Btk at the Pre-B Cell Receptor Checkpoint

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Btk at the Pre-B Cell Receptor Checkpoint

Bruton's tyrosine kinase en het pre-B cel receptor controlepunt

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General introduction

Parts of this chapter are published as a review in Trends in Immunology

1. The immune system

The immune system is the defence mechanism by which the body protects itself against environmental agents that are foreign and possibly dangerous, such as micro-organisms. The first line of defence is non-specific (innate) and consists of physical components, such as the skin and mucous membranes to prevent agents invading the body. The second line of defence is either non-specific or specific and consists of specialized cells whose purpose is to destroy the invader once it has managed to pass the first line of defence. The foreign agent can either be destroyed by cells of the innate immune system (including natural killer (NK) cells, macrophages, granulocytes and the complement system) or it can trigger a chain of events that induces a specific immune response. The latter is also referred to as acquired immunity as the response is specific for the invader only and provides immunity to withstand and resist another exposure to the same agent, also called antigen. There are three major cell types involved in acquired immunity. Two of these cell types arise from a common lymphoid precursor cell, but differentiate in different developmental ways and in separate organs. One cell type matures in the thymus and is called T cell; the other matures in the bone marrow (BM) and is called B cell. Lymphocyte antigen receptors, in the form of immunoglobulin (Ig) molecules or B cell receptors (BCR) on B cells and T-cell receptors (TCR) on T cells, are the means by which lymphocytes sense the presence of antigens in their environment. The receptors produced by each lymphocyte have a unique structure of their antigen-binding site in order to respond to a wide variety of antigens the organism may encounter. The third type of cell participating in the acquired immune response is the antigen-presenting cell (APC), such as the macrophage or the dendritic cell. They take up, process and present the antigen with major histocompatibility complex (MHC) proteins to the specific receptors on T cells.

Immunity to bacterial antigens is mediated by recognition of the antigen by the B cells or antibodies that circulate throughout the body. Once the antigen is recognized and bound to the BCR, the B cell is activated and receives signals to differentiate into a plasma cell and make the secreted form of its BCR, the antibody.

This thesis focuses on the development of B cells and the role of specific signal transduction proteins in this process.

2. The B cell

In mice, B cells are generated from hematopoietic stem cells that commit to the B-cell lineage in response to signals from a variety of soluble factors, called cytokines. The development of B cells takes place in the BM and the spleen. The early B cell development in the BM is ordered into several stages according to the rearrangement status of the immunoglobulin gene loci. After production of a proper BCR on their surface, B cells migrate to the spleen to differentiate into mature B

cells and circulate throughout the body to search for antigens they can respond to. When the BCR recognizes and binds an antigen, B cells differentiate into antibody producing plasma cells or into memory B cells to provide memory and efficiency when the same antigen is encountered again.

2.1 Antibody receptors

Ig molecules or BCRs are composed of two identical heavy (H) chains and two identical light (L) chains, which are bound to each other by a disulfide bridge. Both chains have a constant (C) region and a variable (V) region (Figure 1). The C region of the Ig molecule is involved in membrane expression, complement binding and binding to other cells of the immune system. The V region is the part of the BCR that recognizes and binds antigen and consists of three hypervariable complementary determining regions (CDR). This restricts the antibody to combining only with one particular antigenic structure. Therefore it is necessary to produce a large repertoire of specificities, which is achieved by variation in the amino acid sequence at the antigen-binding site. The V regions of the receptor chains are encoded in several pieces, called the V (variable), D (diversity, only for H chains) and J (joining) gene segments. There are multiple copies of these three V-region gene segments present in the genome. However only one copy of each gene segment is used to assemble a complete V-region in a process called V(D)J recombination.

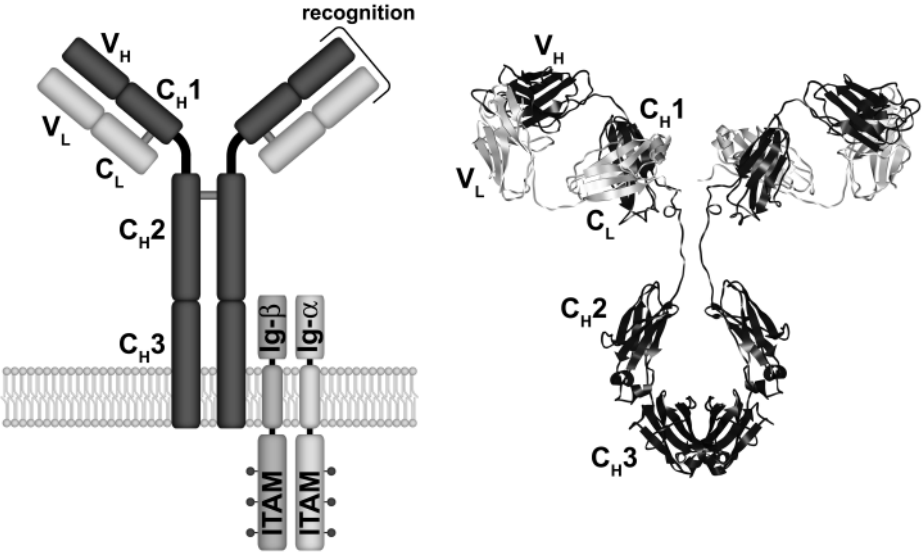

Figure 1. *Schematic (left) and molecular (right) model of human immunoglobulin protein showing the constant (C) and variable (V) regions of the two heavy chains in dark grey (C_HV_H) and the two light chains in light grey (C_LV_L). The heavy and light chain V-region domains are in close contact and function as the antigen-binding part of the molecule. In the schematic drawing, the two signalling molecules Ig-α and Ig-β are also depicted. The molecular model was constructed as described in ref. (1).*

2.2 V(D)J recombination

The Ig gene segments are organized into three clusters or genetic loci, the H chain loci and the κ and λ L chain loci. Each locus consists of separate clusters of V, D or J gene segments and of C-region genes. The DNA rearrangements are guided by conserved non-coding DNA sequences that are found adjacent to the points at which the recombination takes place. These sequences consist of a conserved block of seven nucleotides (the heptamer), a non-conserved region (the spacer, which is either 12 or 23 nucleotides long) and a second conserved block of nine nucleotides (the nonamer). The heptamer-spacer-nonamer sequence is called the recombination signal sequence (RSS).

Recombination only occurs between gene-segments located at the same chromosome. Only a gene segment flanked by an RSS with a 12-basepair (bp) spacer can be joined to another gene segment with a 23bp-spacer RSS. In the H chain locus, the V and J RSS regions contain 23bp-spacers and the D RSS regions contain 12bp-spacers. Therefore it is only possible to recombine D to J segments and V to D segments, but not V to J segments. In contrast, L chain genes only contain V and J regions, with 23 and 12bp-spacer RSSs, respectively.

Two recombination activating gene (RAG) proteins recognize the RSS

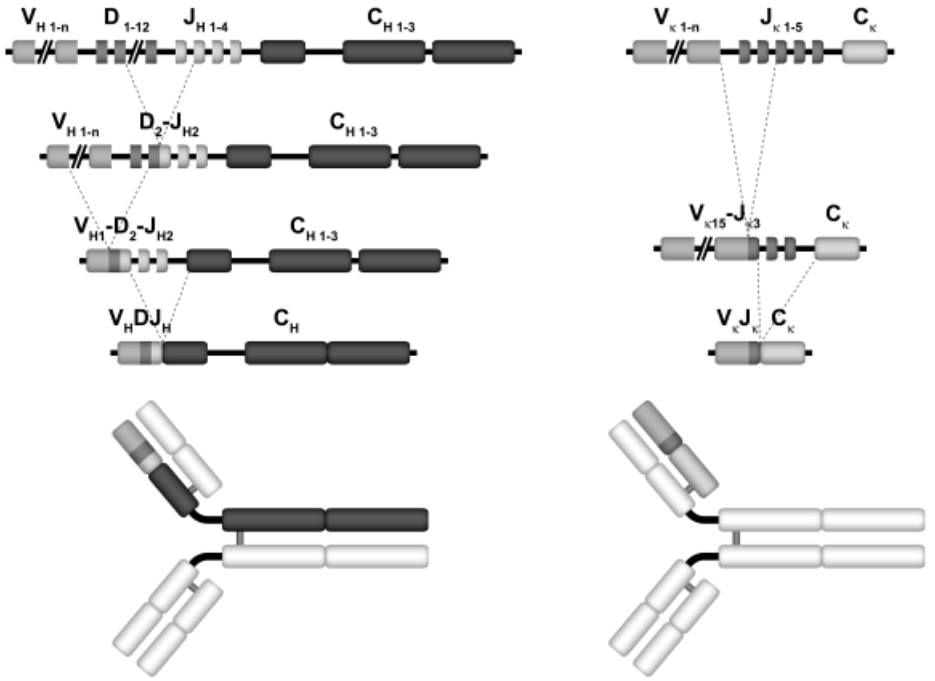

***Figure 2**. Schematic drawing of V(D)J recombination of the heavy (left) and light (right) chain genes, which together form an immunoglobulin protein. See text for details.*

sequences and initiate the rearrangement by introducing DNA double strand breaks (DSBs) at the border of each RSS involved in the rearrangement. The DNA at the ends of the DSBs is left in a hairpin-sealed coding end, which has to be processed before the formation of a coding joint can take place. This DSB repair mechanism is called non-homologous end-joining (NHEJ). At least six factors participate in the rejoining phase of the V(D)J recombination. First the DNA binding proteins Ku70 and Ku86 bind as a complex to the DSBs. The DNA-Ku heterodimer complex can recruit the catalytic subunit (cs) of DNA-dependent protein kinase (DNA-PK_{cs}) and associates with it. Activated DNA-PK_{cs} recruits and activates the X-ray cross-complementation group 4 (XRCC4) and Artemis proteins, of which the latter cleaves the RAG-generated hairpins. Polymerase activity is supplied by DNA polymerase μ (Pol μ) and DNA ligase IV seals the signal and coding joints. The protein terminal deoxynucleotidyl transferase (TdT) can add additional nucleotides in order to make the religation imprecise. A defect in most of the components involved in V(D)J recombination aborts the completion of the process and leads to a complete arrest in B cell development at the pro-B cell stage. The molecular mechanism of V(D)J recombination is illustrated in figures 2 and 3 and is reviewed in references (2) and (3).

The mechanism of DNA rearrangement is similar for the H and L chain loci, although only one joining event is needed to generate a L chain gene and two events to generate a complete H chain gene (Figure 2).

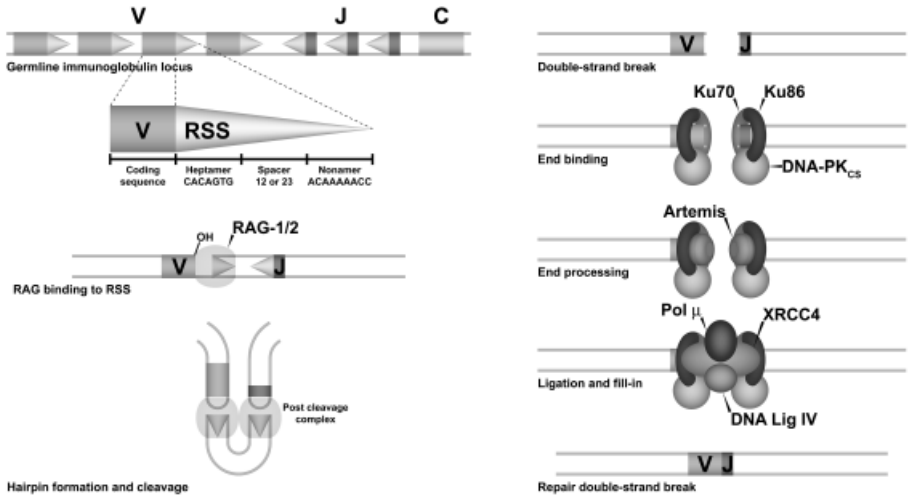

***Figure 3**. V(D)J joining occurs by DNA recombination and non-homologous end-joining (NHEJ). RAG proteins bind to RSS sites and cleave the DNA (left). The resulting double stranded DNA break is repaired by NHEJ involving several factors as indicated (right). For details and abbreviations, see text. Figure adapted from (2).*

2.3 Antibody diversity

As the BCR is very specific for only one particular antigenic structure, the immune system requires a large repertoire of different antibody molecules. Antibody diversity is generated in four ways.

First, there are multiple copies of each of the gene segments that make up an Ig variable region and different combinations of segments can be used in different rearrangement events. A second form of achieving diversity arises from the pairing of the H and L chains to form a complete Ig molecule as the ability of the H and L chains to pair is independent of the variable regions involved. Although there are two chromosomes containing the H chain gene segments and four chromosomes containing the L chain gene segments (the κ and λ L chain loci are located on different chromosomes), only one H chain and one L chain are finally expressed. This restriction is called allelic exclusion. The processes of V(D)J recombination and allelic exclusion are able to generate about $3x10^6$ possible antibody molecules. In the third place, diversity can be introduced at the joins between the different gene segments. By the joining of V, D and J segments, some steps create diversity in the variable regions by inserting or deleting nucleotides (by TdT or exonucleases, respectively) at the joins between the segments. And fourthly, there is somatic hypermutation (SHM), which results in an increased affinity of antibodies for antigen in the secondary immune response. The SHM process introduces point mutations into the V regions of rearranged H and L chain genes by which the specificity of the Ig molecule is altered after antigenic stimulation.

3. B cell development

B-lymphocytes develop from haematopoietic stem cells in the bone marrow through distinct stages that are characterised by differential expression of various cell surface markers and the ordered rearrangement of Ig H and L chain gene segments (Figure 4; see (4, 5) for review). B cell differentiation is initiated by the progression from uncommitted progenitors with multi-lineage potential to a B-lineage restricted stage and is dependent on the combinatorial action of specific transcription factors (reviewed in (6)). This differentiation program is dependent on a series of developmental cell fate decisions at specific antigen-independent checkpoints, controlled by the BCR and its immature form, the pre-BCR. The pre-BCR is transiently expressed on the cell surface of pre-B cells that have performed a productive Ig H chain V(D)J rearrangement, and acts as a key checkpoint to monitor proper expression of functional Ig μ H chain on the cell surface. Pre-BCR signalling inhibits further H chain rearrangements and triggers clonal expansion and developmental progression of Igμ^+ pre-B cells. The pre-B cells rearrange κ or λ L chains that together with the μ H chain form the BCR. These immature B cells are then allowed to go to the spleen to differentiate into mature B cells. Mature B cells recirculate throughout the body and screen for antigenic structures the BCR recognizes.

Transcription factors

Various transcription factors have been shown by loss- and gain-of-function experiments to be required for early B cell development, including Ikaros, PU.1, E2A, Pax5 and IRF-4,8 (reviewed in (7)). Phenotypic analysis of mice with targeted null alleles of genes encoding these factors has been used to order their developmental functions in the B lineage. At the onset of B lymphopoiesis, the transcription factors E2A and EBF coordinately activate the B cell-specific gene expression program (8, 9). Subsequently, activation of Pax5 commits B cell progenitors to the B cell lineage by repressing the transcription of lineage-inappropriate genes and activating B cell lineage-specific genes (10, 11).

The generation of committed lymphocyte precursors is regulated by the Ikaros protein, which belongs to a family of Zinc finger transcription factors that includes two other members, Aiolos and Helios. Ikaros is expressed in stem cells and in mature lymphocytes and Ikaros-like proteins recognize binding sites in many lymphocyte-specific genes including TdT, V_{preB} and $\lambda 5$ (12). In mice with a targeted mutation in the dimerization domain of Ikaros, no B cells in either the fetal or adult phase of life were detected and T cell development was defective (13).

Ets proteins regulate gene expression during a wide variety of developmental and adaptive cellular responses, including cellular differentiation and lymphocyte activation. There are at least 18 different mammalian Ets proteins that bind to purine-rich DNA consensus elements. The Ets protein Spi-B is a hematopoietic-specific transcription factor most closely related to another Ets family member PU.1 (also called Spi-1). PU.1 has been implicated in the myeloid versus lymphoid lineage decision by regulating cytokine-dependent proliferation and differentiation of precursor cells (14, 15). PU.1 is highly expressed in B cells, granulocytes and monocytes and many genes involved in lymphoid and myeloid development have been identified as putative PU.1 target genes. For example: Ig μ H chain (16), Ig κ and λ L chains (17, 18), Ig-α and -β (19), and M-CSF receptor (20). PU.1 is shown to directly regulate the IL-7Rα gene and the activity of the IgH intronic enhancer (21). In PU.1$^{-/-}$ progenitors the IL-7 dependent proliferation is defective (22) and PU.1$^{-/-}$ mice exhibit defects in the development of T and B cells, monocytes and granulocytes and die at gestation day 18.5 (14). In contrast, Spi-B$^{-/-}$ mice are viable, fertile and possess mature B and T cells and Spi-B expression was not able to compensate for the absence of PU.1 in PU.1$^{-/-}$ mice (14).

The initiation of B cell development in the BM critically depends on two transcription factors: the basic helix-loop-helix (bHLH) protein E2A and the early B cell factor (EBF). In the absence of either transcription factor, B cell development is arrested at the earliest stage, even before D-J_H rearrangement of the Ig H chain (23-25). These regulators cooperatively induce the expression of several B cell specific genes, including those encoding the SLC genes $\lambda 5$ and V_{preB}, the signalling molecules Ig-α and Ig-β and the RAG-1 and -2 proteins (8, 9). EBF can directly bind and regulate the Pax5 promoter and encodes a B cell-specific transcription factor that binds DNA as a homodimer using a DNA-binding domain and a dimerization

domain related to the helix-loop-helix (HLH) motif of the bHLH family of transcription factors (26, 27). EBF and E2A heterozygous mice exhibit an ~2 fold decrease in the number of pro-B cells, indicating that normal B cell development depends on two wild-type EBF alleles. E2A is expressed in two splice forms, E12 and E47, which differ in their bHLH domains. E2A binds a DNA sequence termed E-box, which is found in the promoter regions of many different cell type-specific genes. Although E2A is widely expressed, targeted disruption of E2A produces viable animals that have a severe defect in early B cell development, prior to the onset of Ig rearrangement (23, 24). It has been shown that E2A and EBF, next to the RAG proteins are required for initiation of V(D)J recombination in a non-lymphoid cell line. The two transcription factors influence different types of rearrangement. Both E2A and EBF can induce D_H-J_H rearrangement, whereas Vκ-Jκ rearrangement is only induced by E2A and Vλ-Jλ rearrangement by EBF (28).

The Pax family of transcription factors has been shown to play a crucial role in organogenesis and development. The Pax5 gene, encoding the B cell-specific activator protein (BSAP) is expressed exclusively in B cells (29, 30). In Pax5-deficient ($Pax5^{-/-}$) mice, B cell development is arrested at the pro-B cell stage in the BM. Importantly, $Pax5^{-/-}$ pro-B cells still retain a broad lympho-myeloid developmental potential characteristic of uncommitted hematopoietic progenitors. Upon appropriate cytokine stimulation, $Pax5^{-/-}$ pro-B cells are able to differentiate both *in vitro* and *in vivo* into functional T cells, NK cells, dendritic cells, macrophages, osteoclasts and granulocytes (10, 11, 31, 32). $Pax5^{-/-}$ pro-B cells express various B cell-specific genes such as RAG-1 and -2, Ig-α (mb-1), Ig-β (B29), V_{preB} and λ5 as well as sterile transcripts of the H chain locus. $Pax5^{-/-}$ pro-B cells are responsive to IL-7 and have the capacity to grow long-term *in vitro* on stromal cells. However, the cells can not differentiate into immature B cells upon withdrawal of IL-7 from the culture (11, 31). Furthermore, Pax5-deficient pro-B cells were shown to have normal D-J_H but reduced V_H-DJ_H recombination (33). In experiments using conditional Pax5 inactivation in committed pro-B cells it was demonstrated that Pax5 is also required to maintain the B cell transcription program, next to its role to initiate it for B cell commitment. When Pax5 was inactivated, previously committed pro-B cells regained the capacity to differentiate into macrophages *in vitro* and to reconstitute T cell development *in vivo* in $RAG\text{-}2^{-/-}$ mice (34). Pax5 is also required for maintaining the identity and function of mature B cells during late B cell development. This function of Pax5 was demonstrated in a mouse strain where Pax5 was conditionally inactivated by the expression of CD19 (35).

IRF-4 and -8 are two specialized members of the interferon regulatory factor (IRF) family that have been implicated in regulating Ig L chain gene transcription during B cell development (17, 36-39). They are selectively expressed in the lymphoid and myeloid lineages of the immune system. IRF-4 and IRF-8 bind very weakly to DNA-containing IRF sites, but are recruited to their binding sites via interactions with other transcription factors. In particular the related Ets family transcription factors PU.1 and Spi-B have been shown to recruit IRF-4 or IRF-8

to Ets-IRF composite elements (EICE) in Ig κ 3' and λ enhancers (17, 36, 38). It remains to be determined whether PU.1/Spi-B regulates Ig L chain gene expression during the pre-B to immature B cell transition. IRF-4$^{-/-}$ mice exhibit profound defects in B and T cell activation, which are manifest in the failure to generate antibody, cytotoxic or anti-tumor responses. Loss of either IRF-4 or -8 does not block B cell development, however the combined loss of IRF-4 and -8 (IRF-4,8$^{-/-}$) causes a lineage-specific block in early B cell development at the large pre-B cell stage (40). IRF-4 and -8 were shown to coordinate the pre-B to B cell transition in part by downregulation SLC gene expression, thereby facilitating cell cycle exit and upregulation of RAG gene and protein expression. Concomitantly, IRF-4,8 directly induces enhancer-dependent L chain transcription and recombination, thereby enabling pre-B cell differentiation (40).

All the transcription factors discussed above can influence each other's activity. For example, Ikaros and PU.1 appear to act upstream of E2A, EBF and Pax5 (12-15). E2A, EBF and Pax5 directly control transcription of early B cell genes, such as Ig-α, Ig-β, V_{preB}, λ5, RAG-1 and RAG-2. E2A appears to directly regulate

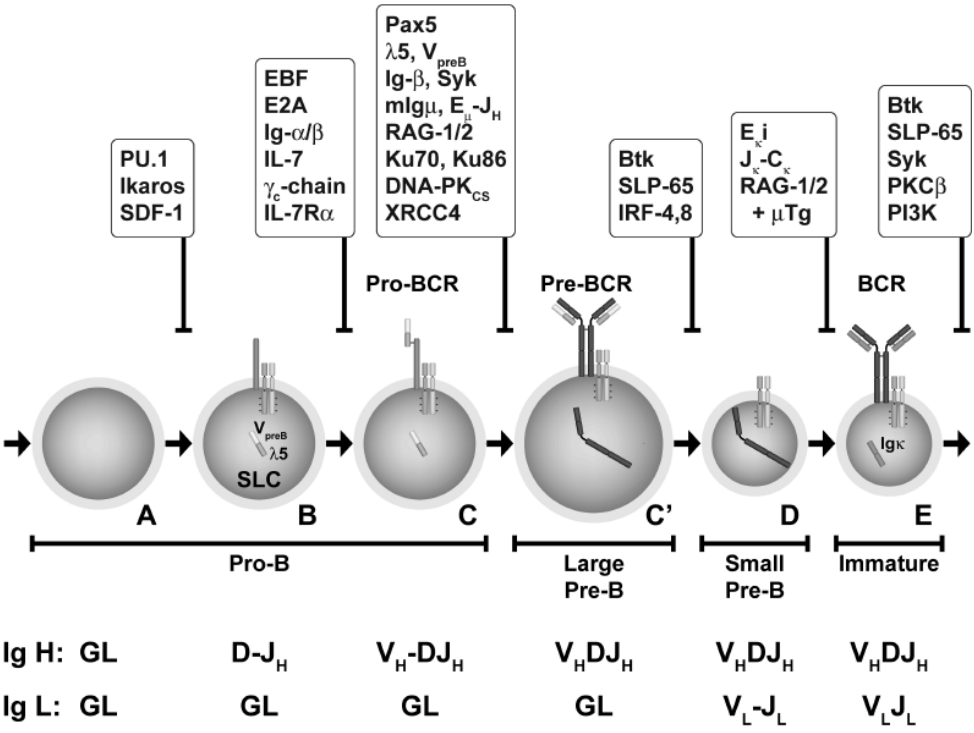

Figure 4. *B cell development in mouse BM. Blocks in different stages of B cell development by deficiency of various molecules is indicated. BCR: B cell receptor; SLC: surrogate light chain; Ig H, Ig L: configuration of heavy (H) or light (L) chain gene loci, respectively; GL: germ-line configuration.*

transcription of the EBF gene (9) and EBF in turn is implicated in activating expression of the Pax5 gene (7).

Chemokines, Cytokines and cytokine receptors

The chemokine stromal cell-derived factor 1 (SDF-1) has complex effects on the migration, proliferation and differentiation of leukocytes. Chemokines are important in directing emigration of specific leukocyte subsets into sites of infection as well as in determining trafficking patterns of recirculating lymphocytes. SDF-1 is involved in directing progenitor cells into appropriate microenvironments to receive expansion and differentiation signals. SDF-1 regulates B cell maturation and can stimulate pre-B cell proliferation (41). Mice deficient in SDF-1 have severely reduced B cell lymphopoiesis and show no bone marrow myelopoiesis. SDF-1 deficient mice die perinatally (42). In addition, SDF-1 functions as a chemo-attractant for T cells and monocytes and for $CD34^+$ hematopoietic progenitors (43).

The cytokine interleukin-7 (IL-7) is a 25 kD protein produced by stromal cells that promotes the proliferation and survival of T- and B-lineage precursors in the mouse, but does not appear to play a role in human B cell development (Reviewed in (44, 45)). The IL-7R is composed of two chains, the IL-7Rα ligand-binding chain and the common γ (γc) signalling chain, which are both required for proper signalling (46, 47). The IL-7Rα chain also comprises part of the thymic stromal lymphoprotein (TSLP) receptor (48), while the γc chain is shared by the receptors for IL-2, -4, -9, -15 and -21 (49, 50). Lymphopoiesis is impeded at an early stage when mice are treated with neutralizing antibodies against IL-7 (51) or against the IL-7Rα chain (52). Mice with a targeted deletion in IL-7 or its receptor (IL-7R) show a block in B cell development at the pro-B cell stage, supporting the fact that IL-7 is very important in this stage of B cell development (53-55).

In IL-7R$\alpha^{-/-}$ mice, D to J_H recombination is not affected, but distal V_H segments do not become accessible to the RAG proteins, resulting in impaired V_H to DJ_H rearrangement (54). In IL-7 or γc chain-deficient mice, the proliferative expansion of $Ig\mu^+$ pre-B cells is abolished. Both pro-B and pre-B cells can be induced to proliferate *in vitro* in the presence of IL-7, but cells expressing a pre-BCR have a substantial proliferating advantage in limiting concentrations of IL-7 (56). It has been proposed that the location of B-lineage precursors in discrete micro-environmental niches in the BM may expose them to gradients of IL-7 that influence signalling through the pre-BCR (45). In this model, developing B cells move centrally toward and through sinusoids before migrating to the periphery. High levels of IL-7 promote an early proliferative phase of pro-B cells undergoing V_H-gene rearrangements, while an adjacent area with limited amounts of IL-7, allows for the selective expansion of $Ig\mu^+$ pre-B cells (45, 56). The transition of large cycling into small resting pre-B cells is accompanied by down-regulation of pre-BCR expression and loss of IL-7 responsiveness.

Within the B lymphocyte lineage, only early B cells express the IL-7R, therefore IL-7 is very useful to study pre-B to immature B cell transitions in an *in vitro* culture

system. When BM cell suspension are cultured in the presence of IL-7 only pro- and pre-B cells are proliferating and immature and mature B cells and other cells that do not express the IL-7R will not survive. Upon removal of IL-7 from the culture system, pre-B cells are induced to differentiate into sIgM$^+$ immature B cells as they stop dividing, activate the RAG proteins and initiate L chain rearrangements (57).

3.1 Pro-B cells

Ig gene recombination starts in pro-B cells with the rearrangement of D_H to J_H gene segments and subsequently V_H gene segments join to the D_HJ_H composites. Upon productive $V_H(D)J_H$ rearrangement, membrane Igμ protein associates with the non-rearranging λ5 and V_{preB} surrogate light chains (SLC) and the Ig-α/CD79a and Ig-β/CD79b signalling components to form the pre-BCR complex. Signalling initiated via the pre-BCR induces clonal expansion of Igμ^+ pre-B cells, whereby IL-7 acts as a crucial pre-B cell-specific proliferation factor. As a result, pro-B cells differentiate to the next compartment of large cycling pre-B cells (Figure 4).

On the surface of pro-B cells the SLC proteins are expressed in a complex, which contains a non-classical cadherin and several as yet unknown proteins (58). A second type of pro-BCR is composed of the Ig-α/Ig-β heterodimer and calnexin (59). This receptor is potentially functional, as its cross-linking with anti-Ig-β induces rapid phosphorylation of several intracellular signalling molecules, including Syk and Btk, as well as the expression of pre-B cell-specific cell surface markers (60, 61).

A third type of immature antigen receptor expressed on the cell surface of pro-B cells consists of Dμ, a truncated H chain product translated from the D_HJ_H gene rearranged in a particular D_H reading frame, RF2 (62, 63). Although D_H segments can be joined to J_H segments in any one of three reading frames, only RF2 leads to production of Dμ protein. This Dμ protein associates with the SLC and the Ig-α and Ig-β proteins and has therefore in principle the capability to transmit a signal that is similar to the pre-BCR (64). As mature B cells in the mouse that have D segments in RF2 are significantly underrepresented, it has been concluded that Dμ protein expression inhibits further B cell development, possibly by inhibiting V_H to DJ_H recombination. This was confirmed by the finding of severely reduced $V_H(D)J_H$ rearrangements, accompanied by an arrest at the pro-B to pre-B transition in Dμ transgenic mice (65). However, the mechanism by which Dμ protein expression blocks further B cell differentiation remains unknown and cannot be explained by the inability of Dμ protein to pair with L chains as the developmental block occurs before the stage of L chain expression.

3.2 Pre-B cells

Signalling initiated via the pre-BCR induces clonal expansion of Igμ^+ pre-B cells, which is dependent on IL-7. As a result, pro-B cells differentiate to the next compartment of large cycling pre-B cells (Figure 4). In these cells, the recombination-activating genes RAG-1 and RAG-2 are transiently down-regulated

to terminate further Ig H chain rearrangements (66), ensuring allelic exclusion. Because of the intrinsic transient nature of pre-BCR expression, this also governs the next step in B cell development: after a limited number of cell divisions the large pre-B cells stop cycling and differentiate into small resting pre-B cells. This transition is associated with reactivation of the rearrangement machinery for the initiation of V_L to J_L rearrangements and significant changes in cell surface marker profiles (Figures 5 and 6). Functional L chain gene recombination in pre-B cells leads to BCR expression and small pre-B cells are transferred to the immature B cell population (4, 5)

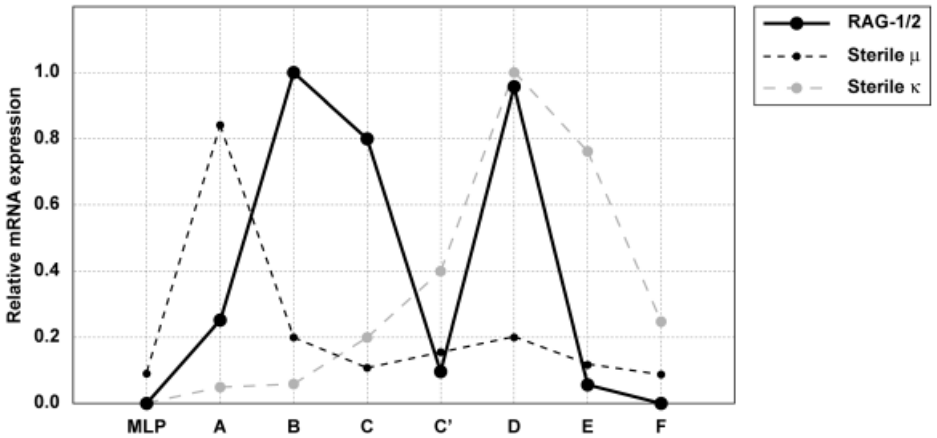

Figure 5. *Profile of Ig rearrangement-related gene expression. Relative mRNA levels were assayed by RT-PCR on cells from the different B cell developmental stages. There is biphasic expression of RAG genes of which the first wave is accompanied by sterile μ transcription for H chain rearrangement and the second wave is related to sterile κ transcription when most L chain rearrangements take place. MLP: multilineage progenitor; capitals indicate developmental stage of B cells. Adapted from (67).*

Activation of the pre-BCR involves phosphorylation of the cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) of the Ig superfamily members Ig-α and Ig-β (Figure 7) and the formation of a lipid raft-associated calcium-signalling module. This complex contains the phosphorylated tyrosine kinases Lyn, Syk, and Btk, as well as other phosphorylated proteins including the adapter molecule SLP-65 (also known as BLNK (B cell linker) or BASH (B cell adaptor containing src homology 2 domain), phospho-inositide 3-kinase (PI3K), Vav and phospholipase Cγ2 (PLCγ2) (61, 68). Syk is strongly activated by binding to the phosphorylated ITAM tyrosines of Ig-α or Ig-β and most likely acts as a positive allosteric enzyme, thereby initiating a positive feedback loop at the pre-BCR (69). The apparent redundancy among the Src-like family members Lyn, Fyn, and Blk has complicated the clarification of the role of the individual enzymes. Nevertheless, the ability of an activated Blk mutant to increase Ig-β and

Syk phosphorylation indicates a proximal point of action of Src-like kinases (70). One important substrate of Syk is SLP-65. Phosphorylation of SLP-65 provides docking sites for Btk and PLCγ2 SH2 domains (Figure 7; (71)). Activated Btk then phosphorylates PLCγ2, which leads to its full activation. Full activation of PLCγ2 induces calcium signalling and Nuclear Factor κB (NF-κB) activation (61, 68, 72). This model is challenged by the recent description of a separate pre-BCR signalling pathway in which NF-κB activation is Syk-independent, but involves Src-family protein tyrosine kinases and protein kinase C-λ (73).

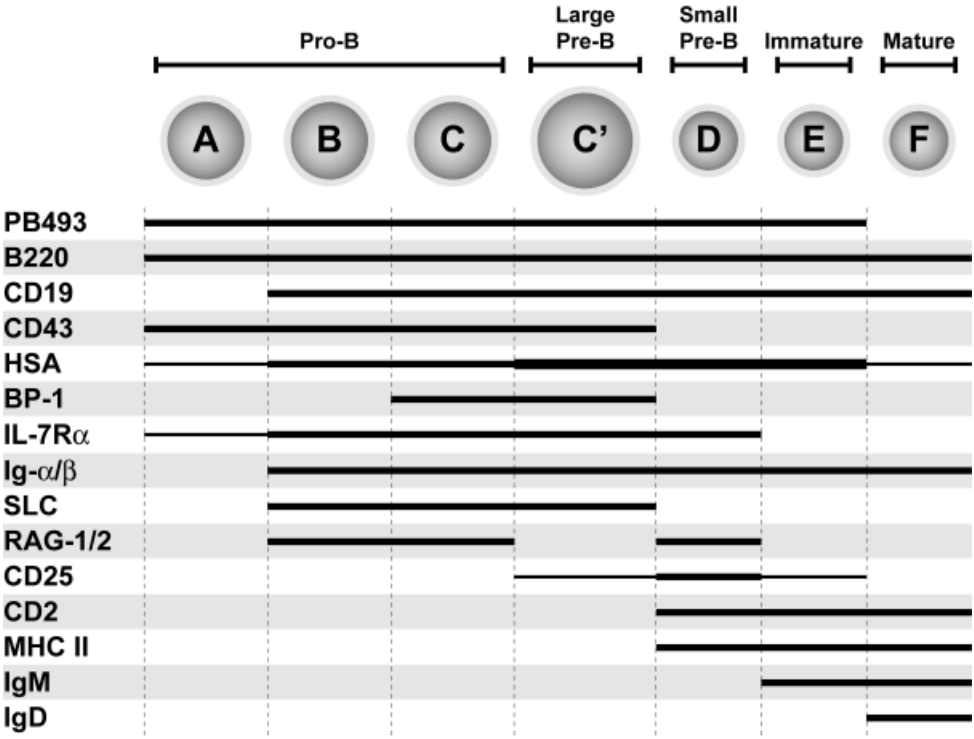

Figure 6. *Diagram of B cell development showing cell surface phenotype or B cell-specific gene expression. Relative expression levels are indicated by line thickness. PB493 is also known as AA4.1, HSA (heat stable antigen) as CD24 and CD25 as IL-2R. SLC, surrogate light chain; RAG-1/2, recombination activating genes 1 and 2; MHC II, major histocompatibility complex type II. Figure adapted from ref. (77).*

The transcription factor NF-κB mediates the regulation of some of the genes important in early B cell differentiation such as the Ig L chain genes. It also plays a role in signals mediated by growth factors and thereby is involved in prevention of apoptosis of precursor cells. NF-κB is a heterodimer of p50 and p65 subunits, whose activity is regulated by interaction with the inhibitory protein IκB. Both

subunits of NF-κB belong to the Rel family of transcription factors, which also includes p52, RelB and c-Rel. Nuclear localization signal (NLS) sequences in NF-κB are masked by binding to IκB that localize NF-κB dimers to the cytoplasm (74). Activation of IκB kinase (IKK) regulates IκB phosphorylation, which in turn targets the inhibitory protein for degradation. The degradation of IκB reveals the NLS in the NF-κB subunits and promotes the translocation into the nucleus. Once in the nucleus, NF-κB dimers are further regulated through phosphorylation of the Rel proteins. The amplitude and duration of the calcium response in B cells differentially regulate the transcription factors NF-κB and NFAT. While NF-κB is activated by large transient calcium responses, NFAT is efficiently activated by a low sustained calcium flux. Therefore, modulators of signalling that affect the quantity and quality of the cytoplasmic calcium response, such as PI3K, Btk and PLCγ2 activation, can change the pattern of gene expression and may determine subsequent B cell fate (75, 76).

Cell-autonomous pre-BCR signalling

Whereas the mature BCR initiates signalling through interaction with its cognate antigen, it is less clear how the pre-BCR is able to start the signalling cascade. It is possible that a ligand exists on stroma cells, on adjacent pre-B cells or on the membrane of the same cell. Two recent findings would support that interaction with a stroma cell ligand triggers pre-BCR activity. First, a pre-BCR/galectin-1 interaction is required to induce Ig-α/β phosphorylation in the human pre-B cell line Nalm6 (78). Second, soluble murine or human pre-BCR molecules show specific binding to stroma cell-associated heparan sulfate, controlled by the non-Ig-like unique tail of λ5 (79).

By contrast, other experiments indicate that pre-BCR signalling does not need a ligand, but specifically depends on the presence of λ5 protein. No cytokine or stroma cell support is needed for *in vitro* proliferation of single wild type (but not λ5-deficient) pre-B cells (80). Using a panel of Abelson virus-transformed Igμ$^+$ pre-B cell lines, Ohnishi and Melchers (81) showed that the non-Ig-like unique tail of λ5 mediates cell-autonomous pre-BCR signalling, resulting in constitutive pre-BCR internalisation and phosphorylation of Ig-α. Two models were proposed: either the pre-BCR serves as its own ligand, whereby neighbouring pre-BCRs directly interact with each other, or alternatively pre-B cells express a molecule on their cell surface that has binding sites for the non-Ig-like unique region of λ5 (81). As in these experiments clonal populations of pre-B cell lines were used, they strongly support the idea that the pre-BCR does not require an exogenous ligand. Nevertheless, it cannot be excluded that λ5-mediated interaction of a pre-BCR with heparan sulfate could rescue pre-BCR signalling in some pre-B cells in which the pre-BCR is expressed at levels too low to initiate ligand-independent signalling (82).

The issue of ligand-independent signalling was previously addressed in a series of experiments in which truncated or chimeric forms of Igμ proteins that cannot associate with SLC were expressed as transgenes (83, 84). As in these

models, allelic exclusion, pre-B cell surface marker changes and germline κ locus transcription was induced, it was concluded that SLC is not required for pre-BCR function. However, this does not necessarily imply that truncated Igμ induces signals that mimic pre-BCR signalling. The levels of surface expression of the truncated Igμ far exceeded those of the wild-type Igμ (83), which would again support the hypothesis that binding to λ5 is essential for pre-BCR internalisation. In these ligand-independent signalling systems not only pre-BCR but also BCR function is bypassed, allowing Ig-negative cells to colonize peripheral lymphoid organs. Taken together, these observations are consistent with a model of cell-autonomous pre-BCR signalling that is crucially dependent on λ5.

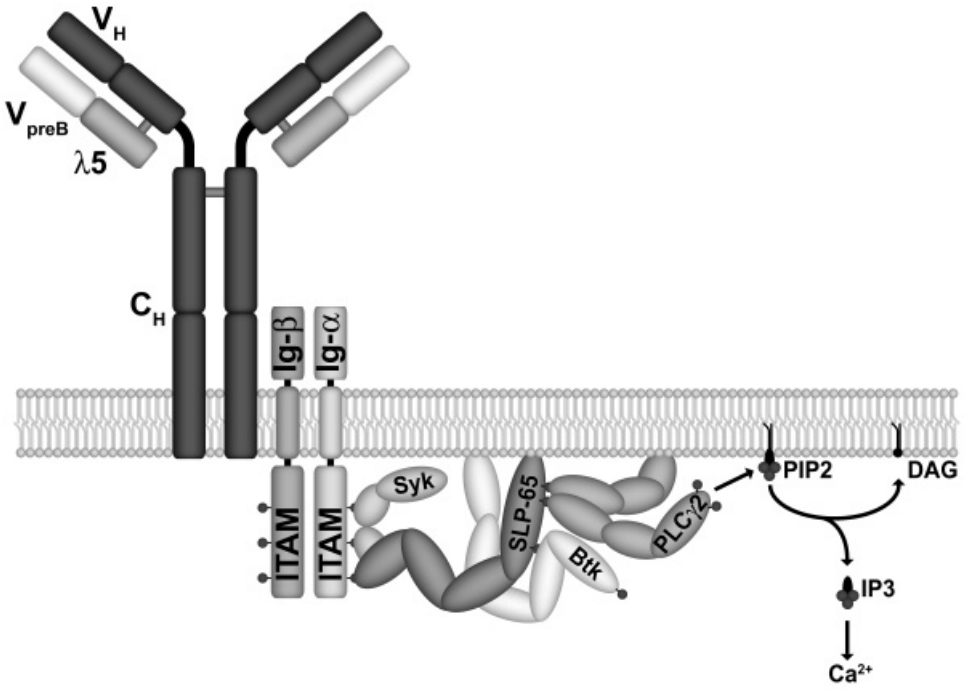

Figure 7. *Pre-B cell receptor signalling model. Activation of the pre-BCR involves phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the Ig-α and Ig-β scaffold proteins, thereby recruiting and activating Syk tyrosine kinase. A prominent substrate of Syk is the adapter protein SLP-65. Phosphorylated SLP-65 provides binding sites for Btk, (which is activated by Syk-mediated phosphorylation of Tyr551 in its kinase domain) and phospholipase Cγ2 (PLCγ2) SH2 domains. In an alternative pathway, PLCγ2 can be activated via the homologous proteins ZAP70, SLP-76, LAT and Tec (not depicted). Btk: Bruton's tyrosine kinase. PIP2: phosphatidyl-inositol-4,5-biphosphate; IP3: inositol-trisphosphate; DAG: diacylglycerol.*

Differential role of the pre-BCR complex and signalling components

In the last decade analyses of gene-targeted mice have defined differential roles of components of the pre-BCR complex or its downstream signalling pathways (Table 1). The pre-BCR utilizes sequential activation of three distinct families of non-receptor protein tyrosine kinases (PTKs), such as Src, Syk and Tec families for initial activation (85). Deficiencies in any one of these PTK families result in defective or aberrant B cell function and development (86). Characterization of the substrates of these activated PTKs is helpful for understanding the mechanism of how BCR activation is regulated. The phosphorylation events performed by these PTKs modulate catalytic activity of effector enzymes and mediate protein-protein interactions that bring together signal transduction elements. A group of cellular proteins, called adapter proteins, regulate the interaction of effector enzymes with the BCR and their targets, thereby integrating multiple signalling pathways (87-89). PTK families, adapter proteins and other molecules involved in pre-BCR signalling have structural similarities as depicted in figure 8.

As outlined in Table 1, the formation of large cycling pre-B cells is dependent on structural components of the pre-BCR (Igμ, SLC, Ig-α and Ig-β) and Syk tyrosine kinase, whereas the signalling molecule SLP-65 is important to limit pre-B cell expansion and for the differentiation into small resting pre-B cells. Targeted

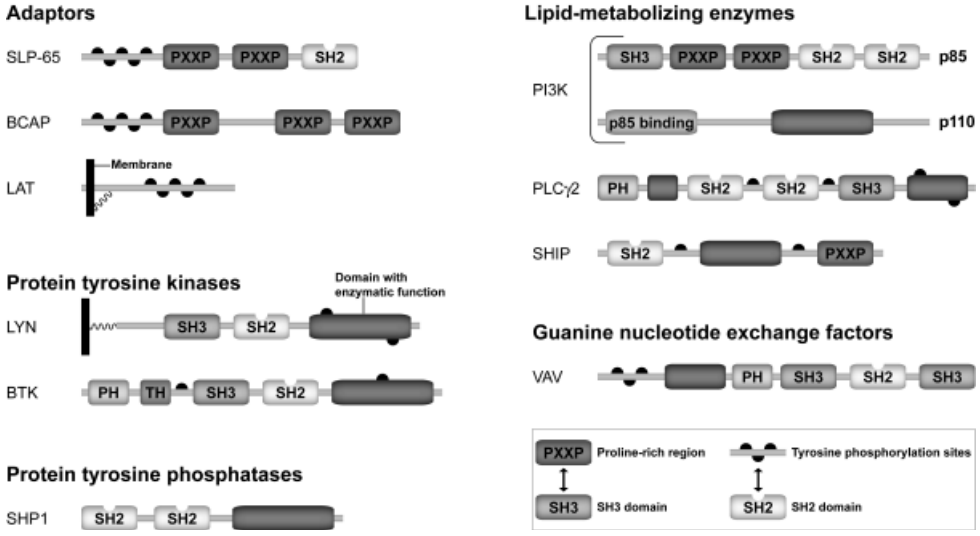

Figure 8*. Molecular structure of signalling molecules involved in the (pre-) BCR signalling pathway. Adapter proteins are defined commonly as proteins that posses protein–protein or protein–lipid interaction domains but do not have enzymatic activity. BCAP, B-cell adapter protein; BTK: Bruton's tyrosine kinase; LAT: linker for activation of T cells; PH: pleckstrin homology; PI3K: phosphoinositide 3-kinase; PLCγ2: phospholipase Cγ2; SH2: SRC-homology 2; SH3: SRC-homology 3; SHIP: SH2-domain-containing inositol 5 phosphatase; SHP1: SH2-domain-containing protein tyrosine phosphatase 1; TH, TEC-homology. Figure adapted from (90).*

***Table 1**. Phenotype of gene-targeted mice with respect to the pre-B cell receptor checkpoint*

Targeted gene	Dμ RF2 counter-selection	Allelic exclusion	Pre-B cell proliferation	Pre-B cell maturation	References
μMT[a]	Absent	Absent	Absent	Absent	(63, 91)
SLC[b] (λ5, V_{preB1}, V_{preB2})	Absent	Normal	Absent	Impaired	(97-99)
Ig-α or Ig-β	Absent in Ig-$\beta^{-/-}$	Absent in Ig-$\beta^{-/-}$	Absent in Ig-$\alpha^{-/-}$ or Ig-$\beta^{-/-}$	Absent in Ig-$\alpha^{-/-}$ or Ig-$\beta^{-/-}$	(92, 93, 100)
Ig-αΔc or Ig-βΔc[c]	N.a.[e]	Normal	Impaired (more severe in Ig-αΔc)	Impaired (more severe in Ig-αΔc)	(101, 102)
Ig-αΔc/ Ig-βΔc[d]	N.a.	Absent	Absent	Absent	(101, 102)
Syk	Absent	Impaired	Impaired	Impaired	(103, 104)
Syk/ ZAP70[d]	N.a.	Absent	Absent	Absent	(104)
SLP-65	Absent	Normal	Enhanced	Impaired	(105-109)
SLP65/ LAT[d]	N.a.	N.a.	Enhanced	Absent	(110, 111)
SLP-65/ CD19[d]	Absent	Normal	Enhanced	Absent	(112)
Btk	N.a.	Normal	Normal	Normal	(113-115)
IRF-4,8[d]	N.a.	N.a.	Enhanced	Impaired	(40)

[a] μMT: deletion of Ig μ membrane exon. [b] Targeted deletion of λ5, V_{preB1}/V_{preB2} or all of these resulted in very similar phenotypes. [c] Ig-αΔc or Ig-βΔc: deletion of cytoplasmic tails of Ig-α or Ig-β. [d] Double mutant. [e] N.a.: Not analysed.

disruption of the Igμ membrane exon (μMT mice), Ig-α or Ig-β in the mouse causes a block of B cell development at the pro-B cell stage (91-93). In μMT mice the pre-B cells cannot deposit Igμ on the cell surface, they do not enter the cell cycle and allelic exclusion is lost. Conversely, transgenic Igμ inhibits endogenous Ig H chain rearrangement, which feature is dependent on the ability of Igμ to associate with the Ig-α/Ig-β heterodimer (92, 94, 95). Nevertheless, B cell development is not completely blocked in μMT mice, as some κ L chain gene rearrangement is still observed and IgA^+ cells can be detected in spleen and Peyer's patches (91, 96).

Mice deficient in the SLC components λ5, V_{preB1}/V_{preB2} or all of these also lack pre-B cell expansion and show decreased peripheral B cell numbers. However, they exhibit a more leaky phenotype than μMT mice, as allelic exclusion is not affected and mature B cells accumulate with age (91, 99). As κ L chain rearrangement may precede H chain rearrangement in up to 15% of normal pro-B cells, the leaky phenotype of SLC-deficient mice could be explained by premature expression of conventional L chains, which would then substitute for the SLC in the pre-BCR complex. However, there is no evidence to suggest that the rescued B cells in

SLC deficient mice have rearranged their L chains at the pro-B cell stage, as a significant fraction of $\lambda5^{-/-}$ B cells express λ L chain, and N regions are not inserted in V_L-J_L joints in $\lambda5^{-/-}$ B cells (discussed in (99)). In addition, a pre-rearranged $V\kappa J\kappa$ gene segment targeted into the Ig κ locus failed to rescue B cell development in $\lambda5^{-/-}$ mice (116). It was also hypothesised that cell surface expression of the pre-BCR is not required and Igμ signals from the endoplasmatic reticulum (ER), but the recent finding that ER-trapped Ig L chain-nonpairing Igμ does not induce tyrosine phosphorylation of Ig-α supports the idea that a pre-BCR must be transported to the cell surface to signal (117). As it appears that a substantial fraction of the Igμ proteins can be transported to the pre-B cell surface in the absence of SLC (118-120), this would provide a likely mechanism by which allelic exclusion and developmental progression is rescued in $\lambda5^{-/-}$ mice.

Additional mouse models provided evidence that clonal expansion and allelic exclusion are not only dependent on the correct pre-BCR assembly but also on pre-BCR signalling. One cytoplasmic domain of either Ig-α or Ig-β is sufficient to signal developmental progression of pre-B cells, but mice in which the ITAMs of both Ig-α and Ig-β are mutated manifest an absolute block of B cell development at the pro-B cell stage (101, 102). Mice deficient in Syk show a partial block at this step, but mice mutant in both Syk and ZAP-70, the only other known Syk family kinase, show a complete block at the pro-B cell stage (104). This requirement for ZAP-70 was unexpected, as ZAP-70 was previously reported to be expressed exclusively in T and NK cells but not in B cells, and shows that Syk and ZAP70 are essential to regulate H chain allelic exclusion and proliferative expansion of pre-B cells. Furthermore, $\lambda5$, Ig-α, Ig-β and Syk are involved in various additional immature BCR forms in particular for Dμ RF2 counterselection in pro-B cells (Table 1).

The pre-BCR signalling component SLP-65

Mice deficient for the adapter protein SLP-65 (SH2-containing linker protein of 65 kD) show a partial block at the large cycling pre-B cell stage (Table 1; (105-108, 113)). Consistent with direct down-regulation of SLC expression by pre-BCR signalling (66), pre-B cells that lack SLP-65 manifest elevated levels of SLC in the cytoplasm and on the membrane (109). Therefore, it seems likely that pre-B cells lose their proliferative capacity because they have used up the previously synthesised SLC for surface pre-BCR formation. Furthermore, the transition of large cycling into small resting pre-B cells is accompanied by down-regulation of IL-7R expression. The finding that SLP-65 is involved in the transcriptional repression of the IL-7Rα gene upon pre-BCR signalling (72, 112), indicates that IL-7Rα expression is controlled by the pre-BCR. It is therefore possible that, in addition to down-regulating pre-BCR expression, SLP-65 may partly prevent excessive cell divisions of Igμ^+ pre-B cells by regulating IL-7 responsiveness.

Importantly, SLP-65$^{-/-}$ mice spontaneously develop pre-B cell lymphomas expressing large amounts of pre-BCR on their cell surface. It was hypothesised that in SLP-65$^{-/-}$ mice, large cycling pre-B cells are trapped in a positive feedback

loop as they are arrested in development and cannot efficiently down-regulate pre-BCR or IL-7R expression, which signal for proliferation (109, 112).

The incomplete block in SLP-65-deficient mice prompted the investigation of alternative adapter proteins that could partially replace SLP-65 in pre-BCR signalling. The homologue of SLP-65 in T cells, SLP-76, and LAT (linker for activation of T cells), which is an integral membrane adapter protein essential in T cell development, can complement the function of SLP-65 in BCR stimulation (121, 122). Recently, two LAT-like molecules named LAB (linker for activation of B cells) and NTAL (non-T cell activation linker) were identified in the B cell lineage and were shown to be constitutively localized in lipid-rafts and are phosphorylated upon BCR stimulation, but their role in pre-BCR signalling is currently unknown (123, 124).

Remarkably, SLP-76 and LAT are also expressed in pre-B cells, where they are recruited to the pre-BCR, associate with Ig-α and become phosphorylated upon pre-BCR stimulation (110, 111). Concomitant absence of LAT enhances the pre-B cell defects in SLP-65-deficient mice, while reconstitution of LAT expression in SLP-65/LAT double mutant pre-B cells restored their ability to mobilize calcium, to down-regulate pre-BCR expression and to initiate Ig κ L chain expression (110). Likewise, the Btk homologue Tec has the ability to compensate defective Btk function in pre-B cells *in vivo* (125), and ZAP-70 can compensate for Syk (as discussed above) (104). Taken together, these data suggest that PLCγ2 can be activated in pre-B cells by two parallel pathways, one containing Syk, SLP-65, Btk, and one containing ZAP70, SLP-76, Tec and LAT (110).

3.3 Immature B cells

Differentiation into IgM$^+$ immature B cells is dependent on the quality of the L chains produced. Each L chain must be able to pair with the μ H chain already expressed, thereby enabling the cell surface expression of a BCR. In addition, the newly formed BCR should not recognize self-antigens.

Light chain rearrangements

The pre-BCR selects for those cells that produce μ H chains that are able to pair with the SLC by inducing proliferative expansion of pre-BCR expressing cells. Cells with non-productively rearranged H chain alleles, or cells expressing non-pairing μ H chains do not enter the proliferation step and are thereby negatively selected. Similarly, μ H chain-expressing small pre-B cells are allowed to develop into immature and thereafter into mature B cells when a pairing L chain is expressed, provided that the BCR formed does not recognize self-antigens (126).

