants also represent activated forms of Btk in murine B cells. Tg expression of E41K-Btk (i) enhances blast formation of splenic B cells in culture (40), (ii) drives peripheral B cells efficiently into IgM plasma cell differentiation *in vivo* (21), and (iii) induces the expression of the early

activation antigen CD69 on splenic B cells (R.W.H., unpublished). In addition, Tg E41K-Y223F-Btk manifests increased tyrosine phosphorylation in resting mature splenic B cells and has the ability to prevent tumor formation in Slp-65$^-$ pre-B cells (8). This observation indicates that E41K-Y223F-Btk may not only mimic activation of Btk, but may also transmit signals that substitute for Slp-65 function.

In this report, we demonstrated that E41K-Btk was able to rescue the defects in Btk$^-$ pre-B cells, and to induce premature developmental progression and Ig L chain recombination and expression in Ig H chain negative pro-B cells. Similar effects were also found for the E41K-Y223F Btk mutant (R.W.H., unpublished). We did not detect any *in vivo* additional effects of the Y223F mutation on the phenotype of E41K-Btk mice, further supporting our previous conclusion that Y223 autophosphorylation-dependent interactions are not essential for Btk function during B cell development (27).

Approximately 10-15% of all E41K-Btk pro-B cells expressed detectable levels of κ L chain proteins in the cytoplasm. Since for each κ L chain allele 1 out of 3 L chain rearrangements is expected to be productive (and assuming that in the absence of Ig μ H chain protein κ^+ and κ^- pro-B cells do not manifest differences in survival, expansion or differentiation capacities), we conclude that V-to-J rearrangements may be present in up to ~30% of the total pro-B cell fraction in E41K-Btk mice.

Our experiments do not discriminate whether signals transmitted by Btk/Slp-65 directly induce opening of the Ig L chain loci for recombination, or whether these signals only act indirectly by inducing the pre-B cell differentiation program. However, Ig L chain locus activation and pre-B cell maturation are probably closely connected, as transcription factors such as E2A, PU.1, Spi-B, IRF-4/8, are implicated both in the expression of *e.g.* MHC class II or SLC and in opening of Ig L chain loci (41-46). This would be consistent with recent quantitative analyses of κ 3' enhancer chromatin structure and protein association, which indicate that this enhancer is activated progressively through multiple steps as cells mature (47). The transcriptional enhancers within the κ and λ L chain loci contain binding sites for the transcription factors E2A and EBF. Interestingly, transient transfection of E2A and EBF transcription factors into a non-lymphoid cell line was sufficient to activate V(D)J recombination at the Ig κ and λ loci, respectively (42). However, these transcription factors also activate transcription of the SLC genes λ5 and VpreB (41), which are silenced when Ig L chain rearrangement is initiated. Moreover, expression of Tg E41K-Btk in pro-B cells concurrently induced activation of Ig κ and λ L chain transcription and downregulation of SLC expression (Figures 6 and 7). In RT-PCR analyses, expression of E41K-Btk in pro-B cells did not appear to result in a significant increase in the expression levels of EBF or E2A (R.W.H., unpublished

results). It remains however possible that Btk or Slp-65 are involved in the induction of EBF or E2A activity by stage-specific regulation of protein stability or expression of partner molecules that interfere with the action of these transcription factors, such as Id proteins or Ikaros (46). Further experiments are required to investigate if Btk/Slp-65 signaling pathways converge on EBF and E2A.

Recently, the transcription factors IRF-4/8 and Spi-B were shown to be important for downregulation of the λ5 and Vpre-B genes and for the induction of germ-line Ig κ transcription in pre-B cells (43, 44). However, Tg expression of E41K-Btk and E41K-Y223F-Btk in pro-B cells did not appear to result in a significant increase in the expression levels of IRF-4 or Spi-B transcripts (R.W.H., unpublished results), indicating that these transcription factors may not function as nuclear effectors of Btk/Slp-65 signaling. This would be supported by recent findings demonstrating that changes in PU.1 and IRF-4 association during κ locus activation are independent of changes in protein expression levels, but rather reflect changes in chromatin accessibility and histone acetylation (47). Already in pro-B cells intermediate levels of the transcriptional activators PU.1 and IRF-4 associate with the κ 3' enhancer, which is moderately accessible at this stage. Therefore, it is likely that pre-BCR signaling results in Ig L chain chromatin structure changes, which are linked to epigenetic marking and affect transcription factor binding site occupancy in the 3' κ enhancer (47). Likewise, it is possible that pre-BCR signaling may downregulate the expression of the SLC chain genes by affecting the epigenetic status of the recently identified regulatory region within the λ5-VpreB locus, which is associated with a tightly localized epigenetic mark of H3 acetylation and histone H3 K4 methylation (46).

At the immature B cell stage, RAG transcripts are downregulated as the cells acquire higher levels of surface IgM (1, 16)**.** It was recently shown that interruption of basal IgM signaling in immature B cells leads to the induction of the recombination machinery and new endogenous L chain rearrangements (48). Our finding that Btk$^-$ B cells fail to efficiently downregulate RAG-1 expression during the pre-B to immature B cell transition in culture (Figure 1B), implicate Btk signaling in the termination of V(D)J recombination activity upon productive Ig L chain rearrangement in immature B cells. This would also explain the finding that peripheral B cells from XLA patients, which have mutations in the Btk gene, show a distinct antibody repertoire consistent with extensive secondary V(D)J recombination (49). It is attractive to speculate that Btk may in this context act through activation of nuclear factor NF-κB, as NF-κB-deficient cells overexpress RAG and undergo exaggerated receptor editing (50) and Btk has been shown to be required for NF-κB activation in response to B cell receptor engagement in mature B cells (51, 52). The role for Btk signaling in downregulating RAG1 expression is apparently limited to the

immature B cell stage, because Btk^+ and Btk^- cultured pre-B cells expressed similar levels of RAG-1 transcripts (Figure 1) and expression of the E41K-Btk transgene did not affect the levels of RAG-1/2 transcripts in pro-B cells (unpublished data).

In summary, we conclude that expression of E41K-Btk, which mimics pre-BCR signaling, results (i) in the modulation of cell surface markers that signify pre-B cell maturation, and (ii) in the induction and successful completion of Ig L chain rearrangements in pro-B cells. It is therefore unlikely that in pre-B cells Btk/Slp-65 signaling is only required for the termination of pre-B cell expansion or for survival of small resting pre-B cells. Rather, we conclude that Btk and Slp-65 transmit signals that induce cellular maturation and Ig L chain rearrangement in pre-B cells.

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CHAPTER 3

Bruton's tyrosine kinase cooperates with the B-cell linker protein Slp-65 as a tumor suppressor in pre-B cells

Bruton's tyrosine kinase cooperates with the B-cell linker protein Slp-65 as a tumor suppressor in pre-B cells

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SUMMARY

Expression of the pre-B cell receptor (pre-BCR) leads to activation of the adapter molecule Slp-65 and the cytoplasmic kinase Btk. Mice deficient for one of these signaling proteins have an incomplete block in B cell development at the stage of large cycling pre-BCR^{+}CD43^{+} pre-B cells. Our recent findings of defective Slp-65 expression in ~50% of childhood pre-B acute lymphoblastic leukemias and spontaneous pre-B cell lymphoma development in Slp-65$^{-/-}$ mice demonstrate that Slp-65 acts as a tumor suppressor. To investigate cooperation between Btk and Slp-65, we characterized the pre-B cell compartment in single and double mutant mice, and found that the two proteins have a synergistic role in the developmental progression of large cycling into small resting pre-B cells. We show that Btk/Slp-65 double mutant mice have a dramatically increased pre-B cell tumor incidence (~75% at 16 weeks of age), as compared to Slp-65 single deficient mice (<10%). These findings demonstrate that Btk cooperates with the Slp-65 as a tumor suppressor in pre-B cells. Furthermore, transgenic low-level expression of a constitutive active form of Btk, the E41K-Y223F mutant, prevented tumor formation in Btk/Slp-65 double mutant mice, indicating that constitutive active Btk can substitute for Slp-65 as a tumor suppressor.

INTRODUCTION

B lymphocytes develop in the bone marrow (BM) through distinct stages that are characterized by differential expression of various cell surface markers and the ordered rearrangement of immunoglobulin (Ig) heavy (H) and light (L) chain gene segments (1, 2). In pro-B cells, productive V(D)J recombination of the Ig H chain gene leads to surface expression of the pre-B cell receptor (pre-BCR), which acts as a checkpoint in early B cell development to monitor the expression of a functional Ig μ H chain. The pre-BCR is transiently expressed on the cell surface and is essential for the proliferative expansion of cytoplasmic μ H chain positive pre-B cells and for the induction of developmental progression into small pre-B cells in which Ig L chain rearrangement occurs (1, 2). The pre-BCR complex is comprised of μ H chain, the non-rearranging VpreB and λ5 surrogate light chain (SLC) proteins and the Ig-α/CD79a and Ig-β/CD79b signaling components. The importance of the pre-BCR checkpoint function is evidenced by an arrest of B cell differentiation at the pro-B to pre-B cell transition both in agammaglobulinemia patients and in mice with mutations in any of these pre-BCR components (1-3).

Cell surface expression of the pre-BCR in the absence of a ligand appears to be sufficient to activate downstream signaling pathways (4, 5). This activation involves the formation of a lipid raft-associated calcium signaling module, composed of the tyrosine phosphorylated signaling molecules Lyn, Syk, Slp-65 (also known as B cell linker protein BLNK or BASH), phosphoinositide 3-kinase (PI3K), Bruton's tyrosine kinase (Btk), Vav and Phospholipase Cγ2 (PLCγ2). As a result, the activation of PLCγ2 induces calcium signaling and subsequently NF-κB activation (6-8). Additional checkpoints follow when functional L chain gene recombination in pre-B cells results in the expression of the B cell receptor (BCR) and when the resulting immature B cells progress to mature B cells. Mice deficient for any of the (pre-)BCR signaling proteins exhibit essentially similar immunological phenotypes, characterized by reduced numbers of mature peripheral B cells, absence of B-1 B cells, reduced levels of serum IgM and IgG3, lack of *in vivo* responses to T cell independent type II antigens and reduced *in vitro* responses to anti-IgM and LPS stimulation (9).

Several lines of evidence show that the adapter molecule Slp-65 and the cytoplasmic kinase Btk are crucially involved in the regulation of the developmental program of pre-B cells, in particular by limiting pre-B cell expansion and promoting differentiation of large cycling to small resting pre-B cells. Both Slp-65-deficient and Btk-deficient mice show a partial block at the pre-B cell stage, characterized by an impaired developmental progression from large cycling $CD43^+$ into small resting $CD43^-$ pre-B cells (10-14). In an analysis of the kinetics of pre-B cell differentiation *in*

vivo, Btk-deficient cells manifested a specific developmental delay within the small pre-B cell compartment, when compared with wild-type cells (14). By introduction of a transgenic BCR into Slp-65-deficient mice, it was shown that in the absence of Slp-65 the production of κ light chain is decreased and cellular maturation of developing B cell is delayed (15). Reintroduction of Slp-65 into Slp-65-deficient pre-B cells led to pre-BCR downregulation and enhanced differentiation (16). Furthermore, Slp-65$^{-/-}$ and Btk-deficient pre-B cells show enhanced proliferative expansion *in vitro* in the presence of IL-7, when compared with wild-type cells (14, 16-18). A synergistic role of Slp-65 and Btk in B cell development was demonstrated by the almost complete block in B cell development at the CD43^{+} pre-BCR^{+} pre-B cell stage in Slp-65/Btk double-mutant mice (19).

We recently reported that Slp-65$^{-/-}$ mice spontaneously develop pre-B cell lymphomas expressing large amounts of pre-BCR on their cell surface (16). Moreover, also ~50% of human childhood pre-B acute lymphoblastic leukemias (ALL) showed a complete loss or a drastic reduction of Slp-65 expression. Injection of murine Slp-65$^{-/-}$ pre-B cells into immunodeficient mice resulted in tumor development, while reconstitution of Slp-65 expression in these cells eliminated their tumorgenic capacity (19). Tyr_{96}, which is the binding site for Btk, was identified as a crucial residue for the Slp-65 tumor suppressor function. Although Btk-deficient mice do not develop pre-B cell tumors, the possibility remains that Btk cooperates with Slp-65 as a tumor suppressor in pre-B cells. To investigate cooperation between Btk and Slp-65, we characterized the pre-B cell compartment in single and double mutant mice in detail. We show that Btk/Slp-65 double mutant mice have a high incidence of pre-B cell lymphoma and that transgenic expression of low levels of the constitutive active E41K-Y223F Btk mutant prevents tumor formation in Slp-65/Btk double mutant mice.

MATERIALS AND METHODS

Mice

Btk-deficient mice (20) were on the C57BL/6 background. Btk WT alleles were identified by an exon 9 forward primer (5'-CACTGAAGCTGAGGACTCCAT AG-3') and an exon 10 reverse primer (5'-GAGTCATGTGCTTGGAATAC CAC-3'). For Btk KO alleles, primers were within the *LacZ* reporter (20), forward: 5'-TTCACTGGCCGTCGTTTTACAACGTCGT GA-3', and reverse: 5'-ATGTGAGCGAGTAACAACCCGTCGGATTCT-3'. Slp-65-deficient mice (10) were on the Balb/c background and genotyped with the following primers: *Neo2A* 5'-CGGAGAACCTGCGTGCAATC- 3' and *gxBr* 5'-GAGTCCGAATGTTCATCTG-

3' (KO allele) and *WtI* 5'-TCAAACCTGGGTCTCAGAA-3' and *gxBr* (WT allele). The presence of the Btk^{Act} transgene was evaluated by PCR, using the following primers: CD19prom: 5'-TGCAATTAGTGGTGAACAAC-3' and hmBtk.65R: 5'-AGATGCCAGGACTTGGAA GG-3'.

The Btk^{Act} transgene consist of a ~6.3 kb genomic fragment containing the CD19 promoter region, a 0.3 kb fragment with the first three exons of human Btk as cDNA sequence, as well as a 27.1 kb genomic DNA fragment, encompassing the Btk exons 3-19 (21). Using double stranded site-directed mutagenesis (Stratagene, La Jolla, CA) the Y223F mutation, the replacement of AT by TC in exon 8, was introduced into the construct that was previously used to generate CD19-Btk^{E41K} mice. The ~34 kb MluI-NotI insert from the E41K-Y223F-Btk construct was excised from the vector, gel-purified and micro-injected into the pronuclei of FVB fertilized oocytes. Transgenic founder mice were identified by Southern blotting of BamHI digests using a partial human Btk cDNA probe (bp 133-1153), as described previously (20, 21), and crossed with Btk null mice. All mice were bred and maintained at the Erasmus MC animal care facility under specific pathogen free conditions. Statistical analyses of Kaplan-Meier tumor-free survival estimates of the various mouse groups were performed using SPSS 10.1.0 (SPSS Inc. Chicago, Illinois).

Cell culture and flow cytometry

IL-7 driven BM cultures and determination of IL-7 dependent proliferative responses of total BM cells have been described previously (14, 22). Preparations of single-cell suspensions, standard and intracellular flow cytometry, and conjugated monoclonal antibodies (Becton Dickinson Pharmingen, Sunnyvale, CA) have been described previously (14, 20). The anti-SLC hybridoma LM34 (23) was kindly provided by A. Rolink (Basel, Switzerland); antibodies were purified using protein G columns and conjugated to biotin according to standard procedures.

Western blotting analysis

For analysis of Btk expression and protein phosphorylation, single-cell suspensions from spleen were depleted of erythrocytes by NH_4Cl lysis and enriched for B cells by AutoMACS purification, using biotinylated antibodies to Gr-1, Ter119, CD4, CD8 and CD11b and magnetic streptavidin MicroBeads (Miltenyi Biotec, Sunnyvale, CA) for negative selection. B-lineage cells were purified from BM by AutoMACS using anti-B220 MicroBeads for positive selection. Splenic B cell fractions were stimulated with 10 μg/ml $F(ab')_2$ fragment of polyclonal goat-anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA) in RPMI1640 at 37° C for 5 minutes. Western blotting was performed as described (20), using anti-Btk C-20, anti-Erk1/2 SC-094 (SantaCruz Biotechnology, Santa Cruz, CA), or anti-phosphotyrosine P-Tyr-100 (Cell Signaling Technology, Beverly, MA).

RESULTS AND DISCUSSION

Pre-B cell maturation defects in Btk, Slp-65 and double mutant mice

Btk or Slp-65 single mutant mice have a partial block, while double mutant mice have an almost complete arrest at the pre-B cell stage in the BM (10-14, 19). As a result, the reduction of the numbers of mature B cells in the spleen of double-mutant mice is much more drastic, when compared with Btk or Slp-65 single mutant mice (Ref. 18; Figure 1A). We have previously shown that Btk-deficient cells fail to efficiently modulate the expression of developmentally regulated cell surface markers during the transition of large cycling into small resting cytoplasmic μ^+ pre-B cells (14). In particular, in Btk-deficient small pre-B cells the downregulation of the pro-B/large pre-B stage-specific markers SLC, the sialoglycoprotein CD43 and the metallopeptidase BP-1 and the upregulation of CD2 and CD25/interleukin-2 receptor, which are first expressed on small pre-B cells, are impaired (14). To analyze the effect of Slp-65 inactivation or the concomitant deficiency of Slp-65 and Btk on pre-B cell maturation, we compared the expression of these developmentally regulated markers in wild-type, Btk-deficient, Slp-65-deficient and double mutant mice by flow cytometry. Since MHC class II expression is also initiated at the pre-B cell stage (24), we included surface expression of MHC class II of cytoplasmic μ^+ pre-B cells in the analyses.

When compared with Btk-deficient cells, Slp-$65^{-/-}$ pre-B cells manifested a slightly more pronounced defect in the downregulation of CD43, BP-1 and SLC and in the upregulation of CD25, CD2, and MHC class II expression (Figure 1B). Consistent with the reported almost complete arrest at the pre-BCR^+ stage (19), Slp-65/Btk double mutant pre-B cells had high forward scatter characteristics, were positive for CD43, BP-1 and SLC expression and showed very low surface expression of CD25, CD2, and MHCII (Figure 1B).

Collectively, these findings show that Slp-65 and Btk have a synergistic role in the developmental progression of large cycling into small resting cytoplasmic μ^+ pre-B cells.

Deficient differentiation of Btk and Slp-65 mutant pre-B cells in vitro

Cytoplasmic μ^+ pre-B cells undergo rapid cell division in response to IL-7 *in vitro*, whereby subsequent removal of IL-7 strongly induces exit from cell cycle and further differentiation into surface IgM^+ B cells (25). We previously reported that Btk and Slp-65 single mutant pre-B cells manifest an enhanced proliferative response to IL-7 (14, 16). When we compared total BM cells from Btk and Slp-65 single mutants and the double mutant, these three groups of mice showed similar [^{3}H] thymidine incorporation values after 5 days of culture in the presence of 100 U/ml IL-7 (Figure 2A). When total BM cell suspensions from wild-type mice were cultured in the

presence of IL-7 for 7 days, the majority of cells consisted of B220^{+} cytoplasmic μ^{+} pre-B cells that were surface μ^{-} or μ^{low}, while a significant fraction (~20-30%) performed productive κ L chain rearrangements and matured to surface IgM^{+}IgD^{-} or IgM^{+}IgD^{+} B cell stages (Figure 2B, thin lines). In contrast, the IL-7 driven BM cultures from Btk and Slp-65 single or double mutant mice consisted almost exclusively of

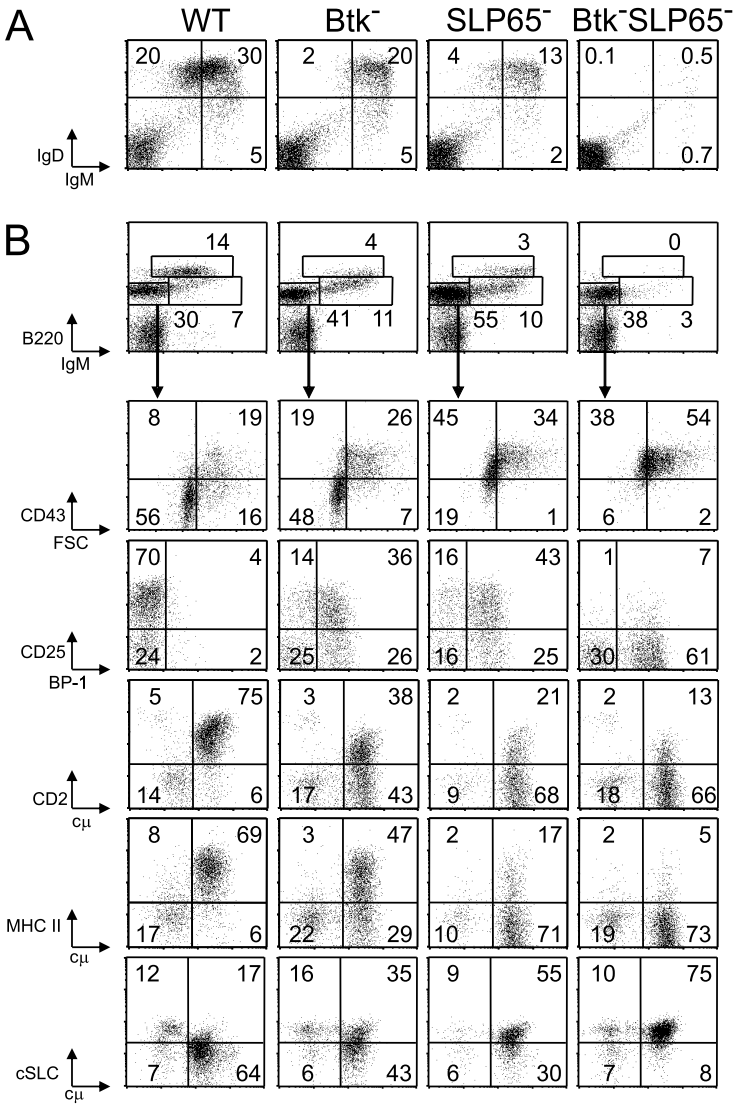

Figure 1. Impaired pre-B cell maturation in Btk-deficient, Slp-65-deficient and Btk/Slp-65 double mutant mice.

(A) Flow cytometric analysis of surface IgM/IgD expression on total lymphoid cells in the spleen. (B) Expression profiles of B220 and IgM on total lymphoid cells in the BM (*top*). The B220^{+}IgM^{-} pro-/pre-B cell fraction was gated and analyzed for the indicated markers (*bottom*). Data are displayed as dot plots and the percentages of cells within the indicated quadrants or gates are given. Data shown are representative of 4 mice examined within each group.

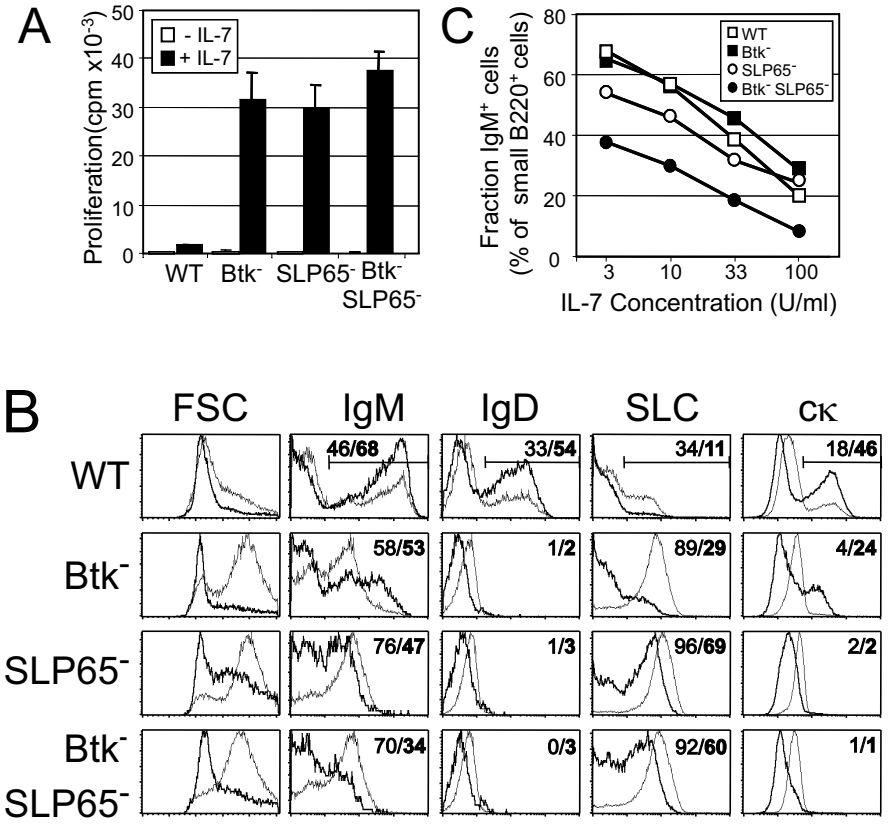

Figure 2. Analysis of IL-7 driven BM cultures from Btk and Slp-65 mutant mice.
(A) Proliferative response to 100 U/ml IL-7, as determined by [^{3}H]thymidine incorporation after 5 days of culture. Bars represent mean cpm and SEM of tripiclate cultures (B) Forward scatter (FSC) values and expression profiles of IgM, IgD, SLC and cytoplasmic κ L chian of IL-7 driven BM cultures from the indicated mice. Data are displayed as histogram overlays of B220$^+$ cells, either cultured under proliferating conditions (with 100U/ml IL-7 for 7 days, *thin lines*) or under differentiating conditions (after 5 days of culture with IL-7 and subsequently without IL-7 for 2 days, *bold lines*). The percentages shown represent the fractions of the cells that are within the indicated marker under the two different culture conditions. (C) Percentage of surface IgM$^+$ cells within the fraction of small FSC B220$^+$ cells after 7 days of culture in the presence of the indicated concentrations of IL-7. Data are representative of four mice per group.

large μ$^+$ SLC$^+$ pre-B cells which did not express κ L chains in their cytoplasm (Figure 2B, thin lines).

To analyze the differentiation capacity of pre-B cells, BM cells were cultured in the presence of IL-7 for 5 days and subsequently for 2 days on S17 stroma cells in the absence of IL-7. Under these conditions, wild-type cells acquired low FSC characteristics and ~50% of the cells showed productive κ L chain rearrangement and differentiated into IgM$^+$IgD$^+$ cells. In contrast, the cultures from

Btk-deficient mice contained no IgD positive cells, a smaller fraction (~25%) of cytoplasmic κ^+ cells, and a significant proportion of SLC^+ cells. The defects were more pronounced in the cultures from Slp-$65^{-/-}$ and Btk/Slp-65 double mutant mice, which were characterized by an inefficient exit from cell cycle and a dominance of $SLC^+\kappa^-$ pre-B cells. In this respect, these two groups of mice were not significantly different.

Only when BM cells were cultured in the presence of limiting concentrations of IL-7 for 7 days, a difference between Slp-65 single mutant and Slp-65/Btk double mutant mice was noticed (Figure 2C). At low IL-7 concentrations, pre-B cell division was limited and cells rapidly differentiated into surface IgM^+ B cells. Within the population of small non-cycling $B220^+$ cells, the percentage of surface IgM^+ cells was lower in Slp-65/Btk double mutant mice, when compared to WT, Btk or Slp-65 single mutant mice. These results indicate that, in the absence of Slp-65, Btk functions to enhance differentiation to $sIgM^+$ B cells at low IL-7 concentrations.

Taken together, these findings show that in the presence of IL-7 Btk, Slp-65 and double mutant pre-B cells show increased proliferation and enhanced pre-BCR expression, when compared with wild-type pre-B cells. The ability to downregulate SLC expression and to differentiate into IgM^+ B cells upon IL-7 withdrawal, is mildly affected in Btk-deficient mice, more severely in Slp-$65^{-/-}$ mice and even more so in Slp-65/Btk double mutant mice.

Btk/Slp-65 double mutant mice have a high incidence of pre-B cell lymphoma

Slp-$65^{-/-}$ mice develop spontaneous pre-B cell tumors, with a frequency of <10% at 16 weeks of age (16). When we followed a panel of Slp-65 single mutant mice up to 16 weeks, approximately 5% (3 out of 66) developed a lymphoma. Remarkably, when Slp-65/Btk double mutant mice were followed for 16 weeks, a significantly enhanced frequency of tumor development was noticed, because 75% of these mice (12 out of 16) developed a pre-B cell lymphoma. In these mice external examination revealed either directly the presence of solid tumors, mainly close to the scapula, or indirectly as the mice displayed pareses of the hind limbs. In general, the mice also developed splenomegaly, and enlargement of the lymph nodes. Comparable tumors were not found in wild-type or Btk-deficient mice (less than 1/4000 mice). The Kaplan-Meier tumor-free survival curves for the Slp-65 single and Slp-65/Btk double mutant mice are shown in Figure 3A.

These results demonstrate that deficiency of Btk strongly increases the frequency of tumor formation in Slp-$65^{-/-}$ mice, indicating that Btk cooperates with Slp-65 as a tumor suppressor in pre-B cells.

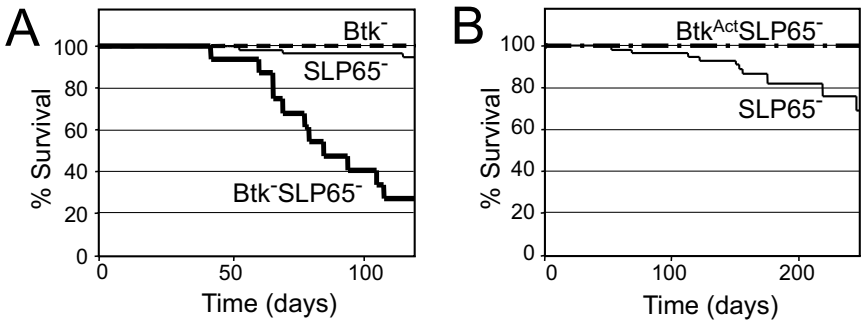

Figure 3. (A) Kaplan-Meier tumor-free survival estimates for Btk-deficient (dotted line), Slp-65$^{-/-}$ (thin line) and Btk^{-}Slp-65$^{-/-}$ mice (bold line). Tumor-free survival in Btk^{-}Slp-65$^{-/-}$ mice was significantly reduced ($p<0.0001$ by log-rank) compared to Slp-65$^{-/-}$ mice. (B) Kaplan-Meier tumor-free survival estimates for Slp-65$^{-/-}$ (thin line) and Btk^{Act}Slp-65$^{-/-}$ mice (dotted line). Tumor-free survival in Btk^{Act}Slp-65$^{-/-}$ mice was significantly enhanced ($p=0.04$ by log-rank) compared to Slp-65$^{-/-}$ mice.

