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Role of Pre-B Cell Receptor Signaling Molecules in B Cell Differentiation and Tumor Suppression

De rol van pre-B cel receptor signaleringseiwitten in B cel differentiatie en tumor suppressie

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Chapter I

General introduction

Pre-B cells in context

In this thesis I aimed to unravel the molecular mechanism involved in the origin of acute lymphoblastic leukemia (ALL). This malignant disease is characterized by oncogenic transformation of progenitors of the B cell lineage in the bone marrow. To study the basis of this disease I have used a mouse model: mice lacking the signaling protein Slp65. This is an important transducer of signals at the precursor (pre)-B cell stage, which reflects a crucial checkpoint for proliferation and differentiation in the bone marrow. Although Slp65-deficient mice do not show increased sizes of any B-lineage subpopulation in the bone marrow, a substantial portion of mice develop ALL. To understand why these Slp65-deficient cells are prone to oncogenic transformation, knowledge on the function of Slp65 in pre-B cells is required. In the introduction to my thesis, I first describe the development of B lymphocytes and in particular I focus on the pre-B cell stage. We are interested in the functions depending on the signaling protein Slp65, this involves cellular maturation and differentiation, regulation of V(D)J recombination and termination of proliferation.

This introduction will help to understand the basis of the mechanisms possibly involved in the malignant transformation of Slp65-deficient pre-B cells (Figure 1).

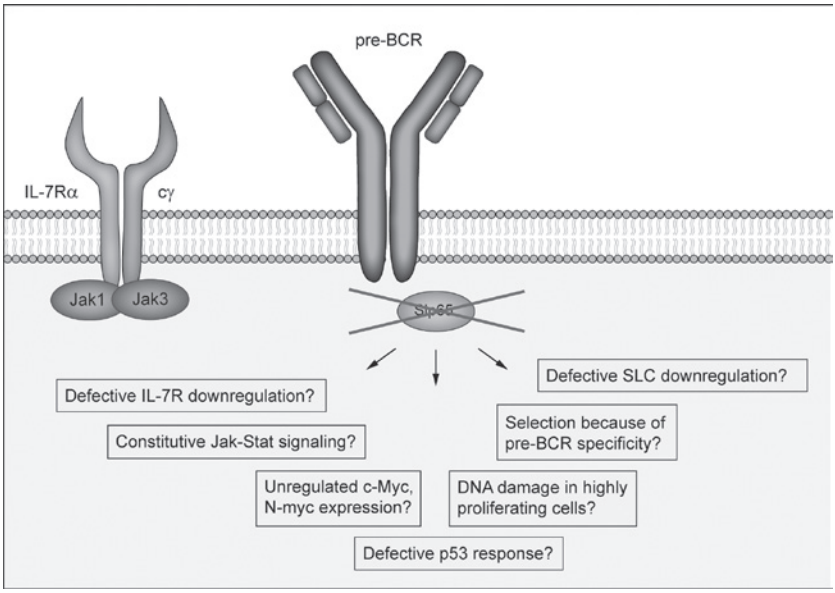

Figure 1. Schematic diagram of mechanisms possibly involved in malignant transformation of Slp65-deficient cells.
At the pre-B cell stage, signaling via the pre-BCR and interleuking-7 receptor (IL-7R) play central roles in the regulation of survival, proliferation, differentiation and V(D)J recombination.

General introduction

The immune system is a complex of cells, tissues and organs that work together to protect the body against attacks by invaders, such as bacteria and viruses. Our immune system saves us from certain death by infection. Many of the responses of the immune system initiate the destruction and elimination of invading organisms and certain toxic molecules produced by them. The key to a healthy life is the remarkable ability of the immune system to distinguish foreign molecules from 'self' molecules. Occasionally, the immune system fails to make this distinction and reacts destructively against the host's own molecules, leading to autoimmune disease.

B lymphocytes contribute to the immune system by producing antibodies and therefore provide humoral immunity, whereas T lymphocytes are responsible for cell-mediated immunity. Both types of lymphocytes can generate immunological memory, which gives protection upon re-infection with the same pathogen. The antigen receptors carried by B cells (B cell receptor (BCR) or immunoglobulin (Ig) and T cells (T cell receptor) are highly variable in their antigen specificity, enabling an individual to respond to millions of different foreign antigens. The receptors provide the means by which lymphocytes can bind and recognize antigens (any substance capable of eliciting an immune response). The antigen binding sites of these receptors have a unique structure, so they can bind and recognize antigens. In most species Igs have a four chain structure and are composed of two identical heavy (H) chains and two identical light (L) chains (Figure 2). The two heavy chains and the heavy and light chains are held together by disulfide bonds and non-covalent interactions. Both Ig H and L chains are composed of two regions; the variable (V) region and the constant (C) region, responsible for antigen recognition and determinining of the effector function, respectively. The V region has three specific sites that determine antigen recognition specificity which are called complementary determining regions (CDR) 1, 2 and 3 which directly interact with the antigen. Igs are subdivided into 5 different groups on the basis of the Ig H chain composition (IgM, IgD, IgG, IgA and IgE). This diverse repertoire of BCRs is generated during the development of B cells from their uncommitted precursors. The mechanisms which generate such diverse antibody specificity in B cells do come with a cost. The generation of DNA breaks and mutations during antibody diversification can give rise to genomic instability and potentially contribute to malignancy.

B lymphocytes develop in mammals in the adult bone marrow. Antigen recognition by BCRs on the surface of B cells trigger adaptive immune responses and control a series of antigen-independent checkpoints during B cell development. In addition to cellular selection, the immune system is shaped by receptor selection. Multiple proteins are critical for proper B cell development. Deficiency of one of these proteins leads to a marked reduction or absence of serum immunoglobulins, which is

seen in patients and mice with primary immunodeficiencies.

This thesis focuses on the role of the precursor pre-BCR signaling molecules Btk and Slp65 in early B cell development and the aberrations that lead to malignancies in mice lacking Btk and Slp65. The pre-B cell therefore takes a central position in the introduction below. In particular, the focus will be on the role of the pre-BCR as a checkpoint in early B cell development and the signaling pathways involved in proliferation, differentiation and the induction of Ig L chain recombination.

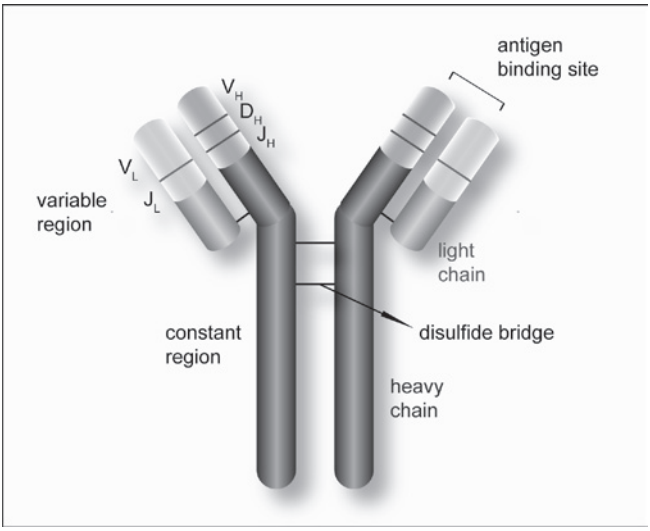

Figure 2. Schematic drawing of an immunoglobulin molecule.
An immunoglobulin molecule is composed of two identical heavy chains and two identical light chains which are held together by disulfide bonds. Each chain consists of a constant region (darker part of an H or L chain) and a variable region (lighter part of an H or L chain). The antigen binding sites are formed by a complex of the variable regions of both light and heavy chains. ***See Appendix for full-color figure.***

Control of B cell lineage commitment by transcription factors

Hematopoietic development is initiated from long-term hematopoietic stem cells (HSC) that are multipotent and have the potential to self-renew (1-2). One of the earliest differentiated precursors is the multipotential progenitor (MPP) expressing Flt3 (3) which can give rise to the erythroid and megakaryocytic progeny (4). Lymphoid primed MPP (LMPP) give rise to primarily the lymphoid lineage as well as macrophage and granulocytic cell lineages. MPP can differentiate into common myeloid progenitors (CMP) or early lymphoid progenitors (ELP) (5). ELP start to express recombination activating gene 1 (Rag1) and Rag2 (6) and can further differentiate into thymic precursors of the T cell lineage or into common lymphoid progenitors in the bone marrow