The majority of B cell progenitors functionally rearrange either the κ or λ gene to generate κ or λ expressing immature B cells. However, the process of isotype exclusion makes sure that no cell expresses both κ and λ L chains. Most κ-expressing cells retain their λ loci in germ-line configuration, whereas λ-expressing cells have non-functional κ rearrangements on both alleles (127-129). This indicates that the κ locus is rearranged earlier than the λ locus during B cell development. However,

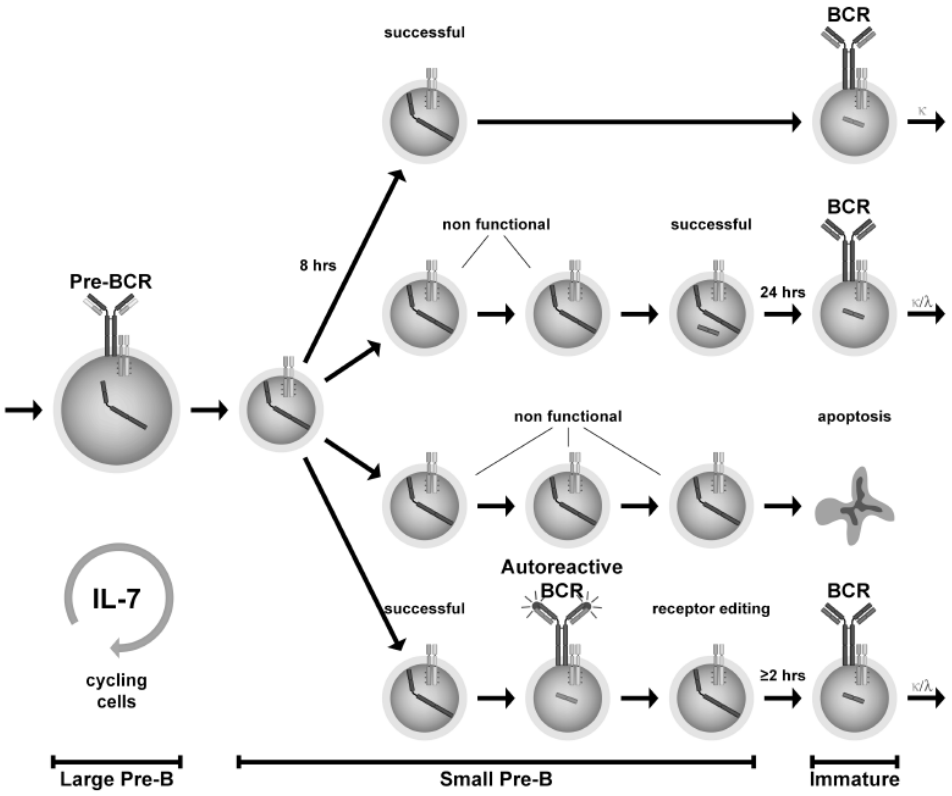

Figure 9. *In small pre-B cells, κ and λ L chain gene rearrangement is initiated. Only when a non-autoreactive functional BCR is expressed, the small pre-B cell is transferred to the immature B cell compartment. The lambda locus is opened 24h after the cell has become a small pre-B cell. Receptor editing takes at least 2h extra. See text for detailed description about rearrangements and receptor editing.*

in mice where κ rearrangement is not possible (iEκT mice), λ-expressing cells are generated, indicating that λ rearrangements are independent of κ rearrangements (130, 131). To study the kinetics of κ or λ expression on the surface of immature B cells, mice were injected intraperitoneally (i.p.) with Bromodeoxyuridine (BrdU). The BrdU, in which brome is coupled to the thymidine–analogue uridine, is incorporated into DNA when it is replicated during the cell cycle. In the BM, the large pre-B cell population is the cycling population and incorporates BrdU into its DNA and subsequently differentiates into small pre-B cells. The small pre-B cells then undergo L chain rearrangement and cells can be tracked back by using an antibody specific for BrdU. The probability of productive L chain rearrangements determines the rate of output of $BrdU^+\kappa^+$ or λ^+ B cells in the BM (132, 133). By performing these experiments it was found that in normal mice as well as in iEκT mice, the λ^+ cells appear ~24h after BrdU injection, whereas the first κ^+ cells appear

after ~8h. This implies that the λ locus is opened at a fixed time point after the pre-B cells have stopped cycling and is not dependent on rearrangements of the κ loci.

Enhancer sequences located in the L chain loci have been shown to regulate the gene rearrangement (131, 134). Different regulatory sequences having different kinetics and strength of activity at the κ and λ loci are likely to account for the earlier initiation of rearrangements at the κ locus. There are two enhancer elements associated with control of transcription and rearrangement of the κ locus. The onset of Jκ germ-line transcription and Vκ-Jκ rearrangement requires the induction of the transcription factor NF-κB (135), which binds to the Igκ intronic enhancer (iEκ) located between the Jκ cluster and the Cκ exon (136, 137). The 3'κ enhancer, located downstream of the Cκ exon (138, 139), also influences κ gene transcription and rearrangement and can be activated by binding of Pax5 (140), PU.1 (17), CRE and PIP factors (37). In contrast to the regulation of Igκ rearrangements, little is known about the transcription factors and DNA elements that regulate recombination of the Igλ gene segments. It has been found that NF-κB signalling is required to induce germ-line transcription and, next to EBF, for recombination of murine Vλ and Jλ gene segments in precursor B cells (28, 141). Only recently it was found that in IL-7 dependent PU.1$^{-/-}$Spi-B$^{-/-}$ cμ^{+} pre-B cell lines, IgH transcription is increased and Igλ transcription is reduced upon IL-7 withdrawal, when compared to wild-type pro-B cells. This indicates a role for PU.1 and/or Spi-B in Igλ transcription and VλJλ recombination (142).

In small pre-B cells the κ and λ L chain gene rearrangement is initiated and the κ locus is five times more frequently rearranged than the λ locus in mice. L chain rearrangements can result in out-of-frame non-productive rearrangements or in-frame productive rearrangements producing L chains that (a) cannot pair with the μ H chains; (b) form an autoreactive BCR or (c) form a non-autoreactive BCR. When the rearrangement is successful and the L chain can pair with the μ H chain and the resulting BCR is not autoreactive, the cells are transferred to the immature B cell compartment. In situations where the rearrangement or pairing is not successful or the resulting BCR is autoreactive, cells are able to perform secondary rearrangements, a process called receptor editing. If these secondary rearrangements result in a pairing L chain, which combines with the μ H chains to form a non-autoreactive BCR, the cells are transferred to the immature B cell compartment. If not, the small pre-B cell keeps trying further rearrangements, also on the other alleles of the κ or λ loci. If none of these rearrangements yields a useful L chain, the cell dies by apoptosis (Figure 9; (143)).

Receptor editing

The progression from the immature to mature B cell stage represents another checkpoint in B cell development. This process has been examined in a number of autoreactive transgenic mouse models that have shown that when immature B cells encounter an antigen capable of crosslinking the BCR, this leads to one of three results: (a) cells are eliminated (clonal deletion) (144-147), (b) cells become

non-responsive (anergic) (148) or (c) cells revise the L chains of their BCR to eliminate self-reactivity (receptor editing) (149-152). It appears that the strength of the interaction between BCR and antigen regulates the choice of either one of these three options. High-affinity interactions with membrane-bound antigen result in deletion, whereas lower-affinity interactions and soluble antigens allow receptor editing or result in anergy.

In the process of successfully generating L chains that can pair with the μ H chains there is a possibility that the BCR that is generated recognizes self-antigens. The process of receptor editing can alter the V-regions of the L chains and thereby lose the self-reactive nature of the BCR. Receptor editing can continue on both alleles and eventually, when rearrangement is unsuccessful at the κ locus, proceed to the λ locus (150). Besides increased usage of λ L chains, another sign of receptor editing is the use of downstream Jκ segments (149). Primary rearrangements at the κ chain locus are well suited for further rearrangements because unrearranged Vκ and Jκ gene segments usually flank the rearranged VκJκ gene segments. Depending on the orientation of the edited Vκ relative to the Jκ segment, the secondary rearrangement may delete the primary VκJκ or separate it from the Cκ by inversion (149) (Fig. 10).

Receptor editing is not likely to take place at the H chain, as V(D)J rearrangements remove all available unrearranged D gene segments on the same allele. Moreover, secondary V_H rearrangements to downstream J_H gene segments are not compatible with the 12-23 spacer rule that governs the joining of the RSSs. Several studies have suggested that secondary L chain gene rearrangements can be readily observed and that they take place when initial rearrangement is not productive.

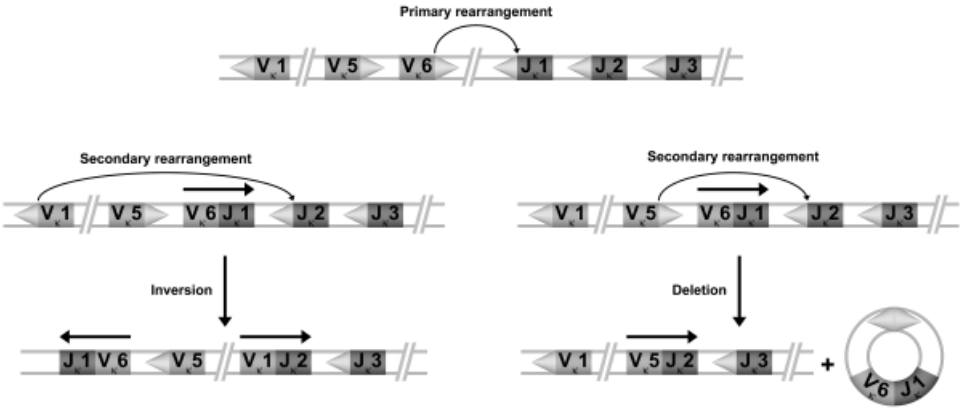

Figure 10. *Receptor editing can take place by secondary rearrangements on the same L chain allele by deletion (left) or inversion (right). After a primary rearrangement (upper part) upstream Vκ segments and downstream Jκ segments may be recombined in a secondary rearrangement event (lower part).*

Direct insight in receptor editing came from experiments in mice expressing a transgene encoding a BCR that recognizes mouse strain-specific MHC I molecules that are expressed on every cell of the body. These so-called 3-83μδ transgenic mice carry a BCR transgene consisting of pre-rearranged μ and δ heavy chain genes as well as a κ L chain gene (153). Because the expression of the 3-83μδ transgene is under the control of the Ig H chain enhancer, the 3-83μδ BCR is expressed from the pro-B cell stage onwards. The 3-83μδ BCR specifically recognizes MHC class I molecules of the H2-$K^{k/b}$ allotype (153), and thus reflects an innocuous BCR in mice that have an H2-K^{d} background. Mice with such a non-autoreactive background (also called non-deleting mice) contain a virtually monoclonal B cell population. As the prematurely expressed 3-83μδ BCR is able to promote B cell development, it appears that this BCR can functionally replace the pre-BCR and consequently B cell development is accelerated (153-155). On the other hand, when the 3-83μδ BCR is expressed in mice of the MHC class I H2-K^{b} background, all B cells generated are autoreactive and are targeted for receptor editing in order to survive (150, 151, 156). By using BM from 3-83μδ transgenic mice, it was found that self-antigen induces receptor editing in $IgM^{low}IgD^{-}$ and rapid apoptosis in $IgM^{high}IgD^{-}$ B cells (155). As IgM expression is also correlated to developmental progression, these results indicate that as B cells advance in development their competence to undergo tolerance-induced receptor editing decreases, whereas sensitivity to antigen-mediated cell death increases. This antigen-induced apoptosis was inhibited by enforced expression of the anti-apoptotic protein Bcl-2 (155).

Immature B cells from the BM of 3-83μδ transgenic deleting mice expressed elevated levels of RAG genes and produced higher amounts of endogenous λ chains in peripheral B cells (150). The increased L chain rearrangements led to the displacement of the transgenic L chains by endogenous L chains and resulted in BCRs with edited specificities that no longer resulted in B cell deletion (150, 157). As the receptor editing process includes recombination of the residual L chain gene segments, B cell development in these mice is delayed by ~ 2h (158, 159).

Usually λ L chain genes rearrange after κ genes. Therefore, the extent of receptor editing in a normal, non-transgenic immune system was determined in $Igλ^{+}$ cells by analysing how frequently in-frame κ genes had failed to suppress λ gene rearrangements. It was found that nearly half of the inactivated VκJκ joins were in-frame, indicating that receptor editing occurs at a surprisingly high frequency in normal B cells (160). In another approach, the extent to which editing occurs in developing B cells was measured using a generated allelic polymorphism of the mouse κ constant region (mCκ) by replacing it with the human counterpart (hCκ), the expression of which correlates with receptor editing (158). It was found that ~25% of all antibody molecules are produced by gene replacement. Furthermore, it was found that B cells with innocuous receptors spend little or no time in the small pre-B cell stage, whereas B cells undergoing receptor editing are specifically delayed at this stage for at least 2h (158). Furthermore, overexpression of Bcl-2 increased the relative frequency of λ versus κ expressing B cells, suggesting that

the Bcl-2 transgene promotes receptor editing (161).

3.4 Late B cell differentiation in the spleen

Transitional B cells

Immature B cells that emigrate from the BM to the periphery are referred to as transitional B cells. Transitional B cells can be distinguished from mature B-2 cells by differential surface expression of a variety of molecules, including IgM, IgD, CD23, CD21, CD24/HSA and PB493 (Figure 11). The transitional B cells were subdivided into two separate subpopulations, termed transitional 1 (T1) and T2 B cells on the basis of their surface phenotype and functional characteristics (162-165). Transitional B cells are short-lived and only 10-30% of these cells enter the long-lived mature peripheral B cell compartment (166-168).

Stimulation of the BCR on mature B cells leads to activation and proliferation, whereas the same signals on transitional immature B cells lead to anergy or cell death. Although transitional B cells migrate to the spleen, they are excluded from the lymph nodes (162). Lack of surface CD62L (162, 169) or fibronectin (170) might mediate this exclusion. The two transitional B cell subsets are also different in their localization in the spleen. The T1 cells appear to be limited to the outer periarteriolar lymphoid sheath (PALS), whereas the T2 cells are found within the B cell follicles (162, 163). Next to different localization, it was found that the T1, T2 and mature

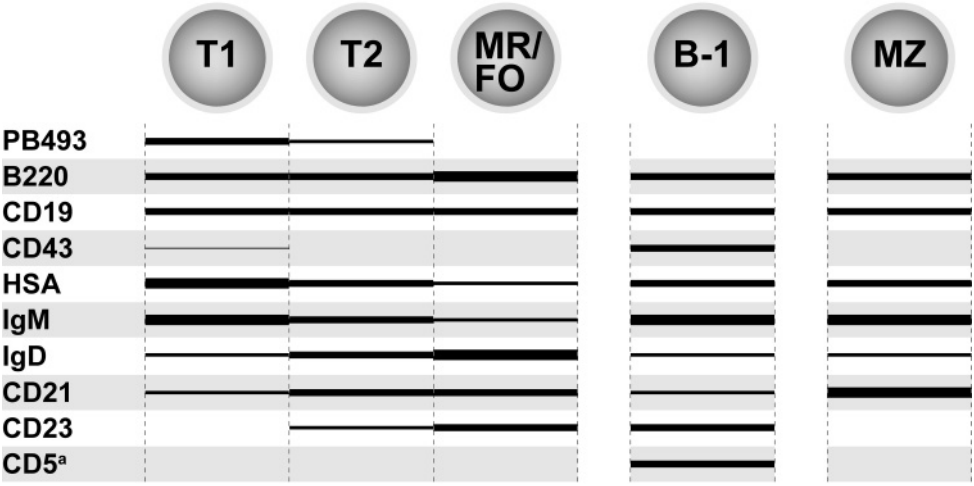

Figure 11. *Diagram of peripheral B cell populations showing cell surface phenotype. Relative expression levels are indicated by line thickness. B cells differentiate subsequently from T1 to T2 into MR or FO B cells, whereas the differentiation into B-1 or MZ B cells is not yet fully understood. PB493 is also known as AA4.1 and HSA (heat stable antigen) as CD24.* [a] *A part of B-1 cells is* $CD5^+$*, another part is* $CD5^-$*. T1, 2: transitional type 1 or 2 B cells; MR: mature recirculating B cells; FO: follicular B cells; MZ: marginal zone B cells. Figure is adapted from ref. (77).*

B cell subsets in the spleen are differentially responsive to BCR signalling (171). T2 B cells express higher basal and BCR-induced levels of activation markers and proliferate as well as mature B cells. In contrast to T1 cells, BCR stimulation in T2 cells induces expression of the pro-survival protein Bcl-x_L and induces phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2), p38-MAPK and Akt, whereas these inductions were only moderate in T1 cells. This enhanced response to BCR stimulation contributes to the positive selection and progression of T2 B cells into the long-lived mature B cell pool (171).

Follicular and Marginal Zone B cells

Newly formed B-lymphocytes enter the spleen via the blood, from which they migrate to the B cell area in the follicle or the marginal zone (MZ) in the white pulp. The MZ, located at the border of the white pulp, is a highly organized region whose function is not yet fully understood. It contains a unique population of B cells, the marginal zone B cells, which do not recirculate. These cells appear to be resting mature B cells and they have a different set of surface proteins then the major follicular population of B cells (Figure 11). For example, they express low levels of CD23, a C-type lectin, and high levels of MHC class I molecules, CD1 and the complement C3-fragment receptor CD21/35 (Figure 11). Furthermore, MZ B cells may have restricted antigen specificities. The MZ also contains dendritic cells as well as three different types of macrophages, two types of MZ macrophages expressing either MARCO or ER-TR9 and the MOMA-1^+ metallophilic macrophages (172-174).

The peripheral lymphoid tissues have a highly organized architecture, with distinct areas for B and T cells. The organization and the survival of newly formed lymphocytes are determined by interactions between lymphocytes and other cell types in the lymphoid tissues, such as macrophages and dendritic cells. The spleen collects antigen from the blood. The bulk of the spleen is composed of red pulp, which is the site of red blood cell disposal that is collected from the blood. The lymphocytes surround the arterioles entering the organ forming areas of white pulp, the inner region of which is divided into the PALS containing mainly T cells and a flanking B cell area containing follicles.

Within the splenic follicles specialized stromal cells, the follicular dendritic cells, play an important role in the development of antibody responses and the survival of B cells. B cells that manage to migrate into follicles and become part of the long-lived mature B cell pool subsequently migrate from the follicles into the efferent lymphatic vessels and so return to the circulation. The system of lymphatic vessels collects extracellular fluid from tissues and returns it to the blood. Lymph nodes are sites where lymphatic vessels converge. The afferent lymphatic vessels that can drain fluid from the tissues also carry antigens from sites of infection in most parts of the body to the lymph nodes, where they are trapped. Within the lymph nodes, B cells are localized in follicles, with T cells more diffusely distributed in surrounding paracortical areas. Some of the B cell follicles contain central areas called germinal

centers, where B cells are undergoing intense proliferation and Ig H chain class switch after encountering antigen.

B-1 cells

B-1 B cells represent a separate subset of mature B cells with distinct origins, cell surface phenotype, functional properties and anatomical distribution. The B-1 cells were first identified by the surface expression of the protein CD5 and they are also characterized by high levels of IgM and low levels of IgD, even when mature (Figure 11). Initially these cells were shown to arise from precursors in the fetal liver, and were therefore called B-1 cells, whereas the conventional mature B cells are also referred to as B-2 cells. However, V-region sequence analysis indicated that the V-region repertoire in B-1 cells was very restricted, no somatic hypermutations were found and the isotype expressed was primarily IgM. More recently, studies using various lines of BCR transgenic mice demonstrated that the mature phenotype of the B cell population is dependent on the specificity of the expressed BCR or on the strength of the BCR signal (175-178). Mutations in positive regulators of BCR signalling, such as CD19, SLP-65, PI3K and Btk, result in decreased numbers of B-1 cells. And in contrast, an increase in B-1 B cell numbers is seen in the absence of negative regulators of BCR signalling, such as CD22, Lyn and the tyrosine phosphatase SH2 domain-containing protein-1 (SHP-1) (77, 175, 178). Regardless of how B-1 cells originate, they are certainly expanded and maintained by interaction with self-antigens or non-self antigens normally present in the body, such as those of the bacterial gut flora. In adult animals, the population of B-1 cells is maintained by continued division in peripheral sites such as the peritoneal and pleural cavities, a process that requires IL-10 in addition to BCR stimulation (179).

Little is yet known about the function of B-1 cells. Their location suggests a role in defending the body cavities, while their restricted repertoire of receptors appears to equip them for a function in the early, non-adaptive phase of an immune response. In practice it is found that B-1 cells contribute little in adaptive immune responses to most protein antigens, but contribute strongly to some responses against carbohydrate antigens. Moreover, a large proportion of the IgM that normally circulates in the blood of non-immunized mice is derived from B-1 cells. The existence of these so-called “natural antibodies”, which are highly cross reactive and bind with low affinity to both microbial and self-antigens, suggests that B-1 cells are partially activated as they are selected for self-renewal by ubiquitous self- and foreign antigens. The most compelling evidence for the role of self-antigens in positive selection of B-1 cells has become apparent in studies using the carbohydrate epitope of the glycoprotein Thy-1 as a self-antigen (180). Expression of Thy-1 did not interfere with the generation and accumulation of B-1 cells expressing anti-Thy-1 antibody. In contrast, no anti-Thy-1 expressing B-2 cells were generated. However, the lack of the self-antigen in Thy-$1^{-/-}$ mice abrogated the development and selection of anti-Thy-1 expressing B-1 nut not B-2 cells (180).

B cell responses

The surface Ig that serves as the antigen receptor on B cells has two roles in B cell activation. First, it transmits signals directly into the cell when it binds antigen. Second, via receptor-mediated endocytosis the B cell can ingest antigen in order to degrade it into smaller peptides, which can be bound to MHC class II molecules. In this way the B cell functions as an antigen-presenting cell (APC) because it expresses the peptide-MHC II complex on its cell surface, which can be recognized by antigen-specific helper T (T_h) cells. These cells produce cytokines that cause the B cells to proliferate and differentiate into plasma cells. Some microbial antigens can activate B cells directly in the absence of T cell help. Those antigens are often polysaccharides and are called thymus independent (TI) antigens. There are two classes of TI antigens, the TI-I and TI-II antigens, which activate B cells by different mechanisms. In contrast, antibody responses against protein antigens require antigen-specific T cell help and are therefore called thymus dependent (TD) antigens.

When naive B cells are activated they differentiate into IgM-producing plasma cells or they undergo a so-called germinal center (GC) reaction. GCs are specialized micro-environments in peripheral lymphoid organs, where B cells undergo affinity maturation through somatic hypermutation (SHM) and class switch recombination (CSR). During CSR the $C\mu$ is removed from the recombinated V(D)J gene and is replaced by a new downstream C_H region resulting in the generation of different antibody isotypes, like IgG, IgA or IgE. The specificity of the C_H region determines the effector function of the antibody. CSR occurs by a deletional recombination between switch regions located upstream of each C_H gene segment (181). CSR is activated by specific cytokines resulting in production of different isotypes upon different stimulations of the BCR. The B cell-specific growth factor IL-4, for example, promotes the expression of IgE and IgG1, whereas transforming growth factor β (TGFβ) promotes switching to IgA (182).

The CSR and SHM processes require the activation-induced cytidine deaminase (AID) protein as profound defects were found in AID-deficient mice (183-185). Identical defects are seen in patients with a homozygous defect in the human AID gene, a condition known as the hyper-IgM syndrome 2. These patients have elevated levels of serum IgM because their B cells are impaired in CSR (186).

4. Bruton's tyrosine kinase

Bruton's tyrosine kinase (Btk) is a cytoplasmic non-receptor protein tyrosine kinase (PTK) involved in regulating B cell development and function (187-194). PTKs are known to play a pivotal role in hematopoietic cell growth, differentiation and lymphocyte signalling pathways (195). Btk is a 659 amino acid 77kDa protein and belongs to a subfamily of tyrosine kinases, the Tec family kinases (196). The Btk protein is expressed in B cells, myeloid, erythroid and mast cells but not in T

cells. Similar levels of Btk expression was observed during B cell development from the pro-B cell to mature B cell stages, but not in plasma cells (191, 197). Btk has been implicated as a mediator of signals from various receptors in several different cell types (Table 2).

The Tec family kinases are the second largest family of non-receptor tyrosine kinases and include Tec, Btk, Itk/Emt, BMX and TXK/Rlk (198, 199). The Tec kinases are structurally similar to Src family kinases and consist of Src homology (SH) 3 and SH2 protein-interaction domains and a tyrosine-kinase catalytic domain (Figure 8, (200, 201)). The Tec kinases also possess a Tec homology (TH) domain, just upstream of the SH3 domain, consisting of a GTP-ase-activating-protein (GAP)-Btk homology (BH) region and a proline-rich (Pr) sequence that is implicated in interaction with SH3 domains and kinase regulation (202). Most Tec kinases also possess an N-terminal pleckstrin homology (PH) domain that binds the products of PI3K, helping to recruit the Tec kinases to the plasma membrane upon PI3K activation (203-205) (Figure 8).

Tec family kinases are activated after stimulation of a variety of cell-surface molecules, for example, antigen receptors, receptor tyrosine kinases, cytokine receptors and G-protein-coupled receptors. The most intensely studied is the signalling pathway downstream the antigen receptors, which activates Btk and Tec in B cells. Activation of most Tec family kinases by antigen receptors requires two key steps: firstly membrane association, a process that requires interaction of the PH domain with the products of PI3K (203, 206) and secondly phosphorylation of a tyrosine in the kinase domain of the Tec kinase by a Src family kinase, such as Syk (207). Transactivation of Btk leads to auto-phosphorylation of a tyrosine in the SH3 domain (Y223) (208). Phosphorylation of this site changes the affinity of the SH3 domain for certain binding partners (209) and facilitates an intramolecular

***Table 2**. Btk is involved in signalling pathways of different receptors in various cell types.*

Receptor pathway	Cell type(s)	References
Pre-BCR	Pre-B cells	(61, 210)
BCR	B cells	(115, 210-212)
CD38	Activated T- and B cells	(213)
Epo-R	Erythrocytes	(214)
FcεR	Mast cells, basophils	(215)
GPVI	Platelets	(216)
IL-5R	B cells, eosinophils, basophils	(217)
IL-6R	Activated B cells, plasmacells, leukocytes	(218)
IL-10R	Activated T- and NK cells	(219)
TLR4	Phagocytic cells	(220, 221)
TRAIL-R1	Erythrocytes	(214)

R: receptor; Epo: Erythropoietin; GPVI: collagen receptor glycoprotein VI; IL: Interleukin; TLR4: Toll like receptor 4. TRAIL: tumor necrosis factor (TNF)-related apoptosis-inducing ligand.

interaction between the SH3 domain and the proline-rich TH domain that has been reported for Itk (202).

Regulation of Btk activation through phosphoinositide metabolism and membrane microdomains play an important role in optimal activation of the kinase. Btk kinase activity can be inhibited by the inositol phosphatase SHIP-1, leading to decreased phosphoinositol-3,4,5-trisphosphate (PIP3) levels (222-226). Phosphopeptide mapping of stimulated Btk has identified two sites of tyrosine phosphorylation. Y551 corresponds to the regulatory tyrosine in the kinase domain (207, 227) and Y223 at the ligand-binding surface of the SH3 domain (228). The SH2 domain of Btk is required for induction of calcium mobilization and binds to the phosphorylated adapter molecule SLP-65 (Figure 8; (225, 229-231)).

Defects in the Btk protein result in the B cell differentiation defects X-linked agammaglobulinaemia (XLA) in man and X-linked immunodeficiency (*xid*) in mice.

4.1 X-linked agammaglobulinaemia (XLA)

XLA is one of the most frequent inherited immunodeficiency diseases in man and was first described by Dr. Ogden C. Bruton in 1952 (232). Most boys with XLA remain well during the first 6-9 months of age by virtue of maternally transmitted IgG antibodies (233). Thereafter, they repeatedly acquire infections with extracellular pyogenic bacteria such as pneumococci and streptococci unless they are given prophylactic antibiotics or gamma globulin therapy. Chronic fungal infections or pneumonia rarely occur, unless there is an associated neutropenia (234). Female carriers of XLA are healthy and demonstrate no abnormalities of the immune system as B cells expressing the mutated gene are selected against (235). Patients with XLA have less than 1% of the normal number of peripheral B cells resulting in an apparent absence of immunoglobulins in peripheral blood and therefore lack an antibody response and fail to clear microbial antigens. An international registry for XLA shows that mutations in all domains of the Btk gene cause the disease (236). However, no correlation has been described between the position of the mutation and phenotypic variables, such as the age at the time of diagnosis or severity of the disease (237, 238). XLA is a heterogeneous disease, even within one pedigree, and this heterogeneity might be related to difference in the nature of the mutations in combination with other genetic or environmental factors. A point mutation in the Btk gene can result in a change of the reading frame of Btk mRNA. This may result in the formation of a premature stop codon, leading to the formation of an instable or truncated protein, or no protein at all (187, 238, 239).

In XLA patients the B cell differentiation arrest occurs in the BM, but the exact location of the blockade has been controversial for some time as the number of pre-B cells in XLA BM are detectable in heterogeneous amounts (240-242). However, all patients had substantial numbers of pro-B cells resulting in an increased ratio of pro- to pre-B cells (242-245). The B cells present in the periphery have an immature $IgM^{high}IgD^{high}$ phenotype and show an increased κ to λ ratio within the IgM^{+} B cell population compared to controls (246).

Detailed flow cytometric studies, using specific antibodies for human V_{preB}, TdT, CD19, CD10, CD34 and cμ have shown that six B cell differentiation stages are distinguishable in human BM. On the basis of differential expression of the rearrangement status of the Igμ H loci and cytoplasmic or surface Igμ expression, human B cells can be subdivided in pro-B, large and small pre-B I, pre-B II, immature and mature B cells. Recently it was shown that the major blockade in XLA patients was at the transition from pro-B ($c\mu^-SLC^{bright}$) into large pre-B I ($c\mu^{low}SLC^{bright}$) cells (243). Patients had a relatively high number of pro-B cells and a low number of pre-B I cells. Normally the pre-B I population consists of large and small pre-B cells. However, in XLA patients the pre-B I cells were mainly small, suggesting that Btk is necessary for the proliferative expansion and the survival of $c\mu^+$ pre-B cells.

Some XLA cases have been shown to exhibit a mild phenotype, manifested by a delayed disease recognition, mild bacterial infections and higher than expected levels of serum Ig (247-249). Another group of investigators tried to correlate the B cell differentiation defect to the specific Btk mutation in nine XLA patients (250). They used flow cytometry to unravel the precise B cell differentiation arrest and found that in the majority (eight out of nine) of the patients, ~80% of the IgM^- precursor B cells in the BM were $c\mu^-$ pro-B cells. Most of these eight patients (seven out of eight) did not express functional Btk protein, suggesting that Btk is required for the expansion of $c\mu^+$ pre-B cells by signalling via the pre-BCR (250). The finding that Btk is phosphorylated in a human pre-B cell line supports this idea (210). This group also found that one patient with low-level expression of Btk mRNA in peripheral blood granulocytes had normal numbers of $c\mu^+$ pre-B II cells in the BM. In this patient however, the number of mature B cells was reduced. This suggests that low amounts of Btk are sufficient for pre-BCR signalling, whereas BCR signalling needs higher levels of Btk. This particular patient had a G99A mutation in exon 3 of the Btk gene, which resulted in the formation of a premature stopcodon at position L119 in the PH domain of the Btk protein. The defects found in this patient were very similar to the xid phenotype in mice, which is discussed below. As the other patients, each with a different mutation, showed quite similar blockades, no obvious correlation was found between the mutation in the Btk gene and the phenotype of the XLA patients analysed (250).

Although approximately 80-90% of patients with an agammaglobulinemia have mutations in the Btk gene, mutations in other genes, like μ H chain (251), λ5 (244), Ig-α (245) and SLP-65 (252), also result in a clinical phenotype resembling XLA.

4.2 X-linked immunodeficiency (xid)

The animal model for human XLA is the X-linked immunodeficiency (Xid) mutation in the CBA/N strain of mice (115). These xid mice have a missense mutation at a conserved arginine residue (R28) within the Btk PH domain (Figure 8; (189, 190, 253)). This R28C mutation changes the conformation of the Btk protein in such a way that it is not able to bind to the cellmembrane (113, 115). Targeted deletion of the Btk gene also causes a xid phenotype in mice (113, 115). Although

the same gene is affected, the B cell differentiation defect is less severe in xid mice than that seen in human XLA (Table 3).

Studies in these xid mice revealed that the murine xid gene defects result in a reduced B cell number of ~50%. Those B cells that are present have an immature phenotype ($IgM^{high}IgD^{low}$). The mice have low levels of serum IgM and IgG3, whereas the levels of other isotypes are normal and peritoneal B-1 B cells are absent (115). Peripheral B cells from xid mice are functionally impaired, as they do not respond to thymus-independent type 2 polysaccharide antigens, such as NP-Ficoll, but respond normally to thymus-dependent antigens (253, 254). Furthermore, xid B cells show a high rate of spontaneous apoptosis *in vitro* and they hardly respond to stimuli such as Ig-receptor crosslinking, IL-5, IL-10, CD38 and LPS (213, 219, 255)

Despite obvious differences in severity of the phenotype, Btk appears to be a conserved factor involved in both murine and human B cell development. Btk has been shown to be involved in BCR signal transduction pathways in both human (211) and murine (212, 256) B cells. Because xid mice have such a milder antibody deficiency than boys with XLA, it was speculated that XLA patients with mutations in the PH domain-encoding part of the Btk gene may have less severe immunodeficiency. However, patients with classic XLA have been identified with mutations affecting the same residue as in CBA/N xid mice (211).

The genomic organization of the murine Btk gene is very homologous to the human Btk gene and the Btk proteins share 99.3% homology (187). Transgenic human Btk could fully compensate the absence of murine Btk (197, 257-259), indicating that the essential sites for Btk interaction with other signalling molecules

Table 3. *Comparison between human XLA and murine xid phenotypes.*

Differences		
Phenotype	XLA	xid
• Pre-B to immature B cell transition	Almost complete arrest	Mild selective disadvantage for Btk-deficient cells
• Peripheral B cell population	Almost absent	~50% reduction
• T cell dependent antibody responses	Low but detectable	Normal
• Ig levels in serum	Levels of all isotypes are very low	Reduced levels of IgM and IgG3, normal levels of other isotypes
Similarities		
• Intrinsic B cell defect, other hematopoietic lineages unaffected		
• Early B cell development up to the pre-B cell stage in BM is normal		
• Residual peripheral B cells have an immature IgM^{high} surface phenotype		
• T cell independent antibody responses are lacking		
• Heterozygous female carriers are normal, but have non-random X-chromosome inactivation in mature cells		

References (113, 191, 246, 253, 263, 264).

is very similar in man and mice. Studies done in human and murine heterozygous female carriers of Btk mutations have shown that Btk-deficient B cell precursors have a selective disadvantage in proliferation, differentiation and survival compared to normal B cell precursors (235, 260-263).

The absolute number of pre-B cells that are generated in the BM is similar in xid and wild-type mice, indicating that commitment to the B cell lineage is normal. Furthermore, Btk-deficient B cell precursors in the BM have normal kinetics and turnover (114, 253). By examining the size, renewal and production rates within the B cell lineage subsets in xid and wild-type mice using BrdU, it was found that xid mice showed significant cell loss at the early pre-B cell stage, placing the initial effects of murine xid and human XLA at the same stage of B cell development (265). However, normal numbers of immature B cells were found in BM and periphery of xid mice, indicating that compensation mechanisms are acting at this stage of B cell development. At the transition of immature into mature B cells, a Btk-mediated deficit was observed, which reduced the rate of mature B cell production and thus the number of mature B cells. Xid immature B cells in the periphery were shown to have defects in their recruitment into the long-lived mature B cell pool. Ectopic expression of the anti-apoptotic protein Bcl-2 was not able to correct the pro-/pre-B cell defects, but significantly increased the numbers of BM pre-B cells as well as immature and mature peripheral B cells in xid/Bcl-2 mice, suggesting that the peripheral defects in xid mice are mainly caused by lack of survival (266).

Tec and Btk are members of the Tec kinase family. While Tec single knockout mice have no obvious immune defects, Tec/Btk double deficient mice have a severe B cell deficiency due to a partial block at the pro- to pre-B cell transition in B cell development (125). This strongly suggests that Btk is required for this transition but that in the absence of Btk, its family member Tec can take over its function. Other genetic analyses are consistent with the data that Btk is required for pro- to pre-B cell transition. The adapter proteins SLP-65, as well as the kinases Syk and PI3K, are directly involved in Btk activation and function (267), especially for the Btk-dependent sustained flux of calcium after BCR stimulation (225, 268). Targeted mutations of SLP-65 or the p85a subunit of PI3K resulted in phenotypes resembling xid (106, 269, 270). Developmental defects in these mice are first seen at the pro- to pre-B cell transition, reducing the number of pre-B cells.

To determine the exact stage of B cell development in which the function of Btk is required, a mouse model was generated in which the *Btk* gene was inactivated by insertion of a β-galactosidase (*lacZ*) reporter gene (263). The xid phenotype in these mice was similar to the findings in Btk null-mutant mice generated in other labs (113). The presence of the lacZ reporter enabled the determination of Btk expression profiles *in vivo*. It was found that Btk expression was not restricted to the B cell lineage. In the BM, the ER-MP20^{high} monocyte precursors showed high *lacZ* expression. Using the *LacZ* activity, Btk expression could be studied *in vivo*. The phenomenon of X-chromosome inactivation in Btk$^{+/-}$ heterozygous female carriers enabled the evaluation of the *in vivo* competition between B cells expressing the

wild-type Btk and those expressing the Btk$^-$/*LacZ* allele in each successive step of B cell development. The model detected even subtle defects of Btk-deficient cells, characterized by only moderate selective disadvantages, which were not recognized in the affected males. Although Btk was already expressed in pro-B cells, the first selective disadvantage only became apparent at the transition from small pre-B cells to immature B cells in the BM. A second differentiation defect was found during the maturation from IgM^{high}IgD^{low} (T2) to IgM^{low}IgD^{high} (mature) stages in the periphery (263).

4.3 Transgenic mice expressing Btk-mutants

Transgenic mice have been generated in which B cell-specific promoters drive expression of Btk in order to correct the *xid* phenotype in Btk-deficient mice. For example, mice that express transgenic human *Btk* (h*Btk*) under the control of the class II MHC Ea gene locus control region provided transgene expression in myeloid cells and B cells from the immature B cell stage onwards (257). When the MHCII-h*Btk* transgenic mice were mated with Btk-deficient mice, Btk protein expression was restored to normal levels in the spleen and the Btk-deficient phenotype was corrected. B cells now differentiated into IgM^{low}IgD^{high} mature B cells; peritoneal CD5$^+$ B-1 cells were present and serum levels of all Ig classes and *in vivo* responses to TI-II antigens were in normal ranges. These results indicate that this transgenic system is suitable for Btk re-expression in the B cell lineage. Complete correction of all *xid* features was also observed by transgenic expression of h*Btk* under the control of the B cell-specific human CD19 promoter region (197). Expression of a hCD19 transgene was reported to be completely restricted to the B cell lineage from the pro-B cell stage onwards. This transgenic model is therefore useful for studying Btk expression in earlier stages of B cell development compared to the MHCII-h*Btk* model where Btk is expressed from the pre-B cells stage onwards. By introducing various mutations in the h*Btk* construct, various transgenic mouse models were generated. The mutations were located in different domains of the Btk gene, which enabled the analysis of the function of each particular Btk domain *in vivo*.

Interestingly, the PH domain gain-of-function mutant E41K (Glu-to-Lys) shows increased membrane localization and phosphorylation of Btk in quiescent cells, independent of PI3K activity, bringing Btk in close proximity of other signalling molecules involved in downstream signalling from the BCR (204, 228, 271). The E41K mutation induces transformation of 3T3 fibroblasts in soft agar cultures, and this capacity is augmented by mutation of the main auto-phosphorylation site (Y223F; (271)). Expression of E41K-Btk in Ramos B cells enhances the sustained increase in intracellular calcium following BCR cross-linking (225). In transgenic mice, which use either the B-cell specific CD19 promoter or the MHC class II Ea locus control region to express E41K-Btk, B cell development is arrested in the BM or the spleen, respectively (197, 258). These findings suggest that expression of the E41K-Btk mimics BCR occupancy by autoantigens.

Several additional mutant mouse strains were generated expressing h*Btk* under the control of the CD19 promoter with mutations at the Y223 autophosphorylation site in the SH3 domain (Y223F-Btk) or at the ATP-binding site in the kinase domain (K430R-Btk). These mutant mice were crossed onto a Btk-deficient background (263) to analyse the role of the individual Btk domains *in vivo*.

5. Aim of the thesis

In XLA patients there is an almost complete arrest of the pre-B to immature B cell transition, whereas only a mild selective disadvantage for this transition was observed in Btk-deficient cells in *xid* mice (Table 2). Biochemical studies in cultured B cells and fibroblasts have provided important insights into the molecular mechanism of BCR-mediated Btk activation. However, these systems have not allowed for the investigation of the involvement of Btk in pre-BCR checkpoint function *in vivo*.

This thesis focuses on the role of Btk in early B cell development and its role in pre-BCR checkpoint function in the mouse. For the studies in this thesis, the Btk^-/ LacZ mouse model was used to identify the function of Btk in various cellular processes.

One of the events initiated upon pre-BCR signalling is the rearrangement of the κ and λ L chains in small pre-B cells. Therefore, we analysed the role for Btk in λ L chain usage (chapter 1.1) and in activation of the V(D)J recombination at the L chain loci (chapter 1.2).

To find out if Btk is involved in pre-BCR signalling, we analysed pre-BCR checkpoint functions, such as clonal expansion of $c\mu^+$ pre-B cells and the transition of large into small pre-B cells in Btk-deficient mice (chapter 2.1). Furthermore, we examined if Btk functions solely as a kinase protein or is also able to function as an adapter molecule in transgenic mice expressing the kinase-dead Btk mutant K430R (chapter 2.2). Next, we analysed the role of Btk in pro-BCR signalling by using transgenic mice expressing the gain-of-function mutant E41K (chapter 2.3).

As it is known that Btk interacts with the adapter protein SLP-65, we analysed their synergistic role in Btk/SLP-65 double deficient mice (chapter 3.1) and investigated whether Btk was able to function as an adapter molecule in the absence of SLP-65 (chapter 3.2).

By using 3–83$\mu\delta$ transgenic mice, which prematurely express a complete B cell receptor (BCR), we analysed the role of Btk in accelerated B cell development and in the receptor editing process (chapter 4).

Summarizing, this thesis provides detailed information about the role of the Btk protein during early and late B cell development in the mouse. Understanding the role of the Btk protein in mice provides important insights in its implication in human B cell development.

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Chapter 1

Btk and Ig L chain rearrangement

1.1 Bruton's tyrosine kinase regulates the activation of gene rearrangements at the λ light chain locus in precursor B cells in the mouse

1.2 Evidence for the involvement of Bruton's tyrosine kinase in the regulation of V(D)J recombinase activity at the Ig L chain loci

Chapter 1.1

Bruton's tyrosine kinase regulates the activation of gene rearrangements at the λ light chain locus in precursor B cells in the mouse

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Bruton's tyrosine kinase regulates the activation of gene rearrangements at the λ light chain locus in precursor B cells in the mouse

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Abstract

Bruton's tyrosine kinase (Btk) is a nonreceptor tyrosine kinase involved in precursor B (pre-B) cell receptor signaling. Here we demonstrate that Btk-deficient mice have an ~50% reduction in the frequency of immunoglobulin (Ig) λ light chain expression, already at the immature B cell stage in the bone marrow. Conversely, transgenic mice expressing the activated mutant Btk^{E41K} showed increased λ usage. As the κ/λ ratio is dependent on (a) the level and kinetics of κ and λ locus activation, (b) the life span of pre-B cells, and (c) the extent of receptor editing, we analyzed the role of Btk in these processes. Enforced expression of the Bcl-2 apoptosis inhibitor did not alter the Btk dependence of λ usage. Crossing 3-83μδ autoantibody transgenic mice into Btk-deficient mice showed that Btk is not essential for receptor editing. Also, Btk-deficient surface Ig^+ B cells that were generated in vitro in interleukin 7-driven bone marrow cultures manifested reduced λ usage. An intrinsic defect in λ locus recombination was further supported by the finding in Btk-deficient mice of reduced λ usage in the fraction of pre-B cells that express light chains in their cytoplasm. These results implicate Btk in the regulation of the activation of the λ locus for V(D)J recombination in pre-B cells.

Key Words: Btk • B lymphocytes • Ig L chain • receptor editing • V(D)J rearrangements

Introduction

During early B cell development in the bone marrow, Ig H and L chain variable region genes are assembled from component V, (D), and J gene segments (for reviews, see references (1-3). V(D)J recombination is a highly ordered process, initiated by DNA rearrangements at the H chain in pro-B cells. The expression of a functional μ H chain is monitored through the formation of a (pre-) B cell receptor (BCR) complex, together with λ5 and $V_{pre\text{-}B}$ proteins. Signaling through the pre-BCR results in feedback inhibition of H chain VDJ recombination to ensure allelic exclusion and IL-7–dependent proliferative expansion. The rapidly proliferating pre-B cells then exit the cell cycle and perform Ig L chain rearrangements, leading to the deposition of complete Ig molecules on the cell surface (2, 3). The murine κ L chain locus contains 70–90 functional V_κ gene segments, present in both orientations relative to the four functional J_κ elements. By contrast, the λ locus in the mouse is very small; it consists of only three functional V_λ and three functional J_λ segments. Ig κ and λ L chain rearrangements occur independently and at different kinetics, with sequential activation of the κ and λ loci (4-6). The privileged activation of the κ locus is thought to determine the final ratio of κ^+ to λ^+ mature B cells of 95:5 in wild-type (WT) mice.

PCR analyses of the Ig κ L chain locus in single developing B cells in the bone marrow have demonstrated the presence of multiple in- and out-of-frame rearrangements in small

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Abbreviations used in this paper: BCR, B cell receptor; Btk, Bruton's tyrosine kinase; PH, pleckstrin homology; WT, wild-type; Xid, X-linked immunodeficiency; XLA, X-linked agammaglobulinemia

pre-B cells (6). These findings indicated that the BCR does not signal allelic or isotypic exclusion of the Ig κ or λ L chain loci, allowing secondary L chain rearrangements to occur. Cells with a first productive rearrangement on one allele are rapidly selected to enter the immature B cell compartment (6). The rearrangement machinery remains active in immature B cells and is only turned off at the transition to mature B cells (7-9). If an immature B cell expresses an Ig that has reactivity with an autoantigen in the bone marrow, continued Ig L chain rearrangement can be induced to rescue autoreactive B cells from tolerance elimination, a phenomenon called receptor editing (10-12).

One of the molecules that is involved in pre-BCR signaling and thereby directs B cell development is the Tec family nonreceptor Bruton's tyrosine kinase (Btk; for a review, see reference 13). Upon BCR stimulation, Btk phosphorylation and kinase activity are increased (14-16) probably by the activity of Src family kinases (13, 17). Concomitantly, Btk is targeted to the plasma membrane by the binding of its pleckstrin homology (PH) domain to the second messenger phospatidylinositol-(3,4,5)triphosphate (18). Binding of Btk to the linker protein BLNK/SLP-65 is crucial for phosphorylation and activation of phospholipase Cγ2 by Btk, implicating Btk as a mediator of BCR-induced Ca^{2+} mobilization (19).

Mutations in Btk lead to X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (*xid*) in mice (for reviews, see references (20-23). XLA is characterized by an almost complete arrest of B cell development at the pre-B cell level (24, 25). As a result, XLA patients have profoundly reduced numbers of B cells in the peripheral blood, and serum Ig levels of all classes are very low (24). Btk-deficient mice display a milder phenotype, mainly reflecting poor survival of peripheral B cells. B cell numbers in the spleen and lymph nodes are reduced by 50%, and IgM and IgG3 levels in the serum are low (20, 26). Nevertheless, mouse Btk-deficient cells also showed an impaired transition from pre-B to immature B cells by analysis of competition in vivo between Btk^+ and Btk^- cells in females heterozygous for a targeted mutation in the Btk gene (27).

Both in XLA patients and in *xid* mice the absence of Btk appeared to result in a decrease of Ig λ L chain usage in peripheral B cells (24, 28, 29). The reduced frequency of Ig λ L chain–expressing cells could either reflect an intrinsic feature of the L chain rearrangement process in the absence of Btk signaling or could alternatively be secondary to an altered antigen-dependent peripheral repertoire selection in Btk-deficient mice. To distinguish between these two possibilities, we determined the proportions of Ig λ^+ B cells during B cell differentiation in Btk-deficient mice, as well as in transgenic mice that express a constitutively activated form of Btk, the E41K PH domain mutant (27, 30). As the κ to λ ratio is dependent on (a) the level and kinetics of the activation of the κ to λ loci for recombination, (b) the life span of pre-B cells that are in the process of L chain rearrangement, and (c) the extent of receptor editing of autoreactive B cells, we analyzed the involvement of Btk in these processes in detail.

Materials and Methods

Mice. *Btk⁻/lacZ* mice (27) were crossed on a C57BL/6 background for over five generations. CD19-h*Btk*^{WT} and CD19-h*Btk*^{E41K} transgenic mice that express WT and E41K-mutated human Btk (hBtk), respectively, under the control of the CD19 promoter region have been described previously (30) and were on a mixed background containing 129/Sv, C57BL/6, and FVB. For the generation of λ5-h*Btk*^{E41K} mice we used a 1.5-kb NotI-BglII fragment containing the murine λ5 promoter and a 6.5-kb ClaI-NotI fragment with the 3' locus control region of the λ5-V_{preB1} locus (31). The promoter and locus control region were cloned into the unique SwaI and SmaI sites, respectively, present in the cosmid vector pTL5 containing a BglII-NotI-XhoI-SwaI-PvuI-KpnI-SmaI-NotI-BglII polylinker (32). Next, an ~27-kb PvuI-NotI fragment containing the E41K-mutated h*Btk* cDNA/genomic DNA fragment (32) was cloned into the λ5/pTL5 vector, using the PvuI and KpnI sites in the polylinker. The ~35-kb NotI insert of the λ5-h*Btk*^{E41K} construct was excised from the vector and purified, using standard methods. DNA (~2–4 ng/μl) was injected into the pronuclei of FVB fertilized oocytes, which were subsequently implanted into pseudopregnant foster mice. Founder mice were crossed with *Btk⁻/lacZ* mice. E_μ*-2-22 Bcl-2* transgenic mice (33) and 3-83μδ mice (10) were on a C57BL/6 and a B10.D2 background, respectively. All mice were bred and maintained in the animal care facility at the Erasmus University Rotterdam.

Mouse Genotyping. To determine the *Btk* genotype and score the presence of the CD19-

h*Btk*^{WT} or CD19-h*Btk*^{E41K} transgenes, tail DNA was analyzed by Southern blot analysis of BamHI digests, as described previously (27, 30). The presence of the E_{μ}-*2-22 Bcl-2* transgene was evaluated by PCR, using primers that are specific for the SV40 DNA sequences flanking the Bcl-2 transgene: 5'-GGCACTATACATCAAATA TTC-3' and 5'-TGAAGGAACCTTACTTCTGT-3'. The presence of the 3-83μδ transgene was identified by Southern blot analysis of BamHI or EcoRI digests, using a J_{κ}-specific probe as described previously (34).

Flow Cytometric Analyses. Preparation of single cell suspensions, flow cytometry, and determination of β-galactosidase activity by loading cells with fluorescein-di-β-galactopyranoside substrate have been described previously (27, 32). Events (5 x 10^4–5 x 10^5) were scored using a FACSCalibur™ flow cytometer and analyzed by CELLQuest™ software (Becton Dickinson). The following mAbs were obtained from BD Pharmingen: FITC-conjugated anti-B220–RA3-6B2, anti-κ–R5-240, and anti-IgM, PE-conjugated anti-CD19, anti-CD43, and anti-H2–K^d; and CyChrome-conjugated anti-B220–RA3-6B2 and biotinylated anti-CD19, anti-λ_1 and λ_2–R26-46, anti–IgM, and anti-H2–K^b. PE-conjugated anti-IgD was from Soutern Biotechnologies, Inc. The anti–3-83 clonotype 54.1 antibody has been described previously (35). Secondary antibodies were PE-conjugated goat anti–rat, Tri-color, or allophycocyanin-conjugated Streptavidin, purchased from Caltag. Affinity-purified polyclonal rabbit anti-Btk (BD Pharmingen) was used for intracellular flow cytometric detection of cytoplasmic Btk protein using FITC-conjugated goat anti–rabbit total Ig (Nordic) as a secondary antibody (32).

IL-7–driven Bone Marrow Cultures. Primary pre-B cell cultures were performed as described previously (36). In brief, bone marrow cells were depleted of erythrocytes by standard ammonium-chloride lysis, and subsequently IgM^- B cells were purified by negative selection. Cell suspensions were labeled with biotinylated anti-IgM (BD Pharmingen) and incubated with Streptavidin-coated microbeads (Miltenyi Biotec). After cell separation using MACS column purification, the IgM^- fraction was collected and purity was confirmed by flow cytometry. Cells were cultured in IMDM medium, supplemented with 10% heat-inactivated FCS at 2 x 10^6 cells/well in 24-well plates at 37°C in the presence of 100 U/ml of recombinant murine IL-7 (R&D Systems). After 5 d of culture, cells were washed and recultured on S17 stromal cells with or without 100 U/ml IL-7 for 48 h.

Results

Reduced Ig λ L Chain Usage throughout B Cell Development in Btk-deficient Mice. The expression of Ig λ L chain was investigated in Btk-deficient mice, in which the Btk gene is inactivated by a targeted insertion of a *lacZ* reporter (27). Total splenic cell suspensions were analyzed by three-color flow cytometry, using an antibody specific for the Ig λ_1 and λ_2 L chain constant regions in conjunction with anti-B220 and anti-CD19. The proportions of B cells that expressed Ig λ L chain on the cell surface were 4.9% ± 0.2 (n = 7) and 5.0 ± 0.2 (n = 6) in the spleen of Btk^+ and $Btk^{+/-}$ control mice, respectively. By contrast, we observed a significant reduction of this proportion in Btk^- mice (2.3 ± 0.4; n = 10).

Next, λ expression was determined in individual B cell subpopulations in bone marrow and spleen. We performed four-color flow cytometry experiments, using anti-λ L chain antibodies in combination with mAbs specific for the B220, IgM, and IgD surface markers, which define successive stages of B cell development (Fig. 1). In Btk^+ mice, 10–16% of cells within the immature B cell subpopulations, including immature IgM^+IgD^- and transitional IgM^+ IgD^{low} B cells in the bone marrow and immature IgM^{high} IgD^{low} B cells in the spleen, were found to express Ig λ L chain. By contrast, in the Btk^- mice only 5–7% of cells within these subpopulations were λ^+, i.e., about half the number found in WT mice. In the mature B cell subpopulations, including the IgM^+IgD^{high} cells in bone marrow and spleen, Ig λ L chain was expressed in 4–5% of cells in Btk^+ mice and in 2.5–3.5% of cells in Btk^- mice. Finally, we found that in the subpopulation of large lymphoblastoid cells in the spleen of Btk^+ and Btk^- mice 8–12% and 4–6% were λ^+, respectively (Fig. 1). In $Btk^{+/-}$ heterozygous mice, intermediate values of λ^+ cells were found in the bone marrow immature cells (data not shown).

These findings demonstrate that the absence of Btk leads to a significantly reduced frequency of λ usage, already from the immature B cell stage onwards.

Reduced λ L Chain Usage Is an Intrinsic Feature of Btk-deficient B Cells. Although we demonstrated that Ig L chain isotype usage is determined in the bone marrow, the possibility remained that the reduced λ usage did not

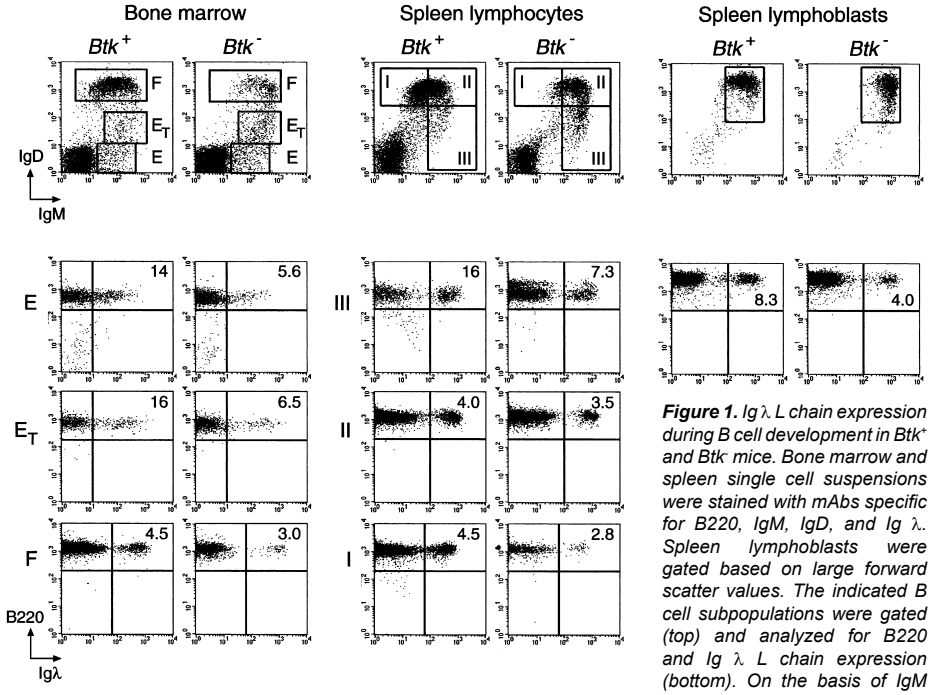

Figure 1. *Ig λ L chain expression during B cell development in Btk⁺ and Btk⁻ mice. Bone marrow and spleen single cell suspensions were stained with mAbs specific for B220, IgM, IgD, and Ig λ. Spleen lymphoblasts were gated based on large forward scatter values. The indicated B cell subpopulations were gated (top) and analyzed for B220 and Ig λ L chain expression (bottom). On the basis of IgM and IgD expression, B cell subpopulations were defined in the bone marrow: E, IgM⁺IgD⁻ immature B cells; E_T, IgM⁺IgD^{low} transitional immature B cells; F, IgM⁺IgD⁺ mature recirculating B cells (reference (47)). In the spleen: I, mature IgM^{low} IgD^{high}; II, IgM^{high}IgD^{high}; III, immature IgM^{high}IgD^{low} B cells. Data are displayed as dot plots, and the percentages of λ⁺ cells of the B220⁺ B cell population are indicated. Data shown are representative of 10 Btk⁺ and 8 Btk⁻ mice animals examined.*

reflect an intrinsic feature of Btk-deficient B cells. Alternatively, this phenomenon could originate from the *xid* immune status of the Btk-deficient mice, which may, directly or indirectly, affect selection events at the pre-B to B cell progression.