Constitutive active Btk^{Act} prevents pre-B cell tumor formation in Btk/Slp-65 double mutant mice

The finding that Btk apparently cooperated with Slp-65 as a tumor suppressor prompted us to investigate whether transgenic expression of a constitutive active Btk mutant could prevent tumor development in Slp-65-deficient mice. The PH domain gain-of-function mutant E41K shows increased membrane localization and phosphorylation in quiescent cells, independent of PI3K activity and induces transformation of 3T3 fibroblasts in soft agar cultures (26, 27).This capacity is augmented by mutation of the main autophosphorylation site in the Btk SH3 domain (Y223F; (28)). In Ramos B cells expression of E41K-Btk enhances the sustained increase in intracellular [Ca^{++}] following BCR cross-linking (29). Thus, the E41K mutant and the E41K-Y223F double mutant represent activated forms of Btk. When E41K-Btk was expressed at physiological levels in transgenic mice under the control of the B-cell specific CD19 promoter, B cell development was arrested at the immature B cells in the BM (probably because the E41K-Btk mutant mimics BCR occupancy by auto-antigens), while residual B cells were efficiently driven into IgM plasma cell differentiation (21). We recently generated a panel of 7 independent E41K-Btk (n=3) or E41K-Y223F-Btk (n=4) transgenic mouse lines, which were crossed onto the Btk null background. Expression of the two different mutants resulted in parallel phenotypes, whereby the deletion at the immature B cell stage was dose-dependent (S.M. and R.H., submitted). From this panel we selected a low-copy E41K-Y223F-Btk transgenic mouse strain (Btk^{Act}), in which the extent of the B cell arrest was limited, while the finding of enhanced protein tyrosine phosphorylation in splenic B cells and significantly increased serum IgM levels provided evidence for the constitutive active nature of Btk^{Act} *in vivo* (See below; Figure 4A-C).

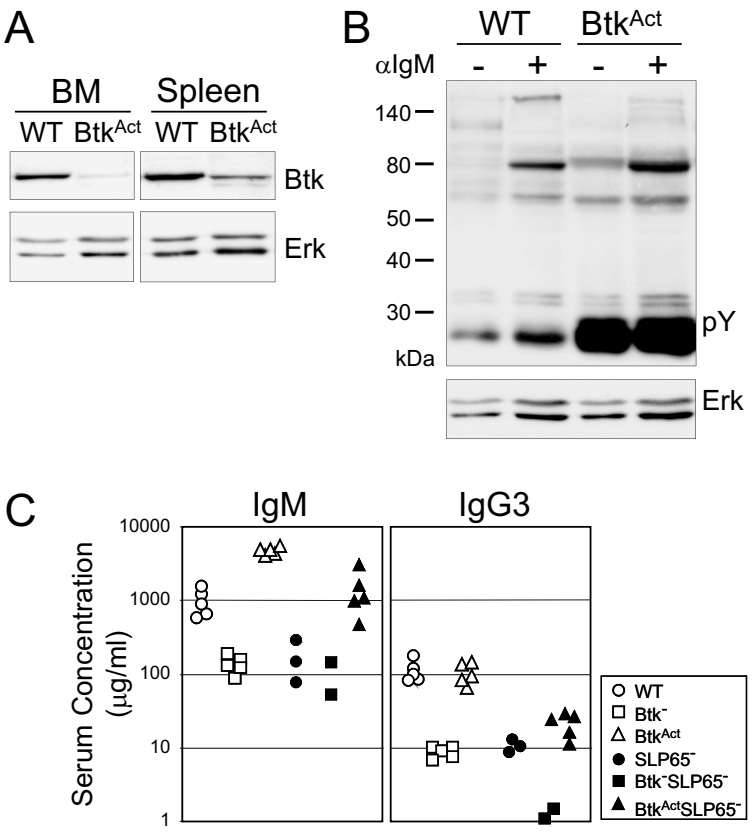

Figure 4. Low-level Btk^{Act} expression can partially substitute for the absence of Btk and Slp-65.
(A) Western blotting analysis of Btk expression in wild-type and Btk^{Act} B cells from BM and spleen. Membrane was re-blotted with anti-Erk. (B) Protein tyrosine phosphorylation in extracts of untreated and anti-IgM stimulated wild-type or Btk^{Act} splenic B cells, analyzed by immunoblotting with a phosphotyrosine (pY)-specific antibody. Membrane was re-blotted with anti-Erk. (C) Serum concentrations of IgM and IgG3 in the indicated mutant mouse strains. Mice were 2 months of ages and each symbol represents an individual animal. (D) Flow cytometric analysis of surface IgM/IgD expression on total lymphoid cells in the spleen of the indicated mice. (E) Expression profiles of B220 and IgM on total lymphoid cells in the BM of the indicated mice (*top*). The B220^{+}IgM^{-} pro-/pre-B cell fractions were gated and analyzed for CD43/FSC and cytoplasmic SLC and μ H chain (*bottom*). Data are displayed as dot plots and the percentages of cells within the indicated quadrants or gates are given. Data shown are representative of 4 mice examined within each group.

Btk^{Act} mice were crossed onto the Btk/Slp-65 double mutant background and a panel of 20 transgenic mice was followed for 8 months. At this age, the fraction of Slp-65$^{-/-}$ mice that developed a pre-B cell lymphoma increased to 18% (12 out of 66) (Figure 3B). In contrast, none of the Btk^{Act} transgenic mice on the Btk/Slp-65 double mutant background developed lymphoma (Figure 3B). When these Btk^{Act} mice were sacrificed at 8 months, no evidence for ongoing pre-B cell proliferation was found: pre-B cell numbers in the BM were not increased and splenomegaly or

pre-B cell infiltrations into the spleen were absent.

These findings show that substitution of endogenous Btk by low levels of constitutively activated Btk prevents tumor formation in Slp-65-deficient mice. Therefore, we conclude that Btk^{Act} can substitute for Slp-65 as a tumor suppressor in pre-B cells.

Low-level BtkAct expression can partially substitute for the absence of Btk and Slp-65

Btk^{Act} protein expression was analyzed by western blotting of purified B cell fractions from BM and spleen, in which Btk protein was visible as a single ~77 kDa band (Figure 4A). Quantification of Btk signals using Erk1/2 as a loading control showed that Btk^{Act} expression was low (up to ~20% of the physiological levels). This was confirmed in intracellular flow cytometry experiments by comparison of median fluorescence intensities of transgenic Btk^{Act} and endogenous Btk (data not shown). The analysis of unstimulated and anti-IgM stimulated splenic B cells by immunoblotting with a phosphotyrosine-specific antibody revealed that Btk^{Act} B cells exhibit enhanced protein tyrosine phosphorylation *in vivo* (Figure 4B).

To evaluate the capacity of Btk^{Act} to drive peripheral B cells into plasma cell differentiation, we determined serum IgM and IgG3 levels in 2-month-old Btk^{Act} mice, both on a Btk-deficient and on a Btk/Slp-65 double deficient background, with non-transgenic WT, Btk^{-}, Slp-$65^{-/-}$ and Btk/Slp-65 double deficient mice as controls. Consistent with previous reports, the serum levels of IgM and IgG3 in mice deficient for Btk or Slp-65 were significantly decreased (10-12, 30). In Btk/Slp-65 double mutant mice IgM levels equally low and IgG3 levels were even more reduced (Figure 4C). When Btk^{Act} was expressed on the Btk-deficient background, the levels of IgM in the serum were increased with a factor of ~5-10 and IgG3 levels were in the normal range, when compared with wild-type mice. Also the frequencies of IgM-producing cells in spleen and BM, as determined in an Elispot assay, were increased in these Btk^{Act} mice (data not shown). Expression of Btk^{Act} completely corrected IgM levels and significantly restored IgG3 levels in Btk/Slp-65 double mutant mice (Figure 4C).

Flow cytometric analyses of the spleen revealed that low-level expression of Btk^{Act} did not rescue the decrease in splenic B cell numbers in Btk-deficient mice (Figure 4D). On the other hand, Btk^{Act} expression also did not result in significant deletion of peripheral B cells, in contrast to our previous findings in mice expressing physiological levels of the E41K-Btk mutant (21). Expression of Btk^{Act} partially restored the almost complete absence of splenic B cells in Btk/Slp-65 double mutant mice, whereby their surface IgM/IgD profiles were similar to those in Slp-65 single mutant mice (See Figures 1A and 4D). In the bone marrow, expression of the

Btk^{Act} transgene corrected the maturation defects of Btk-deficient $c\mu^+$ pre-B cells, *i.e.* the downregulation of CD43 and SLC expression and the upregulation of CD2, CD25 and MHC class II expression (S.M. and R.H., manuscript submitted; shown for CD43 and SLC in Figure 4E). In Slp-$65^{-/-}$ mice, substitution of endogenous Btk by very low levels of constitutively activated Btk was associated with a reduction of the percentages of SLC^+ and $CD43^+$ $c\mu^+$ pre-B cells (Figure 4E).

Taken together, comparison of Slp-$65^{-/-}$ and Btk^{Act} Slp-$65^{-/-}$ mice with respect to serum Ig concentration and the maturation of pre-B cells *in vivo* indicate that low-level Btk^{Act} expression can partially substitute for the absence of Slp-65.

Characterization of pre-B cell tumors

All pre-B cell tumors characterized expressed high levels of SLC and Ig μ H chain in their cytoplasm, irrespective of the Btk genotype of the Slp-$65^{-/-}$ mice, (Figure 5A). The two $Btk^{+/-}$Slp-$65^{-/-}$ mice present in our panel of mutant mice also rapidly developed pre-B cell tumors. Because of the presence of a *LacZ* reporter in the targeted Btk allele, we could evaluate the X-chromosome inactivation status of the lymphoma cells (20). As a result of the process of random X-chromosome inactivation in $Btk^{+/-}$ heterozygous females, each X chromosome is active in about half of the pre-B cells. Similar to findings in lymphoma cells from male Btk$^-$Slp-$65^{-/-}$ mice, we observed that the majority of lymphoma cells from $Btk^{+/-}$Slp-$65^{-/-}$ mice were $LacZ^+$. These results indicate that the $Btk^{+/-}$ lymphoma cells carried the disrupted Btk allele on the active X chromosome and therefore were functionally Btk-deficient (Figure 5B).

As both Btk and Slp-65 are crucially involved in the modulation of pre-B cell surface makers and the initiation of Ig L chain gene rearrangement during the transition of large cycling into small resting cytoplasmic μ^+ pre-B cells, we were interested in the phenotype of pre-B cell lymphoma cells. Most pre-B cell lymphomas contained a (minor) fraction of cells that co-expressed κ L chain and SLC in their cytoplasma and showed variable surface expression of the developmentally regulated markers, such as CD43 and CD2 (Figure 5C). In general, the expression of SLC and cell surface markers remained stable when the tumor cells were cultured in the presence of IL-7. Some of these cell lines became IL-7 independent or lost expression of the IL-7R, as detectable by flow cytometry (data not shown). When we cultured pre-B lymphoma cells derived from different tissues from a single Btk/Slp-65 double mutant mouse *in vitro*, we found that a BM-derived cell culture mainly consisted of κ L chain positive cells, while a lymph node-derived cell culture was essentially κ L chain negative. Nevertheless, both cultures expressed high levels of CD43 and low levels of CD2.

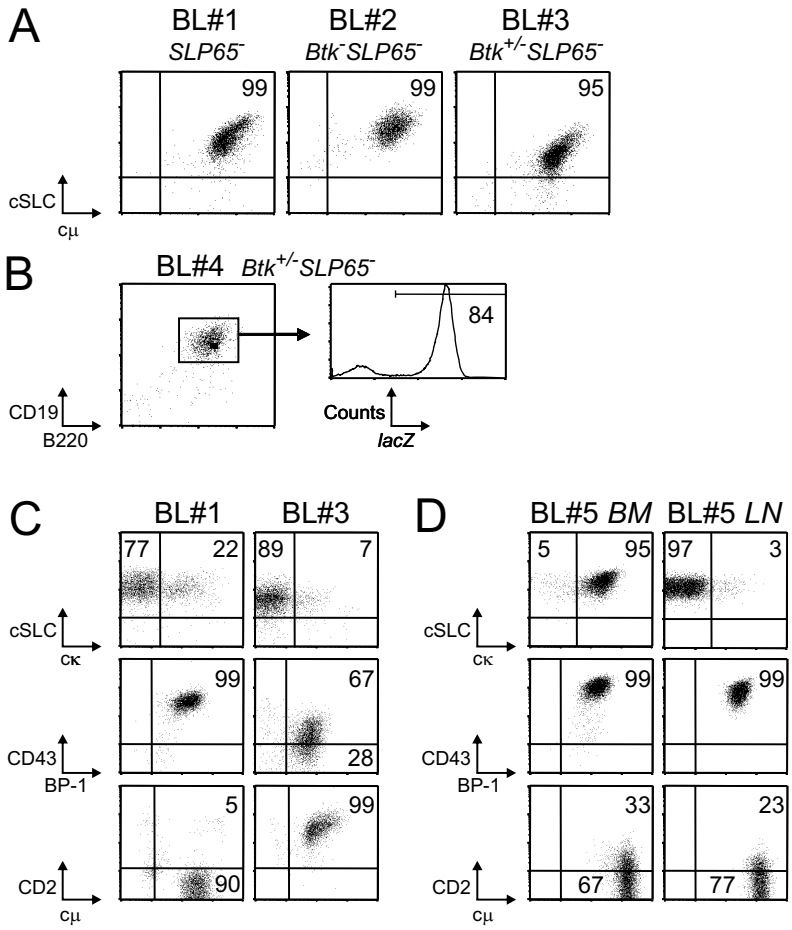

Figure 5. Characterization of pre-B cell tumors by flow cytometry
(A) Dot plots for cytoplasmic SLC and μ H chain in gated B220+ cells from tumor samples from the indicated mice, grown for 1 to 3 weeks in the presence of IL-7. (B) Flow cytometric analysis of *lacZ* expression in gated CD19+B220+ pre-B lymphoma cells in a lymph node from a Btk+/-Slp-65-/- mouse. (C) Phenotype of two pre-B cell lymphoma cultures, showing variable expression of cytoplasmic κ L chain, CD43 and CD2. Cell suspensions were stained for the indicated markers in combination with B220, and the results are displayed as dot plots of gated B220+ cells. (D) Phenotype of two separate tumor cell suspensions derived from BM and mesenteric lymph node from a single mouse, which were cultured in the presence of Il-7 for 7 days.

Collectively, these results show that in the tumors the ordered differential expression of stage-specific pre-B cell markers (CD43, SLC and BP-1 on large cycling pre-B cells but CD2, CD25 and MHCII on small resting pre-B cells) is lost. Furthermore, the finding of variable percentages of κ L chain expressing cells in the pre-B cell lymphomas suggests that in the absence of Slp-65 Ig L chain locus rearrangements are initiated in large cycling pre-BCR+ cells.

Btk and Slp-65 cooperate as tumor suppressors

By crossing Slp-65$^{-/-}$ mice with mice that were either Btk-deficient or expressed a constitutive active form of Btk we have demonstrated in this report that (i) Btk and Slp-65 have synergistic roles in the developmental progression of large cycling into small resting pre-B cells, (ii) the concomitant deficiency of Btk significantly enhanced tumor formation in Slp-65$^{-/-}$ mice, and (iii) expression of Btk^{Act} prevents tumor formation in Btk/Slp-65 double mutant mice. We therefore conclude that Btk^{Act} compensates for loss of Slp-65 tumor suppressor function, either by promoting pre-B cell differentiation or limiting pre-B cell expansion independent of Slp-65.

In contrast to Slp-65$^{-/-}$ mice, Btk/Slp-65 double mutant mice exhibit an almost complete arrest at the large cycling pre-BCR^{+} pre-B cell stage. Thus, it is conceivable that the increased frequency of malignant transformation in Btk/Slp-65 double mutant mice reflects the increased pool size of proliferating pre-B cells with a reduced ability to progress into CD43^{-} small resting pre-B cells, when compared with Slp-65 single mutant mice. Alternatively, the absence of Btk may alter the proliferative capacities of Slp-65$^{-/-}$ pre-B cells in a signaling pathway different from the pre-BCR. In this respect, the IL-7R pathway would be an attractive candidate, as Btk-deficient RAG-1$^{-/-}$ pro-B cells were shown to have an increased responsiveness to IL-7, when compared with Btk^{+} RAG-1$^{-/-}$ pro-B cells (14). A third possibility would be that the concomitant absence of Btk and Slp-65 precludes the efficient down-regulation of RAG enzyme levels upon pre-BCR signaling (1, 2). The finding of SLC and κ L chain co-expression in the pre-B cell tumors suggests that Ig L chain rearrangements have occurred in large pre-BCR^{+} cycling pre-B cells. This implies that the V(D)J recombination machinery is active in Slp-65$^{-/-}$ cycling pre-B cells, which may cause DNA damage contributing to the induction of secondary mutations required for malignant transformation. Further experiments comparing Btk^{+} and Btk^{-} Slp-65-deficient pre-B cell tumor cells should identify the nature of the cooperation between Btk and Slp-65 as tumor suppressors. In this context, we found that Btk expression levels vary considerably between different pre-B cell lines established from Slp-65$^{-/-}$ mice. Btk was not detectable in 1 out of 6 Slp-65$^{-/-}$ pre-B cell lines by western blotting, suggesting that Btk could be a target for secondary mutations in Slp-65$^{-/-}$ mice (R.H., unpublished). Since Slp-65 is implicated in childhood pre-B ALL (19), the finding of cooperation between Btk and Slp-65 may also have important implications for our understanding of the etiology of this malignancy in humans.

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CHAPTER 4

Tumor suppressor function of Bruton's tyrosine kinase is independent of its catalytic activity

Tumor suppressor function of Bruton's tyrosine kinase is independent of its catalytic activity

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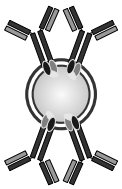
SUMMARY

During B cell development in the mouse, Bruton's tyrosine kinase (Btk) and the adaptor protein Slp-65 limit the expansion and promote the differentiation of pre-B cells. Btk is thought to mainly function by phosphorylating phospholipase Cγ2, which is brought into close proximity of Btk by Slp-65. However, this model was recently challenged by the identification of a role for Btk as a tumor suppressor in the absence of Slp-65 and by the finding that Btk function is partially independent of its kinase activity. To investigate if enzymatic activity is critical for the tumor suppressor function of Btk, we crossed transgenic mice expressing the kinase-inactive K430R-Btk mutant onto a Btk/Slp-65 double deficient background. We found that K430R-Btk expression rescued the severe developmental arrest at the pre-B cell stage in Btk/Slp-65 double deficient mice. Moreover, K430R-Btk could functionally replace wild-type Btk as a tumor suppressor in Slp-65$^-$ mice: at 6 months of age, the observed pre-B cell lymphoma frequencies were ~15% for Slp-65$^-$ mice, ~44% for Btk/Slp-65 deficient mice, and ~14% for K430R-Btk transgenic mice on the Btk/Slp-65 deficient background. Therefore, we conclude that Btk exerts its tumor suppressor function in pre-B cells as an adaptor protein, independent of its catalytic activity.



INTRODUCTION

Early in B cell development productive V(D)J recombination leads to the synthesis of Ig μ H chain protein, which is expressed on the cell surface, together with the non-rearranging VpreB and λ5 surrogate light chain (SLC) proteins and forms the pre-B cell receptor (pre-BCR) (reviewed in Ref. (1, 2)). It appears that the pre-BCR has a ligand-independent signal transducing capacity, inducing phosphorylation of the Igα/CD79a and Ig-β/CD79b signaling components and subsequently proliferative expansion and differentiation into large cycling pre-B cells (3, 4). Pre-BCR signaling involves the formation of a lipid raft-associated signaling module, which is composed of the tyrosine phosphorylated proteins Syk, the Src-family tyrosine kinase Lyn, SH2 domain-containing leukocyte protein of 65 kDa (Slp-65, also known as BLNK or BASH), phosphoinositol 3-kinase (PI3K), Bruton's tyrosine kinase (Btk), Vav and phospholipase Cγ2 (PLCγ2) (5-7). Activation of PLCγ2 by Btk results in the production of the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG), which activate calcium signaling and protein kinase C (PKC), respectively (8, 9). The pre-BCR is transiently expressed on the cell surface, whereby the signaling molecules Slp-65 and Btk are important for the developmental progression of large cycling into small resting pre-B cells in which Ig L chain rearrangement occurs (reviewed in Ref. (10)).

By analogy with the BCR in mature B cells, Btk is thought to be phosphorylated by Syk or Lyn upon recruitment to the pre-B cell membrane through binding of its PH domain to the PI3K product phosphatidyl-inositol trisphosphate (PIP3) (11, 12). In this signaling pathway phosphorylated Slp-65 plays an essential role as it provides docking sites for phosphorylated Btk as well as PLCγ2 and thus brings Btk in close proximity with PLCγ2 (13, 14). However, this model is challenged by several independent observations.

Firstly, Btk can also act as adaptor molecule, independent of its catalytic activity. Expression of kinase-inactive Btk mutants have been shown to partially or fully reconstitute BCR-induced calcium mobilization in Btk-deficient chicken DT40 and human A20 mature B cell lines (8, 15, 16). In addition, phosphorylation of PLCγ2 upon BCR stimulation is apparently unaffected in human Btk-deficient B cell lines (17). We have recently shown that during *in vivo* B cell development Btk function is in part independent of its catalytic activity, as transgenic expression of the kinase-inactive K430R-Btk mutant partially restored pre-B cell and B cell defects in Btk-deficient mice (18).

Secondly, in mature A20 B cells Btk is essential for the recruitment of phosphatidylinositol phosphate 5-kinase (PIP5K) to the membrane, whereby enzymatic activity of Btk is not required for its association with PIP5K (15). Activation

of PIP5K leads to local synthesis of PIP2, which is a common substrate shared by both PI3K and PLCγ2. As a result, a positive feedback loop is initiated that allows Btk to stimulate the production (by PI3K) of PIP3, which is required for sustained Btk localization to the plasma membrane. At the same time, the shuttling function of Btk also provides substrate for sustained PLCγ2 activity (12, 15).

Thirdly, Btk/Slp-65 double deficient mice show an almost complete block in B cell development at the pre-B cell stage, when compared with the partial block at this stage in Slp-65 or Btk single mutant mice (19-21), indicating that Btk and Slp-65 partially function in parallel pathways. Both Btk and Slp-65 are essential for the regulation of pre-B cell development, in particular by limiting pre-B cell expansion at the transition of large cycling into small pre-B cells (22-26). Slp-65^{-} mice spontaneously develop pre-B cell lymphomas expressing high levels of the pre-BCR on the cell surface (20, 27). Although Btk-deficient mice do not develop pre-B cell tumors, we recently found that Btk cooperates with Slp-65 as a tumor suppressor, because the incidence of pre-B cell lymphomas was significantly higher in Btk/Slp-65 double mutant mice, when compared with Slp-65 single deficient mice. Moreover, transgenic expression of the constitutive active E41K-Y223F Btk mutant, which shows enhanced membrane localization (28, 29), prevented tumor formation in Btk/Slp-65 double deficient mice (21).

Importantly, the finding of defective Slp-65 expression in ~50% of childhood pre-B acute lymphoblastic leukaemias (ALL) indicated that Slp-65 also acts as a tumor suppressor in pre-B cells in human (30). The loss of SLP-65 protein was apparently due to the incorporation of alternative exons into Slp-65 transcripts, leading to premature stop codons (30). Moreover, in human Bcr-Abl^{+} pre-B ALL, the activity of the Bcr-Abl1 kinase was found to be linked to defective pre-B signaling and the expression of the same aberrant SLP-65 transcripts (31). It was also observed that a large fraction of childhood pre-B ALL cases manifested aberrant Btk transcripts predicted to encode Btk proteins with a substantial kinase domain deletion (32).

The mechanism by which Btk exerts its tumor suppressor function in pre-B cells independent of Slp-65 is currently unknown. By analogy with the findings in mature A20 B cells, Btk may function in pre-B cells as an adaptor molecule to localize PIP5K to the plasma membrane. However, it remains possible that this PIP5K shuttling mechanism that allows Btk to stimulate PLCγ2 is dependent on the presence of Slp-65, *e.g.* as a scaffold molecule to bring Btk or other tyrosine kinase proteins, such as Syk or Src-family kinases, in close proximity with PLCγ2.

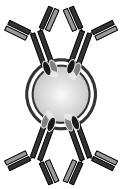
In this study, we investigated whether Btk tumor suppressor function is independent of its kinase activity, and at the same time whether Slp-65 is required for adaptor function of Btk. To this end, we crossed transgenic mice expressing the

kinase inactive Btk mutant K430R onto a Btk/Slp-65 double deficient background and found that kinase-inactive Btk was able to functionally replace wild-type Btk as a tumor suppressor in the absence of Slp-65.

MATERIALS AND METHODS

Mice and genotyping

Btk-deficient mice (33) and Slp-65-deficient mice (22) were on the C57BL/6 and Balb/c background, respectively. Wild-type and targeted alleles were identified as described previously (21, 26). Btk-K430R transgenic mice, in which B-cell specific expression of human Btk is under the control of the CD19 promoter region, were on a mixed C57Bl/6 x FvB background. The presence of the Btk-K430R transgene was evaluated by PCR (18).



Flow cytometry and Ig ELISA

Standard and intracellular flow cytometry and conjugated monoclonal antibodies (BD Biosciences, Mountain View, CA) have been described previously (26, 33). The anti-SLC hybridoma LM34 (34) was kindly provided by A. Rolink (University of Basel, Basel, Switzerland). Levels of Ig subclasses in serum were measured by sandwich ELISA (35).

Cell culture and western blotting

Total bone marrow cells or pre-B lymphoma cells were cultured in the presence of 100U/ml IL-7 (Sigma-Aldrich, St. Louis, MO), as described (26). For analysis of Btk phosphorylation, cells were stimulated with 10 μg/ml $F(ab')_2$ fragment of polyclonal goat-anti-mouse IgM (Jackson Immuno Research, Westgrove, PA) in RPMI1640 at 37°C. Total cell lysates of unstimulated and stimulated cells were immunoprecipitated overnight at 4°C with anti-phosphotyrosine (α-pTyr-100, Cell Signaling Technology, Beverly, MA) and samples were blotted using standard SDS-PAGE procedures (36). Western blots were stained with anti-Btk C-20 (SantaCruz Biotechnology, Santa Cruz, CA) to detect Btk protein. Routinely, a small fraction of the total cell lysates were blotted with α-pTyr-100 to verify pre-BCR stimulation (21).

Ca^{2+} mobilization assay

Cells were loaded with 6μg/ml indo-1 acetoxymethylester (indo-1 AM; Molecular Probes Europe, Leiden, The Netherlands) at 37°C for 45 min. and subsequently stained with PE-labeled anti-CD4, anti-NK1.1, anti-Ter119, anti-CD11b, and FITC-labeled anti-CD8 monoclonal antibodies (BD Biosciences). Cells were stimulated

by 20 μg/ml $F(ab')_2$ fragment of polyclonal goat-anti-mouse IgM (Jackson Immuno Research) and subsequently by 2 μg/ml ionomycin as a positive control. Fluorescence ratios (FL-5/FL-4) were measured on a FACS-VantageSE/DiVa (BD Biosciences) and data for B cells ($CD4^-CD8^-$ $NK1.1^-$ $Ter119^-CD11b^-$) were analyzed by FlowJo (Tree Star, Inc., Ashland, OR) multiparameter flow application.

RESULTS

Kinase-inactive Btk stimulates calcium mobilization in mature B cells

We previously showed that transgenic expression of the kinase-dead Btk mutant K430R partially rescued Btk function *in vivo,* including the induction of NF-κB and the expression of Bcl-x_L and cyclin D2 in splenic B cells upon BCR stimulation (18). To investigate whether these effects are mediated by the ability of K430R-Btk to potentiate calcium flux, we evaluated calcium mobilization in splenic B cells in response to BCR engagement in K430R-Btk transgenic mice on a Btk-deficient background, in comparison with wild-type and Btk-deficient mice (Figure 1A). Btk-deficient B cells showed a significantly reduced BCR-mediated Ca^{2+} signal, when compared with wild-type B cells. Transgenic K430R-Btk provided complete correction of the Ca^{2+} response in Btk-deficient B cells, indicating that Btk stimulates calcium flux independent of its catalytic activity.

Slp-65 is not required for pre-BCR-induced tyrosine phosphorylation

If Btk and Slp-65 can function independently in parallel pathways in pre-B cells, this implies that Btk can be activated in the absence of Slp-65. It was previously shown that in $Slp\text{-}65^-$ pre-B cell lines, established by long-term IL-7 dependent bone marrow culture, the pre-BCR is signaling competent, whereby pre-BCR stimulation led to phosphorylation of Ig-α, Syk, Lyn, PI3K and PLCγ2 (20). We evaluated phosphorylation of Btk in stable Slp-65-deficient pre-B cell lines expressing high levels of pre-BCR on the cell surface, which we established from $Slp\text{-}65^-$ pre-B lymphoma cells (21). Pre-B cells were stimulated with antibodies specific for Ig μ H chain and tyrosine phosphorylated proteins were immunoprecipitated from total cellular lysates using pTyr-specific monoclonal antibodies and Btk protein was identified by blotting with a Btk-specific antibody. As shown in Figure 1B, pre-BCR stimulation in Slp-65 deficient pre-B cells resulted in Btk phosphorylation. Similar results were obtained in pre-BCR stimulation of short-term IL-7-dependent bone marrow-derived cultured pre-B cells from tumor-free $Slp\text{-}65^-$ mice (data not shown). These findings show that in murine pre-B cells, Btk can be phosphorylated upon pre-BCR stimulation in the absence of Slp-65.