(CLP) (7-8). CLP contain extensive Ig H chain DJ rearrangements (9) and their rapid progression to early stages of B lineage development suggests that most CLP are early B cell progenitors that retain residual potential for generation of alternate lineages. CLP are defined as Lin-Sca1^{+}CD117^{+}CD127^{+} and also represent the major intermediates en route to become NK cells (7, 10-11). Recently the group of Weissman (12) have identified Ly6d which divides CLP into two distinct populations: one that retains full in vivo lymphoid potential (Ly6d^{-}) and produces more thymocytes at early timepoints than LMPP and another that behaves essentially as a B cell progenitor (Ly6d^{+}). Expression of the B cell marker B220 by a subset of CLPs coincides with their entry into the B cell differentiation pathway (5). The earliest committed B cell precursors are pre-pro-B cells corresponding with Hardy fraction A (13) (Figure 3). These cells have Ig H chain alleles which are largely in germline configuration and can be distinguished from other earlier precursors by their ability to differentiate into B cells but not other lymphoid cells. Pre-pro-B cells have low expression of Rag1 and Rag2 (6, 14-15).

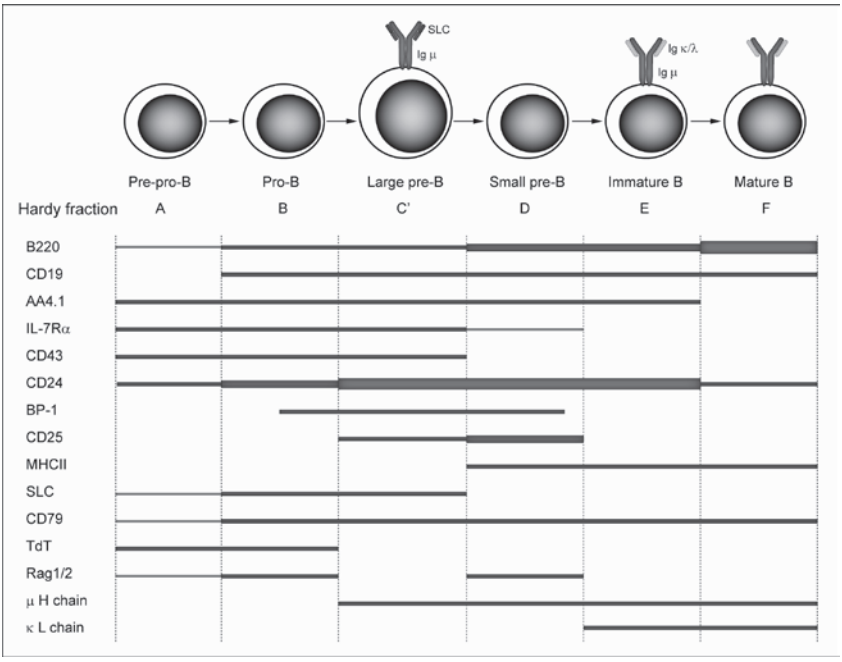

Figure 3. Framework of B cell lineage development based on ordered changes in cell surface molecules.

Relative expression levels are indicated by line thickness. AA4.1 is also known as CD93 and PB493; CD24 as HSA (heat stable antigen); CD25 as IL-2R; MHCII, major histocompatibility complex type II; CD79, Igα and Igβ; SLC, surrogate light chain (λ5 and VpreB). TdT and Rag1/2 (recombination activating gene) expression assessed by analysis of mRNA. Figure adapted from ref. (11). ***See Appendix for full-color figure.***

B cell development requires a hierarchy involving many transcription factors. In CLP, the helix-loop-helix protein E47, an E2A isoform, induces the expression of early B cell factor 1 (Ebf1) (16-18). Ebf1 in turn activates expression of the transcription factor Pax5 (19). The Pax5 gene, encoding the B cell specific activator protein (BSAP) is expressed exclusively in B cells and activates B lineage specific genes and represses B lineage inappropriate genes (20). Pax5 expression is regulated by the concerted activities of ETS-family transcription factor PU.1 and the interferon regulatory factors IRF-4 and IRF-8 (19, 21). In addition to E2A, Ebf1 and Pax5, other transcriptional regulators that modulate the developmental progression of B lineage cells have been identified. Prominent among these are Ikaros, Bcl-11 and Foxo1. Ikaros-like proteins recognize binding sites in many lymphocyte-specific genes including TdT, VpreB and λ5 (see below) whereby Ikaros proteins control the expression of λ5 in a developmental specific manner (22-24). B cell development in Bcl11a-deficient mice is arrested at a stage similar to that described for E2A- or Ebf1-deficient mice and expression of Bcl11a is activated by E2A proteins (25-26). Foxo1-deficient mice show a block at the pro-B cell stage, and Foxo1 directly activates the expression of Rag1 and Rag2 (27). Collectively, these factors form the transcriptional machinery that promotes commitment to the B cell lineage, suppresses the expression of genes associated with alternative cell fates and coordinates cellular population expansion with developmental progression (11, 21).

Generation of pre-B cells and Ig H chain gene recombination

Pro-B cells are distinguished from earlier precursors by a series of cell surface markers (11, 28). B lineage cells are identified by coexpression of B220, the high molecular weight isoform of Ptprc (CD45), together with leukosialin, CD43 (13). These early B lineage cells are further subdivided based on expression of CD24, the heat stable antigen (HSA), and BP-1, a zinc-dependent cell surface metallopeptidase (Figure 3). Furthermore, the first step in generating the required antibody diversity is initiated in this stage.

V(D)J recombination is the process which assembles previously scattered variable (V), diversity (D) and joining (J) encoding gene segments to assemble a (pre-) BCR (Figure 4) (28-31). The reaction is initiated by the lymphoid-specific factors Rag1 and Rag2 (14-15), which recognize recombination signal sequences (RSS) that flank all V, D and J gene units and introduce a DNA double strand break (DSB) at the border of the RSS. The resulting DNA DSB is resolved by the DNA repair machinery known as non-homologous end-joining (NHEJ) (32-33). If these DSB are not properly repaired, they may result in chromosomal translocations and lymphoid malignancies (34-36). V(D)J recombination is strictly regulated at many different levels. Specifically, V(D)J recombination is tissue-specific, lineage-specific and developmental stage-specific. Furthermore, V(D)J recombination is temporally ordered and cell cycle regulated,

whereby recombination is restricted to the G0-G1 stage of the cell cycle. In large cycling pre-B cells, Rag proteins are transiently downregulated to terminate further Ig H chain gene rearrangement. Moreover, as ongoing recombinase activity during mitosis will interfere with faithful transmission of the genome to daughter cells, Rag2 proteins are degraded during cell division, thus linking V(D)J recombination to the cell cycle (37-39).