To address this issue, we analyzed heterozygous Btk$^{+/-}$ female mice, which do not manifest the *xid* phenotype due to the selective advantage of B cells that have the intact *Btk*$^+$ allele on their active X chromosome (27). Because of the process of random X chromosome inactivation, ~50% of the B cell progenitors have the disrupted *Btk*$^-$*/lacZ*$^+$ allele on the active X chromosome. When cells reach a differentiation stage at which Btk is required, their further development is hampered. As a consequence of this selective disadvantage, the proportions of *Btk*$^-$*/lacZ*$^+$ cells decrease below the value of 50%, finally to undetectable levels in the mature peripheral B cells (27).

We analyzed splenic cell samples from Btk$^{+/-}$ heterozygous females for λ usage in four-color flow cytometry experiments, using fluorescein-di-β-galactopyranoside as a fluorogenic substrate in conjunction with surface labeling to define the immature IgD^{low}B220$^+$ B cell subpopulation in the spleen. In this fraction, ~30% of the cells expressed *lacZ*, enabling a separate analysis of the *Btk*$^+$*/lacZ*$^-$ and *Btk*$^-$*/lacZ*$^+$ B cell populations. We found that the proportion of Ig λ$^+$ cells within the Btk$^-$ immature IgD^{low}B220$^+$ subpopulation was reduced to 3.6% ± 0.7, when compared with Btk$^+$ immature B cells (6.3% ± 0.9; Fig. 2).

These results show that reduced λ usage is an intrinsic feature of Btk-deficient B cells, which is independent of the *xid* immune status of the mice.

Bcl-2 Overexpression Does Not Alter the Btk Dependence of Ig L Chain Isotype Usage. The finding of reduced λ expression in Btk-deficient cells may be explained by a role for Btk signal transduction in extending the life span of pre-B cells that are in the process of L

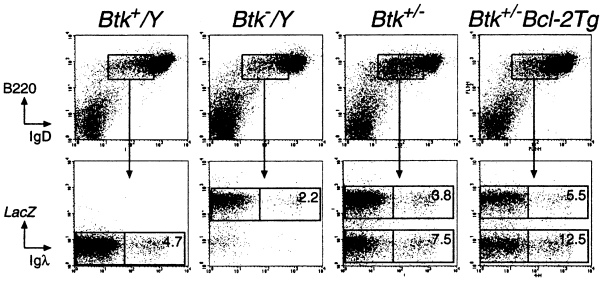

Figure 2. *Separate effects of Btk and Bcl-2 on Ig λ L chain usage. Four-color flow cytometric analyses of spleen cells of the indicated mice. The immature IgD^{low}B220^{+} compartment was gated (top) and analyzed for lacZ and λ L chain expression (bottom). The percentages of λ^{+} cells within the indicated lacZ^{+} or lacZ^{-} subpopulations are given. Data are shown as dot plots representative of the 4–10 mice examined.*

chain rearrangement. Enforced expression of the anti-apoptotic *Bcl-2* gene results in elevated Ig λ expression in mature B cells (37). As the Ig κ and λ loci are sequentially activated for V_L-J_L recombination, *Bcl-2* gene is assumed to provide an extended time window per cell for Ig L chain rearrangement (37). To investigate a role for Btk in pre-B cell survival, we have crossed *Btk*$^{-}$*/lacZ* mice onto an E_μ-*Bcl-2* transgenic background (33). We investigated λ usage in E_μ-*Bcl-2* transgenic Btk$^{+/-}$ female mice, in which the Btk^{+} and Btk^{-} immature splenic IgD^{low}B220^{+} B cell population can be analyzed within a single animal. In these mice the Btk^{+} subpopulation had a significantly higher percentage (12.8% ± 1.3; *n* = 4) of λ^{+} B cells, compared with the Btk^{-} subpopulation (7.3% ± 1.5; Fig. 2).

These analyses demonstrate that even if the life span of pre-B cells is extended by the presence of the *Bcl-2* transgene, the Btk-deficient B cell population still manifests an ~50% reduction in λ usage. Therefore, we conclude that protection of pre-B cells from apoptosis does not alter the Btk dependence of the frequency of λ usage.

Expression of a Constitutively Activated Btk Mutant Increases λ Usage. To further test the involvement of Btk signaling in the mechanism that sets the κ to λ isotype ratio in vivo, we examined mice that express the constitutively activated Btk^{E41K} mutant. This Glu-to-Lys PH domain Btk mutant shows increased membrane localization in quiescent cells, independent of phosphatidylinositol 3-kinase activity (38, 39). In CD19-*hBtk*^{E41K} transgenic mice, which express Btk^{E41K} under the control of the CD19 promoter region, B cell development is almost completely arrested at the immature B cell stage in the bone marrow, probably because the Btk^{E41K} mutant mimics BCR occupancy by autoantigens (30; Fig. 3).

In four-color flow cytometry experiments using antibodies to B220, IgM, IgD, and Ig λ, the CD19-*hBtk*^{E41K} transgenic mice manifested a significant increase in the frequency of λ usage (~11% in the IgM^{+}IgD$^{-/low}$ immature B cell fraction in the bone marrow compared with ~6% in nontransgenic littermates; Fig. 3). This increase was specifically associated with the expression of the *Btk*^{E41K} mutation, as the control CD19-*hBtk*^{WT} transgenic mice in which the CD19 promoter drives expression of WT hBtk contained normal proportions of λ^{+} B cells (Fig. 3). We conclude that the presence of constitutively activated Btk enhances λ usage.

Ig λ Usage in CD19-hBtk^{E41K} E_μ-Bcl-2 Double Transgenic Mice. To confirm that Btk-mediated signaling increases λ usage, independent of the life span of pre-B cells, we crossed CD19-*hBtk*^{E41K} and CD19-*hBtk*^{WT} transgenic mice onto the E_μ-*Bcl-2* background. The enforced expression of Bcl-2 partially prevented central deletion of B cells in CD19-*hBtk*^{E41K} transgenic mice, as was evident from the presence of substantial numbers of mature B cells in bone marrow and spleen of CD19-*hBtk*^{E41K} E_μ-*Bcl-2* double transgenic mice (Fig. 3). We analyzed Ig λ L chain expression in the B cell populations in bone marrow and spleen from E_μ-*Bcl-2* transgenic, CD19-*hBtk*^{E41K} E_μ-*Bcl-2,* and CD19-*hBtk*^{WT} E_μ-*Bcl-2* double transgenic mice (Fig. 3). Consistent with previous reports (37), E_μ-*Bcl-2* mice or CD19-*hBtk*^{WT} E_μ-*Bcl-2* double transgenic mice had significantly higher percentages of λ^{+} cells in the immature IgM^{+}IgD$^{-/low}$ B cell population in the bone marrow (~24%), when compared with nontransgenic or CD19-*hBtk*^{WT} littermates (~7%). Most importantly, in CD19-*hBtk*^{E41K} E_μ-*Bcl-2* double transgenic mice we found even higher proportions of Ig λ^{+} cells (~35–40%). Similar significant differences were found in the mature recirculating IgM^{+}IgD^{+} B cell population in the bone marrow and in the spleen (data not shown, and Fig. 3). Therefore, we conclude that the Btk^{E41K}-mediated increase of λ usage cannot be explained by an effect of this mutant on pre-B cell survival.

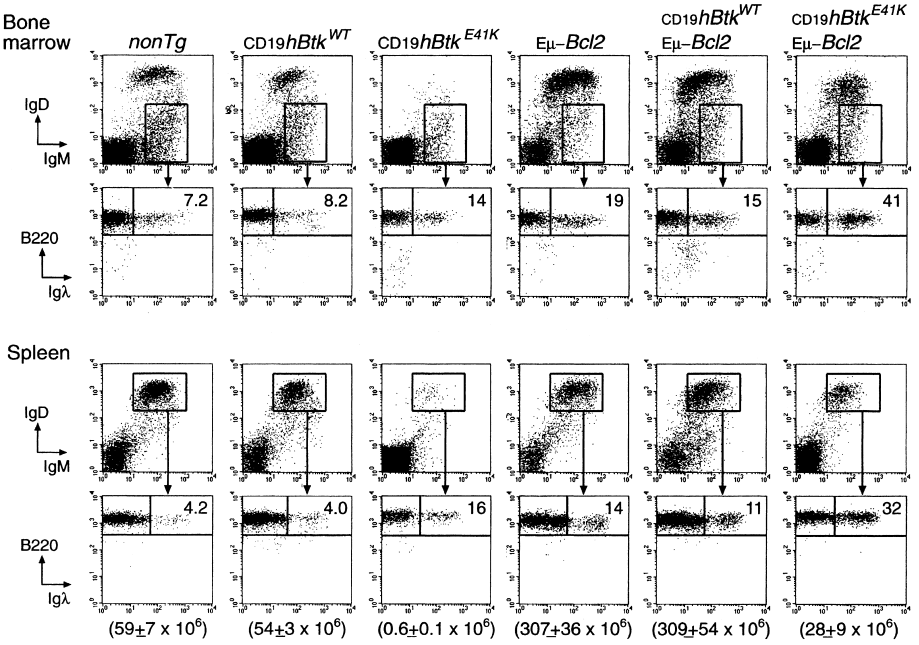

Figure 3. *Increased λ usage in CD19-hBtk^{E41K} mice independent of cell survival. Four-color flow cytometric analyses of bone marrow and spleen of the indicated mice. Single cell suspensions were stained with mAbs specific for B220, IgM, IgD, and Ig λ. On the basis of IgM and IgD expression, the immature IgM$^+$IgD$^{-/low}$ B cell population in the bone marrow (top, fraction E+E$_{\mu}$; see legend to Fig 1) and the total IgM$^+$IgD$^+$ B cell population in the spleen (bottom) were gated and analyzed for λ usage. Data shown are representative of five to nine mice examined per group. At the bottom, the total splenic B cell numbers of the indicated animals are given as mean values ± SD (determined by flow cytometric analysis using mAbs to B220 and CD19).*

Btk Signaling Is Not Essential for Receptor Editing. As receptor editing may occur frequently during normal B cell development and is accompanied by increased λ usage (10, 12, 40), we tested whether the reduced λ expression in Btk-deficient B cells results from the inability of these cells to perform receptor editing. Therefore, Btk-deficient mice were crossed with 3-83μδ transgenic mice bearing rearranged H and L chain genes encoding an antibody that specifically recognizes MHC class I H-2K^{k,b} (10). On a nondeleting H-2K^d background, the 3-83μδ mice contain a virtually monoclonal B cell population bearing the 3-83μδ BCR, as identified by the antiidiotypic antibody 54.1 (Fig. 4). In contrast, centrally deleting 3-83μδ/H2-K^b mice exhibit the phenotype of central B cell tolerance in which idiotype-positive B cells are present in the bone marrow (Fig. 4 A), but are efficiently deleted from the spleen and lymph nodes (10). Only small numbers of B cells are present in the spleen and lymph nodes and most of these lack the autoreactive specificity (>94% and >99%, respectively; Fig. 4, B and C). These B cells, which have performed receptor editing, express the transgenic H chain together with endogenous L chain, a large fraction (~50%) of which is λ (10; Fig. 4, B and C). Btk-deficient 3-83μδ/H2-K^b mice manifested similar B cell numbers in the spleen and lymph nodes, when compared with Btk$^+$ littermates. Deletion of autoreactive 3-83μδ–expressing B cells occurred in the absence of Btk, but was less efficient in the spleen than in lymph nodes (~70 and ~97% of B cells were 54.1$^-$, respectively; Fig. 4, B and C). In the Btk-deficient 3-83μδ/H2-K^b mice, significant numbers of idiotype-negative B cells were present, 25–40% of which expressed Ig λ L chain. Therefore, we conclude that receptor editing can occur in Btk-deficient autoreactive immature B cells.

Reduced λ Usage in Btk-deficient B Cells Generated in Bone Marrow Cultures In Vitro. To further investigate the relation between Btk signaling and λ chain rearrangement, we

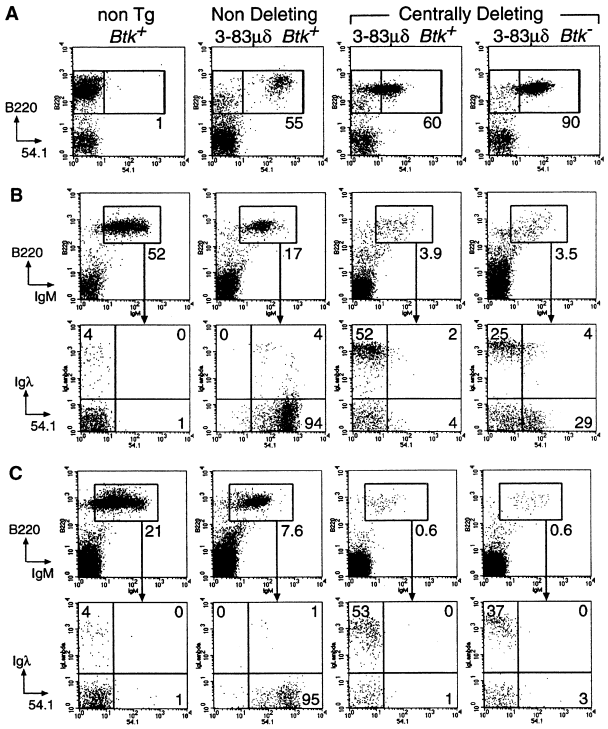

Figure 4. *Analysis of receptor editing in 3-83μδ transgenic Btk⁺ and Btk⁻ mice. Single cell suspensions were stained with mAbs specific for B220, IgM, Ig λ, as well as the clonotype-specific rat mAb 54.1 that detects idiotype-positive B cells in 3-83μδ transgenic mice. The Btk dependence of receptor editing was analyzed in Btk⁺ and Btk⁻ centrally deleting 3-83μδ transgenic mice on an H-2K^d/H-2K^b F_1 background. As controls, nondeleting 3-83μδ transgenic mice (H-2K^d) and nontransgenic (H-2K^b) Btk⁺ mice are shown. (A) Presence of 54.1⁺ B cells in the bone marrow of the indicated mice. In the spleen (B) and mesenteric lymph node (C), the B cell population was gated (numbers indicate the percentages of B220⁺IgM⁺ cells from total lymphoid cells) and analyzed for the expression of λ and the 54.1 idiotype. The numbers in the quadrants indicate the percentages of B220⁺IgM⁺ cells that are λ⁺54.1⁻, λ⁺54.1⁺, and λ⁻54.1⁺. Data shown are representative of the mice examined.*

performed IL-7–driven bone marrow culture experiments as described previously (7, 41). When surface IgM⁻ bone marrow cell suspensions from WT or Btk-deficient mice were cultured for 5 d in the presence of IL-7, the majority of cells consisted of B220⁺IgM⁻ pre-B cells that expressed μ H chain in their cytoplasm. In these cultures, a considerable fraction of the WT B220⁺ cells (~15%) matured to surface IgM⁺ B cells, while <5% of Btk-deficient B220⁺ cells were surface IgM⁺, suggesting that the progression from pre-B cell to sIg⁺ B cell is hampered in Btk-deficient mice. When cells were subsequently cultured without IL-7 on S17 stroma cells for 48 h, allowing the cells to exit from the cell cycle and further differentiate (36), significant fractions of the WT (~40%) and Btk-deficient (~30%) B220⁺ cells were surface IgM⁺. In these cultures, in vitro–generated Btk-deficient surface IgM⁺ B cells showed significantly reduced usage of λ L chains, compared with WT B cells (Fig. 5 A).

Reduced Cytoplasmic L Chain Expression in Btk-deficient Pre-B Cells. About one fifth of all pre-B cells have been reported to express μ H chain and L chains in their cytoplasm without depositing IgM molecules on their surface (3). In the bone marrow of Btk⁺ and Btk⁻ mice, we determined the proportions of sIgM⁻ cells that express κ and λ L chain in their cytoplasm. In four-color flow cytometry experiments, intracellular staining for H or L chain expression was combined with surface labeling to define the IgM⁻IgD⁻B220⁺ pre-B cells. In Btk⁺ and Btk⁻ mice, the proportions of sIgM⁻ B cells that expressed cytoplasmic μ H chain were similar. By contrast, the percentage of sIgM⁻ B cells that were positive for κ or λ L chains in their cytoplasm was significantly reduced in Btk-deficient mice, compared with WT mice (Fig. 5 B). The absence of Btk had a much stronger effect on λ expression, resulting in decreased frequencies of λ usage in Btk-deficient sIgM⁻ cells (1.9 compared with 5.8% in WT mice).

Transient Expression of Btk^{E41K} in Pre-B Cells Increases λ Usage. To confirm that the *Btk*^{E41K} mutant exerts its role on λ L chain usage at the pre-B cell stage, we generated transgenic mice in which *Btk*^{E41K} expression was driven by the λ5 promoter and the 3' λ5-$V_{pre\text{-}B1}$ locus control region. As shown in Fig. 6 A, the transgene was selectively expressed in early CD43⁺B220⁺ B

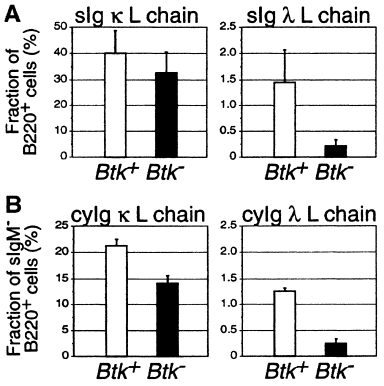

Figure 5. *Decreased λ L chain usage in in vitro–generated Btk⁻ B cells and in Btk⁻ pre-B cells in vivo. (A) Fraction of B220⁺ cells that express κ and λ L chain on the cell surface in bone marrow cultures from Btk⁺ and Btk⁻ mice (n = 10). Cells were grown in IL-7 for 5 d and subsequently recultured on S17 stroma in the absence of IL-7 for 48 h. Cells were stained with mAbs specific for B220, IgM, IgD, and either κ or λ. (B) Fraction of sIgM⁻ sIgD⁻B220⁺ cells that express L chains in their cytoplasm in the bone marrow of Btk⁺ and Btk⁻ mice (n = 8).*

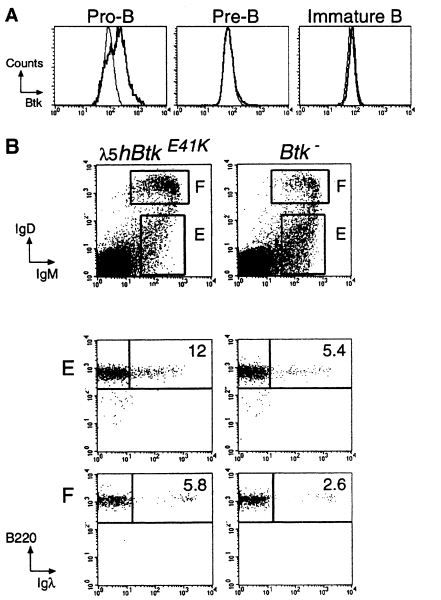

Figure 6. *Increase of λ usage by transient Btk^{E41K} expression in λ5-hBtk^{E41K} mice. (A) Intracellular Btk expression in B cells from λ5-hBtk^{E41K} transgenic mice on a Btk⁻ background. Btk expression profiles are displayed as histograms for CD43⁺B220⁺IgM⁻ pro-B cells, CD43⁻ B220⁺IgM⁻ pre-B cells, and B220⁺IgM⁺IgD⁻ immature B cells of λ5-hBtk^{E41K} transgenic mice (black lines), together with those of Btk⁻ mice, which served as background stainings (thin lines). (B) Four-color flow cytometric analyses of bone marrow of the indicated mice. On the basis of IgM and IgD expression (top), the immature IgM⁺IgD$^{-/low}$ B cell population (fraction E+E$_T$; see legend to Fig.1) and the IgM⁺IgD⁺ recirculating B cell population (fraction F) were gated and analyzed for B220 and λ usage (bottom). Data shown are representative of the mice examined.*

cell precursors. In contrast to the CD19-*hBtk*^{E41K} transgenic mice, the λ5-*hBtk*^{E41K} mice did not manifest an arrest of B cell development, as the sizes of the B cell subpopulations in bone marrow and peripheral organs were in the normal ranges (Fig. 6, and data not shown). When λ5-*hBtk*^{E41K} transgenic and nontransgenic mice on a Btk⁻ background were compared, expression of the transgene was found to increase λ usage. The frequencies of λ⁺ cells in the IgM⁺IgD$^{-/low}$ immature B cell population were 4.9 ± 0.3 in Btk⁻ mice (*n* = 4) and 8.9 ± 0.7 in λ5-*hBtk*^{E41K} transgenic Btk⁻ mice (*n* = 4; Fig.6 B). This finding showed that transient expression of Btk^{E41K} in CD43⁺ pro- and pre-B cells increased the frequency of λ usage.

Discussion

In this report we show that the absence of Btk during murine B cell development results in a 50% reduction of the proportion of Ig λ L chain–expressing cells. Conversely, expression of the constitutively activated Btk^{E41K} mutant significantly promotes λ usage. The observed λ expression profiles of Btk-deficient, CD19*hBtk*^{E41K}, and λ5-*hBtk*^{E41K} mice during B cell development show that the effect of Btk signaling is at the level of the Ig L chain rearrangement events in pre-B cells, and not a result of antigen-dependent selection processes in the periphery. Moreover, the comparison of Btk⁺ and Btk⁻ B cells in the spleen of heterozygous females shows that reduced λ usage is an intrinsic feature of Btk-deficient B cells and not associated with the immunodeficient status of Btk-deficient mice. We conclude that the regulation of V_λ-J_λ recombination events in the bone marrow is partially dependent on Btk signal transduction.

It has been shown that the κ to λ ratio of 95:5 in murine cells reflects a developmental hit-and-run program of B cells in which κ gene rearrangements precede λ rearrangements, whereby λ usage is also dependent on the pre-B cell life span and the extent of receptor editing in immature B cells (4-6, 37).

The finding of increased λ L chain usage in Bcl-2 transgenic animals indicates that the extent of secondary rearrangement events in pre-B cells with a nonfunctional L chain rearrangement is dependent on their life span (37). Although Btk is involved in the survival of mature peripheral B cells (20, 42), a role for Btk in the survival of pre-B cells in the mouse is not very likely. The absolute numbers of pre-B cells that are generated in the bone marrow of Btk-deficient mice are normal, and Btk-deficient B cell precursors in the bone marrow have the same kinetics of turnover (20, 26, 27, 43). Moreover, our analyses in Btk-deficient and Btk^{E41K} mice on an E_{μ}-*Bcl-2* transgenic background show that protection of pre-B cells from apoptosis by the expression of the Bcl-2 transgene did not alter the Btk dependence of the frequency of λ usage (Figs. 2 and 3). Therefore we conclude that the influence of Btk on λ usage is not at the level of the regulation of the time window for Ig L chain rearrangements in pre-B cells.

As our findings argue against a role of Btk in pre-B cell survival, the relationship between Btk activity and λ usage would point at a role for Btk either in the regulation of the initiation of gene rearrangement at the λ L chain locus or in the induction of receptor editing. As comparable numbers of 54.1 idiotype-negative B cells were present in Btk^+ and Btk^- centrally deleting 3-83μδ autoantibody transgenic mice, we conclude that Btk signaling is not essential for receptor editing. Nevertheless, a 50% reduction of λ usage was found in the spleen and lymph nodes of Btk-deficient 3-83μδ transgenic mice (Fig. 4). This finding may indicate that in Btk-deficient mice the mechanism of receptor editing is intact, but λ usage is decreased due to impaired initiation of λ L chain rearrangement, or alternatively that the overall level of Ig L chain replacement is reduced. Recent experiments indicated that receptor editing represents a major force in shaping the antibody repertoire in the mouse, as it was found that ~25% of all Ig L chains are produced by gene replacement (12). Whether all of these replacements are induced by self-reactive or nonpairing receptors is currently unknown. Thus, it is possible that the 15–20% of all small pre-B cells that have been reported to express Ig L chains in their cytoplasm (44) may have deposited IgM on the membrane, but sIgM expression has subsequently been downregulated due to binding of an autoantigen. Such cells would only be present in the bone marrow for a very short time, as receptor editing has been estimated to take only 2 h (12). In this model, the reduced cytoplasmic λ L chain expression observed in Btk-deficient pre-B cells may reflect reduced receptor editing in the absence of Btk signaling.

Alternatively, the pre-B cells that express μ H chain and L chains in their cytoplasm, but not on their surface, may produce Ig L chains that are not capable of pairing with the μ H chain in that cell (6). On the basis of these findings, it could be assumed that the κ to λ ratio in cytoplasmic L chain–positive pre-B cells reflects the intrinsic probabilities for productive rearrangements on the κ and λ loci. In this alternative model, the observed significant reduction of the frequencies of cytoplasmic λ L chain expressing pre-B cells would point at an intrinsic defect in λ gene rearrangement in Btk-deficient cells. Such a role for Btk in the initiation of λ gene rearrangement would, together with the observation of hampered in vitro progression of Btk-deficient pre-B cells into surface IgM^+ B cells in IL-7–driven bone marrow cultures, implicate Btk in pre-BCR signaling in the mouse. In this context, impaired L chain rearrangement of Btk-deficient cells would be consistent with their selective disadvantage at the transition from small pre-B to immature B cell, as we previously observed in an in vivo competition assay (27). Obviously, in this model Btk signaling could mediate developmental progression to a stage of B cell development in which λ L chain rearrangements are initiated, or alternatively Btk signaling could directly control the rate or efficiency of L chain rearrangement. The observed increase in coding and signal broken DNA ends at multiple gene segments in the Ig κ locus across the pro-B to pre-B cell transition supported the hypothesis that the role of pre-BCR signaling is not limited to expanding the population undergoing L chain gene rearrangement, but actually increases the activity of V(D)J recombination at the Ig κ locus (45). During the rounds of DNA replication that follow pre-BCR expression, pre-BCR signaling could then, e.g., induce alterations of the chromatin structure of the L chain loci to provide accessibility to the V(D)J recombinase system (1, 46). As Btk does not appear to influence the size of the pre-B cell population or its kinetics of turnover in the mouse (20, 26, 27, 43), we would propose that Btk is most likely involved in the activation of the L chain loci, in particular the λ locus, for recombination. In this context, the differential effect of Btk signaling on the κ and

λ loci would reflect a different regulation of the activation of the two loci for recombination. This is to be expected, as the loci become accessible for recombination at different stages of B cell development, i.e., large cycling pre-B cells for the κ locus and small quiescent pre-B cells for the λ locus (5).

A role for Btk in the initiation of λ L chain rearrangement rather than receptor editing would be supported by the finding of increased Ig λ usage, when the constitutively activated Btk^{E41K} mutant was selectively expressed in early $CD43^+$ pro- and large pre-B cells, but not in $CD43^-$ small pre-B cells (Fig. 6). Normally, both the initiation of λ L chain rearrangement and receptor editing take place at the small pre-B cell stage (5, 12) where the Btk^{E41K} mutant is no longer expressed in the λ5-*hBtk*^{E41K} mice. Therefore, the increased Ig λ usage in λ5-*hBtk*^{E41K} mice may reflect premature initiation of $V_λ$-$J_λ$ recombination. However, as our findings do not provide direct evidence for the presence of a Btk-mediated signaling pathway controlling the initiation of L chain rearrangements, further experiments are required to directly demonstrate whether Btk signaling activates the λ locus for recombination, e.g., by changing its accessibility for the V(D)J recombinase.

In summary, the observed correlation between Btk activity and the frequency of λ usage indicates a role for Btk-mediated signaling in the activation of Ig λ L chain locus for V(D)J recombination. As a result, Btk signaling contributes significantly to the mechanism that sets the κ/λ isotype ratio in the mouse. Although we have shown that Btk is not essential for receptor editing in the 3-83μδ autoreactive model, it remains possible that the absence of Btk signaling results in a decrease in the extent of L chain replacement events, and thereby in decreased λ usage in Btk-deficient B cells. Alternatively, Btk could be involved in the activation of the λ L chain locus for recombination. A role for Btk in the initiation of L chain rearrangements may also explain the developmental arrest in those XLA patients with normal pre-B cell numbers that nevertheless show impaired maturation to surface Ig^+ B cells (23).

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Chapter 1.2

Evidence for the involvement of Bruton's tyrosine kinase in the regulation of V(D)J recombinase activity at the Ig L chain loci

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Work in progress

Evidence for the involvement of Bruton's tyrosine kinase in the regulation of V(D)J recombinase activity at the Ig L chain loci

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Abstract

Bruton's tyrosine kinase is important for the developmental progression of large cycling into small resting pre-B cells, whereby it limits proliferative expansion and regulates the modulation of cell surface marker expression. As in Btk-deficient mice the appearance of κ or λ^+ immature B cells is delayed and λ usage is reduced, we investigated whether Btk signalling is involved in the regulation of V(D)J recombination activity at the L chain loci in pre-B cells. We compared the frequency of L chain-specific signal broken ends (SBE), produced in the process of V(D)J recombination, in Btk^+ and Btk^- mice. Whereas in wild-type mice the frequency of κ SBE was strongly increased in $CD2^+$ pre-B cells, in Btk-deficient mice the κ SBE were equally present in $CD2^-$ and $CD2^+$ pre-B cells. This pattern is consistent with the previously observed delay in surface CD2 upregulation and suggests that in the absence of Btk the V(D)J recombinase activity at the κ locus is slightly reduced. In contrast, λ-specific SBE were low in all Btk-deficient (pre-)B cell populations analysed. We conclude that Btk is involved in the regulation of L chain locus-specific V(D)J recombinase activity, particularly in the induction of λ L chain locus in pre-B cells.

Introduction

The immunoglobulin heavy (H) and light (L) chains are assembled from the variable (V), diversity (D) and joining (J) gene segments through a process called V(D)J recombination during the early stages of B cell development (1, 2). V(D)J recombination proceeds in a tightly regulated and sequential manner, starting with DNA rearrangements at the H chain in pro-B cells. Productive H chain rearrangement is monitored by expression of the pre-BCR complex, which consists of the μ H chain and the λ5 and V_{preB} surrogate L chains (SLC) associated with the Ig-α/Ig-β heterodimer (3, 4). Expression of the pre-BCR induces allelic exclusion and proliferative expansion, which marks the progression of pro-B cells into large cycling pre-B cells (5, 6). Subsequently, the proliferation stops and the cells differentiate into small pre-B cells in which Ig L chain rearrangement takes place. There are two isotypes of L chains, κ and λ, and L chain rearrangement involves the recombination of V and J gene segments. The κ L chain locus contains ~80 functional Vκ gene segments and four Jκ elements, while the λ locus consists of only three functional Vλ and three Jλ gene segments. Rearrangements of the κ allele are thought to occur earlier than λ rearrangements based on (a) the earlier appearance of κ^+ than λ^+ immature B cells in kinetic studies using BrdU labelling, (b) the observation that $Ig\kappa^+$ cells mostly retain their λ locus in germline configuration and $Ig\lambda^+$ cells carry unproductive κ rearrangements or have the κ loci deleted (8), and (c) the finding that germ-line κ transcription precedes germ-line λ transcription (9).

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Abbreviations used in this paper: BCR, B cell receptor; BM, bone marrow; Btk, Bruton's tyrosine kinase; LM-PCR: ligation-mediated PCR.

Primary rearrangements mostly involve Jκ1, which is most proximal to the Vκ segments (8). However, productive VκJκ rearrangement does not inhibit secondary rearrangements at the rearranged locus or further rearrangement at the unrearranged κ allele. The secondary rearrangements, which involve upstream Vκ and downstream Jκ gene segments, lead to the deletion of previously rearranged VκJκ (10, 11) and are thought to play a role in autoantigen-directed changes in BCR specificity during receptor editing (12-14).

The timing and efficiency of rearrangement of the Ig loci must be determined by the activation of the proteins involved in the V(D)J recombination process (15, 16). The pre-BCR checkpoint is thought to regulate expression of the recombination activating genes RAG-1 and RAG-2 and opening the L chain loci makes them accessible for RAG and transcription factors. Germline transcripts of the κ locus are first detected in large cycling CD25$^+$ pre-B cells, confirming a role for pre-BCR signalling in the opening of the L chain loci for recombination (9, 17, 18). One of the molecules involved in pre-BCR signalling is the Tec family nonreceptor protein Bruton's tyrosine kinase (Btk) (19).

Mutations in Btk lead to X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (xid) in mice (20-23). XLA is characterized by an almost complete arrest in B cell development at the pre-B cell level. (24, 25), resulting in very low peripheral B cell numbers and low serum Ig levels of all classes (25). Btk-deficient mice show an impaired transition from pre-B into immature B cells (26) and have a more profound defect in the survival of peripheral B cells. In Btk-deficient mice, peripheral B cell numbers are reduced by ~50% and IgM and IgG3 levels in serum are low (20, 27).

Interestingly, both in XLA patients and xid mice, deficiency in Btk results in a reduced frequency of Ig λ L chain usage already in the immature B cells in the BM (22, 28). The decrease in λ usage could either result from a defect in receptor editing or from a decreased rate or efficiency of λ L chain rearrangement. By crossing 3-83μδ autoantibody transgenic mice with Btk$^-$ mice, we have shown that Btk is not essential for receptor editing. Furthermore, BrdU-labelling studies revealed a delay of ~3h at the small pre-B cell stage in Btk-deficient mice supporting a role for Btk in the regulation of the initiation of L chain rearrangement events (29). However, it is unknown whether Btk signalling directly regulates the activation of L chain loci or whether Btk acts indirectly, e.g. by mediating developmental progression into the small pre-B cell stage in which L chain rearrangement is initiated, by regulating the survival of pre-B cells or by regulating the extent of receptor editing.

In this study we addressed the question whether Btk is involved in the regulation of the V(D)J recombinase activity at the Ig L chain loci. Therefore, we performed ligation-mediated polymerase chain reactions (LM-PCR) to detect signal broken ends (SBE) at specific Jκ and Jλ gene segments that are formed during primary as well as secondary rearrangements.

Materials and Methods

Mice and genotyping. Btk-deficient mice (26) were crossed onto the C57BL/6 background for >8 generations. Endogenous Btk WT and Btk knockout alleles (30) were identified as described previously.

Flow cytometric analysis. Preparations of single-cell suspensions, standard and intracellular flow cytometry and all mAbs used have been described previously (26, 28, 29).

MACS column purification. IL-7 driven BM cultures were performed as described before (28, 29). In short, BM cell suspensions were cultured for 5 days in the presence of 100U/ml IL-7 (R&D systems) and subsequently recultured for two consecutive days without IL-7. Cultured cells were harvested and labelled with biotinylated Igκ (187.1) and Igλ (R26-46) antibodies (BD Phamingen). Incubation with streptavidin-coated microbeads (Miltenyi Biotec) enabled separation by MACS column purification and purity was confirmed by flow cytometry.

Cell sorting. BM cell suspensions from Btk$^+$ and Btk$^-$ mice from 4-5 mice per group were pooled and purified for B-lineage cells by incubation with anti-B220-coated magnetic microbeads and subsequent MACS column purification. The B220$^+$ cell fractions were labelled with antibodies to CD19, IgM and CD2 and were either stored at 4ºC for sorting the next day or the labelled cells were fixed with 0.25% PFA on ice for 60 min. Fixed cells were then washed and kept at 4ºC overnight. The next day, fixed cells were permeabilized twice by incubation at 37ºC for 15 min with 0.2%Tween-20/PBS and incubated with polyclonal μ H antibody in permeabilization buffer. Cells were sorted using a FACS Vantage Cell Sorter (BD Pharmingen) and purity was confirmed

by flow cytometry.

Ligation-mediated PCR. All purified cell fractions were snap frozen before DNA isolation by use of a mammalian DNA purification kit (Sigma). Isolated DNA was precipitated to a concentration of 0.2μg/μl and 2μg was linkerligated with a BW-1 linker (GCGGTGACCCGGGAGATCTG AATTC) for >16h at 16ºC using T4 DNA ligase (Promega). LM-PCRs were performed by two consecutive rounds of PCR, the first containing a locus-specific outside primer and the second a locus-specific inside primer. Both rounds of PCR contained the linker-specific primer BW-H (CCGGGAGATCTGAATTCCAC). Second round PCR products were run out on a 1.4% agarose gel for 90 min at 150V before blotting under alkaline conditions to a nitrocellulose membrane. Blots were hybridised with γ-AT^{32}P end-labelled locus-specific internal oligonucleotides and analysed using a PhosphorImager.

The following primer sets were used for PCR and hybridisation: Jκ1: inside κ0^5 (GCCCAAG CGCTTCCACGCATGCTTGGAG), outside and hybridisation probe κ0^3 (TCCACGCATGCTT GGAGAGGGGGTT); Jκ2: outside κ0^3, inside Jκ910F (GGGAATAGGCTAGACATGTTCTC), hybridisation probe Jκ1-2 (GTGTCCCTTCAC TCAACCCCCATAC); Jκ5: outside Jκ1794F (ACTTGTGACGTTTTGGTTCTG), inside Jκ1847F (GCCATTCCTGGCAACCTGTGCA TCA) and hybridisation probe Jκ2000F (TAG TTGGACTGGCTTCACAGGCA); Jλ2 and 3: outside Jλ2-3A (TCAAGTGAGGTCAYAGCT CCACCCA), inside Jλ2-3B (ACCCATTGTAGC TAGCTAGTAGTTTGA) and hybridisation probe Jλ2-3C (CAGCTGTGAGAGAACAGGMCCAG). For the analysis of secondary rearrangements, the first round PCR was performed with the outside probe VκS (CCGAATCGSTTCAGTGG CAGTGGRTCWGGRAC) and the inside probes for Jκ2 (Jκ910F), Jκ4 (Jκ1474F: GGTCCCAT TGTGTCCTTTGTATGAGTTTGTGG) and Jκ5 (Jκ1847F). The accompanying hybridisation probes were Jκ1-2, Jκ1568F (TCTAGCTACT GTACAAGCTGAGCAAACAGAC) and Jκ2000F, respectively (31-34).

Linker-ligated DNA samples were amplified with specific forward (TTACGTCCATCGTGGACAGC) and reverse (TGGGCTGGGTGTTAGTCTTA) primers for the Myogenin (Myo) gene as a control for DNA concentration.

Results and discussion

LM-PCR assay to analyse L chain specific SBE. The initial step in V(D)J recombination involves the precise cleavage between a coding gene segment and its flanking RSS (35, 36). The level of these broken-ended DNA intermediates, which are detected by means of a LM-PCR assay, can be used as a measure of V(D)J recombinase activity at a specific Ig locus (Fig. 1A; (33, 34, 37, 38)). The LM-PCR technique also allows for the detection of secondary rearrangements when the V?S outside primer, which is designed to recognize any of the Vκ elements used during earlier rearrangements, is employed. This Vκ element is then in close proximity of the remaining Jκ elements and when the RSS of a downstream Jκ element is used for secondary rearrangements, PCR products are formed (Fig 1B).

Analysis of κ L chain specific SBE in cultured pre-B cells. The role of Btk in the initiation of L chain rearrangement was investigated in IL-7 driven BM cultures. In these cultures (29, 39), pre-B cells that express a productive μ H chain undergo rapid proliferation in response to IL-7 and after 5 days, the majority of cells are B220$^+$IgM$^-$ pre-B cells. When cells are subsequently cultured without IL-7, they exit the cell cycle, initiate L chain rearrangement and further differentiate into Ig$^+$ B cells (39). We have previously shown that Btk has an inhibitory effect on the proliferation of pre-B cells as Btk-deficient cells manifest increased IL-7 driven proliferation and reduced progression into Ig$^+$ immature B cells (29).

Total BM cells from Btk$^+$ and Btk$^-$ mice were cultured for 5 days in the presence of IL-7, recultured for 2 subsequent days without IL-7 in order to induce L chain rearrangement and IgM/IgD profiles were determined by flow cytometry (Fig. 2A). In agreement with previous results (29), in both Btk$^+$ and Btk$^-$ cultures ~30% expressed Igs on their surface, but the expression of IgD was significantly reduced in Btk-deficient cultures (12%, compared with 23% in Btk$^+$ cultures). We separated Ig$^-$ pre-B cells from Ig$^+$ immature B cells by MACS sorting, using antibodies specific for κ and λ L chains (Fig 2A). Equal amounts of DNA from the sorted populations (which were >90% pure) were subjected to ligation with a double stranded BW linker. Linker-ligated DNA was used for amplification by PCR using a linker-specific primer and a pair of nested locus-specific primers (34). Control amplification of the non-rearranging Myogenin (Myo) gene confirmed that all samples contained comparable amounts of amplifiable

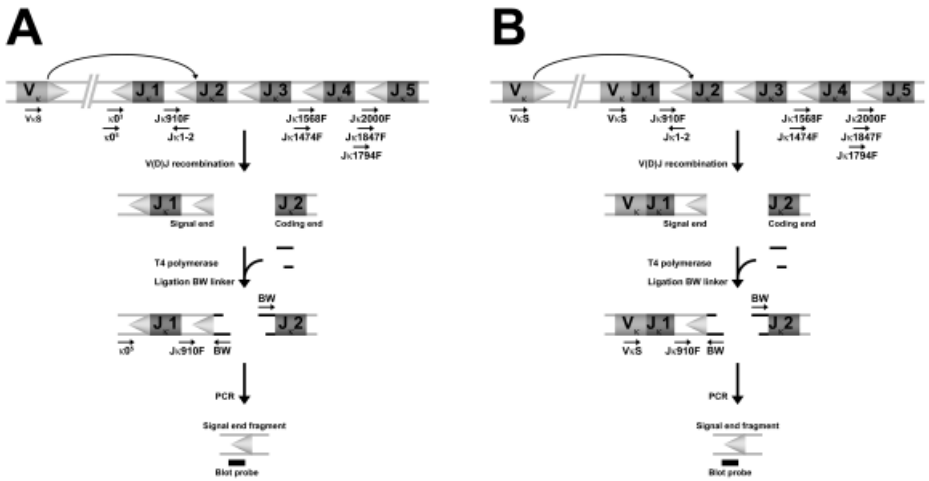

Figure 1. *Diagram of the LM-PCR assay for the detection of L chain-specific signal broken ends (SBE) produced during the process of V(D)J recombination. (A) Example of Jκ2-specific SBE detection after a primary rearrangement event. (B) Example of Jκ2-specific SBE detection after a secondary rearrangement event (VκS-Jκ2). The horizontal arrows represent the linker-specific primer (BW) and the inside and outside primers used, as described in the Materials and Methods section.*

DNA and thymus (T) DNA was used as a negative control. As the detection of signal broken ends (SBE) is not strictly quantitative, we used template dilution controls, confirming that the differences in the hybridization signals represent the differences in the initial frequency of the corresponding signal broken ends (34).

As expected, in Btk$^+$ cultures κ L chain-specific V(D)J recombination activity was present in the IgM$^-$ pre-B cell fractions, and rearrangement was reduced when cells expressed κ or λ L chain as indicated by a reduction in the hybridisation signal in IgM$^+$ B cells. In contrast, in Btk$^-$ cultures V(D)J recombination activity appeared to be slightly lower in IgM$^-$ pre-B cells and slightly higher in IgM$^+$ B cells, when compared with the corresponding B cell populations in wild-type cultures. These results suggest that Btk may play a role both in the induction and in the downregulation of the L chain rearrangement process.

Analysis of κ L chain specific SBE in sorted pre-B cell fractions. B220-purified BM cells were sorted into three separate fractions using mAbs for IgM and CD2. The populations isolated were IgM$^-$CD2$^-$ pro-and large pre-B cells, IgM$^-$CD2$^+$ small pre-B cells and IgM$^+$CD2$^+$ immature B cells (Fig 3A). We have previously reported that Btk-deficient pre-B cells are impaired in the upregulation of CD2 (29). Therefore, the absolute numbers of CD2$^-$ pre-B cells in the BM is increased, whereas the absolute number of CD2$^+$ pre-B cells is decreased. However, the total number of large cycling pre-B cells is similar in Btk$^+$ and Btk$^-$ mice, and they are all part of the CD2$^-$cμ$^+$ pre-B cell fraction. This implies that in Btk$^-$ BM the CD2$^-$cμ$^+$ fraction contains elevated numbers of small resting cells that are delayed in surface CD2 expression.

With respect to the initiation of L chain rearrangements in Btk-deficient pre-B cells, two possibilities exist. V(D)J recombination activity may be induced as soon as Btk-deficient cells progress from large cycling into small resting pre-B cells. As a result, the frequency of κ locus-specific SBE products in CD2$^-$ pre-B cells would be enhanced in Btk-deficient mice when compared with Btk$^+$ mice. Or alternatively, V(D)J recombination activity is induced only when the Btk-deficient cells have progressed to the CD2$^+$ stage and in that case, the frequency of κ-specific SBE would be highest in the CD2$^+$ B cell population.

We found that Btk$^+$ IgM$^-$CD2$^-$ pro- and large pre-B cells contained a low frequency of total κ-specific SBE, including secondary rearrangements at the κ locus (Fig. 3B). As expected, the frequency of κ-specific SBE was high in IgM$^-$CD2$^+$ small pre-B cells and was reduced upon IgM expression in

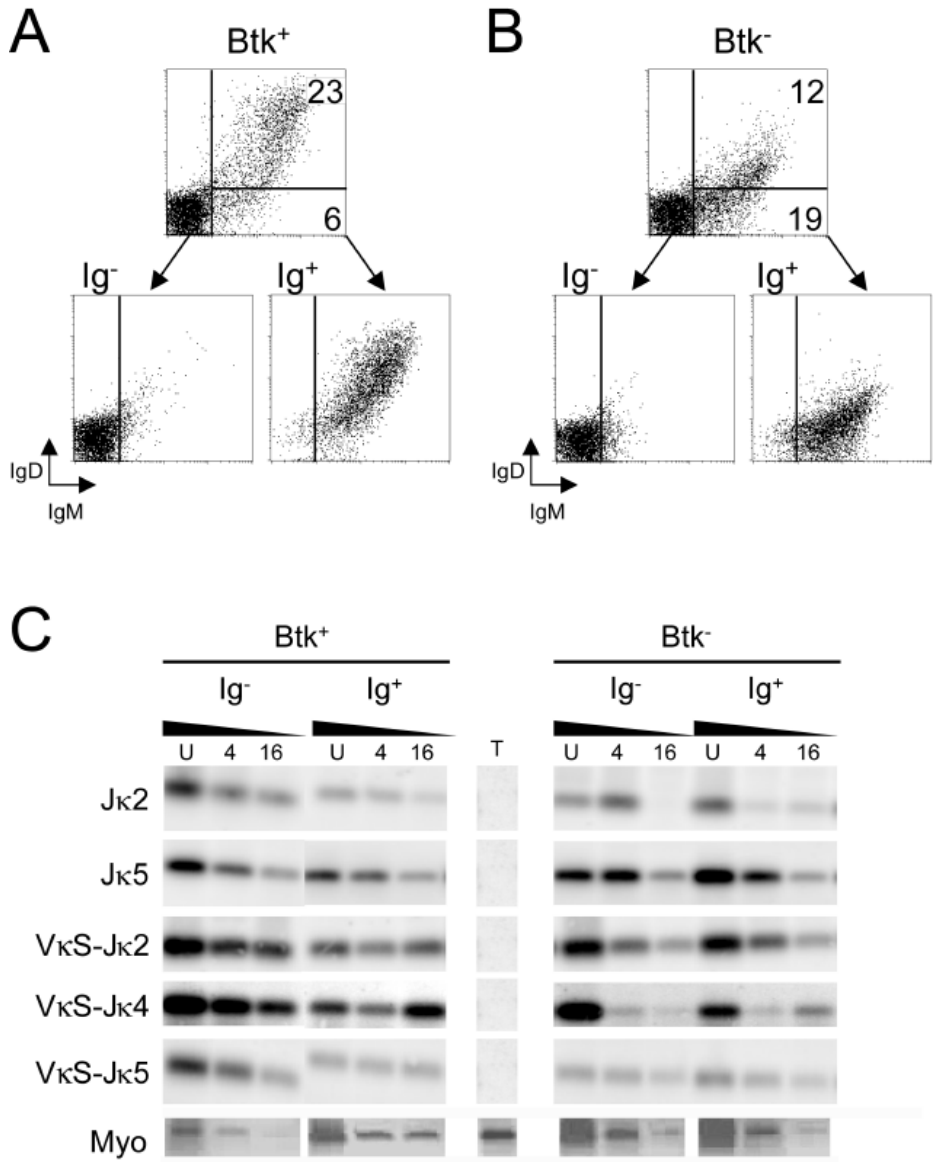

Figure 2. *Reduced κ L chain specific signal broken ends (SBE) in Btk-deficient pre-B cells. IgM/IgD profile of IL-7 driven BM cultures from Btk⁺ (A) and Btk⁻ (B) mice. BM was cultured for 5 days in the presence of IL-7 subsequently recultured for 2 days without IL-7 (upper part) and MACS-sorted using Igκ and Igλ antibodies to separate Ig⁻ pre-B cells from Ig⁺ immature B cells (lower part). Sorted fractions were >90% pure. Percentages of cells within the indicated gates are given. (C) DNA was isolated from the Ig⁻ and Ig⁺ cell fractions and ligated with BW-1 linker. Linker-ligated DNA was analysed for the frequency of specific SBE of the indicated Jκ elements. Secondary rearrangements were analysed by use of a Vκ-specific inside primer. Template dilution controls (U, 4, 16: undiluted, 4 and 16 times diluted, respectively) confirmed that differences in hybridisation signal reflected the frequency of the specific SBE. DNA from thymus (T) was used as a negative control. Data are representative for 3 mice per group.*

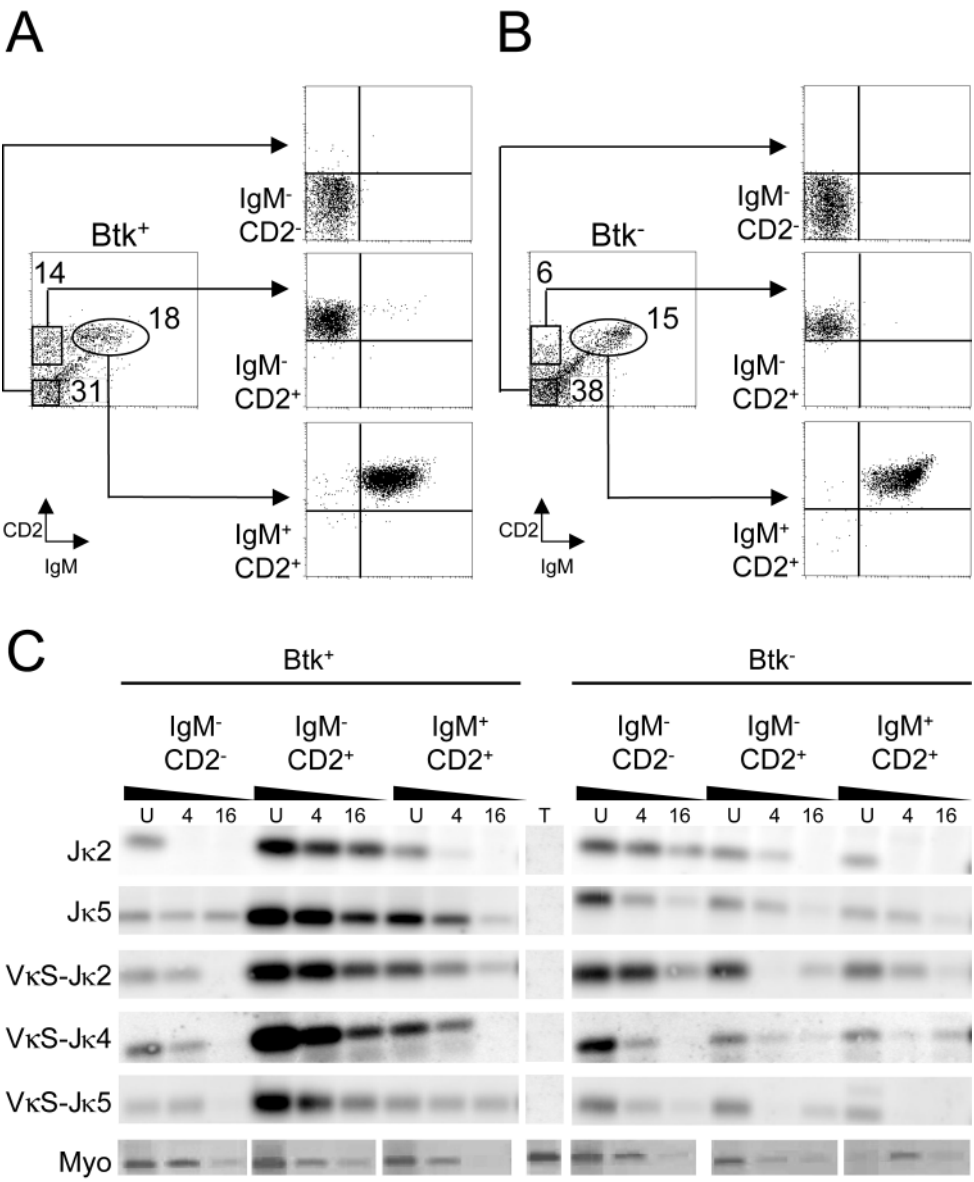

Figure 3. *Btk-deficient mice manifest an altered distribution of κ-specific SBE over B cell subpopulations in the BM. (A-B) BM cells from 5 Btk$^+$ (A) and 5 Btk$^-$ (B) mice were pooled and MACS-sorted for B220$^+$ cells. B220$^+$ cells were stained for CD19, IgM and CD2 and sorted for pro- and large pre-B cells (IgM$^-$CD2$^-$), small pre-B cells (IgM$^-$CD2$^+$) and immature B cells (IgM$^+$). Sorted fractions were >90% pure. (C) DNA was isolated from the different fractions and ligated with the BW-1 linker. Linker-ligated DNA was analysed for the frequency of specific SBE involved in the indicated Jκ rearrangements. Secondary rearrangements were analysed using a Vκ-specific inside primer. Template dilution controls (U, 4, 16: undiluted, 4 and 16 times diluted, respectively) confirmed that differences in hybridisation signal reflected the frequency of the specific SBE. DNA from thymus (T) was used as a negative control.*

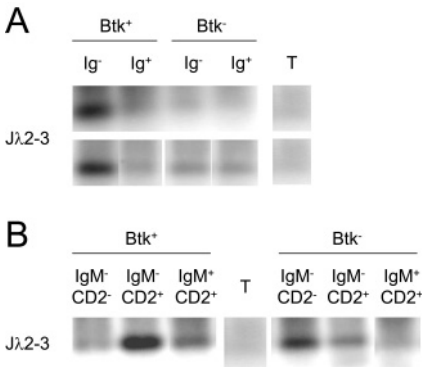

Figure 4. *Impaired λ L chain rearrangement in Btk-deficient mice (A) Linker-ligated DNA of Ig⁻ and Ig⁺ cells from Btk⁺ and Btk⁻ BM cultures (as described in Fig. 2) were analysed for the frequency of Jλ2 and 3-specific SBE products (Jλ2-3). Hybridisation signals for two mice per group are shown. (B) Linker-ligated DNA from sorted B cell fractions of Btk⁺ and Btk⁻ mice (as described in Fig. 3) were analysed for the frequency of Jλ2-3-specific SBE products. Thymus (T) DNA was used as a negative control. The Myo control amplifications are shown in Figs 2 and 3.*

immature B cells. In contrast, in Btk-deficient mice the frequency of κ-specific SBE was only slightly higher in IgM⁻CD2⁻ pre-B cells when compared with the IgM⁻CD2⁺ pre-B and IgM⁺ immature B cell fractions.

Taken together, the analyses show that there is an apparent difference between Btk⁺ and Btk⁻ mice in the distribution of κ L chain-specific SBE frequencies over the three cell populations analysed. This difference could partly be explained by the fact that the CD2⁻ pre-B cell population in Btk-deficient mice contains a high proportion of small pre-B cells that have not yet expressed CD2 on their cell surface. Therefore, we conclude that the initiation of Ig κ L chain rearrangement does not appear to be delayed in Btk-deficient mice, but is induced as soon as Btk-deficient cells progress from large cycling into small resting cells, regardless of their delayed cellular maturation into CD2⁺ pre-B cells. Nevertheless, the hybridisation signals for the κ-specific SBE in these CD2⁺ pre-B cell fractions are significantly lower in Btk⁻ than in Btk⁺ BM. As in both Btk⁺ and Btk⁻ mice, the CD2⁺ pre-B cell population consists only of small resting pre-B cells, we conclude that Btk is required for full V(D)J recombination activity at the κ locus.

Btk is required for V(D)J recombination activity at the λ L chain locus. Previous studies revealed that the expression of cytoplasmic κ and λ L chains was reduced in Btk-deficient surface IgM⁻ pre-B cells, both in vivo and in IL-7 driven BM cultures, when compared to Btk⁺ IgM⁻ pre-B cells (28). In these analyses, the frequency of cλ⁺ cells was much more decreased than the frequency of cκ⁺ cells in Btk⁻ pre-B cells. Furthermore, it was found that the frequency of λ-expressing immature B cells was reduced by ~50% in Btk-deficient mice (28).