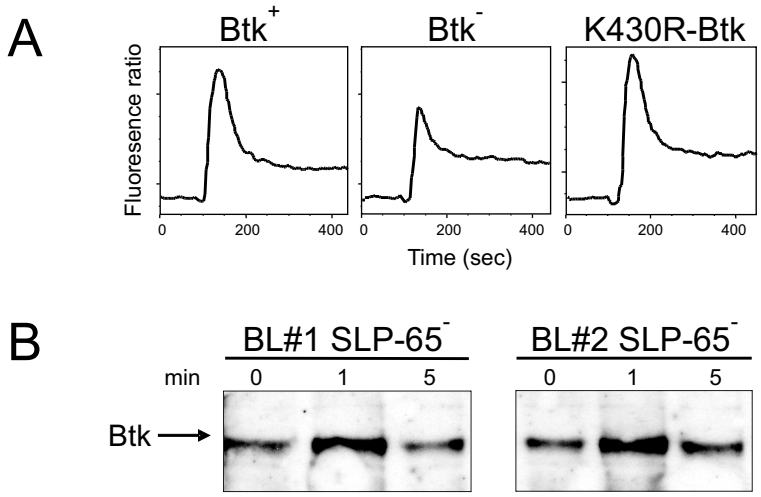
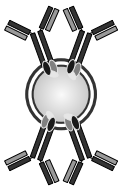


Figure 1. Kinase domain independent and Slp-65 independent function of Btk.
(A) K430R-Btk stimulates calcium flux in response to BCR activation. Indo-1 AM loaded splenic B cells of the indicated mice were stimulated with anti-IgM F(ab')$_2$ fragments and calcium flux was monitored. The plots are representative for 2-4 mice of each genotype. (B) Btk phosphorylation in the absence of Slp-65. Cultured cells from two different Slp-65$^-$ pre-B cell lymphomas (BL#1 and BL#2) were either not stimulated or stimulated for 1 or 5 min with polyclonal anti-IgM F(ab')$_2$ fragments. The presence of Btk in anti-phosphotyrosine immunoprecipitates from total cellular lysates was analyzed by western blotting using Btk-specific antibodies.



Transgenic K430R-Btk expression partially corrects the defects in B cell development in Btk/Slp-65 double mutant mice

Mice deficient for either Btk or Slp-65 have a partial block in B cell development at the pre-B cell stage in the BM, while Btk/Slp-65 double deficient mice have an almost complete block at this stage (19, 22-26). We have previously shown that expression of transgenic kinase-inactive K430R-Btk is able to partially rescue the mild differentiation defects at the pre-B cell stage and the more severe block in peripheral B cell maturation in Btk-deficient mice (18). To investigate to what extent the K430R-Btk transgene can reconstitute the almost complete pre-B cell differentiation block in Btk/Slp-65 double deficient mice, we compared the size of the B cell subpopulations in spleen and BM by flow cytometry in six different mouse groups: Btk$^+$, Btk$^-$, or K430R-Btk transgenic on a Btk$^-$ background, each of which were either Slp-65$^+$ or Slp-65$^-$.

In agreement with our reported findings (18), reconstitution with K430R-Btk partially overcame the block in peripheral B cell maturation present in Btk-deficient mice (See Table 1; Figure 2A). Also, consistent with previous findings (19, 21), the size of the total splenic B cell population in Btk/Slp-65 double deficient mice was severely reduced (~1.7 x 10^6 cells), when compared with wild-type or each

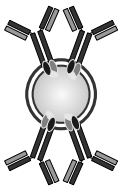
of the single deficient mice (Table 1). Expression of K430R-Btk in Btk/Slp-65 double deficient mice increased the size of the total B cell population in the spleen with a factor of ~4 (to 7.1 x 10^6 cells). This reconstitution was only partial, since the total numbers of splenic B cells in Slp-65$^-$ mice were significantly higher (~22 x 10^6 cells) (Table 1). Nevertheless, the residual B cells present, which failed to differentiate into IgM^{low}IgD^{high} B cells in Btk/Slp-65 double deficient mice, in part differentiated to IgM^{low}IgD^{high} mature B cells when the K430R-Btk transgene was present (Figure 2A).

In the bone marrow, the Btk$^-$, K430R-Btk and Slp-65$^-$ mice differed from wild-type mice by a ~50% reduction in the size of the mature IgM$^+$IgD$^+$ population of recirculating B cells (Table 1). By contrast, the Btk/Slp-65 double deficient mice manifested a dramatic reduction in the total numbers of both immature IgM$^+$IgD$^-$ and mature IgM$^+$IgD$^+$ B cells. Importantly, the size of the immature B cell population was similar in K430R-Btk/Slp-65$^-$ mice (~2.6 x 10^6) and Slp-65$^-$ mice (~3.1 x 10^6). Thus, in the absence of Slp-65, kinase inactive K430R-Btk could functionally replace Btk at the developmental progression from pre-B cells into immature B cells. From the analysis of IgM/IgD profiles of the B220$^+$ population (Figure 2C), it is clear that Slp-65$^-$ and K430R-Btk/Slp-65$^-$ mice contained similar fractions of IgM$^+$IgD$^-$ immature B cells (~12%). Expression of the K430R-Btk transgene only partly restored the reduction of the size of the mature IgM$^+$IgD$^+$ B cell population observed in Btk/Slp-65$^-$ mice (Figure 2BC, Table 1).

Table 1. Absolute numbers of B cell subpopulations in spleen and bone marrow

		Spleen	Bone marrow			
			Pro-B cells	Pre-B	Immature B	Mature B
Mouse strain	*n* [a]	B220$^+$ [b]	B220$^+$cμ$^-$	B220$^+$cμ$^+$IgM$^-$	IgM$^+$IgD$^-$	IgM$^+$IgD$^+$
Btk$^+$	14 (5)	61.0 ± 9.3	2.6 ± 0.3	13.8 ± 1.1	3.9 ± 1.3	3.7 ± 0.7
Btk$^-$	13 (5)	11.9 ± 1.3	3.0 ± 0.3	11.9 ± 1.0	3.9 ± 0.3	2.0 ± 0.2
K430R-Btk	5	23.1 ± 4.5	2.8 ± 0.4	12.8 ± 2.0	3.8 ± 0.8	2.0 ± 0.7
Slp-65$^-$	5	21.7 ± 5.0	3.0 ± 0.5	12.8 ± 2.3	3.1 ± 0.6	1.5 ± 0.4
Btk$^-$Slp-65$^-$	5	1.7 ± 0.3	3.7 ± 0.3	8.2 ± 1.6	1.1 ± 0.3	0.1 ± 0.0
K430R-Btk/Slp-65$^-$	7	7.1 ± 1.6 [c]	2.8 ± 0.3	10.4 ± 1.6	2.6 ± 0.4	0.7 ± 0.2

a) Number of mice analyzed; the numbers in parentheses indicate that the B cell population in spleen was analyzed in 5 mice per group.
b) Absolute number as average ± SEM (x 10^6).
c) The absolute numbers of splenic B220$^+$ cells in K430R-Btk/Slp-65$^-$ mice were significantly different from the values in Slp-65$^-$ mice (P=0.008) and in Btk/Slp-65 double deficient mice (P=0.025).



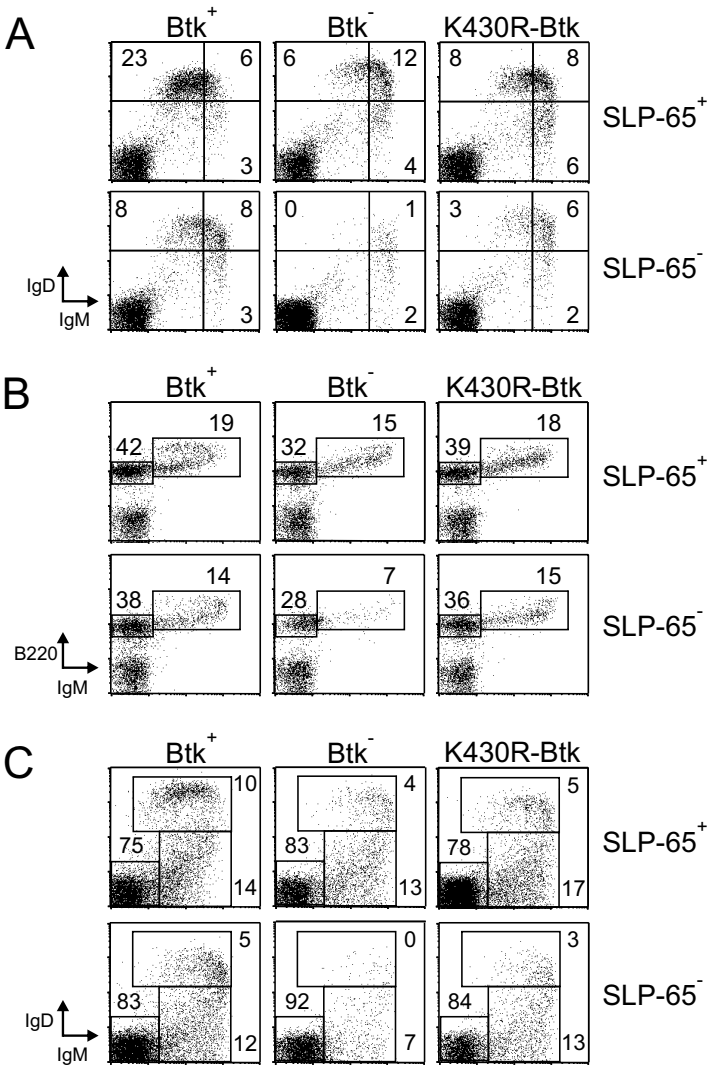


Figure 2. K430R-Btk partially corrects the defects in B cell development in Btk/Slp-65 double mutant mice.

Flow cytometric analysis of (A) spleen and (B) bone marrow of Btk$^+$, Btk$^-$ and K430R-Btk mice on either the Slp-65$^+$ or the Slp-65$^-$ background. (A) IgM/IgD profiles of total splenic lymphoid cells, (B) IgM/B220 and (C) IgM/IgD profiles of total bone marrow lymphoid cells. Lymphoid cells were electronically gated on the basis of forward and side scatter characteristics. Data are presented as dot plots and the percentages within the indicated quadrants or gates are given. The plots are representative for 5-14 mice of each genotype.

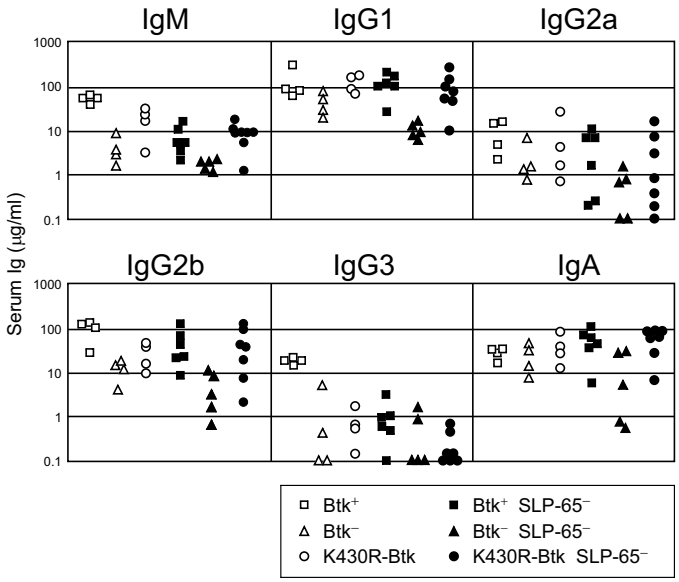


Figure 3. K430R-Btk restores serum Ig levels in Btk/Slp-65 double mutant mice.
Serum concentrations of IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA, as determined by ELISA, are show for the indicated mouse strains. Mice were 2 mo of age and each symbol represents an individual animal.

In summary, these flow cytometric analyses show that expression of kinase-inactive Btk can partially correct the severe B cell differentiation defect in Btk/Slp-65 double deficient mice, whereby the level of reconstitution is more complete in immature B cells than in mature B cells. Therefore, these results indicate that in Slp-65 independent signaling pathways, Btk kinase activity is not critical for the transition of pre-B cells into immature B cells and apparently more important for the maturation of peripheral B cells.

Btk kinase activity is specifically required for IgG3 production

To further investigate if the Btk kinase domain is essential for the synergistic role of Btk and Slp-65 in mature B cells, we investigated the serum Ig levels in the six mouse groups. When compared to wild-type mice, Btk^- and $Slp\text{-}65^-$ mice have severely decreased levels of IgM and IgG3, while other isotypes are generally not affected (22-25, 37). We have previously found that kinase-inactive Btk could significantly restore the decreased levels of IgM in serum of Btk-deficient mice, but only a limited correction of serum IgG3 was observed (18). As shown in Figure 3, Btk/Slp-65 double deficient mice have reduced serum levels of all isotypes, when compared to each of the single mutant littermates. Serum Ig levels in K430R transgenic Btk/Slp-65 double deficient mice were restored to levels similar to those

found in Slp-65 single deficient mice (Figure 3), except for IgG3. In fact, serum IgG3 levels in Btk/Slp-65 double deficient and K430R-Btk/Slp-65$^-$ mice were very low, close to the lower detection limit of the ELISA.

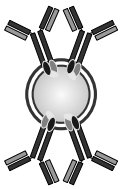
Taken together, these results indicate that with respect to late B cell and plasma cell differentiation, in the absence of Slp-65 Btk mainly acts as an scaffolding protein. Only for the differentiation of B cells into IgG3 producing plasma cells, Btk kinase activity is essential.

K430R-Btk can functionally replace wild-type Btk in cellular maturation of Slp-65-deficient pre-B cells

We have previously shown that Btk and Slp-65 are required for efficient transition of large cycling into small resting cytoplasmic μ^+ pre-B cells (21, 26). In Btk or Slp-65 mutant mice the downregulation of the pro-B/large pre-B cell-specific expression of SLC, CD43 and BP-1 and the upregulation of CD2, CD25 and MHC class II on small pre-B cells are impaired. A synergistic role for Btk and Slp-65 in this context is clear from the more pronounced defects in the modulation of these markers in Btk/Slp-65 double deficient mice (21, 26). We also reported that kinase-inactive Btk is able to partially restore the defects at the pre-B cell stage in Btk-deficient mice (18).

To investigate the effects of K430R-Btk on pre-B cell maturation in the absence of Slp-65, we compared the surface IgM$^-$ pro-B/pre-B cell fraction of Btk$^+$, Btk$^-$, and K430R-Btk transgenic mice, either on a Slp-65$^+$ or a Slp-65$^-$ background. We analyzed the expression of various developmentally regulated markers, including SLC, CD25 and MHC class II by flow cytometric analysis. In wild-type cells only a small proportion of μ^+ pre-B cells expressed SLC (~7%) (Figure 4A). As previously reported(18),(21, 26), transgenic K430R-Btk was able to restore the impaired downregulation of SLC expression in Btk-deficient mice, while Btk/Slp-65 double mutant mice manifested a much more severe defect in SLC downregulation (~71% SLC$^+$ pre-B cells). Interestingly, the levels of SLC expression in K430R-Btk/Slp-65$^-$ and Slp-65$^-$ single deficient pre-B cells were similar (~43% SLC$^+$ pre-B cells, Figure 4A), indicating that K430R-Btk could completely replace wild-type Btk function with respect to SLC downregulation. Likewise, the levels of CD25 and MHC class II induction in K430R-Btk/Slp-65$^-$ pre-B cells (~38 and ~32%, respectively) were comparable to those in Slp-65$^-$ pre-B cells (~56 and ~21%), and significantly higher than those in Btk/Slp-65 double mutant mice (~18 and 6%).

In summary, these analyses show that transgenic expression of kinase-inactive Btk is able to reduce the severe pre-B cell maturation defects in Btk/Slp-65 double deficient mice to a level that is comparable with Slp-65 single deficient pre-B cells. Therefore, we conclude that in the absence of Slp-65, Btk signaling contributes to cellular maturation of pre-B cells, independent of its kinase activity.



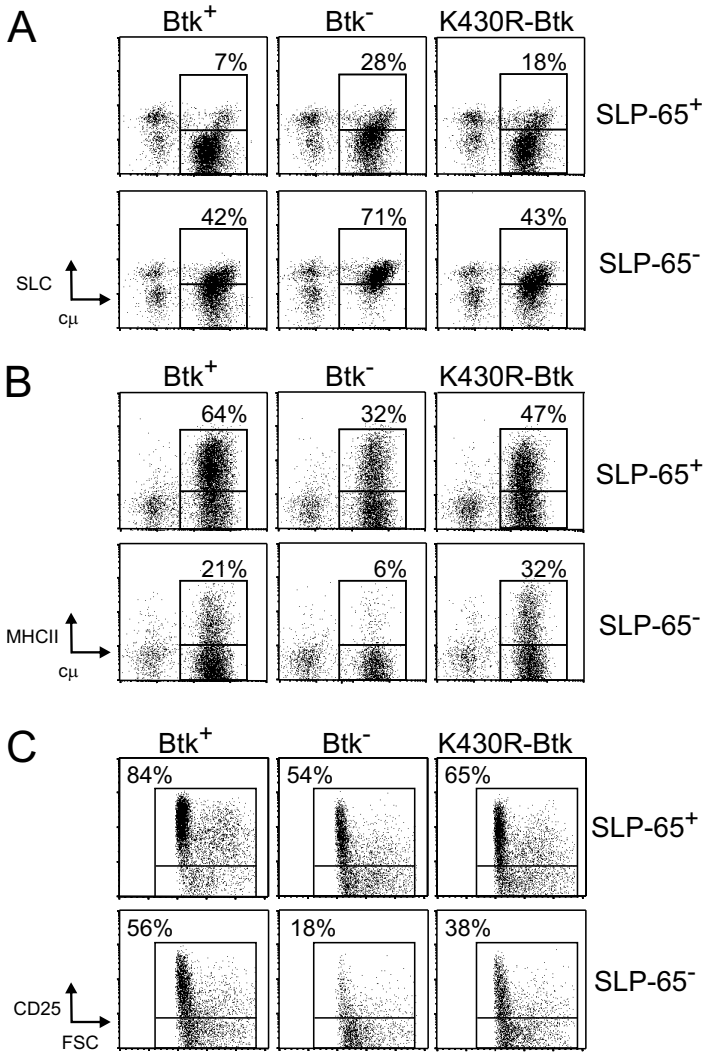


Figure 4. K430R-Btk corrects pre-B cell maturation in Btk/Slp-65 double mutant mice.
Flow cytometric analysis of gated surface IgM-negative B220$^+$ pro-/pre-B cells of Btk$^+$, Btk$^-$ and K430R-Btk mice on either a Slp-65$^+$ or the Slp-65$^-$ background. B220$^+$IgM$^-$ cells were gated and analyzed for the expression of (A) cμ/SLC, (B) cμ/MHC class II, and (C) FSC/CD25. Data are displayed as dot plots and the percentage of cells within the indicated gates are given. Data shown are representative of 5-14 mice analyzed within each group.

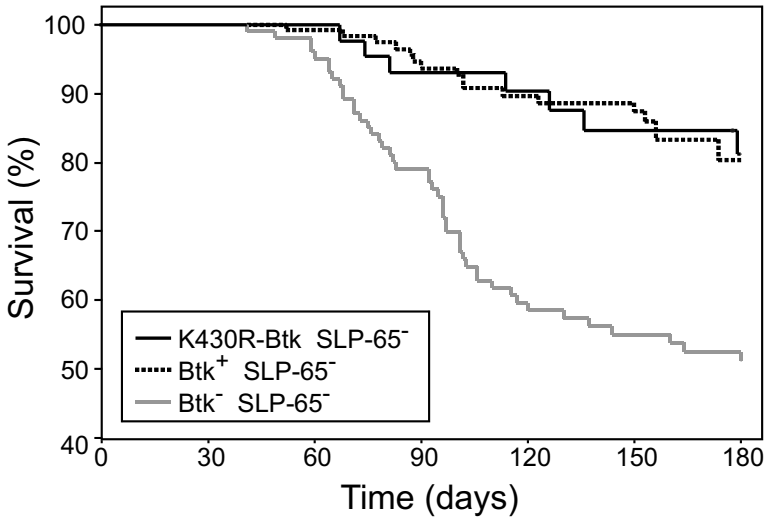
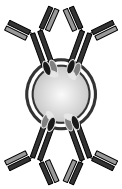


Figure 5. Tumor suppressor function of Btk is not dependent on its kinase activity.
Kaplan-Meier tumor-free survival estimates for Slp-65$^-$ (n=123), Btk$^-$Slp-65$^-$ (n=107) and K430R-Btk mice on the Btk$^-$Slp-65$^-$ background (n=51). Tumor-free survival in Btk/Slp-65 double deficient mice was significantly reduced compared with Slp-65$^-$ mice (P<0.00001, by log-rank) and to K430R-Btk/Slp-65$^-$mice (P<0.0008). Tumor-free survival in the Slp-65$^-$ and K430R-Btk/Slp-65$^-$ groups of mice was not significantly different.



K430R-Btk can functionally replace wild-type Btk as a tumor suppressor in Slp-65– mice

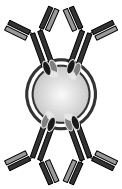
Concomitant deficiency of Btk strongly increases the frequency of pre-B cell lymphoma formation in Slp-65$^-$ mice, demonstrating that Btk cooperates with Slp-65 as a tumor suppressor in pre-B cells (21). To investigate whether the tumor suppressor function of Btk is dependent on its kinase activity, we examined the capacity of K430R-Btk to substitute for Btk by following a panel of 44 K430R transgenic mice for 180 days. At this age, 18 out of 123 (~15%) Slp-65 single deficient mice developed a pre-B cell lymphoma, while the Btk/Slp-65 double deficient mice showed a frequency of ~44% (47 out of 107). In contrast, only 7 out of 51(~14%) K430R-Btk/Slp-65-deficient mice developed a pre-B cell lymphoma (Figure 5). From these results we conclude that transgenic kinase-inactive Btk can substitute for the endogenous wild-type Btk as a tumor suppressor in Slp-65 deficient mice, and therefore that the tumor suppressor function of Btk in pre-B cells is independent of its kinase activity.

DISCUSSION

The finding that the block in B cell differentiation and the associated pre-B cell lymphoma formation in Slp-65^{-} mice is much more severe when Btk is concomitantly mutated, suggested that the two proteins do not only function in a common signal transduction pathway. In this study, we demonstrate that during pre-B cell development in the mouse, Btk functions as an adaptor molecule in a Slp-65-independent pathway.

We found that transgenic expression of the kinase-inactive K430R-Btk mutant was able to rescue the severe defects present in Btk/Slp-65 double deficient pre-B cells to such an extent that the resulting phenotype was similar to that of Slp-65 single deficient mice. In particular, we found that in the absence of Slp-65, Btk can act as an adaptor molecule that signals the modulation of SLC, CD25 and MHC class II expression in pre-B cells. Importantly, the Btk kinase activity is also not required for its function as a tumor suppressor in Slp-65 deficient pre-B cells. Expression of the K430R-Btk transgene also restored the severe defects in mature B cells, although total B cell numbers in the spleen and serum IgG3 levels were still reduced in K430R-Btk transgenic mice on the Btk/Slp-65 double deficient background, compared with Slp-65 single deficient mice. This partial inability of K430R-Btk to substitute for Btk in mature B cells could reflect their absolute dependence on Btk kinase activity. Alternatively, this might be related to the expression pattern of the K430R-Btk transgene, which is under the control of the CD19 promoter region. While the expression level is in the physiological range in the bone marrow (1-2 x endogenous levels), this increases significantly as immature B cells differentiate to mature peripheral B cells (~15 x overexpression). Therefore, it is possible that transgenic K430R-Btk expression partly has dominant-negative effects *in vivo*, which would be supported by our previous finding of reduced serum IgG3 levels and T cell independent IgM responses in K430R-Btk transgenic mice on a Btk wild-type background (18).

The mechanism by which Btk functions as an adaptor molecule in pre-B cells is not known, but it is attractive to hypothesize that equivalent to the findings in mature A20 B cells, Btk may associate with PIP5K and recruit this enzyme to the plasma membrane (15). As overexpression of PIP5K was reported to lead to an increase in the sustained calcium response upon BCR activation (12), it is assumed that by providing the substrate PIP2 for PLCγ2, PIP5K recruitment by Btk is sufficient to induce production of IP3 and DAG by PLCγ2. This mechanism would then explain the ability of kinase-inactive Btk to induce calcium mobilization in B cells upon BCR stimulation (Figure 1A). In this model, Btk should be recruited to the membrane and activated in a Slp-65 independent fashion. Although it has been proposed that Btk



phosphorylation would be Slp-65 dependent (13), other data including ours favor a model in which Btk activation is Slp-65 independent (Ref. (38, 39) and Figure 1B).

Taken together, our findings provide the biochemical basis for a parallel Slp-65-independent escape route when Btk is not able to phosphorylate PLCγ2 directly: upon pre-BCR expression, Lyn, Syk and PI3K are activated, resulting in phosphorylation and membrane translocation of Btk. Activated Btk is able to recruit PIP5K, which provides substrate for both PLCγ2 and PI3K. This mechanism may also explain the phenotype of CD19/Slp-65 double deficient mice, in which pre-B cell differentiation was also almost completely blocked (27), quite similar to the phenotype of Btk/Slp-65 double deficient mice (19, 21). From the findings in CD19/Slp-65 double deficient mice, it was concluded that the pre-B cell transition, including cell cycle progression, downregulation of RAG-2 protein and SLC expression, as well as transcriptional activation of the Ig κ locus, which is principally mediated by Slp-65 can also be (inefficiently) executed by CD19 (27). In addition, in reconstituted myeloma cells, CD19 has been shown to be necessary for efficient BCR-mediated activation of Btk (40), most likely through the PI3K pathway and the production of PIP3 in concert with Lyn- or Syk-mediated phosphorylation of Btk (41, 42). Therefore, we propose that the Slp-65-independent parallel PLCγ2 activation pathway in pre-B cells requires both Btk (as an adaptor protein) and CD19-dependent PIP3 synthesis by PI3K. In this model, a direct interaction between Btk and PLCγ2 is not required and Btk and PIP5K would interact in the absence of Slp-65.

A possibility remains that Slp-65 deficiency can be compensated by the presence of redundant proteins, because the concomitant absence of LAT in Slp-65^{-} mice resulted in an almost complete block at the large pre-B cell stage (43). Furthermore, it was shown that upon pre-BCR engagement LAT recruits PLCγ2 to the pre-BCR by association with Ig-α and the Slp-65 homologue Slp-76. These results indicate that LAT/Slp-76 can rescue PLCγ2 activation in the absence of Slp-65 (43). However, in this model the capacity of LAT/Slp-76 to replace Slp-65 function would be dependent on the presence of Btk as an adaptor molecule. Although Slp-65 or LAT/Slp-76, together with Btk, may serve as a scaffold to bring other kinase proteins in close proximity to PLCγ2, phosphorylation of two out of four regulatory tyrosines in PLCγ2 appear to be entirely dependent upon Btk/Tec family kinases, while Syk/ZAP-70 fail to modify these sites (44). Further experiments are required to investigate if Btk can interact with LAT or Slp-76.

In summary, our results show that Btk not only activates PLCγ2 by phosphorylation in a complex with Slp-65, but also has a scaffolding function in a Slp-65 independent pathway in pre-B cells. This kinase-independent Btk function provides the molecular basis of the observed cooperation of Btk with Slp-65 as a tumor suppressor in pre-B cells. Based on recent findings in mature B cells (15),

we hypothesize that Btk tumor suppressor function involves its capacity to recruit PIP5K to the membrane, which allows Btk to provide substrate for PLCγ2.

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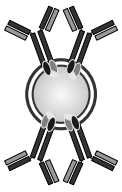

CHAPTER 5

Role of V(D)J recombination in leukemic transformation of Slp-65-deficient pre-B cells

Role of V(D)J recombination in leukemic transformation of Slp-65-deficient pre-B cells.

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Work in progress.

SUMMARY

The two pre-B cell receptor signaling components Slp-65 and Btk cooperate to limit proliferation and induce differentiation of pre-B cells in the mouse, thereby acting as tumor suppressors. Slp-65-deficient mice spontaneously develop pre-B cell leukemia. As Slp-65-deficient leukemia's express high levels of the recombination activating genes Rag 1/2 and manifest ongoing immunoglobulin light chain rearrangement, we hypothesized that malignant transformation may be dependent on deregulated V(D)J recombination. We show that combined loss of Slp-65 and the cell cycle checkpoint protein p53 transformed pre-B cells very efficiently, showing that unrepaired DNA damage possibly resulting from abortive recombination enhances malignant transformation of Slp65-deficient pre-B cells. To further analyze the involvement of V(D)J recombination in tumor formation Btk/Slp-65-deficient mice were crossed with V_H81X Ig heavy chain (IgHC) transgenic mice. The V_H81X IgHC transgene allowed progression to the pre-B cell stage and likely reduces the duration of the pro-B cell stage in which V(D)J recombination occurs. We found that expression of the V_H81X H chain transgene prevented pre-B cell tumor development in Btk/Slp-65-deficient mice. Our results suggest that deregulated V(D)J recombination activity may contribute to malignant transformation of Slp-65-deficient pre-B cells.

INTRODUCTION

B cells are produced in the BM from haematopoietic stem cells through a complex process of cellular differentiation, characterized by the ordered rearrangement of Ig Heavy and Light chain (IgHC and IgLC) gene segments encoding the B cell antigen receptor (BCR) (1-3). This process is initiated in pro-B cells with the rearrangement of IgHC V, D and J gene segments by Rag-1 and Rag-2, which are uniquely expressed in lymphoid cells. Productive V(D)J recombination results in deposition of IgHC on the surface of pre-B cells, together with the non-rearranging surrogate light chain (SLC) components λ5 and VpreB. Signaling from this pre-BCR monitors proper expression of a functional IgHC and triggers clonal expansion, whereby pre-B cells acquire the capacity to respond to low concentrations of the proliferation factor IL-7 (4-6). In the resulting large cycling pre-B cells, Rag proteins are transiently downregulated to terminate further Ig H chain rearrangement, thus ensuring that only one functional IgHC is assembled, a phenomenon termed allelic exclusion (1-3,7). Developmental progression from large cycling into small resting pre-B cells is associated with reactivation of the V(D)J recombination machinery for IgLC gene rearrangement and with cell surface marker changes (1-4).