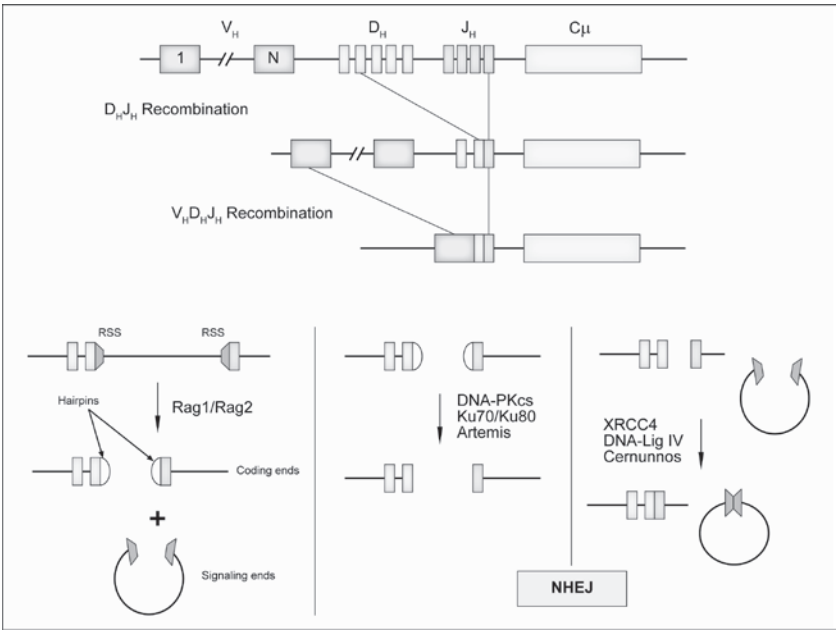

Figure 4. VDJ recombination.
Schematic representation of the Ig H chain locus and V(D)J recombination process. The V(D)J reaction can be divided into three steps. First, the Rag1/2 complex introduces a DNA DSB at the border between D_H and J_H segments and their respective recombination signal sequences (RSS), creating hairpin-sealed coding ends and blunt signaling ends. Artemis, which is recruited and phosphorylated by the DNA-PKcs/Ku70/Ku80 complex, opens the hairpins through its endonuclease activity. The XRCC4/ Cernunnos/DNA-Ligase IV complex finally seals coding and signal joints. NHEJ, non-homologous end-joining. Figure adapted from ref. (31). ***See Appendix for full-color figure.***

The RSS that serve as recognition element for the V(D)J recombinase consist of a conserved heptamer, a spacer of either 12 or 23 nucleotides and a conserved nonamer. Only gene segments flanked by RSS with dissimilar spacer lengths can efficiently rearrange with one another (30, 40). The NHEJ pathway that resolves V(D)J recombinase-mediated DSB can be schematically divided into three steps: (1) the Ku70/Ku80 heterodimer is recruited to and interacts with the DNA DSB; (2) the DNA-dependent serine/threonine kinase DNA-PKcs and the nuclease Artemis are

recruited and activated to modify the DNA ends when needed; and (3) the XRCC-4/ DNA-Ligase IV complex together with Cernunnos terminates the reaction by rejoining the broken DNA ends (32, 41). Imprecise joining of the broken DNA ends contributes considerably to diversity and includes duplication of palindromic sequences (P nucleotides) and deletion or insertion of non templated nucleotides (N nucleotides) by terminal deoxynucleotidyl transferase (TdT) (42-43). In eukaryotic cells there are two major pathways of DSB repair, NHEJ and homologous recombination (HR) (44). HR repairs DSB using information on the undamaged sister chromatid. NHEJ and HR have overlapping roles in repairing DNA DSBs (45-47).

In the murine Ig H chain locus there are approximately 195 V genes, 13 D genes and 4 J genes dispersed over ~2.5 Mb of DNA on chromosome 12 (Figure 5). Close to half the murine V_H genes belong to the J558 family. The majority of these are located at the 5' end of the V_H cluster, though several are also found interspersed amongst other gene families throughout the locus. The extreme 3' end of the V_H locus comprises of the 7183 family (48-49). Although not all VH genes are functional, random recombination of all V, D and J gene segments and random association of heavy and light chains still produce an enormous diverse repertoire. However, V,D and J genes are not used equally in the pre-immune repertoire, thus limiting the theoretical estimates of combinatorial diversity (50-52).

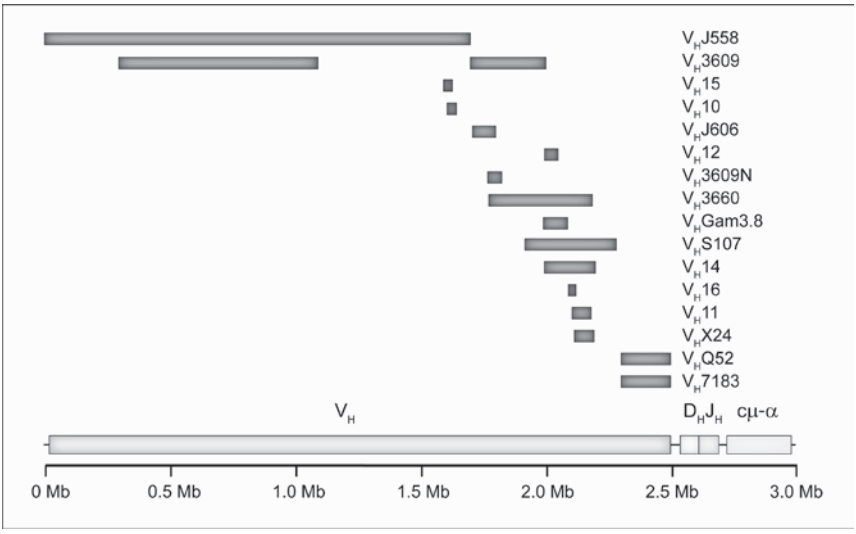

Figure 5. Organization of Ig H chain locus on chromosome 12.
Schematic map of V_H gene family distribution within the mouse Ig H chain V region according to IMGT (www.imgt.org). The length of boxes indicates the distance spanned by members of the gene families and is not related to the number of genes. ***See Appendix for full-color figure.***

Ig gene recombination occurs in a stepwise fashion and begins with D_H to J_H junction segment rearrangement in pro-B cells (28, 53). Due to an inexact joining mechanism, the D_H segments can be joined to the J_H in any of the three reading frames (RF). D_H segments are preferentially used in RF1, while RF2 and RF3 are counter selected on the basis of expression of a truncated μ chain protein and stop codons, respectively (54). After D_HJ_H rearrangement, V_H genes become accessible to the V(D)J recombinase and V_H to D_HJ_H joining takes place. V_H gene accessibility is dependent on transcriptional regulatory elements and is associated with the onset of germline transcription of V_H genes (55-56). Furthermore, the Ig H chain locus relocates from the periphery to the center of the nucleus in pro-B cells which facilitates $V_HD_HJ_H$ recombination (57) and locus contraction brings the distal V_H gene segments into close proximity with the D_HJ_H region (57-59). Several proteins have been linked to the control of chromatin accessibility of the Ig H chain locus in pro-B cells or with the regulation of the Rag genes, including Pax5, Yy1, Ezh2, FoxP1 and IL-7R signaling via Stat5 (60-64). Correlating with the activation of the Ig H chain locus are antisense transcripts originating in intergenic regions between V_H gene segments. These transcripts are turned off when the early pro-B cells reach the late pro-B cell stage (Hardy fraction C) (65). However, the role of these antisense transcripts has not been elucidated yet.

Productive in-frame V(D)J recombination in pro-B cells gives rise to the Ig μ chain, which is expressed by pre-B cells as part of the pre-BCR complex and marks the transition to the pre-B cell stage (66-68).

Pre-B cells and the pre-BCR checkpoint

The pre-BCR comprises of two Ig μ chains and two surrogate light chains (SLC) associated with the signaling subunits Igα and Igβ. The SLC substitutes the not yet rearranged Ig L chains and is a heterodimer composed of two germline-encoded invariant proteins: λ5, which resembles the constant region of conventional λ light chains and VpreB (Figure 6) (68-70). The pre-BCR is transiently expressed but marks an important checkpoint in B cell development when Ig μ H chains are tested for their ability to associate with the SLC. The primary functions of the pre-BCR are triggering of B cell differentiation, clonal expansion and H chain allelic exclusion. Pre-B cells which express the pre-BCR acquire the capacity to respond to low concentrations of the proliferation factor interleukin (IL)-7 (66-68).

In 1957 Burnet proposed that all B lymphocytes carry unique cell surface receptors for antigen (71). Each cell expresses on its surface just one kind of antibody and during immunization, antigen selects cells with the corresponding specificity for multiplication and differentiation into antibody-secreting cells. The process which guarantees that each B cell only produces a single antigen receptor is called allelic exclusion (72-73).