To test if Btk signalling influences V(D)J recombination activity at the λ locus, we studied the frequency of λ L chain specific SBE in both Ig⁻ and Ig⁺ cells from the IL-7 driven BM cultures and the sorted B cell fractions as described above. Figure 4A shows the hybridisation signals of the SBE specific for the Jλ2 and 3 (Jλ2-3) gene segments, analysed in two different mice per group. Similar to our findings for κ-specific SBE, we observed a high frequency of λ-specific SBE in the surface Ig⁻ pre-B cell fraction and a low frequency in Ig⁺ B cells in Btk⁺ BM cultures 2 days after IL-7 withdrawal. In strong contrast, λ-specific SBE were hardly detectable in the Ig⁻ or Ig⁺ fractions of Btk-deficient cultured BM cells.

Analysis of the sorted large and small pre-B and immature B cell fractions showed that in the presence of Btk, recombination activity at the Jλ2-3 gene segments is high in Btk⁺ IgM⁻CD2⁺ small pre-B cells (Fig. 4B). Similar to the distribution of κ-specific SBE signals over the three cell populations analysed, as described above, we found that also the frequencies of λ-specific SBE signal in Btk-deficient mice were more prominent in CD2⁻ pre-B cells and significantly reduced in CD2⁺ pre-B cells, when compared with Btk⁺ mice.

Again, both in Btk⁺ and Btk⁻ BM the CD2⁺ pre-B cell fraction contains only small resting pre-B cells. Therefore, we conclude that in the absence of Btk V(D)J recombination at the λ L chain locus is significantly reduced. As we have previously shown that the cellular maturation defects associated with Btk-deficiency affects λ⁺ immature B cells to a lesser extent than κ⁺ immature B cells (40), the observed reduction in λ-locus specific V(D)J recombination activity cannot be explained by defective CD2 upregulation.

In summary, these LM-PCR analyses demonstrate the presence of κ and λ L chain-specific SBE already in the CD2⁻ pre-B cell fractions of Btk-deficient mice. Assuming that these SBE are present in small resting pre-B cells that were delayed in surface CD2 expression, our findings indicate that the absence of Btk does not significantly affect the kinetics of the

initiation of L chain rearrangement *in vivo*. However, the observation that the frequencies of L chain-specific SBE were generally lower in Btk-deficient $CD2^+$ small pre-B cells, when compared to Btk^+ cells $CD2^+$ small pre-B cells, indicates that Btk is involved in the regulation of the level of V(D)J recombination activity in pre-B cells. We therefore propose that Btk signalling regulates the accessibility of L chain loci, in particular of the λ locus. Further experiments are needed to find out if Btk regulates RAG activity and which DNA-binding factors required for L chain locus opening are controlled by Btk-dependent pathways.

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Chapter 2

Btk and pre-B cell differentiation

2.1 Impaired precursor B cell differentiation in Bruton's tyrosine kinase-deficient mice

2.2 Function of Bruton's tyrosine kinase during B cell development is partially independent of its catalytic activity

2.3 Expression of the constitutive active Bruton's tyrosine kinase mutant E41K mimics pre-B cell receptor signalling in pro-B cells

Chapter 2.1

Impaired precursor B cell differentiation in Bruton's tyrosine kinase-deficient mice

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Impaired precursor B cell differentiation in Bruton's tyrosine kinase-deficient mice

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Abstract

Bruton's tyrosine kinase (Btk) is a cytoplasmic signaling molecule that is crucial for precursor (pre-B) cell differentiation in humans. In this study, we show that during the transition of large cycling to small resting pre-B cells in the mouse, Btk-deficient cells failed to efficiently modulate the expression of CD43, surrogate L chain, CD2, and CD25. 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 of ~3 h, when compared with wild-type cells. Likewise, in in vitro bone marrow cultures, Btk-deficient large cycling pre-B cells showed increased IL-7 mediated expansion and reduced developmental progression into noncycling $CD2^+CD25^+$ surrogate L chain-negative small pre-B cells and subsequently into Ig-positive B cells. Furthermore, the absence of Btk resulted in increased proliferative responses to IL-7 in recombination-activating gene-1-deficient pro-B cells. These findings identify a novel role for Btk in the regulation of the differentiation stage-specific modulation of IL-7 responsiveness in pro-B and pre-B cells. Moreover, our results show that Btk is critical for an efficient transit through the small pre-B cell compartment, thereby regulating cell surface phenotype changes during the developmental progression of cytoplasmic μ H chain expressing pre-B cells into immature IgM^+ B cells.

Introduction

Bruton's tyrosine kinase (Btk) belongs to the Tec family of cytoplasmic protein tyrosine kinases and plays an essential role in B lymphocyte development and function (1, 2). In mature B cells, Btk is tyrosine phosphorylated and its kinase activity is increased upon B cell receptor (BCR) stimulation (3-5).

Mutations in the *Btk* gene lead to X-linked agammaglobulinaemia (XLA) in humans and X-linked immunodeficiency (*xid*) in the mouse (6-9). XLA is characterized by recurrent bacterial infections, and very low serum Ig levels of all classes due to the lack of plasma cells in the secondary lymphoid organs. There is a severe deficiency of peripheral B cells, and those B cells that are present have an aberrant IgM^{high} phenotype (10). In the bone marrow (BM) of XLA patients, the numbers of cytoplasmic μ H chain (cμ)-expressing pre-B cells are variable, but in most cases reduced; those pre-B cells present are mainly nonproliferating small cells (11-13). The absence of Btk apparently results in deficient expansion of the earliest cμ-expressing pre-B cells. Therefore, the XLA disease phenotype most likely reflects defective signaling through the pre-BCR, by which the expression of a functional μ H chain normally is monitored, together with surrogate L chain (SLC; Ref. 14). As most patients have substantial numbers of pro-B cells in their BM, XLA generally results in an increased ratio of pro-B to pre-B cells (11-13).

In contrast, Btk deficiency in the mouse is associated with an impairment of peripheral B cell maturation, without a major early B cell developmental block (15-17). In *xid* mice the mature $IgM^{low}IgD^{high}$ B cell populations in spleen

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Abbreviations used in this paper: Btk, Bruton's tyrosine kinase; BCR, B cell receptor; BM, bone marrow; FSC, forward scatter; WT, wild type; *xid*, X-linked immunodeficiency; XLA, X-linked agammaglobulinemia; SLC, surrogate L chain; RAG, recombination-activating gene; sIgM, surface IgM; BrdU, 5-bromo-2'deoxyuridine; cμ, cytoplasmic μ H chain; 7-AAD, 7-aminoactinomycin-D.

and lymph nodes are severely reduced, B-1 B cells are absent, and serum IgM and IgG3 levels are low. *Xid* B cells do not proliferate upon surface IgM (sIgM) stimulation. The most obvious difference between the BM B cell compartment of wild-type (WT) mice and Btk-deficient mice is the reduction in mature recirculating cells (15-17). The absolute numbers of pre-B cells that are generated in the BM of Btk-deficient mice are normal, and Btk-deficient B cell precursors in the BM have the same kinetics of turnover (18, 19). Nevertheless, several lines of evidence point at a role for Btk at the transition of pre-B cells into immature surface Ig^+ B cells, thereby indicating the involvement of Btk in pre-BCR signaling in the mouse. Using an in vivo competition assay in heterozygous $Btk^{+/-}$ female mice, we identified a small but significant selective disadvantage of Btk-deficient cells to contribute to the IgM^+IgD^- immature B cells stage in the BM (17). Our recent finding of an intrinsic reduction of Ig λ L chain usage in Btk-deficient B cells in the mouse implicated Btk in the activation of gene rearrangements at the λ L chain locus (20). Furthermore, while mice deficient for another Tec kinase family member, Tec, showed normal B cell development, an almost complete block at the $CD43^+B220^+$ stage of B cell development was observed in Btk/Tec double-deficient mice (21). These results indicated that Btk is critically involved in pre-BCR-mediated signaling in the mouse and that Tec is able to compensate for the loss of Btk during early B cell development. A role for Btk in pre-BCR signaling would be consistent with the recent finding that Btk is able to function in CD79b (Ig-β)-mediated signaling in recombination-activating gene (RAG)-2-deficient pro-B cells (22).

To examine the role of Btk at the pre-BCR checkpoint in vivo, we compared the expression of early B cell surface markers in the BM from Btk-deficient and WT control mice. In this report, we show that Btk is involved in the pre-BCR-dependent induction of cell surface phenotype changes during the progression of large cycling $c\mu^+$ pre-B cells into surface Ig^+ immature B cells, thereby affecting the transit time through the small pre-B cell compartment. In addition, we performed IL-7-driven BM cultures, which point at a role for Btk in the differentiation stage-specific modulation of IL-7 responsiveness in early murine B cell development, even in stages preceding cμ expression.

Materials and Methods

Mice and genotyping. *Btk*$^-$*/lacZ* mice (17) were crossed onto a C57BL/6 background for >6 generations. Specific experiments were additionally performed with Btk mice on a mixed C57BL/6 x 129 or C57BL/6 x FVB background, but no significant influence of the genetic background was observed. *RAG-1*$^{-/-}$ mice (23) were on a 129/Sv background. All mice were bred and maintained in the animal care facility at the Erasmus Medical Center, Rotterdam (Rotterdam, The Netherlands). To determine the Btk genotype, tail DNA was analyzed by Southern blotting of *Bam*HI digests, as described (17). The *RAG-1* genotype was determined using a 1.9-kb partial *RAG-1* probe (encoding amino acid positions 387-1008, kindly provided by D. van Gent, Erasmus Medical Center) to hybridize to *Bam*HI-*Nco*I genomic digests (23).

Flow cytometric analysis. Preparations of single-cell suspensions, flow cytometry, and determination of β-galactosidase activity by loading cells with fluorescein-di-β-D-galactopyranoside substrate (Molecular Probes Europe, Leiden, The Netherlands), have previously been described (17, 24). Events (1-3 x 10^5) were scored using a FACSCalibur flow cytometer and analyzed by CellQuest software (BD Biosciences, Mountain View, CA). The following mAbs were obtained from BD PharMingen (San Diego, CA): FITC-conjugated anti-BP-1/6C3, anti-B220/RA3-6B2, anti-κ (R5-240), and anti-IgM (II/41); PE-conjugated anti-CD2, anti-κ (187.1), anti-CD25, and anti-CD43; CyChrome-conjugated anti-B220/RA3-6B2 and biotinylated anti-IL7R, anti-κ?(187.1), anti-λ_1/λ_2(R26-46), and anti-IgM (II/41). PE-conjugated IgD was obtained from Southern Biotechnology Associates (Birmingham, AL). Biotinylated anti-SLC Ab LM34 (25) was kindly provided by A. Rolink (Basel Institute for Immunology, Basel, Switzerland). Secondary Abs were PE-, tricolor-, or APC-conjugated streptavidin, purchased from Caltag Laboratories (Burlingame, CA). For intracellular flow cytometric detection of cytoplasmic Ig H or L chain or SLC, the following Abs were applied: FITC-conjugated anti-Igκ (R5-240, BD PharMingen) or polyclonal anti-Ig μ H chain (Jackson Immunoresearch Laboratories, West Grove, PA) and biotinylated anti-λ_1/λ_2 (BD PharMingen), or anti-SLC (LM34). Cells were first stained for cell surface markers and subsequently fixed in 2% paraformaldehyde and permeabilized using 0.5% saponin.

Cell cycle analysis. To analyze cell cycle status of cultured cell suspensions, cells were incubated in ice-cold ethanol for >2 h. Subsequently, cells were incubated at room temperature for 30 min in PBS containing 0.02 mg/ml propidium iodide, 0.1% v/v Triton X-100, and 0.2 mg/ml RNase, left overnight at 4°C, and analyzed with a FACSCalibur flow cytometer. Doublet cells were excluded from the analysis by measuring peak area and width. For simultaneous analysis of cell surface markers, cμ expression, and DNA content, we used a method described by Constantinescu and Schlissel (26). Cells were sequentially stained for surface CD2 expression using PE-labeled anti-CD2, for cμ using polyclonal anti-μ H chain, and 7-aminoactinomycin-D (7-AAD, Molecular Probes Europe).

IL-7-driven BM cultures. Primary pre-B cell cultures were essentially performed as described previously (20), using erythrocyte-depleted total BM cell suspensions. For specific experiments, BM cell suspensions were depleted of sIgM$^+$ cells, using biotinylated anti-IgM and streptavidin-coated microbeads, as described (20). Recombinant murine IL-7 was from R&D Systems (Minneapolis, MN). To measure IL-7-dependent proliferative responses, cells were cultured in flat-bottom 96-well plates for 5 days with various amounts of IL-7 at different cell concentrations (0.25–1 x 10^5 cells/well). Cultures were pulsed for 24 h with 0.5 μCi/well of [^{3}H]thymidine, harvested on glass-fiber filters, and the incorporated radioactivity was determined using a beta counter, according to standard procedures.

In vivo 5-bromo-2'-deoxyuridine (BrdU) labeling. BrdU (Sigma Aldrich, St. Louis, MO) was dissolved in PBS at 2 mg/ml. Mice were injected i.p. with 200 μl, and sacrificed at various time points. Total BM cell suspensions were analyzed by flow cytometry for BrdU incorporation, using the BrdU flow kit (BD PharMingen) in conjunction with cell surface marker expression.

Results

Btk-cμ$^+$ pre-B cells show a defective surface marker expression profile. To investigate the involvement of Btk in pre-BCR signaling, we compared the pre-B cell compartment in the BM of Btk$^+$ and Btk$^-$ mice. Consistent with published findings (16, 17, 21), we did not observe a major early B cell developmental block in Btk$^-$ mice, but only a small increase in the fraction of CD43$^+$ cells within the population of sIgM$^-$B220$^+$ pro-/pre-B cells: ~20–25% in Btk$^+$ and ~50–55% in Btk$^-$ mice (Fig. 1*A*).

About one-fifth of all pre-B cells have been reported to express μ H chain and L chains in their cytoplasm, without depositing IgM on the cell surface (27). These cells may have down-regulated sIgM expression due to autoantigen binding, or they may produce Ig H and L chains that do not pair (14, 27). As previously described (20), surface Ig$^-$ B cell precursors of Btk$^-$ mice showed reduced expression of cytoplasmic L chain. Surface Ig$^-$ B cells (16 ± 3%) expressed κ L chain in the cytoplasm, compared with 23 ± 2% in Btk$^+$ mice, while the frequency of cytoplasmic λ expression pre-B cells was reduced by a factor ~3 (Fig. 1*B*). These findings suggest reduced or delayed Ig L chain rearrangement.

Signaling through the pre-BCR initiates proliferative expansion of those cells with a productively rearranged Ig H chain. Subsequently, these cells progress into small resting pre-B cells that have changed their cell surface phenotype and initiate κ and λ L chain rearrangement (14). Therefore, we analyzed IgM$^-$B220$^+$ B cell precursors for the expression of several cell surface markers in four-color flow cytometric experiments (Fig. 1*C*). In Btk$^-$ mice, the IgM$^-$B220$^+$ compartment contained a unique subpopulation of CD43^{low} cells which manifested increased expression of the BP-1 molecule, a metallopeptidase that is selectively induced coincident with IL-7-driven pre-B cell proliferation (28). As soon as a pre-BCR is expressed, the synthesis of SLC is turned off (29). However, Btk$^-$ cells had an increase in the fraction of IgM$^-$B220$^+$ cells that expressed SLC in their cytoplasm, as compared with Btk$^+$ cells (Fig. 1*C*). In a specific analysis of cμ$^+$ pre-B cells, Btk$^-$ mice showed significantly reduced expression of the IL-2R CD25 (30 ± 6%), and the CD2 adhesion molecule (32 ± 4%), as compared with Btk$^+$ mice (66 ± 7% and 70 ± 6%, respectively). These molecules were previously reported to be upregulated upon pre-BCR signaling (30, 31).

Collectively, these findings indicated that the absence of Btk resulted in a defective progression of SLC$^+$CD43$^+$CD2$^-$CD25$^-$ into SLC$^-$CD43$^-$CD2$^+$CD25$^+$cμ$^+$ pre-B cells. Analysis of the absolute numbers of the various B-lineage subpopulations in the BM (Table I) revealed that in Btk$^-$ mice the size of the total population of CD2$^-$ cμ$^+$ early pre-B cells was increased with a factor of ~2.4, whereas the total population of

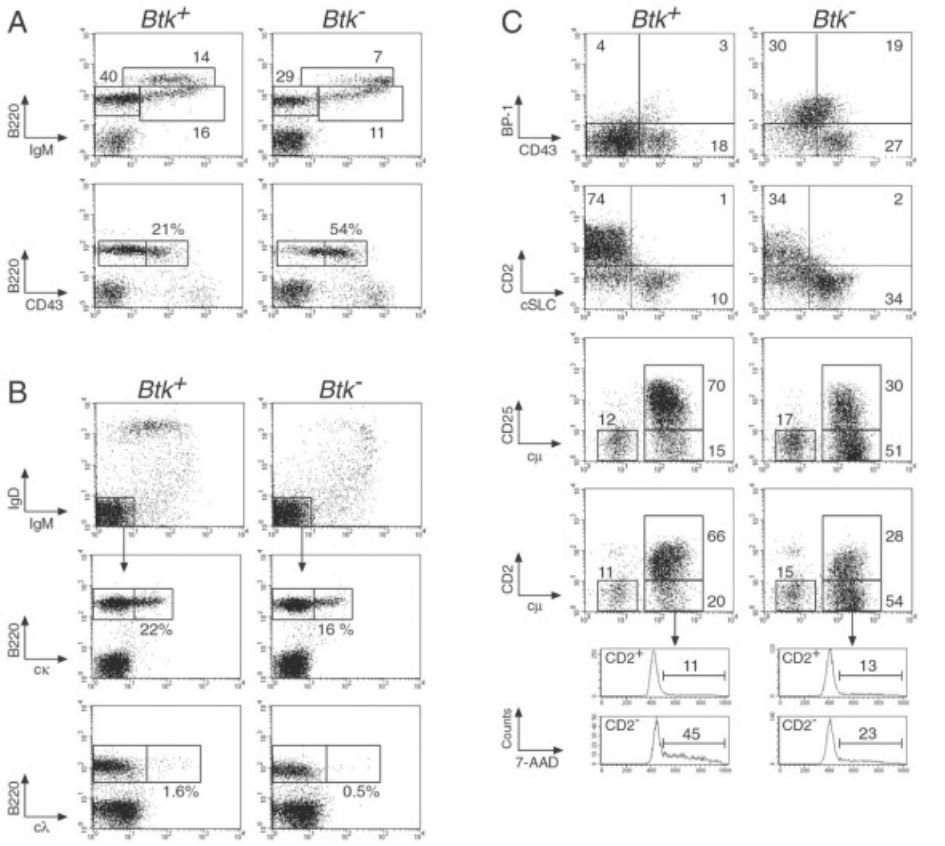

Figure 1. *Characterization of the pre-B cell compartment in Btk$^+$ and Btk$^-$ mice. A, BM cell suspensions were stained with mAbs to B220, CD43, and IgM. The expression profiles of B220 and sIgM are shown (top). sIgM$^-$ cells were analyzed for B220 and CD43 (bottom). Percentages of CD43$^+$ cells within the indicated B220$^+$ gate are given. B, BM cell suspensions were stained with mAbs to detect surface expression of IgM, IgD, B220, and cytoplasmic (c) expression of κ or λ L chain. The percentages of cκ$^+$ and cλ$^+$ cells within the IgM$^-$IgD$^-$ B220$^+$ subpopulation are given. C, Four-color flow cytometric analyses of BM cell suspensions, using mAbs to B220 and IgM, together with various additional mAbs. B220$^+$IgM$^-$ pro-B/pre-B cells were electronically gated and analyzed for the indicated markers. Cell cycle status was determined in cμ$^+$CD2$^+$ and cμ$^+$CD2$^-$ cells, using 7-AAD. The percentages of cycling cells (S, M, or G_2 phase) are indicated. Data shown are representative of three to eight mice examined.*

CD2$^+$ cμ$^+$ late pre-B cells was decreased with a similar factor.

The developmental progression to CD2$^+$ late pre-B cells normally correlates with a change in cell cycle status, from large cycling into small resting pre-B cells (26). This was confirmed by the finding in Btk$^+$ mice of ~45% of CD2$^-$ and ~10% of CD2$^+$cμ$^+$ pre-B cells in S, M, or G_2 phase, using 7-AAD staining for DNA content (Fig. 1*C*). In contrast, the CD2$^-$cμ$^+$ pre-B cell population in Btk$^-$ mice contained only ~23% of cycling cells, i.e., about half the number found in Btk$^+$ mice. However, as the size of the total population of CD2$^-$cμ$^+$ pre-B cells was increased with a factor ~2.4 in Btk$^-$ mice, we conclude that Btk$^+$ and Btk$^-$ mice contain comparable numbers of large cycling cμ$^+$ cells (~0.8 x 10^6 cells per hind leg).

In summary, the absence of Btk in pre-BCR signaling resulted in defective down-regulation of CD43 and SLC, defective up-regulation of CD2 and CD25, augmented expression of BP-1, whereas in vivo proliferative expansion of pre-B cells appeared to be unaffected.

Btk⁻ cells show a developmental delay within the small pre-B cell compartment in vivo. The inefficient induction of cell surface phenotype changes in Btk⁻ mice at the pre-B cell stage was still noticeable at the next developmental stage of IgM⁺ immature B cells. As shown by flow cytometric analysis (Fig. 2*A*), IgM⁺B220^{low} immature B cells in Btk⁻ mice manifested lower expression of CD2 (66 ± 5%, *n* = 9) and CD25 (19 ± 1%, *n* = 12), compared with immature B cells in Btk⁺ animals (CD2⁺: 90 ± 3%, *n* = 8, and CD25⁺: 36 ± 1%, *n* = 12). In addition, considerable fractions of Btk⁻ immature B cells expressed surface BP-1 (45 ± 3%, *n* = 6) or cytoplasmic SLC (13 ± 3%, *n* = 8), which were not detected in Btk⁺ immature B cells (<3%; Fig. 2*A*).

To investigate whether the inefficient induction of cell surface phenotype changes in Btk⁻ pre-B and immature B cells would be associated with a delayed developmental progression into IgM⁺ immature B cells, we estimated the transit time through the small pre-B cell compartment. Btk⁺ and Btk⁻ mice were injected with a single dose of the BrdU thymidine analog, which is specifically incorporated into the DNA of large cycling pre-B cells (32-34). BM cells were stained with an anti-BrdU Ab, in conjunction with cell surface analysis for B220, IgD, and κ or λ L chain, and analyzed by flow cytometry. Twelve hours after injection, ~30–35% of Ig⁻ B cell precursors incorporated BrdU, both in Btk⁺ and Btk⁻ mice. At various time points after BrdU injection, we analyzed BrdU incorporation within κ⁺ and λ⁺ IgD⁻ immature B cells. As shown for the 48-h time point in Fig. 2*B*, the fractions of BrdU⁺ cells within the surface κ⁺ or λ⁺ immature B cells were decreased in Btk⁻ mice when compared with Btk⁺ mice. As small pre-B cells are noncycling cells, the first appearance of BrdU⁺ cells within the surface Ig⁺ immature B cells reflects the minimum transit time through the small pre-B compartment. When for κ⁺ immature B cells, the data from six time points were subjected to linear regression analysis (33), it was found that BrdU⁺κ⁺ immature B cells emerged ~8 h after BrdU injection in Btk⁺ mice and only after ~11 h in Btk⁻ mice (Fig. 2*C*). In agreement with reported experiments (33, 34), BrdU⁺λ⁺ immature B cells only emerged ~30 h after BrdU injection. Likewise, linear regression analysis revealed a delay of ~3.5 h for Btk-deficient λ⁺ immature B cells (~32.5 h), as compared with Btk⁺ cells (~29 h).

In summary, we conclude that in Btk⁻ mice the inefficient induction of cell surface phenotype changes in μ⁺ pre-B cells was accompanied by a specific developmental delay within the small pre-B cell compartment of ~3 h. In addition, the immature B cells generated manifest an aberrant

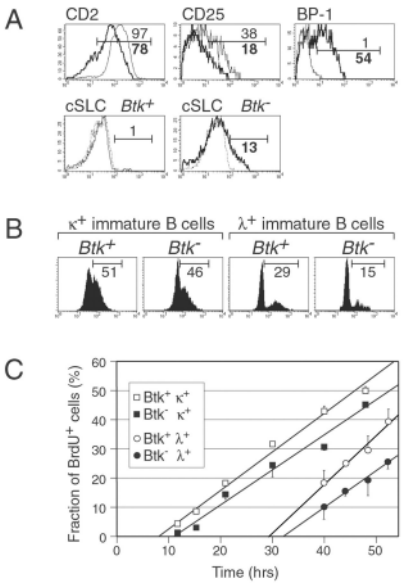

Figure 2. *Absence of Btk results in delayed developmental progression into immature B cells. A, Expression of CD2, CD25, cytoplasmic SLC (cSLC), and BP-1 on B220^{low}IgM⁺ cells in BM of Btk⁺ (thin lines) and Btk⁻ mice (bold lines). For cSLC analysis, histogram overlays of gated B220⁻ cells are shown as negative control stainings (dotted lines). The percentages of positive cells are indicated above the marker line (Btk⁺) and below the marker line in bold (Btk⁻). B, BrdU incorporation 48 h after BrdU injection in Btk⁺ and Btk⁻ mice. BM cells were stained for surface expression of B220, IgD, and κ or λ, in conjunction with BrdU. Histograms show BrdU staining in B220⁺IgD⁻ immature B cells, with the percentages of BrdU⁺ cells. C, Linear regression analysis of the appearance of BrdU⁺ κ⁺ and λ⁺ immature B cells over time in Btk⁺ (open symbols) and Btk⁻ mice (filled symbols). Each point represents BrdU staining of B220⁺IgD⁻κ⁺/λ⁺ BM cells from three to six mice per group (except for Btk⁻ mice, 30 h: n = 2). Entry points and slope values were 8 h and 1.3 (κ, Btk⁺), 11 h and 1.2 (κ, Btk⁻), 29 h and 1.7 (λ, Btk⁺), and 32.5 h and 1.3 (λ, Btk⁻), respectively. As evaluated by Student's t test, the fractions of BrdU⁺ cells were significantly lower in κ⁺ Btk⁻ cells as compared with Btk⁺ cells, with p values of <0.01 (12, 16, and 40 h), <0.02 (48 h), and 0.05 (21 h). For the 30-h time point, the number of measurements was insufficient for a statistical evaluation. For λ⁺ B cells, the p values ranged from <0.0001 to <0.01 for the four time points shown.*

surface phenotype.

Btk⁻ pre-B cells show deficient developmental progression in vitro. To further investigate the role of Btk in the developmental progression of cμ⁺ pre-B cells and the initiation of Ig L chain expression, IL-7-driven BM culture experiments were performed, as described (35, 36). Pre-B cells that have a productive Ig H chain rearrangement undergo rapid cell division in response to IL-7. When IgM⁻ BM cell suspensions from Btk⁺ mice were cultured in the presence of 100 U/ml IL-7 for 5 days, the majority of cells consisted of B220⁺IgM⁻ cells, while a significant fraction matured to sIgM⁺IgD⁻ (~10%) or IgM⁺IgD⁺ B cell stages (~5%) (Fig. 3). This differentiation into IgM⁺ cells did not result from an IL-7 insufficiency in culture, as it was also present when higher IL-7 concentrations were used. In contrast, only <5% and <0.5% of the Btk⁻ cells exhibited an IgM⁺IgD⁻ or IgM⁺IgD⁺ profile in culture, respectively. Therefore, these findings indicated that Btk signaling supported the progression from pre-B cell to surface Ig⁺ B cell in vitro in the presence of IL-7. Similar differences were observed, when cells were subsequently cultured for 48 h on S17 stroma cells in the presence of IL-7: ~30% of Btk⁺B220⁺ cells were surface Ig⁺, but only ~5% of Btk⁻B220⁺ cells (Fig. 3). After removal of IL-7, which strongly induces the cells to exit from cell cycle and to further differentiate (36), significant numbers of sIgM⁺ B cells were generated in the Btk⁻ BM cultures. Nevertheless, when compared with Btk⁺ B cells, the maturation of Btk⁻ B cells to the IgM⁺IgD⁺ stage was reduced (Fig. 3). Therefore, the analyses of IL-7-driven BM cultures showed that Btk signaling promotes the maturation of pre-B cells into surface Ig⁺ B cells in the presence of IL-7 in vitro.

Btk⁻ pre-B cells show enhanced IL-7-driven expansion in vitro. Next, we analyzed the kinetics of in vitro B cell development in IL-7-driven BM cultures, using total BM cells from Btk⁺, Btk⁻, and Btk⁺/⁻ mice, without S17 stroma. Similar results

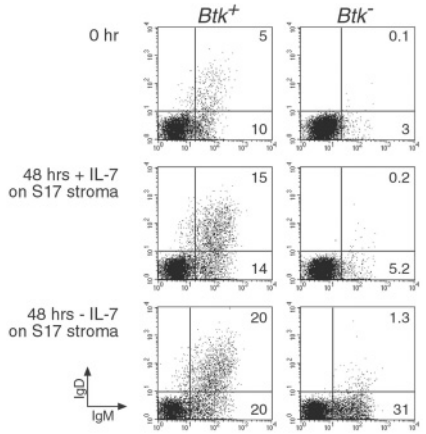

Figure 3. *Developmental progression of Btk⁺ and Btk⁻ cells in IL-7-driven BM cultures. BM cells were cultured in the presence of IL-7 for 5 days and subsequently analyzed ("0 h") or recultured on S17 stroma cells in the presence or absence of IL-7 for 48 h. Cultured cells were stained for B220, IgM, and IgD. B220⁺ cells were gated and analyzed for the expression of IgM and IgD. Data shown are representative of 10 mice examined in each group.*

were obtained in IL-7-driven BM cultures in the presence of S17 stromal cells, either throughout the 7-day culture period or from day 5 onwards (data not shown).

In these experiments, we used biotinylated Abs to detect sIgM expression, which resulted in higher fluorescence intensity values (up to 2 x 10^3), as compared with the experiments described in Fig. 3 (up to 5 x 10^2). We observed that most Btk⁻B220⁺ cells stained with the anti-μ H chain Ab after culture with IL-7 (fluorescence intensities ranging from 20 to 100; Fig. 4*A*). These cells were cμ positive, surface, or cytoplasmic κ or λ negative, and expressed SLC both in their cytoplasma and on the cell surface (see *Results*; Fig. 5). Therefore, we concluded that these cells

Table 1. *B-lineage subpopulations in Btk⁺ and Btk⁻ mice.*

Cell Population	Btk⁺ (*n* = 7)	Btk⁻ (*n* = 7)	*p* Values
Total B220⁺ cells	13.8 ± 2.19[a]	11.1 ± 2.78	NS
Total sIgM⁻B220⁺ cells	8.70 ± 1.58	7.63 ± 2.35	NS
Pro-B (cμ⁻)	1.16 ± 0.36	1.24 ± 0.50	NS
Early pre-B (cμ⁺CD2⁻)	1.56 ± 0.75	3.82 ± 1.41	0.0028
Late pre-B (cμ⁺CD2⁺)	5.63 ± 1.29	2.32 ± 0.72	<0.0001
Immature B cells (sIgM⁺sIgD⁻)	2.22 ± 0.51	2.01 ± 0.57	NS
Mature B cells (sIgM⁺sIgD⁺)	1.83 ± 0.54	0.86 ± 0.37	0.0022

[a] *Absolute number of cells (mean ± SD x 10^6) per hind leg*

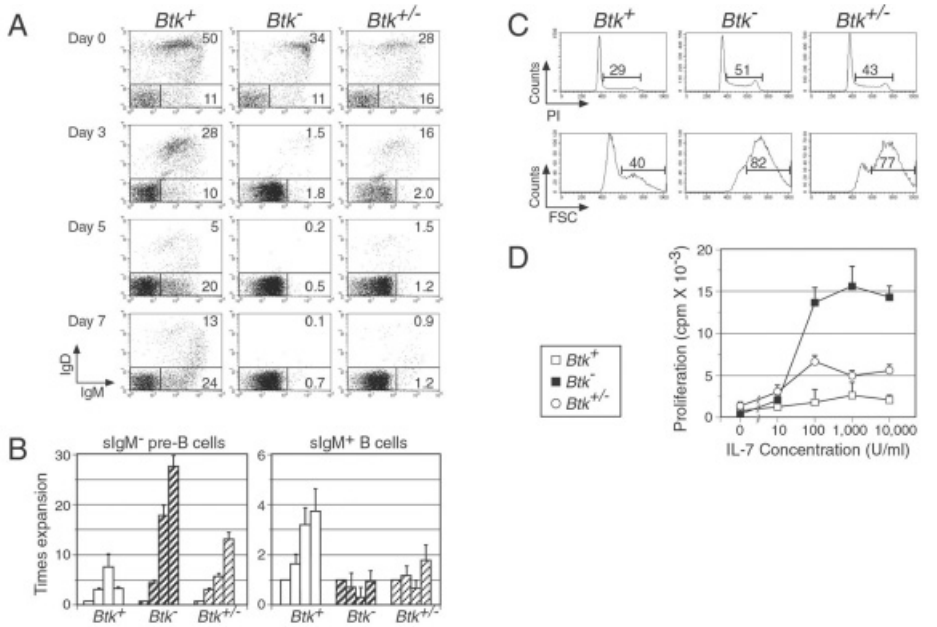

Figure 4. *Analysis of IL-7-driven BM cultures from Btk⁺, Btk⁻, and Btk⁺/⁻ heterozygous female mice. A, IgM/IgD expression profile of B220⁺ cells in uncultured BM samples, as well as after the indicated days of culture in the presence of IL-7. The gates for the pre-B cells (sIgM⁻/low), immature B cells (IgM⁺IgD⁻), and mature B cells (IgD⁺) are indicated, as well as the percentages of cells within the B cell gates. Note that the pre-B vs immature B gate settings for Btk⁺ and Btk⁻ mice at days 3, 5, and 7 are almost one log different, as they are based on differential expression of κ, λ, and SLC in parallel stainings (see Results). Dot plots are representative of 8–11 mice per group. B, Expansion of IgM⁻ pre-B cells and IgM⁺ B cells during culture with IL-7. The four bars per group indicate the expansion at days 3, 5, and 7, as compared with the IgM⁻ and IgM⁺ (pre-)B cell numbers at the start of the culture, which were set to one. The plot is representative of six experiments using 2–4 mice per group. Error bars are the SD from three mice within every group. C, Cell cycle status and cell size after 5 days of culture with IL-7 from Btk⁺, Btk⁻, and Btk⁺/⁻ mice. DNA content of total cultured cells was examined by propidium iodide staining. The percentages of cycling cells (S, M, or G_2 phase) are indicated. Cell size was examined in B220⁺IgM⁻IgD⁻ pre-B cells. Numbers indicate the percentage of cells with a FSC value >600. Histograms are representative of five to six mice per group. D, Proliferative responses to the indicated concentrations of IL-7, as determined by [³H]thymidine incorporation after 5 days of culture. Error bars are the SD from three mice within every group. The plot is representative of three experiments.*

were pre-B cells, and that the anti-μ H chain Ab staining in Btk⁻ cells reflected high surface pre-BCR expression. In contrast, the Btk⁺ cells with anti-IgM fluorescence intensities ranging from 20 to 100 were surface κ⁺ cytoplasmic SLC⁻ immature B cells (see *Results*; Fig. 5).

When compared with Btk⁺ cultures, Btk⁻ and Btk⁺/⁻ cultures were characterized by a more rapid loss of mature surface Ig⁺ cells (Fig. 4*A*, day 3), and a reduced appearance of newly formed Ig⁺ B cells (Fig. 4*A*, days 5 and 7). From the total cell counts and the flow cytometric analyses of the cells, the expansion rates of sIgM⁻ and sIgM⁺B220⁺ cells during culture were calculated. As shown in Fig. 4*B*, pre-B cell expansion was significantly higher in the cultures from Btk⁻ mice (~30x) when compared with Btk⁺ mice (<10x). Only for Btk⁺ BM cells, the further differentiation of pre-B cells generated in the cultures in the presence of IL-7 resulted in an expansion of mature sIgM⁺ B cells (~4x).

Increased proliferation of Btk⁻ cells was confirmed by analysis of cell cycle and cell size (Fig. 4*C*). After 5 days of culture, 53 ± 3% of the Btk⁻ cells (n = 6) and only 25 ± 3% of the Btk⁺ (n = 6) were in the S, M, or G_2 phase of the cell cycle. In these experiments, the Btk⁻ BM cultures contained higher proportions (77 ± 6%) of large cells with high forward scatter (FSC) values than the Btk⁺ BM cultures (42 ± 7%). To

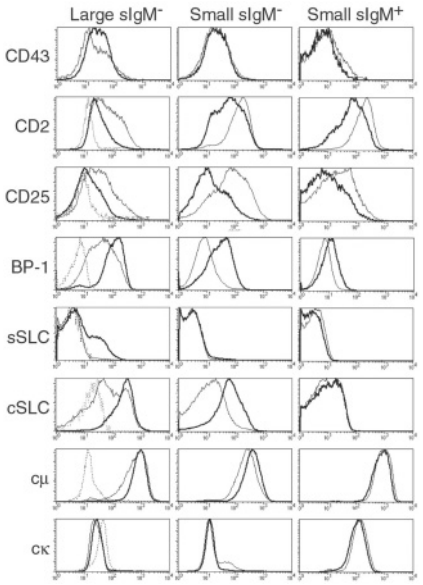

Figure 5. *Phenotypic characterization of IL-7-driven BM cultures from Btk+ and Btk- mice. The expression profiles of the indicated surface and intracellular markers are displayed as histogram overlays of Btk+ (thin lines) and Btk- mice (bold lines). The histogram plots of the first column (the large sIgM- cells) include the background stainings of B220- (S17 stromal) cells, as negative controls (dotted lines). This is not shown for CD43, as the B220- fraction in the cultures expressed CD43. Large and small IgM- cells were gated on the basis of FSC characteristics. Results shown are representative of 2–6 mice per group.*

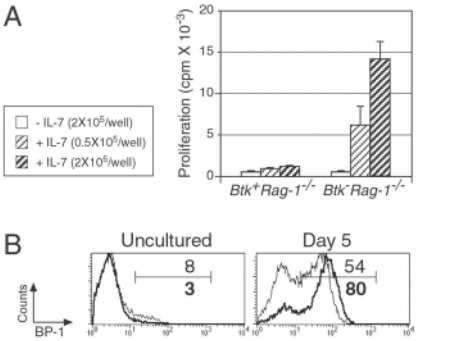

Figure 6. *Btk affects IL-7 responsiveness in pro-B cells. A, Proliferative responses of Btk+ and Btk- RAG-1-/- B cell precursors to IL-7, as determined by [3H]thymidine incorporation after 7 days of culture. B, BP-1 expression profiles are displayed as histograms for B220+ cells, either uncultured or after 5 days of culture in the presence of IL-7, from Btk+ (thin lines) and Btk- (bold lines) RAG-1-/- BM. Data are representative of four experiments using two mice per group.*

further quantify cell proliferation in the presence of various concentrations of IL-7, we performed [3H]thymidine incorporation experiments. After 5 days of culture, Btk- cells showed significantly higher proliferative responses to IL-7 when compared with Btk+ cells (Fig. 4*D*). These findings in Btk+ and Btk- BM cultures demonstrated that in the absence of Btk, IL-7-driven pre-B cell proliferation in vitro is increased.

Enhanced IL-7-driven expansion and decreased developmental progression is intrinsic to Btk- pre-B cells. The observed differences in IL-7-driven proliferation between Btk+ and Btk- pre-B cells cannot easily be explained by increased surface expression of IL-7R on these cells in Btk- mice. In flow cytometric experiments, the proportions of IL-7Rα chain positive cells within the large pre-B cell population in Btk+ and Btk- mice were 77 ± 4% and 87 ± 2%, respectively, while the mean fluorescence intensities of IL-7Rα+ cells were comparable.

To answer the question of whether the phenotype of Btk- cells in IL-7-driven BM cultures is an intrinsic feature of Btk- pre-B cells, or alternatively, originates from a defective BM microenvironment in Btk- mice, we also investigated BM cultures from Btk+/- mice. Due to random X chromosome inactivation, the pre-B cell compartment in these mice consists of two functionally different populations, i.e., Btk+ and Btk- (17, 37, 38). In Btk+/- heterozygous mice, IL-7-driven pre-B cell proliferation was intermediate between Btk+ and Btk- mice, as measured by pre-B cell expansion, cell cycle status, and [3H]thymidine incorporation (Fig. 4, *B–D*). At days 5 and 7, the surface Ig expression profiles and the FSC values in Btk+/- cultures were similar to those of Btk- cells (Fig. 4, *A* and *C*), indicating a proliferative advantage of Btk- over Btk+ cells. The presence of a *lacZ* reporter in the *Btk-* allele enabled a separate evaluation of the expansion of Btk+/*lacZ-* and Btk-/*lacZ+* cells in Btk+/- cultures. At days 5 and 7, the proportions of *lacZ+* cells in Btk+/- and Btk- cultures were comparable, indicating an intrinsic selective advantage of Btk-deficient cells in culture. In these cultures, the majority of IgM+ cells were Btk+/*lacZ-*. Thus, the increased expansion and reduced differentiation in IL-7-driven pre-B cell cultures is an intrinsic feature of Btk- cells, which is independent of the

xid BM microenvironment in vivo, or coculture of Btk-deficient stromal cells in the BM cultures.

Aberrant phenotype of Btk⁻ cells in IL-7-driven BM cultures. Next, we investigated whether the inefficient pre-BCR-mediated induction of cell phenotype changes in Btk⁻ pre-B and immature B cells in vivo were paralleled in the BM cultures in vitro. Large and small IgM⁻ cells were obtained from total BM cultures in the presence of IL-7 for 7 days. Small IgM⁺ cells were obtained from total BM cell suspensions that were cultured in the presence of IL-7 for 5 days and subsequently in the absence of IL-7 on S17 stromal cells for 2 days. In agreement with our findings in vivo (Fig. 1), we found by flow cytometric analysis that in Btk⁻ cultures down-regulation of CD43 and up-regulation of CD2 and CD25 at the large to small pre-B cell transition was impaired (Fig. 5). The expression of BP-1 was elevated in all three stages analyzed. Most importantly, we noticed that in the absence of Btk, SLC synthesis is not efficiently turned off, resulting in significantly enhanced cytoplasmic and surface SLC expression in Btk⁻ large pre-B cells (96 ± 1% and 29 ± 2%, n = 3, respectively), compared with 67 ± 5% and 2.9 ± 0.9% (n = 3) in Btk⁺ cells. Also in small pre-B cells, cytoplasmic SLC expression was enhanced in Btk⁻ cultures (62 ± 5%), compared with Btk⁺ cultures (17 ± 3%; Fig. 5). A small but reproducible increase in the expression level of cμ was present in Btk-deficient large and small pre-B cells. In addition, significant fractions of cytoplasmic κ L chain-positive pre-B cells were only present in Btk⁺ cultures (~20% of small sIgM⁻ pre-B cells, compared with <2% in Btk-deficient pre-B cells).

Taken together, these findings in IL-7-driven BM cultures confirm the involvement of Btk signaling in the pre-BCR-mediated down-regulation of CD43 and SLC and the induction of CD2 and CD25 expression, and the initiation of Ig L chain rearrangements.

Btk⁻ pro-B cells show significantly increased IL-7-driven proliferation in vitro. To investigate whether Btk-dependent modulation of IL-7 responsiveness in the mouse is specific for the pre-B cell stage, or alternatively, also occurs in earlier stages of B cell development, we crossed the Btk-deficient mice onto a RAG-1$^{-/-}$ background. In these mice, B cell development is arrested at the CD43⁺ pro-B cell stage, in which the BCR-linked CD79a/CD79b heterodimers are expressed on the cell surface without associated μ H chains (39). Btk⁺ and Btk⁻RAG-1$^{-/-}$ mice contained similar numbers of CD43⁺B220⁺ pro-B cells in vivo (data not shown), but the expression of IL-7R was slightly different (~62% in Btk⁺ and ~79% in Btk⁻RAG-1$^{-/-}$ mice). In [^{3}H]thymidine incorporation experiments, IL-7-induced proliferation was significantly enhanced in Btk⁻RAG-1$^{-/-}$ pro-B cells, when compared with control Btk⁺RAG-1$^{-/-}$ pro-B cells (Fig. 6*A*). Whereas we did not detect significant differences between the two groups of mice in the in vivo expression of the BP-1, the expression was elevated in the Btk⁻RAG-1$^{-/-}$BM cultures (Fig. 6*B*). Collectively, these results indicate that Btk-signaling can modulate the responsiveness to IL-7 of early B cell precursors, even before pre-BCR expression.

Discussion

Btk and pre-BCR signaling. Signaling through the pre-BCR complex mediates the checkpoint function of μ H chain by inducing cell cycle entry and rapid down-regulation of the rearrangement machinery, thereby ensuring allelic exclusion (14, 29). Subsequently, the proliferating pre-B cells exit the cell cycle, change the expression profile of various cell surface markers, and perform Ig L chain rearrangements with sequential activation of the κ and the λ loci (14). In humans, mutations in the *Btk* gene result in XLA, which reflects an almost complete block in B cell development at the pre-B cell stage (12, 13, 40). Similar to findings in mice deficient for the SLC component λ5 or the membrane form of the Ig μ H chain (41, 42), most XLA patients lack the compartment of large cycling pre-B cells (13). Therefore, the XLA disease phenotype implicates Btk in the pre-BCR signaling-mediated expansion of those pre-B cells with a productive μ H chain rearrangement in humans.

In contrast, Btk is not essential for the selective expansion of cμ⁺ pre-B cells in the mouse, as in Btk-deficient mice the pre-B cell population is normal in size and contains normal proportions of cycling cells (16-19). In this report, we demonstrate that the mouse Btk is involved in later phases of pre-B cell differentiation. We compared the pre-B cell compartment in Btk⁺ mice and Btk⁻ mice both in vivo and in vitro in IL-7-driven BM cultures. The absence of Btk resulted in (a) defective down-regulation of CD43 and SLC expression, (b) impaired up-regulation of CD2 and CD25, (c) aberrant expression of BP-1, (d) a 3-h delay in B cell development in vivo at the small pre-B cell stage and reduced progression to surface Ig⁺ B cells in vitro, and (e)

a significant increase in IL-7-driven proliferative expansion of pro-B and pre-B cells in vitro. Because Btk has been reported to be in the pre-BCR signaling pathway in the mouse (5, 22), our findings implicate pre-BCR signaling in the control of CD2, CD25, CD43, BP-1, and SLC expression, IL-7 responsiveness, and the transit time through the small pre-B cell stage.

As pre-B cell production in Btk$^-$ mice is normal, we conclude that the inefficient down-regulation of BP-1, CD43, and SLC expression and up-regulation of CD2 and CD25 do not affect the proliferative capacities of pre-B cells in vivo. Nevertheless, Btk$^-$ large pre-B cells manifested increased proliferative expansion in vitro, when compared with Btk$^+$ cells. The finding of significantly increased surface SLC expression on these Btk$^-$ large pre-B cells (when compared with Btk$^+$ cells) would support the hypothesis by Melchers et al. (14) that large cμ^+ pre-B cells stop cycling when they run out of a sufficient number of assembled pre-BCR molecules per cell. This is not necessarily in conflict with our finding that the increased cytoplasmic SLC expression in Btk$^-$ pre-B cells did not result in increased proliferation in vivo. It is very possible that only the level of membrane SLC expression may correlate with the proliferative capacity of pre-B cells, and we were unable to detect differences between Btk$^+$ and Btk$^-$ large pre-B cells in vivo in membrane SLC expression levels.

Btk and L chain rearrangement. We have previously found that Btk$^-$ mice show reduced λ L chain usage, which could either result from a defect in receptor editing or alternatively from a decreased rate or efficiency of λ L chain rearrangement (20). Although by crossing 3-83$\mu\delta$ autoantibody transgenic mice into Btk$^-$ mice we have shown that Btk is not essential for receptor editing (20), a possibility remained that the extent of L chain replacement events is reduced in Btk$^-$ mice. However, our BrdU-labeling experiments, in which we identified a 3-h delay at the small pre-B cell stage in Btk$^-$ mice, clearly argue against this possibility. Reduction of the level of receptor editing in Btk$^-$ mice would have the opposite effect of a more rapid transit through the small pre-B cell compartment, as B cells targeted for receptor editing were recently shown to be specifically delayed in this compartment for at least 2 h (33). Therefore, the finding of a 3-h delay supports a role for Btk in the regulation of the initiation of L chain rearrangement events. As both κ^+ and λ^+ Btk$^-$ cells are equally delayed, this cannot explain the observed reduced λ usage in Btk$^-$ mice (20). However, we conclude that the absence of Btk specifically affects the production rate of λ^+ immature B cells, as our linear regression analysis (Fig. 2*C*) revealed a lower slope value for λ^+ cells in Btk$^-$ mice (1.3) than in Btk$^+$ mice (1.7), while the values for κ^+ cells were similar (1.2 and 1.3).

In this context, it is unknown whether Btk signaling directly or indirectly regulates the activation of the L chain loci, or alternatively, whether Btk acts by mediating developmental progression to a pre-B cell stage in which L chain rearrangements are initiated. The latter possibility would be consistent with the delayed modulation of the expression of SLC, BP-1, CD43, CD2, and CD25. The finding that the expression of germline κ transcripts is first detected in vivo in large cycling CD25$^+$ pre-B cells would be consistent with a role for pre-BCR signaling the opening of the L chain loci for rearrangement (29, 43). However, additional experiments would be needed to address this issue, as it has been shown that productive L chain rearrangement can also be induced by IL-7 withdrawal of pre-BCR-deficient $\lambda5^{-/-}$ or $J_H^{-/-}$ pre-B cells in vitro (14, 44).

The size of the immature B cell pool and the turnover of immature B cells were reported to be essentially normal in *xid* or Btk$^-$ mice (16-18, 45). Nevertheless, we previously observed a selective disadvantage of Btk$^-$ cells at the transition from pre-B cells to immature B cells in the BM, which we determined in an in vivo competition analysis in Btk$^{+/-}$ heterozygous female mice (17). In the immature B cell subset, we found *lacZ* expression values of ~30%, whereas in the absence of a competitive disadvantage, Btk$^-$/*lacZ*$^+$ cells would be expected to represent ~50% of any B cell subpopulation. The finding of a 3-h delay in production of immature B cells in Btk$^-$ mice would explain the observed selective disadvantage of Btk-deficient cells in Btk$^{+/-}$ female mice.

Btk and IL-7 responsiveness. An inhibitory effect of Btk on proliferation of B-lineage cells in long-term Whitlock-Witte BM cultures has previously been reported (46). However, analysis of growth kinetics of cultures established from mixtures of WT and *xid* cells indicated that the observed differences resulted from changes in the BM microenvironment associated with the *xid* mutation. In contrast, our separate analyses of *lacZ*$^+$ and *lacZ*$^-$ cells in the BM cultures from Btk$^{+/-}$ mice showed that increased IL-7 responsiveness is an intrinsic feature of Btk$^-$ pre-B cells. This

would be consistent with the finding of an increased frequency of CFU responsive to IL-7 by limiting dilution analysis of day 15 fetal liver cells from CBA/N *xid* mice (47).

Finally, crosses of Btk-deficient and RAG-1-deficient mice showed that IL-7-dependent expansion in vitro is constrained by an inhibitory signal mediated by Btk, even before the expression of μ H chain. Cross-linking of the Igβ signaling component of the (pre-)BCR, which is expressed on the cell surface in $c\mu^-$ pro-B cells in association with calnexin induced a rapid phosphorylation of several intracellular signaling molecules, including Btk (22, 48). Therefore, it is possible that Btk-mediated Igβ/calnexin signaling would serve to stop pro-B cell proliferation to facilitate V(D)J recombination, which is exclusively initiated in the G_0/G_1 cell cycle phase (49). This hypothesis may be supported by a significant reduction of the level of V_H to DJ_H rearrangements observed in Igβ-deficient mice (50), which would then be explained by a reduced signal to make pro-B cells competent to undergo V_H-DJ_H rearrangement (48). In this context, it has been shown previously that in later stages of B cell development, modulation of the IL-7 responsiveness is dependent on pre-BCR and BCR signaling (51, 52). However, it is very possible that in pro-B cells, Btk may function in a pathway that is unrelated to the Igβ/calnexin complex, as Btk has been implicated in signaling of various other receptors, including IL-5R, CD38, IL-10R, and FcεRI (1, 2).

Taken together, our findings implicate Btk in two different pathways in early B cell development. First, Btk is required for the efficient developmental progression of cytoplasmic μ^+ pre-B cells, as Btk was shown to be involved in BP-1, CD43, and SLC down-regulation, CD2 and CD25 up-regulation, and the rate of transit through the small pre-B cell compartment. Additional experiments are required to show whether Btk-mediated pre-BCR signaling directly or indirectly influences the expression of SLC, germline κ, or λ transcripts or the RAG genes. Second, the IL-7-driven BM culture experiments indicate a role for Btk as a pro- and pre-BCR associated negative regulator of IL-7 expansion. Although XLA patients may occasionally have a significantly increased pro-B cell compartment (12, 53), in general there is no absolute pro-B expansion in XLA. Obviously, both in the mouse and in humans, other signaling molecules or pathways must be able to compensate for the loss of Btk in pro-B cells in vivo.

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Chapter 2.2

Function of Bruton's tyrosine kinase during B cell development is partially independent of its catalytic activity

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Function of Bruton's tyrosine kinase during B cell development is partially independent of its catalytic activity

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Abstract

The Tec family member Bruton's tyrosine kinase (Btk) is a cytoplasmic protein tyrosine kinase that transduces signals from the pre-B and B cell receptor (BCR). Btk is involved in pre-B cell maturation by regulating IL-7 responsiveness, cell surface phenotype changes, and the activation of λ L chain gene rearrangements. In mature B cells, Btk is essential for BCR-mediated proliferation and survival. Upon BCR stimulation, Btk is transphosphorylated at position Y551, which promotes its catalytic activity and subsequently results in autophosphorylation at position Y223 in the Src homology 3 domain. To address the significance of Y223 autophosphorylation and the requirement of enzymatic activity for Btk function in vivo, we generated transgenic mice that express the autophosphorylation site mutant Y223F and the kinase-inactive mutant K430R, respectively. We found that Y223 autophosphorylation was not required for the regulation of IL-7 responsiveness and cell surface phenotype changes in differentiating pre-B cells, or for peripheral B cell differentiation. However, expression of the Y223F-Btk transgene could not fully rescue the reduction of λ L chain usage in Btk-deficient mice. In contrast, transgenic expression of kinase-inactive K430R-Btk completely reconstituted λ usage in Btk-deficient mice, but the defective modulation of pre-B cell surface markers, peripheral B cell survival, and BCR-mediated NF-κB induction were partially corrected. From these findings, we conclude that: 1) autophosphorylation at position Y223 is not essential for Btk function in vivo, except for regulation of λ L chain usage, and 2) during B cell development, Btk partially acts as an adapter molecule, independent of its catalytic activity.

Introduction

Bruton's tyrosine kinase (Btk) is a member of the Tec family of cytoplasmic protein tyrosine kinases (PTK) and plays an essential role in B lymphocyte development and function (1, 2). Together with two other types of nonreceptor PTK, Syk and the Src family member Lyn, Btk acts as an important transducer of signals originating from the pre-B cell receptor (pre-BCR) and the BCR.

The pre-BCR complex, which is comprised of μ H chain, the nonrearranging VpreB and λ5 surrogate L chain (SLC) proteins, and the Ig-α/CD79a and Ig-β/CD79b signaling components, is a key checkpoint in B cell development to monitor the expression of a functional Ig μ H chain (3, 4). Pre-BCR expression is essential for the proliferative expansion of cytoplasmic μ H chain-positive pre-B cells and the induction of progression into small pre-B cells in which Ig L chain rearrangement occurs (3, 4). Pre-BCR engagement leads to the formation of a lipid raft-associated calcium signaling module composed of the tyrosine-phosphorylated signaling molecules Lyn, Syk, B cell linker protein (BLNK)/

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Abbreviations used in this paper: Btk, Bruton's tyrosine kinase; BCR, B cell receptor; BLNK, B cell linker protein; BM, bone marrow; MFI, median fluorescence intensity; PH, pleckstrin homology; PLC, phospholipase C; PTK, protein tyrosine kinase; SH, Src homology; SLC, surrogate L chain; TFII-I, transcription factor II-I; TH, Tec homology; TI-II, T cell-independent type II; TNP, trinitrophenol; WASP, Wiskott Aldrich syndrome protein; WT, wild type; *xid*, x-linked immunodeficiency; XLA, X-linked agammaglobulinemia.

SLP-65, phosphoinositide 3-kinase, Btk, Vav, and phospholipase Cγ2 (PLC-γ2) (5).