In human, mutations in the pre-BCR downstream molecules Bruton's tyrosine kinase (Btk) or the SLP-65 adapter result in defective pre-B cell proliferation and an almost complete developmental arrest at the pro-B to pre-B cell transition (Reviewed in (4)). In contrast, in mice Slp-65 and Btk regulate pre-B cell differentiation by limiting the expansion of large pre-B cells, by downregulating the expression of SLC and the IL-7 receptor and by inducing cellular maturation (8-10). Slp-65-deficient pre-B cells are partially arrested in development at the large cycling pre-B cell stage. Importantly, Slp-65-deficient mice develop pre-B cell leukemia expressing high levels of pre-BCR (9,10). Btk/Slp-65 double deficient mice show an almost complete block at the large pre-B cell stage and have an increased tumor incidence (11,12). Therefore, Btk and Slp-65 cooperate as tumor suppressors apparently independent of Btk its kinase activity (12,13). As it has been reported that in a fraction of childhood pre-B cell acute lymphoblastic leukemia (ALL) cases SLP-65 expression is defective due to aberrant splicing (12,14,15), loss of SLP-65 and the accompanying pre-B cell differentiation arrest may be one of the primary causes of pre-B ALL in human.

Recently it has been shown that loss of Btk and its family member Tec accelerated B cell tumor formation in c-myc transgenic mice significantly (16). Additionally, Nussenzweig et al showed that transgenic expression of a pre-rearranged IgHC reduced tumor formation in c-Myc transgenic mice (17). Therefore, likely c-Myc and defective pre-BCR signaling, with Btk, Tec and Slp-65

as intermediates contribute to tumor formation. One of the possibilities explaining the tumor suppressive effect of transgenic IgHC was accelerated pro-B cell development, thereby possibly reducing the time window for V(D)J recombination.

Here, we examined the contribution of V(D)J recombination to malignant transformation of Slp-65-deficient pre-B cells. V(D)J recombination involves DNA double-strand breaks (DSB) that, if not resolved properly may give rise to chromosomal alterations and lymphoid tumors (18). The presence of unrepaired DNA DSBs generally leads to activation of the cell cycle checkpoint protein p53, which is followed by cell cycle arrest and apoptosis (19,20). Thus, it may be possible that a substantial fraction of Slp-65-deficient pre-B cells harboring Rag-mediated unresolved DNA DSBs with oncogenic potential are normally eliminated through p53-dependent apoptosis. To investigate the effect of p53-deficiency on the formation of pre-B cell tumors, we have crossed Slp-65-deficient mice onto a p53-deficient background. As it is likely that mutations have accumulated during the time of V(D)J recombination activity at the pro-B cell stage, we also studied the effect of limiting the time spent in the pro-B cell stage, using mice that express a functionally pre-rearranged IgHC transgene (21). Tumor development was followed in a panel of Btk/Slp-65-deficient mice, which did or did not carry the V_H81X transgene.

MATERIALS AND METHODS

Mice and genotyping

Btk-deficient (22), Rag-1-deficient (23) and p53-deficient (19) mice were on the C57BL/6 background. Slp-65-deficient (Balb/c) mice (24) and V_H81X transgenic mice (25) were provided by H. Jumaa (Freiburg, Germany) and J.F. Kearney (Birmingham, AL), respectively. The different composite genotypes were on a mixed background and in single experiments littermates were compared. Mice were bred and maintained in the Erasmus MC animal care facility. For mouse genotyping, genomic DNA was analyzed by PCR, as described (12,25). Rag-1 genotyping was performed using standard primers (www.jax.org). PCR products were separated by electrophoresis on 1% agarose 1x TBE gel and visualized by ethidium bromide.

Flow cytometric analyses

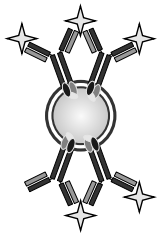
Preparations of single-cell suspensions, flow cytometry procedures, and mAbs have been described previously (8,12,22). The anti-SLC hybridoma LM34 was provided by A. Rolink (University of Basel, Basel, Switzerland). For intracellular staining of cytoplasmic proteins, cells were first stained for cell surface markers and subsequently fixed in 2% paraformaldehyde and permeabilized using 0.5%

saponin. Events (1–5 x 10^5) were scored using a FACSCalibur flow cytometer and were analyzed using CellQuest software (BD Biosciences).

Cell culture

BM cell suspensions were depleted of erythrocytes by standard ammonium chloride lysis and IL-7 driven cultures were performed as described previously (8). For proliferation assays, serial dilutions of cells (0.25-1 x 10^6 cells/well) were cultured in the presence of 100 U/ml IL-7 (R&D Systems, Minneapolis) for 5 days. Cultures were pulsed for 24 hours with 0.5 μCi/well of ^{3}H-thymidine, harvested on a glass-fiber filter, and the incorporated radioactivity was determined using a β-counter, according to standard procedures.

RESULTS AND DISCUSSION



Ongoing IgLC gene recombination in Slp-65 deficient pre-B cell leukemia's

We have previously reported that pre-B cell leukemia's from Slp-65 deficient or Btk/Slp-65 double deficient mice contained cells that co-express κ IgLC and SLC. To investigate this in more detail, we determined IgLC expression in a panel of 13 Slp-65 deficient and 17 Btk/Slp-65 double deficient pre-B cell tumors. In these leukemia samples, the majority of cells expressed high surface levels of pre-BCR (unpublished data). With the exception of two samples, all leukemia samples co-expressed SLC with surface κ IgLC, whereby the proportions of κ IgLC chain positive cells were variable (FigureE 1a). λ IgLC was generally not detectable or present in only a very small fraction of leukemic cells. Consistent with the expression of κ IgLC protein, various clonal V_κ-J_κ rearrangement patterns were detected next to the germline J_κ restriction fragment in Southern blotting analyses using a J_κ-specific probe (Figure 1, b and c). In most of the tumors analyzed, we observed multiple J_κ restriction fragments, indicating oligoclonality. IgLC recombination is generally initiated in small resting pre-B cells that have terminated SLC expression (1,4, 5). Therefore, the finding that Slp-65- and Btk/Slp-65-deficient pre-B cell leukemias contain oligoclonal κ^+ B cell fractions co-expressing SLC indicated that IgLC gene rearrangement occurred after leukemic transformation of pre-B cells. As leukemic transformation likely generates only one dominant clone and probably takes place before the IgLC positive immature-B cell stage these data indicate continued rearrangement activity at the IgLC loci in leukemic B cells.

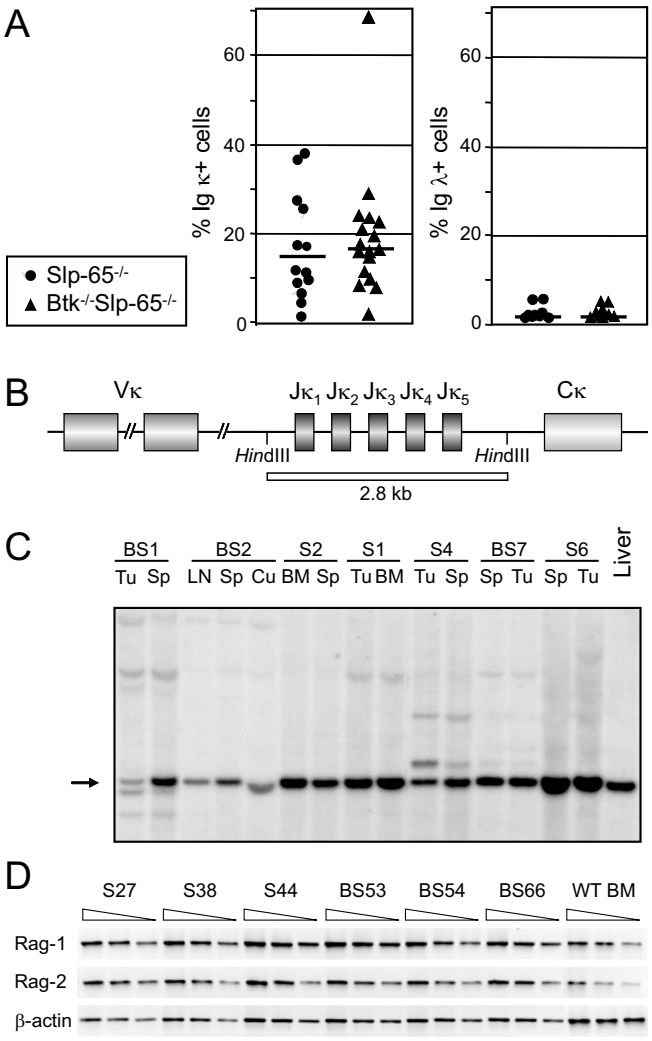
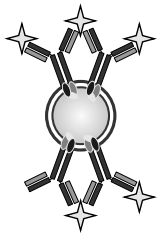


Figure 1. Ongoing V(D)J recombinase activity in tumors.
(A) Flow cytometric characterization of Slp-65-deficient and Btk/Slp-65-double deficient Slp-65-single deficient and Btk/Slp-65-double deficient tumors. $B220^+Ig\mu^+SLC^+$ tumor cells were gated and analyzed for the expression of Ig L chains. (B) Schematic structure of the germline Ig κ L chain locus. Restriction sites of HindIII and the 2.8 kb κ germline fragment are shown. (C) Southern blotting analysis of HindIII digests of genomic DNA from the indicated tissues from Slp-65-single deficient (S) and Btk/Slp-65-double deficient (BS) tumors. κ IgLC chain gene rearrangements were analyzed using a J_κ probe. The arrow indicates the 2.8 kb κ germline band. Tu= tumor; Sp= spleen; Cu= cultured cells from tumors. (D) Expression of Rag-1 and Rag-2 in pre-B cell tumors. RNA was reverse transcribed, diluted serially threefold, and used as a template for PCR amplification, using β-actin (bottom) as a control.



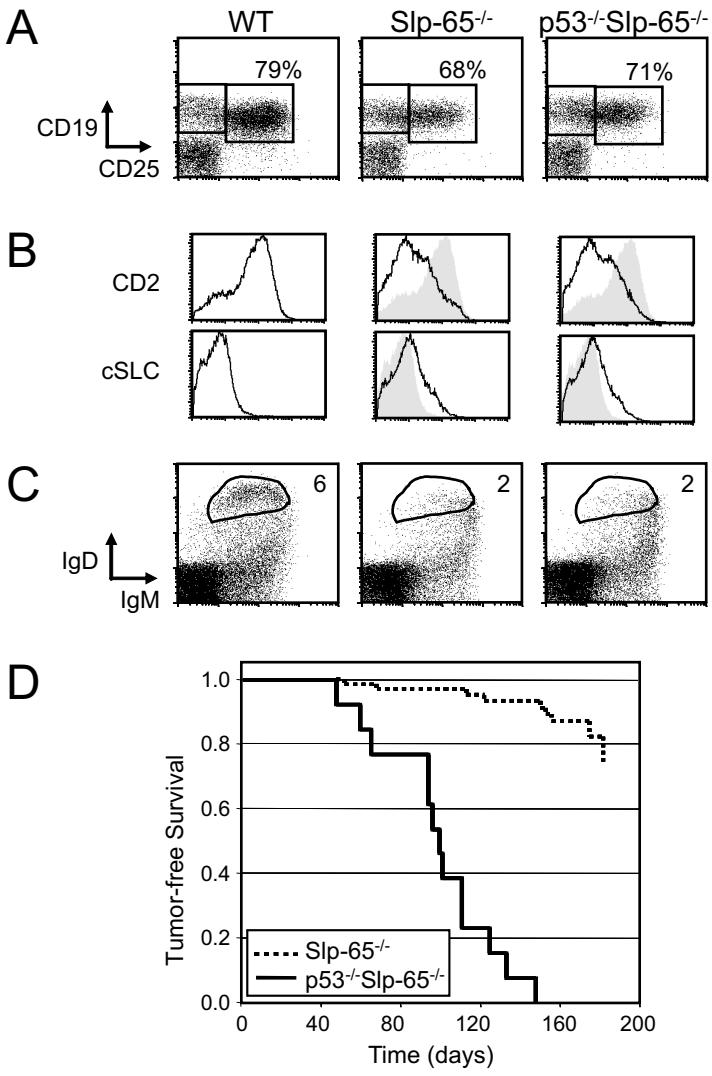


Figure 2. Slp-65 and p53 cooperate as tumor suppressors in pre-B cells

(A) Flow cytometric analysis of BM cell suspensions obtained from the indicated mice. Lymphoid cells were gated on the basis of FSC and SSC characteristics and CD19/CD25 profiles are shown. Percentages indicate the proportions of $CD25^+$ cells within the $CD19^+$ gate. (B) $CD19^+$ B-lineage cells were gated and analyzed for CD2 and cSLC expression. In the Slp-65-single deficient and p53/Slp-65-double deficient mice, results are displayed as histogram overlays (black lines), compared with the wild-type (shaded histograms). (C) $CD19^+$ cells were gated and displayed as IgM/IgD dot plots. The proportions of mature recirculating IgM^+IgD^+ cells are indicated. Four five-week-old non-tumor bearing mice were analyzed per group and representative plots are shown. (D) Kaplan-Meier tumor-free survival estimates for Slp-65-single deficient mice (n=26) and p53/Slp-65-double deficient mice (n=17).

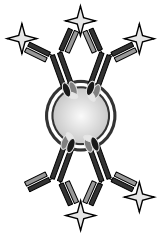
During normal B cell development, V(D)J recombination activity is downregulated upon pre-BCR expression and therefore large cycling pre-B cells contain low levels of Rag transcripts (2). In contrast, when we analyzed Slp-65 single deficient or Btk/Slp-65 double deficient pre-B cell leukemias by RT-PCR we found that Rag-1 and -2 were highly expressed (Figure 1 d). The observed presence of Rag transcripts supports the notion that the V(D)J recombination machinery is active in Slp-65 single deficient or Btk/Slp-65 double deficient pre-B cell leukemias.

P53 and Slp-65 cooperate as tumor suppressors

B cell precursors harboring Rag-mediated unresolved DNA damage will be eliminated through activation of the p53 cell cycle checkpoint. Therefore, we investigated the effect of p53-deficiency on pre-B cell tumor formation by crossing Slp-65-deficient mice onto a p53-deficient background. The absence of p53 did not markedly affect the initial viability of Slp-65-deficient mice. In flow cytometric experiments, no significant differences were found between the sizes of the individual B-lineage subpopulations in Slp-65 deficient mice and p53/Slp-65 double deficient mice: the B-lineage cells remained partially arrested at the $CD2^{low}SLC^{+}$ pre-B cell stage in the BM (Figure 2, a and b) and mature recirculating $IgM^{low}IgD^{+}$ B cells were severely reduced (Figure 2 c). The in vitro proliferative capacity of Slp-65-deficient pre-B cells in IL-7 driven BM cultures was not affected by the concomitant absence of p53 (unpublished data). Whereas ~25% of the Slp-65 deficient mice developed pre-B cell leukemia before the age of 6 months, all p53/Slp-65 deficient mice developed pre-B cell leukemia within 150 days after birth (Figure 2 d). The pre-B cell tumors in p53/Slp-65 deficient mice were positive for Ig μ H chain and SLC, similar to those normally found in Slp-65 deficient mice. In contrast, p53-deficient mice on this genetic background occasionally developed T cell lymphoma (only 1 out of 20 mice, at 26 weeks of age). Thus, loss of Slp-65 on the p53 deficient background results in the rapid onset of pre-B cell tumors before the age at which T cell tumors usually arise in p53 deficient mice. Therefore Slp-65 and p53 cooperate as tumor suppressors and may do so by limiting DNA damage.

B cell development in V_H81X IgHC transgenic mice

To investigate if insufficiently controlled V(D)J recombination contributes to malignant transformation of Slp-65-deficient pre-B cells, we employed mice carrying the functionally pre-rearranged IgHC transgene V_H81X. The expression of such an IgHC transgene allows the formation of a pre-BCR and progression to the pre-B cell stage. Likely, the expression of the transgenic IgHC protein accelerates the passage of B cell precursors through the pro-B cell stage (21), possibly limiting the time window of V(D)J recombination activity and the generation of DNA DSBs.



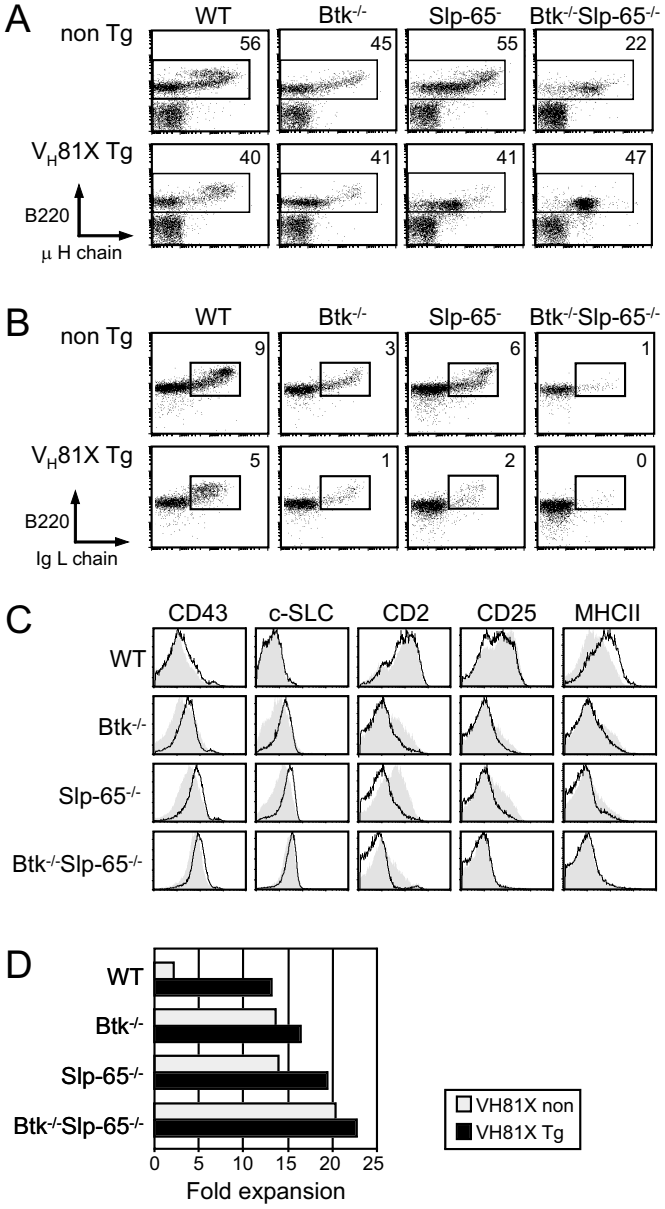


Figure 3. The V_H81X transgene does not effect the pre-B cell arrest in Btk or Slp-65 single and double deficient mice.

(A) Flow cytometric analysis of BM lymphoid cells from the indicated mouse groups, either non-V_H81X transgenic (upper panels) or carrying the V_H81X transgene (lower panels). B220/μ H chain profiles are displayed as dot plots. Total $B220^+$ B-lineage cells were gated and percentages within the indicated gates are shown. Data are representative for 4 independent experiments. (B) Cytoplasmic μ H $chain^+$ B-lineage fractions from total BM were analyzed for κ/λ IgLC chain surface expression. Data are shown as dot plots,

whereby the numbers indicate the proportions of cytoplasmic μ^+ surface κ/λ^+ $B220^+$ cells from total BM cells. (C) $B220^+$ Ig κ/λ^- pro-/pre-B cells were gated and analyzed for the expression of CD43, cSLC, CD2, CD25 and MHCII. The results are displayed as histogram overlays of non-V_H81X transgenic (shaded histograms) and V_H81X transgenic (black lines) pro-/pre-B cell fractions of the mice indicated on the left. Plots are representative for 4-5 animals of each genotype. (D) Effect of the V_H81X transgene on pre-B cell proliferation. Total BM cells were cultured for 5 days in the presence of IL-7 and proliferative responses were determined by calculation of fold expansion, whereby the pre-B cell numbers at the start of the culture where set to one. Data are from one out of two independent experiments.

To ascertain that the V_H81X transgene did not affect the generation of large and small pre-B cell compartments, we compared 8 groups of mice by flow cytometry: WT, Btk-deficient, Slp-65-deficient and Btk/Slp-65-double deficient mice, which either did or did not carry the V_H81X transgene (Figure 3). In agreement with reported findings (11,12), in the absence of Btk or Slp-65 the proportions of $B220^{high}Ig\mu^+Ig\kappa^+$ recirculating B cells in the BM were reduced, while in Btk/Slp-65 double deficient mice B cell development was almost completely blocked at the $B220^{high}Ig\mu^+Ig\kappa^-$ pre-B cell stage (Figure 3a, b). These phenotypes were not significantly altered by the presence of the V_H81X transgene. In mice that carried the V_H81X transgene, the proportions of surface Ig positive B cells were slightly reduced in the bone marrow (Figure 3b) and spleen (unpublished data), when compared with mice of the same genotype that did not carry the transgene.

A detailed analysis of the fraction of $B220^+IgLC^-$ cells showed that in the absence of Btk or Slp-65 pre-B cells failed to efficiently downregulate CD43 and SLC, and to efficiently initiate CD2, CD25 and MHC II expression (Figure 3c; as reported previously (8,12). For all four genotypes, the presence of the V_H81X transgene did not significantly affect the expression of these developmentally regulated markers (Figure3 c). The presence of the V_H81X transgene resulted in enhanced IL-7 responsiveness of wild-type pre-B cells *in vitro* (14-fold expansion in V_H81X Tg versus 3-fold in non-V_H81X Tg), but the high proliferative capacities of Btk-, Slp-65- or Btk/Slp-65-deficient pre-B cells (8,12,24) were not significantly increased by the presence of the V_H81X transgene (Figure 3 d).

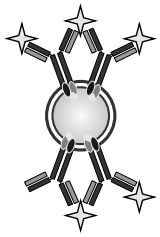
We conclude that the phenotypical abnormalities of Btk-, Slp-65- and Btk/Slp-65-deficient pre-B cells, which reflect their impaired cellular maturation from large cycling into small resting pre-B cells, were preserved in the presence of the pre-rearranged V_H81X Ig H chain transgene.

Expression of the V_H81X IgHC transgene prevents pre-B cell leukemia in Btk/Slp-65-deficient mice

To address the effect of IgHC transgene expression on pre-B cell leukemia formation, we followed panels of Btk/Slp-65-deficient mice, which did or did not carry the V_H81X IgHC transgene. At 6 months of age, 16 out of 20 Btk/Slp-65-deficient mice developed pre-B cell leukemia (Figure 4). Strikingly, expression of the V_H81X IgHC

transgene prevented pre-B cell leukemia formation in Btk/Slp-65-deficient mice. Also when we examined BM and spleen of aged mice by flow cytometry we did not find evidence for lymphoproliferative disease. Therefore, we conclude that the expression of the pre-rearranged V_H81X IgHC transgene in early B cells prevents oncogenic transformation of Btk/Slp-65-double deficient pre-B cells.

Our findings show that oncogenic transformation of Slp-65-deficient pre-B cells does not exclusively result from the arrest of B cell differentiation at the large cycling pre-B cell stage. Instead, it appears that mutations have accumulated before this stage, since early expression of a functional IgHC is known to considerably shorten or even bypass pro-B cell development (21). It is thought that acceleration of the passage of B cell precursors through the pro-B cell stage prevents the oncogenic transformation. Supporting this view very few pro-B cells were present in V_H81X transgenic mice (data not shown). The tumor suppressive effect of the V_H81X transgene was similar to the previously reported finding that expression of an IgHC transgene greatly reduced tumor incidence in c-myc transgenic mice (17). An alternative explanation, that the tumor-preventing effect of V_H81X H chain is due to its signaling incompetence, is very unlikely, because (i) the V_H81X H can pair efficiently with IgLC, (ii) the V_H81X pre-BCR induces strong proliferation (Figure 3 d), and (iii) the presence of the V_H81X transgene does not significantly affect B cell differentiation (Figure 4).



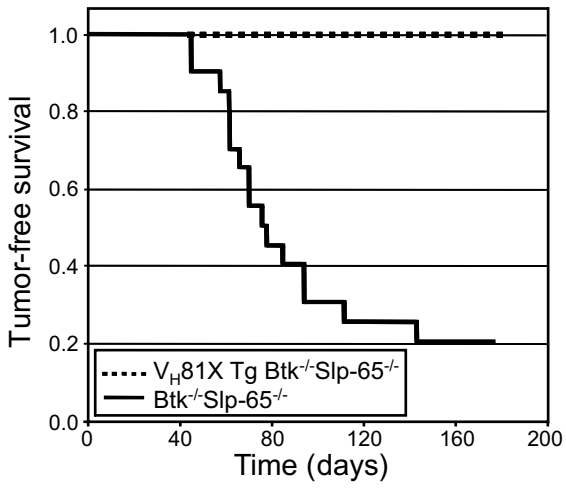


Figure 4. The V_H81X transgene prevents tumor formation in Btk/Slp-65-deficient mice.
Kaplan-Meier tumor-free survival estimates for Btk/Slp-65-deficient mice (n=20) and V_H81X transgenic Btk/Slp-65-deficient mice (n=26).

Obviously, our findings do not directly indicate that V(D)J recombinational activity plays an important role in Btk/Slp-65-deficient oncogenesis. Reports on deranged V(D)J recombinational and nonhomologous DNA end-joining activity in pro-B cells, such as e.g. found in p53/artemis-double deficient mice, described that the progenitor B lymphomas that developed, harbored common chromosomal abnormalities, involving translocation and amplification of the IgHC gene locus, together with the N-Myc or c-Myc loci (26,27). Although we detected N-myc locus amplification in three Slp-65 pre-B cell tumor cases, we did not find common chromosomal abnormalities involving the IgHC gene locus by spectral karyotyping or Southern blotting analysis (V.T., P.t.B. J.P.v.H., R.K., unpublished findings) Alternatively, our results from V_H81X transgenic mice may be explained by a developmental stage-dependent transformation by IL-7 R signaling. Furthermore, p53-deficiency may not only point to chromosomal instability as the p53 pathway can be activated by multiple other triggers such as heat shock protein release, osmotic shock en cytokine signaling.

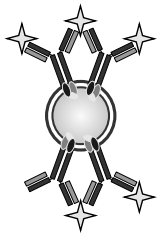
CONCLUSIONS

Several findings indicate that V(D)J recombination is ongoing in Slp-65-deficient large pre-B cells. First, Slp-65-deficient and Btk/Slp-65-double deficient mice pre-B cell tumors mostly co-express IgHC, SLC, IgLC and Rag transcripts. Second, pre-B cell fractions from CD19/Slp-65-deficient mice show increased expression of Rag-2 protein (10). Third, In human pre-B cell leukemia SLP-65 deficiency correlates with RAG expression and ongoing V_H gene rearrangement activity (28).

In this report, we show that expression of the V_H81X IgHC transgene prevents leukemic transformation of Btk/Slp-65-double deficient pre-B cells and that loss of p53 increases tumor development in Slp-65-deficient mice. The tumor preventing mechanism of the IgHC transgene may be based on its effect on V(D) J recombination activity in the context of allelic exclusion and by limiting the time window for V(D)J recombination activity. Thus, although some of our data point in the direction of deranged V(D)J recombinational activity, the mechanism and definitive proof await further experiments. In any case, we have identified the pro-B cell stage as critical stage in the oncogenic transformation process of Btk/Slp-65-deficient pre-B cells. Therefore, we conclude that developmental stage related factors and deregulated V(D)J recombination activity may contribute to malignant transformation of Btk/Slp-65-deficient pre-B cells.

ACKNOWLEDGEMENTS

We thank T. van Os, B. Beverloo, E. Zijlstra (Erasmus MC, Rotterdam) for their assistance at various stages of the project. This work was partially supported by the Netherlands Organisation for Scientific Research NWO-Mozaiek, the Dutch Cancer Society and the Association for International Cancer Research.



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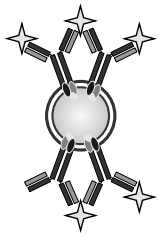

CHAPTER 6

Bruton's Tyrosine Kinase and Phospholipase Cγ2 Mediate Chemokine-Controlled B Cell Migration and Homing

Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing

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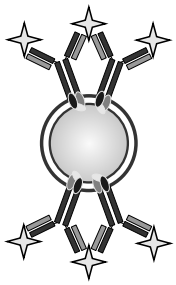
SUMMARY

Control of integrin-mediated adhesion and migration by chemokines plays a critical role in B cell development, differentiation, and function; however, the underlying signaling mechanisms are poorly defined. Here we show that the chemokine SDF-1 induced activation of Bruton's tyrosine kinase (Btk) and that integrin-mediated adhesion and migration in response to SDF-1 or CXCL13, as well as in vivo homing to lymphoid organs, was impaired in Btk-deficient (pre-)B cells. Furthermore, SDF-1 induced tyrosine phosphorylation of Phospholipase Cγ2 (PLCγ2), which, unlike activation of the migration regulatory GTPases Rac or Rap1, was mediated by Btk. PLCγ2-deficient B cells also exhibited impaired SDF-1-controlled migration. These results reveal that Btk and PLCγ2 regulate chemokine-controlled migration, thereby providing insights into the control of B cell homeostasis, trafficking and function, as well as into the pathogenesis of the immunodeficiency disease X-linked agammaglobulinemia (XLA).

INTRODUCTION

Integrin-mediated cell adhesion and migration play a critical role in a wide variety of processes underlying proper organization and function of the immune system, including B cell development and differentiation. During early B cell development, the consecutive generation of pro-, pre-, and immature B cells requires their retention in defined microenvironments in the bone marrow (BM), which is controlled by interactions of integrin $\alpha4\beta1$ with fibronectin (FN) in the extracellular matrix (ECM) and with vascular cell adhesion molecule-1 (VCAM-1)-expressing BM stromal cells (1-4). In mature B cells, integrins $\alpha4\beta1$ and leukocyte function-associated antigen-1 (LFA-1) mediate high endothelial venule (HEV) attachment and transendothelial migration required for recirculation and homing , and they control cell compartmentalization in peripheral lymphoid tissue (3, 5-8). Integrins $\alpha4\beta1$ and LFA-1 also play a key role in the T cell-dependent humoral immune response, being involved in migration of naive B cells into B-cell follicles and in the interaction of germinal center (GC) B cells with antigen-presenting follicular dendritic cells (FDCs) during antigen-specific B cell differentiation (2, 3, 9-12).