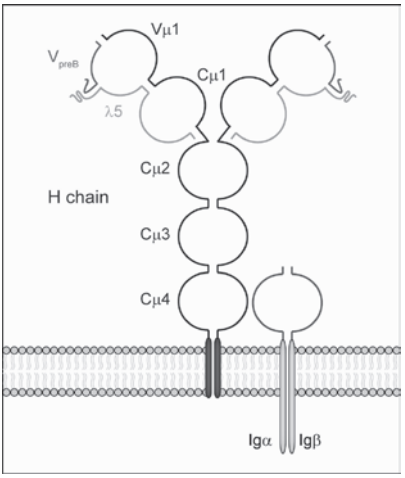

Figure 6. Structure of the pre-BCR.
The pre-BCR complex consists of the heterodimeric Igα/Igβ signal transducer and two covalently associated Ig H chains which are paired with the surrogate light chain, consisting of the invariant proteins λ5 and VpreB. The SLC contains two oppositely charged non-Ig-like tails located at the C-terminus of VpreB and the N-terminus of λ5. The unique tails protrude from the pre-BCR at the position where the CDR3 (complementarity-determining region) of a conventional L chain is located in the BCR.
See Appendix for full-color figure.

Allelic exclusion is dependent on correct pre-BCR assembly and pre-BCR signaling (74-75). Large-scale contraction of the Ig H chain locus following recombination is instrumental in establishing allelic exclusion. The second allele is repositioned at centromeric heterochromatin at the pre-B cell stage while Ig L recombination takes place (58). Allelic exclusion of the Ig H chain is initiated in the early embryo when the Ig receptor loci become asynchronously replicating in a stochastic manner with one early and one late allele in each cell (76). Molecular components including histone modification, nuclear localization and DNA demethylation leads to rearrangement on a single allele and a feedback mechanism that inhibits recombination on the second allele once recombination of the first allele has been successful (72).

Pre-BCR signaling pathways

Currently, it still remains controversial as to how pre-BCR mediated signals are initiated. Pre-BCR signaling could be triggered by ligand binding or by cell autonomous aggregation of pre-BCR complexes on the cell surface. The non-Ig-like tail of the SLC component λ5 induces ligand-independent pre-BCR cross-linking and as a result cell-autonomous signaling for pre-B cell expansion (77). Recently, Jumaa and colleagues have shown that functional pre-BCR formation and autonomous signaling requires the

N-linked glycosylation site in the C_H1 domain of Ig μ H chain (N46) (78). However, ligand mediated crosslinking seems possible since the pre-BCR shapes the V_H repertoire at the transition from pro-B to large pre-B cells (79). Besides, several groups have identified interactions between the non-Ig tail of λ5 of the pre-BCR to galectin-1 (80) and to stromal-cell-associated heparin sulphate (81). Furthermore, the pre-BCR is a poly-reactive receptor and capable of recognizing multiple (self-)antigens, including DNA, LPS and insulin, via the non-Ig part of λ5 (Kohler 2008). Thus, pre-BCR auto-reactivity may serve to clonally expand those cells that produce a functional μ H chain and ensures that this selection can occur in the absence of foreign antigens. In support of this idea, in SLC-deficient mice mainly autoreactive pre-B cells are selected, resulting in the accumulation of autoreactive antibodies (82). But also in the presence of SLC, more than half of the antibodies expressed in early B cell compartments of healthy individuals are polyreactive (83).

Activation of pre-BCR signaling involves phosphorylation of immunoreceptor tyrosine based activation motifs (ITAMs) in the cytoplasmic domains of Igα and Igβ by the Src family kinases (Fyn, Lyn and Blk) and the cytosolic protein tyrosine kinase Syk (Figure 7) (67, 84). This is the first step in the formation of a lipid-raft associated calcium signaling module (85-86). The binding of Syk to the phosphorylated Igα and Igβ places the active Syk in the right position to allow further phosphorylation of neighbouring ITAM sequences. This results in further Syk recruitment and activation (87-88). The SH2 domain-containing leukocyte protein of 65 kD, Slp65 (also known as Bash or Blnk) is one of the most prominent targets of Syk kinase activity and is phosphorylated by Syk on several tyrosines. Slp65 and its close relative in T cells (Slp76) are adaptor proteins which lack intrinsic enzymatic function but regulate the assembly and localization of signaling complexes. They are able to regulate the availability of a substrate to an enzyme and create a scaffold for bridging signaling cascades (89). Slp65 is expressed in B cells and myeloid cells and has been mainly reported to function under the (pre-) BCR (67).

Phosphorylated Slp65 provides docking sites for several proteins including the Tec family kinase Btk, Grb2, Vav, Nck, Plc-γ2 (88-90). Btk is a member of the Tec kinase family, which also includes Tec, Itk, Rlk and Bmx. These molecules have a structure containing a pleckstrin homology domain, followed by a short Tec homology domain, src homology domains and a kinase domain (91-92). Btk is expressed in B cells, platelets and myeloid cells and activated under a large variety of receptors such as BCR, Fcε, TLR2, TLR4, CD38, CCL5, IL-6R and IL-10R.

Activation of Plc-γ2 by Syk and Btk results in the production of inositol triphosphate (IP3) and diacylglycerol (DAG), both of which are second messengers required for cellular responses (88, 90). Models of targeted disruption of components of the pre-BCR or downstream signaling proteins have shown the importance of

these molecules in pre-BCR signaling (66, 88). Additionally, active Syk induces phosphorylation and activation of the lipid-modifying kinase phosphoinositide 3 kinase (PI3K), which regulates diverse biological processes, including cell growth, survival, proliferation, migration and metabolism (67, 93).

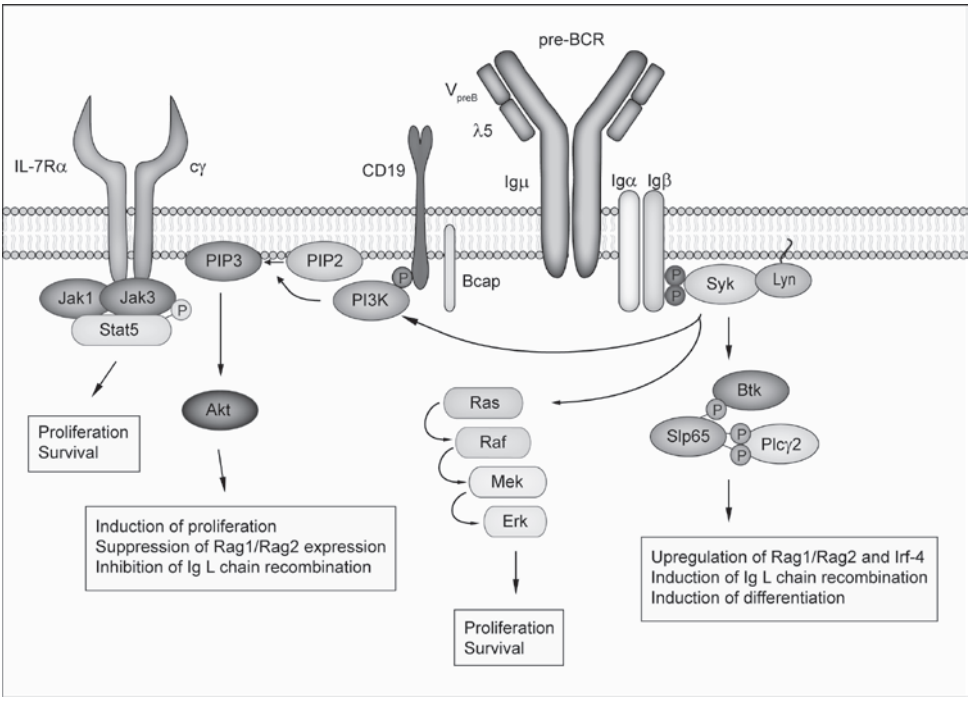

Figure 7. Signaling pathways in pre-B cells.
Pre-BCR activation results in activation of Syk which together with Src family protein kinases, such as Lyn, phosphorylate downstream signaling proteins resulting in pathways involved in proliferation, differentiation and induction of Ig L chain recombination. Activation of Slp65 recruits and activates Btk and Plcγ2 resulting in upregulation of Rag1/Rag2 and Irf-4, induction of Ig L chain recombination and induction of differentiation. An important pathway for proliferation and survival is the activation of the PI3K pathway. Phosphorylation of the co-receptor CD19 and the adaptor protein BCAP (B cell PI3K adaptor) recruit and activate PI3K resulting in the generation of the second messenger PIP3 (Phosphatidylinositol (3,4,5)-trisphosphate) from PIP2 (Phosphatidylinositol 4,5-bisphosphate). PIP3 recruits Akt which is the dominant mediator for controlling cellular proliferation. Ongoing signaling through the PI3K-Akt pathway induces proliferation, suppresses the Rag1/Rag2 genes and blocks Ig κ gene recombination. Proliferation in pre-B cells is also induced via the pathway involving Erk MAP kinase. In addition to the role of the pre-BCR, signaling via the interleuking-7 receptor (IL-7R) plays a central role in controlling the survival and proliferation of pre-B cells. ***See Appendix for full-color figure.***