The importance of Btk in pre-BCR checkpoint function in humans is evidenced by an almost complete arrest of B cell differentiation due to defective expansion of the earliest cytoplasmic μ H chain-positive pre-B cells in X-linked agammaglobulinemia (XLA) patients with mutations in the Btk gene (6, 7). XLA is characterized by an almost complete absence of peripheral B cells and plasma cells; serum levels of all Ig classes are very low. In contrast, mice with a targeted mutation in the Btk gene or CBA/N mice, which carry an R28C point mutation in the Btk pleckstrin homology (PH) domain, exhibit a milder disorder, x-linked immunodeficiency (*xid*), mainly reflecting poor survival of peripheral B cells (8-10). Btk-deficient mice manifest a specific arrest of peripheral B cell development within the immature B cell pool at the progression of IgM^{high} to IgM^{low} $AA4^{+}CD23^{+}$ transitional B cells (8-12). In these mice, the numbers of pre-B cells that are generated in the bone marrow (BM) are normal (8, 9, 13). Nevertheless, Btk is crucially involved in the regulation of the developmental progression of pre-B cells by limiting the IL-7-driven expansion of large cycling pre-B cells (14, 15). In in vitro BM cultures, Btk-deficient pre-B cells showed increased IL-7-driven expansion and reduced developmental progression of large cycling into small resting pre-B cells and subsequently into Ig^{+} B cells (14). In addition, we recently demonstrated that Btk cooperates with the BLNK/SLP adapter molecule as a tumor suppressor that limits pre-B cell expansion (15). During the transition of large cycling to small resting cytoplasmic μ^{+} pre-B cells in the mouse, Btk-deficient cells fail to efficiently modulate the expression of SLC, the metallopeptidase BP-1, the adhesion molecule CD2, the IL-2R CD25, and the membrane sialoglycoprotein CD43 (14). Btk-deficient cells manifest a specific developmental delay within the small pre-B cell compartment of ~3 h in vivo. The finding of reduced λ L chain usage in Btk-deficient mice implicates Btk in the regulation of the activation of the λ locus for V(D)J recombination in pre-B cells (16).

Btk contains five distinct domains: an N-terminal PH domain, a Tec homology (TH) domain, a Src homology 3 (SH3) domain, an SH2 domain, and a C-terminal catalytic domain (Fig. 1*A*). Upon BCR stimulation, Btk activation is initiated by targeting the kinase to the plasma membrane through interactions of its PH domain with phosphatidylinositol-3,4,5-trisphosphate, a second messenger generated by phosphoinositide 3-kinase (17, 18). In concert, the Lyn and Syk kinases are activated, resulting in transphorylation of Btk at position Y551, which promotes the catalytic activity of Btk and subsequently results in its autophosphorylation at position Y223 in the SH3 domain (Fig. 1*A*) (19, 20). Although Y223 phosphorylation has little discernible influence on Btk catalytic activity, it prevents binding to Wiskott Aldrich syndrome protein (WASP) and increases the affinity to Syk (21). Concomitantly, Syk activation results in phosphorylation of BLNK/SLP65, which allows for the association of this adapter molecule with the Btk SH2 domain and with PLC-γ2 (2, 22). These interactions are critical to the activity of Btk and result in PLC-γ tyrosine phosphorylation, inositol trisphosphate production, and calcium mobilization.

Although biochemical studies in cultured B cells and fibroblasts have provided important insights into the molecular mechanism of BCR-mediated Btk activation, these systems have not allowed for the investigation of the role of the individual Btk domains in (pre-) BCR checkpoint functions in vivo. In this study, we addressed the functional significance of Btk Y223 autophosphorylation and investigated whether Btk can also act as an adapter molecule, independent of its catalytic activity. Therefore, we generated transgenic mouse strains that express the autophosphorylation site mutant Y223F-Btk and K430R kinase-inactive Btk, under the control of the B cell-specific CD19 promoter region. We crossed these mice onto a Btk null background (10), and examined the in vivo effects of the Y223F and K430R mutations, in particular with respect to Ig λ L chain usage, pre-B cell surface marker modulation, and the proliferative response of pre-B cells to IL-7.

Materials and Methods

Generation of Btk-transgenic mice. Wild-type (WT)-Btk transgenic mice have been described previously (23). The human Btk transgenes 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 (23). Using double-stranded site-directed mutagenesis (Stratagene, La Jolla, CA), the Y223F and K430R mutations were introduced

into the constructs that were previously used to generate the WT-Btk mice (23).

The Y223F mutation was created by replacement of AT by TC in exon 8 of human Btk in a 2.8-kb *Cfr10*I-Asp718 fragment (containing exons 8–11) in pBlueScript, using a mutation primer (5'-GCTGAAAAAGGTTGTGGCCCT TTTCGATTACATG CCAATG-3') and a 39-bp *Kpn*I-*Bgl*II selection primer. The mutated *Cfr10*I-Asp718 fragment was subsequently cloned into the WT-Btk transgene construct. The A-to-G replacement mutation K430R in exon 14 was created in a 2.1-kb Asp718-*Mun*I fragment encompassing human Btk exon 12–14. A 40-bp mutation primer, 5'-CCAGTAGGACGTGGCCA TCAGGATGATCAAAGAAGGCTCC-3' and the 39-bp *Kpn*I-*Bgl*II selection primer was used. The mutated 2.1-kb Asp718-*Mun*I fragment was ligated to a 9.2-kb *Mun*I-Asp718 fragment, to yield a 11.3-kb Asp718 human Btk fragment, which was used to replace the 11.3-kb Asp718 fragment in the WT-Btk construct (23).

The ~34-kb *Mlu*I-*Not*I inserts from the Y223F-Btk and K430R-Btk constructs were excised from the vector, gel purified, and microinjected into the pronuclei of FVB-fertilized oocytes. Transgenic founder mice were identified by Southern blotting and crossed with Btk null mice (10), which were bred onto a C57BL/6 background for more than eight generations. Sequence fidelity of the mutated fragments was confirmed, both in plasmid DNA before subsequent cloning steps and in genomic DNA obtained from the F_1 transgenic mice generated. All mice were bred and maintained at the Erasmus MC animal care facility under specific pathogen-free conditions.

Mouse genotyping. Tail DNA was analyzed by Southern blotting of *Bam*HI digests using a partial human Btk cDNA probe (bp 133-1153), as described previously (10, 23). Alternatively, the presence of Btk transgenes was evaluated by PCR, using the following primers: *CD19prom*, 5'-TGCAATTAGTGGTGAACAAC-3', and *hmBtk.65R*, 5'-AGATGCC AGGACTTGGAAGG-3'. Endogenous mouse Btk WT alleles were identified by an exon 9 forward primer (5'-CACTGAAGCTGAGGACTCCATAG-3') and an exon 10 reverse primer (5'-GAGTCATGTGCTTGGAATACCAC-3'). For Btk knockout alleles, primers were within the *LacZ* reporter (10): forward, 5'-TTCACTGGCCGT CGTTTTACAACGTCGTGA-3', and reverse, 5'-ATGTGAGCGAGTAACAACCC GTCGGATTCT-3'.

Flow cytometric analyses. Preparations of single-cell suspensions, standard and intracellular flow cytometry, and conjugated mAbs (BD PharMingen, Sunnyvale, CA) have been described previously (10, 14). The anti-SLC hybridoma LM34 (24) was kindly provided by A. Rolink (Basel, Switzerland); Abs were purified using protein G columns and conjugated to biotin according to standard procedures. Mice were analyzed at the age of 8–16 wk. Transgenic Btk expression levels were quantified by intracellular flow cytometry, using affinity-purified polyclonal rabbit anti-Btk (BD PharMingen) and FITC-conjugated goat anti-rabbit Ig (Nordic, Capistrano Beach, CA). Median fluorescence intensity (MFI) values were obtained by CellQuest software (BD Biosciences, San Diego, CA), whereby values for transgenic human Btk and endogenous murine Btk were corrected for background fluorescence, as detected in Btk null mutant mice. Subsequently, the ratio of the MFI values of transgenic Btk over endogenous Btk was calculated.

In vitro (pre-) B cell cultures. IL-7-driven BM cultures and determination of IL-7-dependent proliferative responses of total BM cells have been described previously (14, 16). Mature B cell fractions were purified from spleen, using standard NH_4Cl lysis to deplete erythrocytes, followed by complement-mediated T cell lysis, as described previously (25). The enriched B cell fractions were cultured in vitro in the presence or absence of LPS, 5 μg/ml anti-CD40, or polyclonal goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL), as described previously (16). To measure DNA synthesis, cells were pulsed with [^{3}H]thymidine for 16–20 h, harvested, and counted using standard methods. For apoptosis assays, DNA content was determined in ethanol-fixed cells, using propidium iodide.

B cell stimulation and Western blotting analyses. For analyses of nuclear c-Rel expression, single-cell suspensions from spleen were depleted of erythrocytes by NH_4Cl lysis and enriched for B cells by AutoMACS purification, using biotinylated Abs to Gr-1, Ter119, CD4, CD8, and CD11b and magnetic streptavidin MicroBeads (Miltenyi Biotec, Sunnyvale, CA) for negative selection. Cells were stimulated with 10 μg/ml $F(ab')_2$ of polyclonal goat anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA) in RPMI 1640 at 37°C for 4 h. Total nuclear and cytoplasmic protein extracts were prepared according to Andrews and Faller (26).

Samples from equivalent cell numbers were resolved by SDS-PAGE, and proteins were transferred to nitrocellulose membrane, as described (10). Membranes were blocked with 5% milk in TBS/0.05% Tween 20 for 1 h and incubated overnight with anti c-Rel (SC-071; Santa Cruz Biotechnology, Santa Cruz, CA), and subsequently with HRP-conjugated swine anti-rabbit Ig. Densitometry analysis was conducted using ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA).

For analyses of Bcl-x_L and cyclin D2 induction, total splenocytes were depleted of erythrocytes and stimulated with 10 μg/ml polyclonal goat anti-mouse IgM (Southern Biotechnology Associates) in RPMI 1640/10% FCS at 37°C. Total cell lysates were subjected to SDS-PAGE using standard procedures (27). Immunoblotting Abs used included: anti-Bcl-x_L (2762; Cell Signaling Technology, Beverly, MA), anti-cyclin D2 (M-20; SC-593; Santa Cruz Biotechnology), and anti-extracellular signal-regulated kinase 1/2 (SC-094; Santa Cruz Biotechnology).

For analysis of Btk expression and Btk in vitro kinase assays, single-cell suspensions from spleen were depleted of erythrocytes and enriched for B cells by AutoMACS purification. B cell fractions were stimulated, as described above, for 5 and 10 min. Anti-Btk C-20 (Santa Cruz Biotechnology) was used for immunoblotting, while a polyclonal rabbot anti-Btk (kindly provided by V. Tybulewicz, London, U.K.) was used for immunoprecipitation. In vitro kinase assays were performed, as described previously (27).

Ig detection and in vivo immunizations. Levels of Ig subclasses in serum were measured by sandwich ELISA, as described previously (25). To measure thymus-independent responses, mice were injected i.p. with 50 μg of trinitrophenol (TNP)-Ficoll in PBS, and TNP-specific IgM and IgG3 were analyzed at day 7 in a TNP-specific ELISA (28).

Results

Expression of Btk mutants in transgenic mice. We have previously described transgenic mice that express WT human Btk under the control of the CD19 promoter region (23). When these mice were mated onto a Btk-deficient background, correction of all *xid* features was observed, indicating that in this system the Btk gene was appropriately targeted to both conventional and $CD5^+$ B-1 cells. We now generated mouse strains, expressing Y223F (n = 5) and K430R (n = 2) mutant Btk. These mice were crossed onto the Btk null background, and transgenic Btk protein expression was quantified by intracellular flow cytometry. Median fluorescence intensities of transgenic Btk were compared with endogenous Btk in B-lineage subpopulations (Fig. 1*B*; Table I). The different mouse lines exhibited a wide range of Btk protein expression levels, which were directly correlated with the transgene copy number, as estimated by Southern blotting analyses of genomic DNA samples from the mice. The expression of transgenic Btk increased as the B cell progenitors in the BM matured to surface Ig-expressing peripheral B cells (Table I). Expression of transgenic Btk protein was confirmed by Western blotting analysis of BM and spleen cell suspensions, in which Btk protein was visible as a single ~77-kDa band for all transgenic

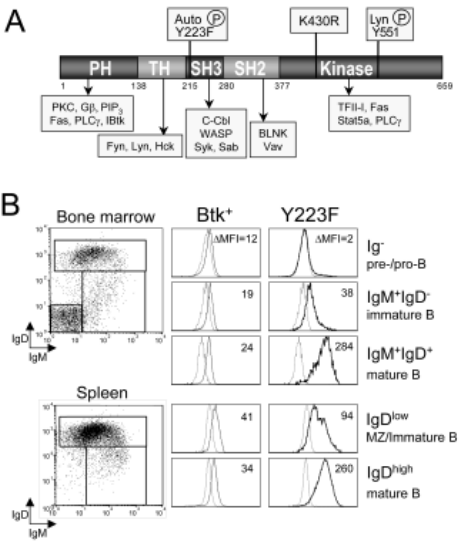

Figure 1. *Expression of Btk mutant transgenes during B cell development. A, Domains of Btk. The positions of the mutations and Btk-associated molecules are shown. The numbers represent the amino acid positions of the domain boundaries. B, Intracellular Btk expression in the indicated B-lineage subpopulations in BM and spleen. $B220^+$ cells were gated and B-lineage subpopulations were defined on the basis of surface IgM/IgD expression (left) and analyzed for intracellular Btk expression (right). The results are displayed as histograms of a wild-type mouse (Btk^+; thin lines) and a Y223F-Btk mutant transgenic mouse (AM-02; bold lines), compared with the background staining, as determined in Btk^- mice (dashed lines). The values for the differences in MFI (ΔMFI) are given. MZ = marginal zone.*

Table I. *Overview of transgenic lines expressing mutant Btk*

Btk Mutant	Tg Line	Relative Expression Level[a]		
		Pro-/pre-B	Immature B	Mature B
WT	NR-02[b]	<0.2	1.2	6.9
Y223F	AM-05	<0.2	<0.2	<0.2
	AM-03	<0.2	0.45	2.7
	AM-02	0.37	1.9	10
	TK-20	0.40	2.6	11
	UK-21	5.8	14	37
K430R	YK-25	<0.2	0.20	0.54
	XK-24	<0.2	0.92	17

[a] *As determined by intracellular flow cytometry. The median fluorescence intensities in the different B-lineage cell fractions of wild-type mice were set to 1.0.* [b] *These mice were described previously (23).*

lines generated (data not shown). Because we aimed to investigate the role of Btk in particular at the pre-B cell to immature B cell progression, we mainly focused on those mouse strains that expressed physiological levels of transgenic Btk (1–2 x endogenous levels) in immature B cells in the BM. These transgenic lines, AM-02 (Y223F) and XK-24 (K430R), expressed high Btk levels in mature IgD$^+$ peripheral B cells (Table I).

B cell development in Btk mutant mice. To determine the effect of the Btk mutations on B cell development, we examined the size of the B cell subpopulations in spleen, peritoneal cavity, and BM in the mutant mice by flow cytometry. In these experiments, we also included WT-Btk transgenic mice, as well as nontransgenic Btk$^+$ and Btk$^-$ littermates, which served as controls.

The *xid* phenotype in Btk$^-$ mice is characterized by a specific deficiency of mature IgM^{low}IgD^{high} cells in the spleen and lack of CD5$^+$ B-1 cells in the peritoneal cavity (8-10) (see Fig. 2, *A* and *B*). The only obvious difference between the BM B cell subpopulations of Btk-deficient and control Btk$^+$ mice is that the subpopulation of mature B220^{high}-recirculating cells is reduced and has an aberrant IgM^{high} phenotype (Fig. 2*C*). We previously reported (23) that CD19 promoter-driven human WT-Btk expression restores these defects. We did not detect a significant effect of the Y223 autophosphorylation site mutation, as also the expression of Y223F-Btk appeared to restore B cell numbers completely in spleen, peritoneal cavity, and BM (Fig. 2, *A*–*C*). Expression of low levels of Y223F-Btk (line AM-05) (<20% of normal in immature B cells, not detectable with our flow cytometric assay) on the Btk null background rescued the presence of peritoneal CD5$^+$ B-1 cells, but not the numbers or surface profile of splenic B cells (data not shown). Considerable overexpression of Y223F-Btk (line UK-21, ~14x in immature B cells) was associated with deletion of IgM^{high} immature B cells in the BM

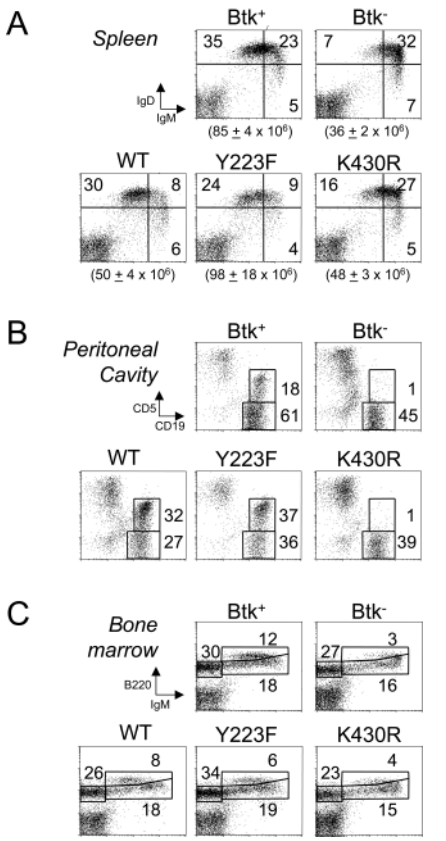

Figure 2. *Flow cytometric analysis of B cell populations in Btk mutant mice. A, Surface IgM/IgD expression in the spleen with total splenic B cell numbers as mean values ± SEM. B, Surface CD19/CD5 expression in the peritoneal cavity. C, Surface IgM/B220 expression in the BM. Total lymphocytes were gated on the basis of forward and side scatter characteristics. Data are displayed as dot plots, and the percentages of cells within the indicated gates are given. Data shown are representative of 5–20 animals examined within each group.*

(data not shown), similar to our previous findings in transgenic mice expressing the constitutive active Btk-mutant E41K (23). Interestingly, reconstitution with kinase-inactive K430R-Btk partially overcame the block in peripheral B cell maturation, when significantly overexpressed. In these mice, the fraction of mature IgM^{low}IgD^{high} B cells in the spleen was partially restored (~16%, compared with ~7% in Btk$^-$ mice and 30% in WT transgenic mice, Fig. 2*A*), and B220^{high}IgM^{low}-recirculating cells appeared in the BM (Fig. 2*C*). However, we still did not detect CD5$^+$ B-1

cells in the peritoneum (Fig. 2*B*). When present on the Btk$^+$ background, overexpression of K430R-Btk did not appear to result in adverse effects on B cell development (data not shown), indicating that in this respect the K430R-Btk did not act as a dominant-negative mutant.

Collectively, these findings show that expression of Y223F-Btk restored B cell development, indicating that Y223 autophosphorylation is not essential for the function of Btk signaling in directing B cell development. The limited, but detectable, correction of the *xid* phenotype in K430R transgenic mice indicates that Btk function is partly independent of its intrinsic enzymatic activity. Finally, the analyses showed that B-1 B cell development is supported by low levels of Y223F-Btk, but not by kinase-inactive Btk.

Ig λ L chain usage in Btk mutant mice. We previously showed that B cells from Btk-deficient mice have a ~50% reduction in the frequency of Ig λ L chain expression, already at the immature B cell stage in the bone marrow (16). This finding implicated Btk in the regulation of the activation of the λ locus for V(D)J recombination in pre-B cells (16).

To investigate the effects of the Y223F and K430R mutations on the ability of Btk to regulate λ usage, we analyzed immature B220^{low}IgM$^+$IgD$^-$ B cells from the BM of a panel of Btk$^+$, Btk$^-$, and transgenic Btk mutant mice by four-color flow cytometry (Table II). Consistent with our previous report (16), we found reduced λ usage in Btk$^-$ mice, when compared with Btk$^+$ or WT-Btk transgenic mice (Table II). In Y223F-Btk mice, λ usage was still significantly reduced, when compared with Btk$^+$ mice (Table II) or with WT-Btk transgenic mice, which showed normal proportions of λ$^+$ B cells (16). Thus, transgenic expression of physiological levels of Y223F-Btk could only partially correct λ usage in Btk$^-$ mice. Unexpectedly, expression of K430R-Btk fully reconstituted λ usage, as in K430R-Btk mice the proportions of λ L chain-expressing cells were similar to those in control Btk$^+$ mice.

In summary, these findings indicate the differential requirement for Btk domains in the regulation of λ L chain usage: the Y223 autophosphorylation site is important, but kinase activity does not appear to be critical. We conclude that in the context of the control of λ usage in early B cell development, Btk mainly functions as an adapter molecule, independent of its enzymatic activity.

Pre-B cell maturation in Btk mutant mice. We previously showed that during the developmental progression of large cycling into small resting cytoplasmic μ$^+$ pre-B cells, Btk-deficient cells fail to efficiently down-regulate the expression of BP-1 and SLC and up-regulate the expression of surface CD2 and CD25/IL-2R (14). To investigate the requirement for the different Btk domains in the modulation of these phenotypic markers, we analyzed their expression profile in pre-B cells and immature B cells from Btk mutant mice by flow cytometry.

Table II. *Ig λ L chain usage in Btk mutant mice*

Btk Mutant	*N*[a]	Ig λ$^+$ Immature B Cells (%)	*p* Values[b]
Btk$^+$	32	7.7 ± 0.5[c]	
Btk$^-$	24	2.9 ± 0.3	$<10^{-9}$
Y223F	12	4.9 ± 0.5	0.0017
K430R	19	7.5 ± 0.7	NS

[a] *Number of mice per group*
[b] *Values of p for difference with the group of Btk$^+$ mice*
[c] *Mean values ± SEM*

As shown in Fig. 3*A*, Btk-deficient pre-B cells have significantly higher levels of cytoplasmic SLC and BP-1, but lower expression levels of CD2 and CD25. This aberrant marker profile was fully corrected in transgenic mice expressing WT or Y223F Btk (Fig. 3*A*). By contrast, expression of K430R-Btk only partially restored Btk function in pre-B cell differentiation, as the expression levels of BP-1, cSLC, CD2, and CD25 in pre-B cells were intermediate between the values for Btk$^+$ and Btk$^-$ mice (Fig. 3, *A* and *B*). Also, at the next developmental stage of immature B cells, the expression profiles of the BP-1, cSLC, CD2, and CD25 markers were fully corrected in WT and Y223F Btk mice and partially corrected in K430R-Btk mice (data not shown, and Fig. 3*C*).

In summary, these findings demonstrate that the function of Btk in vivo in the induction of cell surface phenotype changes in cytoplasmic μ$^+$ pre-B and immature B cells is not affected by loss of the Y223 autophosphorylation site and is only partially dependent on its kinase activity.

Proliferative responses to IL-7 in Btk mutant mice. Btk has an inhibitory effect on the proliferation of B cell precursors in long-term Whitlock-Witte BM cultures and in IL-7-driven cultures of BM or fetal liver cells (14, 29, 30). In [^{3}H]thymidine incorporation experiments, after 5 days of culture in the presence of IL-7, Btk-deficient total BM cells showed significantly higher proliferative responses when compared with Btk$^+$ cells (Fig. 4*A*). Expression of WT or Y223F Btk exhibited a similar inhibitory effect

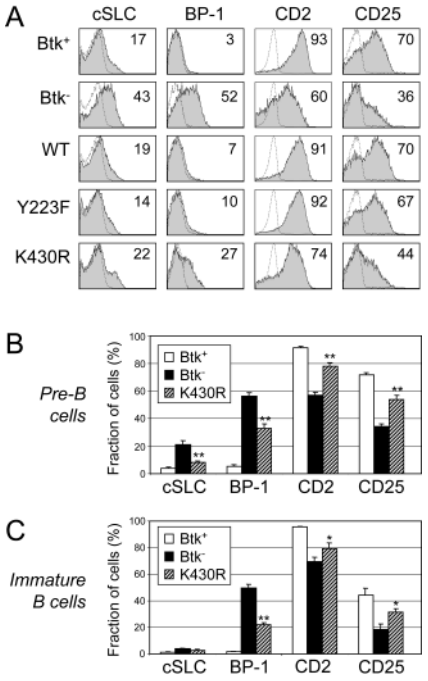

Figure 3. *Phenotypic characterization of pre-B and immature B cells in Btk mutant mice. A, Expression profiles of cytoplasmic SLC, surface BP-1, CD2, and CD25 in pre-B cells of the indicated mice. Flow cytometry data are displayed as filled histograms, with background stainings of B220$^-$ cells (dashed lines) as negative controls. The percentages of positive cells are indicated. Data shown are representative of 5–20 animals examined within each group. B and C, The K430R-Btk transgene partially corrects the expression of the indicated markers in pre-B cells (B) and B220^{low}IgM$^+$ immature B cells (C). Differences between Btk$^+$ and Btk$^-$ pre-B and immature B cells were significant for all markers (t test; p < 0.005). Asterisks indicate significance of differences between K430R-Btk and Btk$^-$ mice (*, p < 0.05; **, p < 0.0005). Cytoplasmic SLC and CD2 expression was investigated in B220^{low}IgM$^-$cμ$^+$ pre-B cells, while BP-1 and CD25 were analyzed in total B220^{low}IgM$^-$ pro-B/pre-B cell fractions (pro-B cells have very low proportions of BP-1$^+$ or CD25$^+$ cells).*

on IL-7-driven proliferation as intact endogenous Btk. Proliferative responses to IL-7 in K430R mutant mice were only slightly higher than those found in Btk$^+$ mice. Therefore, we conclude that the function of Btk in down-regulation of IL-7-driven proliferation is not critically affected by loss of Y223 autophosphorylation or kinase activity of Btk.

Developmental progression of Btk mutant pre-B cells in vitro. In Btk-deficient pre-B cells, the increased IL-7-driven proliferation in vitro is accompanied by reduced developmental progression from large cycling CD2$^-$ pre-B cells into noncycling small resting CD2$^+$ pre-B cells, and subsequently into surface Ig$^+$ B cells (14). To analyze the function of Btk domains in this context, total BM cell suspensions from the panel of mutant mice were cultured in the presence of 100 U/ml IL-7. At day 5, the distribution of B220$^+$ cells over six developmental fractions, μ$^-$ pro-B cells, CD2$^-$ μ$^+$ large cycling pre-B cells, CD2$^-$ μ$^+$ small pre-B cells, CD2$^+$ μ$^+$ small pre-B cells, IgM$^+$IgD$^-$ immature B cells, and IgM$^+$IgD$^+$ mature B cells, was evaluated by flow cytometry (Fig. 4*B*). Consistent with our previous findings (14), we noticed that in the absence of Btk: (a) growth of cytoplasmic μ$^-$ cells was hardly supported, (b) progression from the CD2$^-$ into the CD2$^+$ pre-B cell stage was impaired, and (c) the proportions of IgM$^+$ immature and mature B cells were reduced, when compared with wild-type mice. The BM cultures from WT and Y223F Btk mice exhibited a subpopulation distribution that was similar to that in Btk$^+$ control mice. Expression of the K430R-Btk transgene partly restored Btk function, as in the K430R cultures significant fractions of μ$^-$ pro-B cells were found and CD2 expression was more efficiently induced on μ$^+$ small pre-B cells, when compared with cultures from Btk-deficient mice (Fig. 4*B*).

Taken together, these results show that the role of Btk signaling in the induction of the developmental progression of CD2$^-$ into CD2$^+$ small pre-B cells and subsequently to surface Ig$^+$ B cells in vitro is completely independent of Y223 autophosphorylation. In this context, kinase-inactive Btk is apparently partially active.

Kinase-independent adapter function of Btk in mature B cells. Because kinase-inactive Btk could partially restore developmental progression of pre-B cells, we examined mature B cell function in K430R-Btk mice to evaluate the overall ability of kinase-inactive Btk to complement other features of the *xid* phenotype.

Btk-deficient mice have severely decreased levels of IgM and IgG3 in the serum and do not mount specific Ab responses to T cell-independent type II (TI-II) Ags in vivo, and *xid* B cells undergo apoptosis instead of proliferation in response to in vitro stimulation (8, 9, 31-33). The K430R-Btk transgene provided a significant correction of the decreased levels of IgM in

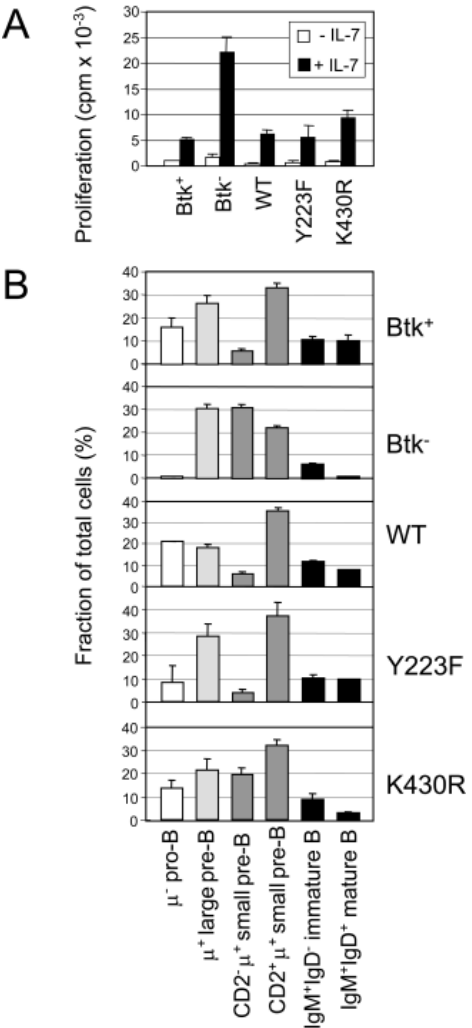

Figure 4. *Analysis of IL-7-driven BM cultures from Btk mutant mice. A, Proliferative response to IL-7, as determined by [^{3}H]thymidine incorporation after 5 days of culture in the presence or absence of 100 U/ml IL-7. B, The distribution profile over the indicated B-lineage subpopulations of day 5 IL-7-driven total BM cultures of the Btk mutant mice. Error bars are the SEM values from four to seven mice per group.*

the serum of Btk-deficient mice, while only a modest increase in IgG3 was observed (Fig. 5*A*). Expression of K430R-Btk in Btk-deficient mice did not reconstitute the in vivo responsiveness of B cells to the TI-II Ag TNP-Ficoll. Moreover, K430R-Btk expression on a wild-type background even showed a dominant-negative effect on the production of TNP-specific IgM (Fig. 5*B*).

When purified splenic B cell suspensions were stimulated in vitro, K430R-Btk completely restored the proliferative responses to anti-CD40 and LPS (Fig. 6*A*), which are known to be affected by defective Btk function (8, 9, 34). In contrast, the rescue of the proliferative response to anti-IgM stimulation was modest (Fig. 6*A*). To determine the effect of the K430R-Btk transgene on B cell survival, purified splenic B cells from Btk$^+$, Btk$^-$, WT-Btk, and K430R-Btk mice were cultured in vitro in medium alone, or in the presence of anti-CD40, LPS, or anti-IgM. After 40 h, cells were stained with propidium iodide and examined by flow cytometry to determine the fraction of apoptotic cells, i.e., cells with sub-G_1 amounts of DNA. The presence of the K430R-Btk transgene on the Btk null background strongly reduced the proportion of apoptotic cells, in all four culture conditions (Fig. 6*B*). Apoptosis levels of K430R-Btk B cells stimulated with anti-IgM were intermediate between the values for wild-type and Btk-deficient mice. Taken together, these data indicate that kinase-inactive Btk can partially rescue the compromised cell cycle induction and cell survival of Btk-deficient mature B cells.

Kinase-inactive Btk induces NF-κB activation

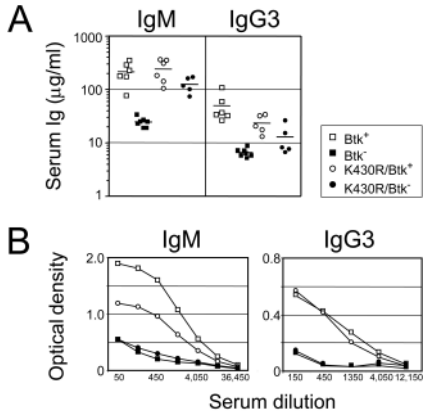

Figure 5. *Partial correction of mature B cell function in K430R-Btk mice. A, Serum concentrations of IgM and IgG3 in Btk$^+$ and Btk$^-$ mice, as well as K430R-Btk mice on the Btk$^+$ and Btk$^-$ background. Mice were 2 mo of age, total Ig levels were determined by ELISA, and each symbol indicates an individual mouse (n = 5–7). B, Serum concentrations of TNP-specific IgM and IgG3 7 days after in vivo TNP-Ficoll injection. For each serum dilution, the ODs are shown as mean values from 11–23 mice in each group.*

upon BCR stimulation. To explore the mechanism underlying the kinase-independent adapter function of Btk, we further investigated kinase-inactive Btk function in BCR signal transduction.

Consistent with a previous report (19), in vitro kinase assays of immunoprecipitated Btk from purified splenic B cell fractions stimulated with anti-IgM Abs confirmed that K430R-mutated Btk did not have any detectable autophosphorylation activity, even though high levels of K430R-Btk were expressed (Fig. 7*A*).

Btk is known to have a critical role in BCR-directed cell cycle induction (32, 33, 35), as well as nuclear translocation of NF-κB, which induces expression of the antiapoptotic survival protein Bcl-x_L (31, 33, 36, 37). We thus evaluated the ability of K430R-Btk to activate NF-κB and induce Bcl-x_L and cyclin D2 expression in response to BCR engagement. Western blot analysis of purified splenic B cells that were stimulated with anti-IgM Abs for 4 h showed that, while induction of nuclear c-Rel was negligible in Btk-deficient B cells, the fold inductions in K430R-Btk were close to those observed in Btk$^+$ control mice (Fig. 7*B*). Under these conditions, the amounts of cytoplasmic c-Rel were similar in all three groups of mice. Furthermore, the induction of Bcl-x_L and cyclin D2 after BCR stimulation, which

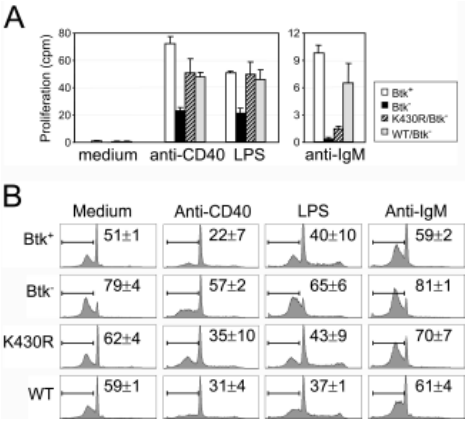

Figure 6. *Partial correction of mature B cell proliferation and survival in K430R-Btk mice. A, Proliferation, determined by [^{3}H]thymidine incorporation, of cells cultured in medium alone, in the presence of LPS, anti-CD40, or anti-IgM. B, Propidium iodide DNA content analysis of B cells following stimulation in vitro. Numbers indicate the proportion of cells ± SEM in the sub-G_1 fraction (%) in cell cycle analysis. Data (with mean ± SEM values from two to four mice per group) are representative of three independent experiments.*

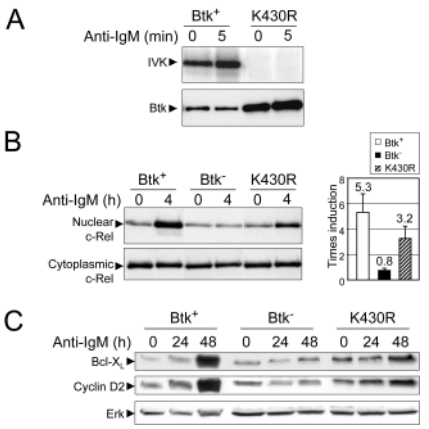

Figure 7. *K430R-Btk induces NF-κB activation upon BCR stimulation. A, SDS-PAGE of in vitro phosphorylated Btk immunoprecipitates from purified splenic B cells (12 x 10^6) from wild-type and K430R-Btk mice were left untreated or stimulated with anti-IgM for 5 min. For quantification of Btk expression, equal proportions of the immunoprecipitates were analyzed by Western blotting using Btk-specific Abs. In agreement with a previous report (42), wild-type Btk showed detectable kinase activity also in unstimulated splenic B cells. B, Western analysis of nuclear and cytoplasmic c-Rel expression. Equal numbers of purified splenic B cells from the indicated mice were left untreated or stimulated with anti-IgM for 4 h (left). Induction of nuclear c-Rel was quantified by densitometry, and results are shown as mean values and SEM from four mice per group (right). C, Induction of Bcl-x_L and cyclin D2. Total splenocytes of the indicated mice were stimulated with anti-IgM and evaluated for the expression of Bcl-x_L and cyclin D2 by immunoblotting using specific Abs. Membrane was reblotted with anti-extracellular signal-regulated kinase. Data are representative of four mice analyzed per group.*

was blocked in Btk-deficient B cells, was partially restored in K430R-Btk transgenic mice (Fig. 7*C*).

In summary, these results show that in mature B cells, K430R-Btk is able to transmit BCR signals that can activate NF-κB and induce Bcl-x_L and cyclin D2 expression, leading to cell cycle entry, proliferation, and rescue from apoptosis.

Discussion

In vivo structure-function analysis for Btk. The characterization of 400 unique human Btk mutations, 155 of which represent missense mutations, in XLA families could in principle have allowed the identification of in vivo structure-function effects of Btk. However, there is phenotypical heterogeneity among patients,

even from single XLA pedigrees, and to date no correlation has been described between the type and position of the mutations and clinical or immunological XLA phenotype (38). Furthermore, the almost complete pre-B cell arrest in most XLA patients (6) complicates the analysis of the function of the individual Btk domains in developmental stages beyond the large μ^+ pre-B cell. As a result, the role played by the various Btk domains in vivo in humans remained largely unknown.

Analyses in the mouse to date have focused on the in vivo function of the Btk PH domain, which was shown to be absolutely essential in mature B cells. This was inferred from the finding of the same *xid* phenotype in CBA/N mice with the classical R28C PH domain mutation and in mice with a complete ablation of Btk protein generated by gene targeting (8-10). Apparently, the R28 residue in the PH domain is also essential for Btk function in pre-B cells, as we observed that, comparable to Btk-deficient pre-B cells, also *xid* CBA/N pre-B cells manifested an impaired modulation of the phenotypic markers BP-1, SLC, CD2, and CD25, and an increased proliferative response to IL-7 (S. Middendorp and R. Hendriks, unpublished results).

By using transgenic mice expressing Y223F and K430R mutant Btk, we now demonstrate a differential role of Y223 phosphorylation and Btk catalytic activity in developmental progression of cytoplasmic μ^+ pre-B cells in vivo. In particular, we analyzed λ L chain usage; the modulation of BP-1, SLC, CD2, and CD25 expression; and the IL-7 responsiveness of pre-B cells, all of which were previously shown to be Btk dependent (14, 16). In this study, we demonstrate that: (a) the Y223 autophosphorylation site is not essential for signaling developmental progression of pre-B or B cells, except for the role of Btk in the regulation of λ L chain usage; (b) in pre-B cells, Btk functions to a large extent as an adapter molecule; (c) in mature B cells kinase-inactive Btk can induce the activation of NF-κB upon BCR stimulation.

The Y223 autophosphorylation site in the SH3 domain. Although phosphorylation of Y223F has little discernible influence on Btk catalytic activity, it is generally thought that full activity of Btk is dependent on phosphorylation of Y223. Y223 is located on the surface of the SH3 domain, and its phosphorylation prevents binding to WASP and increases the affinity to Syk (21). The Y223F mutation dramatically potentiates the transforming activity of the gain-of-function mutant E41K-Btk in fibroblasts (19). Moreover, the SH3 domain has specificity for the N-terminal proline-rich region of the Btk TH domain, and therefore it has been hypothesized that intra- or intermolecular interaction between the TH and SH3 domain might have an important regulatory function (1). With the exception of λ L chain usage, we found that expression of Y223F-mutated Btk fully corrected all features of the Btk-deficient phenotype, including pre-B cell maturation, differentiation of peripheral B cells, and B-1 cell development. Furthermore, we observed reconstitution of B cell function, both in total serum IgM and IgG3 levels, TI-II responses in vivo, and in proliferative responses to anti-IgM stimulation in vitro (R. Hendriks, unpublished). Taken together, these results indicate that Y223 autophosphorylation-dependent interactions are not essential for B cell development and mature B cell function. This is consistent with previous observations that reconstitution of Btk-deficient chicken DT 40 B cells with Y223F and WT Btk resulted in a similar rescue of BCR-induced PLC-γ phosphorylation and calcium flux (39). Our findings may also explain the remarkable absence of missense mutations in the Btk SH3 domain in a total of 155 missense mutations identified to date in XLA families (38), while on the basis of SH3 domain size ~10 of these should be located within the SH3 domain. Nevertheless, the Y223 autophosphorylation site appears to be involved in the regulation of λ L chain usage. In this context, it is tempting to speculate that transcription factor II-I (TFII-I) is involved in the activation of the λ L chain locus for recombination, as the Y223F-Btk mutation also abrogates phosphorylation of a Btk substrate, the NF BAP-135/TFII-I (40). Obviously, additional experiments are required to demonstrate whether TFII-I plays any role in the regulation of gene rearrangements at the λ L chain locus.

It is intriguing that mutation of the Y223 Btk autophosphorylation site partially affected λ L chain usage, while expression of kinase-inactive Btk restored λ usage completely. This may imply that the Y223F mutation does not only change the interaction of Btk with proteins that bind to the SH3 domain when phosphorylated at Y223, but also with proteins that bind to nonphosphorylated Y223, such as c-Cbl and WASP (21). Alternatively, the obtained results could be explained by a contribution of other kinases to phosphorylation of Btk at position Y223 in vivo, once Btk is recruited to the pre-BCR

signaling complex.

The kinase-inactive K430R-Btk mutant. The K430R mutation destroys the ATP-binding site, resulting in kinase-inactive Btk (19, 20). Expression of physiological levels of kinase-inactive Btk in early B cell development normalized λ L chain usage and partially reconstituted the impaired pre-B cell differentiation and IL-7 responsiveness in Btk-deficient mice. These findings imply that Btk partially functions as an adapter molecule, independent of its kinase activity, possibly in a larger complex together with other molecules that interact with Btk (Fig. 1*A*). Such a complex might include PTK that either bind directly to Btk, such as Fyn, Lyn, Hck, and Syk, or indirectly through the BLNK/SLP65 linker molecule, which binds to the Btk SH2 domain. It is therefore possible that Syk may partially compensate for kinase-inactive Btk and phosphorylate PLC-γ2 in a complex that contains activated BLNK/SLP65 and Btk. Our data indicate that, especially in the activation of the λ L chain locus for V(D)J recombination in pre-B cells, Btk mainly acts as an adapter molecule. Likewise, in mature B cells, the role of Btk in proliferation and survival upon LPS and anti-CD40 stimulation in vivo was found to be fully independent of Btk enzymatic function. Nevertheless, the (incomplete) induction of NF-κB, Bcl-x_L, and cyclin D2 by K430R-Btk cannot fully rescue B cell development, as is particularly clear from the lack of reconstitution of the B-1 cell compartment and in vivo TI-II responses. In this context, it was recently shown that Btk participates in NF-κB induction by Toll-like receptor 4, whereby K430R-Btk acts as a dominant-negative inhibitor of the LPS-induced activation of NF-κB in an astrocytoma and a monocytic cell line (41). Taken together, these findings illustrate that the importance of the Btk kinase domain varies between the different Btk-mediated signaling pathways during B cell development and between the different signal transduction pathways.

In summary, our findings demonstrate the differential requirement for Btk domains in the developmental progression of cytoplasmic μ H chain-positive pre-B cells. We show that the regulation of Ig λ L chain usage is Y223 dependent, but K430 independent, and that the modulation of pre-B cell marker expression is Y223 independent, but partially K430 dependent. None of the two residues appear to be essential for Btk function in the regulation of IL-7 responsiveness. We therefore conclude that the control of Ig λ L chain usage, pre-B cell marker phenotype, and IL-7 responsiveness involve different downstream signaling pathways or molecules interacting with Btk.

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Chapter 2.3

Expression of the constitutively active Bruton's tyrosine kinase mutant E41K mimics pre-B cell receptor signalling in pro-B cells

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Expression of the constitutively active Bruton's tyrosine kinase mutant E41K mimics pre-B cell receptor signalling in pro-B cells

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Abstract

We have previously reported that Bruton's tyrosine kinase (Btk)-deficient mice showed impaired pre-B cell maturation, implicating Btk in the regulation of IL-7 responsiveness, cell surface marker expression and the activation of light chain gene rearrangements in pre-B cells. Although Btk-mediated signalling may be directly involved in all of these processes, a possibility remains that Btk is only required for the developmental progression of large pre-B cells into a small pre-B cells stage in which surface marker changes and L chain rearrangements are induced in a Btk-independent fashion. To address this question, we analysed the effects of the expression of the constitutively active Bruton's tyrosine kinase mutant E41K on pro-B cells. Expression of E41K-Btk in μ^- pro-B cells resulted in the induction of CD2 and CD25, as well as in significant κ light chain expression in the cytoplasm. We conclude that expression of the E41K-Btk mutant mimics pre-B cell receptor signalling in pro-B cells. Therefore, these findings indicate that Btk-mediated signalling directly induces cellular maturation and the initiation of L chain rearrangements in pre-B cells.

Introduction

The precursor B cell receptor (pre-BCR) is a key checkpoint in B cell development to monitor the expression of a functional Ig μ H chain (1-3). 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 signalling components. Pre-BCR expression is essential for the proliferative expansion of cytoplasmic μ H chain positive pre-B cells and the induction of progression into small pre-B cells in which Ig L chain rearrangement occurs (1-3). It is thought that the pre-BCR signals in a ligand-independent fashion, resulting in pre-BCR internalisation and the activation of various downstream signal transduction proteins. Mice deficient for pre-BCR components, such as μ, Ig-α, Ig-β, SLC or the Syk tyrosine kinase exhibit a specific block in B cell development at the pre-B cell stage, reflecting the inability of pre-B cells to proliferate and progress into large cycling pre-B cells. In contrast, disruption of (pre-)BCR signalling elements Bruton's tyrosine kinase (Btk) and SLP-65 (also known as BASH or BLNK) are crucially involved in the regulation of the developmental progression of pre-B cells by limiting the IL-7 driven expansion of large cycling pre-B cells (4, 5).

During the transition of large cycling into small resting cytoplasmic μ^+ pre-B cells in the mouse, Btk-deficient cells show defective down-regulation of SLC, the metallopeptidase BP-1 and the sialoglycoprotein CD43 and defective upregulation of the adhesion molecule CD2, the IL-2 receptor CD25 and MHC class II. Moreover, 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 of ~3 h, when compared with wild-type cells. In in vitro BM cultures, Btk-deficient large cycling pre-B cells

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Abbreviations: BCR, B cell receptor; Btk, Bruton's tyrosine kinase; SLC, surrogate light chain.

showed increased IL-7 driven expansion and reduced developmental progression into non-cycling $CD2^+CD25^+SLC^-$ small pre-B cells and subsequently into Ig^+ B cells (5). Furthermore, the finding of reduced λ L chain usage in Btk-deficient mice implicates Btk in the regulation of the activation of the λ locus for V(D)J recombination in pre-B cells (6).

Although these findings suggest that Btk is directly involved in the regulation of IL-7 responsiveness, cell surface marker expression and the activation of light chain gene rearrangements in pre-B cells, it remains possible that Btk is only required for the developmental progression of large pre-B cells into a small pre-B cells stage in which subsequently surface marker changes and L chain rearrangements are induced in a Btk-independent fashion. To distinguish between these two possibilities, we employed transgenic mice expressing the constitutive active Btk mutants E41K or E41K-Y223F to investigate whether Btk signalling can induce cell surface marker modulation and L chain rearrangements, independent of the differentiation stage, e.g. also in μ H chain negative pro-B cells.

The gain-of-function mutant E41K shows increased membrane localisation and phosphorylation in quiescent cells, independent of phosphatidyl-inositol 3-kinase (PI3K) activity (7, 8). Normally, Btk is targeted to the plasma membrane through interactions of its pleckstrin homology (PH) domain with the second messenger phosphatidyl-inositol-3,4,5-trisphosphate (PIP_3) generated by PI3K, which is activated by BCR engagement (9, 10). Upon BCR stimulation, Btk is transphosphorylated at Y551 in the SH3 domain, possibly by Syk or the Src-like kinase Lyn (11, 12). This phosphorylation promotes its catalytic activity, and subsequently results in Btk autophosphorylation at Y223. (12, 13). Concomitantly, Syk activation results in phosphorylation of the adapter molecule SLP-65, which allows the association of SLP-65 with the Btk SH2 domain and with phospholipase Cγ2 (PLCγ2) (14, 15). These interactions are critical to the activity of Btk and result in PLCγ2 tyrosine phosphorylation, inositol trisphosphate production and calcium mobilisation. It has been shown that E41K-Btk induces transformation of 3T3 fibroblasts in soft agar cultures, and this capacity is augmented by mutation of the main autophosphorylation site (Y223F; (7, 13)). Expression of E41K-Btk in Ramos B cells enhances the sustained increase in intracellular calcium following BCR cross-linking (16). In transgenic mice, which use either the B-cell specific CD19 promoter or the MHC class II Ea locus control region to express E41K-Btk, B cell development is arrested in the BM or the spleen, respectively (17, 18). These findings suggest that expression of the E41K-Btk mimics BCR occupancy by autoantigens. Moreover, we recently demonstrated that low-level expression of an E41K-Y223F-Btk double mutant transgene could partially rescue the pre-B cell arrest in SLP-65-deficient pre-B cells and prevent pre-B cell tumour formation in $SLP\text{-}65^{-/-}$ mice. This observation indicates that, in the absence of SLP-65, expression of E41K-Y223F-Btk enhances pre-BCR signalling (19).

To examine the effects of the E41K and E41K-Y223F Btk mutations in pro- and pre-BCR signalling in vivo, we analysed transgenic mice expressing these gain-of-function Btk mutants under the control of the CD19 promoter region. In addition to the mice described previously (18, 19), we generated mice that express various levels of the E41K-Y223F double mutant Btk and crossed these mice onto the Btk null background (20). In particular, we analysed the effects of these constitutively active Btk mutants on cell surface marker and L chain expression in cytoplasmic μ H chain negative pro-B cells.

Materials and Methods

Generation of Btk-transgenic mice. The WT and E41K transgenic mice have been previously described (18). The human Btk transgenes 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 (18). Using double stranded site-directed mutagenesis (Stratagene, La Jolla, CA) the Y223F mutation was introduced into the construct that was previously used to generate the $CD19\text{-}Btk^{WT}$ and $CD19\text{-}Btk^{E41K}$ mice (18). The Y223F mutation was created by replacement of AT by TC in exon 8. 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 fertilised oocytes. Transgenic founder mice were identified by Southern blotting and crossed with Btk null mice (20), which were bred onto a C57BL/6 background for >8 generations. Sequence fidelity of the mutated fragments was confirmed, both in plasmid DNA prior to

subsequent cloning steps and in genomic DNA obtained from the F_1 transgenic mice generated. Mice were bred and maintained at the Erasmus MC animal care facility under specific pathogen free conditions.

Mouse Genotyping. Tail DNA was analysed by Southern blotting of BamHI digests using a partial human Btk cDNA probe (bp 133-1153), as described previously (18, 20). Alternatively, the presence of Btk transgenes, endogenous mouse Btk WT or Btk KO alleles were evaluated by PCR, as described previously (19, 21).

Flow cytometric analyses. Preparations of single-cell suspensions, standard and intracellular FCM, and conjugated monoclonal antibodies (Becton Dickinson Pharmingen, Sunnyvale, CA) have been described previously (5, 20). The anti-SLC hybridoma LM34 ((22) was kindly provided by A. Rolink, Basel Institute for Immunology, Basel, Switzerland); antibodies were purified using protein G columns and conjugated to biotin according to standard procedures. In vivo 5'-bromo-2'deoxyuridine (BrdU) labelling and FCM BrdU detection has been previously described (5). Mice were analysed at the age of 8-16 weeks. Transgenic Btk expression levels were quantified, using median fluorescence intensity (MFI) values obtained by CellQuest software (BD), whereby values for transgenic human Btk and endogenous murine Btk were corrected for background fluorescence, as detected in Btk null mutant mice. Subsequently, the ratio of the MFI values of transgenic Btk over endogenous Btk was calculated.

In vitro (pre-)B cell cultures. IL-7 driven BM cultures and determination of IL-7 dependent proliferative responses of total BM cells have been described previously (5, 6).

Results

Constitutively active Btk induces a developmental arrest at the immature B cell stage. We previously reported the generation of transgenic mice, which express wild-type (WT), Y223F (n=5 independent lines), E41K (n=3 independent lines), and E41K-Y223F (n=1) human Btk under the control of the CD19 promoter region (18). We now generated three additional E41K-Y223F transgenic lines, expressing higher levels of this double mutant and crossed these mice onto the Btk null background.

To determine the effect of expression of the individual Btk mutations on B cell development, we examined the size of the individual B-lineage subpopulations in the BM by FCM. Figure 1A shows that Btk-deficiency mainly affects the $B220^{high}$ population of mature recirculating cells, which is reduced in size and has an IgM^{high} phenotype (5, 20, 23). As reported previously (18, 21), transgenic expression of WT-Btk or Y223F-Btk corrected this defect, while expression of the E41K-Btk mutant resulted in an almost complete block of B cell development at the transition of IgM^{low} to IgM^{high} $B220^{low}IgD^{-}$ immature B cell stage in the BM (Figure 1A). We now observed that expression of the E41K-Y223F-Btk double mutant was also associated with an arrest at the immature B cell stage.

The extent of B cell deletion in the E41K single and the E41K-Y223F double mutants varied between the individual transgenic lines generated (Figure 1B). A correlation was observed with the expression levels of the Btk transgenes, as quantified in the immature B cell population by comparing median fluorescence intensities of transgenic human Btk with endogenous Btk (21) (Figure 1C). Differences in expression levels of transgenic Btk protein were confirmed by western blotting analysis of BM and spleen cell suspensions, in which Btk protein was visible as a single ~77 kDa band for all transgenic lines generated (data not shown). These results demonstrate that the effects of the E41K and the E41K-Y223F mutants are dose-dependent and that deletion of IgM^{high} immature B cells in the BM already occurs when mutant Btk is expressed at low physiological levels (~20-40% of normal in immature B cells).

Since we aimed to investigate the role of Btk in early B cell development, we focused on those transgenic lines that expressed physiological levels of transgenic Btk in immature B cells in the BM. However, as expression of transgenic Btk increased as B cell progenitors matured, these transgenic lines, LF-03 (E41K) and AM-06 (E41K-Y223F), expressed low Btk levels in pro-B/pre-B cells (~20-60% of normal) and high levels in mature peripheral B cells (up to 4 times normal values).

Deletion of immature B cells in E41K-Btk mice was further substantiated in kinetic studies with the thymidine analogue BrdU, which is specifically incorporated into the DNA of large cycling cytoplasmic μ^+ pre-B cells (24, 25). BM cells were stained with an Ab specific for BrdU, in conjunction with cell surface marker analysis for B220, IgD and κ L chain. At various time points after injection, the proportions of the pre-B cell

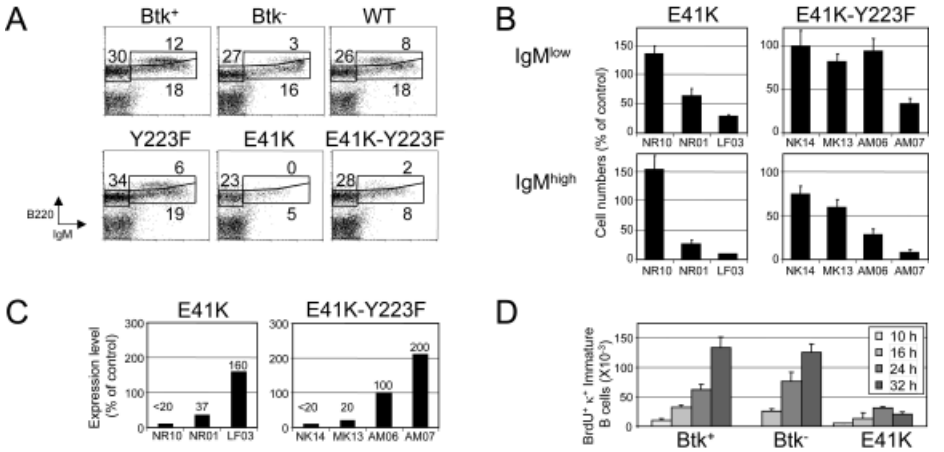

Figure 1. *B cell developmental arrest in transgenic mice expressing constitutively active Btk. (A) Flow cytometric analysis of surface expression of IgM and B220 on total lymphocytes in the BM, which were gated on the basis of forward and side scatter characteristics. Percentages of total lymphocytes within the indicated gates are given. Data shown are representative of 3-20 animals examined within each group. (B) Dose-dependent decrease of the IgM^{low} and IgM^{high} immature B220^{low}IgD^{-} B cell subpopulations in E41K and E41K-Y223F transgenic mice. Absolute cell numbers of the two immature B cell subpopulations in Btk^{+} control mice were set to 100%. Error bars are SEM values from 4-11 mice per group. (C) Tg expression levels in immature B cells, as determined by FCM. B220^{+}IgM^{+}IgD^{-} cells were analyzed for intracellular Btk expression and the differences in median fluorescence intensities between the indicated mice and background values (in Btk^{-} mice) were calculated and compared to endogenous Btk expression in wild type mice, which was set to 100%. (D) Production of κ^{+} immature B cells over time, as calculated from the total numbers of κ^{+} immature B cells and the percentages of BrdU^{+} cells in this population. Error bars are SEM values from 3-9 mice per group. As the transit time through the small pre-B cell stage is ~11 hrs in Btk^{-} mice (as compared with ~8 hrs Btk^{+} mice), no significant population of BrdU^{+} κ^{+} immature B cells was found in Btk^{-} mice 10 hrs after BrdU injection.*

subpopulations that incorporated BrdU were similar in the Btk^{+}, Btk^{-} and E41K-Btk mice (data not shown), indicating that pre-B cell expansion is comparable in the three groups of mice. However, the absolute numbers of κ^{+} immature B cells generated in the BM of E41K-Btk mice were limited and decreased after 24 hours (Fig. 1D), indicating a short life span of E41K-Btk immature B cells, consistent with the arrest of B cell development found at this stage.