Chemokines play a prominent role in controlling integrin-mediated adhesion and migration (13, 14). As such, the so-called 'homeostatic' chemokines SDF-1 (CXCL12) and CXCL13 (BLC or BCA-1) and their respective G-protein-coupled receptors CXCR4 and CXCR5, play a major role in B cell homeostasis, trafficking and function (5, 6, 8, 15). CXCR4 is expressed by all B cell subsets (16), and SDF-1 is highly expressed by stromal cells in the BM and GCs, by reticulum cells aligning the GCs, in the splenic red pulp and lymph node medullary cords, and is present on HEV in lymph nodes and Peyer's patches (5, 6, 17, 18). SDF-1-CXCR4 signaling plays a critical role in a variety of processes underlying proper B cell development and function, including development and retention of precursor B cells in the BM, homing of mature B cells to secondary lymphoid organs, trafficking and homing of plasma cells to BM, GC organization, and T-independent humoral immune responses (4-6, 17-21). CXCR5 is mainly expressed by mature B cells (16), and CXCL13 is produced in follicles and is present on HEV of lymph nodes and Peyer's patches (8). CXCL13-CXCR5 signaling is required for migration of naive B cells into follicles and for GC organization (6, 15, 17, 18, 22, 23).

Despite the important role of chemokine-controlled integrin-mediated migration in B cell development and function, the underlying signal transduction mechanisms are as yet poorly defined (13, 14). Interestingly, many of the B cell defects in mice deficient in SDF-1-CXCR4, CXCL13-CXCR5, VCAM-1 or $\alpha4$ integrin, such as impaired development and retention of B cell precursors in the BM, and impaired B cell differentiation and immune responses (1, 2, 17, 18, 20-24), are similar to

the defects observed in the immunodeficiency diseases X-linked immunodeficiency (Xid) in mice and XLA in men, caused by loss-of-function germline mutations in the cytoplasmic tyrosine kinase Btk (25-31). Combining this notion with our recent finding that Btk is required for the control of integrin-mediated adhesion by the B cell antigen receptor (BCR) (12), we hypothesized that Btk may be involved in the signaling mechanism underlying chemokine-controlled integrin-mediated migration. Here, we demonstrate that SDF-1- or CXCL13-controlled integrin-mediated adhesion and migration, as well as in vivo homing of pre-B and B cells, was indeed mediated by Btk. Furthermore, Btk mediated SDF-1-induced phosphorylation of PLCγ2, and PLCγ2 mediated SDF-1-controlled migration as well. This function for Btk implies that impaired chemokine-controlled migration may contribute to the developmental and functional B-cell defects observed in XLA and Xid.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for complete methods.

Materials

The following reagents were used in this study: phosphorylation state-specific antibodies phospho-p44/42 MAP kinase [T202/Y204], phospho-Akt [S473], phospho-Btk [Y223], phospho-PLCγ2 [Y759], and phospho-PLCγ2 [Y1217] (Cell Signaling Technology); phosphotyrosine antibodies PY20 (BD Biosciences) and 4G10 (Upstate Biotechnology); rabbit polyclonal antibodies against ERK2 (C-14), Akt1/2 (H-136), Btk (C20), PLCγ2 (Q20 and H160) (Santa Cruz Biotechnology), and CXCR4 (AB 1846) (Chemicon); mouse monoclonal antibodies against T7 (Novagen), Btk (G149-11), Rap1 and Rac1 (BD Biosciences); mouse monoclonal IgG1 antibodies HP2/1 against integrin subunit α4 (Immunotech), Act-1 against integrin subunit β7 (kindly provided by Dr. A. Lazarovits), TS2/16 against integrin subunit β1 (kindly provided by Dr. F. Sanchez-Madrid), CSAT IgG2b against the chicken integrin subunit β1 (DSHB, University of Iowa); rat and hamster monoclonal antibodies PS/2-biotin and OXM718-FITC against mouse integrin α4 and β1, respectively (Chemicon), and 2G8-biotin against CXCR5 (BD Biosciences); anti-CD45R(B220) microbeads (Miltenyi Biotec); mouse anti-human IgM (MH15) (Sanquin, Amsterdam, the Netherlands), goat anti-chicken IgM (Bethyl Laboratories); anti-B220-FITC (Leinco Technologies), anti-IgM-biotin (BD Biosciences), Streptavidin-PE, Streptavidin-FITC, goat-anti mouse-Biotin, goat anti-mouse-PE (Southern Biotechnology Associates), Swine anti-Rabbit-FITC (DAKO), and rabbit anti-goat-Biotin (Vector); pharmacological inhibitors PD-98059, LY-294002, Wortmannin, U-73122 (Biomol); recombinant human sVCAM-1, SDF-

1α and BCA-1/CXCL13 (R&D Systems), human plasma FN, and BSA (fraction V) (Sigma); CMFDA and CMTMR (Molecular Probes).

Isolation of tonsillar B cells, murine pre-B cells and splenic B cells

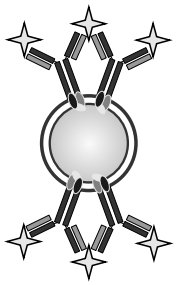
Human tonsillar B cells, mouse splenic B cells from Btk-deficient mice and WT littermate controls, and mouse pre-B cells (IL-7-driven primary BM cultures of WT and Btk-deficient mice) were obtained essentially as described (12, 30, 32).

Cell migration assay

Migration assays were performed in triplicate using Transwells (Costar) coated with 1 μg/ml sVCAM-1 or 10 μg/ml FN. The lower compartment contained 100 ng/ml SDF-1 or 500 ng/ml CXCL13, and the cells were applied to the upper compartment and allowed to migrate for 5 hrs (DT40 and Namalwa), 3.5 hrs (tonsillar B cells), 2.5 hours (splenic B cells), or 1 hr (pre B cells) at 37°C (39.5°C for DT40), unless otherwise indicated. The amount of viable migrated cells was determined by FACS and expressed as a percentage of the input. Unless otherwise indicated, the migration of non-pretreated WT cells on VCAM-1-coated Transwells in the presence of SDF-1 or CXCL13 was normalized to 100%, and the bars represent the means ±SD of at least 3 independent experiments, each assayed in triplicate.

Cell adhesion assays

The static cell adhesion to VCAM-1 was assayed essentially as described (Spaargaren *et al.*, 2003), except that 150 ng/ml SDF-1 was co-immobilized with 1 μg/ml VCAM-1 on the plates, the plates were spun directly after applying the cells to the plate, and the cells were allowed to adhere for 2 min.



The under flow cell adhesion to HUVECs was assayed essentially as described (da Costa Martins *et al.*, 2006). Briefly, primary HUVECs were overlaid with 10 ng/ml SDF-1 prior to perfusion, DT40 cells were perfused at 0.8 dyn/cm^2 for 5 min, and 20 randomized images recorded between 2 and 5 min were analyzed to determine the average amount of adhering cells per field.

In both assays, the adhesion of WT cells in the presence SDF-1 was normalized to 100%.

Homing assay

Cells from IL-7-driven BM cultures of WT and Btk-deficient mice were labeled either with CMFDA or CMTMR, mixed (1:1) and injected intraveneously in C57Bl/6 mice. Each WT/Btk$^-$ combination was analyzed by adoptive transfer of 2 recipient mice, which included a dye-swap. After 3 hrs, lymphoid organs were collected and FACS-analyzed to identify pre-B and immature B cells by their B220/IgM profile and to

quantify dye-labeled cells. The homing ratio (Btk-deficient/WT cells) was corrected for the input ratio (which was normalized to 1).

Immunoprecipitation and immunoblotting

For analysis of Btk and PLCγ2 tyrosine phosphorylation, cells were stimulated with 200 ng/ml SDF-1 or 10 µg/ml anti-IgM and immunoprecipitated with anti-Btk (C20), anti-T7, anti-P-Btk, anti-PLCγ2 (Q20), or anti-phosphotyrosine (PY20 or 4G10). Immunoprecipitates and total lysates were analyzed by SDS-PAGE and immunoblotting, using anti-P-Btk, anti-Btk (G149-11), anti-P-PLCγ2, anti-PLCγ2 (H160), or anti-phosphotyrosine (4G10).

For analysis of ERK and PKB phosphorylation, cells were stimulated with 100 ng/ml SDF-1, and immunoblotted with anti-phospho-MAPK and anti-phospho-PKB, as described (12).

Rac and Rap1 activity pull-down assays

Cells were stimulated with SDF-1 (100 ng/ml) and cell lysates were prepared and used immediately for GTPase pull down assays using GST-RalGDS-RBD or GST-PAK-RBD fusion protein for Rap and Rac, respectively. Bound proteins were eluted with sample buffer, separated by 15% SDS-PAGE, and immunoblotted with anti-Rap1 or anti-Rac1.

Statistical analysis

The unpaired two-tailed Student's t-test was used to determine the significance of differences between means. Unless otherwise indicated, all relevant comparisons (*e.g.*, control versus inhibitors or WT versus gene-deficient cells) were significantly different ($p<0.05$)

RESULTS

SDF-1 and CXCL13 induce integrin-mediated migration of B cells

To study the control of integrin-mediated B cell migration by the chemokines SDF-1 and CXCL13, murine BM-derived pre-B cells, murine splenic B cells and human tonsillar B cells were assayed for migration towards these chemokines in a Transwell system of which the membrane was either uncoated or coated with VCAM-1 or FN. Although SDF-1 strongly induced the migration of these primary B cells on uncoated membranes, migration towards SDF-1 was more pronounced (~3-fold increase) on membranes coated with VCAM-1 (Figures 1A-C). Similar results were obtained for migration on FN (not shown) and for migration of murine splenic B cells and tonsillar B cells towards CXCL13 (Figure 1B and C).

VCAM-1 and FN (not shown) also enhanced migration towards SDF-1 of the human GC B cell-like cell line Namalwa (Figure 1D), and the chicken DT40 B cells (Figure 1E), by approximately 3-fold. As shown in Figure 1F, the integrin $\alpha4\beta1$ blocking antibody HP2/1 and the $\alpha4\beta1$ activating antibody TS2/16 completely abolished VCAM-1-mediated migration of Namalwa cells towards SDF-1, whereas no effect was observed with either an isotype control IgG1 or the integrin $\alpha4\beta7$ blocking antibody Act-1. Furthermore, antibody CSAT, directed against the chicken integrin $\beta1$ subunit, strongly suppressed SDF-1-induced migration of DT40 cells on VCAM-1 (Figure 1G). Taken together, our data demonstrate that SDF-1 and CXCL13 control integrin ($\alpha4\beta1$)-mediated migration of primary B cells and B cell lines.

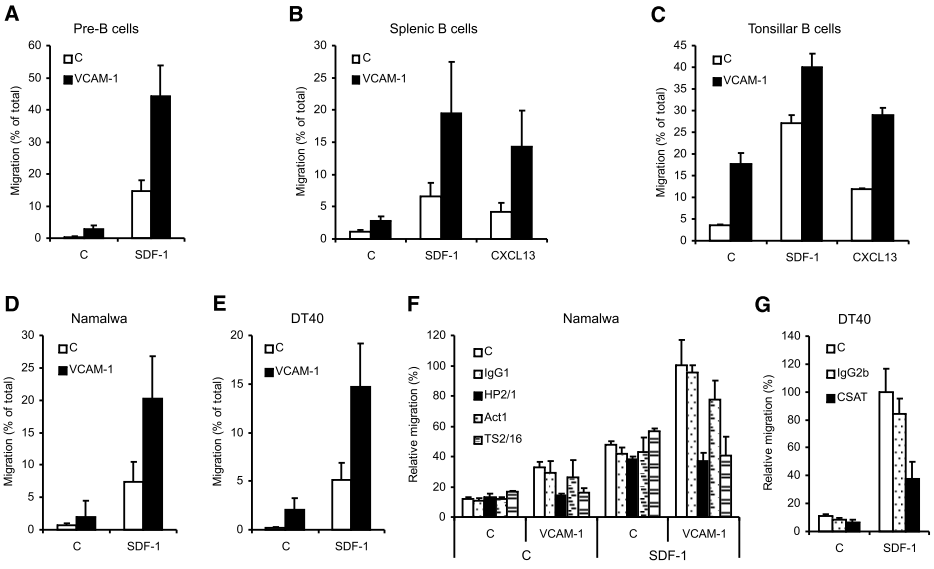


Figure 1. **SDF-1 and CXCL13 induce integrin-mediated B cell migration on VCAM-1**
(A-E) Mouse pre-B cells (A), mouse splenic B cells (B), human tonsillar B cells (C), Namalwa cells (D), and DT40 cells (E) were allowed to migrate in the absence or presence of 100 ng/ml SDF-1 (SDF-1) or 500 ng/ml CXCL13 in Transwells that were either uncoated or coated with 1 μg/ml VCAM-1, as indicated. The bars represent the means ±SD of a representative experiment (C), or of 6 (A,B and D) or 14 (E) independent experiments, each assayed in triplicate, and presented as percent migration of total cells.
(F) Namalwa cells were preincubated for 1 hour at 4°C with medium alone (none), 10 μg/ml of an IgG1 isotype control (IgG1), or antibody Act-1 blocking $\alpha4\beta7$ (Act-1), HP2/1 blocking $\alpha4\beta1$ (HP2/1), or antibody TS2/16 activating integrin $\alpha4\beta1$ (TS2/16). Subsequently, cells were allowed to migrate for 2 hrs in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in Transwells that were either uncoated or coated with 1 μg/ml VCAM-1, as indicated.
(G) DT40 cells were preincubated for 1 hour at 4°C with medium alone (none), or 10 μg/ml of an IgG2b isotype control (IgG2b) or antibody CSAT against integrin subunit $\beta1$ (CSAT). Subsequently, cells were allowed to migrate for 2 hrs in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in Transwells that were either uncoated or coated with 1 μg/ml VCAM-1, as indicated.
(*F,G*) The migration was normalized to 100% for the cells not pretreated with antibody allowed to migrate in the presence of SDF-1 in Transwells coated with 1 μg/ml VCAM-1. The experiments shown are representative for 3 independent experiments, each assayed in triplicate.

Btk mediates chemokine-controlled migration

As many of the defects observed in mice deficient in SDF-1-CXCR4, CXCL13-CXCR5, VCAM-1 or α4 integrin are also observed in *Xid* or Btk-deficient mice and XLA patients (as elaborated in the Discussion), and as we have recently established that Btk is required for the control of integrin-mediated adhesion by the BCR (Spaargaren *et al.*, 2003), we hypothesized that Btk might be involved in the signaling mechanism underlying chemokine-controlled integrin-mediated migration.

In support of a possible role for Btk in chemokine-controlled migration, we observed SDF-1-induced tyrosine phosphorylation of Btk in DT40 cells, Namalwa cells and human tonsillar B cells (Figure 2A). Phosphorylation of Btk could be detected using either a general phosphotyrosine antibody or a phospho-specific antibody for the autophosphorylation site Y223, reflecting Btk activation. Typically, SDF-1 induced Btk phosphorylation in a fast and transient fashion with optimal phosphorylation being observed between 30 sec and 2 min. A similar degree of Btk phosphorylation was observed after suboptimal duration of BCR stimulation (*i.e.*, 2 min) with anti-IgM (Figure 2A), however, optimal 5 min BCR stimulation resulted in stronger Btk phosphorylation (not shown).

To directly examine a possible role for Btk in SDF-1-controlled integrin-mediated migration we used pre-B cells derived from Btk-deficient mice. Interestingly, as shown in Fig. 2B, Btk-deficient pre-B cells exhibited ~55% reduced SDF-1-induced migration on VCAM-1 in comparison to WT pre-B cells. Notably, surface expression of CXCR4, integrin α4 and β1 was similar on WT and Btk-deficient pre-B cells (Figure S1 available as Supplemental Figure online). In DT40 cells, Btk-deficiency also resulted in a reduction (by ~45%) of SDF-1-controlled migration (Figure 2C). This defect was not due to clonal variation since it could be completely restored by stable transfection of a Btk expression construct (Figure 2C). Furthermore, as shown in Fig. 2D, whereas SDF-1-induced activation of the MAP kinase ERK2 (MAPK1) was slightly reduced, activation of PKB (Akt) was not affected in the Btk-deficient cells, demonstrating that the impaired migration is not due to a general signaling defect. To examine the role of Btk in the control of B cell migration by CXCL13 we used splenic B cells, since pre-B cells and DT40 cells do not respond to CXCL13 (not shown). As shown in Fig. 2E, Btk-deficient B cells also exhibited impaired migration on VCAM-1 towards CXCL13 (by ~30%). Notably, surface expression of CXCR5 and integrin β1 was similar on WT and Btk-deficient splenic B cells (Figure S2).

In conclusion, these data identify Btk as a component of the SDF-1-CXCR4 signaling cascade and reveal a regulatory function for Btk in chemokine-controlled integrin-mediated migration of (pre-)B cells.

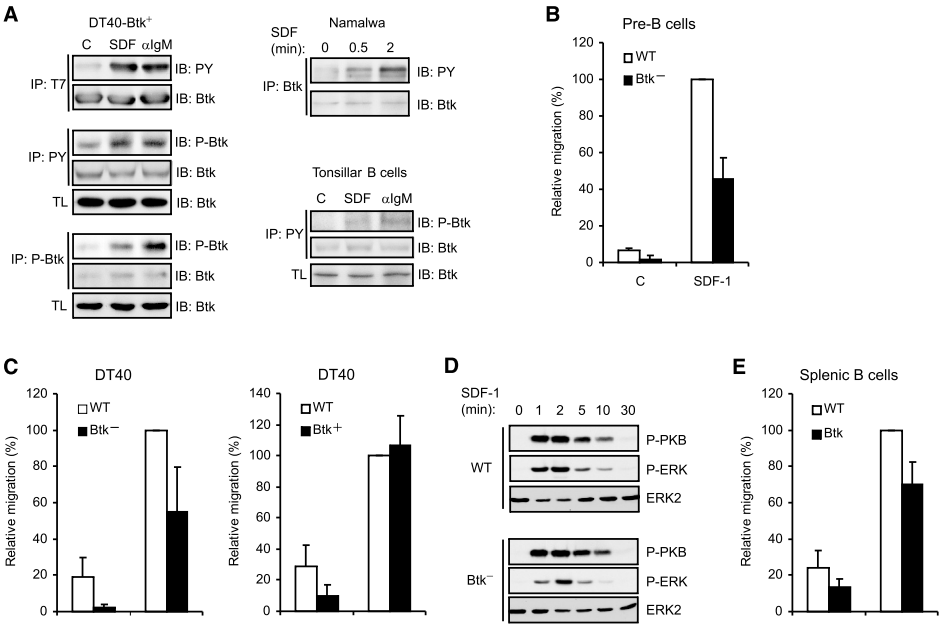


Figure 2. Btk mediates chemokine-controlled migration

(**A**) Btk-deficient DT40 cells reconstituted with T7-tagged Btk (Btk$^+$), Namalwa cells and human tonsillar B cells, were stimulated with SDF-1 or anti-IgM for 2 min. Lysates were immunoprecipitated (IP) by means of anti-Btk (Btk), anti-T7 (T7), anti-phospho-Btk (P-Btk), or anti-phosphotyrosine (PY), and the IPs were immunoblotted (IB) with anti-phospo-Btk or anti-phosphotyrosine, as indicated. As a control, the immunoprecipitates and total lysates (TL) were (re-)probed with anti-Btk.

(B) Wild type (WT) or Btk-deficient (Btk$^-$) mice pre-B cells were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in Transwells coated with 1 μg/ml VCAM-1(n=6).

(**C**) Wild type (WT), Btk-deficient (Btk$^-$), or Btk-deficient DT40 cells reconstituted with Btk (Btk$^+$) were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in Transwells coated with 1 μg/ml VCAM-1 (n=8).

(D) Wild type (WT) or Btk-deficient (Btk$^-$) DT40 cells were stimulated with SDF-1 for the indicated period of time (min), immunoblots are probed with anti-phospho-PKB (P-PKB) and anti-phospho-MAPK (P-ERK) and reprobed with anti-ERK2, as indicated.

(E) Wild type (WT) or Btk-deficient (Btk$^-$) mice splenic B cells were allowed to migrate in the absence (C) or presence of 500 ng/ml CXCL13 in Transwells coated with 1 μg/ml VCAM-1 (n=3).

SDF-1-controlled adhesion is impaired in Btk-deficient B cells

Chemokine-controlled cell migration is a complex process that involves proper coordination of cell polarity, cytoskeletal reorganization, and control of integrin localization and activity. As we have previously demonstrated that Btk mediates BCR-controlled integrin-mediated adhesion of B cells (12), we examined whether Btk is also involved in SDF-1-controlled integrin-mediated adhesion. Using a static adhesion assay, adhesion to VCAM-1 in the presence of SDF-1 was found to be reduced by ~55% in the Btk-deficient DT40 cells in comparison to the WT DT40

cells (Figure 3A). In a more physiological assay, *i.e.*, under flow adhesion to SDF-1-presenting primary HUVECs (at a shear stress of 0.8 dyn/cm^2), the stimulation of adhesion by SDF-1 was completely abolished in Btk-deficient cells (Figure 3B). Taken together, these results demonstrate that Btk also mediates SDF-1-controlled integrin-mediated adhesion, which may underlie the impaired chemokine-controlled migration of Btk-deficient B cells.

***In vivo* homing is impaired in Btk-deficient (pre-)B cells**

B cell homing is critically dependent upon the differential expression and coordinated action of B cell integrins, endothelial cell adhesion molecules, and specific chemokines and their cognate receptors, including SDF-1-CXCR4 and CXCL13-CXCR5 (5, 6, 8, 15). Therefore, to study the *in vivo* consequence of impaired chemokine-controlled adhesion and migration due to Btk-deficiency, we investigated the homing capacity of pre-B and B cells from Btk-deficient mice versus WT mice. Given the B cell developmental arrest in Btk-deficient mice, we used an IL-7-driven BM culture system to obtain better developmentally matched WT and Btk-deficient pre-B and immature B cell populations. 3 hrs after adoptive transfer, lymphoid organs were collected to determine the homing ratio of the Btk-deficient B cells compared to WT cells (Figure 4A). Interestingly, the accumulation of the Btk-deficient pre-B cells was reduced in axillary- (by ~45%), inguinal- (~55%) or mesenteric lymph nodes (~60%), the spleen (~25%), and in BM (~25%) (Figure 4B). Btk-deficient IgM$^+$ immature B cells also exhibited impaired homing to the peripheral- (by ~40-45%) and mesenteric lymph nodes (~70%), but not to the spleen or BM (Figure 4C). Consistent with a homing defect, and opposing a possible survival disadvantage, elevated numbers of Btk-deficient B cells were recovered from the peripheral blood (Figure 4). These results clearly demonstrate that Btk-deficient pre-B and immature B cells suffer from an intrinsic defect in the in vivo homing to (secondary) lymphoid organs, characteristic for impaired chemokine-controlled migration.

The role of Lyn and Syk tyrosine kinases and PI3K in SDF-1-controlled migration

Recent studies have revealed an important role for cytoplasmic tyrosine kinases in signaling by G protein-coupled receptors, and the cytoplasmic tyrosine kinases Lyn and Syk play a prominent role in BCR-controlled activation of Btk (33). Consistent with a putative similar role for these kinases in SDF-1-induced activation of Btk, SDF-1-controlled migration on VCAM-1 was reduced by ~50% in DT40 cells deficient in both Lyn and Syk (Figures S3A and Supplemental Results).

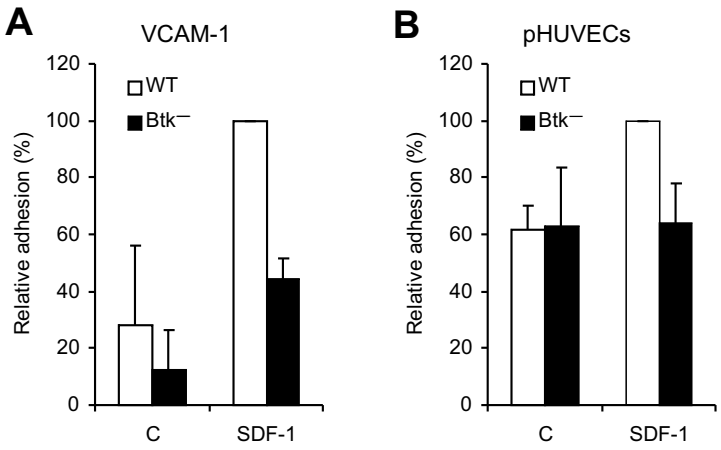


Figure 3. SDF-1-controlled adhesion is impaired in Btk-deficient B cells
(A) Wild type (WT) or Btk-deficient DT40 cells (Btk⁻) were allowed to adhere to wells coated with 1 μg/ml VCAM-1 co-immobilized without (C) or with 150 ng/ml SDF-1 (SDF-1) (n=4; each in triplicate).
(B) Wild type (WT) or Btk-deficient DT40 cells (Btk⁻) were perfused at a shear stress of 0.8 dyn/cm² over primary HUVECs overlaid without (C) or with 10 ng/ml SDF-1 (SDF-1) (n=3; each in duplicate).

Previous studies have also implicated PI3K in chemokine-controlled migration (13, 14), and in activation of Btk (33). However, in comparison to the reduced migration in Btk-defcient DT40 cells (~45%) (Figure 2C), pretreatment of DT40 or Namalwa cells with the unrelated PI3K inhibitors Wortmannin (WM) and LY294002 (LY) caused only a minor reduction of SDF-1-induced migration (~20%) (Figure S4B and Supplemental Results). Yet, the residual migration of the Btk-deficient cells towards SDF-1 could be further reduced by ~60% upon PI3K inhibition (Figure S4B). Taken together, these results suggest that PI3K and Btk mediate SDF-1-controlled migration in a parallel fashion, independent of each other.

Btk mediates SDF-1-induced phosphorylation of PLCγ2 but not activation of Rap1 or Rac

Next, we wished to explore which signaling molecules may control chemokine-controlled migration downstream of Btk. The small GTPases Rap1 and Rac have both been implicated in SDF-1-controlled migration of B lymphocytes (13, 34). Indeed, GTPase pull-down assays revealed that SDF-1 stimulation of DT40 cells resulted in enhanced amounts of GTP-bound Rac and Rap1; however, similar Rap1 and Rac activation was observed upon SDF-1 stimulation of Btk-deficient DT40 cells (Figure 5A). Thus, SDF-1-induced activation of neither Rap1 nor Rac is mediated by Btk.

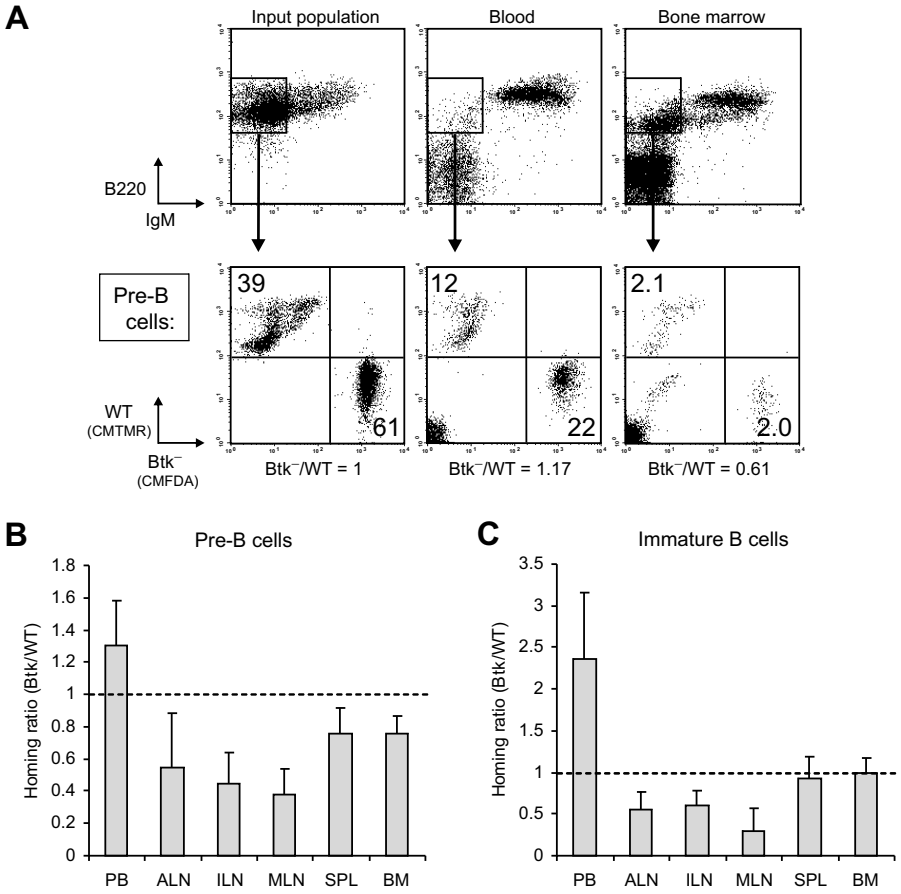


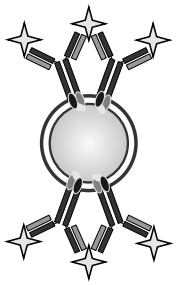
Figure 4. In vivo homing is impaired in Btk-deficient pre-B and immature B cells
(**A-C**) Cells from IL-7-driven cultures of BM from wild type (WT) or Btk-deficient (Btk⁻) mice were labeled with CMFDA or CMTMR, mixed and injected into C57Bl/6 mice. After 3 hours, peripheral blood (PB), inguinal lymph nodes (ILN), axillary lymph nodes (ALN), mesenteric lymph nodes (MLN), spleen (SPL) and bone marrow (BM) were collected and FACS-analyzed.
(**A**) An example of the FACS analysis of the input population, peripheral blood (PB) and BM of a representative mouse. The B220⁺IgM⁻ pre-B cell fractions were gated and analyzed for the two dye-labeled populations. The percentages of labeled cells are given within the quadrants of the dot plots. The homing ratio of Btk-deficient (Btk⁻) versus WT B cells was corrected for the input ratio (normalized to 1).
(**B**) The homing ratio of Btk⁻ versus WT B220⁺IgM⁻ pre-B cells in the indicated tissues (n=5; *i.e.*, 5 Btk/WT-combinations analyzed in 10 mice, including a dye swap). P-values were <0.05 for ALN, spleen and BM, and <0.005 for ILN and MLN.
(**C**) The homing ratio of Btk⁻ versus WT B220⁺IgM⁺ immature B cells in the indicated tissues (n=7; *i.e.*, 14 recipient mice). P-values were <0.01 for blood, ALN, ILN and MLN.