Pre-B cell proliferation

Expansion of pre-B cells is severely impaired in mice with targeted disruption of pre-BCR components, including μ H chain, the SLC components λ5 and VpreB, Igα and Igβ and Syk (74, 94-98). Overexpression or constitutive activation of Syk functions as an oncogene by promoting growth factor independent proliferation of pre-B cells and by inhibiting differentiation (67). In mice deficient for components of the pre-BCR complex, including Igα, Igβ, SLC or Syk, Ig μ chain positive pre-B cells do not proliferate (66).

In contrast, deficiency of Slp65 or Btk shows an increased proliferative response to IL-7 in vitro, indicating that Slp65 and Btk are crucially involved in the termination of IL-7 driven proliferation (90). Slp65 inhibits IL-7R signaling through direct binding to Jak3 in a Btk-independent manner (99) and also by downregulating IL-17R expression (100).

Downstream of the pre-BCR, Syk and the Src family protein tyrosine kinases induce phosphorylation of the co-receptor CD19 and/or the adaptor protein Bcap resulting in the recruitment and activation of PI3K (67, 93). PI3K activation results in phosphorylation and thereby activation of the serine/threonine kinase Akt, also known as protein kinase B (PKB). Akt inhibits the activities of the forkhead (Foxo) transcription factors (which are mediators of apoptosis and cell-cycle arrest), resulting in cell proliferation and survival (101-102). Tight regulation of pre-BCR induced signaling is important to avoid abnormal Akt activity, which can result in uncontrolled cellular expansion and malignant transformation. The tumor-suppressor phosphatase with tensin homology (Pten) is the most important negative regulator of the cell survival signaling pathway initiated by PI3K (103).

In addition to the role of the pre-BCR signaling, IL-7 receptor signaling plays a central role in cellular survival, proliferation and maturation. Mice deficient in components of IL-7R signaling have an arrest of B cell development at the pro-B cell stage and impaired V_H to D_HJ_H recombination (104). Expression of the pre-BCR by pro-B cells upregulates IL-7R expression and thereby increases the responsiveness of these cells to IL-7 (90, 104). Integration of IL-7R signaling and pre-BCR dependent signals to expand the pool of pre-B cells occurs via the D-type cyclin, cyclin D3 (105).

Another downstream signaling cascade involves the mitogen activated protein kinase (MAPK) pathway which functions independently of signaling via Slp65 and Btk (90). This signaling pathway leads from the Ras GTPase to the extracellular signal regulated kinases (Erk1 and Erk2) (106). Erk activity has been shown to be important for the pre-BCR to collaborate with IL-7 signaling to drive pre-B cell proliferation (104). Expression of genes dependent on Erk activation includes genes encoding the proliferation-associated transcription factors c-Myc, c-Fos, Egr-1, Egr-2, Egr-3, Fra-1 and Fra-2 (106-107).

From large cycling pre-B cells to small resting pre-B cells

Upon pre-BCR signaling, cells initially undergo a proliferative burst accompanied by down regulation of SLC components and downregulation of Rag1/Rag2 protein expression, followed by exit from the cell cycle and transition from large pre-B cells to small pre-B cells. In small resting pre-B cells Ig L chain rearrangement is initiated. Termination of pre-BCR signaling occurs by downregulation of λ5 gene expression and consequently the termination of SLC expression. The pre-BCR activates a negative feedback loop that prevents continuous pre-BCR signaling (67, 108-109).

Pre-BCR signaling leads to activation of Syk, which then phosphorylates Slp65 and thereby regulates pre-B cell differentiation. Slp65 is required for the downregulation of λ5, which terminates SLC expression and the activation of Ig κ L chain recombination by induction of the expression of the transcription factors Aiolos, Irf-4, Foxo3a and Foxo1 (22, 110-111). At the transition from large cycling into small resting pre-B cells up- and downregulation of specific cell surface markers occurs (Figure 3). Small pre-B cells downregulate expression of sialoglycoprotein CD43, SLC, metallopeptidase BP-1 and the IL-7R and upregulate expression of CD2, CD25, MHCII (11, 90). Other transcription factors which are important for Ig κ rearrangement are E2A, Pax5, Irf-8 and Spi-B (111-113). Germline transcription of the Ig L chain loci is associated with the regulation of accessibility of the Ig loci to the VDJ recombinase, because germline transcription precedes VDJ recombination of the Ig L chain (114). There are two type of light chains, Ig κ and Ig λ, which are expressed at a ratio of approximately 20:1 in mice (112). Ig κ genes are activated and undergo recombination before Ig λ, which contributes to the preferential usage of the Ig κ L chain in mature B cells (112, 115).

Beyond the pre-B cell stage: immature and mature B cells

After successful rearrangement of the Ig H and L chain locus, the immature B cells are the first B lineage cells to express surface BCR, they display surface IgM but little or no IgD. Expression of the BCR is a second key checkpoint in B cell development for monitoring the production of a functional Ig L chain and to eliminate autoreactive BCR. The receptor consists of randomly selected Ig H and L chains and has an unpredictable specificity that includes the ability to bind self. Indeed, it has been found that about 50% of BCR are autoreactive in humans and mice (83, 116-117). Induction of B cell tolerance can occur by clonal deletion, cell inactivation (anergy) or receptor editing. Receptor editing is the process that alters antigen receptors by allowing secondary rearrangements of mainly the Ig L chain. This process is the dominant tolerance mechanism for developing B cells (117-119). When autoreactive B cells encounter self-antigen that induces signaling through the BCR developmental progression is blocked and secondary VDJ recombination can take place. Immature B cells differ from mature B cells in that they are particularly susceptible to BCR-induced apoptosis. Thus the

BCR is an essential regulator of immature B cell development.

Immature B cells that emigrate from the bone marrow (BM) to the periphery are referred to as transitional B cells. Transitional B cells can be distinguished from mature B cells by a series of cell surface markers. Transitional B cells are short-lived and only 10-30% of these cells enter the long-lived mature peripheral B cell compartment. Transitional B cells are unable to proliferate and are unable to generate an immune response upon BCR crosslinking (11, 120). The transitional cells that lack autoreactivity enter the pool of mature recirculating B cells in the spleen. These cells are termed follicular B cells because of their localization to the B cell follicle region (121). This is the final maturation stage of developing BM B cells (Figure 8).