Collectively, these findings show that expression of the E41K-Btk, either on its own or in combination with the Y223F mutation, resulted in a dose-dependent arrest of B cell development at the immature B cell stage in the BM.

Constitutively active Btk corrects pre-B cell maturation defects in Btk-deficient mice. We previously showed that during the developmental progression of large cycling into small resting cytoplasmic μ^{+} pre-B cells, Btk-deficient cells fail to efficiently downregulate the expression of BP-1 and SLC and upregulate the expression of surface CD2 and CD25/IL2-R (5). To analyse whether transgenic expression of E41K-Btk or E41K-Y223F-Btk could correct these defects, we analysed the SLC, BP-1, CD2 and CD25 expression profile in pre-B cells from Btk mutant and control mice. We performed four-color FCM experiments of BM cell suspensions, using monoclonal antibodies to B220 and IgM or Ig κ L chain, together with various additional antibodies. As shown in Fig. 2, Btk-deficient pre-B cells have significantly higher levels of cytoplasmic SLC and BP-1, but lower expression levels of CD2 and CD25. This aberrant marker profile was fully corrected in transgenic mice expressing WT, E41K or E41K-Y223F double mutant Btk (Fig. 2). Complete reconstitution was also found in mice with low-level transgene expression, such as the E41K-Btk line NR01 or the E41K-Y223F-Btk line MK13 (data not shown).

In summary, these findings demonstrate that the *in vivo* function of Btk as a regulator of cellular maturation of pre-B cells is neither enhanced nor reduced by the E41K or E41K-Y223F mutation.

Constitutively active Btk supports IL-7 driven expansion of pro-B cells in vitro. Btk has an inhibitory effect on the proliferation of pre-B cells

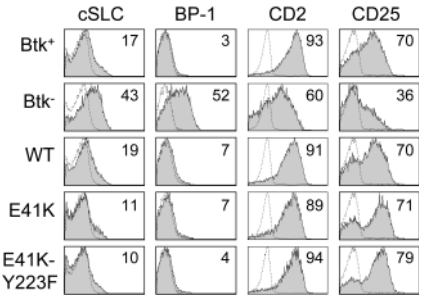

***Figure 2**. Phenotypic characterization of pre-B cells in Btk mutant mice. Expression profiles of cytoplasmic SLC, surface BP-1, CD2 and CD25 in B220$^+$IgM$^-$ pre-B cell subpopulations of the indicated mice. Data are displayed as filled histograms, with background stainings of B220$^-$ cells (dashed lines) as negative controls. The percentages of positive cells are indicated. Data shown are representative of 3-20 animals examined within each group. Cell populations analysed were cytoplasmic μ^+ surface IgM$^-$ pre-B cells (for CD2 and SLC) or total surface IgM$^-$ pro-B and pre-B cells (for BP-1 and CD25).*

in long-term Whitlock-Witte BM cultures and in IL-7 driven cultures of BM or fetal liver cells (5, 26, 27). In [^{3}H] thymidine incorporation experiments after 5 days of culture in the presence of IL-7, Btk-deficient total BM cells showed significantly higher proliferative responses when compared with Btk$^+$ cells. This defect was largely corrected when E41K-Btk or E41K-Y223F-Btk was expressed (Fig. 3A).

To analyse the effects of constitutive active Btk in IL-7 driven BM cultures in more detail, total BM cell suspensions from the panel of mutant mice were cultured in the presence of 100 U/ml IL-7 and at day 5, the distribution of B220$^+$ cells over six developmental fractions, μ^- pro-B cells, μ^+ large cycling pre-B cells, CD2$^-$ μ^+ small pre-B cells, CD2$^+$ μ^+ small pre-B cells, IgM$^+$IgD$^-$ immature B cells and IgM$^+$IgD$^+$ mature B cells, was evaluated by FCM (Fig. 3B). Consistent with our previous findings (5), we noticed that in the absence of Btk (i) growth of cytoplasmic μ^- cells is hardly supported, (ii) progression from the CD2$^-$ into the CD2$^+$ pre-B cell stage was impaired, and (iii) the proportions of IgM$^+$ immature and mature B cells were reduced, when compared with wild-type mice. The BM cultures from WT and E41K-Y223F (line AM06) transgenic mice exhibited a subpopulation distribution that was similar to that in Btk$^+$ control mice.

In contrast to the selective outgrowth of cytoplasmic and surface μ^+ cells generally found in IL-7 BM cultures (5, 28, 29), we noticed substantial growth of cytoplasmic μ^- pro-B cells in cultures from E41K and E41K-Y223F mutant mice (Fig. 3B). This phenomenon was dose dependent, as BM cultures from the mouse strains expressing lower levels of E41K-Btk or E41K-Y223F (e.g. NR01 or AM06) contained pro-B cell fractions similar to cultures from wild-type mice (data not shown). The preferential outgrowth of μ^- pro-B cells in E41K-Btk or E41K-Y223F BM cultures reflected an increased expansion of μ^- pro-B cells in absolute numbers and was observed both at high (100U/ml) and low (10U/ml) IL-7 concentrations (Shown for E41K-Btk in Fig. 3C). As it has been reported that the expression of an Ig H chain transgene increases the IL-7 responsiveness of μ^- pro-B cells (29), our findings are consistent with the hypothesis that expression of constitutively active Btk can mimic pre-BCR signalling in this respect.

To investigate whether E41K-Btk expression would also support the expansion of pro-B cells *in vivo*, we crossed E41K-Btk mice with RAG-1$^{-/-}$ mice, in which B cell development is arrested at the CD43$^+$B220$^+$ pro-B cell stage. However, the size of the CD43$^+$B220$^+$ pro-B cell populations in E41K-Btk *RAG-1$^{-/-}$* and non-transgenic *RAG-1$^{-/-}$* mice were comparable (data not shown), indicating that E41K-Btk expression does not signal substantial proliferation of pro-B cells *in vivo*.

E41K-Btk supports premature cell surface marker changes and L chain expression in μ^- pro-B cells. To examine the ability of activated Btk to mimic pre-BCR activation and consequently signal premature cellular maturation of cytoplasmic μ^- pro-B cells *in vivo*, we compared CD19$^+$B220$^+$ cytoplasmic μ^- pro-B cells in the BM from Btk$^+$, Btk$^-$, E41K-Btk, and E41KY223F mutant mice. In these cells, we analysed the expression of SLC and κ L chain in the cytoplasm, and CD2, CD25, MHC class II and BP-1 on the cell surface (Fig. 4). E41K-Btk pro-B cells showed reduced expression of SLC and increased levels of CD2, CD25, MHC class II and κ L chain, when compared with Btk$^+$ or Btk$^-$ pro-B cells (Fig. 4). In all four groups of mice, the pro-B cells lacked significant BP-1 expression. A similar effect on the expression of CD2, CD25, MHC class II and κ L chain in pro-B cells was also observed in mice expressing high levels of the E41K-Y223F double mutant (AM-07), but not in WT-Btk mice (data not shown). These phenotypic marker changes induced by E41K-Btk in pro-B

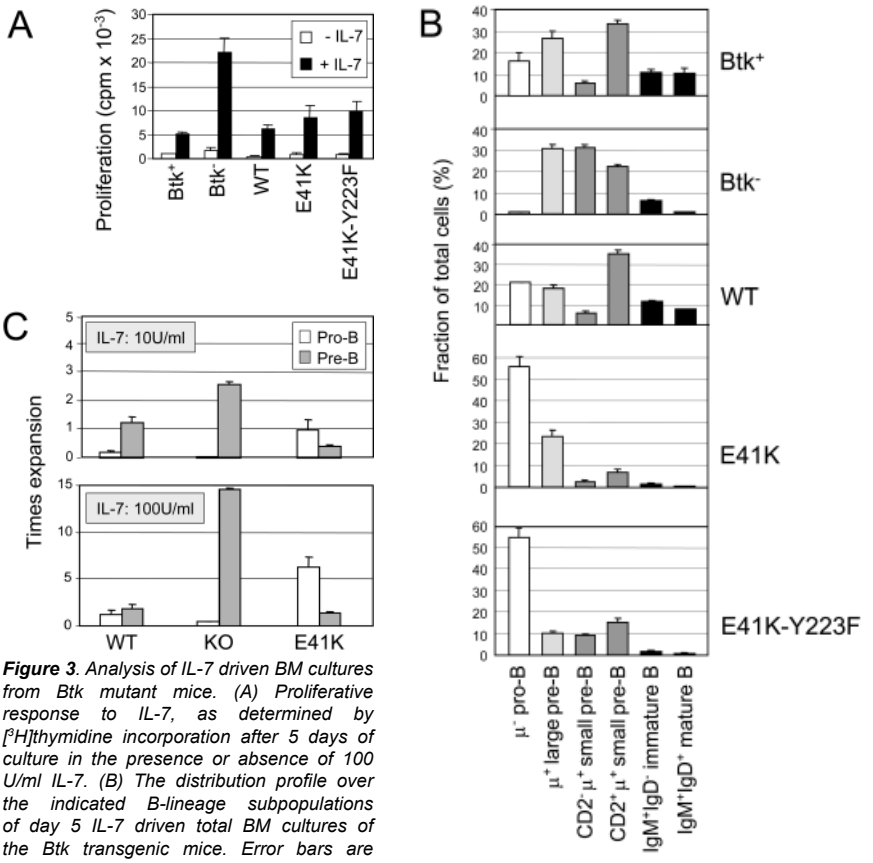

***Figure 3**. Analysis of IL-7 driven BM cultures from Btk mutant mice. (A) Proliferative response to IL-7, as determined by [^{3}H]thymidine incorporation after 5 days of culture in the presence or absence of 100 U/ml IL-7. (B) The distribution profile over the indicated B-lineage subpopulations of day 5 IL-7 driven total BM cultures of the Btk transgenic mice. Error bars are SEM values from 3-7 mice per group. (C) Expansion of cytoplasmic μ^- pro-B and $c\mu^+$ pre-B cells during culture with 10 or 100 U/ml IL-7. The bars indicate the expansion at day 5, as compared with the IgM$^-$ B220$^+$ pro/pre-B cell numbers at the start of the culture, which were set to one.*

cells reflected the phenotypic changes that are normally induced by pre-BCR signalling during the developmental progression of large cycling to small resting pre-B cells. These findings therefore indicate that expression of E41K-Btk or E41K-Y223F can mimic pre-BCR signals.

Discussion

Several lines of evidence show that the E41K gain-of-function mutation, which is associated with enhanced Btk membrane localization, activation and calcium-signalling in cultured cell lines (7, 8, 13, 16), represents an activated form of Btk in murine B cells *in vivo*. Expression of E41K (a) enhances blast formation of splenic B cells in culture (17), (b) drives peripheral B cells efficiently into IgM plasma cell differentiation *in vivo* (18), (c) induces the expression of the early activation antigen CD69 on peripheral B cells (R.W.H., unpublished), and (d) significantly increases Ig λ usage (6). In addition, unstimulated E41K-Y223F-Btk expressing mature splenic B cells showed increased tyrosine phosphorylation (19) and the presence of E41K-Y223F-Btk prevented tumour formation in SLP-65-deficient pre-B cells. Apparently, E41K-Y223F-Btk breaks the positive feedback loop of SLP-65-deficient large cycling pre-B cells which are arrested in development and cannot efficiently down-regulate pre-BCR expression, which signals for their proliferation (19).

In this report, we found that E41K-Btk and E41K-Y223F-Btk had no additional effects in pre-B cells, in terms of the cell surface marker

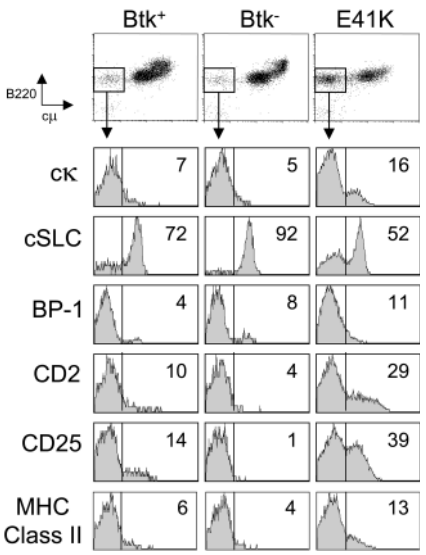

***Figure 4**. E41K-Btk induces phenotypic changes in cytoplasmic μ⁻ pro-B cells. Expression profiles of the indicated markers in pro-B cells of Btk⁺, Btk⁻ and E41K-Btk mice. Flow cytometric analysis of surface B220 and cytoplasmic μ H chain expression on total CD19⁺ B-lineage cells (upper part). CD19⁺B220⁺cμ⁻ pro-B cells were gated and analyzed for the expression of cytoplasmic κ L chain and SLC, and surface BP-1, CD2, CD25 and MHC class II (lower part). The results are displayed as filled histograms in which the percentages of positive cells are indicated. Data shown are representative of 7-11 animals examined within each group.*

profile changes or proliferative response to IL-7. The finding of similar effects of E41K-Btk, E41K-Y223F-Btk and wild-type Btk would be consistent with the hypothesis that the pre-BCR signals in constitutively active way (1-3, 30). In contrast, E41K-Btk or E41K-Y223F-Btk expression in μ⁻ pro-B cells signalled their developmental progression *in vivo* in terms of cell surface expression of CD2 and CD25 and L chain rearrangements, all of which are normally induced upon pre-BCR signalling. Y223 is located on the surface of the SH3 domain and its mutation dramatically potentiates the transforming activity of E41K-Btk in fibroblasts (13). However, we were unable to detect any *in vivo* potentiating effects of the Y223F mutation on the phenotype of E41K-Btk mice, when combined in E41K-Y223F-Btk double mutant mice. This further supports our previous conclusion that Y223 autophosphorylation-dependent interactions are not essential for Btk function during B cell development (21).

We found that ~16% of all E41K-Btk pro-B cells expressed detectable levels of κ L chain proteins in the cytoplasma. Since only 1 out of 3 L chain rearrangements will be productive (and assuming that in the absence of Ig H chain μ protein κ⁺ and κ⁻ pro-B cells do not manifest differences in survival, expansion or differentiation capacities) we conclude that V-to-J rearrangements will be present in approximately half of the total pro-B cell fraction in E41K-Btk mice. It is currently not clear whether E41K-Btk and E41K-Y223F signalling affect V(D)J recombination activity, κ locus accessibility or both. However, it is tempting to speculate that the transcription factor IRF-4 acts as a downstream target of Btk, as IRF-4 was recently shown to be important for the down-regulation of pre-BCR expression in large cycling pre-B cells and for the induction of germ-line Ig κ transcription in Abelson-transformed pre-B cell lines (31, 32). Given that κ transcription is also impaired in SLP-65⁻/⁻ immature B cells (33), further experiments should demonstrate the connection between Btk/SLP-65 signalling, IRF-4 and Ig κ rearrangements.

We conclude that E41K-Btk and E41K-Y223F-Btk reflect constitutively active forms of Btk that mimic pre-BCR signalling in pro-B cells, resulting in the induction and successful completion of Ig κ L chain rearrangements as well as surface expression of the maturation markers CD2 and CD25. It is therefore unlikely that in the pre-BCR checkpoint Btk-mediated signalling is only required for the progression of pre-B cells into a stage in which surface marker changes and L chain rearrangements are induced in a Btk-independent fashion. In contrast, our findings strongly support the hypothesis that Btk-mediated signalling directly induces cellular maturation and the initiation of L chain rearrangements in pre-B cells.

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Chapter 3

Btk and SLP-65 deficiency

Chapter 3.1

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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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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Abstract

Expression of the pre-B cell receptor (pre-BCR) leads to activation of the adaptor 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 wk of age), as compared with SLP-65 single deficient mice (<10%). These findings demonstrate that Btk cooperates with 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.

Key Words: Btk • lymphoma • precursor-B cell • SLP-65/BLNK • tumor suppressor

Introduction

B lymphocytes develop in the BM through distinct stages that are characterized by differential expression of various cell surface markers and the ordered rearrangement of 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 nonrearranging VpreB and λ5 surrogate light chain (SLC) proteins and the Igα/CD79a and Igβ/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,

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Abbreviations used in this paper: BCR, B cell receptor; Btk, Bruton's tyrosine kinase; SLC, surrogate light chain.

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 nuclear factor (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 adaptor 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 WT 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 down-regulation 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 WT cells (14, 16-17). 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^+ preB cell stage in SLP-65/Btk double mutant mice (18).

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 preB 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'-CACTGAAGCTGAGGACTCCATAG-3') and an exon 10 reverse primer (5'-GAGTCATGTGCTTGGAATACCAC -3'). For Btk KO alleles, primers were within the LacZ reporter (20), forward: 5'-TTCACTGGCCGTCGTTTTAC AACGTCGTGA-3', and reverse: 5'-ATGTGAGC GAGTAACAACCCGTCGGATTCT-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'-TCAAACCTG GGTCTCAGAA-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'-AGATGCCAGGACTTGG AAGG-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) 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.).

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) have been described previously (14, 20). The anti-SLC hybridoma LM34 (23) was kindly provided by A. Rolink (University of Basel, 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) 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 Laboratories) in RPMI1640 at 37°C for 5 min. Western blotting was performed as described (20), using anti-Btk C-20, anti-Erk1/2 SC-094 (Santa Cruz Biotechnology, Inc.), or anti-phosphotyrosine P-Tyr-100 (Cell Signaling Technology).

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, 18). 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 (reference 18; Fig. 1 A). 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 down-regulation of the pro-B/large pre-B stage-specific markers SLC, the sialoglycoprotein CD43, and the metallopeptidase BP-1 and the up-regulation of CD2 and CD25/interleukin-2 receptor, which are first expressed on small preB 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 WT, Btk-deficient, SLP-65-deficient, and double mutant mice by flow cytometry. As 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 down-regulation of CD43, BP-1, and SLC and in the up-regulation of CD25, CD2, and MHC class II expression (Fig. 1 B). Consistent with the reported almost complete arrest at the pre-BCR$^+$ stage (18), 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 (Fig. 1 B).

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

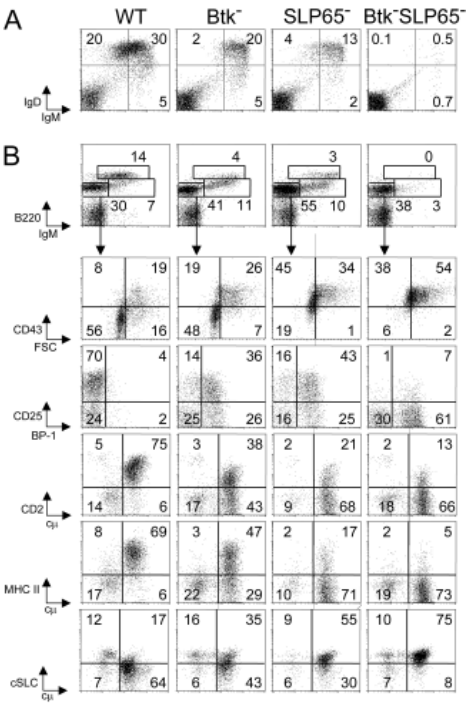

Figure 1. *Impaired pre-B cell maturation in Btk-deficient, SLP-65-deficient, and Btk/SLP-65 double mutant mice. (A) Flow cytometric analysis of surface IgM/IgD expression on total lymphoid cells in the spleen. (B) Expression profiles of B220 and IgM on total lymphoid cells in the BM (top). The B220$^+$IgM$^-$ 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 four mice examined within each group.*

single mutants and the double mutant, these three groups of mice showed similar [^{3}H]thymidine incorporation values after 5 d of culture in the presence of 100 U/ml IL-7 (Fig. 2 A). When total BM cell suspensions from WT mice were cultured in the presence of IL-7 for 7 d, 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 (Fig. 2 B, thin lines). In contrast, the IL-7 driven BM cultures from Btk and SLP-65 single or double mutant mice consisted almost exclusively of large μ^+ SLC$^+$ pre-B cells which did not express κ L chains in their cytoplasm (Fig. 2 B, thin lines).

To analyze the differentiation capacity of pre-B cells, BM cells were cultured in the presence of IL-7 for 5 d and subsequently for 2 d on S17 stroma cells in the absence of IL-7. Under these conditions, WT 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 d, a difference between SLP-65 single mutant and SLP-65/Btk double mutant mice was noticed (Fig. 2 C). At low IL-7 concentrations, preB cell division was limited and cells rapidly differentiated into surface IgM$^+$ B cells. Within the population of small noncycling B220$^+$ cells, the percentage of surface IgM$^+$ cells was lower in SLP-65/Btk double mutant mice, when compared with 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 WT pre-B cells. The ability to down-regulate 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 wk of age (16). When we followed a panel of SLP-65 single mutant mice up to 16 wk, ~5% (3 out of 66) developed a lymphoma. Remarkably, when SLP-65/Btk double mutant mice were followed for 16 wk, 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

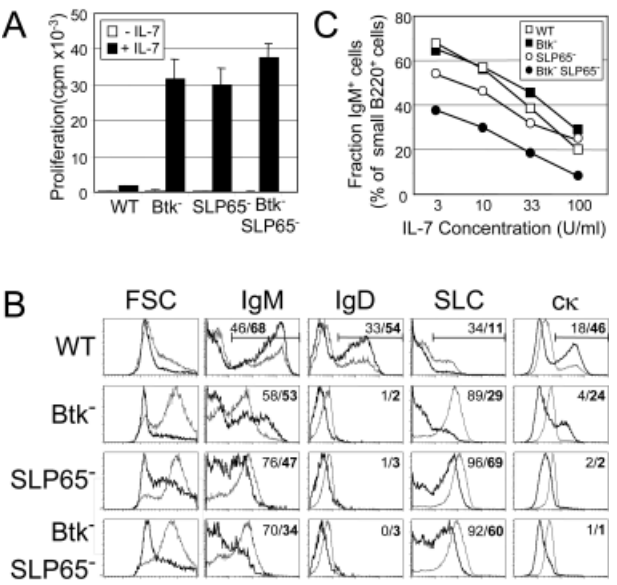

Figure 2. *Analysis of IL-7 driven BM cultures from Btk and SLP-65 mutant mice. (A) Proliferative response to 100 U/ml IL-7, as determined by [³H]thymidine incorporation after 5 d of culture. Bars represent mean cpm and SEM of triplicate cultures. (B) Forward scatter (FSC) values and expression profiles of IgM, IgD, SLC, and cytoplasmic κ L chain 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 100 U/ml IL-7 for 7 d, thin lines) or under differentiating conditions (after 5 d of culture with IL-7 and subsequently without IL-7 for 2 d, 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 d of culture in the presence of the indicated concentrations of IL-7. Data are representative of four mice per group.*

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 WT or Btk-deficient mice (less than 1/4,000 mice). The Kaplan-Meier tumor-free survival curves for the SLP-65 single and SLP-65/Btk double mutant mice are shown in Fig.3 A.

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 preB cells.

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; reference 28). In Ramos B cells expression of E41K-Btk enhances the sustained increase in intracellular [Ca^{2+}] after BCR cross-linking (29). Thus, the E41K mutant and the E41K-Y223F double mutant represent activated

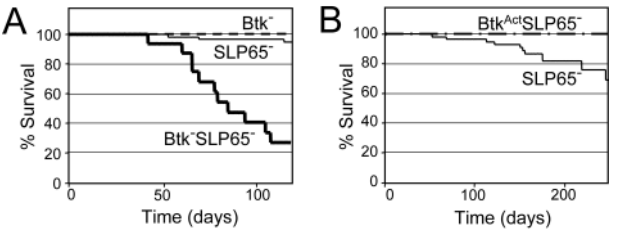

Figure 3. *(A) Kaplan-Meier tumor-free survival estimates for Btk-deficient (dotted line), SLP-65$^{-/-}$ (thin line), and Btk^{-}SLP-65$^{-/-}$ mice (bold line). Tumor-free survival in Btk^{-}SLP-65$^{-/-}$ mice was significantly reduced ($P < 0.0001$ by log-rank) compared with SLP-65$^{-/-}$ mice. (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 with SLP-65$^{-/-}$ mice.*

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 (unpublished data). 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; Fig. 4, A-C).

Btk^{Act} mice were crossed onto the Btk/SLP-65 double mutant background and a panel of 20 transgenic mice was followed for 8 mo. At this age, the fraction of SLP-$65^{-/-}$ mice that developed a preB cell lymphoma increased to 18% (12 out of 66; Fig. 3 B). In contrast, none of the Btk^{Act} transgenic mice on the Btk/SLP-65 double mutant background developed lymphoma (Fig. 3 B). When these Btk^{Act} mice were killed at 8 mo, 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 Btk^{Act} 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 kD band (Fig. 4 A).

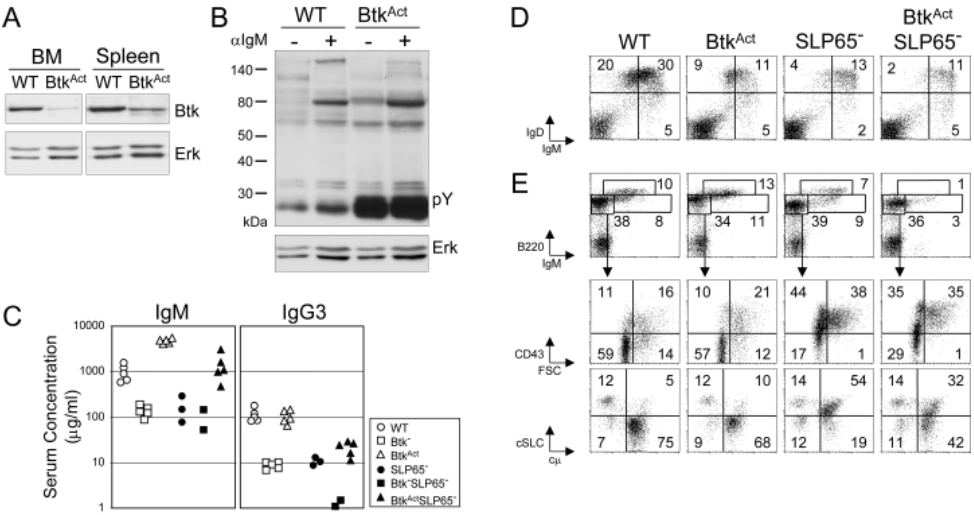

Figure 4. *Low-level Btk^{Act} expression can partially substitute for the absence of Btk and SLP-65. (A) Western blotting analysis of Btk expression in WT and Btk^{Act} B cells from BM and spleen. Membrane was reblotted with anti-Erk. (B) Protein tyrosine phosphorylation in extracts of untreated and anti-IgM stimulated WT or Btk^{Act} splenic B cells, analyzed by immunoblotting with a phosphotyrosine (pY)-specific antibody. Membrane was reblotted with anti-Erk. (C) Serum concentrations of IgM and IgG3 in the indicated mutant mouse strains. Mice were 2 mo 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 four mice examined within each group.*

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 (unpublished data). 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 (Fig. 4 B).

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-mo-old Btk^{Act} mice, both on a Btk-deficient and on a Btk/SLP-65 double-deficient background, with nontransgenic WT, Btk^{-}, $SLP\text{-}65^{-/-}$, 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 (Fig. 4 C). 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 WT 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 (unpublished data). Expression of Btk^{Act} completely corrected IgM levels and significantly restored IgG3 levels in Btk/SLP-65 double mutant mice (Fig. 4 C).

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 (Fig. 4 D). 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 Figs. 1 A and 4 D). 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 down-regulation of CD43 and SLC expression and the up-regulation of CD2, CD25, and MHC class II expression (unpublished data; shown for CD43 and SLC in Fig. 4 E). In $SLP\text{-}65^{-/-}$ mice, substitution of endogenous Btk by very low levels of constitutively activated Btk was associated with a reduction of the percentages of SLC^{+} and $CD43^{+}$ $c?^{+}$ pre-B cells (Fig. 4 E).

Taken together, comparison of $SLP\text{-}65^{-/-}$ and Btk^{Act} $SLP\text{-}65^{-/-}$ 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 $SLP\text{-}65^{-/-}$ mice (Fig. 5 A). The two $Btk^{+/-}$ $SLP\text{-}65^{-/-}$ 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

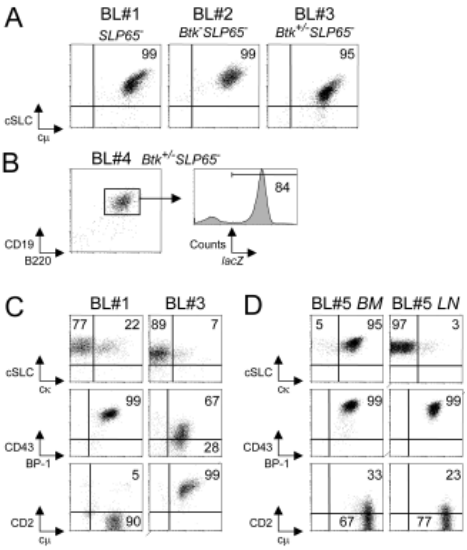

Figure 5. *Characterization of pre-B cell tumors by flow cytometry. (A) Dot plots for cytoplasmic SLC and μ H chain in gated $B220^{+}$ cells from tumor samples from the indicated mice, grown for 1 to 3 wk in the presence of IL-7. (B) Flow cytometric analysis of lacZ expression in gated $CD19^{+}B220^{+}$ pre-B lymphoma cells in a lymph node from a $Btk^{+/-}SLP\text{-}65^{-/-}$ 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 d.*

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 (Fig. 5 B).

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 coexpressed κ L chain and SLC in their cytoplasma and showed variable surface expression of the developmentally regulated markers, such as CD43 and CD2 (Fig. 5 C). 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 (unpublished data). 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 (Fig. 5 D).

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 (a) Btk and SLP-65 have synergistic roles in the developmental progression of large cycling into small resting pre-B cells, (b) the concomitant deficiency of Btk significantly enhanced tumor formation in SLP-65$^{-/-}$ mice, and (c) 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 coexpression 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 preB 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 (unpublished data). As 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 3.2

Tumor suppressor function of Btk in SLP-65-deficient pre-B cells is independent of its kinase activity

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To be submitted

Tumor suppressor function of Btk in SLP-65-deficient pre-B cells is independent of its kinase activity

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Abstract

Bruton's tyrosine kinase (Btk) and the B cell linker protein SLP-65 have a synergistic role at the transition of large cycling into small resting pre-B cells. SLP-65/Btk double deficient mice manifest an almost complete block in B cell development at the large cycling pre-B cell stage, which is associated with a high incidence of pre-B cell lymphoma. We have recently shown that Btk partially acts as an adapter molecule, independent of its catalytic activity. Here, we investigated whether Btk adapter function is dependent on the presence of SLP-65, which in this context might serve as a scaffold molecule to bring other kinase proteins in close proximity to Btk and its substrates. To this end, we crossed transgenic mice expressing the kinase-inactive K430R-Btk mutant onto a SLP-65-deficient background. We observed that kinase-inactive Btk was able to rescue the severe developmental arrest at the pre-B cell stage present in SLP-65/Btk double deficient mice, indicating that SLP-65 is not required for Btk adapter function. Moreover, expression of kinase-inactive Btk significantly reduced the high frequency of pre-B cell lymphoma found in SLP-65/Btk double deficient mice. Therefore, we conclude that in SLP-65-deficient pre-B cells Btk exerts its tumor suppressor function as an adapter protein.

Introduction

A mature B cell receptor (BCR) is composed of two Immunoglobulin (Ig) heavy (H) and two light (L) chains. During B cell development, first the μ H chain is rearranged in pro-B cells and together with the non-rearranging VpreB and λ5 surrogate light chain (SLC) proteins and the Ig-α/CD79a and Ig-β/CD79b signalling components, the pre-BCR complex is formed. Expression of a pre-BCR on the cell surface acts as a checkpoint to monitor the expression of a functional Ig μ H chain, resulting from successful V(D)J recombination [1,2]. The pre-BCR is transiently expressed on the cell surface and is essential for the induction of proliferative expansion of pre-B cells and their developmental progression into small pre-B cells in which Ig L chain rearrangement occurs. It appears that the pre-BCR has a ligand-independent signal transducing capacity which involves the formation of a lipid raft-associated signalling module, composed of the tyrosine phosphorylated proteins Lyn, Syk, B cell linker protein SLP-65 (also known as BLNK or BASH), phosphoinositol 3-kinase (PI3K), Bruton's tyrosine kinase (Btk), Vav and phospholipase Cγ2 (PLCγ2) [3-5]. Activation of PLCγ2 induces hydrolysis of phosphatidylinositol bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG), which activate calcium signalling and protein kinase C (PKC), respectively.

By analogy with the BCR in mature B cells, Btk is most likely phosphorylated in pre-B cells by Syk

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Abbreviations used in this paper: BCR, B cell receptor; BM, bone marrow; Btk, Bruton's tyrosine kinase; SLC, surrogate light chain.

or the Src-family tyrosine kinase Lyn and recruited to the membrane by binding of the Btk-PH domain to the PI3K product phosphatidylinositol trisphosphate (PIP3) [6]. In this signalling pathway SLP-65, when phosphorylated by Syk, plays an essential role as it provides docking sites for Btk as well as PLCγ2, thereby bringing Btk in close proximity with PLCγ2. This model was recently challenged by the observation that in mature B cells Btk is essential for the recruitment of phosphatidylinositol phosphate 5-kinases (PIP5K) to the membrane [7]. Activation of PIP5K leads to local PIP2 synthesis by phosphorylation of phosphatidylinositol phosphate (PIP). As PIP2 is the substrate for both PLCγ2 and PI3K, Btk was found to be the regulator of a positive feedback loop by providing the substrate for both its up- and downstream targets [7].

Although Btk phosphorylates and activates PLCγ2 [8], several lines of investigation have shown that Btk can also act as adaptor molecule independent of its catalytic activity. Expression of kinase-inactive Btk mutants have been shown to partially [7,9] or fully [10] reconstitute BCR-induced calcium mobilization in Btk-deficient chicken DT40 and human A20 mature B cell lines. In addition, phosphorylation of PLCγ2 upon BCR stimulation appears to be unaffected in human Btk-deficient B cell lines [11]. More recently, we have shown that transgenic expression of the kinase-inactive K430R-Btk mutant in vivo partially restored Btk-dependent defects in the transition of large into small pre-B cells in BM of Btk-deficient mice [12]. Furthermore, the association of Btk with PIP5K was shown to be independent of the enzymatic activity of Btk in A20 B cells that were infected with viruses expressing kinase-dead Btk [7].

Recently we and others have shown that both SLP-65 and Btk 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 [13-17]. A synergistic role of SLP-65 and Btk was demonstrated by the almost complete block in B cell development at the pre-BCR$^+$ pre-B cell stage in SLP-65/Btk double deficient mice, compared to the partial block at this stage in SLP-65 or Btk single mutant mice [18-20]. It was reported that SLP-65$^-$ mice spontaneously develop pre-B cell lymphomas expressing high levels of the pre-BCR on the cell surface [19,21]. Although Btk-deficient mice do not develop pre-B cell tumors, we recently found that Btk cooperates with SLP-65 as a tumor suppressor as the incidence of pre-B cell lymphomas was significantly higher in SLP-65/Btk double mutant mice (~75% at 16 weeks of age), when compared with SLP-65 single deficient mice (<10%). Moreover, transgenic expression of the constitutive active E41K-Y223F Btk mutant, which shows enhanced membrane localisation [22,23], prevented tumor formation in Btk/SLP-65 double deficient mice [20].

The mechanism by which Btk exerts its function in pre-B cells independent of its catalytic activity is currently unknown. Kinase-inactive Btk may act as an adapter protein by using SLP-65 as a scaffold molecule to bring other tyrosine kinase proteins, such as Syk, in close proximity with PLCγ2. Alternatively, it is possible that the adapter function of Btk is independent of SLP-65, since activated kinase-inactive Btk has the capacity to recruit PIP5K to the membrane, thereby activating the positive feedback loop for synthesis of the PLCγ2 substrate PIP2. In this study, we investigated if SLP-65 is required for the adapter function of Btk and investigated developmental progression of pre-B cells and the frequency of pre-B cell tumor formation in K430R-Btk transgenic mice on a Btk/SLP-65 double deficient background.

Materials and Methods

Mice and genotyping. Btk-deficient mice [24] and SLP-65-deficient mice [13] were on the C57BL/6 and Balb/c background, respectively. WT or KO alleles were identified as described previously [17, 20]. The presence of the Btk-K430R transgene was evaluated by PCR [12].

Cell culture and Flow cytometry. Preparations of single-cell suspensions, primary pre-B cell BM cultures, determination of IL-7 dependent proliferative responses of total BM cells and standard and intracellular flow cytometry have been described previously [17,24,25]. The hybridoma LM34 (anti-SLC; [26]) was kindly provided by A. Rolink (University of Basel, Basel, Switzerland).

Ig detection in serum. Levels of Ig subclasses in serum were measured by sandwich ELISA, as described previously [27].

Western blotting. BM cells from SLP-65-deficient mice were cultured for 5 days in the presence of 100U/ml IL-7 (Sigma-Aldrich, St. Louis, MO). Cells from SLP-65$^{-/-}$ pre-B cell lymphomas were cultured in IMDM/10% FSC. 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. Lysates of unstimulated or stimulated cells were blotted with anti-phosphotyrosine (α-pTyr-100, Cell Signaling Technology, Beverly, MA) or incubated overnight at 4°C with α-pTyr-100 before samples were blotted using standard SDS-PAGE procedures [28]. Anti-Btk C-20 (SantaCruz Biotechnology,Santa Cruz, CA) was used for Btk-specific immunoblotting.

Results

SLP-65 is not required for pre-BCR-induced tyrosine phosphorylation of Btk. The finding that Btk and SLP-65 have a synergistic function in the developmental progression of large cycling into small resting pre-B cells, suggests that the two proteins can at least partially act in separate pathways. This would however be in apparent conflict with the observation that SLP-65 is required for BCR-induced tyrosine phosphorylation and subsequent activation of Btk in chicken DT40 B cells [29]. To address this issue, we investigated Btk phosphorylation in the absence of SLP-65 by stimulation of cultured pre-B cells from SLP-65 deficient mice with antibodies directed against the Igμ H chain protein. Blotting total cellular lysated with a pTyr-specific monoclonal antibody was used to monitor the stimulation of the cells. Tyrosine phosphorylated proteins were immunoprecipitated from total cellular lysates using pTyr-specific mAb and Btk protein was identified by blotting with a Btk-specific antibody. As shown in Fig. 1A, Igμ stimulation in SLP-65 deficient pre-B cells resulted in Btk phosphorylation. Similarly, we analysed Btk-specific phosphorylation in pre-B lymphoma cell lines derived from SLP-65 deficient mice (Fig. 1B). These findings show that, in contrast to previous findings in chicken B cells, Btk can be phosphorylated in the absence of SLP-65 upon pre-BCR stimulation in murine pre-B cells.

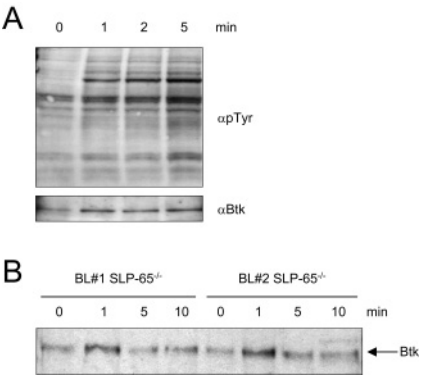

Figure 1. *Btk is phosphorylated in the absence of SLP-65. (A) BM B cells from SLP-65 mice was cultured for 5 days in the presence of IL-7. Cells were harvested and stimulated with polyclonal anti-IgM F(ab')$_2$ fragments for the time indicated. Stimulation was analysed by Western blotting of total lysates using anti-pTyr mAbs. Total lysates were immuno-precipitated with anti-pTyr and tyrosine phosphorylation of Btk was analysed by Western blotting using Btk-specific Abs. (B) Cultured cells from two different SLP-65$^{-/-}$ pre-B cell lymphomas (BL#1 and BL#2) were analysed for Btk-specific phosphorylation as described in (A).*

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

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

		Spleen	Bone marrow			
Mouse strain	*N*	B220$^+$ [a, b]	Total B cells B220$^+$	Pro- and pre-B IgM$^-$ [c]	Immature B IgM$^+$IgD$^-$	Mature B IgM$^+$IgD$^+$
Btk$^+$	3	49.8 ±21.2	18.2 ± 7.9	12.1 ± 5.3	3.5 ± 1.8	2.2 ± 1.5
Btk$^-$	3	8.6 ± 1.5	16.0 ± 4.0	10.6 ± 3.6	3.7 ± 0.9	1.5 ± 0.5
SLP-65$^-$	3	10.5 ± 2.1	12.4 ± 7.0	9.2 ± 4.7	2.4 ± 1.4	0.9 ± 0.5
Btk$^-$SLP-65$^-$	3	1.3 ± 0.8	5.9 ± 3.0	5.2 ± 2.7	0.5 ± 0.3	0.1 ± 0.0
K430R-Btk/SLP-65$^{-/-}$	3	3.6 ± 1.4	9.0 ± 4.2	4.9 ± 3.6	2.2 ± 1.1	0.8 ± 0.5

[a] Absolute number as mean ± SEM (x 10^6). [b] The absolute numbers of splenic B220$^+$ cells in K430R-Btk/SLP-65$^{-/-}$ was significantly different from the value in SLP-65 single deficient mice (P=0.008) and in Btk/SLP-65 double deficient mice (P=0.002). [c] pro- and pre-B, immature and mature B cell subsets of BM B220$^+$ cells.

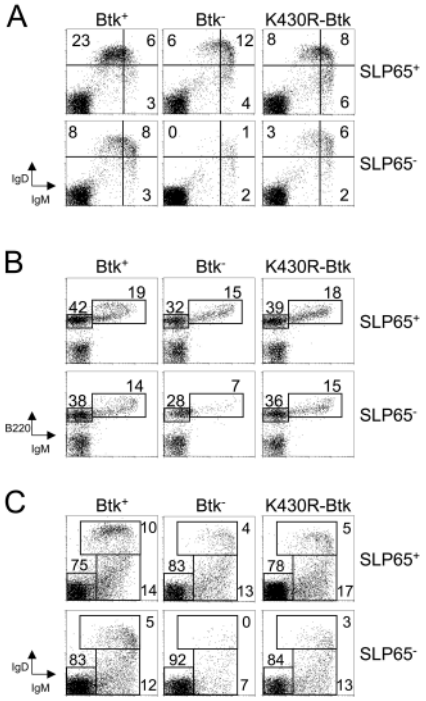

Figure 2. *K430R-Btk partially corrects the defects in B cell development in Btk/SLP-65 double mutant mice. The phenotypic characterization of the B-lineage compartment in spleen and BM of K430R-Btk/SLP-65$^{-/-}$ mice is compared to Btk- and SLP-65 single deficient and Btk/SLP-65 double deficient mice. (A) Splenic lymphoid cells were electronically gated on the basis of forward and side scatter and surface expression of IgM/IgD is plotted. (B-C) Lymphoid cells from BM were gated and analysed for (B) B220/IgM and (C) IgM/IgD expression. Data are presented as dot plots and the percentages within the indicated quadrants or gates are given. The plots are representative for 3-7 mice of each genotype.*

an almost complete block at this stage [13-18]. As a result, the reduction in mature B cell numbers in double mutant mice is much more severe, when compared to Btk or SLP-65 single mutant mice [18,20]. We have previously shown that expression of transgenic kinase-inactive K430R-Btk is able to partially rescue the block in peripheral B cell maturation in Btk-deficient mice, indicating that Btk function is in part independent of its kinase activity.

To investigate whether SLP-65 is required for Btk adapter function, we replaced the endogenous wild-type Btk by kinase-inactive Btk by crossing K430R-Btk transgenic mice onto a Btk/SLP-65 double deficient background. We analysed the size of the B cell subpopulations in spleen and BM by flow cytometry. In agreement with reported findings [18,20], we observed a severe reduction in the size of the peripheral B cell population in Btk/SLP-65 double deficient mice, when compared with wild-type mice or either of the single deficient mice (Table 1; Fig. 2A). In addition, the residual B cells present in the spleen differentiated into IgM^{high}IgD^{high} B cells, but failed to downregulate IgM (Fig. 2A). We found that the fractions of IgM^{high}IgD^{high} (~6% in K430R-Btk/SLP-65$^{-/-}$ mice, compared with ~8% in SLP-65$^{-/-}$ mice and ~1% in Btk/SLP-65 double deficient mice) and IgM^{low}IgD^{high} mature B cells (~3%, ~8% and undetectable, respectively, in these three groups of mice) in the spleen were partially restored (Fig. 2A). However, the absolute B cell numbers in the spleen of K430R-Btk/SLP-65$^{-/-}$ mice are still low (~4 x 10^6) when compared with SLP-65 single deficient mice (~11 x 10^6; Table 1).

In the BM, the proportions of IgM$^+$ B cells in K430R-Btk/SLP-65$^{-/-}$ (~15%) were similar to those in SLP-65 single deficient mice (~14%) and were increased when compared with Btk/SLP-65 double deficient mice (~7%; Fig. 2B). In a more detailed analysis of the B220$^+$ population in which we analysed IgM/IgD expression profiles, we found that K430R-Btk/SLP-65$^{-/-}$ mice contained a significantly larger fraction of IgM$^+$IgD^{high} recirculating B cells (~6%), when compared with Btk/SLP-65 double deficient mice (~1%, Fig. 1C).

In summary, these results show that expression of kinase-inactive Btk is able to partially rescue the severe B cell differentiation defect in Btk/SLP-65 double deficient mice, resulting in a phenotype that is similar to that of SLP-65 deficient mice. Therefore, these data indicate that the role of Btk as an adapter protein is at least in part independent of SLP-65.

Transgenic K430R-Btk expression partially corrects the pre-B cell defects in Btk/SLP-65 double mutant mice. We have previously shown that Btk and SLP-65 are required for efficient transition of large cycling into small resting cytoplasmic μ^+ (cμ^+) pre-B cells [17,20]. In particular, 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, were impaired in Btk and SLP-65 single mutant mice. A synergistic role for Btk and SLP-65 in this context is clear from the more pronounced defects in the modulation

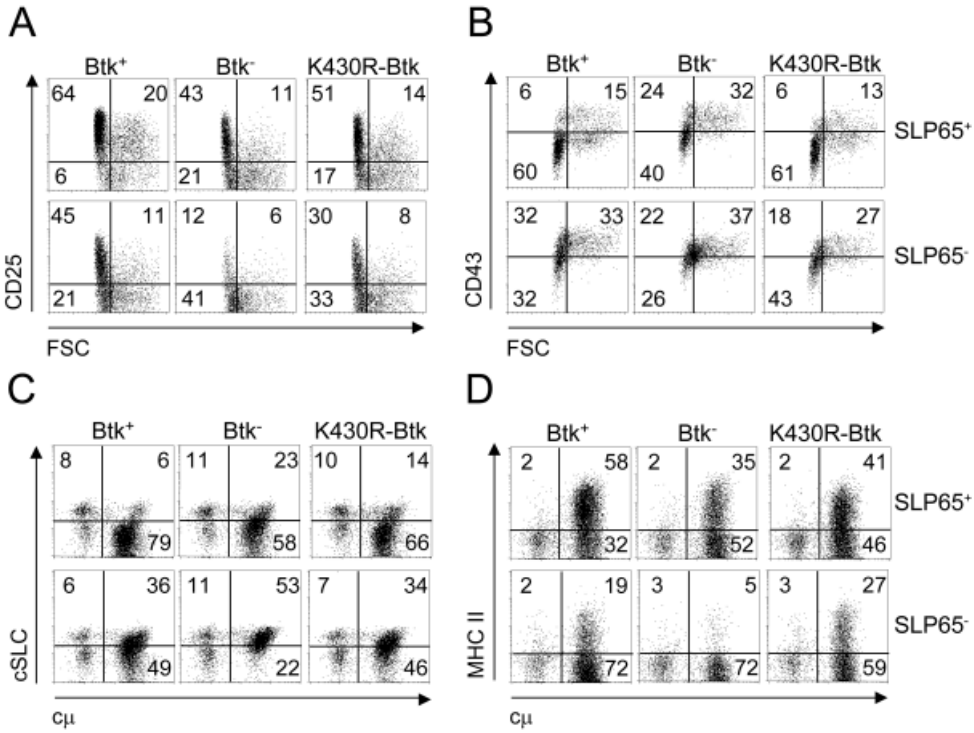

Figure 3. *K430R-Btk partially corrects the pre-B cell defects in Btk/SLP-65 double mutant mice. The B220$^+$IgM$^-$ pro-/pre-B cell population in BM of Btk$^+$, Btk$^-$, or K430-Btk on a SLP-65$^+$ or SLP-65$^-$ background was gated and analysed for (A) CD25/FSC, (B) CD43/FSC, (C) cSLC/cμ and (D) MHC class II/cμ expression profiles. Data are displayed as dot plots and the percentage of cells within the indicated quadrants is given. Data shown are representative of 3-7 mice analysed within each group.*

of most of these markers in Btk/SLP-65 double deficient mice, perhaps with the exception of BP-1 and CD2 [17,20]. In addition, we reported that kinase-inactive Btk is able to partially restore the defects at the pre-B cell stage in Btk-deficient mice [12]. To analyse the effects of kinase-inactive Btk on pre-B cell maturation in the absence of SLP-65, we now compared the expression of developmentally regulated markers by flow cytometric analysis of 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 (Fig. 3). These analyses show that kinase-inactive Btk is able to rescue the defects found in IgM$^-$cμ$^+$ pre-B cells of Btk/SLP-65 double deficient mice, as the observed expression levels of cSLC, CD43, CD25 and MHC class II are comparable in K430R-Btk SLP-65$^{-/-}$ mice (34%, 45%, 38%, and 27%, respectively) and SLP-65$^{-/-}$ single deficient mice (36%, 65%, 60% and 19%) (Fig. 3).

In summary, these results show that transgenic expression of kinase-inactive Btk is able to reduce the severe developmental defects in Btk/SLP65 double deficient pre-B cells to a level present in SLP-65 single deficient mice. Therefore, we conclude that the adapter function of Btk in pre-B cells in vivo apparently does not require SLP-65.

The effects of K430R-Btk expression on serum Ig levels. Btk- and SLP-65- deficient mice have severely decreased levels of IgM and IgG3, while other isotypes are not or only moderately affected, when compared with wild-type levels [13,20,30]. Furthermore, we have found that kinase-inactive Btk could significantly restore the decreased levels of IgM in serum of Btk-deficient mice, but only a modest increase in IgG3 was

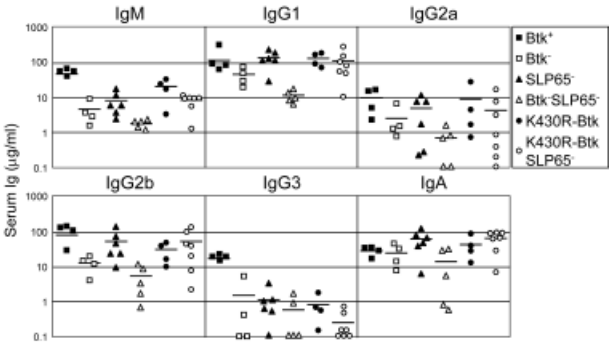

Figure 4. *Kinase-inactive Btk restores the low serum Ig levels in Btk/SLP-65 double mutant mice. Serum concentrations of IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA are shown for the indicated mouse strains. Mice were 2 mo of age and each symbol represents an individual animal.*

observed [12]. As shown in Fig. 4, serum levels of all isotypes are lower in Btk/SLP-65 double deficient mice, when compared to either of the single mutant littermates. In accordance with the previous findings that the kinase activity of Btk is not absolutely required for plasma cell differentiation [12], we now found that serum levels of IgM, IgG1, IgG2a, IgG2b and IgA in K430R transgenic Btk/SLP-65 double deficient mice are similar to the levels found in SLP-65 single deficient mice (Fig. 4). Only the levels of IgG3 in Btk$^-$, SLP-65$^-$, Btk/SLP-65 double deficient and K430R-Btk^i/SLP-65$^{-/-}$ mice are all lower than wild-type levels and close to the lower threshold of the ELISA. These results are in agreement with previous findings in K430R-Btk mice [12] and further indicate that also with respect to late B cell and plasma differentiation, in the absence of SLP-65 Btk mainly acts as an adapter protein.

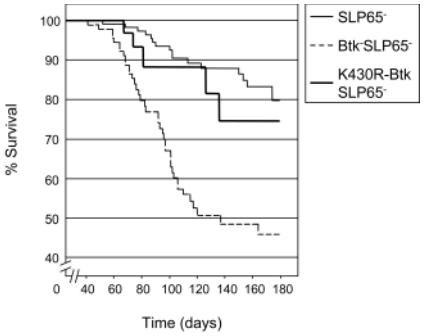

Figure 5. *Tumor suppressor function of Btk is not dependent on its kinase activity in SLP-65-deficient pre-B cells. Kaplan-Meier tumor-free survival estimates for SLP-65$^{-/-}$, Btk/SLP-65 double deficient and K430R-Btk/SLP-65$^{-/-}$ mice. Tumor-free survival in Btk/SLP-65 double deficient mice was significantly reduced compared with SLP-65$^-$ mice (P<0.00001, by log-rank) and to K430R-Btk/SLP-65$^{-/-}$ mice (P<0.035). Tumor free survival in SLP-65$^-$ and K430R-Btk/SLP-65$^{-/-}$ was not significantly different.*

Kinase-inactive Btk functions as a tumor suppressor in SLP-65-deficient pre-B cells. We recently reported that concomitant deficiency of Btk strongly increased the frequency of pre-B cell lymphoma formation in SLP-65$^-$ mice, indicating that Btk cooperates with SLP-65 as a tumor suppressor in pre-B cells [20]. 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 43 K430R transgenic mice for ~30 weeks. At this age, the fraction of SLP-65$^-$ mice that developed a pre-B cell lymphoma was ~15% (17 out of 118), whereas the Btk/SLP-65 double deficient mice showed a frequency of ~40% (39 out of 97). In contrast, only six out of 43 (~14%) K430R-Btk/SLP-65-deficient mice died of a pre-B cell lymphoma (Fig. 5). From these results we conclude that kinase-inactive Btk can substitute for 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

In this study we examined the adapter function of Btk in the absence of SLP-65. To this end, we crossed kinase-inactive K430R-Btk mutant mice with SLP-65-deficient mice and examined B cell development in BM and spleen and the marker modulation during the transition of large into small pre-B cells *in vivo* by detailed flow cytometric analysis. Furthermore, we analysed serum Ig levels and the incidence of pre-B cell tumor formation. We found that expression of transgenic kinase-inactive Btk was largely able to rescue the severe defects present in SLP-65/Btk

double deficient mice to such an extent that the resulting phenotype was similar to that of SLP-65 single deficient mice. In particular, we found that SLP-65 is not required for the function of Btk as an adapter molecule that signals the upregulation of CD25 and MHC class II expression and the downregulation of CD43 and SLC chain expression and acts as a tumor suppressor in pre-B cells. Therefore, we conclude that the Btk kinase domain is not required for its function as a tumor suppressor in SLP-65 deficient pre-B cells.

The mechanism by which kinase-inactive Btk may act as a tumor suppressor in pre-B cells remains unknown. However, together with recently published findings, our data would support the presence of a parallel SLP-65-independent escape route in pre-B cells in which Btk activates PLCγ2 by recruiting PIP5K, thereby providing substrate for PLCγ2 [7]. Engagement of the pre-B cell receptor leads to activation of Src family kinases, phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on Ig-α and Ig-β and recruitment, phosphorylation and activation of Syk. By analogy with the BCR in mature B cells, in a SLP-65 dependent pathway, Syk also phosphorylates SLP-65 and Btk is activated by Src kinases or Syk by phosphorylation at Y551 and subsequent autophosphorylation at Y223 [31,32]. Activated Btk then induces tyrosine phosphorylation of PLCγ2 [9]. Production of IP3 and DAG by PLCγ2 induces calcium mobilization and PKC activation, respectively. The mobilization of intracellular calcium is one of the major targets of BCR signaling and is critical for the regulation of multiple transcription factors such as NFκB and Nuclear Factor of Activated T cells (NFAT) [33-36].

However, it recently became apparent that, next to this pathway, kinase-inactive Btk is able to recruit PIP5K, thereby activating a positive feedback loop for PIP2 synthesis, the substrate for PI3K and PLCγ2 [7]. PI3K products are necessary to localize Btk to the plasma membrane after its activation and inhibition of PI3K blocks Btk-dependent PLCγ2 activation [37]. Furthermore, in reconstituted myeloma cells, CD19 has been shown to be necessary for efficient BCR-mediated activation of Btk [38], most likely through the PI3K pathway and the production of PIP3 in concert with Lyn-mediated phosphorylation of Btk [39,40].