Another candidate is PLCγ2, since it has been identified as a direct substrate for Btk (35). Furthermore, we have recently established an important role

for PLCγ2 in BCR-controlled integrin-mediated adhesion (12). Interestingly, SDF-1 stimulation resulted in enhanced tyrosine phosphorylation of PLCγ2 in Namalwa cells, human tonsillar B cells, and DT40 cells (Figure 5B and C). SDF-1-induced phosphorylation of PLCγ2 could be detected using a general phosphotyrosine antibody as well as by phospho-specific PLCγ2 antibodies directed against Y1217 and the Btk substrate siteY759. Importantly, SDF-1-induced tyrosine phosphorylation of PLCγ2 was severely reduced in the Btk-deficient DT40 cells, and could be completely restored by stable transfection of a Btk expression construct (Figure 5C). Most likely due to higher Btk expression, PLCγ2 phosphorylation was even more pronounced in these reconstituted cells. Furthermore, SDF-1-induced phosphorylation of PKB (analyzed in the same cell lysates) was not affected by the absence of Btk (Figure 5C), thereby emphasizing the specificity of the defect in PLCγ2 phosphorylation. Thus, these data demonstrate that Btk mediates SDF-1-induced phosphorylation of PLCγ2.

PLCγ2 mediates SDF-1-controlled migration

To examine a possible role for PLCγ2 in SDF-1-controlled migration, we used PLCγ2-deficient DT40 cells. As shown in Figure 6A, PLCγ2-deficient DT40 cells showed a reduction of SDF-1-controlled migration on VCAM-1 by ~55%. This defect could be completely restored by gene complementation with a PLCγ2 expression construct (Figure 6A), demonstrating that the impaired migration is not due to clonal variation. Similar to Btk-deficient DT40 cells (Figure 5C), SDF-1-induced phosphorylation of PKB was not affected but phosphorylation of ERK2 was reduced in PLCγ2-deficient DT40 cells (Figure 6B). Interestingly, treatment of Namalwa or DT40 cells with the PLC-inhibitor U73122 almost completely abolished SDF-1-induced migration on VCAM-1 (Figure 6C). These data indicate that PLCγ2, together with other isoforms of PLC, plays an important role in SDF-1-CXCR4 signaling and SDF-1-controlled B cell migration.



DISCUSSION

Our results reveal that Btk and PLCγ2 mediate chemokine-controlled integrin-mediated migration. Besides providing several answers and new insights, this study also raises some new questions and challenges for future studies.

How do chemokines control Btk activity? In BCR signaling, the PI3K-product PIP3 is implicated in membrane recruitment of Btk, enabling activation of Btk by Lyn, whereas Syk, through phosphorylation of the adaptor BLNK (Slp-65 or BASH), facilitates activation of PLCγ2 by Btk (Kurosaki, 2002). Consistent

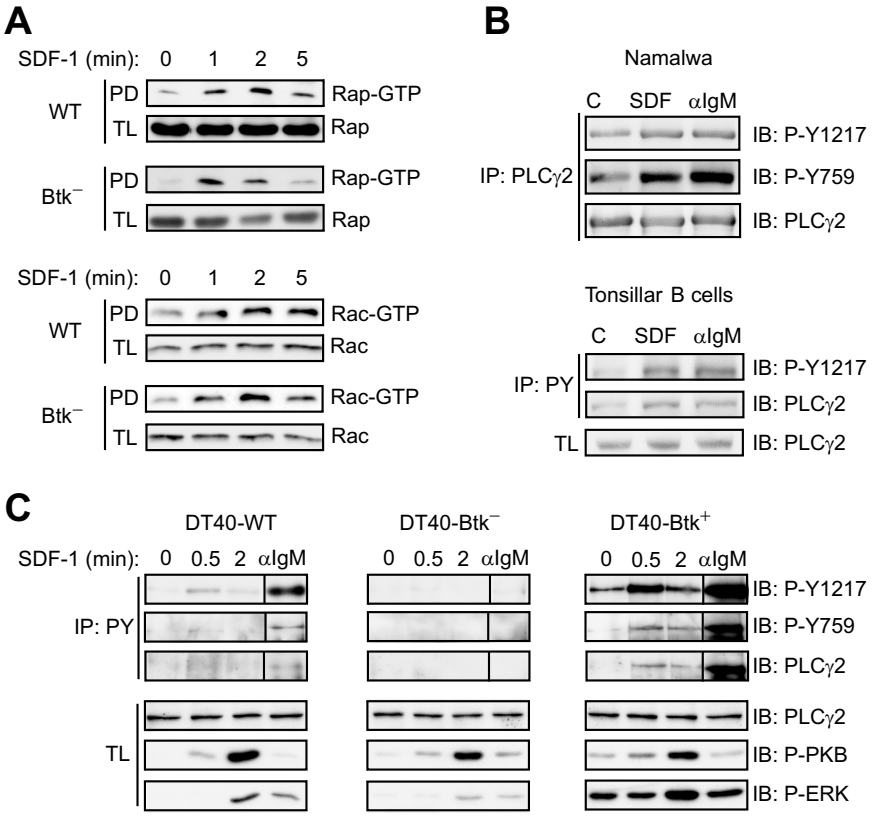


Figure 5. Btk mediates SDF-1-induced phosphorylation of PLCγ2 but not activation of Rac or Rap1
(**A**) WT DT40 cells (WT) or Btk-deficient DT40 cells (Btk⁻) were stimulated with SDF-1 for 1, 2 or 5 min and activation of the GTPases was determined by GTPase pull-down and immunoblotting with anti-Rap1 (Rap-GTP) or anti-Rac (Rac-GTP). In parallel, total lysates were immunoblotted with anti-Rap1 (Rap) or anti-Rac1 (Rac).
(**B,C**) Namalwa cells or tonsillar B cells (B), and WT DT40 cells (WT), Btk-deficient DT40 cells (Btk⁻), or Btk-deficient DT40 cells reconstituted with Btk (Btk⁺) (C), were stimulated with SDF-1 or anti-IgM for 2 min. The lysates were immunoprecipitated (IP) by means of anti-PLCγ2 (PLCγ2) or anti-phosphotyrosine (PY), and the IPs were immunoblotted (IB) with anti-phospo-PLCγ2 (P-Y1217 or P-Y759), as indicated. As a control, the immunoprecipitates and total lysates (TL) were (re-)probed with anti-PLCγ2. Phosphorylation of PKB and ERK2 in the cell lysates was analyzed as in Figure 2D.

with a putative role for these tyrosine kinases in activation of Btk and/or PLCγ2 by SDF-1, SDF-1-controlled migration was impaired in B cells deficient in Lyn and Syk. In contrast, our observations suggest that PI3K does not mediate activation of Btk by SDF-1. Yet, SDF-1-induced membrane recruitment of a Btk-GFP fusion protein in Hela cells was reported to be sensitive to PI3K inhibition (36). However, supporting our results, and opposing the general perception that activation of Btk is strictly dependent upon its PI3K-mediated membrane recruitment, BCR-controlled

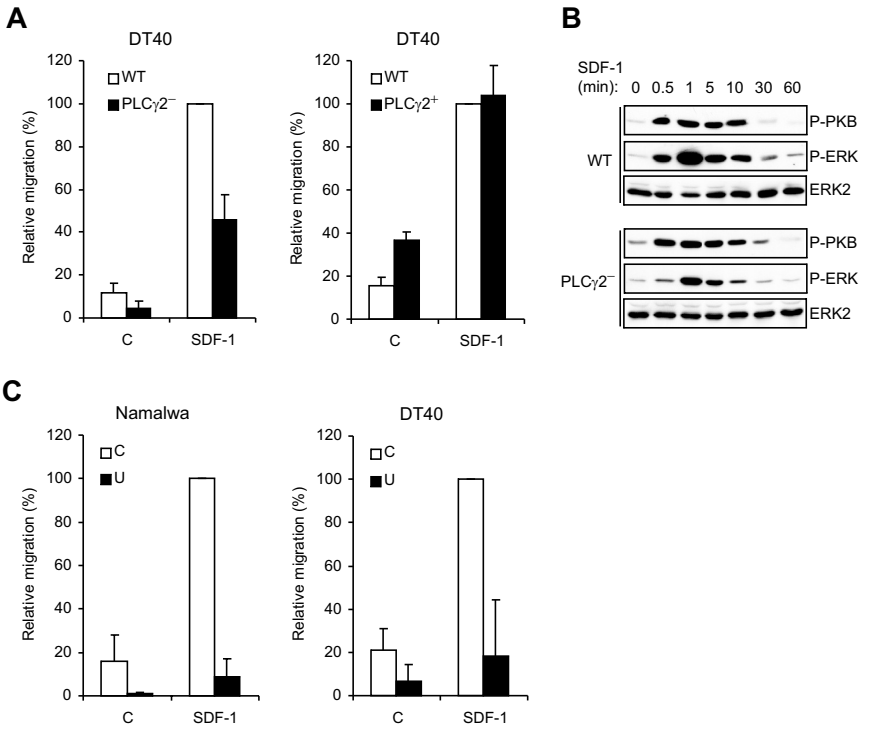
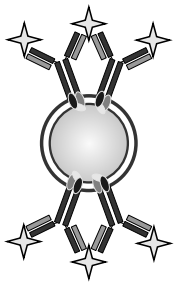


Figure 6. PLCγ2 mediates SDF-1-controlled migration
(**A**) Wild type (WT), PLCγ2-deficient (PLCγ2⁻), or PLCγ2-deficient DT40 cells reconstituted with PLCγ2 (PLCγ2⁺) were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in Transwells coated with 1 μg/ml VCAM-1, as indicated (n=6).
(**B**) Activation of PKB and ERK in wild type (WT) or PLCγ2 (PLCγ2⁻)-deficient DT40 cells after stimulation with SDF-1 for the indicated period of time (min). The blots are probed as in Figure 2D.
(**C**) Namalwa cells (left) or DT40 cells (right) were pretreated with 2.5 μM U73122 (U) or left untreated for 30 min, and subsequently cells were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in Transwells coated with 1 μg/ml VCAM-1 (n=5).

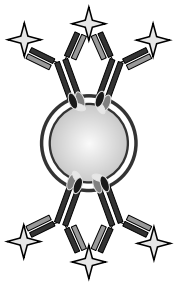


phosphorylation and activation of Btk (and PLCγ2) is not affected in B cells deficient in the regulatory PI3K subunit p85α or the catalytic subunit p110δ, nor in primary B cells treated with LY or WM (37, 38). Likewise, in accordance with the minor effect of PI3K inhibition on SDF-1-induced B cell migration, several recent studies have challenged the general concept that PI3K activation is indispensable for chemokine-controlled cell migration (39-41). Nevertheless, the residual migration of Btk-deficient cells was largely PI3K-dependent. Most likely, this involves the catalytic p110δ and adapter p85α subunits, which, in contrast to the catalytic subunit p110γ, have been implicated in chemokine-controlled B cell migration (39, 42). Recently, B cells were shown to require the scaffolding protein DOCK2 for efficient SDF-1- and CXCL13-

controlled migration and integrin-mediated adhesion to ICAM-1 and VCAM-1, respectively (13, 39). Since DOCK2, like Btk, mediates chemokine-controlled migration in a largely PI3K-independent fashion (39), it is tempting to speculate that DOCK2 may be involved in chemokine-induced Btk or PLCγ2 activation (Figure 7). Alternatively, by analogy to BCR signaling (33), the adaptor protein BLNK may be involved. Finally, as previously established for Gα (αq and α12) and Gβ/γ subunits (43,44), the membrane recruitment and activation of Btk may be mediated by the direct interaction of chemokine receptor-activated G protein subunits (Figure 7).

How do Btk and PLCγ2 control chemokine-induced migration? Our results strongly indicate that Btk mediates chemokine-controlled migration through PLCγ2, which is highly expressed in all B cell subsets (Figure 7). To our knowledge, the only previous evidence for a possible role for PLC-γ in chemokine signaling is the observation that SDF-1 induces tyrosine phosphorylation of PLC-γ (isotype undefined) in hematopoietic progenitor cells (45). In addition, since SDF-1-induced migration could be completely abolished with the general PLC inhibitor U-73122, other PLC isotypes, such as PLCγ1, which is highly expressed in pro- and pre-B cells, or PLCγ, may be involved as well (Figure 7). Similar to our observations, CXCR3-mediated migration of T lymphocytes is relatively insensitive to PI3K inhibition, but can be completely abolished by PLC inhibition (40). The observed critical role of PLC, including PLCγ2, in chemokine-controlled migration points towards an important downstream role for calcium- and/or DAG-dependent signaling molecules. Likely candidates are the classical and novel isoforms of PKC, and the RasGRP (CalDAG-GEF) family of exchange factors, which act on different Ras family GTPases, including Ras and Rap1 (13). Indeed, Rap1 was shown to be involved in SDF-1-induced migration of B lymphocytes (34, 46). However, SDF-1-induced activation of Rap1 was not impaired in Btk- or PLCγ2-deficient B cells. Similarly, activation of Rap1 by SDF-1 is not impaired in Jurkat T cells deficient in PLCγ1, the major PLC-γ isotype in T cells (47). The activation of Rac, another GTPase implicated in chemokine-controlled migration, can occur through PI3K-dependent and PI3K-independent mechanisms (13,41). Noteworthy, DOCK2 also mediates activation of Rac (and Rap) (39, 48). However, SDF-1-induced activation of Rac was not impaired in Btk- or PLCγ2-deficient B cells. Taken together, our data indicate that Btk, Rap1 and Rac act in parallel signaling pathways in chemokine-controlled B cell migration (Figure 7).

What about the role of other Tec family kinases in chemokine-controlled migration? Several members of the Tec family of tyrosine kinases, consisting of Tec, Btk, Itk, Rlk, and Bmx, have previously been implicated in cytoskeletal reorganization, integrin-mediated adhesion or migration (49). In B cells we have previously shown that Btk mediates BCR-controlled α4β1-mediated adhesion,



which involves cytoskeletal reorganization (12). Similarly, in T cells Itk mediates TCR-controlled actin polymerization and activation of β1 integrins (49). Moreover, two recent studies revealed a critical role for the Tec family kinases Itk and Rlk in chemokine-controlled migration of T cells (50,51). Furthermore, in neutrophils the chemotactic peptide fMLP induces the membrane recruitment and activation of Tec, Btk and Bmx (52), which, like the chemotactic response, can be suppressed by the pharmacological Tec family kinase inhibitor LFM-A13 (53). Recently, LFM-A13 was also reported to inhibit SDF-1-induced B cell migration and homing, and solely based upon the use of this inhibitor the authors propose a role for Btk in these processes (54). We would like to emphasize, however, that LFM-A13 is not a specific Btk inhibitor: it is a potent inhibitor of the other Tec-family kinases as well (53, 55, 56), and LFM-A13 is an equally efficient inhibitor of the non-related kinase JAK2 (57), which has also been implicated in SDF-1-induced lymphocyte migration (58,59). Yet, combining all of the above with our current findings, Tec family kinases appear to play an important role in chemokine-controlled adhesion and migration, at least in lymphocytes. Given the observed partial redundancy of Btk with Tec in mice B cells (27), it would be interesting to determine whether Tec (or another Tec family member) is responsible for the residual migration observed in Btk-deficient (pre-)B cells. Furthermore, by analogy to the role of Btk and PLCγ2 in chemokine-controlled B cell migration, it would be interesting to determine whether chemokine-controlled migration of T lymphocytes involves Itk- or Rlk-mediated phosphorylation and activation of PLCγ1, the major PLC-γ isotype in T cells.

What are the implications for XLA and Xid? Our study demonstrates a direct role for Btk (and PLCγ) in signaling by CXCR4 and in chemokine-controlled adhesion, migration and homing. Interestingly, loss-of-function germline mutations in the *BTK* gene give rise to the B-cell immunodeficiency disease XLA in humans and Xid in mice. XLA patients show a severe reduction in mature B cell numbers (>99%) (26,31), and in Xid- and the phenotypically identical Btk-deficient mice, mature B cell numbers are ~50% reduced (29). The earliest role for Btk occurs during B cell development at the progression from pre-B to immature B cells, which is severely impaired in XLA patients and to a lesser extent in Xid and Btk-deficient mice (26, 28, 30). In XLA this involves a proliferation defect of the μH-chain positive pre-B cells (31), and in Xid mice this involves a defect in cellular maturation of pre-B cells (30). Furtermore, Xid mice have been reported to display a defect in the retention of immature B cells in the BM (25). Interestingly, α4 integrin-deficient pre-B cells also display a proliferation defect (1), and SDF-1 was originally identified as a pre-B cell growth-stimulating factor. Moreover, our results as well as previous studies (4, 16) show that SDF-1 controls α4β1-mediated adhesion and migration of pre-B and immature B cells, and mice deficient in SDF-1-CXCR4, VCAM-1, or α4 integrin,

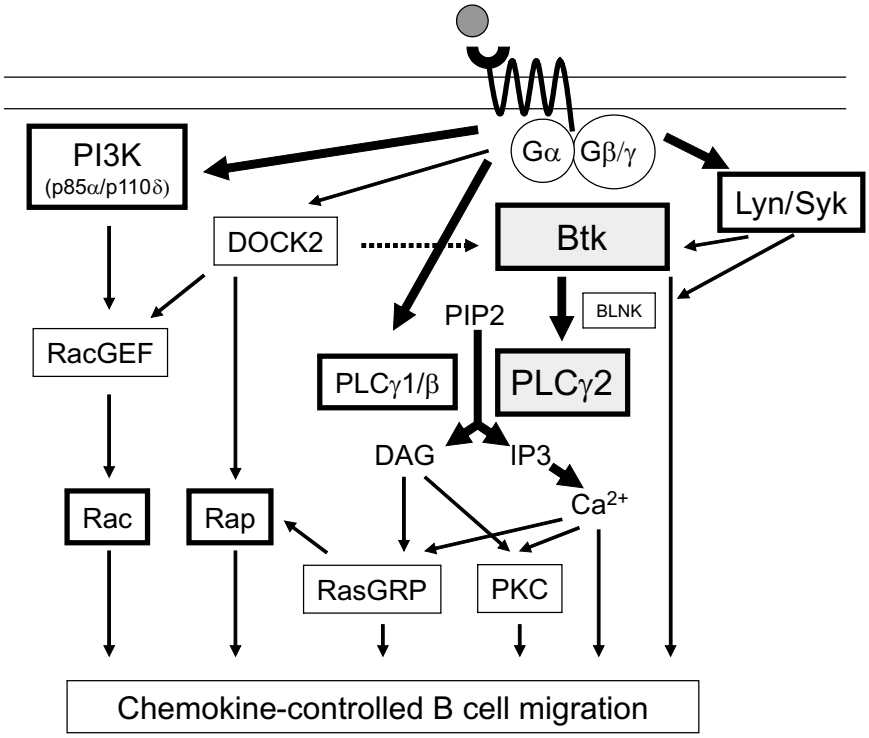


Figure 7. The role of Btk and PLCγ2 in the signaling cascades underlying chemokine-controlled integrin-mediated migration.
A schematic representation of the (putative) signaling pathways underlying chemokine-controlled integrin (α4β1)-mediated migration is shown. The proteins studied and connections established in this study are represented by the bold boxes and bold arrows, respectively. See the Discussion section for further detail.

display defects in development and retention of precursor (pro-, pre- and immature) B cells in the BM (1,2,19,21,60). In the mature B cell population, Btk-deficient mice reveal a defect in follicular entry of long-lived recirculating follicular cells, in follicle and GC formation, in T-independent immune response, and a strongly reduced primary and variably affected secondary T-dependent immune response, whereas both responses are absent in Btk/Tec double-deficient mice (27-29,61,62). Similarly, SDF-1- or CXCR4- and CXCL13- or CXCR5-deficient mice display defects in migration of naïve B cells into follicles, GC B cell migration and GC organization (17, 18,22,23), CXCR4-deficient mice show a loss of T-independent immune response (21), and mice lacking either VCAM-1 expression on FDCs or expression of integrin β1 in the hematopoietic system exhibit an impaired T-dependent immune response (2, 7, 9). Thus, based upon our current findings, impaired chemokine (SDF-1 or CXCL13)-controlled adhesion and migration, required for localization of pre-B and

immature B cells in the appropriate BM niches and for emigration of immature B cells from the BM into the blood and to secondary lymphoid organs, may very well contribute to the defects in early B cell development in XLA and Xid, and to the partial defects in localization, differentiation and responses of mature B cells in Xid-mice.

In conclusion, our results demonstrate that Btk and PLCγ2 mediate chemokine-controlled B cell adhesion and migration, which plays an important role in B cell development and function as well as in the pathogenesis of B cell malignancies and chronic inflammatory or autoimmune diseases. Furthermore, our results indicate that impaired adhesion and migration, due to loss-of-function germline mutations of *BTK*, may contribute to the developmental and functional B cell defects observed in XLA patients and Xid mice.

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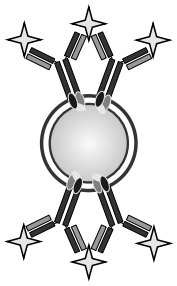

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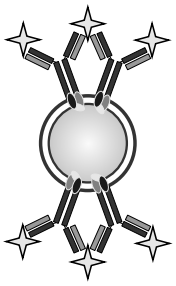


CHAPTER 6

Supplemental Results

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell lines and generation of reconstituted DT40 cells

The Burkitt's lymphoma cell line Namalwa, and the chicken bursal lymphoma B cell line DT40 and DT40 cells deficient in both Lyn and Syk, Btk, or PLCγ2, obtained from Riken Cell Bank (Tsukuba Science City, Japan) with permission from Dr. T. Kurosaki, were cultured as previously described (4). The reconstitution of the Btk- or PLCγ2-deficient DT40 cell lines was performed by electroporation, using pApuro expression vectors containing the T7-tagged cDNAs for human Btk and rat PLCγ2 (kindly provided by Dr. T. Kurosaki), exactly as described (4).

All DT40 cells, i.e., WT, gene-deficient and reconstituted, showed similar expression of surface IgM and integrin β1, as determined by FACS analysis, using goat anti-chicken IgM (10 μg/ml) and rabbit anti-goat-Biotin/Streptavidin-PE, or CSAT (3 μg/ml) and goat anti-mouse-PE, respectively, analyzed with a FACSCalibur™ (Beckton Dickinson) using CELLQuest Pro software.

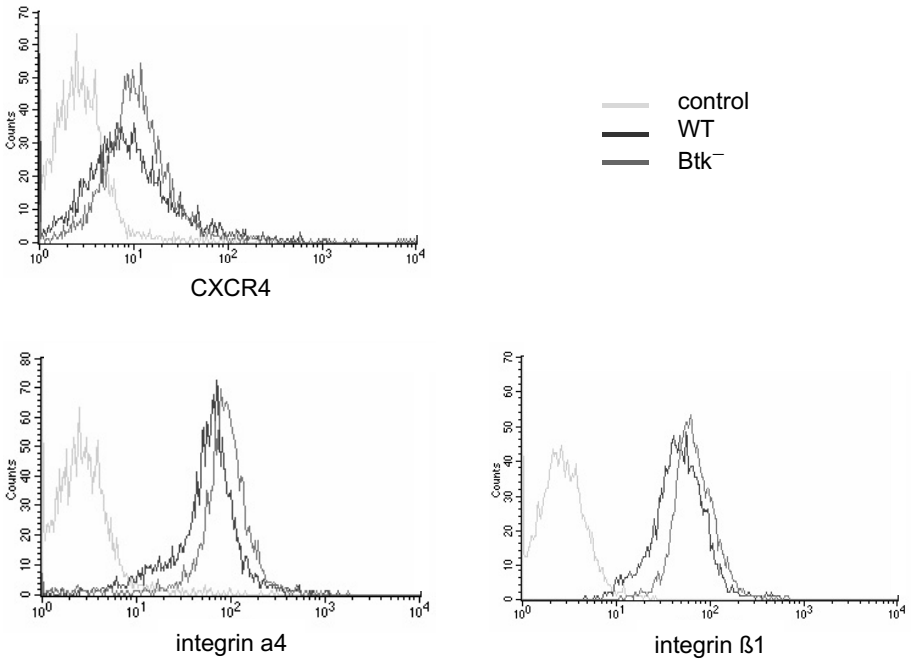


Figure S1. Similar expression levels of CXCR4, integrin α4 and β1 on WT and Btk-deficient pre-B cells

WT and Btk-deficient mice pre-B cells were FACS-analyzed for expression of CXCR4, integrin α4 and β1, using AB 1846, PS/2-biotin, and OXM718-FITC antibodies, respectively.

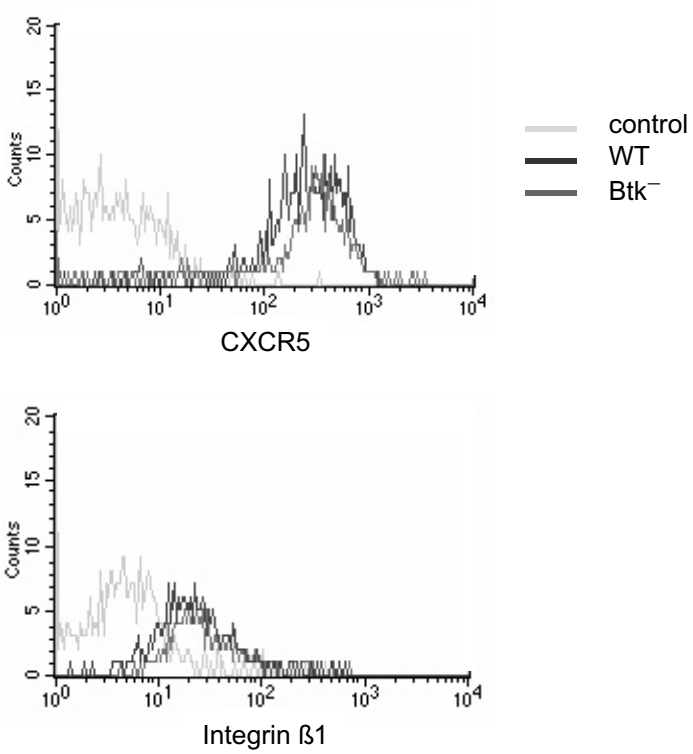
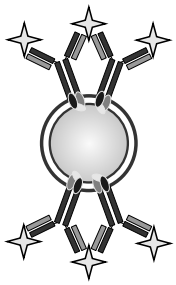


Figure S2. Similar expression levels of CXCR5 and integrin β1 on WT and Btk-deficient splenic B cells
WT and Btk-deficient splenic B cells were FACS-analyzed for expression of CXCR5 and integrin β1, using 2G8-biotin and OXM718-FITC antibodies, respectively. A representative result is shown.



Isolation of tonsillar B cells, murine pre-B cells and splenic B cells

Human tonsillar B cells were isolated essentially as previously described (4, 5). Typically, the obtained cell population contained > 97% B cells as determined by FACS analysis. Isolated B cells were maintained in RPMI containing 10% FCS and were used immediately or after overnight storage at 4°C.

Mouse splenic B cells were obtained from C57BL/6 mice, bred and maintained at the animal care facility of the Academic Medical Center. In addition, mouse splenic B cells were from Btk-deficient mice, i.e., Btk⁻/LacZ mice crossed onto a C57BL/6 background for over 6 generations and Btk genotyped as described (6), and littermate controls, bred and maintained at the animal care facility of the Erasmus MC (Rotterdam, the Netherlands). The splenic B cells were isolated using the MACS system (Miltenyi Biotec) by positive selection with anti-CD45R (B220) microbeads, essentially according to manufacturer's instruction. Expression levels

of CXCR5 and integrin β1 were similar on WT and Btk-deficient splenic B cells as determined by FACS analysis using 2G8-biotin (1:50) and OXM718-FITC (1:20) antibodies, respectively.

To obtain mouse pre-B cells, IL-7-driven primary BM cultures of WT and Btk-deficient mice were performed essentially as described (4, 7). The IL-7 driven BM cultures of the WT or Btk-deficient mice typically resulted in >98% $B220^+$ cells and approximately 80% and 95% pre-B cells ($B220^+/IgM^-$), respectively. Expression levels of CXCR4, integrin α4 and β1 were similar on WT and Btk-deficient mice pre-B cells as determined by FACS analysis, using AB 1846 (1:25), PS/2-biotin (1:80), and OXM718-FITC (1:20) antibodies, respectively.

Cell migration assay

Migration assays were performed in triplicate using 5 µm (DT40 and primary B cells) or 8 µm (Namalwa) pore size Transwells (Costar), coated overnight at 4°C with PBS containing 1 µg/ml sVCAM-1 or 10 µg/ml FN, and washed twice with PBS and blocked for 1 hr at 37°C with 0.5% BSA in RPMI 1640. The lower compartment was filled with 600 µl 0.5% BSA/RPMI containing 100 ng/ml SDF-1 or 500 ng/ml CXCL13, and 5 x 10^5 cells in 100 µl were applied to the upper compartment and allowed to migrate for 5 hrs (DT40 and Namalwa), 3.5 hrs (tonsillar B cells), 2.5 hours (splenic B cells), or 1 hr (pre B cells) at 37°C (39.5°C for DT40), unless otherwise indicated. Where indicated, cells were pretreated with pharmacological inhibitors for 30 min at 37°C, or with anti-integrin antibodies Act-1 (3 µg/ml), TS2/16 (1:5) and HP2/1 (1 µg/ml) for 1 hour at 4°C, in RPMI with 1% BSA, and inhibitors and antibodies were also present in the Transwells during migration. The amount of viable migrated cells was determined by FACS and expressed as a percentage of the input, i.e., the number of cells applied directly into the lower compartment in parallel wells. Unless otherwise indicated, the migration of non-pretreated WT cells on VCAM-1-coated Transwells in the presence of SDF-1 or CXCL13 was normalized to 100%, and the bars represent the means ±SD of at least 3 independent experiments, each assayed in triplicate.