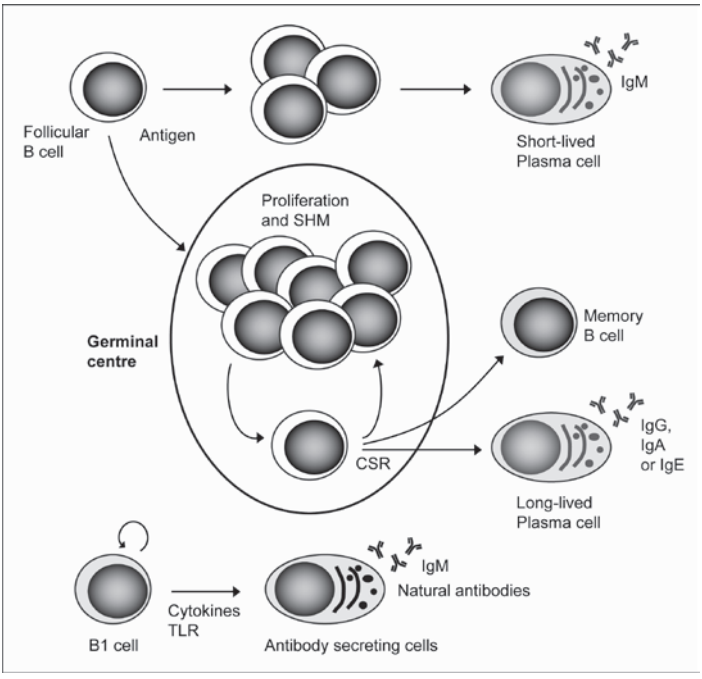

Figure 8. Activation of B cells.
Productive interaction of follicular B cells with antigen results in proliferation and differentiation. The primary response generates pre-germinal centre plasma cells that are short-lived and usually secrete IgM. Some activated follicular B cells form a germinal centre. Antigen-activated lymphoblasts that enter a germinal centre are subjected to multiple rounds of SHM and antigen selection. Cells that express high-affinity antigen receptors are selected for survival with subsequent differentiation to memory B cells or post-germinal centre plasma cells. Post-germinal centre plasma cells that undergo Ig H chain CSR typically home to the bone marrow where they reside and become long-lived (122).
Upon activation of a distinct population of B cells, B-1 cells, by cytokine and/or TLR activation antibody secreting cells are formed which secrete natural IgM antibodies, which often recognize bacterial antigens. SHM, somatic hypermutation; CSR, class switch recombination. ***See Appendix for full-color figure.***

Marginal zone (MZ) B cells are a second population of mature splenic B lymphocytes which are specifically located in the marginal zone of the spleen (123). MZ B cells are preselected to express a BCR repertoire that is biased and they respond rapidly to antigenic challenge.

Another B cell subset is the B-1 B cells, which are found in the peritoneal and pleural cavities, spleen and gut. B-1 cells persist largely by self renewal. In contrast to follicular and MZ B cells, B-1 cells are primarily generated from progenitors in the fetal liver and neonatal BM and can be divided into the $CD5^+$ B-1a and $CD5^-$ B-1 B cells. The most distinctive feature of B-1a cells is the production of 'natural antibodies', autoantibodies with specificities that include glycoproteins, phosphorylcholine, phosphatidylcholine but which are not pathogenic. The choice of differentiation into any of the three subsets is dependent on BCR signaling (11). However, recently in our lab it was found that constitutive active Btk expression did not change follicular, marginal zone, or B-1 B cell fate choice, but resulted in selective expansion and survival of B-1 cells. Therefore, constitutive Btk activation and consequently active BCR signaling results in defective B cell tolerance (124).

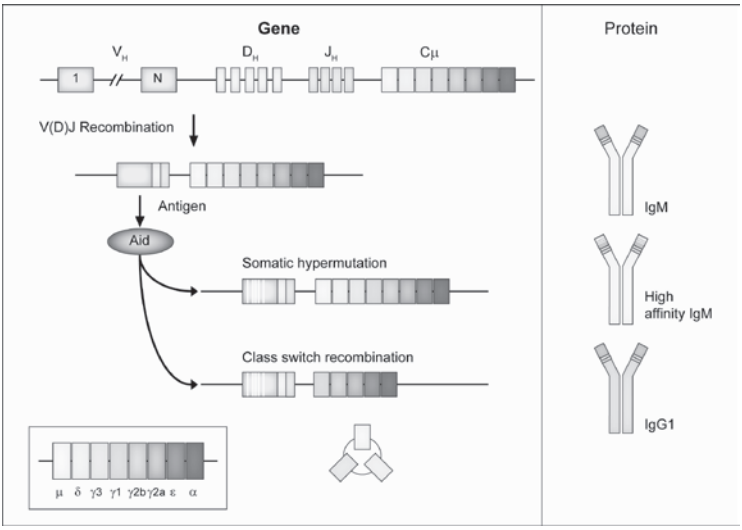

Figure 9. Generating antibody diversity.
Antibodies are encoded by immunoglobulin genes; these include V, D and J regions and C regions. In pro-B cells an immunoglobulin gene includes the full range of V,D, J and C regions. V(D)J recombination generates an antibody (IgM) with a variable region that recognizes a particular antigen. When the B cell encounters antigen, two other processes — both catalysed by the Aid protein — are triggered. Somatic hypermutation generates mutations (thin white lines) in the variable regions, potentially generating an IgM with higher affinity for its antigen. Class switch recombination results in the excision of some of the constant regions, generating antibodies with distinct effector functions (IgG1 is shown here). Figure adapted from ref. (125). ***See Appendix for full-color figure.***

Germinal centre reaction

After being stimulated with antigen, mature B cells undergo two additional processes of antigen receptor diversification. Class switch recombination (CSR) and somatic hypermutation (SHM) occur mainly in specialized structures in secondary lymphoid organs called germinal centres. CSR and SHM are initiated by activation-induced cytidine deaminase (Aicda/Aid) (Figure 9) (126-127). Aid is a single-stranded DNA deaminase that targets cytidine residues and thereby creates uracil-guanine mismatches in DNA that can be processed by many different pathways to produce mutations or DSB (128-130). SHM introduces point mutations in the V_H regions of Ig H and L chains. B cells expressing high affinity Ig are selected by limited amounts of antigen, resulting in affinity maturation. CSR is a recombination reaction that does not affect the antigen binding specificity of an antibody but exchanges the constant region of the Ig H chain and thereby producing antibodies with distinct effector functions. During CSR the Ig H chain isotype switches from IgM to IgG, IgA or IgE. Aid expression is normally restricted to germinal centre B cells but when expressed in non-germinal centre cells, Aid can induce mutation in various highly transcribed genes and thereby acts as a genome-wide mutator (131-133). Furthermore, Aid can initiate chromosomal translocations between c-Myc and the Ig H locus (134). Thus, all three processes responsible for diversification of the antigen receptor genes (V(D)J recombination, SHM and CSR) can initiate the formation of oncogenic translocations (127, 135).

Defects in the pre-BCR checkpoint are associated with primary immunodeficiency disease

Deficiencies in antibody production and function are the hallmark of primary immunodeficiency diseases that involve B cells. There is a markedly decreased serum level of antibodies or an absence of antibody production. There are three major categories of antibody deficiencies: (a) defects in early B cell development; (b) hyper-IgM syndromes (CSR defects, e.g. because of deficiency of AID or in CD40L, leading to defective T-B cel interaction and GC formation); and (c) common variable immunodeficiencies and deficiencies of specific Ig subclasses, in particular IgA-deficiency is relatively frequent. All antibody deficiencies are associated with an increased susceptibility to infection with encapsulated bacteria.

Many genetic defects that result in a failure of B cell development involve signaling through the pre-BCR. Mutations in Btk, components of the pre-BCR and BCR (μ H chain, λ5, Igα and Igβ) or Slp65 account for approximately 90% of patients with defects in early B cell development (136-138). Mutations in Btk, the gene responsible for X-linked agammaglobulinemia (XLA), account for most of the affected patients. XLA is considered the prototype immunodeficiency and was first described in 1952 by Bruton (139). He reported a case of an 8-year old boy with multiple episodes of pneumococcal

sepsis associated with complete absence of serum Ig fractions. Patients with XLA have markedly reduced numbers of B cells in the circulation and low numbers of plasma cells. Serum Ig are very low, but detectable. Hallmark of the disease is frequent and recurrent infection of the upper and lower airways. In 1993, two groups reported that XLA resulted from mutations in a cytoplasmic tyrosine kinase called Btk (140-141). Defects in SLP65 result in an autosomal agammaglobulinemia with a phenotype similar to that found in XLA patients (136).