Taken together, these data provide the biochemical basis for a parallel SLP-65-independent escape route when Btk is not able to phosphorylate PLCγ2 directly: upon activation by Lyn or Syk, Btk is able to bind to PIP3 and PIP5K is recruited and provides substrate for PLCγ2 and PI3K. This mechanism may also explain the phenotype of CD19/SLP-65 double deficient mice [21]. In these mice, pre-B cell differentiation was completely blocked and they manifested a high frequency of pre-B cell lymphoma development, a phenotype quite similar as the phenotype of Btk/SLP-65 double deficient mice [18,20]. Therefore, we propose that the SLP-65-independent parallel PLCγ2 activation pathway requires both Btk (as an adapter protein) and CD19-dependent PIP3 synthesis by PI3K.

The possibility remains that SLP-65 deficiency can be compensated by the presence of redundant proteins, as the concomitant absence of LAT in SLP-65$^{-/-}$ mice resulted in an almost complete block at the large pre-B cell stage [41]. Furthermore, it was shown that LAT recruits PLCγ2 to the pre-BCR by association with Ig-α and the SLP-65 homologue SLP-76 upon pre-BCR engagement. These results indicate that LAT/SLP-76 can rescue PLCγ2 activation in the absence of SLP-65 [41]. 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 adapter molecule. Further experiments are required to investigate if Btk can interact with LAT or SLP-76.

In summary our results, in combination with recent reports about alternative signalling pathways, indicate that membrane recruitment of Btk, leading to activation of PIP5K and PLCγ2 is sufficient for Btk function as a tumor suppressor in pre-B cells. In this SLP-65-independent pathway, Btk functions as an adapter protein, independent of its catalytic activity.

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Chapter 4

Btk and cellular maturation

Chapter 4

Cellular maturation defects in Bruton's tyrosine kinase-deficient immature B cells are amplified by premature B cell receptor expression and reduced by receptor editing

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Cellular maturation defects in Bruton's tyrosine kinase-deficient immature B cells are amplified by premature B cell receptor expression and reduced by receptor editing

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Abstract

In the mouse, Bruton's tyrosine kinase (Btk) is essential for efficient developmental progression of $CD43^+CD2^-$ large cycling into $CD43^-CD2^+$ small resting pre-B cells in the bone marrow and of IgM^{high} transitional type 2 B cells into IgM^{low} mature B cells in the spleen. In this study, we show that the impaired induction of cell surface changes in Btk-deficient pre-B cells was still noticeable in κ^+ immature B cells, but was largely corrected in λ^+ immature B cells. As λ gene rearrangements are programmed to follow κ rearrangements and λ expression is associated with receptor editing, we hypothesized that the transit time through the pre-B cell compartment or receptor editing may affect the extent of the cellular maturation defects in Btk-deficient B cells. To address this issue, we used 3-83$\mu\delta$ transgenic mice, which prematurely express a complete B cell receptor and therefore manifest accelerated B cell development. In Btk-deficient 3-83$\mu\delta$ mice, the IgM^+ B cells in the bone marrow exhibited a very immature phenotype (pre-$BCR^+CD43^+CD2^-$) and were arrested at the transitional type 1 B cell stage upon arrival in the spleen. However, these cellular maturation defects were largely restored when Btk-deficient 3-83$\mu\delta$ B cells were on a centrally deleting background and therefore targeted for receptor editing. Providing an extended time window for developing B cells by enforced expression of the antiapoptotic gene *Bcl-2* did not alter the Btk dependence of their cellular maturation. We conclude that premature B cell receptor expression amplifies the cellular maturation defects in Btk-deficient B cells, while extensive receptor editing reduces these defects.

Introduction

The generation of B cells from progenitor cells is a complex process involving the transit of cells through several critical developmental stages. Developing B cell precursors in the bone marrow pass several checkpoints at which they are subject to choices between survival, proliferation, or cell death dependent on the presence of functional Ig H and L chains. Expression of the pre-B cell receptor (pre-BCR) complex, comprised of μ H chain, the V_{preB} and $\lambda 5$ surrogate L chain (SLC) proteins, and the Igα/CD79a and Igβ/CD79b signaling components, is essential for IL-7-dependent proliferative expansion of cytoplasmic μ H chain positive ($c\mu^+$) pre-B cells (1, 2). In addition, the pre-BCR triggers the inhibition of further H chain rearrangements, known as allelic exclusion, as well as drastic changes of pre-B cell surface phenotype. Furthermore, the down-regulation of proliferation in large cycling pre-B cells and the induction of progression into small resting pre-B cells, in which Ig L chain rearrangement is initiated, are regulated by the pre-BCR. Signaling proteins involved in the downstream pathway of the pre-

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Abbreviations used in this paper: BCR, B cell receptor; SLC, surrogate L chain; BM, bone marrow; Btk, Bruton's tyrosine kinase; Tg, transgenic; WT, wild type; cμ, cytoplasmic μ H chain; BrdU, 5-bromo-2-deoxyuridine; T1/2, transitional type 1 and 2.

BCR include the Lyn, Syk, and Bruton's tyrosine kinase (Btk) tyrosine kinases, phosphoinositide 3-kinase (consisting of p85 and p110 subunits), the B cell linker protein BLNK/SLP-65 and the phospholipase Cγ2. Cytoplasmic μ^+ pre-B cells of mice deficient for the pre-BCR components λ5, V_{preB}, membrane μ H chain, Igα, or Igβ or the pre-BCR signaling molecules Syk or p85α do not enter the expansion phase (reviewed in Ref. 3).

In contrast, absence of the pre-BCR signaling proteins Btk and BLNK/SLP-65 was shown to be associated with defects in cell cycle exit, resulting in a high incidence of pre-B cell tumors (4-8). In SLP-65-deficient mice, and even more so in Btk/SLP-65 double mutant mice, large cycling pre-B cells are trapped in a positive feedback loop as they are arrested in development and cannot efficiently down-regulate pre-BCR expression, which signals for proliferation (7-9). Mutations in the *Btk* gene result in X-linked agammaglobulinemia in humans and x-linked immunodeficiency (*xid*) in the mouse (10). X-linked agammaglobulinemia is characterized by a severe arrest of B cell development at the pre-B cell stage, and consequently peripheral B cells and Abs of all subclasses are almost completely absent. *xid* mice manifest a milder phenotype associated with an impairment of peripheral B cell maturation, without a major early block in B cell development in the bone marrow (BM). As a result, peripheral B cells are present in lower numbers and serum IgM and IgG3 levels are low.

Recently, we have shown that pre-B cell differentiation in Btk-deficient mice is impaired (5, 8). During the transition of large cycling into small resting pre-B cells, Btk-deficient mice fail to efficiently modulate the expression of SLC, the metallopeptidase BP-1, the adhesion molecule CD2, the IL-2R CD25, MHC class II, and the membrane sialoglycoprotein CD43. Moreover, 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 of ~3 h when compared with wild type (WT) cells. Despite the similar absolute numbers of immature B cells generated in both mice, Btk^- immature B cells are phenotypically different from WT immature B cells. The impaired induction of cell surface changes in Btk-deficient small pre-B cells is still noticeable at the immature B cell stage, in particular with regard to the expression levels of BP-1 and CD2 (5). In this respect, we now found that κ L chain-positive immature B cells are more affected by the lack of Btk than λ L chain-positive immature B cells. Given that λ gene rearrangements in pre-B cells are programmed to occur ~24 h later than κ rearrangements, it is possible that the transit time through the pre-B cell compartment may affect the differentiation defects in Btk-deficient B cells. Alternatively, as λ L chain expression is often associated with receptor editing, the BCR signaling events that induce receptor editing may also facilitate cellular maturation.

To distinguish between these possibilities, we modified the kinetics of B cell development by crossing Btk-deficient mice with 3-83μδ-transgenic (Tg) mice, which carry a BCR transgene consisting of prerearranged μ and δ H chain genes as well as a κ L chain gene (11). Because the expression of the 3-83μδ transgene is under the control of the Ig H chain enhancer, the 3-83μδ BCR is expressed from the pro-B cell stage onward. The 3-83μδ BCR specifically recognizes MHC class I molecules of the $H2\text{-}K^{k/b}$ allotype (11), and thus reflects an innocuous BCR in mice that have an $H2\text{-}K^d$ background. Mice with such a nonautoreactive background (also called nondeleting mice) contain a virtually monoclonal B cell population. Since the prematurely expressed 3-83μδ BCR is able to promote B cell development, it appears that this BCR can functionally replace the pre-BCR and consequently B cell development is accelerated (11-13). In contrast, when the 3-83μδ BCR is expressed in mice of the MHC class I $H2\text{-}K^b$ background, all B cells generated are autoreactive and are targeted for continued L chain gene rearrangement, a process known as receptor editing, to survive (14-16). B cells in these centrally deleting mice are delayed in the small pre-B cell stage due to the receptor editing process (17, 18).

By crossing Btk-deficient mice with 3-83μδ Tg mice on a nondeleting or a deleting MHC class I background, we were able to study the effects of Btk on cellular maturation of B cell precursors undergoing accelerated development or receptor editing, respectively. Furthermore, we tested whether providing an extended time window per cell for the induction of cell surface marker changes, by the enforced expression of the Bcl-2 apoptosis inhibitor, would facilitate cellular maturation in Btk-deficient pre-B cells.

Materials and Methods

Mice and genotyping. Btk-deficient mice (19) were crossed onto the C57BL/6 background for more than eight generations. 3-83μδ mice (11) were on a nondeleting B10.D2 background. Endogenous Btk WT alleles were identified as described previously (5). Expression of a 3-83μδ was identified by a 3-83 Vκ forward primer (5'-CAGCTTCCTGCTAATCAGTGCC-3') and a 3-83 Jκ2 reverse primer (5'-TGGTCCCCCCTCCGAACGTG-3') (11). MHC I background was determined by FACS analysis of peripheral blood samples by using mAbs against H2-K^b and H2-K^d (below). The presence of the *Eμ-2–22 Bcl-2* transgene was evaluated by PCR (20).

Flow cytometric analysis. Preparations of single-cell suspensions, standard and intracellular flow cytometry, and determination of β-galactosidase activity by loading cells with fluorescein-β-D-galactopyranoside substrate have been described previously (5, 19, 21). The hybridomas LM34 (anti-SLC; (22)) and PB493 (anti-AA4.1; (23)) were kindly provided by A. Rolink (University of Basel, Basel, Switzerland). The anti-3-83μδ hybridoma 54-1 (15) was kindly provided by D. Nemazee (The Scripps Research Institute, La Jolla, CA). Hybridoma Abs were purified using protein G columns and conjugated to biotin according to standard methods or used unlabeled in combination with PE- or allophycocyanin-conjugated goat anti-rat secondary Abs. The following mAbs were obtained from BD PharMingen (San Diego, CA): FITC-conjugated anti-CD21 (7G6), anti-CD22 (Cy34.1), and anti-CD62L (MEL-14); PE-conjugated anti-MHC class II (M5/114), anti-CD19 (1D3), anti-IgM (R6-60.2), anti-CD23 (B3B4), and anti-H2-K^d (SF1–1.1); PerCP- and allophycocyanin-conjugated anti-B220 (RA3-6B2) and anti-CD19 (1D3); biotinylated anti-Ly6C (ER-MP20), anti-CD23 (B3B4), and anti-H2-K^b (AF6-88.5). Unlabeled anti-CD21 (7G6; BD PharMingen) was biotinylated according to standard methods and FITC-conjugated anti-IgD (11-26) was obtained from Southern Biotechnology Associates (Birmingham, AL). In vivo 5'-bromo-2'deoxyuridine (BrdU) labeling and detection and all other mAbs used were described previously (5).

IL-7-driven BM cultures. Primary pre-B cell BM cultures and determination of IL-7-dependent proliferative responses of total BM cells have been described previously (5, 20).

Results

In Btk-deficient mice, κ^+ and λ^+ immature B cells show differences in cellular maturation. During the progression of large cycling into small resting cμ H chain-positive pre-B cells, Btk-deficient mice manifest defective down-regulation of BP-1, CD43, and SLC and impaired up-regulation of CD2, CD25, and MHC class II, as shown by flow cytometric analysis (Fig. 1, *A* and *B* and Refs. 5 and 8). In this study, we have also included the mAb PB493 recognizing the complement component C1q-like receptor C1qRp, also known as AA4.1 (24). This marker is expressed on all B lineage cells in the BM, but in the spleen only on those B cells that reflect recently immigrated immature B cells (23). In Btk-deficient mice, we found increased proportions of $PB493^+$ cells in the $B220^+IgM^-$ B cell precursor compartment when compared with Btk^+ mice (Fig. 1*B*), indicating that the modulation of expression of this marker is also Btk dependent. As shown in Fig. 1*B*, the proportions of cells that express μ H chain in their cytoplasm within the surface IgM^- B cell precursor fractions are similar in Btk-deficient and WT mice.

We previously found that the inefficient induction of cell surface phenotype changes in Btk-deficient mice was still noticeable in immature B cells, in particular for BP-1 and CD2 (5). In a separate analysis of κ and λ L chain-positive $B220^{low}IgM^+$ immature B cells, we now observed that elevated expression of BP-1 was more pronounced in Btk-deficient κ^+ immature B cells when compared with λ^+ cells. Reduced expression of CD2, MHC class II and CD25 was limited to κ^+ immature B cells, while λ^+ immature B cells manifested essentially normal surface expression of these markers (Fig. 1*C* and data not shown).

In summary, these findings indicate that in Btk-deficient mice the impaired modulation of developmentally regulated markers in small pre-B cells results in very immature phenotype of κ^+ B cells in the BM, while λ^+ B cells appear less affected.

Btk-deficient 3-83μδ mice show amplified B cell differentiation defects. We hypothesized that the observed differences in surface phenotype between Btk-deficient κ^+ and λ^+ immature B cells may reflect the programmed sequential activation of the κ and λ loci. In pre-B cells, λ gene rearrangements are programmed to occur ~24 h later than κ rearrangements, and thus λ^+ immature B cells have resided for a

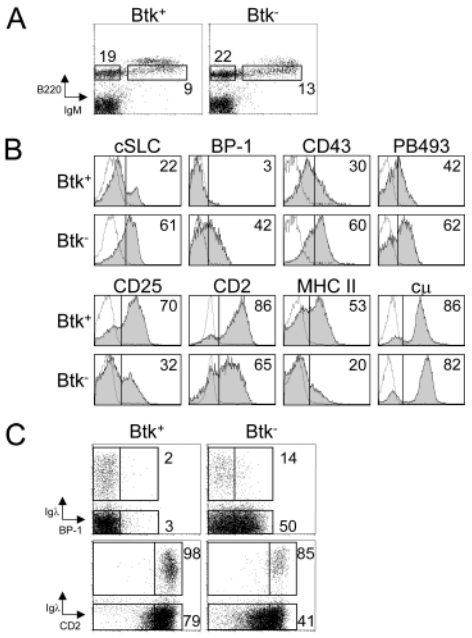

Figure 1. *Phenotypic characterization of the B lineage compartment in BM of Btk⁺ and Btk⁻ mice. A, BM lymphoid cells were electronically gated on the basis of forward and side scatter and expression of B220 vs IgM is plotted. B, B220^{low}IgM^{-} pro- and pre-B cells were gated and expression of several developmentally regulated markers is shown as histograms. Numbers indicate percentage of cells positive for the indicated marker and plots are representative for ~20 mice of each genotype. C, B220^{low}IgM^{+} immature B cells were gated and expression of Igλ and BP-1 or CD2 is shown. Numbers indicate percentage of BP-1- or CD2-positive cells within λ⁺ or κ⁺ immature B cells. Plots are representative for four mice of each genotype.*

longer period of time within the small pre-B cell compartment (25, 26). Therefore, the extent of the differentiation defects in Btk-deficient B cells may correlate with the rate of transit through the pre-B cell compartment. To directly test this hypothesis, we crossed Btk-deficient mice with 3-83μδ Tg mice, which prematurely express a prerecombined BCR that functionally replaces the pre-BCR. As a result, B cells spend little or no time in the small pre-B cell stage and B cell development is accelerated significantly.

Total BM cell suspensions from Btk⁺ and Btk⁻ 3-83μδ mice were analyzed by flow cytometry (Fig. 2). Irrespective of the presence of Btk, the B lineage compartment in 3-83μδ mice mainly consisted of B220^{low} immature B cells that

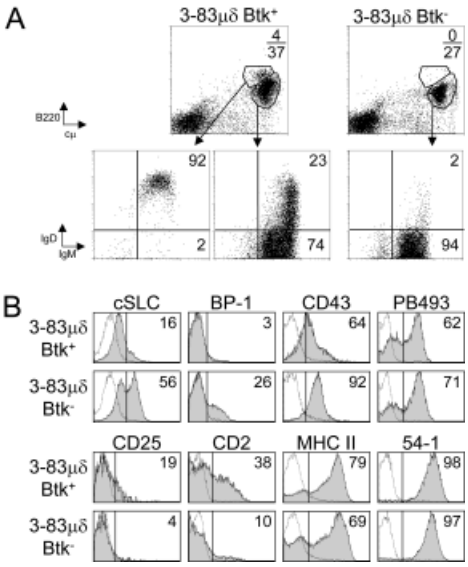

Figure 2. *Phenotypic characterization of the BM B cell compartment in Btk⁺ and Btk⁻ 3-83μδ mice. A, BM lymphoid cells were gated and plotted for B220 and cμ (upper part). B220^{high} and B220^{low} cells were separately gated and IgM/IgD profiles of both populations are shown (lower part). B, The B220^{low} B cell population was further analyzed for expression of various developmentally regulated markers, as shown in the histograms. The percentages of cells within the indicated gates or quadrants are given. Data represent six to eight*

were cμ⁺ and also expressed IgM on the cell surface. However, the B220^{low} immature B cell fraction present in Btk⁻ 3-83μδ mice contained very few IgD⁺ B cells (<2%) when compared with Btk⁺ 3-83μδ mice (23% IgD⁺ cells; Fig. 2*A*). Furthermore, the BM of Btk⁻ 3-83μδ mice did not contain mature B220^{high}IgD^{high} recirculating B cells. Next, the expression pattern of several developmental markers was analyzed in the B220^{low}IgM⁺ immature B cell compartment. In WT mice, immature B cells did not express SLC, BP-1, and CD43, were low for PB493 and CD25, and positive for CD2 and MHC class II (Fig. 1*C* and Ref. 5). However, due to the expression of the 3-83μδ transgene in WT mice, the B220^{low}IgM⁺ compartment had a more immature phenotype: CD43$^{+/low}$, PB493^{high}, CD25^{low}, and CD2^{low}. Notably, Btk-deficient 3-83μδ mice showed significantly impaired B cell maturation, as the B220^{low}IgM⁺ B cells expressed substantial levels of CD43 and SLC and lacked expression of CD2 and CD25 (Fig. 2*B*), when compared with their Btk⁺ 3-83μδ

littermates. The expression of MHC class II on $B220^{low}IgM^{+}$ B cells did not appear to be affected by the premature BCR expression.

These results indicate that a prematurely expressed 3-83μδ BCR was only partially able to functionally replace the pre-BCR, as the modulation of expression of developmentally regulated markers was impaired. In Btk-deficient 3-83μδ mice, this defect was even stronger, as evidenced by an almost complete absence of down-regulation of CD43 and SLC and induction of CD2 and CD25 in IgM^{+} B cells in the BM.

Btk-deficient 3-83μδ *mice manifest a severe reduction of B cell numbers in the spleen.* Total splenic cell suspensions from Btk^{+} and Btk^{-} 3-83μδ mice were analyzed by flow cytometry and compared with non-Tg littermates. The *xid* phenotype of Btk-deficient mice is characterized by a specific deficiency of mature $IgM^{low}IgD^{high}$ B cells in the spleen, resulting in a reduction of the B cell numbers by ~50% (19, 27, 28). As previously described (16), expression of the 3-83μδ transgene on a WT background only marginally affected the splenic B cell numbers. Surprisingly, we observed a profound reduction of the size of the splenic B cell population in Btk^{-} 3-83μδ mice, as <2 x 10^6 B cells were present compared with ~20 x 10^6 B cells in Btk^{+} 3-83μδ mice (Fig. 3*A* and Table I).

This severe reduction of splenic B cells in Btk^{-} 3-83μδ mice may reflect a reduced survival capacity of Btk^{-} 3-83μδ B cells in the BM or in the spleen. Alternatively, it is possible that the absence of Btk affects the signals transmitted by the 3-83μδ BCR in such a way that these B cells are considered autoreactive and are therefore deleted. Autoreactive B cells down-regulate surface IgM expression and are subject to receptor editing at the immature B cell stage in the BM (12, 14, 16, 29). However, flow cytometric analyses did not provide evidence for autoreactive characteristics of Btk^{-} 3-83μδ B cells. The B cells were IgM^{high} and did not edit their BCR, as they were mainly positive for the anti-idiotypic Ab 54-1

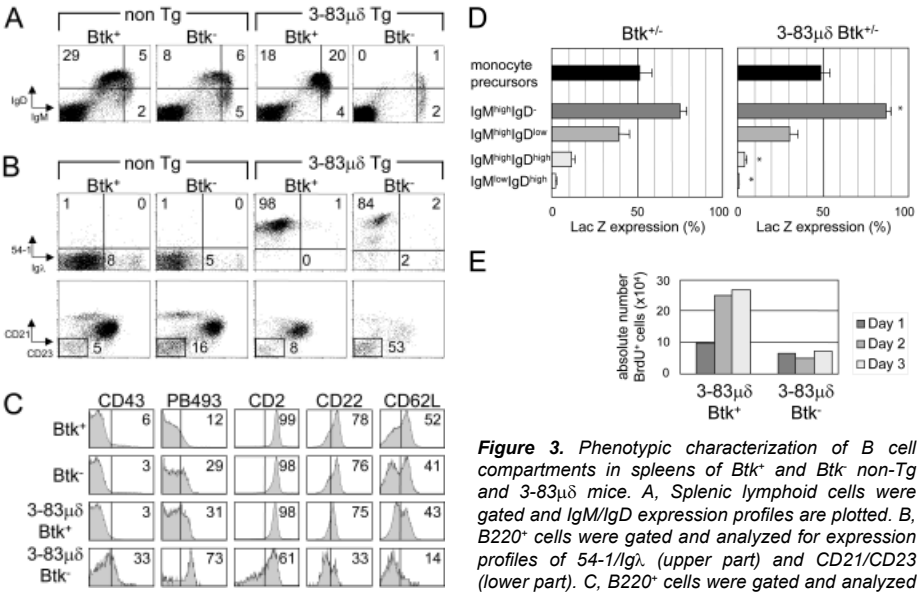

Figure 3. *Phenotypic characterization of B cell compartments in spleens of Btk^{+} and Btk^{-} non-Tg and 3-83μδ mice. A, Splenic lymphoid cells were gated and IgM/IgD expression profiles are plotted. B, $B220^{+}$ cells were gated and analyzed for expression profiles of 54-1/Igλ (upper part) and CD21/CD23 (lower part). C, $B220^{+}$ cells were gated and analyzed for the expression of CD43 and CD2 on IgM^{+} cells and expression of PB493, CD22, and CD62L on $CD19^{+}$ cells. A–C, The percentages of cells within the indicated gates or quadrants are given; data represent three to eight mice analyzed per genotype. D, Expression of lacZ in splenic B cells of $Btk^{+/-}$ non-Tg and 3-83μδ heterozygous female mice. Splenic B cells were gated using IgM and IgD expression profiles, and LacZ expression in these populations was compared with lacZ expression in ER-$MP20^{high}$ BM monocyte precursor cells. Mean percentages ± SD values of lacZ-positive cells within the indicated gates were plotted and significance between non-Tg and 3-83μδ mice was evaluated by Student's t test („ $p \leq 0.01$). Data represent four to five mice analyzed per genotype. E, BrdU incorporation in spleen after a single dose of BrdU injection in Btk^{+} and Btk^{-} 3-83μδ mice. The absolute number of $BrdU^{+}$ cells was calculated within the $B220^{+}IgM^{high}IgD^{-/low}$ B cell population. Mean values of two to six mice per time point were plotted.*

Table I. *Absolute number of B cell subsets in spleen*

Mouse Strain	*N*	B220^{+a}	*p*^{b}	IgM^{high}IgD$^{-/low}$ (T1)^{c}	IgM^{high}IgD^{high} (T2)	IgM^{low}IgD^{high} (Mature)
Btk^{+}	17	37.8 ± 2.4		2.1 ± 0.2	5.5 ± 0.5	25.0 ± 2.0
Btk^{-}	15	16.1 ± 1.5	<1 x 10^{-7}	2.2 ± 0.3	5.5 ± 0.8	5.8 ± 0.6
3-83μδ Btk^{+} nondeleting	19	16.0 ± 1.2		1.3 ± 0.2	6.3 ± 0.8	6.9 ± 0.6
3-83μδ Btk^{-} nondeleting	14	1.2 ± 0.1	<1 x 10^{-10}	0.5 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
3-83μδ Btk^{+} deleting	4	4.0 ± 1.4		0.7 ± 0.3	1.0 ± 0.4	1.8 ± 0.8
3-83μδ Btk^{-} deleting	7	2.1 ± 1.0	NS	0.2 ± 0.1	0.4 ± 0.2	1.0 ± 0.6
3-83μδ/Bcl-2 double Tg Btk^{+} nondeleting	3	28.3 ± 2.8		0.7 ± 0.2	11.7 ± 0.8	11.3 ± 2.6
3-83μδ/Bcl-2 double Tg Btk^{-} nondeleting	3	15.8 ± 4.1	<0.05	5.3 ± 1.3	6.0 ± 1.3	1.9 ± 0.7

[a] *Absolute number as mean ± SEM (x10^{6})*
[b] *Values of P for the difference in absolute numbers of B220^{+} cells compared with Btk^{+} littermates, determined by Student's t test*
[c] *T1, T2, and mature B cell subsets of B220^{+} cells*

and κ L chain, both in BM and in spleen (Figs. 2, *A* and *B*, and 3, *A* and *B*).

The residual B cell population in Btk^{-} 3-83μδ spleens mainly consisted of immature IgM^{high} IgD$^{-/low}$ that were CD21^{-} and CD23^{-}, indicating that these B cells were recent immigrants from the BM arrested at the stage of transitional type 1 (T1) B cells (Fig. 3*B* and Table I; Refs. 30 and 31). This was confirmed by an evaluation of additional developmentally regulated cell surface markers. As shown in Fig. 3*C*, splenic B cells in Btk^{+} and Btk^{-} non-Tg mice and in Btk^{+} 3-83μδ mice are largely CD43^{-}, PB493^{-}, CD2^{high}, CD22^{high}, and CD62L^{+}. In contrast, Btk^{-} 3-83μδ B cells showed significantly increased expression of the immature markers CD43 and PB493 and reduced expression of the maturation markers CD2, CD22, and CD62L. Taken together, only a small number of peripheral B cells reached the stage of T2 cells (IgM^{high}, IgD^{+}, CD21^{+}, CD23^{+}; Refs. 30 and 31) and mature IgM^{low} IgD^{+} cells were virtually absent (Fig. 3 and Table I). The expression levels of SLC, BP-1, CD25, and MHC class II were similar in the four groups of mice (data not shown). Despite the low B cell numbers and their immature phenotype, splenic architecture was not seriously disturbed in Btk^{-} 3-83μδ mice as B/T cell separation was normal and small regions with marginal zone B cells and macrophages were present, as analyzed by immunohistochemistry (data not shown).

In summary, these findings show that peripheral Btk^{+} 3-83μδ B cells differentiated normally. By contrast, Btk-deficient 3-83μδ mice exhibited a severe reduction of B cell numbers in the spleen, whereby the residual B cells present were Id positive and mainly had an immature T1 surface phenotype.

Btk-deficient 3-83μδ *B cells are rapidly deleted upon arrival in the spleen.* The finding that the proportions of B220^{+} B lineage cells in BM of Btk^{+} 3-83μδ mice were not significantly different from those in Btk^{-} 3-83μδ mice (5.5% ± 1.2 and 4.5% ± 1.8, respectively) implied that the numbers of B cells generated in the BM of Btk^{+} and Btk^{-} 3-83μδ mice were comparable. Therefore, the severe decrease in splenic B cell numbers in Btk^{-} 3-83μδ mice may be caused by defective homing of immature B cells from the BM into the spleen or alternatively by rapid deletion upon arrival in the spleen. To distinguish between these two possibilities, we investigated the competition between Btk^{+} and Btk^{-} 3-83μδ B cells in vivo. We analyzed heterozygous Btk$^{+/-}$ female mice, which have the *Btk^{-}lacZ^{+}* allele on the active X chromosome in ~50% of all cells, due to random X chromosome inactivation. However, when in these mice B lineage cells reach a differentiation stage in which Btk is required, the *Btk^{-}lacZ^{+}* cells will be arrested in development and consequently the proportions of *Btk^{-} lacZ^{+}* cells decrease below 50% (19).

BM and spleen cell suspensions from Btk$^{+/-}$ non-Tg and 3-83μδ mice were compared for the proportions of *lacZ*-expressing cells by using fluorescein-di-β-galactopyranoside as a substrate in conjunction with surface labeling of B220, IgM, and IgD. Irrespective of the presence of the 3-83μδ transgene, the spleens of Btk$^{+/-}$ mice contained detectable fractions of *lacZ^{+}* B cells, indicating that homing of B cells from the BM into the spleen was not altered by the expression of the 3-83μδ BCR (Fig. 3*D*). Moreover, the immature IgM^{+}IgD^{-} fraction in 3-83μδ mice contained >50% *lacZ^{+}* cells, showing that Btk-deficient B cells accumulated due to the differentiation arrest. Consistent with previous

findings (19), a dramatic selection against *Btk⁻lacZ⁺* cells occurred during B cell differentiation in the spleen: the immature IgM⁺IgD$^{-/low}$ cell populations still contained substantial proportions of *Btk⁻lacZ⁺* cells, whereas in the IgM^{low}IgD^{high} mature B cell population these cells were virtually absent (Fig. 3*D*).

To confirm that Btk-deficient 3-83μδ B cells were deleted upon arrival in the spleen, we performed BrdU-labeling experiments in vivo. Btk⁺ or Btk⁻ 3-83μδ mice were injected with a single dose of BrdU, which is incorporated into the DNA of large cycling pre-B cells in the BM (17, 25, 32). The amount of BrdU incorporation was found to be similar in BM B cells from both types of mice (data not shown). One, 2, or 3 days after BrdU injection, spleen cell suspensions were stained for B220, IgM, and IgD in conjunction with intracellular staining for BrdU. B220⁺IgM^{high}IgD$^{-/low}$ cells were gated and the absolute number of BrdU⁺ cells was calculated. We found that in Btk⁺ 3-83μδ mice the number of BrdU⁺IgM^{high}IgD$^{-/low}$ B cells accumulated over time (Fig. 3*E*). In contrast, the number of BrdU⁺ cells in Btk⁻ 3-83μδ mice remained low, indicating that the number of cells arriving in the spleen was similar to the number of cells that was deleted.

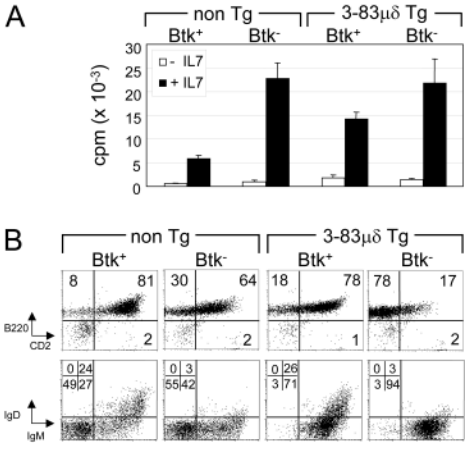

Figure 4. *Analysis of IL-7-driven BM cultures from Btk⁺ and Btk⁻ non-Tg and 3-83μδ mice. A, Proliferative responses of total BM cells as determined by [^{3}H]thymidine incorporation after 5 days of culture with or without IL-7. Means ± SD values for two to four mice per genotype were plotted. B, After 5 days of culture with IL-7, cells were washed and recultured for 48 h on S17 stromal cells in the absence of IL-7. Expression profiles of B220/CD2 on live cells (upper part) and of IgM/IgD on B220⁺ gated cells (lower part) are shown. Data are representative of five mice analyzed per group.*

From these findings, we conclude that Btk⁻ 3-83μδ immature B cells migrated normally from the BM into the spleen, but upon arrival these B cells did not further differentiate and were deleted within 24 h.

Btk⁻ 3-83μδ BM B cells show deficient developmental progression in vitro. To investigate whether the inefficient 3-83μδ BCR-mediated induction of cell phenotype changes in Btk⁻ B cells in vivo were paralleled in BM cultures in vitro, IL-7-driven BM cultures were performed as described previously (5, 33). Total BM cells from Btk⁺ and Btk⁻ non-Tg and 3-83μδ mice were cultured in the presence of 100 U/ml IL-7 for 5 days and proliferation was quantified in [^{3}H]thymidine incorporation experiments (Fig. 4*A*). Consistent with our previous report (5), Btk⁻ non-Tg BM cultures showed significantly higher proliferative responses to IL-7 when compared with Btk⁺ cultures. Proliferative responses to IL-7 were similar in Btk⁺ and Btk⁻ 3-83μδ BM cultures. To evaluate developmental progression in vitro, BM cells were cultured in the presence of IL-7 for 5 days and recultured in the absence of IL-7 on S17 stromal cells for 48 h. As previously described (5), under these conditions, WT pre-B cells differentiated into immature B cells expressing CD2, IgM, and IgD, whereas Btk-deficient pre-B cells were not able to efficiently up-regulate CD2 and IgD expression (Fig. 4*B*). In BM cultures from Btk⁺ 3-83μδ mice, expression of CD2 and IgD was induced on the B cells, but Btk⁻ 3-83μδ B cells were not able to up-regulate CD2 and IgD upon IL-7 withdrawal (Fig. 4*B*).

These data show that also under in vitro conditions, developmental progression of Btk⁻ 3-83μδ BCR-expressing B cells is impaired, as is clear from low CD2 and IgD induction upon IL-7 withdrawal.

Receptor editing can compensate for developmental defects in Btk⁻ 3-83μδ mice. To investigate whether receptor editing and the resulting delay at the small pre-B cell stage in the BM could compensate for the cellular maturation defects in Btk⁻ 3-83μδ mice, we targeted 3-83μδ B cells for receptor editing by crossing the 3-83μδ mice onto a centrally deleting MHC class I background.

When we analyzed the BM of Btk⁺ and Btk⁻ 3-83μδ centrally deleting mice by flow cytometry, we found that B220⁺cμ⁺ cells had down-regulated surface expression of IgM and CD19 and were also low for the anti-idiotypic Ab 54-1, confirming the autoreactive nature of the

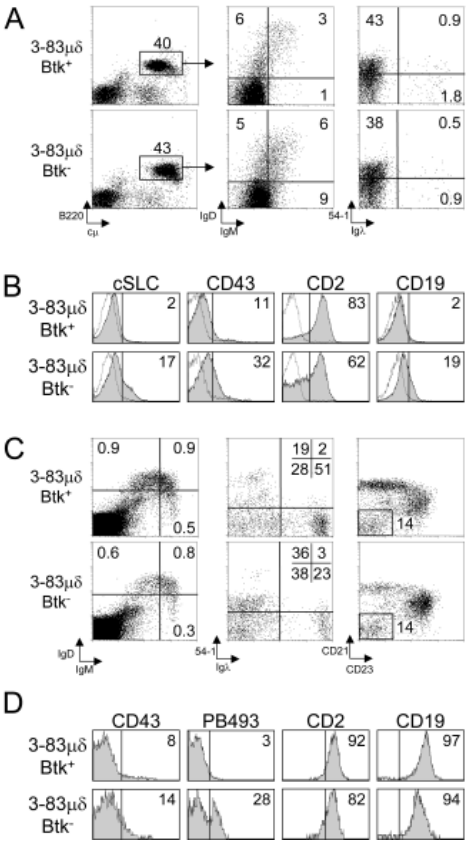

Figure 5. *Phenotypic characterization of the B cell compartment in Btk⁺ and Btk⁻ 3-83μδ mice on a centrally deleting background. A and B, BM lymphoid cells were gated and plotted for the expression of B220 and cμ. B220⁺cμ⁺ cells were gated and plotted for IgM/IgD and 54-1/Igλ expression profiles (A) and analyzed for the expression of the indicated markers (B). C, Splenic lymphoid cells were gated and analyzed for IgM/IgD expression and for CD21/CD23 and 54-1/Igλ expression profiles on B220⁺ gated cells. D, Expression pattern of the indicated markers on B220⁺ cells. The percentages of cells within the indicated gates or quadrants are given. Data represent 8–20 mice analyzed per genotype.*

B cell compartment (Fig. 5*A*). The expression profiles of developmentally regulated surface markers on B220^{low}cμ⁺ cells in Btk⁺ 3-83μδ mice were similar to those found in non-Tg WT immature B cells, i.e., SLC⁻CD43⁻CD2⁺ (Fig. 5*B*). Remarkably, Btk⁻ 3-83μδ centrally deleting mice manifested an almost complete correction of marker expression profiles when compared with Btk⁻ 3-83μδ nondeleting mice: expression of SLC and CD43 was low and CD2 was present on a large fraction of cells (cf Figs. 5*B* and 2*B*). Therefore, we conclude that extended receptor editing in the BM could almost completely correct the Btk-dependent defects in B cell surface marker expression of 3-83μδ immature B cells in the BM.

The spleens of 3-83μδ-deleting mice contained very few B cells, irrespective of the presence of Btk (Fig. 5*C* and Table I). As a result, the total splenic B cell numbers in Btk⁻ 3-83μδ mice were comparable on the nondeleting (~1.2 x 10^6) and the deleting MHC class I background (~2.1 x 10^6; Table I). Those Btk⁺ and Btk⁻ B cells present in the spleen of 3-83μδ-deleting mice have essentially lost 54-1 expression and show increased Ig λ L chain usage due to receptor editing (cf Figs. 5*C* and 3*B*). Remarkably, we found that the residual B cells in the spleens of Btk⁺ and Btk⁻ 3-83μδ-deleting mice had similar IgM/IgD and CD21/CD23 profiles (Fig. 5*C*), had similar expression levels of CD43, CD2, CD19, and MHC class II, and were negative for SLC, BP-1, and CD25 (Fig. 5*D* and data not shown).

In summary, these data indicate that both Btk⁺ and Btk⁻ 3-83μδ B cells that were targeted for receptor editing have reduced maturation defects when compared with Btk⁺ and Btk⁻ 3-83μδ B cells on a nondeleting background. The expression of the developmentally regulated markers is completely corrected in edited Btk⁺ 3-83μδ peripheral B cells, while in edited Btk⁻ 3-83μδ cells the cellular maturation defects are comparable to those found in non-Tg Btk⁻ B cells (Figs. 1 and 2).

*Bcl-2 overexpression does not rescue Btk-dependent marker modulation. W*e investigated whether the observed correction of marker modulation in editing Btk-deficient pre-B cells was dependent on additional signals that follow the engagement of an autoreactive BCR with autoantigen or alternatively resulted from the developmental delay in the small pre-B cell stage associated with receptor editing. To this end, Btk-deficient mice were crossed with Tg mice that express the antiapoptotic *Bcl-2* gene, which is assumed to provide an extended time window per cell for Ig L chain rearrangement (34). When we compared the B cell compartment in Btk⁺ and Btk⁻ Eμ-Bcl-2 Tg mice, we found that enforced expression of Bcl-2 only slightly improved cellular maturation of pre-B and immature B cells in the absence of Btk (cf Figs. 6, *A* and *B*, and 1).

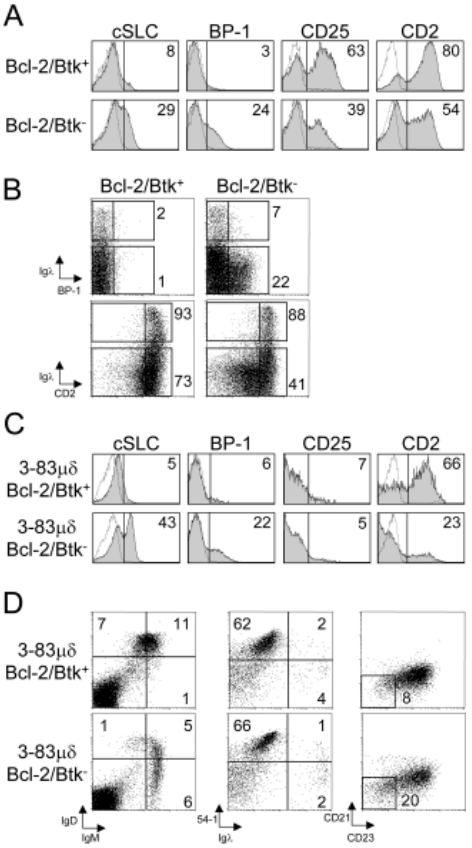

Figure 6. *Bcl-2 expression does not rescue developmental defects in Btk-deficient non-Tg or 3-83μδ mice. A, BM B220^{low}IgM$^-$ pro- and pre-B cells of Btk$^+$ and Btk$^-$ Bcl-2 Tg mice were gated and expression of several developmentally regulated markers is shown as histograms. Numbers indicate percentage of cells positive for the indicated marker and plots are representative for two to four mice of each genotype. B, BM B220^{low}IgM$^+$ immature B cells were gated and expression of Igλ and BP-1 or CD2 is shown. Numbers indicate percentage of BP-1- or CD2-positive cells within λ$^+$ or κ$^+$ immature B cells. C, BM B220^{low} cells of Btk$^+$ and Btk$^-$ 3-83μδ/Bcl-2 double Tg mice were gated and analyzed for expression of several developmentally regulated markers as shown in the histograms. D, Splenic lymphoid cells were gated and analyzed for IgM/IgD expression and for CD21/CD23 and 54-1/Igλ expression profiles on B220$^+$ gated cells. The percentages of cells within the indicated gates or quadrants are given. Data represent three mice analyzed per genotype.*

Next, we investigated whether Bcl-2 expression could rescue the developmental defects observed in Btk$^-$ 3-83μδ nondeleting mice. Therefore, Btk-deficient 3-83μδ mice were crossed with Eμ-Bcl-2 Tg mice on a non-deleting MHC class I background. Overexpression of Bcl-2 did not rescue cellular maturation of Btk$^-$ 3-83μδ immature B cells, as expression of SLC, BP-1, CD43, and PB493 was not down-regulated and expression of CD25, CD2, and IgD was hardly induced (Fig. 6*C* and data not shown). The BM B cells in Btk$^+$ and Btk$^-$ 3-83μδ/Bcl-2 double Tg mice were all 54-1$^+$ (data not shown) and did not show increased λ usage (<0.3%). The absolute number of splenic B cells in Btk$^-$ 3-83μδ/Bcl-2 double Tg mice was corrected when compared with Btk$^-$ 3-83μδ mice and the B cells in the spleen were not arrested at the T1 stage (Fig. 6*D* and Table I). The B cells in Btk$^-$ 3-83μδ/Bcl-2 double Tg spleens were largely IgM^{high}CD21$^+$CD23$^+$, indicating an almost complete block in B cell development from transitional stage T2 into the mature B cell compartment. Compared with their Btk$^-$ 3-83μδ single Tg littermates, a larger fraction of splenic B cells in Btk$^-$ 3-83μδ/Bcl-2 double Tg mice differentiated into CD21$^+$CD23$^+$ B cells (>80% compared with <50%, respectively; Fig. 6*D*).

From these data, we conclude that an extended time window per cell for pre-B cell differentiation by enforced expression of Bcl-2 was not sufficient to rescue the maturation defects in Btk-deficient pre-B cells. However, the expression of Bcl-2 rescued the peripheral B cell arrest at the T1 stage in Btk$^-$ 3-83μδ mice and consequently B cells were arrested at the T2 stage, as in non-Tg Btk-deficient mice.

Discussion

Btk is essential at two distinct steps in B cell development in the mouse (a) at the developmental progression of CD43$^+$CD2$^-$ large cycling into CD43$^-$CD2$^+$ small resting pre-B cells and (b) at the checkpoint for selection of immature IgM^{high} T2 cells into the pool of long-lived IgM^{low} mature B cells (5, 19, 28, 30, 31, 35, 36). In this report, we show that in Btk-deficient mice premature expression of the 3-83μδ BCR amplifies the cellular maturation defects in immature B cells in the BM and results in an arrest of peripheral B cell development at the transition of T1 into T2 cells in the spleen. Both defects are largely restored when Btk-deficient 3-83μδ mice are crossed onto a centrally deleting background where the B cells were targeted for receptor editing. In contrast, enforced expression of the apoptosis inhibitor Bcl-2, which prolongs (pre-)

B cell life span, does not rescue the maturation defects in Btk-deficient 3-83μδ immature B cells in the BM, but does restore the development of T2 B cells in the spleen.

Expression of the pre-BCR triggers IL-7-driven proliferative expansion, allelic exclusion, cellular maturation of pre-B cells, and the initiation of L chain rearrangement (1, 2). We have previously shown that Btk has an important function in the regulation of the substantial changes in cell phenotype that accompanies pre-B cell maturation, including down-regulation of SLC, CD43, and BP-1, and up-regulation of CD2, CD25, and MHC class II (5, 8). However, the functional significance of the modulation of the individual surface markers at the pre-B cell stage is largely unknown, with the exception of SLC. The down-regulation of SLC expression is assumed to limit the capacity of pre-B cells to proliferate. Hence, after several divisions, these cycling cells fall into a resting state where L chain rearrangement is initiated. At this stage of B cell development, Btk and SLP-65 have synergistic roles as tumor suppressors to limit pre-B cell expansion, most likely by signaling the down-regulation of SLC expression (8). Recent analyses of RNA expression profiles identified a major change in gene expression at the transition from cycling into resting pre-B cells, as ~1000 genes were found with differential expression with a magnitude of >2 (37, 38). In flow cytometric analyses, we observed similar differences between Btk-deficient and WT B cells for surface and intracellular expression of CD2, CD25, and MHC class II in $c\mu^+$ pre-B cells (S.M. and R.W.H., unpublished results), indicating that the absence of Btk affects the initiation of the expression of these genes rather than transport of existing protein from cytoplasmic stores to the cell surface. It is therefore possible that Btk is directly or indirectly involved in the regulation of a substantial proportion of the genes required for the initiation of new differentiation programs in resting pre-B cells.

In this context, we found that premature expression of the prerearranged 3-83μδ BCR, which accelerates B cell development (34, 39), is not completely able to replace pre-BCR function, as cellular maturation in these mice was impaired, when compared with non-Tg mice. Nevertheless, the aberrant phenotype of 3-83μδ immature B cells did not seem to significantly affect their capabilities for further maturation in the spleen. In contrast, in the absence of Btk we found a much more defective modulation of marker expression in the BM and a severe decrease in absolute B cell numbers in the spleen, reflecting an almost complete arrest of B cell development at the transition of T1 to T2 immature B cells in the spleen. This contrasts with the arrest in non-Tg Btk-deficient mice, which is at the next developmental transition from T2 into mature B cells.

The cells generated in the BM of the Btk^- 3-83μδ mice were shown to home into the spleen, even in the presence of competitive Btk^+ 3-83μδ B cells. In BrdU-labeling studies, we showed that the numbers of B cells immigrating from the BM in the spleen within 24 h is similar in Btk^+ and Btk^- 3-83μδ mice. However, Btk^- 3-83μδ B cells are efficiently deleted upon arrival in the spleen. It is not likely that the elimination of Btk^- 3-83μδ B cells is due to autoreactivity of the 3-83μδ BCR as (a) the deletion of B cells takes place in the spleen and not in the BM; (b) immature B cells in the BM did not manifest down-regulation of CD19 and IgM surface expression; (c) most splenic B cells express the 3-83μδ Id 54-1, indicating that they did not perform receptor editing; and (d) when the Btk^- 3-83μδ B cells were on a centrally deleting background, the induction of receptor editing even increased survival of T2 peripheral B cells. Instead, our finding that the early T1 to T2 block could be restored by enforced Bcl-2 expression indicates that Btk is required for the survival of T2 cells that carry the 3-83μδ BCR. Interestingly, 3-83μδ Tg and 3-83μδ Bcl-2 double Tg $Lyn^{-/-}$ B cells were also found to be arrested at the T1 and T2 immature B cell stage in the spleen, respectively (40), further supporting synergistic roles for Lyn and Btk in peripheral B cell survival (41). In this respect, the function of CD45 and CD19 in peripheral B cell survival appears to be different, as 3-83μδ Tg $CD45^{-/-}$ and $CD19^{-/-}$ mice showed relatively mild defects in the transitional B cell compartment in the spleen (39, 42, 43).

We have previously shown that lack of Btk did not abolish the receptor editing competence of immature B cells (20). Surprisingly, we found that when Btk-deficient 3-83μδ mice were crossed onto a centrally deleting background and the B cells were targeted for receptor editing, both the cellular maturation defects of immature B cells in the BM and the defective T1 to T2 transition of splenic B cells are restored. It has been shown that B cells with an autoreactive BCR are delayed for 2–12 h in the small pre-B cells compartment where L chain rearrangements

take place (17, 18). This delay may provide an extended time window per cell, which could be sufficient to compensate for the impaired cellular maturation of Btk$^-$ pre-B cells. Alternatively, it is also possible that engagement of the BCR with membrane-bound autoantigens induces distinct Btk-independent signaling pathways that drive cellular maturation of editing immature B cells. However, enforced expression of Bcl-2 did not improve cellular maturation of non-Tg or 3-83$\mu\delta$ Btk-deficient immature B cells, indicating that increased life span is not sufficient to correct the Btk-mediated pre-BCR maturation signals. Therefore, our observations would be consistent with the hypothesis that an autoreactive BCR undergoing receptor editing transmits an additional unique signal that enhances cellular maturation of editing pre-B/immature B cells, next to the signals required for the receptor editing events, such as expression of the recombination-activating gene proteins. Such an additional signal would not be provided by an innocuous BCR, as on a nondeleting MHC class I background 3-83$\mu\delta$ immature B cells showed defective cellular maturation. Additional experiments are required to investigate whether BCR stimulated by autoantigens indeed provide maturation signals for editing cells and whether such signals affect the capacity of immature B cells to continue receptor editing or their sensitivity to Ag-induced apoptosis (13).

In this context, the finding that in Btk-deficient mice κ^+ B cells show a more profound cellular maturation defect than λ^+ B cells may suggest that in these mice λ^+ B cells have performed extensive receptor editing. This may be supported by our previous analysis of the kinetics of pre-B cell differentiation in vivo (5, 20). We observed that in Btk-deficient mice, λ^+ B cells manifest a specific developmental delay within the small pre-B cell compartment of ~3 h, accompanied by a decreased production rate. Therefore, the residual λ^+ B cell population in Btk-deficient mice may develop relatively late and consequently contain more cells that have performed receptor editing. Additional experiments are required to directly determine the extent of receptor editing in Btk-deficient mice.

In conclusion, we have shown that in the absence of Btk 3-83$\mu\delta$ B cells are quite efficiently deleted during their development, whether they are on a nondeleting background or on a centrally deleting background. On the nondeleting background, the premature BCR expression amplifies the cellular maturation defects in Btk-deficient immature B cells, finally resulting in defective survival at the T1 to T2 transition in the spleen. On the autoreactive MHC class I background, most immature B cells are deleted in the BM, but cellular maturation defects are significantly reduced and edited B cells that reach the spleen no longer exhibit the T1 to T2 arrest. Our results suggest that at the immature B cell stage in the BM, BCR stimulation by autoantigens does not only induce receptor editing, but also enhances cellular maturation.

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General discussion

Parts of this chapter are published as a review in Trends in Immunology

1. Introduction

Btk-deficient mice manifest an almost complete block during late B cell development in the spleen, resulting in very low numbers of circulating B cells (1-4). In contrast, XLA patients, who also have defects in the Btk gene, were shown to have an almost complete block in pre-B cell development, resulting in very low numbers of immature B cells already in the BM (5, 6). Previous studies using targeted Btk/*LacZ* mice indicated a role for Btk at an earlier stage in B cell development in the mouse as well, because a selective disadvantage was found for Btk-deficient cells at the pre-B to immature B cell stage transition (7). In addition, studies using a mouse pre-B cell line (2) and Tec/Btk double deficient mice (8)

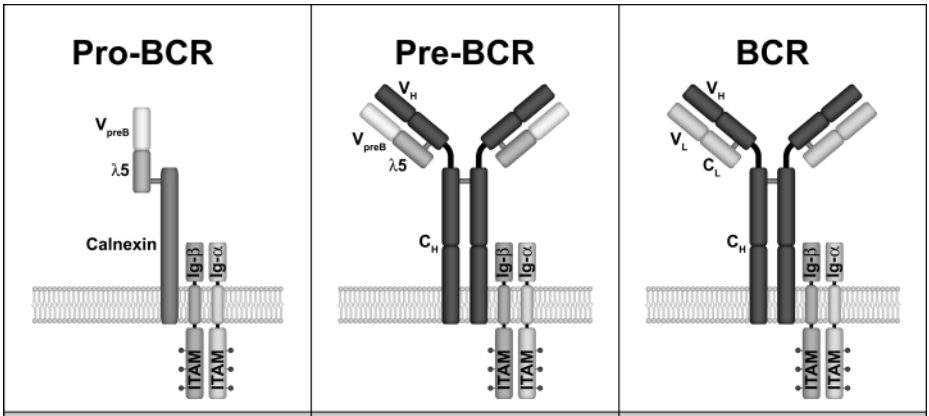

Figure 1. *Summary of the functions of signalling pathways downstream of the BCR and its immature forms in early B cell development in the bone marrow. For the pro-BCR and the pre-BCR on B cell precursors and the BCR on immature B cells, Btk-independent and Btk-dependent functions are indicated.*

indicated a role for Btk downstream the pre-BCR.

To study the role of Btk during pre-B cell development in the mouse in more detail, we made use of the Btk/*LacZ* mouse model, which was generated by inactivation of the *Btk* gene by insertion of a *LacZ* reporter gene (7). From the results presented in this thesis, we can conclude that Btk plays an important role at the pre-BCR checkpoint (Figure 1). Our findings demonstrate that Btk directly regulates L chain rearrangement (Chapter 1), pre-B cell differentiation (Chapter 2) and cellular maturation of immature B cells (Chapter 4). Furthermore, we and others have found that the interaction of Btk with the linker protein SLP-65 is required for proper signalling at the pre-BCR checkpoint and analysis of mice deficient for both proteins revealed a role for Btk as a tumor suppressor (Chapter 3 and Ref. (9)). Next to its role in pre-BCR and BCR signalling, we have found that Btk also acts downstream the pro-BCR (Figure 1; Chapters 2.1 and 2.3).

Table 1. *Phenotype of different Btk mutant mice*

R28C[a] : PH domain, Btk cannot bind to PIP3
Impaired downregulation of pre-BCR-induced proliferation Impaired cellular maturation of pre-B cells Reduced Igλ usage ~50% reduction of peripheral B cells due to survival problem for mature B cells Deficient induction of c-Rel and Bcl-x_L upon BCR stimulation Lack of B-1 B cells Reduced IgM and IgG3 serum-levels, no T-cell independent antibody response
E41K[b] : PH domain, Btk remains bound to PIP3, constitutive activation
Induction of κ L chain, CD2, CD25 and MHC II expression in μ^- pro-B cells Increased IL-7 responsiveness of pro-B cells Normal pre-B cell differentiation Immature B cells in BM are deleted Increased Igλ usage Almost no peripheral B cells
Y223F[b] : SH3 domain, autophosphorylation (auto-P)-site disrupted
Reduced Igλ usage, but no further defects in B cell development or function
E41K/Y223F[b] : PH and SH3 domains, constitutive activation, (auto-P)-site disrupted
Induction of κ L chain, CD2, CD25, and MHC II expression in μ^- pro-B cells Increased IL-7 responsiveness of pro-B cells Normal pre-B cell differentiation Immature B cells in BM are deleted Almost no peripheral B cells
K430R[b] : Kinase domain, ATP-binding site disrupted, kinase activity lost
Reduced downregulation of pre-BCR-induced proliferation Reduced cellular maturation Normal Igλ usage Reduced survival problem of mature B cells Almost normal induction of c-Rel and Bcl-x_L upon BCR stimulation Lack of B-1 cells Reduced IgM and IgG3 serum-levels, no T-cell independent antibody response

[a] Spontaneous mutation in CBA/N mice; similar phenotype as Btk-null mutant mice
[b] Expression of transgenic human Btk mutants under the control of the B cell specific promoter CD19
References: (7, 10-17)

To analyse the function of Btk during B cell development, not only Btk null mutant mice were analysed, but also various transgenic mouse models with specific gain-of-function or loss-of-function mutations were studied. The transgenic human *Btk* constructs were placed under the control of the B cell specific CD19 promoter to ensure expression from the pro-B cell stage onwards. These transgenic mouse models were crossed onto a Btk-deficient background and the effects of the specific point mutations on B cell development were characterized (Table 1). Mice carrying the E41K gain-of-function mutant, the Y223F autophosphorylation mutant, the E41K/Y223F double mutant and the K430R kinase-inactive mutant provided information on the role of the individual domains of Btk in pre-B cell development (Chapters 1.1, 2.2 and 2.3). An interesting and unexpected aspect of our findings is that we have found that Btk can also function as an adapter molecule, next to its role as a tyrosine kinase protein (Chapter 2.2 and 3.2).