Cell adhesion assays

The static cell adhesion to VCAM-1 was assayed essentially as described previously (4), with the following modifications: 96-well flat-bottom high binding plates (Costar) were used; 150 ng/ml SDF-1 was co-immobilized with 1 µg/ml VCAM-1 on the plates; the plates were spun for 30 sec at 400 rpm directly after applying the cells to the plate, and; the cells were allowed to adhere for 2 min and plates were washed twice to remove non-adhering cells. The adhesion of WT cells on SDF-1/VCAM-1-coated wells was normalized to 100%, and the bars represent the means ±SD of 4 independent experiments, each assayed in triplicate.

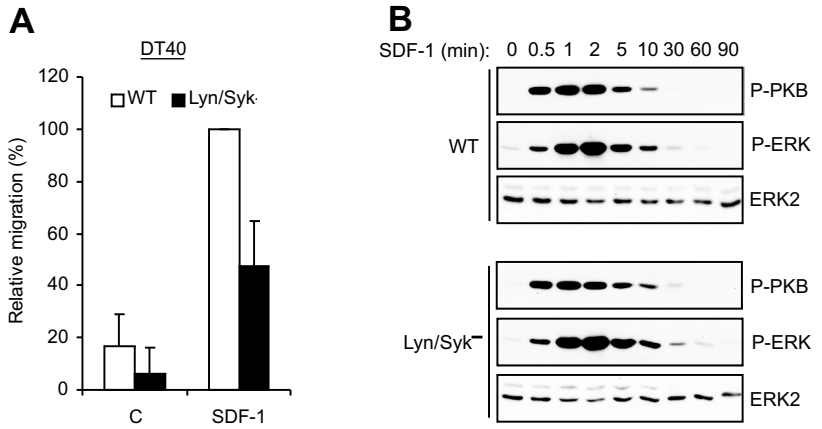
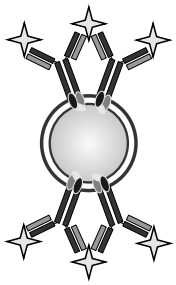


Figure S3. SDF-1-controlled migration is impaired in Lyn and Syk-deficient B cells
(A) Wild type (WT) or Lyn/Syk double-deficient DT40 cells (Lyn^-/Syk^-)were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in Transwells coated with 1 μg/ml VCAM-1 (n=4).
(B) Activation of PKB and ERK in wild type (WT) or Lyn/Syk double-deficient DT40 cells (Lyn^-/Syk^-) after stimulation with anti-IgM for the indicated period of time (min). The immunoblots are probed as in Fig. 2D.

The under flow cell adhesion to HUVECs was assayed essentially as described previously (8). Briefly, primary human umbilical cord vein endothelial cells were isolated, cultured for 3 or 4 passages, and stimulated for 5 hrs with 10 ng/ml TNFa (Pepro Tech); The HUVECs were overlaid with 10 ng/ml SDF-1 for 5 min at 37°C prior to perfusion; DT40 cells (10^6/ml) were perfused at 0.8 dyn/cm^2 for 5 min; images were recorded for 5 sec from 2 to 5 minutes after the start of perfusion; 20 randomized fields were analyzed to determine the average amount of adhering cells per field. The adhesion of WT cells on SDF-1-overlaid HUVECs was normalized to 100%, and the bars represent the means ±SD of 3 independent experiments, each assayed in duplicate.



Homing assay

WT and Btk-deficient pre-B and immature B cells were obtained from IL-7-driven BM cultures: Pre-B cell fractions were harvested after 4 days of culture in the presence of 100 U/ml IL-7, whereas immature B cell fractions were collected after one additional day of culture without IL-7. WT and Btk-deficient cells were labeled for 45 min at 37°C either with 0.25 μM Cell tracker Green (CMFDA) or 2 μM Cell tracker Orange (CMTMR), washed, and WT and Btk-deficient cells (15 x 10^6 cells each) were mixed and injected intraveneously in age- and sex-matched C57BL/6 mice. After 3 hrs, blood, lymph nodes, spleen and BM were collected, and FACS-analyzed to identify pre-B and immature B cells by their B220/IgM profile and to quantify dye-labeled cells. Each combination of cells from a WT and a Btk-deficient mouse was analyzed

by adoptive transfer of 2 recipient mice, which included a dye-swap for each Btk⁻/WT combination to compensate for possible dye-specific effects on homing capacity. The ratio of the input population was determined and normalized to 1 to correct the homing ratio (Btk-deficient cells/WT cells).

Immunoprecipitation and immunoblotting

For analysis of Btk and PLCγ2 tyrosine phosphorylation, 1.5-3 x 10^7 cells, serum starved for 1 hr at 37°C (39.5°C for DT40), were stimulated with 200 ng/ml SDF-1 or 10 µg/ml anti-IgM and immunoprecipitated, essentially as described (9). Briefly, the cells were lysed by addition of an equal volume ice-cold 2 x lysis buffer (20 mM Tris-HCl pH 8, 300 mM NaCl, 2% Nonidet P-40, 20% glycerol, 10 mM EDTA, 4mM Na3VO4, 10 mM NaF, and 2 EDTA-free protease inhibitor cocktail tablets (Roche) per 50ml). After preclearence with protein A-Sepharose beads (Pharmacia), the lysates were incubated with 1-3 µg anti-Btk (C20), anti-T7, anti-P-Btk, anti-PLCγ2 (Q20), or anti-phosphotyrosine (PY20 or 4G10) antibody at 4°C O/N, and immunoprecipitation was carried out by adding protein A- or G-Sepharose beads for 30 min. Immunoprecipitates and total lysates were analyzed by SDS-PAGE and immunoblotting, using anti-P-Btk, anti-Btk (G149-11), anti-P-PLCγ2, anti-PLCγ2 (H160), or anti-phosphotyrosine (4G10). Please note that none of the tested Btk or PLCγ2 antibodies could immunoprecipitate Btk or PLCγ2 from (chicken) DT40 cells with sufficient efficiency for phosphorylation analysis. Therefore, Btk-deficient DT40 cells reconstituted with T7-tagged Btk were used for the Btk phosphorylation studies in DT40 cells.

For analysis of ERK and PKB phosphorylation, after stimulation of 10^7 cells /ml RPMI with 100 ng/ml SDF-1, cells were directly lysed in SDS-PAGE sample buffer. 2 x 10^5 cells were applied on a 10% SDS-PAGE gel and immunoblotted with anti-phospho-MAPK1/2 and anti-phospho-PKB, as described (4).

Rac and Rap1 activity pull-down assays

Cells were resuspended to 0.8 x 10^7cells/0.4 ml RPMI and stimulated with SDF-1 (100 ng/ml). Reactions were terminated by adding 0.4 ml of cold 2 x lysis buffer (100 mM Tris-HCl pH 7.4, 400 mM NaCl, 5 mM $MgCl_2$, 2% Nonidet P-40, 20% glycerol, 2 x EDTA-free protease inhibitor cocktail tablets (Roche) per 50 ml). After 10 min on ice, cell-debris was removed by centrifugation. Cell lysates were used immediately for GTPase pull down assays. For this purpose, glutathione-Sepharose beads (100 µl of a 20% solution per sample) were pre-coupled with GST-RalGDS-RBD or GST-PAK-RBD fusion protein by continuous mixing for 30 minutes at 4°C with bacterial cell lysates from *E. coli* strain BL21 transformed with pGEX4T-RalGDS-RBD96 or pGEX2T-PAK-RBD. After being washed three times with cell lysis buffer,

these pre-coupled beads were added to the cell lysates, and incubated for 30 min at 4°C during continuous mixing. Finally, the beads were washed four times with lysis buffer, bound proteins were eluted with sample buffer, separated by 15% SDS-PAGE, and immunoblotted with anti-Rap1 or anti-Rac1.

pGEX4T-RalGDS-RBD96, encoding a GST fusion protein containing the 96 AA Rap and Ras binding domain of RalGDS (AA 789-884) was generated by subcloning a 300 bp EcoRI-AvaII (blunted) fragment from pGAD-RalGDS-RBD (10) into EcoRI- and SmaI-digested pGEX4T3. The pGEX2T-PAK-RBD plasmid containing the Rac binding domain of PAK (AA 56-272) was kindly provided by Dr. J. Collard (Netherlands Cancer Institute, Amsterdam, the Netherlands).

Statistical analysis

The unpaired two-tailed Student's t-test was used to determine the significance of differences between means. Unless otherwise indicated, all relevant comparisons (*e.g.*, control versus inhibitors or WT versus gene-deficient cells) were significantly different ($p<0.05$).

SUPPLEMENTAL RESULTS

SDF-1-controlled migration is impaired in Lyn- and Syk-deficient B cells

Recent studies have revealed an important role for cytoplasmic tyrosine kinases in signaling by G protein-coupled receptors. Moreover, Lyn, a Src-family tyrosine kinase, and Syk, a ZAP70-family tyrosine kinase, play a prominent role in (BCR-) signaling in B cells, including in activation of Btk (1). To investigate a possible role for Lyn and Syk in the regulation of integrin-mediated migration by SDF, we made use of DT40 cells deficient in both Lyn and Syk. As shown in Figure S2A, in Lyn/Syk double-deficient DT40 cells the SDF-1-controlled migration on VCAM-1 was reduced by ~50%. SDF-1-induced phosphorylation of PKB and ERK2 was not affected in the Lyn/Syk-deficient cells, demonstrating that the reduced migration is not due to a general signaling defect (Figure S2B). Thus, these data demonstrate that Lyn and/or Syk are involved in SDF-1-controlled $\alpha4\beta1$–mediated migration, which is in line with a putative role for these kinases in SDF-1-induced activation of Btk.

Btk and PI3K mediate SDF-controlled migration independent of each other

Previous studies have implicated PI3K in chemokine signaling and chemokine-controlled migration (2, 3). Furthermore, the PI3K product PIP3 has been implicated in membrane recruitment and activation of Btk by binding the PH domain of Btk (1). To

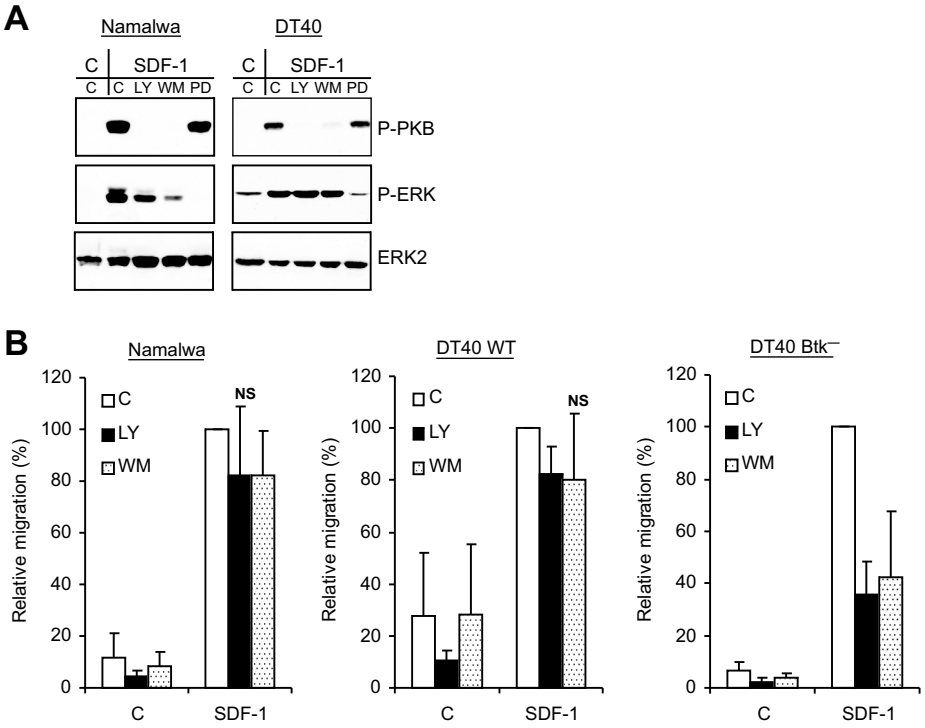
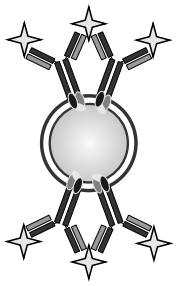


Figure S4. Btk and PI3K mediate SDF-1-controlled migration independent of each other
(A) Activation of PKB and ERK in Namalwa cells (left) or DT40 cells (right) which were pretreated with 20 μM LY294002 (LY), 100 nM Wortmannin (WM), 50μM PD98059 (PD) or left untreated (-) for 30 min, and stimulated with SDF-1 or not (C) for 5 min, as indicated. The blots are probed as in Fig. 2D.
(B) Namalwa cells (left), WT DT40 cells (middle), or Btk-deficient DT40 cells (Btk⁻)(right) were pretreated with 40μM LY294002 (LY), 200 nM Wortmannin (WM), or left untreated for 30 min, and allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in Transwells coated with 1 μg/ml VCAM-1 (n=5). NS = not significant.

investigate the role of PI3K in SDF-1-controlled integrin-mediated migration we used the unrelated PI3K inhibitors Wortmannin (WM) and LY294002 (LY). Pretreatment of Namalwa or DT40 cells with LY and WM, but not with the MEK inhibitor PD98059, completely abolished SDF-1-induced phosphorylation of PKB (Figure S1A). Interestingly, however, inhibition of PI3K caused only a minor reduction of migration by approximately 20% (Figure S1B). Notably, we used as much as 40 μM LY and 200 nM WM, and parallel analysis of SDF-1-induced PKB phosphorylation during the course of the migration experiment confirmed full inhibition of PI3K activity (data not shown).

In comparison to the small effect of PI3K inhibition (~20%), the loss of Btk in DT40 cells resulted in a more severe reduction of migration (~45%) (Figure 3C), indicating that Btk mediates B cell migration in a (largely) PI3K-independent manner.

SDF-1-induced activation of PI3K is not Btk-dependent either, since the completely PI3K-dependent phosphorylation of PKB is not affected in the Btk-deficient cells (Figures 3D and S1A). Moreover, the residual migration of Btk-deficient cells could still be further reduced upon treatment with LY and WM by approximately 60% (Figure S1B). Taken together, these results suggest that PI3K and Btk mediate SDF-1-controlled migration in a parallel fashion, independent of each other.



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CHAPTER 7

GENERAL DISCUSSION

GENERAL DISCUSSION

Part of this chapter was published before as a review in Seminars in Immunology:
Hendriks RW, Kersseboom R. Involvement of Slp-65 and Btk in tumor suppression and malignant transformation of pre-B cells.
Semin Immunol. 2006;18:67-76.

B cell development is a complex process that is characterized by the ordered rearrangement of IgHC and IgLC gene segments encoding the BCR. Moreover, it is strongly dependent on the signal transduction proteins Btk and Slp-65 as these are involved in signaling from a variety of receptors including the pre-BCR and BCR. This thesis aimed to unravel the *in vivo* function of Btk and Slp-65 in B cell development and their involvement in immunodeficiency and malignancy.

To this end, mice expressing various gain-of-function and loss-of-function mutants of Btk under the control of the B cell specific CD19 promoter region were generated. These experiments revealed that Btk and Slp-65 induce cellular maturation and IgLC rearrangements, independent of their role in termination of pre-B cell expansion. Furthermore, we found that part of Btk's role is independent from its kinase function, demonstrating that Btk partly functions as an adapter molecule even in the absence of Slp-65. Finally, we could demonstrate that Btk and Slp-65 cooperate as tumor suppressors in the absence of Btk kinase activity.

1 PRE-BCR SIGNALING

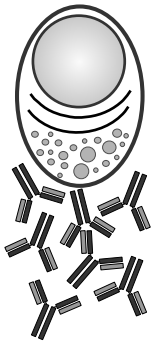
The pre-BCR is a key checkpoint in B cell development to monitor the assembly of a functional IgHC and to terminate further IgHC rearrangements, thus ensuring that only one functional IgHC is synthesized, a phenomenon referred to as allelic exclusion. Pre-BCR expression induces proliferative expansion of cytoplasmic IgHC positive pre-B cells and their progression into small resting pre-B cells in which Ig L chain rearrangement occurs (1,2).

1.1 Btk and Slp-65 limit pre-BCR mediated proliferation

In mice deficient for components of the pre-BCR complex such as Ig-α, Ig-β, SLC, or the downstream tyrosine kinase Syk, IgHC positive pre-B cells were unable to proliferate (reviewed in ref. 3). Specifically, the non-Ig-like unique tail of λ5 was shown to be essential for the activation of downstream signal transduction pathways (4). In

addition to pre-BCR signaling, proliferation is enhanced by signals emanating from the IL-7 receptor. Moreover, it was shown that IL-7 receptor and pre-BCR signaling cooperatively induce proliferation via an Erk dependent pathway (5). Disruption of Btk or the adapter protein Slp-65 showed that these downstream pre-BCR signaling components were dispensable for initiation but crucial for termination of IL-7 driven expansion of large cycling pre-B cells resulting in enhanced *in vitro* proliferative responses (6-8). In line with the latter we and others found that Btk-, Slp-65- and Btk/Slp-65-deficient pre-B cells showed defective downregulation of SLC and IL-7 receptor suggesting that both proliferation inducing pathways remained active (Chapter 3 and ref. 6,7,9-11). It has remained unclear how proliferation of large pre-B cells is limited. On the one hand proliferating large pre-B cells may become resting when SLC is diluted out, thereby limiting pre-BCR expression. On the other hand IL-7 receptor down modulation may also regulate proliferation as well. Very recently van Loo et al. solved part of this conundrum by showing that enforced SLC expression had no effect on pre-B cell population size, proliferation or differentiation (12). Therefore, loss of pre-BCR expression is not essential for the limitation of pre-B cell proliferation. Recently it was shown that the decrease in pre-BCR expression is not merely the result of 'diluting out', but also involves active modulation of λ5 expression by Ikaros and Aiolos (13,14).

Supporting the necessity for adequate regulation of IL-7 receptor signaling, enforced expression of IL-7 resulted in an enlarged large pre-B cell compartment and B cell lymphoma's (15,16). Likely, increased IL-7 responsiveness rather than high surface pre-BCR expression enhances proliferation of Btk- and Slp-65-deficient pre-B cells. Recently, Myc was shown to enhance proliferation and differentiation of pre-B cells suggesting that Myc is an important target of both IL-7R and pre-BCR signaling (17).



1.2 Btk and Slp-65 regulate pre-BCR mediated differentiation and light chain rearrangement

During the transition of large cycling into small resting pre-B cells in the mouse, Btk- or Slp-65-deficient cells show defective downregulation of SLC, the metallopeptidase BP-1 and the sialoglycoprotein CD43, as well as defective upregulation of the adhesion molecule CD2, the IL-2 receptor CD25 and MHC class II (7,9). Btk-deficient cells also manifest a specific ~3 hours developmental delay within the small pre-B cell compartment. Thus, it appears that in addition to their function in the termination of IL-7 driven proliferation, Btk and Slp-65 are also involved in cellular maturation of cytoplasmic IgHC+ pre-B cells.

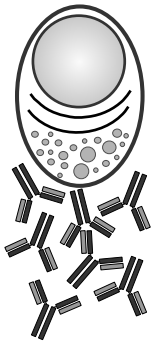
The role of pre-BCR signaling in the induction of pre-B cell differentiation and IgLC rearrangement is controversial (4,18). The hypothesis that pre-BCR signals

are responsible for the redirection of V(D)J recombination activity from the IgHC to the IgLC loci is based on several observations: First, IgHC surface expression correlates with germline transcription, which has been implicated in regulation of accessibility of Ig loci to the V(D)J recombinase. Second, in the absence of pre-BCR function, e.g. in mice with targeted disruption of IgHC, Ig-α, Ig-β, SLC components or Syk, IgLC κ locus rearrangement is diminished. Third, expression of an activated Ras transgene induces IgLC rearrangement in JH-/- pro-B cells, which lack the ability to assemble Ig H chain variable regions (19). Fourth, Slp-65-deficient pre-B cells have reduced IgLC germline transcription and gene rearrangement (10, 20). Fifth, Btk-deficient mice have reduced λ IgLC usage (21,22). These five observations all point to important roles for these signaling molecules in the initiation of IgLC rearrangement. Nevertheless, evidence for a direct involvement of pre-BCR signaling in the induction of L chain V(D)J recombination was lacking. On the contrary, it has also been reported that IgLC rearrangement can occur without IgHC expression. IgLC κ transcription and rearrangement is detectable in IgHC - pro-B cells (23) and is increased by activation of NF-κB by lipopolysaccharide in transformed pro-B cell lines (24). Removal of IL-7 from cultured pro-B cells from JH-/- or λ5-/- mice, which are incapable of expressing a proper pre-BCR, resulted in the apparent differentiation into cells that transcribe and rearrange IgLC loci (25). These results argue for a model in which the initiation of IgLC rearrangement is independent of pre-BCR expression (4).

We found that premature expression of the gain-of-function mutant E41K-Btk induced the pre-B cell developmental program in pro-B cells, including germline transcription and productive rearrangement of IgLC and modulation of cell surface markers. Importantly, these findings indicate that pre-BCR signaling not only acts to terminate proliferation or to increase survival of pre-B cells, but has the intrinsic capacity to signal for cellular maturation and the initiation of IgLC rearrangement (Chapter 2). In support of our results pre-B leukemia cell lines from Slp-65-deficient mice rearranged the IgLC κ gene locus and down-regulated pre-BCR upon PMA (phorbol 12-myristate 13-acetate, which result in Ca2+ release) treatment or Slp-65 reconstitution (26). Additional analyses with specific inhibitors and retroviral transduction of constitutively active PKCη and Raf-1 revealed that a Slp-65 - PKCη - Raf-1 pathway induced IgLC κ rearrangement. In the proposed model pre-BCR mediated activation of the BCR signalosome including Slp-65, Btk and Plcγ2 results in DAG-mediated PKCη activation and subsequent IgLC κ rearrangements (17). Furthermore, reduced IgLC λ usage was found in all B-cell maturation stages from Plcγ2-deficient mice, emphasizing pre-BCR / BCR signalosome pathway involvement in IgLC rearrangements (27). Recently, it was shown that N-myc and c-myc are downstream targets of Btk and Tec, whereby enforced c-myc expression

was able to rescue B cell development up to the IgM+ immature B cell stage in Btk/Tec double-deficient mice (17).

Attractive downstream transcription factor candidates of the pre-BCR signalosome include the interferon regulatory factors IRF-4 and IRF-8. Deficiency of either one results in a phenotype very similar to that found in Btk or Slp-65-deficient mice (28). The phenotype of IRF-4/IRF-8 double-deficient mice strongly resembles that found in Btk/Slp-65 double-deficient mice, resulting in an arrest at the large cycling pre-B-cell stage and failure to down-regulate the pre-BCR (28). In support of a role for IRF-4 and IRF-8 in IgLC rearrangements, expression of IRF-4 in Abelson-transformed pre-B cell lines was sufficient to induce germline IgLC κ transcription (29). Similarly expression of IRF-4 in IRF4/IRF-8 double-deficient pre-B cells induced κ germline transcription, enhanced V(D)J rearrangement activity at the κ locus, lead to histone modifications and enhanced chromatin accessibility at the κ locus, and promoted IgLC rearrangement and transcription (30). Thus, IRF-4 controls pre-B cell development, at least in part, by promoting the activation of the κ locus. Moreover, recent data indicate that IRF-4 and IRF-8 do so by regulating Ikaros and Aiolos (31). However, Tg expression of E41K-Btk and E41K-Y223F-Btk in pro-B cells did not appear to result in a significant increase in the expression levels of IRF-4 or Spi-B transcripts (Chapter 2), indicating that these transcription factors may not function as nuclear effectors of Btk/Slp-65 signaling. Similarly to our results IgLC κ rearrangement was induced upon expression of Slp-65 in a murine Slp-65-deficient tumor cell line (32). However, in this case IgLC κ expression was preceded by some IRF-4 expression. Alternatively Btk and Slp-65 may regulate IRF-4 and IRF-8 function in an as yet not identified manner e.g. involving other transcription factors (PU-1) known to form a complex with IRF-4 (33).



2 BTK AND SLP-65 IN TUMOR SUPPRESSION

2.1 Btk and Slp-65 are tumor suppressors that limit pre-B cell expansion in the mouse

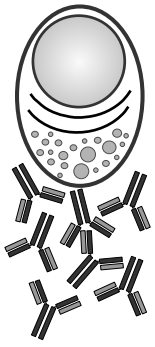
A dramatic consequence of Slp-65-deficiency is the spontaneous development of pre-B cell leukemia with high levels of the pre-BCR on the cell surface in approximately 15% of these mice at the age of 6 months (6,10). Slp-65-deficient pre-B cells cause leukemia 3-5 weeks after injection into immunodeficient RAG-2-/-/γc-/- mice. Conversely, reconstitution of Slp-65 expression stimulated the differentiation of these cells and inhibited their potential to cause leukemia, providing strong evidence for the tumor suppressor role of Slp-65 (34).

Although Btk-deficient mice do not develop pre-B cell tumors, we found that Btk apparently cooperates with Slp-65 as a tumor suppressor, because the incidence of pre-B cell leukemia is significantly enhanced (to ~50-80%) in Btk/Slp-65 double mutant mice, when compared with Slp-65 single-deficient mice (Chapter 3 and ref. 9). Moreover, transgenic expression of the constitutive active E41K-Y223F-Btk mutant, which shows enhanced membrane localization and phosphorylation, prevented tumor formation in Btk/Slp-65 double-deficient mice (Chapter 3 and ref. 9). Because kinase-inactive Btk mutant K430R can functionally replace wild-type Btk as a tumor suppressor in Slp-65-deficient mice, we conclude that Btk exerts its tumor suppressor function in pre-B cells as an adapter protein, independent of its catalytic activity (Chapter 4 and ref. 35). The mechanism by which Btk exerts its tumor suppressor function independent of Slp-65 is currently unknown. It is possible that, by analogy with the findings in mature A20 B cells, Btk functions in pre-B cells as an adapter to recruit phophatidylinositol-4-phosphate-5-kinases (PIP5K) to the plasma membrane (36). These enzymes synthesize PtdIns-4,5,-P2, which is a common substrate shared by PI3K and PLCγ. However, functional studies on the role of PIP5K in pre-BCR signaling have not been reported to date.

Next to the loss-of-function mutations of Slp-65 and Btk, also constitutive signaling by an activated form of the Src-family tyrosine kinase Blk, Y495F-Blk, induces pre-B cell proliferation, enhanced responsiveness to IL-7 and formation of B220+CD43+ B lymphoid tumors (37). Basal tyrosine phosphorylation of Ig-β and Syk was substantially increased in pro-B cells from Y495-Blk transgenic mice. This capacity may be unique to Blk, as expression of a constitutively activated form of the closely related Src-family kinase Lyn does not affect proliferation or IL-7 responsiveness (38).

2.2 Molecular mechanisms of pre-B cell tumor formation in the mouse

Slp-65-deficient pre-B cells are apparently caught in a positive feedback loop, as these cells cannot efficiently downregulate the pre-BCR and the IL-7R, both of which signal for cell survival and/or proliferation. In line with this, Slp-65-deficient mice, or compound mice double-deficient for Slp-65 and either Btk, LAT or CD19, have increased numbers of large pre-B cells (6,9,10,39-44). The dominating population of large pre-BCR+ pre-B cells does not appear to contain large numbers of cycling cells and in CD19/Slp-65 double-deficient mice the pre-B cells were reported to be even almost entirely non-cycling (10,39). Therefore, it appears that pre-B cell accumulation is due to their longevity, which is possibly related to sustained survival signals, involving Bcl-2 or Bcl-xL, emanating from deregulated pre-BCR or IL-7R expression. In agreement with this notion, we found that enhancement of cellular



survival by transgenic overexpression of the anti-apoptotic protein Bcl-2 in the B cell lineage did not increase the incidence of pre-B cell leukemia in Slp-65-deficient mice (R.W.H., unpublished results).

As Btk-deficient mice and a major fraction of Slp-65-deficient mice do not develop leukemia, additional molecular events are required for the induction of genetic changes, finally resulting in malignant transformation of pre-B cells. At present, it is not clear which genes are affected by such secondary mutations and what mechanisms are responsible for the induction of mutations. The finding of elevated protein expression of the V(D)J recombinase component Rag-1 and Rag-2 in Slp-65-deficient pre-B cells would argue for a role of the V(D)J recombinase system in malignant transformation (6,10). Consistent with this notion, pre-B cell leukemias from Slp-65-deficient mice express RAG-1/-2 and contain subpopulations of IgLC-positive cells (Chapter 3 and 5 and ref. 9, 45). Importantly, we found that the expression of a pre-rearranged IgHC, which accelerates B cell development and thereby limits the time window of V(D)J recombination activity, could prevent the formation of pre-B cell leukemia in Slp-65/Btk double-deficient mice (Chapter 5). This capacity of an IgHC transgene to reduce the population of cells susceptible to transformation in Slp-65-deficient pre-B cells parallels previous findings in c-Myc-bearing transgenic mice (46), and may point to the involvement of the V(D)J recombinase system in leukemogenesis. Unlike other mouse models with lymphoid malignancies and involvement of the recombinase system (such as p53/Artemis double-deficient mice, ref. 47) no genomic instability and no specific common chromosomal translocations were found (T.Ta, P. ter Brugge et al., unpublished results). Therefore little evidence exists for a role of the recombinase system in Slp-65-deficient mice as a contributor to genomic instability. Preliminary data however suggest a preference for VH family J558-containing IgHC rearrangements in Slp-65-deficient tumors. These data either suggest preferential outgrowth of pre-leukemic cells expressing VH J558 (etiologically linking specific IgHC composition to oncogenesis) or represent the by-product of other processes. With respect to the latter increased IL-7 receptor signaling in Slp-65-deficient cells might increase VH J558 family usage during IgHC rearrangement (48). Alternatively increased VH J558 family usage may be the result of ongoing rearrangement, as found in some cases of human ALL (49,50).