XLA reflects an almost complete arrest of B cell development at the pre-B cell stage. Cytoplasmic μ postive cells are generated, but they do not proliferate and they are arrested in their development. In contrast to XLA, Btk-deficiency in the mouse is not associated with a major early B cell developmental block but with impaired maturation and poor survival of peripheral B cells. This X-linked immunodeficiency (xid) phenotype is the result of a spontaneous mutation of a CpG site in the Btk gene changing residue 28 from arginine to a cysteine (R28C) in the pleckstrin homology domain (142-143) and is mild compared to XLA patients. Xid mice are characterized by a mild arrest at the large pre-B cell stage, reduction of 50% of the normal numbers of B cells in the periphery, more immature B cells in the spleen ($IgM^{high}IgD^{low}$), a lack of B1-a B cells, shortened survival of MZ B cells and low serum IgM and IgG3 levels. Peripheral B cells from xid mice do not proliferate in response to anti-IgM treatment, and show a reduced proliferative response to LPS. In addition, xid mice are not able to mount thymus-independent type II (TI-II) response to antigens (144).

Like xid mice, Slp65-deficient mice have a less complete block of B cell development than is found in humans. Slp65-deficiency results in a partial arrest at the large pre-B cell stage and leads to more immature B cells in the spleen, fewer mature B cells and a 5-40% reduction in the number of B cells. In addition, Slp65-deficient mice lack B1-a B cells, have low serum levels of IgM and IgG3, respond poorly to TI-II antigens, have reduced Ca^{2+} responses to BCR crosslinking and poor proliferation upon CD40 ligation or LPS stimulation (88, 90).

The molecular mechanisms underlying the difference in the degree of severity in phenotype between XLA and xid is not known. However, in mice Tec can partially replace Btk, while in humans Btk deficiency alone causes severe disease in spite of the presence of Tec in human B lymphocytes (145). Furthermore, in mice other adaptor proteins (Lat and Slp76) can to some extent replace Slp65 and are also expressed in pre-B cells (146). Moreover, the roles of Btk and Slp65 are different in pre-BCR signaling in human and mice with respect to the initiation of proliferation. A crucial difference in this context is the role of IL-7R signaling. In humans, B cell development appears to proceed in the absence of IL-7, but in mice IL-7R signaling plays a central role in governing commitment, survival, proliferation and maturation (104, 147). The presence of normal numbers of circulating B cells in primary immune deficiency patients with a

mutation in genes encoding the IL-7R argues that B cell development does not require IL-7R signaling (148-149). A developmental block at the pre-B cell stage is also present when V(D)J recombination is defective, e.g. in cases of mutations in Rag-1 or Rag-2. However, as V(D)J recombination is also essential for T cell development, such defects do not result in isolated B cell defects, but in severe combined immunodeficiency syndromes (SCID) (136, 138).

Current therapy for immunodeficiencies consists of monthly administration of intravenous Ig and the use of antibiotics (150). This is only partially effective. Furthermore, such treatment is expensive and associated with long-term complications. A definitive therapy for immunodeficiency, and in particular XLA, could be gene therapy. In the past different groups have shown that gammaretroviral vector based HSC gene therapy can provide significant clinical benefits in patients with SCID (151-152). Unfortunately, retroviral vector therapy in SCID has led to proto-oncogene (LMO2) transcriptional activation and development of T-ALL (151-152). Recently, different groups have focused attention on lentiviral vectors as potential delivery platforms for these disorders (153-154). These studies are the first demonstration of successful lentiviral gene therapy in an animal model of XLA. However, additional experiments are still required before proceeding toward clinical application in XLA.

Malignancies

The development of lymphoid neoplasia is a complex multistep process of genetic alterations and cellular transformations. Lymphoid malignancies are characterized by the frequent occurrence of chromosomal abnormalities, often translocations between proto-oncogenes and Ig or TCR loci (135). Many of the most known oncogenes, including ABL, Bcl-1 and Bcl-2, were discovered because they are located at translocation breakpoints in leukemia or lymphomas. This has led to the hypothesis that some of these translocations represent the consequence of misregulated V(D)J recombination or CSR (36). Alarmingly, and despite success achieved during the last decades in the treatment of these malignancies, their incidence is rapidly increasing. The need for further improving therapy requires in-depth understanding of the fundamental nature of the disease process and of the mechanisms by which lymphoid cells undergo malignant transformation and progression. The complexity of the pathogenic mechanism in lymphoid neoplasia is related to the fundamental strategy of the immune system. The mechanisms of V(D)J recombination, SHM and CSR cause genetic instability and impose a constant threat of malignant transformation.

Due to the unique feature of lymphoid cells to somatically rearrange antigen receptor genes, these cells are frequent targets for chromosomal translocations and oncogene activation resulting from recombinase targeting mistakes or incorrect repair of the V(D)J recombination intermediates (34-35). It was demonstrated in the mouse that

chromosomal reinsertion of broken RSS can target cryptic RSS-like elements via a V(D)J recombination-like mechanism (155). Moreover, cryptic RSS sequences immediately internal to the deletion breakpoints in the *IKZF1* locus, encoding the transcription factor *IKAROS*, have been identified in human *BCR-ABL1-positive* ALL (156). Cryptic RSS are estimated to have a density of 1 per 600 bp in the genome and have also been identified in other loci involved in ALL, including *PAX5* and *CDKN2A/B* in humans and *c-Myc* and *Lmo2* in the mouse (157-158). In addition, Rag proteins have the ability to rearrange DNA sequences that do not resemble RSS, as reported in follicular lymphoma (159). Taken together, these findings suggest that gene deletion or translocation arising from aberrant Rag activity contributes to leukemogenesis in human and mouse ALL.

Malignancies of pre-B cells: acute lymphoblastic leukemia

Precursor-B cell leukemia is one of the most common forms of childhood malignancy and reflects clonal proliferation of transformed cells as a result of genetic changes. Extensive molecular characterization of these genetic abnormalities, in particular chromosomal translocations have allowed the development of sensitive assays for the identification of underlying molecular defects, which are applicable to disease diagnosis and to monitor response to treatment. Childhood B-lineage ALL can be divided in different subtypes based on genetic abnormalities. The two main genetic subtypes, *TEL-AML1*-positive and hyperploidy with more than 50 chromosomes, account for 50% of pre-B ALL cases. *BCR-ABL1*-positive ALL, *MLL* and *E2A-PBX1* rearranged ALL each account for less than 5% of pediatric cases of ALL (160).The *BCR-ABL1* fusion gene is a result of the t(9;22)(q34;q11) translocation and represents the most frequent recurrent genetic aberration leading to ALL in adults (161). Next to translocations, a large group of pre-B ALL cases has aberrations in the loci of various genes, identified by genome-wide hybridization studies (156, 162). These include transcription factors such as *Pax5* (in ~30% of cases), *IKAROS*, *E2A*, or *EBF1*, cell cycle proteins (*Cdkan2a/b, Rb*), DNA repair protein *Atm,* the microRNAs Mir-15/16 and the apoptotis regulator *Btg1*. Interestingly, in a small fraction of B-lineage ALL the genes encoding cytkine receptors *Csfr2* (a IL-7R homologue) and *IL-3R* and the signaling molecules *PTEN* and *SLP65* are affected (156, 162). Despite this impressive progress, there is still a deficiency in our understanding of how consecutive genetic abnormalities ultimately subvert the developmental program of normal precursor-B cells.

Deficiency of SLP65 has been found in ~50% of childhood precursor-B cell ALL cases (163). The loss of SLP65 protein was found to be due to defective splicing, leading to premature stop codons. The SLP65 gene contains alternative exons (exon 3a and 3b) located in intron 3 and when these alternative exons are included into the SLP65 mRNA they interrupt the open reading frame of SLP65 and prevent protein expression. Moreover, in precursor-B ALL that where positive for the BCR-ABL

translocation the activity of the fusion protein, the BCR-ABL1 kinase, was linked to the expression of the same aberrant SLP-65 transcripts (164). Because other expression profiling studies with a large number of patients reported a low frequency (156, 165), it is not clear whether the loss of SLP-65 is a common leukemogenic event. Nevertheless, the reported findings indicate that loss of SLP-65 and the accompanying pre-B cell differentiation arrest may be one of the primary causes of precursor-B ALL.