Pre-BCR signalling pathways

Studies in Btk-deficient cells showed that Btk is an important regulator of BCR-induced calcium mobilization (18, 19). The mobilization of intracellular calcium is one of the major targets of BCR signalling and is critical for the regulation of multiple transcription factors such as NF-κB and Nuclear Factor of Activated T cells (NFAT) (20-23). In Btk-deficient cells defects in sustained calcium influx resulted in decreased phosphorylation of phospholipase Cγ2 (PLCγ2) resulting in impaired inositol-trisphosphate (IP3) and diacylglycerol (DAG) production. As a consequence, lymphocytes defective in Tec family kinases show decreased activation of multiple downstream targets dependent on IP3 and DAG production, including activation of NF-κB, NFAT, MAP kinases and PKC (24, 25). However, NF-κB induction might not be essential for pre-BCR checkpoint function, as B cell

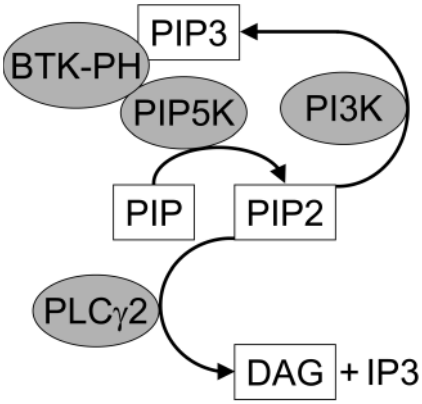

Figure 2*. Activated Btk (or Tec) can bind to PIP3 and PIP5K with its PH domain (Btk-PH). This stimulates a positive feedback loop in BCR signalling by activating PIP5K to increase the hydrolysis of PIP into PIP2, the substrate for both PI3K and PLCγ2. PLCγ2 activation results in production of DAG and IP3, important for calcium mobilization and NF-κB activation. For details and abbreviations, see text.*

development is unimpaired in mice deficient for various NF-κB subunits (26). By contrast, the downstream Ras/Raf/MEK/MAP kinase pathway (Figure 4) is a key regulatory signalling cascade, as its activation can induce proliferation and cellular maturation of pre-B cells, as well as Ig L chain gene rearrangement (Figure 4; (27-29)).

Although Btk has been implicated in direct phosphorylation of PLCγ2, tyrosine phosphorylation of PLCγ2 was still observed in cells deficient for Tec family kinases, despite the calcium defects (18, 19, 24). Overexpression of wild type or kinase inactive Btk has been shown to increase PIP3 levels and to partially rescue calcium mobilization in Btk-deficient DT40 cells (30, 31). This indicates that other molecules also contribute to the phosphorylation and activation of PLCγ2.

Recently, Btk as well as Tec were found to be able to associate directly with phosphatidylinositol-4-phosphate 5-kinase (PIP5K) through their PH domains, thereby acting as a shuttle to bring PIP5K to the plasma membrane and into lipid rafts. PIP5K is the enzyme that generates phosphatidylinositol bisphophate (PIP2), by the phosphorylation of phosphatidylinositol phosphate (PIP), as was observed in

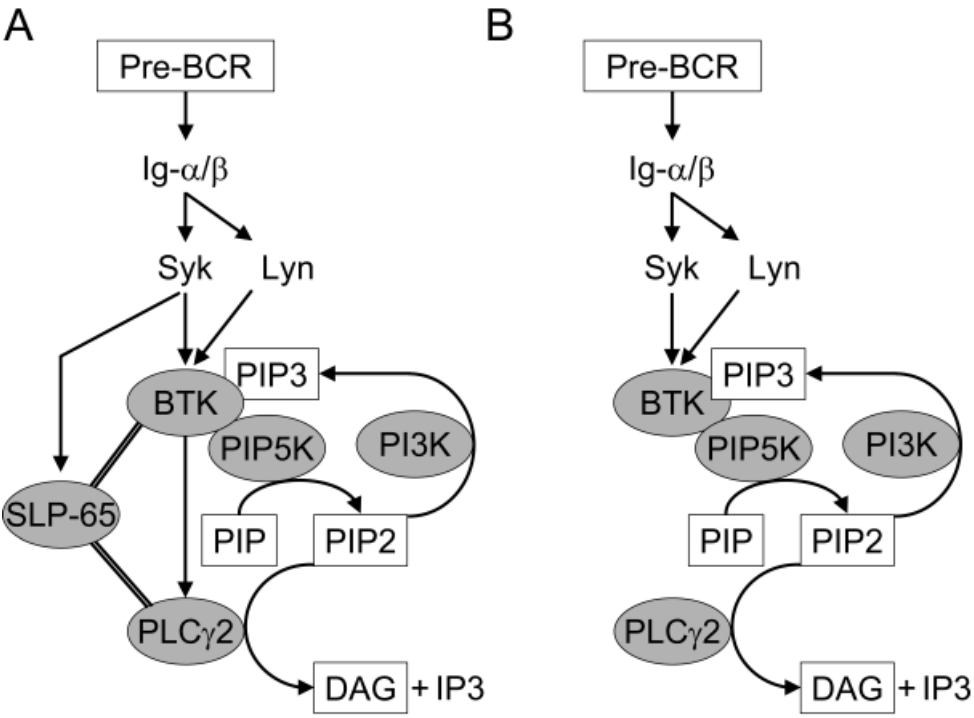

Figure 3. *(A) Signalling pathways downstream the pre-BCR including the SLP-65 dependent route. (B) Signalling pathway in the absence of SLP-65. This pathway is not dependent on kinase activity of Btk. Double lines reflect the link between Btk, SLP-65 and PLCγ2, straight arrows reflect activation and bended arrows reflect enzymatic conversions involving the enzyme attached to the arrow. For details and abbreviations, see text.*

A20 mature B cells (Figure 2; (32)). PIP2 is the substrate for both PLCγ2 and PI3K and PI3K phosphorylates PIP2 to generate PIP3 molecules that bind and activate proteins with PH domains, such as Btk (33). By activating PIP5K, Btk provides more PIP2 substrate for both PI3K, required for Btk activation, and PLCγ2, the downstream target of Btk, generating a positive feedback loop to sustain both Btk activation and PLCγ2 activity (Figure 2). This PIP5K shuttle function of Btk was found to be independent of Btk kinase activity (32).

As shown in chapter 2.2, we found that in the pre-BCR signal transduction pathway, Btk can partially function as an adapter molecule, independent of its kinase activity (17). Expression of kinase-inactive Btk in early B cell development normalized λ L chain usage and partially restored the impaired pre-B cell differentiation and IL-7 responsiveness in Btk-deficient mice (Table 1). From these findings, we concluded that Btk was able to function at least partially as an adapter molecule next to its role as a tyrosine kinase protein. Btk may act as an adapter molecule by using SLP-65 as a scaffold molecule to bring other kinase proteins such as Syk in close proximity with PLCγ2. However, our findings in K430R-Btk transgenic mice on a SLP-65 deficient background indicate that even in the absence of SLP-65, Btk was able to exert its function as an adapter molecule (Chapter 3.2). The recent finding that kinase-inactive Btk was able to activate the positive feedback loop for PIP2 synthesis leads to the suggestion of parallel signalling pathways downstream the pre-BCR as described in Figure 3.

In contrast to the function of Btk in early B cell development, the kinase function of Btk was more important during later stages of B cell development, as T cell independent antibody responses were still defective and B-1 cells were still absent (Table 1). We have shown that although K430R-Btk did not phosphorylate other proteins, the NF-κB pathway was activated leading to induction of Bcl-x_L and cyclin D2 expression (Chapter 2.2; (17)). These results indicate that the requirement for Btk kinase activity varies between the different Btk-mediated signalling pathways during B cell development and between the different signal transduction routes.

2. Pro-BCR signalling

The physiological functions of the pro-BCR (Figure 1) and its signal transduction pathways have not been identified. At the pro-B cell stage, the construction of the Ig μ H chain is initiated first by recombination of D to J_H elements and subsequently V_H elements are recombined with the D-J_H segment. Next, IL-7R signalling regulates further V_H to DJ_H recombination. When a V_H to DJ_H rearrangement is successful, a μ H chain protein is formed and associates with the SLC and Ig-α/β proteins, which together are expressed on the cell surface as the pre-BCR complex. As a consequence of pre-BCR expression, the pro-B cell is transferred to the cμ^+ large pre-B cell compartment.

In mice deficient for the recombination activating genes (RAG)-1 or RAG-2, no rearrangements take place and as a result, B cell development is blocked at

the pro-B cell stage. The finding that Btk^-RAG^- pro-B cells showed an increased proliferation in response to IL-7 when compared with Btk^+RAG^- pro-B cells indicates that Btk is involved in pro-BCR signalling and that pro-BCR signalling can limit pro-B cell proliferation (Chapter 2.1; (16)). Another piece of evidence for the involvement of Btk in pro-BCR signalling is the finding that in $c\mu^-$ pro-B cells of the gain-of-function E41K-Btk mutant mice expression of cytoplasmic κ L chain and surface CD2, CD25 and MHC class II was induced and expression of SLC, BP-1 and CD43 was downregulated (Chapter 2.3). This indicates that constitutive active Btk can induce a pre-B cell-specific differentiation program in pro-B cells. In summary, these results suggest that pro-BCR expression results in a weak downstream signal, activating Btk among other signalling proteins, possibly to regulate low-level IL-7 driven proliferation of pro-B cells.

3. Pre-BCR signalling

Signalling at the pre-BCR checkpoint is required for the induction of clonal expansion of $c\mu^+$ pre-B cells, and subsequently downregulation of this proliferation, cellular maturation by modulating the expression of cell surface and intracellular markers during the transition of large into small pre-B cells and initiation of L chain rearrangement in small pre-B cells.

It could be argued that Btk does not directly regulate the downregulation of IL-7- driven proliferation, modulation of SLC, CD43, BP1, CD2, CD25 and MHC class II expression and activation of L chain gene rearrangements but instead induces the transition of large cycling into small resting pre-B cells, in which Ig L chain rearrangements and cellular maturation take place in an Btk-independent fashion. However, the observations in E41K-Btk mutant mice indicate that activation of Btk can directly regulate pre-BCR checkpoint functions, because we found that L chain rearrangement and induction of CD2, CD25 and MHC class II expression, which normally takes place in small pre-B cells, was already induced in pro-B cells by transgenic expression of constitutively active Btk (Table 1; Chapter 2.3).

Another possible mechanism for the action of Btk would be that Btk signalling affects the general kinetics of pre-B cell differentiation. In such a model, Btk-deficient pre-B cells only need more time for the transition of the large cycling into the small resting pre-B cell stage, as Btk is required for efficient cellular maturation of large pre-B cells (Chapter 2.1; (16)). However, by crossing Btk-deficient mice with mice transgenic for the anti-apoptosis protein Bcl-2, which provides an extended time window per cell, we found that overexpression of Bcl-2 could not rescue the cellular maturation defects in Btk-deficient mice (Chapter 4; (34)). Therefore, Btk does not only affect the kinetics of pre-B cell differentiation, but also provides signals that regulate efficient pre-B cell development. Together these results indicate that Btk directly induces the downregulation of IL-7 driven proliferation, the modulation of SLC, CD43, BP1, CD2, CD25 and MHC class II expression and Ig L chain locus opening upon pre-BCR signalling.

3.1 Termination of proliferation

As SLP-65 or Btk mutant pre-B cells manifest significantly increased proliferative expansion in response to IL-7 (Chapter 2.1; (16, 35)), it can be concluded that the pre-BCR-mediated signalling pathway essential for proliferation, *i.e.* the Ras/Raf/MEK/MAP kinase cascade, should be independent of SLP-65 or Btk. In agreement

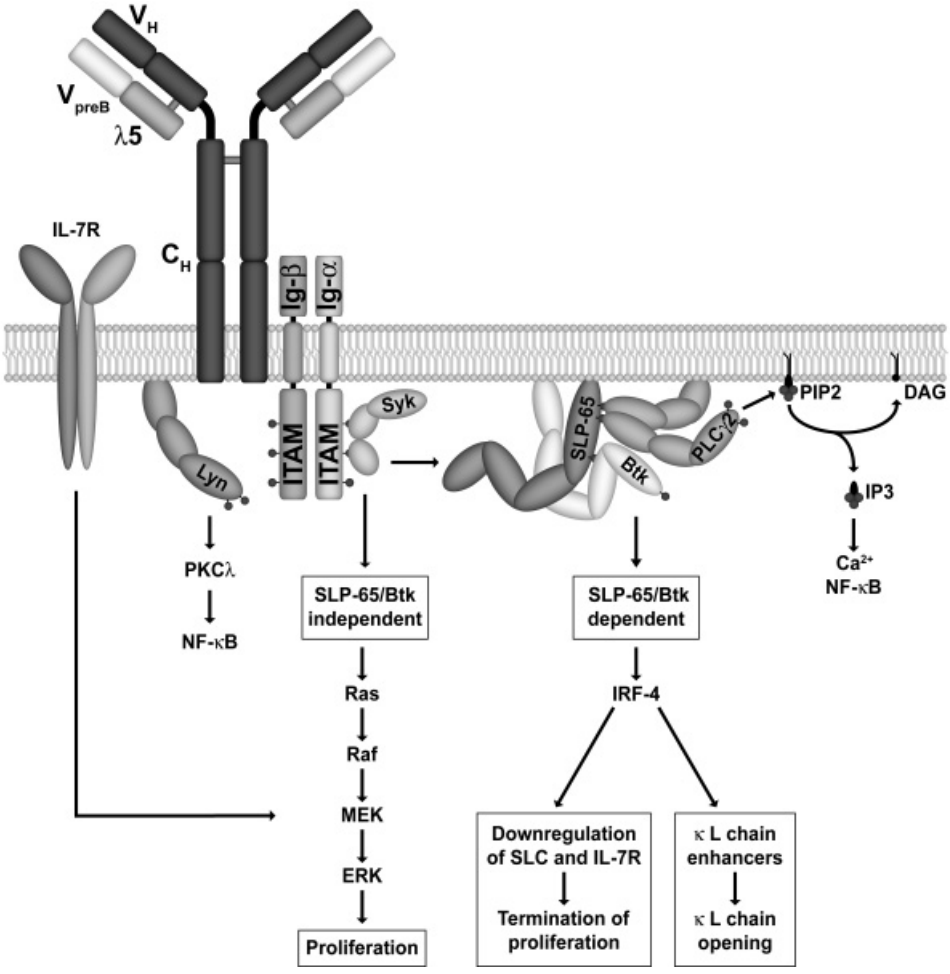

Figure 4. *Pre-B cell receptor signalling model. Proliferation is induced in a SLP-65/Btk-independent pathway involving Erk MAP kinase. Phosphorylated SLP-65 provides binding sites for Btk and PLCγ2 SH2 domains. This SLP-65/Btk-dependent pathway is essential to signal termination of proliferation by down-regulation of surrogate light chain (SLC) and interleukin-7 receptor (IL-7R) expression and κ L chain opening. IRF-4 might regulate the large to small pre-B transition in part by down-regulating SLC gene expression and by direct induction of enhancer-dependent κ L chain locus opening (37).*

with this, pre-BCR engagement induced Erk activation in the absence of SLP-65 or Btk, and inhibition of Erk activation prevented survival of SLP65$^{-/-}$ pre-B cells (35, 36). Therefore, two separate signalling pathways exist downstream the pre-BCR, one proliferative pathway that is independent of SLP-65 and Btk and results in Erk activation, and a second anti-proliferative pathway that involves SLP-65 and Btk (Figure 4).

In cells receiving an additional pre-BCR signal, higher levels of phosphorylated Erk MAP kinase were produced in response to low amounts of IL-7 (38). As Erk activation is implicated in pre-BCR-induced clonal expansion (27, 36), these findings demonstrate that the pre-BCR and IL-7R signalling pathways intersect functionally (Figure 4).

An additional connection between pre-BCR and IL-7R signalling is clear from the observed SLP-65-mediated repression of IL-7Rα transcription and cell surface expression at the transition of large cycling into small resting pre-B cells (39, 40). Furthermore, introduction of a rearranged L chain in an IL-7R positive pre-B cell line resulted in loss of IL-7 responsiveness (41). This inhibition of proliferative signalling through the IL-7R might be mediated through an inhibitory molecule that is recruited by the BCR to the cytoplasmic domain of the IL-7Rα chain. Taken together, these observations indicate that there are multiple levels by which IL-7 responsiveness is regulated by the BCR and its immature forms.

Two recent discoveries have provided new insights into the cell-autonomous mechanism by which the pre-BCR exerts its key checkpoint function. First, evidence was provided that pre-BCR triggering does not require exogenous ligands to induce clonal expansion (42), and second, the downstream component SLP-65 was shown to act as a feedback inhibitor of pre-BCR signalling (35, 40, 43).

Although Btk-deficient mice do not develop pre-B cell tumors, we have shown that Btk co-operates with SLP-65 as a tumor suppressor in pre-B cells (Chapter 3.1; (43)). Btk/SLP-65 double mutant mice have a dramatically increased pre-B cell tumor incidence, as compared to SLP-65 single deficient mice. Conversely, transgenic low-level expression of a constitutive active form of Btk prevented tumor formation in Btk/SLP-65 double mutant mice, indicating that constitutive active Btk can substitute for SLP-65 as a tumor suppressor (Chapter 3.1; (43)). The importance of Btk and SLP-65 for the induction of proliferation upon BCR cross-linking in mature cells has been well documented. However, quite surprisingly, it now appears that SLP-65 and Btk have an opposite role in pre-B cells, where they terminate proliferative expansion and serve as tumor suppressors. Unexpectedly, the tumor suppressor function of Btk was also shown to be independent of the kinase activity of Btk as the incidence of pre-B cell tumor formation was significantly reduced in K430R-Btk SLP-65^{-} mice when compared to Btk/SLP-65 double deficient mice (Chapter 3.2).

3.2 Cellular maturation of pre-B cells

Recent RNA expression profile studies have indicated that a major change in

gene expression takes place at the transition from large cycling into small resting pre-B cells, as proliferation ceases and new differentiation programs are initiated (44). Pro-B cells express the membrane sialoglycoprotein CD43, SLC and the metallopeptidase BP-1, which are downregulated upon pre-BCR signalling. In contrast the adhesion molecule CD2, the IL-2 receptor CD25 and MHC class II are upregulated upon pre-BCR signalling and are expressed on small pre-B cells. The importance of the modulation of the expression of these proteins is not yet clear. However, CD43, CD2 and IL-2 do not appear to be required at the pre-B cell stage as knockout mice for these genes did not show any defects in B cell development (45-47). Although the role of the CD43, BP-1, CD2 and CD25 proteins during B cell development is unknown, their expression pattern provides a way to analyse the efficiency of pre-BCR signalling in respect to the cellular maturation of pre-B cells *in vivo* (48-51).

It was found that the signalling proteins Btk and SLP-65 play a crucial role at the transition of large into small pre-B cells as Btk or SLP-65-deficient mice fail to efficiently modulate the expression SLC, BP-1, CD43, CD2, CD25 and MHC class II (Chapters 2.1 and 3.1; (16, 40, 43)). In addition, SLP-65 reconstitution experiments in $Ig\mu^{+}Pax5^{-/-}$ pre-B cells indicated that SLP-65 is involved in the transcriptional repression of RAG-1, RAG-2, terminal deoxynucleotidyl transferase (TdT) and IL-7Rα and in the posttranslational down-regulation of cell surface c-kit expression (39). Therefore, it can be concluded that pre-BCR signalling regulates cellular maturation of pre-B cells and this signalling requires the activation of both Btk and SLP-65.

Interestingly, targeted inactivation of the transcription factors interferon regulatory factor (IRF)-4 and IRF-8 is associated with a phenotype that is remarkably similar to that of SLP-65-deficient mice: a developmental block at the pre-B cell stage, whereby IRF-4,$8^{-/-}$ pre-B cells are cycling and fail to down-regulate the pre-BCR (37). As IRF-4,$8^{-/-}$ pre-B cells express normal levels of SLP-65, it is attractive to hypothesise that IRF-4 and -8 function as crucial downstream nuclear targets of SLP-65 signalling. Consistent with this hypothesis, it was recently shown that in Abelson-transformed pre-B cell lines, expression of IRF-4 and Spi-B was sufficient to induce germ-line Ig κ transcription (52). Importantly, the presence of the v-Abl kinase in these cell lines represses the expression of IRF-4, Spi-B and several pre-BCR signalling components including SLP-65 (52). SLP65-deficient mice show defects in germline κ transcription and κ gene rearrangement in pre-B cells, while also κ transcription in immature B cells is impaired (40, 53). Concomitant absence of CD19 appears to enhance the defects in transcriptional activation of the κ locus in SLP65-deficient mice. This might explain the virtually complete arrest of B cell development at the pre-B cell transition in SLP-65/CD19 double mutant mice (40). Future experiments should demonstrate the molecular connection between CD19, SLP-65 and Btk signalling, IRF-4 and Ig κ gene rearrangements.

3.3 Ig L chain locus opening

Btk-deficient mice exhibit a specific developmental delay of ~3h within the small pre-B cell compartment and have reduced λ L chain usage, implicating Btk in the regulation of L chain locus accessibility for V(D)J recombination (Chapters 1.1 and 2.1; (15, 16)).

We have analysed the initiation of L chain rearrangement in small pre-B cells and the usage of κ or λ L chain in immature B cells of Btk-deficient mice. We have found that Btk-deficient mice have a reduced λ L chain usage of ~50% (Chapter 1.1; (15)). This reduction in λ usage was shown to be dependent on the autophosphorylation site Y223 and not of the kinase domain of Btk, as λ usage was reduced in Y223F and normal in K430R transgenic mice (Chapter 2.2; (17)). In gain-of-function E41K transgenic mice, λ usage was increased when compared to wild type mice (Chapter 1.1; (15)). These findings implicate a differential role for the individual Btk domains in the regulation of λ L chain rearrangement.

The reduction in λ usage in Btk-deficient mice could be the result of defective receptor editing. However, when we analysed Btk-deficient 3-83μδ transgenic mice on a deleting (autoreactive) MHC class I background, we found comparable numbers of B cells with edited receptors compared to 3-83μδ deleting mice with endogenous Btk (Chapter 1.1; (15)), indicating that Btk is not required for receptor editing.

The finding of increased λ L chain rearrangement in Bcl-2 transgenic mice indicates that the extent of secondary rearrangement events in pre-B cells with a non-functional L chain rearrangement was dependent on the lifespan of the pre-B cells (54). Furthermore, it was shown that the opening of the λ locus takes more time than opening of the κ locus (55). However, the overexpression of Bcl-2 did not alter the Btk-dependent frequency of λ usage (Chapter 1.1; (15)). Therefore, we conclude that the influence of Btk on λ usage is not at the level of regulating the lifespan of pre-B cells.

By analysis of the frequency of signal broken ends (SBE) that are formed during the V(D)J recombination process under the influence of RAG activity, we were able to determine that Btk was involved in the regulation of the accessibility of the L chain loci (Chapter 1.2). We compared surface Ig^- pre-B cells in which L chain rearrangement was initiated by IL-7 withdrawal in IL-7 driven BM cultures from Btk^+ and Btk^- mice. In addition, we analysed the frequency of specific SBE in sorted large and small pre-B and immature B cell populations of both type of mice. In summary, we found that Btk-deficient mice had lower frequencies of Jλ-specific SBE when compared to wild type mice, which supports the hypothesis that Btk signalling affects the regulation of λ L chain recombination.

Taken together, the findings that in Btk-deficient mice (1) the frequency of λ-expressing immature B cells is reduced, (2) Jλ-specific SBE in pre-B cells are low, (3) Bcl-2 expression does not change the Btk dependent λ usage, and (4) receptor editing appears unaffected, indicate that Btk is involved in the regulation of V(D)J recombination at the λ L chain locus.

3.4 Migration and adhesion

Integrin-mediated migration as well as cell-cell and cell-matrix adhesion plays an important role in B cell development and function. Early B cell development in the BM occurs in defined microenvironments and is controlled by integrin $\alpha4\beta1$-mediated interactions with fibronectin in the extracellular matrix and with vascular cell adhesion molecule (VCAM)-1-expressing BM stromal cells (56-59). Integrins also mediate the trans-endothelial migration of mature B cells required for their recirculation and homing and play a key role in antigen-specific B cell differentiation (60-63).

Recently it has been shown that the BCR controls integrin activity through Btk and PLCγ2 (64). In particular, Btk-deficient pre-B cells showed a ~50% lower basal adhesion in comparison to wild type pre-B cells. Consistent with ligand-independent constitutive signalling by the pre-BCR, in wild type cells this basal adhesion could not be enhanced by PMA-stimulation. However, in Btk-deficient pre-B cells the basal adhesion could be enhanced up to the level of adhesion of control pre-B cells. These observations suggest that Btk plays a role in the regulation of pre-BCR-controlled integrin activity in pre-B cells, which may be required for retention of pre-B cells in the proper microenvironment and their transition into immature B cells. The proliferation of $c\mu^+$ pre-B cells is reduced in XLA patients as well as in $\alpha4$ integrin-deficient mice when compared to control pre-B cells. Moreover, as pre-BCR signalling requires the activation of Btk, it was suggested that loss of control of integrin-mediated adhesion by the pre-BCR might contribute to the proliferation defects in XLA pre-B cells (64).

4. BCR signalling in immature B cells

It is established knowledge that Btk plays an important role in the signal transduction pathway downstream the BCR. The most obvious defect in Btk-deficient mice is the reduction of the size of the mature B cell population in the periphery, caused by a block in splenic B cell differentiation. However, analysis in Btk-deficient mice revealed that $B220^{low}IgM^-$ immature B cells still expressed high levels of cSLC and BP-1 and were low in CD2 and CD25 expression (Chapters 2.1 and 2.2; (16, 17)). Together with the findings of a 3h delay in the appearance of immature B cells, these results indicate that Btk also regulates the cellular maturation of immature B cells in the BM.

By analysing the 3-83$\mu\delta$ transgenic mice on a non-deleting background, we found that premature expression of the 3-83$\mu\delta$ BCR amplified cellular maturation defects in immature B cells in the BM of Btk-deficient mice and these cells were able to enter the spleen, but were efficiently deleted within 24h upon arrival. The residual B cells were shown to have a $PB493^+IgM^{high}IgD^{-/low}CD21^-CD23^-$ immature phenotype, reflecting a block at the transition of T1 into T2 B cells (Chapter 4; (34)). This block was rescued by crossing these mice with Bcl-2 transgenic mice, indicating that there was a survival problem for the T2 cells in Btk^- 3-83$\mu\delta$ non-

deleting mice. We also found that in Btk-deficient 3-83μδ transgenic mice on a deleting background the cellular maturation defects of immature B cells in the BM were restored. In addition, the T1 to T2 transition of splenic B cells was corrected. These results suggest that when B cells are targeted for receptor editing, the autoreactive BCR transmits an additional unique Btk-independent signal signal that enhances cellular maturation of immature B cells that are in the process of receptor editing (Chapter 4; (34)).

5. Future prospects

The transition of large into small pre-B cells is a highly organized process that results in a series of events: (1) allelic exclusion, (2) enhanced rearrangement of L chain genes, (3) downregulation of RAG and SLC, (4) alterations in cell surface phenotype and (5) extensive proliferation of the pre-B cell population. Additional studies in both human and murine models of immunodeficiency should help to elucidate the exact control of Btk in these critical events.

Fundamental aspects of pre-BCR signalling

The findings presented in this thesis provide a detailed analysis of the role of Btk in early B cell development in the mouse. Still, for some aspects of B cell development it is not yet clear if they are dependent on Btk activation.

For example, it is not known if Btk is involved in Dμ RF2 counterselection (see General introduction, page 9). The observations that in mice deficient for λ5 or SLP-65, Dμ RF2 counterselection is abolished but allelic exclusion remains intact (40, 65) clearly indicate that the signalling pathways for the two types of inhibition of V_H to DJ_H recombination are not identical. It is therefore attractive to speculate that Dμ RF2 counterselection reflects partial signalling: Dμ protein does not induce developmental progression or proliferation, but it does induce the SLP-65 dependent premature down-regulation of IL-7 responsiveness, which would terminate further V_H gene recombination (66) and strongly reduce their proliferative or survival capacities, when compared with Dμ-negative pro-B cells. In this model, SLP-65-deficient Dμ-expressing pro-B cells would not down-regulate IL-7R expression and therefore RF2 counterselection is abolished. As Btk and SLP-65 are involved in parallel signalling pathways and have a synergistic role at the pre-BCR checkpoint, it is very well possible that Btk also has a role in Dμ RF2 counterselection.

Although we have shown that constitutive active E41K-Btk induced κ L chain rearrangement in pro-B cells and that in Btk-deficient mice λ L chain expression was reduced and Jλ-specific SBE were low, the exact mechanism of how Btk can regulate L chain locus opening is not understood and will be subject of future research. For L chain rearrangement, the RAG genes and transcription factors, such as IRF-4 and Spi-B, have to be activated. It is possible that Btk regulates the activation of these factors indirectly via the activation of other proteins that are downstream in the signal transduction pathway. In this context, it

was demonstrated that the Btk protein is able to associate directly with transcription factors, such as TFII-I (67) and Bright (68) and Btk is able to shuttle into the nucleus where it may directly regulate gene transcription (69).

Further studies are necessary to determine the exact pathway from Btk to the regulation of IL-7 driven proliferation, L chain rearrangement and induction of CD2, CD25 and MHC class II expression upon pre-BCR signalling. One of the models that may be instrumental for this is the E41K gain-of-function mutant mouse. These mice could be crossed with μMT mice, in which B cell development is blocked at the pro-B cell stage, as they are not able to express the μ H chain on the cell surface. In E41K/μMT double mutant mice the induction of L chain rearrangement could be studied in pro-B cells, without the contamination of pre-B cells that are also in the process of L chain rearrangement. It would be possible to examine the initiation of L chain rearrangement in more detail by determining the expression of κ, λ or μ sterile transcripts by RT-PCR and the frequency of signal broken ends of specific H and L chain loci by LM-PCR. Moreover, expression-profiling studies could identity transcription or recombination factors induced by Btk-signalling and proteomic studies could give information about how protein complexes are assembled and connected in the signalling network. These data would provide more insight in the role of Btk in these processes.

Btk and agammaglobulinemia patients

The main future prospect for studies to the role of Btk in mouse B cell development is to correlate the acquired knowledge to the role of Btk in human B cell development.

Recently it was found that defective SLP-65 expression occurred in ~50% of childhood pre-B acute lymphoblastic leukaemias (ALL) demonstrating that SLP-65 acts as a tumor suppressor in pre-B cells also in man and indicates that loss of SLP-65 and the accompanying pre-B cell differentiation arrest may be one of the primary causes of pre-B ALL (70). The loss of SLP-65 protein was found to be due to the incorporation of alternative exons into SLP-65 transcripts, leading to premature stop codons. Likewise, in another study ~50% of childhood pre-B ALL cases manifested aberrant Btk transcripts predicted to encode Btk proteins with a substantial kinase domain deletion (71). As no clear correlation was observed between SLP-65-deficiency, Igμ expression or the presence of additional genetic abnormalities such as translocations (70), further studies are necessary to elucidate the significance of SLP-65- or Btk-deficiency in the process of malignant transformation of pre-B cells in humans. In this context, it is important to note that in contrast to the mouse, deficiency of SLP-65 or Btk in agammaglobulinaemia patients is not associated with increased but with a lack of pre-B cell expansion (72, 73). This makes it difficult to envisage how human pre-B ALL can originate from SLP-65 or Btk-deficient B-cell precursors, which do not proliferate *in vivo.* Moreover, it can be concluded that the function of SLP-65 and Btk in the regulation of pre-B cell proliferation in humans and the mouse is apparently different.

Our findings on the role of Btk in pre-B cell development in Btk-deficient mice made it clear that Btk in man and mouse functions at a similar stage in B cell development. Although we cannot fully explain the differences in severity of disease between the two species, this may be related to the fact that pre-B cell development in the mouse, in contrast to man, is highly dependent on IL-7. The signal from the IL-7R was suggested to activate the Ras/Raf/MEK/MAP kinase pathway independent of the presence of Btk or SLP-65 (Figure 4). The selection of late pro-B cells to microenvironments with low concentrations of IL-7 allows the selective proliferation of pre-BCR-expressing cells as the concomitant expression of the IL-7R and the BCR provides a signal of higher magnitude. Up to this date, no IL-7-like growth factor has been found to regulate early B cell development in man. This may indicate that the selection of pre-BCR-expressing cells in man is absolutely dependent on pre-BCR signalling and the activation of Btk, as there is no alternative pathway to induce proliferation. Mice that are deficient in IL-7 or its receptor have a block in B cell development at the pro- to pre-B cell transition (74-76). In contrast, patients with severe combined immunodeficiency due to mutations in the genes for the α or γ_c chain of the IL-7R, have normal numbers of immature B cells (77, 78). This further indicates that there are significant differences in the regulation of early B cell development in mouse versus man.

Another possible way to explain the differences between the XLA and xid phenotypes is the fact that there is at least one family member of Btk, Tec, which may take over some of the Btk-dependent functions in the absence of Btk in mice. This was indicated by the findings that the Tec/Btk double deficient mouse showed a much more severe defect at the pre-B cell stage than either single deficient mice (8). Although it was shown that Tec is expressed in human B cell lines of different developmental stages (79), the extent of which Tec is able to compensate for Btk-deficiency in human XLA is not known.

Until now, only one patient with agammaglobulinemia was found to have a mutation in the gene encoding SLP-65 (80). The fact that this patient showed a severe block at the pre-B cells stage indicates that the family member of SLP-65, SLP-76, or alternative pathways of B cell differentiation that are independent of SLP-65, can not compensate for the absence of SLP-65 in man.

Although treatment of XLA with antibiotics and gamma globulin therapy is very effective, the repetitive administration of these medicines is not equivalent to a natural immune system. Furthermore, about 10% of patients have significant problems with the therapies and XLA patients are always at risk for developing chronic pulmonary infections (81, 82). Therefore, gene therapy is an attractive possibility for future corrections of the disease.

Using mouse models, the efficiency of gene transfer strategies and the obtained correction of the xid phenotype can be investigated. It was shown that expression of a human cDNA transgene under the control of the MHC class II promoter could correct the defects in Btk-deficient mice (83). Furthermore, in contrast to the fact that certain protein tyrosine kinases have been implicated in tumor formation when

mutated or overexpressed, it was shown that overexpression of Btk did not cause any unfavourable effects (12). In a bone marrow transplantion animal study, Btk-deficient mice showed a correction of the B cell defect even when only a small fraction of donor bone marrow cells expressed normal Btk (84). Recently it was found that B cell defects in unirradiated Btk-deficient mice that have not received any immunosuppressive therapy was completely corrected when a small amount of BM cells from a congenic donor with normal Btk was given (85). These findings suggest that in patients with XLA, the engraftment of a small number of stem cells or B cell precursors from a donor with Btk might provide significant clinical benefit (86). Therefore, it is worthwhile to further develop gene therapy strategies that would cure agammaglobulinemia patients in the near future.

6. References

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Summary

Samenvatting

Summary

Signalling from the BCR or its immature form, the pre-BCR, was shown to be crucial for B cell development. Gene-targeted mice have defined differential roles of components of the (pre-) BCR complex or its downstream signalling pathways. One of the proteins involved in (pre-) BCR signalling is the cytoplasmic protein Bruton's tyrosine kinase (Btk). Mice deficient in Btk have B cell differentiation defects resulting in an X-linked immunodeficiency (xid) phenotype. The xid phenotype is characterized by a reduction in the number of peripheral B cells and the residual B cell have an immature phenotype, indicating that the lack of Btk causes a block in peripheral B cell differentiation. Btk-deficient mice have low levels of serum IgM and IgG3, peritoneal B-1 cells are lacking and these mice are not able to respond normally to infection with T cell independent antigens. In contrast, humans with mutations in the Btk gene develop the much more severe X-linked agammaglobulinemia (XLA), which is characterized by an almost complete block at the pre-B cell stage in the BM, indicating a role for Btk at the pre-BCR checkpoint. Boys with XLA have almost no peripheral B cells and have very low serum levels of all Ig classes. Therefore, XLA patients hardly respond to bacterial antigens, but treatment with antibiotics and gamma globulin therapy is very effective. To determine the exact role of Btk during early B cell development in the mouse bone marrow, we studied pre-BCR checkpoint functions in the Btk-deficient mice.

Pre-BCR signalling regulates the clonal expansion of pre-B cells and subsequent downregulation of the V(D)J recombinase system ensures allelic exclusion. Furthermore, downregulation of surrogate light chain (SLC) and IL-7R expression terminates the proliferation of large pre-B cells and induces differentiation into small pre-B cells when the cells have stopped cycling. Modulating the expression of various cell surface and intracellular proteins regulates cellular maturation during the transition of large into small pre-B cells. In small pre-B cells, the rearrangement machinery is activated again and L chain recombination is initiated. Functional L chain rearrangement leads to the expression of the BCR on the cell surface and the cells are transferred to the IgM^+ immature B cell compartment.

In chapter 1, the role of Btk in Ig L chain rearrangement is described. We found that in Btk-deficient mice the frequency of Ig λ-expressing immature B cells was reduced by ~50%. This reduction in λ usage was not caused by defective receptor editing or by a survival problem for immature B cells in the BM, as transgenic expression of an autoreactive BCR or overexpression of the anti-apoptotic protein Bcl-2, respectively, did not alter the Btk-dependence of λ usage. Analysis of Jλ-specific signal broken ends, that are formed during the process of λ locus recombination, revealed that Btk regulates the V(D)J recombination activity at the λ L chain locus as the frequency of Jλ signal broken ends was very low in Btk-deficient mice, when compared with wild type mice. Taken together, these findings indicate that Btk is involved in the activation of V(D)J recombination at the λ L chain locus.

Chapter 2 covers the main theme of this thesis, i.e. the role of Btk at the pre-BCR checkpoint. By analysis of the pre-BCR checkpoint functions *in vivo* we found that Btk is required for the efficient transition of large cycling into small resting pre-B cells. In this respect, we found that Btk-deficient cells failed to efficiently modulate the expression of CD43, SLC, CD2 and CD25 and that the cells manifested a specific developmental delay within the small pre-B cell stage of ~3h, when compared with wild-type cells. To examine the function of the individual domains of Btk in pre-BCR signalling *in vivo*, we analysed transgenic mice that express various Btk mutants, including the E41K gain-of-function PH domain mutant, the Y223F autophosphorylation site mutant, the E41K-Y223F double mutant, and the K430R kinase-inactive mutant. We crossed these mice onto the Btk null background,

and investigated the role of the individual Btk domains at the pre-BCR checkpoint.

Studies in Btk-deficient mice transgenic for kinase-inactive K430R-Btk indicated that Btk functions at least partially as an adapter protein at the pre-BCR checkpoint, but kinase activity of Btk is absolutely required during later stages of B cell development. The E41K gain-of-function Btk mutant mice revealed that Btk is directly involved in the regulation of κ L chain rearrangement, the induction of cellular maturation and IL-7 responsiveness. Furthermore, these mice exhibited an increased Ig λ usage in immature B cells, further indicating a direct role for Btk in L chain rearrangement. When the autophosphorylation site Y223 of Btk was inactivated, no obvious B cell defects were observed. However, expression of the Y223F-Btk transgene could not fully rescue the reduction of λ L chain usage in Btk-deficient mice. Collectively, the analyses of these mutant Btk transgenic mouse models provided detailed information on the role of the individual domains of Btk in pre-B cell development and revealed that the requirement for Btk varies significantly between the different Btk-mediated signalling pathways during B cell development.

Chapter 3 describes the synergistic role of Btk and SLP-65 during pre-B cell differentiation. Both proteins play a role in parallel pathways downstream of the pre-BCR and it has been shown that concomitant absence of Btk in SLP-deficient mice increased the developmental defects at the pre-BCR checkpoint. We further defined this synergy and identified an unexpected role for Btk as tumor suppressor in SLP-65 deficient mice. We found a dramatically increased pre-B cell tumor incidence in Btk/SLP-65 double mutant mice, when compared with SLP-65 single deficient mice. Furthermore, by crossing the kinase-inactive K430R-Btk mutant with SLP-65 deficient mice, we found that Btk functions as an adapter protein in a SLP-65 independent way.

In chapter 4, we show that the impaired induction of cell surface changes in Btk-deficient pre-B cells was still noticeable in κ^+ immature B cells, but was largely corrected in λ^+ immature B cells. To find out if the transit time through the pre-B cell compartment or receptor editing may affect the extent of the cellular maturation defects in Btk-deficient B cells, we employed 3-83μδ transgenic mice, which prematurely express a complete B cell receptor (BCR) and therefore manifest accelerated B cell development. In Btk-deficient 3-83μδ mice, the immature B cells in the bone marrow exhibited a very immature phenotype (pre-BCR$^+$CD43$^+$CD2$^-$ and were arrested upon arrival in the spleen at the stage of the T1 transitional B cell. However, these cellular maturation defects were largely restored when Btk-deficient 3-83μδ B cells were targeted for receptor editing in mice with a centrally deleting background. We concluded that premature BCR expression amplified the cellular maturation defects in Btk-deficient B cells, while extensive receptor editing reduced these defects.
In conclusion, we obtained important information about the role of Btk at the pre-B cell receptor checkpoint in the mouse. This information will also be helpful for further studies on the functional defects in XLA patients and future strategies to correct these defects. Moreover, our studies defined a role for downstream pre-BCR signalling molecules as tumor suppressors in the context of pre-B cell leukaemia.

Samenvatting voor iedereen

Het immuunsysteem beschermt het lichaam tegen infecties. Wanneer lichaamsvreemde stoffen, antigenen, het lichaam binnendringen kunnen gespecialiseerde cellen deze antigenen rechtstreeks of via de activatie van andere cellen opruimen. Zo zijn er B cellen die na activatie antistoffen kunnen produceren tegen bacteriële antigenen. Een antistof wordt ook wel immuunglobuline (Ig) of B cel receptor (BCR) genoemd en bestaat uit twee zogenaamde zware en twee lichte eiwitketens die het antigeen kunnen binden en daarmee de specificiteit van de BCR bepalen. Omdat er een grote variatie aan antigenen het lichaam binnen kan komen, moeten de antistoffen zo gemaakt worden dat ze een uiteenlopende specificiteit hebben. B cellen ontwikkelen zich volgens een vast patroon via verschillende fases en vindt voor een groot deel plaats in het beenmerg. Tijdens de B cel ontwikkeling worden de eiwitketens van de immuunglobulines gemaakt. Eerst worden er zware ketens in elkaar gezet en voordat de B cel zich verder mag ontwikkelen worden de zware ketens getest. De zware ketens moeten goed kunnen binden met de nog te vormen lichte ketens van de BCR. Daarom worden de zware ketens eerst aan surrogaat lichte ketens gebonden en dit wordt als een complex op de celmembraan van de vroege B cel, de pre-B cel geplaatst. Dit complex van zware en surrogaat lichte ketens wordt ook wel het pre-B cel receptor (pre-BCR) complex genoemd. De pre-BCR is gekoppeld aan eiwitten die signalen kunnen doorgeven naar de celkern en daardoor worden er, zodra de pre-BCR op de membraan staat, signalen doorgegeven naar de celkern dat de cel door kan gaan met uitrijpen. Als de zware ketens niet goed zijn, kunnen ze niet aan surrogaat lichte ketens binden of zullen er geen of te weinig signalen doorgegeven worden en zal de cel niet doorgaan met de ontwikkeling. Omdat het maken van een zware keten een ingewikkeld proces is, zal de cel die hier succesvol in is geweest een aantal keren delen zodat er meer cellen komen met dezelfde zware keten. Daarna komt de cel in een rustfase en worden de lichte ketens van het immuunglobuline gemaakt. Als de lichte eiwitketens goed zijn worden ze aan de zware ketens gebonden en als een B cel receptor (BCR) complex op de membraan van de B cel gezet. De lichte ketens hebben ook een grote variatie in structuur en daarmee krijgt iedere B cel een unieke BCR met een unieke specificiteit. B cellen die een BCR op hun membraan hebben worden immature B cellen genoemd. De BCR geeft de immature B cel signalen door om naar de milt te gaan om daar verder uit te rijpen tot een antistofproducerende B cel.

Er zijn veel verschillende eiwitten betrokken bij de ontwikkeling van de B cel. Vooral eiwitten die het pre-BCR complex vormen of betrokken zijn bij het doorgeven van signalen vanaf de pre-BCR zijn erg belangrijk voor een goede B cel ontwikkeling. Eén van de betrokken eiwitten is het Bruton's tyrosine kinase (Btk) eiwit. Btk is een zogenaamd signaleringseiwit en speelt een rol in het doorgeven van de signalen vanaf de pre-BCR en de BCR.

Bij mensen komt een erfelijke ziekte voor waarbij een mutatie in het gen dat codeert voor het Btk eiwit resulteert in een niet-functionerend Btk eiwit. Deze ziekte heet X-gebonden agammaglobulinemia (XLA), of de ziekte van Bruton. Het gen voor Btk ligt op het X-chromosoom en de aandoening komt daarom alleen voor bij jongens. Als het Btk eiwit niet werkt, kunnen de B cellen niet volledig uitrijpen tot antistof-producerende cellen en de XLA patiëntjes hebben daardoor bijna geen antistoffen in hun bloed. De patiëntjes hebben nauwelijks weerstand tegen bacteriën en ze hebben vooral last van infecties in de bovenste luchtwegen. Door behandeling met antibiotica en cocktails met algemene antistoffen kunnen de patiëntjes prima met de ziekte leven, maar ze zijn wel volledig afhankelijk van deze behandeling. Nader onderzoek heeft uitgewezen dat de B cel ontwikkeling bij XLA patiënten bijna volledig geblokkeerd is op het pre-B cel stadium in het beenmerg. De cellen die een

goede zware keten hebben gemaakt kunnen het pre-BCR complex wel op de membraan zetten, maar omdat signalen vanaf de pre-BCR niet goed worden doorgegeven door de afwezigheid van Btk, ondergaat de cel geen celdelingen. Daardoor is de ontwikkeling van de B cel ernstig verstoord en zijn er maar zeer weinig B cellen die zich tot antistofproducerende cel ontwikkelen.

Bij muizen heeft Btk een vergelijkbare rol in de B cel ontwikkeling. Ook bij muizen veroorzaakt een mutatie in Btk een probleem in de B cel ontwikkeling en hebben de muizen een X-gebonden immunodeficiëntie (xid) fenotype. Echter, bij xid muizen ontstaan de grootste problemen pas later tijdens de B cel ontwikkeling, namelijk pas tijdens de uitrijping in de milt. Ook xid muizen hebben bijna geen rijpe B cellen en hebben zeer weinig antistoffen in het bloed. Hoewel de rol van Btk in xid muizen minder opvallend is tijdens de vroege B cel ontwikkeling, heeft eerder onderzoek aangetoond dat Btk hier wel een rol speelt. Om de exacte rol van Btk in de vroege B cel ontwikkeling nader te bestuderen is in ons lab een muis gemaakt waarbij het Btk gen verstoord is. Deze muizen kunnen geen normaal Btk eiwit maken en hebben daardoor een xid fenotype.

De signalen vanaf de pre-BCR reguleren de celdeling van pre-B cellen met een goede zware keten en het stoppen van deze delingen om vervolgens te beginnen met het maken van de lichte ketens. De overgang van delende naar rustende pre-B cel gaat gepaard met de modulatie van de expressie van vele eiwitten, sommige worden op de membraan gezet terwijl andere eraf gehaald worden. Door te kijken welke eiwitten er wel of niet op de membraan staan, kan de B cel ontwikkeling gevolgd worden en door deze resultaten in Btk-deficiënte muizen te vergelijken met controle muizen kan de rol van Btk bepaalt worden in de ontwikkeling van de pre-B cel.

In hoofdstuk 1 wordt de rol van Btk in het maken van de lichte ketens beschreven. We hebben gevonden dat de hoeveelheid cellen die een zogenaamde lambda lichte keten tot expressie brengen met 50% verminderd is in Btk-deficiënte muizen ten opzichte van controle muizen. Ons onderzoek heeft aangetoond dat Btk betrokken is bij het toegankelijk maken van het gen dat codeert voor deze lambda lichte ketens hetgeen de expressie van lambda kan beïnvloeden.

Hoofdstuk 2 beschrijft de rol van Btk in het pre-BCR complex, het controlepunt voor de zware ketens. We hebben gevonden dat Btk vereist is voor de goede overgang van delende naar rustende pre-B cel. Btk-deficiënte cellen kunnen de modulatie van de verschillende oppervlakte eiwitten niet efficiënt reguleren en daardoor zijn de pre-B cellen ongeveer 3 uur vertraagd in hun ontwikkeling in vergelijking met controle pre-B cellen.

Het Btk eiwit bestaat uit 5 domeinen, ieder met een eigen functie. Zo is het PH-domein betrokken bij de binding van Btk aan het membraan van de cel. De SH-domeinen zijn betrokken bij de binding van andere signaleringseiwitten en het kinase-domein kan andere eiwitten activeren. Door mutaties in de verschillende domeinen aan te brengen en deze als transgen in muizen tot expressie te laten brengen kan de functie van ieder domein in detail onderzocht worden. De resultaten van het onderzoek met deze transgene muizen is beschreven in hoofdstuk 2 en samengevat blijkt dat de noodzakelijkheid voor de aanwezigheid van Btk erg verschilt tussen de verschillende Btk-afhankelijke signaleringsroutes tijdens de vroege en late B cel ontwikkeling. Zo is de functie van het kinase-domein van groter belang in de signaalroute vanaf de BCR dan vanaf de pre-BCR.

Een ander eiwit dat betrokken is in de signaleringsroute vanaf de pre- en BCR is het verbindingseiwit SLP-65. Muizen die SLP-65 niet hebben, hebben een vergelijkbaar defect in de vroege B cel ontwikkeling als Btk-deficiënte muizen. Het defect in SLP-65 deficiënte muizen wordt vooral veroorzaakt doordat de pre-B cellen niet makkelijk kunnen stoppen

met delen. Waarschijnlijk ontstaan er hierdoor in 10% van deze muizen pre-B cel tumoren. Er is aangetoond dat in de afwezigheid van zowel Btk als SLP-65 de defecten op het pre-B cel stadium zijn vergroot. In ons lab zijn de defecten nader onderzocht en hebben we een onverwachte rol voor Btk gevonden als tumor onderdrukker in SLP-65 deficiënte muizen. De frequentie van de ontwikkeling van pre-B cel tumoren was ernstig toegenomen Btk/SLP-65 deficiënte muizen in vergelijking met muizen die alleen het SLP-65 eiwit niet hebben.

In hoofdstuk 4 laten we zien dat de vertraagde modulatie van eiwitexpressie in immature Btk-deficiënte cellen minder evident is in cellen die de lambda lichte keten tot expressie brengen in vergelijking met cellen die de kappa lichte keten hebben gebruikt. Aangezien de lambda-positieve cellen langer in het beenmerg verblijven dan kappa-positieve cellen, hebben we onderzocht of de tijd waarin een B cel zich ontwikkelt van belang is voor de maturatie van Btk-deficiënte cellen. We hebben gevonden dat specifieke receptor signalen ervoor zorgen dat de maturatie van Btk-deficiënte cellen beter gaat dan wanneer cellen niet zo'n specifiek signaal krijgen.

Samenvattend hebben we veel belangrijke informatie verzameld over de rol van Btk in het pre-BCR complex in de muis. Deze informatie kan behulpzaam zijn voor verder studies naar de functionele defecten die XLA patiënten ondervinden en voor toekomstige strategieën om deze defecten te corrigeren met bijvoorbeeld gentherapie. Verder hebben we gevonden dat signalerings moleculen onder de pre-BCR als tumor onderdrukkers kunnen functioneren in relatie tot pre-B cel leukemie.

List of abbreviations

BCR	B cell receptor
BLNK	B cell linker protein (see also SLP-65)
BM	Bone marrow
BrdU	Bromodeoxyuridine
Btk	Bruton's tyrosine kinase
DAG	Diacylglycerol
H	Immunoglobulin heavy chain
Ig	Immunoglobulin
IL-7	Interleukin-7
IL-7R	Interleukin-7 receptor
IP3	Inositol trisphosphate
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
κ	Immunoglobulin kappa light chain
L	Immunoglobulin light chain
λ	Immunoglobulin lambda light chain
μ	Immunoglobulin μ heavy chain
MHC	Major histocompatibility complex
NFAT	Nuclear factor in activated T cells
NF-κB	Nuclear factor κB
PI3K	Phosphatidylinositol 3-kinase
PIP	Phosphatidylinositol phosphate
PIP2	Phosphatidylinositol biphosphate
PIP3	Phosphatidylinositol trisphosphate
PIP5K	Phosphatidylinositol phosphate 5-kinases
PKC	Protein kinase C
PLCγ2	Phospholipase Cγ2
Pre-BCR	Precursor B cell receptor
RAG	Recombination activating gene
SLC	Surrogate light chain
SLP-65	SH2-domain leukocyte protein of 65 kD (see also BLNK)
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

Dankwoord

Zo, dat zit erop. Ik zeg altijd maar zo: je moet er wel wat voor doen, maar dan heb je ook wat. Gelukkig heb ik dit werk niet alleen gedaan en wil ik een aantal mensen bedanken voor hun betrokkenheid. Ga er maar even voor zitten, want ik ben niet kort van stof.

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Sabine

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1998-1999	Differentially expressed mRNAs and proteins in undifferentiated and differentiating type A spermatogonia in the rat. Afdeling Celbiologie, Academisch Ziekenhuis Utrecht.
1999	Scriptie: The mechanisms of tolerance in the B-cell system. Afdeling Immunologie, Erasmus Universiteit Rotterdam.
31 augustus 1999	Doctoraal examen Fundamentele Biomedische Wetenschappen
1999-2004	Promotie-onderzoek: The role of Bruton's tyrosine kinase during early B cell development in the mouse. Afdeling Immunologie, Erasmus Universiteit Rotterdam. Promotor: Prof.dr. R. Benner Co-promotor: Dr. R. W. Hendriks
April-mei 2002	Werkbezoek University of California, Berkeley, USA Department of Molecular and Cellular Biology Prof.dr. M.S. Schlissel and Dr. S. A. Muljo.
15 januari 2003	Presentatie in de workshop "BCR signaling" tijdens het Keystone symposium "B cells and antibodies: laboratory to clinic". 14-19 Januari 2003, Keystone Resort, Keystone, Colorado, USA
19 mei 2004	Promotiedatum

List of publications

1. Steerenberg, P. A., J. A. Dormans, C. C. van Doorn, **S. Middendorp**, J. G. Vos, and H. van Loveren. 1999. A pollen model in the rat for testing adjuvant activity of air pollution components. *Inhal Toxicol 11:1109*.
2. Dingjan, G. M., **S. Middendorp**, K. Dahlenborg, A. Maas, F. Grosveld, and R. W. Hendriks. 2001. Bruton's tyrosine kinase regulates the activation of gene rearrangements at the λ light chain locus in precursor B cells in the mouse. *J Exp Med 193:1169*.
3. **Middendorp, S.**, G. M. Dingjan, and R. W. Hendriks. 2002. Impaired precursor B cell differentiation in Bruton's tyrosine kinase- deficient mice. *J Immunol 168: 2695*.
4. Van Loo, P. F., P. Bouwman, K. W. Ling, **S. Middendorp**, G. Suske, F. Grosveld, E. Dzierzak, S. Philipsen, and R. W. Hendriks. 2003. Impaired hematopoiesis in mice lacking the transcription factor Sp3. *Blood 3:3*.
5. Kersseboom, R., **S. Middendorp**, G. M. Dingjan, K. Dahlenborg, M. Reth, H. Jumaa, and R. W. Hendriks. 2003. Bruton's tyrosine kinase cooperates with the B cell linker protein SLP-65 as a tumor suppressor in pre-B cells. *J Exp Med 198:91*.
6. **Middendorp, S.**, G. M. Dingjan, A. Maas, K. Dahlenborg, and R. W. Hendriks. 2003. Function of Bruton's tyrosine kinase during B cell development is partially independent of its catalytic activity. *J Immunol 171:5988*.
7. Spaargaren, M., E. A. Beuling, M. L. Rurup, H. P. Meijer, M. D. Klok, **S. Middendorp**, R. W. Hendriks, and S. T. Pals. 2003. The B cell antigen receptor controls integrin activity through Btk and PLCγ2. *J Exp Med. 198: 1539.*
8. **Middendorp, S.**, and R. W. Hendriks. 2004. Cellular maturation defects in Bruton's tyrosine kinase-deficient immature B cells are amplified by premature B cell receptor expression and reduced by receptor editing. *J Immunol 172: 1371*.
9. Hendriks, R. W., and **S. Middendorp**. 2004. The pre-B cell receptor checkpoint as a cell-autonomous proliferation switch. *Trends in Immunology, in press.*
10. **Middendorp S.**, A.J.E. Zijlstra, R. Kersseboom, G.M. Dingjan, H. Jumaa and R.W. Hendriks. Tumor suppressor function of Btk in SLP-65-deficient pre-B cells is independent of its kinase activity. *To be submitted.*
11. **Middendorp S.**, R. Kersseboom, G.M. Dingjan, M. van der Zee, A. Maas and R.W. Hendriks. Expression of the constitutive active Bruton's tyrosine kinase mutant E41K mimics pre-B cell receptor signalling in pro-B cells. *To be submitted.*