Another genotoxic factor possibly contributing to oncogenesis in Slp-65-deficient B cells includes activation-induced cytidine deaminase (AID). Normally AID is only expressed in germinal center B cells, allowing SHM and CSR but also exposing these cells to genotoxic stress and possible oncogenic hits. In malignancies originating from such cells (human Burkitt's lymphoma) chromosomal translocations between the Ig switch region and c-myc are a frequent feature. In fact, it was shown

that AID was required to generate these translocations in IL-6 induced mature B cell lymphomas in mice (51,52). Recently AID was also shown to be a mutator in human BCR-ABL1 transformed ALL cells, likely contributing to the unfavorable prognosis of this leukemia subset (53). To investigate whether AID also contributed to oncogenesis in Slp-65-deficient B cells, Slp-65-deficient mice were crossed onto the AID-deficient background. Preliminary results indicate that pre-B cell tumors can arise in Slp-65/AID double-deficient mice, indicating that (premature) AID activity is not essential for tumor formation in Slp-65-deficient mice (T. Ta, unpublished results).

Btk and Slp-65 single and double-deficient pre-B cells express high cell-surface levels of pre-BCR and IL-7 receptor (Chapter 3 and ref. 7, 9, 40, 41). As both are involved in proliferation they may contribute to the transformation of Slp-65-deficient pre-B cells, allowing oncogenic hits to accumulate during successive cell cycles. The importance of pre-BCR signaling was recently addressed in transgenic mice expressing pre-BCR components under the B-cell specific CD19 promoter (12). In this model enforced expression of pre-BCR was unable to induce pre-B cell leukemia *in vivo*. This was in agreement with the finding that sustained pre-BCR expression could not prevent cell cycle exit of large pre-B cells in this model. It could be argued that the higher pre-BCR expression in Btk/Slp-65 double-deficient mice compared to Btk and Slp-65 single-deficient mice might explain the difference in tumor frequency between Btk/Slp-65 double-deficient and Slp-65 single-deficient mice (Chapter 3 and 5). However, no increase in tumor frequency in Slp-65-deficient mice crossed onto a pre-BCR transgenic background was observed (P.F. vanLoo, unpublished results). Therefore, increased pre-BCR expression is not sufficient to increase tumor frequency in Slp-65-deficient mice. We conclude that the increased tumor frequency of Btk/Slp-65 double-deficient mice (as compared to Slp-65 single mutant mice) results from either increased IL-7R signaling or an increased pool size of the population at risk. On the other hand, it is obvious that the findings by Van Loo et al. do not rule out a role for pre-BCR signaling in the initiation of proliferation. Thus pre-BCR signaling-induced proliferation is likely to be an essential step in the process of malignant transformation of pre-B cells.

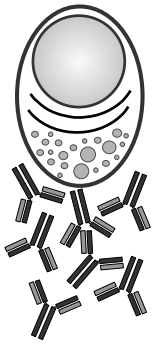
In pre-B cells, IL-7 rapidly and dramatically induces transcription of the proto-oncogenes N-myc and c-myc (54), and upregulates levels of the survival proteins Bcl-2 and Bcl-xL (55). Transgenic expression of N-myc, c-myc, Bcl-2 and Bcl-xL have all been shown to result in fatal pre-B cell leukemias (56-58). Although IL-7 expression by itself is not sufficient for transformation of pre-B cells *in vitro* (16), prolonged expression in transgenic mice does promote the development of tumors of both B and T cell lineages (15). Interestingly, one of the common integration sites in the inbred SL/Kh mouse strain, which has a high incidence of pre-B leukemia

induced by an endogenous murine leukemia virus is located in the Stat5a gene (59). The Stat5a protein is an essential signaling molecule activated by IL-7R signaling. In these pre-B cell leukemias constitutive activation of Stat5a is associated with significant upregulation of c-myc, pim-1 and Bcl-xL, confirming the oncogenic potential of the IL-7R/Stat5a/myc/Bcl pathway.

Although pre-BCR and IL-7 receptor signaling are both involved in initiation of proliferation it was unclear how these two pathways cooperated. Therefore, it was of great importance that recently myc was identified as a common target of both signaling pathways (17). Enforced expression of c-myc in Btk/Tec double-deficient mice was able to drive B cell development past the large pre-B cell stage, up to the IgM+ immature B cell stage. Moreover, loss of Btk and Tec significantly accelerated B cell tumor formation in c-myc transgenic mice and the proliferative response to IL-7. Likely, sustained IL-7 receptor expression in Btk/Tec double-deficient mice cooperates with transgenic c-myc to increase the number of cycling cells at risk for genotoxic stress. Likewise, Slp-65-deficient pre-B cells are probably caught in a positive feedback loop because negative feedback on the IL-7R signaling pathway by pre-BCR signaling is impaired. Therefore, both IL-7 receptor and pre-BCR signaling pathways stimulate proliferation via a myc-dependent pathway, while inhibition of proliferation occurs via a Tec/Btk/Slp-65 dependent pathway. Thus IL-7 receptor signaling and pre-BCR signaling are critical factors in transformation of Slp-65-deficient tumors.

Interestingly, IL-7 can reduce apoptosis and cell cycle arrest that occur upon inactivation of the oncogenic v-Abl kinase protein (55). The v-Abl kinase is an activated form of the c-Abl nonreceptor tyrosine kinase, encoded by the Abelson murine leukemia virus (A-MuLV), and has the capacity to transform pro-B or early pre-B cells (60). It is attractive to hypothesize that the mechanism by which the absence of Slp-65 ultimately subverts normal pre-B cell development is related to the mechanism of malignant transformation of pre-B cells by c-Abl. This would be supported by several findings indicating that v-Abl expression interferes with pre-BCR signaling. First, A-MuLV transformed cells fail to maintain IgHC allelic exclusion, initiate IgLC rearrangement or CD25 expression (24,29,60,61). Second, recent DNA microarray analyses show that in the mouse v-Abl downregulates the expression of several pre-BCR components including Slp-65 (29). Third, in human BCR-ABL1+ pre-B acute lymphoblastoid leukemias (ALL) the kinase activity of the oncogenic BCR-ABL1 fusion protein is linked to defective pre-BCR signaling and expression of truncated forms of Btk and Slp-65 (62-64).

Therefore, factors that contribute to cellular transformation or inhibition of differentiation of Slp-65-deficient pre-B cells may overlap with genes repressed by v-Abl (e.g. the Ku70, BRCA1 and Rb tumor suppressor genes) or induced by v-Abl (e.g. the proto-oncogenes c-Myc and N-myc) (29).



3 FUTURE DIRECTIONS

3.1 Btk and Slp-65 in oncogenesis

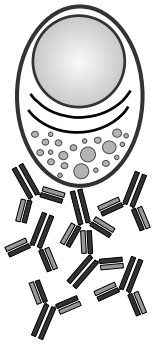
One of the issues of which our understanding has greatly progressed, is that of Btk and Slp-65 as critical regulators of pre-B cell proliferation and differentiation. Obviously, future experiments should identify the nuclear targets of Btk/Slp-65-mediated signaling and the molecular mechanism of malignant transformation of Slp-65-deficient pre-B cells. Studies in human ALL have provided new insight into the role of pre-BCR components in oncogenesis. Although Slp-65-deficiency was found in ~50% of pediatric leukemia's by a German group, this could not be confirmed in an American study. However, in the latter study protein expression was not studied in great detail. To solve this controversy the role of Slp-65 in human leukemias should be studied more extensively in a separate study. Recently, essential roles for truncated kinase-inactive Btk isoforms in BCR-ABL1+ ALL were described, but many challenges remain. At the moment it is not known how and if truncated Btk isoforms contribute to transformation in ALL that do not express the BCR-ABL1 fusion protein. A likely model would be that other constitutively activated tyrosine kinases might have a similar capacity to activate Btk, e.g. ZAP-70, which was recently found to be constitutively expressed and phosphorylated in various human B-lineage ALL (65). Furthermore, it appears that Slp-65 and Btk are not the only targets for aberrant splicing in B-lineage leukemia's. Leukemic cells from pediatric ALL patients show aberrant mRNA species encoding abnormal Syk proteins with a missing or truncated catalytic domain (66). Additionally dominant negative ikaros forms were found that may contribute to oncogenesis by blocking differentiation and enhancing survival (67-71). Further studies are required to determine whether truncated Syk and ikaros isoforms play a pathophysiological role in B-lineage ALL and whether mechanistic parallels exist with the Btk splicing defects in BCR-ABL1$^+$ ALL.

The role of Btk appears to be critical for transformation by BCR-ABL1, as specific inhibition of Btk induces apoptosis in the leukemia cells. Therefore, Btk activity represents an obvious target to be considered for therapeutic intervention in BCR-ABL1$^+$ ALL. This is of particular interest as a means to circumvent resistance to the potent BCR-ABL1 inhibitor STI571, which may arise due to the appearance of point mutations in the BCR-ABL1 gene that impair drug binding. Likely cocktails of inhibitors will be the answer to the development of resistance in BCR-ABL1 positive leukemia's. Thus it is critical to identify new signaling proteins or transcriptional targets involved in pre-B cell leukemia.

3.2 Therapeutic strategies for XLA

Although current therapy for XLA is highly effective, it still lays a heavy burden on patients. They have to visit the hospital very frequently for intravenous or intramuscular immunoglobulin replacement (although some receive this at home) and have to take prophylactic antibiotics lifelong. Still then patients are at increased risk for developing infections. Although life expectancy seems quite good at the moment long-term data are not available for this treatment regimen. Therefore, it is still worthwhile searching for more definitive solutions for children with this disease. One of the options is gene therapy. It has previously been shown that high level expression of Btk under the control of the MHCII promoter was able to rescue B cell development without side-effects (72). Additionally BM transplantation in Btk-deficient mice that did or did not receive BM conditioning was able to fully correct the Btk-deficient phenotype (73,74). Unfortunately preliminary tests of non-conditioned BM transplants in XLA patients failed to rescue B cell development (75). Possibly the BM niches for B cells were occupied by autologous B cell precursors that prevented engraftment of the transplant (76). Therefore, likely pre-transplant BM conditioning is essential for efficient engraftment. Unfortunately, pre-transplant BM conditioning and autologous BM transplant exposes patients with a relatively mild disease such as XLA to severe infectious complications and possibly graft-versus-host disease.

Therefore, currently gene-therapy is investigated as an alternative approach to cure patients. Likely when stable engraftment and gene transfer is required, pre-transplant BM conditioning will be necessary as well. However, in this case no risk of Graft-versus-host disease will be present as autologous stem cells can be used. On the other hand patients might be exposed to oncogenic transformation (77). As an alternative gene-therapy using short-term Btk reconstitution might result in satisfactory B-cell reconstitution and function.



3.3 Concluding remarks

One of the basic goals of studying the role of Btk and Slp-65 in B cells was to improve our understanding of normal B cell physiology and to deepen our understanding of the pathophysiology of B cell diseases. One of the key questions that was posed already early the previous century was the origin of the B cell defect found in XLA patients. Studies by us and many others have greatly deepened our understanding of the defects found in Btk-deficient mice and allowed better insight into XLA. Despite these advances many questions remain. As such it will be challenging to uncover the (pre-)B cell receptor signaling network and the downstream transcription factors involved at the several stages of B cell development. Importantly for XLA and Xid this concerns the developmental progression of pre-B cells and the initiation of

IgLC rearrangement. In this respect, the E-Btk transgenic mouse model might be instrumental as it is able to induce IgLC rearrangement in pro-B cells (Chapter 2). Comparison of E-Btk-transgenic pro-B cells with WT pro-B cells by microarray might allow identification of transcriptionally regulated proteins involved in IgLC rearrangement and cellular maturation independent from those involved in proliferation. The genes identified in this way should then be investigated in more detail in other experimental settings. It is expected that candidate gene approaches in which specific selected genes are either deleted, downregulated (e.g. by RNAi techniques) or overexpressed will contribute significantly to our understanding. It will be increasingly difficult to fit all signaling pathways in one picture as connections are unlikely to be linear and will involve feedback loops. Moreover, considerable redundancy will be present within these pathways (as is obvious for Btk and Tec, Slp-65 and Slp-76/LAT, Plcγ1 and Plcγ2, etc), resulting in false negative results in single-gene-deficient mice. Therefore, studies using compound signaling protein-deficient mice are essential to improve our understanding. Furthermore, as complexity increases this may necessitate use of mathematical models to understand consequences of aberrancies in signaling pathways (78).

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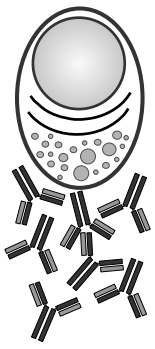

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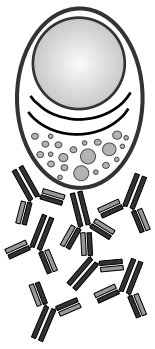

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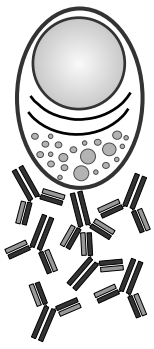
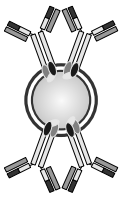


CHAPTER 8

SUMMARY & SAMENVATTING VOOR NIET INGEWIJDEN



SUMMARY

B cell development is a complex process that is characterized by the ordered rearrangement of immunoglobulin heavy chain (IgHC) and immunoglobulin light chain (IgLC) gene segments encoding the BCR. Moreover, it is strongly dependent on the signal transduction proteins Btk and Slp-65, as these are involved in signaling from a variety of receptors including the pre-BCR and BCR. Humans deficient for Btk or Slp-65 virtually lack circulating Ig due to a severe arrest of B cell development at the pre-B cell stage and a resulting B cell deficiency. Accordingly, patients with either X-linked agammaglobulinemia or autosomal agammaglobulinemia suffer from recurrent infections of the respiratory tract and digestive system but may even develop life-threatening disease as well. In contrast, mice deficient for Btk have a milder phenotype characterized by a partial arrest at the pre-B cell stage, failure to efficiently generate peripheral B cells and a block of B cell differentiation at the immature B cell stage. Despite these obvious differences between humans and the mouse, a pre-B cell defect is still present in the mouse. Therefore, studying mouse models is very informative. This thesis aimed to unravel the *in vivo* function of Btk and Slp-65 in B cell development and their involvement in immunodeficiency and malignancy.

In **Chapter 2** we show that Btk- and Slp-65-deficient murine pre-B cells have a specific defect in the transcription of unrearranged λ IgLC genes (germline transcription). In Btk/Slp-65 double-deficient murine pre-B cells both κ and λ germline transcripts were severely reduced. Although these observations pointed to an important role of Btk and Slp-65 in the initiation of light chain gene rearrangement in the mouse, a possibility remained that these signaling molecules were only required for termination of pre-B cell proliferation or for pre-B cell survival, whereby differentiation and IgLC rearrangement was subsequently initiated in a Btk/Slp-65 independent fashion. Because transgenic expression of the anti-apoptotic protein Bcl-2 did not rescue the developmental arrest of Btk/Slp-65 double deficient pre-B cells, we conclude that defective IgLC opening in Btk/Slp-65-deficient small resting pre-B cells is not due to their reduced survival. Next, we analyzed transgenic mice expressing the constitutively active Btk mutant E41K. Expression of E41K-Btk in IgHC negative pro-B cells induced (i) surface marker changes that signify cellular differentiation, including downregulation of surrogate light chain and upregulation of CD2, CD25 and MHC class II, and (ii) premature rearrangement and expression of κ and λ IgLCs. From these data we concluded that Btk and Slp-65 transmit signals that induce cellular maturation and IgLC rearrangement, independent of their role in termination of pre-B cell expansion.

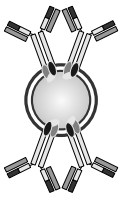
In **Chapter 3** we show that Btk and Slp-65 have synergistic roles in the developmental progression of large cycling into small resting pre-B cells and cooperate as tumor suppressors as Btk/Slp-65 double mutant mice had a dramatically increased pre-B cell tumor incidence (~75% at 16 weeks of age), when compared with Slp-65 single deficient mice (<10%). Furthermore, transgenic low-level expression of a constitutive active form of Btk, the E41K-Y223F mutant, prevented tumor formation in Btk/Slp-65 double mutant mice, indicating that constitutive active Btk can substitute for Slp-65 as a tumor suppressor. Furthermore, **Chapter 4** describes that the kinase-dead mutant K430R-Btk was able to suppress tumor formation as well as wild-type Btk. Therefore, Btk exerts its tumor suppressor function in pre-B cells as an adaptor protein, independent of its catalytic activity.

In **Chapter 5** we investigated genomic instability and deregulated V(D)J recombination as contributors to malignant transformation. As Slp-65-deficient leukemias expressed high levels of the recombination activating genes Rag 1/2 and manifested ongoing IgLC rearrangement, we hypothesized that malignant transformation may be dependent on deregulated V(D)J recombination. We demonstrated that combined loss of Slp-65 and the cell cycle checkpoint protein p53 transformed pre-B cells very efficiently, showing that unrepaired DNA damage enhances malignant transformation of Slp65-deficient pre-B cells. To further analyze the involvement of V(D)J recombination in tumor formation, Btk/Slp-65-deficient mice were crossed with VH81X IgHC transgenic mice. The VH81X IgHC transgene allowed progression to the pre-B cell stage and likely reduces the duration of the pro-B cell stage in which V(D)J recombination occurs. We found that expression of the VH81X IgHC transgene prevented pre-B cell tumor development in Btk/Slp-65-deficient mice. Our results suggest that deregulated V(D)J recombination activity may contribute to malignant transformation of Slp-65-deficient pre-B cells.

In **Chapter 6** the function of Btk in migration towards the chemokines SDF-1 and CXCL13 and in *in vivo* homing was assessed. An *in vivo* homing assay demonstrated that Btk deficient pre-B cells and immature B cells had impaired homing to secondary lymphoid organs. Our results provided new insights into the role of Btk in the control of B cell homeostasis and trafficking.

In **Chapter 7** the findings from the previous chapters and the current literature were discussed. Our findings in the mouse have shed new light on the molecular mechanism underlying XLA. The data indicate that XLA patients do not only suffer from decreased generation of sufficient pre-B cell numbers but that also defects in pre-B cell differentiation and IgLC rearrangements may contribute. Additionally, defects in migration may be important in human XLA. Furthermore we demonstrated that Btk cooperates with Slp-65 as a tumor suppressor in the mouse. Possibly increased IL-7 receptor and pre-BCR signaling and deranged V(D)

J recombinational activity contributed to pre-B cell leukemia formation. Our findings in the mouse have contributed considerably to fundamental knowledge on pre-BCR signaling. Furthermore, our results suggest a role for Btk in human leukemias. In fact, it has been shown that Btk was necessary for survival of leukemia cells, thus making Btk an attractive target for therapeutic intervention with kinase inhibitors.



SAMENVATTING VOOR NIET-INGEWIJDEN

B cellen zijn witte bloedcellen die onderdeel uitmaken van het immuunsysteem. Na contact met een ziekteverwekker kunnen ze uitrijpen tot plasmacellen, die verantwoordelijk zijn voor het maken van beschermende antistoffen. Antistoffen uit het bloed van gevaccineerde paarden werden bv. ook wel gebruikt als antiserum bij mensen gebeten door giftige slangen. B cellen ontwikkelen zich in het beenmerg vanuit de haematopoëtische stamcel. Tijdens dit proces zijn er diverse fasen te onderscheiden waarin de voorloper-B cellen een groot aantal celdelingen doormaken, afgewisseld met fases waarin de cellen zich niet delen en juist een specifiek differentiatieprogramma doormaken. Het bestuderen van B cellen en hun ontwikkeling is belangrijk vanwege hun betrokkenheid bij verschillende ziektes waaronder leukemie en problemen in de afweer (immuundeficiënties).

De voorloper-B cel, Btk en Slp-65

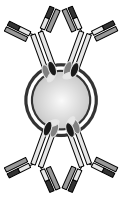
B cellen worden gekenmerkt door het tot expressie brengen van een op de celmembraan gebonden B cel receptor (BCR), die bestaat uit twee immuunglobuline (Ig) zware en lichte ketens. Om de productie van Ig mogelijk te maken, zijn DNA herschikkingen van de Ig genen nodig, waarbij op een nauwkeurig gereguleerde manier breuken in het erfelijk materiaal, het DNA, worden aangebracht door de speciale eiwitten, RAG-1 en RAG-2. Tijdens deze zogenoemde V(D)J recombinatie worden door de combinatie van verschillende V, D, en J gensegmenten de genen van de Ig zware en lichte keten gevormd, waarbij de DNA breuken worden hersteld door DNA schadeherstel eiwitten. Het vroegst herkenbare B cel stadium, het pro-B cel stadium, herschikt zijn Ig zware keten gensegmenten totdat succesvolle herschikking leidt tot expressie van de Ig zware keten. Samen met de reeds aanwezige surrogaat lichte ketens (SLC) vormt deze Ig zware keten de pre-BCR, die op het cel oppervlak tot expressie wordt gebracht. Expressie van de pre-BCR functioneert als een controlepunt en induceert proliferatie en voortgang naar het grote pre-B cel stadium. Na een aantal celdelingen ontstaat vervolgens de kleine pre-B cel waarin Ig lichte keten herschikkingen plaatsvinden. Succesvolle herschikking van de Ig lichte keten gensegmenten leidt tot de expressie van de BCR. De hierbij ontstane immature B cellen verlaten het beenmerg en migreren naar de milt waar zij zich verder ontwikkelen tot functioneel verschillende B cel subtypes.

Btk en Slp-65 zijn twee intracellulaire signaleringseiwitten, die signalen doorgeven vanaf de pre-BCR en de BCR. Mutaties in het Btk gen zijn verantwoordelijk voor de meest voorkomende ernstige primaire immuundeficiëntie bij de mens: X-gebonden agammaglobulinemie (XLA). De eerste beschrijving van dit ziektebeeld dateert uit 1952 toen Bruton een achtjarige patiënt beschreef die meerdere keren

met een bloedvergiftiging opgenomen was geweest. Het was opmerkelijk dat in het serum van deze patiënt de concentratie van antistoffen sterk verlaagd was. Meestal hebben deze patiënten last van frequente infecties van bovenste en onderste luchtwegen met eventueel ernstige levensbedreigende infecties. Ook afwezigheid van Slp-65 in de mens leidt tot agammaglobulinemie. Agammaglobulinemie wordt gekarakteriseerd door een bijna complete blokkade op het pre-B cel stadium in het beenmerg met een probleem in proliferatie. Ook Btk en Slp-65-deficiënte muizen vertonen milde defecten op de overgang van het grote naar het kleine pre-B cel stadium waarbij zij verminderd in staat zijn om proliferatie te stoppen en verder te differentiëren. Verrassend genoeg bleek Slp-65 in de muis ook als een tumor suppressor eiwit te functioneren aangezien ~10% van de Slp-deficiënte muizen pre-B cel leukemie ontwikkelde. Dit was een van de eerste bewijzen dat pre-BCR signaleringsdefecten, betrokken konden zijn bij het ontstaan van leukemie. Acute lymfatische leukemie is een van de meest voorkomende vormen van kanker bij kinderen. Hierbij treedt er een woekering op van de voorlopercellen van B of T lymfocyten. Hoewel er verschillende genetische afwijkingen bij leukemieën zijn gevonden, blijft er over het algemeen nog weinig bekend over de precieze oorzaak van dergelijke tumoren. Er zijn aanwijzingen dat in ~50% van de voorloper-B cel leukemieën bij kinderen SLP-65 niet tot expressie komt.

Om de rol van Btk en Slp-65 bij het ontstaan van immuundeficiënties en maligniteiten beter te begrijpen, bestudeerden wij het effect van afwezigheid van Btk en Slp-65 en aanwezigheid van mutante vormen van Btk, op de B cel ontwikkeling en het ontstaan van immuundeficienties en leukemieën.

Ons onderzoek



In **Hoofdstuk 2 en 3** werd bestudeerd hoe de samenwerking van Btk en Slp-65 was bij de differentiatie van grote delende pre-B cellen tot kleine rustende pre-B cellen in de muis. Hiervoor werd het pre-B cel compartiment in Btk/Slp-65 enkel en dubbel-deficiënte muizen geanalyseerd. Wij vonden dat Btk en Slp-65 samenwerken om van grote naar kleine pre-B cel te ontwikkelen waardoor in Btk/Slp-65 dubbel-deficiënte muizen een bijna complete blokkade op het grote pre-B cel stadium ontstaat. De vraag was of deze blokkade het directe gevolg was van defecten in de pre-B cel ontwikkeling (differentiatie) en de κ en λ lichte keten gen herschikkingen. Een andere mogelijkheid zou namelijk zijn dat Btk en Slp-65 enkel nodig zijn voor het stoppen van de proliferatie in het grote pre-B cel stadium of voor de overleving van pre-B cellen waarna ontwikkeling en lichte keten gen herschikkingen onafhankelijk van Btk en Slp-65 verder zou gaan. Daarom hebben wij in een transgeen muizenmodel het effect van een constitutief actieve Btk mutant op lichte keten genherschikkingen in het zeer vroege pro-B cel stadium onderzocht. Onafhankelijk van proliferatie

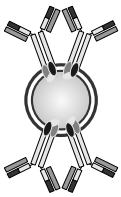
en overleving induceerde het constitutief actieve Btk differentiatie en lichte keten herschikkingen. Hiermee toonden wij aan dat Btk en Slp-65 signalen doorgeven die differentiatie en lichte keten herschikkingen induceren onafhankelijk van deling en overleving.

Slp-65-deficiente muizen ontwikkelen spontaan voorloper B cel leukemie, terwijl afwezigheid van Btk nooit in verband is gebracht met tumoren. Daarom onderzochten we in **Hoofdstuk 3** verder of de afwezigheid van Btk effect had op de tumorvorming in Slp-65-deficiente muizen. Om deze vraagstelling te kunnen beantwoorden werden Btk/Slp-65 dubbel-deficiënte muizen bestudeerd. Zeer onverwacht, omdat afwezigheid van Btk alleen nooit in verband is gebracht met maligniteiten, bleken deze een dramatisch toegenomen tumor frequentie te hebben (~75%) t.o.v. Slp-65-deficiënte muizen (~10%). Het belang van de verschillende gedeeltes (eiwitdomeinen) van Btk, voor zijn functie als tumor suppressor, werd onderzocht in Btk/Slp-65 dubbel-deficiënte muizen die verschillende specifieke mutanten van Btk, transgeen, gedurende de B cel ontwikkeling tot expressie brachten. Transgene expressie van een constitutief actieve Btk mutant (in alle B cel stadia in de muis) kon de vorming van pre-B cel leukemieën in de Slp-65-deficiënte muizen voorkomen. Daarom trokken wij de conclusie dat constitutieve activiteit van Btk de activiteit van Slp-65 als tumor suppressor kon vervangen. Verder lieten we in **Hoofdstuk 4** zien dat transgene expressie van een Btk-mutant zonder enzymatische activiteit (kinase-dood) de hoge frequentie van tumoren in Btk/Slp-65 dubbel-deficiënte muizen drastisch kon reduceren tot de frequentie zoals deze in Slp-65-deficiënte muizen werd gevonden. Daarom concluderen wij dat Btk in de muis zijn tumor suppressor functie onafhankelijk van zijn enzymatische kinase activiteit kan uitvoeren.

In **Hoofdstuk 5** bestudeerden wij of het V(D)J recombinatiesysteem bij de vorming van pre-B cel leukemieën in Slp-65-deficiënte muizen betrokken was. Dit werd gesuggereerd doordat voortgaande Ig lichte keten recombinatie activiteit werd gevonden in pre-B cel leukemieën. Zo vertonen pre-B leukemie cellen grote hoeveelheden Rag-1/2 transcripten, co-expressie van SLC en κ-lichte keten op de celmembraan, en oligoklonale (bestaande uit enkele verschillende groepen leukemiecellen) κ lichte keten herschikkingen. Daarnaast zorgde afwezigheid van het oa. door DNA-schade geactiveerde p53, voor een sterke toename in het aantal leukemieën in Slp-65-deficiënte muizen. Bovendien kon introductie van een herschikt Ig zware keten transgen in Btk/Slp-65 dubbel-deficiënte muizen de ontwikkeling van leukemie voorkomen. Het transgen zorgt zeer waarschijnlijk voor een verkorting van de duur van het Ig zware keten recombinerende pro-B cel stadium. Daarom denken wij dat gedereguleerde V(D)J recombinatie activiteit bijdraagt aan de maligne transformatie van Slp-65-deficiënte pre-B cellen.

In **Hoofdstuk 6** lieten we zien dat Btk naast signalering onder de pre-BCR en BCR ook bij de beweging (migratie) van B cellen betrokken is. Meer specifiek zagen we dat migratie richting de twee chemokines SDF-1 en CXCL13 *in vitro* gestoord was. Verder vertoonden Btk-deficiënte pre-B cellen en immature B cellen ook in het lichaam een gestoorde migratie naar voornamelijk lymfeklieren. Onze resultaten laten zien dat Btk meerdere functies kan hebben binnen dezelfde cel, hetgeen mogelijk een aantal aspecten van het Btk-deficiënte ziektebeeld in zowel muis als mens zou kunnen verklaren.

Tenslotte werden in **Hoofdstuk 7** alle resultaten uit eerdere hoofdstukken bij elkaar gezet en binnen de context van de huidige literatuur geplaatst. Onze resultaten geven aan dat er bij XLA patiënten waarschijnlijk niet alleen een probleem is in de vorming van voldoende pre-B cellen, maar ook dat de aanwezige B cellen niet voldoende in staat zijn hun Ig lichte keten gensegmenten te herschikkingen. Daarnaast zouden ook defecten in migratie hierbij een rol kunnen spelen. Verder toonden wij aan dat Btk in de muis functioneert als een tumor suppressor in B cellen waarbij mogelijk persisterend cytokine receptor en pre-BCR signalering en het V(D)J recombinatiesysteem een rol spelen in de leukemie vorming. Naast dat onze bevindingen belangrijke nieuwe inzichten hebben geleverd in fundamentele kennis over de pre-BCR signalering, suggereren ze ook dat Btk potentieel betrokken is in humane leukemieën. Inderdaad heeft men laten zien dat Btk in een bepaald type leukemie in de mens nodig is voor overleving van leukemiecellen wat in de toekomst mogelijk nieuwe interessante therapeutische mogelijkheden kan bieden met bv. Btk-remmers.



DANKWOORD &
ABOUT THE AUTHOR

DANK DANK DANK.

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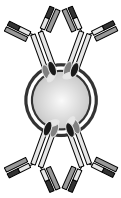

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