Research in this area is strongly facilitated by the availability of an animal model for this disease: SLP-65-deficient mice spontaneously develop precursor-B cell tumors that show a similar phenotype as found in humans (166-167). Although Btk-deficient mice do not develop pre-B cell leukemia, Btk and Slp65 cooperate as tumor suppressors whereby Btk exerts its tumor suppressor function independently of its kinase activity (168-169). Combined deficiency of Slp65 and Btk result in a more complete arrest at the pre-B cell stagev(168) and a higher incidence of pre-B cell leukemia than compared with single deficient mice (88, 166-167, 170) suggesting that the developmental block is one of the tumor-promoting factors. In addition, expression of the pre-BCR is also essential for the development of leukemia as mice which are Rag-deficient or which cannot express the Ig μ H chain on the surface (e.g. because of λ5-deficiency), due to a disruption of the membrane exon of the Ig μ H chain constant region (μMT), are arrested at the pro-B cell stage but do not develop leukemia (66, 74, 166). Furthermore, Slp65 has a specific function that suppresses malignant transformation because in Btk/Plc-2 and Irf-4/Irf-8 double-deficient mice with a nearly complete arrest at the large pre-B cell stage no leukemias have been reported (171-172).

Several lines of evidence have indicated a role for unregulated Rag activity in Slp65-deficient pre-B cell leukemia. First, Rag proteins are expressed in strongly proliferating Slp65-deficient pre-B cells (166-167). Second, Slp65-deficient pre-B cell leukemias mostly co-express Ig H chain, SLC and Ig L chain (168). Third, in human pre-B cell leukemia SLP65 deficiency correlates with RAG expression and ongoing V_H gene rearrangement activity (173).

Similar to Slp65-deficient mice Eμ-*myc* transgenic mice, which express c-Myc under the control of the IgH intronic enhancer, develop rapid-onset pre-B cell malignancies (174). The c-Myc proto-oncogene is a broadly expressed transcription factor that is implicated in various cellular processes – cell growth, loss of differentiation and apoptosis. Elevated or deregulated myc expression has been found in a wide range of human cancers, and is often associated with aggressive, poorly differentiated tumors. Such cancers include breast, colon, cervical, small-cell lung carcinomas, osteosarcomas, gliobastomas, melanomas and myeloid leukemias (175). In Burkitt's lymphoma c-MYC is oncogenically activated as a result of a translocation between chromosome 8 and the Ig H chain locus or the Ig κ or λ L chain locus (176-177). Interestingly, the incidence of lymphomas in Eμ-*myc* mice is greatly reduced by the introduction of a human IgH

transgene (178). In addition, loss of Btk or PLC-γ2 synergizes with deregulation of c-Myc during lymphoma formation in Eμ-*myc* mice (179-180). The presence of the Eμ-*myc* transgene substantially increases the proliferative potential of B cell progenitors in response to IL-7 (179-180). Activation of c-Myc is required for progression of quiescent cells into the S phase of the cell cycle, but c-Myc can also induce the p53 protein, which protects against oncogenic transformation of proliferating cells (181). c-Myc activates the $p19^{Arf}$ tumor suppressor that interferes with the E3 ubiquitin protein ligase Mdm2 and thereby stabilizes and activates p53, resulting in cell-cycle arrest or apoptosis. In Myc-induced lymphomagenesis the $p19^{Arf}$–Mdm2–p53 circuitry is often disrupted, indicating that c-Myc activation strongly selects for spontaneous inactivation of this pathway (182).

Aim of the thesis

B lymphocytes are necessary to provide humoral immunity against a wide variety of possible antigens. A prerequisite for the generation of an effective humoral response is the diversity and specificity of the antibody repertoire. Signaling by membrane immunoglobulin governs a number of distinct checkpoints in B cell differentiation that shape the antibody repertoire by a combination of cellular and molecular selection. A central checkpoint is the pre-BCR and its signaling pathways involved in proliferation, differentiation and Ig L chain recombination.

This thesis describes studies aimed at understanding the pre-BCR checkpoint and the role of the signaling proteins Btk and Slp65 in pre-B cell differentiation, proliferation and the suppression of malignant transformation. In particular, we aimed to identify the molecular mechanisms involved in oncogenic transformation of pre-B cells lacking functional Slp65. One of the events initiated upon pre-BCR signaling is the rearrangement of Ig κ and λ L chains in small pre-B cells. **Chapter II** describes the role of Btk and Slp65 in pre-B cells in the initiation of Ig L chain rearrangements and developmental progression.

The development of lymphoid neoplasia is a complex multistep process of genetic alterations and cellular transformations. The complexity of the pathogenic mechanisms in lymphoid cells is likely to be related to the fundamental strategy of the immune system, in which somatic gene rearrangement ensures diversity, specificity and optimal function of the B cell receptor. The cost, however, is genomic instability and its potential contribution to malignancy. Similar to Slp65-deficient mice Eμ-*myc* transgenic mice, which express c-Myc under the control of the IgH intronic enhancer, develop rapid-onset pre-B cell malignancies. Myc-induced lymphomagenesis often disrupts the $p19^{Arf}$–Mdm2–p53 circuitry, indicating that c-Myc activation strongly selects for spontaneous inactivation of this pathway. In Chapter III we investigated whether malignant transformation of Slp65-deficient pre-B cells also requires loss of the protective checkpoint function of the $p19^{Arf}$–Mdm2–p53 pathway.

Pre-B cell leukemias in Slp65-deficient mice have high-level pre-BCR expression and this high level of expression is thought to contribute to their strong proliferative capacity. In Chapter IV, we investigated whether particular pre-BCR specificities may predispose these cells to malignant transformation.

A majority of B- and T-cell leukemias and lymphomas are associated with chromosomal abnormalities that bear the hallmarks of aberrant V(D)J or class-switch recombination. While leukemic Slp65-deficient pre-B cells express the recombination activating genes and manifest ongoing immunoglobulin light chain rearrangement, it has been hypothesized that deregulated recombinase activity contributes to their malignant transformation. DNA double stranded breaks initiated by V(D)J recombination are repaired by the non-homologous end joining pathway, which is active throughout the

cell cycle. Due to deregulated V(D)J recombination activity in strongly cycling Slp-65-deficient pre-B cells, Slp65-deficient pre-B cells may also have DSBs in cycling cells, leading to genomic instability. In **Chapter V** we investigated whether Rag-induced DNA damage and illegitimate repair by the non-homologous end joining repair pathway is involved in the oncogenic transformation of Slp65-deficient B cells.

In Slp65-deficient mice, pre-B cell tumors arise from large, cycling pre-B cells which are highly proliferative. It is therefore conceivable that cell division in these cells may be accompanied by genomic instability or mutations, ultimately leading to oncogenic transformation. Therefore, we investigated in **Chapter VI** whether homologous recombination, the pathway which repair DSB in cycling cells, plays a role in the malignant transformation of Slp65-deficient pre-B cells.

In **Chapter VII** we aimed to accomplish sporadic SV40 T gene expression in B-cell lineage in mice by introducing the SV40 T gene without its promoter and in opposite transcriptional orientation between the Ig H chain D and J_H segments. By achieving sporadic SV40 T expression in B-lineage cells we aimed to investigate whether the V(D)J recombination system active in pro-B cells manifests in vivo transposition activity, leading to leukemic formation as soon as the SV40 gene is expressed in a new genomic context.

The General discussion (**Chapter VIII**) deals with the implications of the studies described in this thesis in context of the currently available literature and with directions for future research.

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Chapter II

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

Kersseboom R, Ta VBT, Zijlstra AJE, Middendorp S, Jumaa H, van Loo PF, Hendriks RW. J Immunol. 2006 Apr

Abstract

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

Introduction

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

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

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

The role of pre-BCR signaling in the induction of pre-B cell differentiation and Ig L chain rearrangement is controversial (2, 4). The hypothesis that pre-BCR signals are responsible for the redirection of V(D)J recombination activity from the Ig H chain

to the L chain loci is based on the observations that Ig H chain surface expression correlates with transcription of unrearranged Ig L chain gene segments. This so-called germline transcription has been implicated in regulation of accessibility of Ig loci to the V(D)J recombinase, because it precedes or coincides with V(D)J recombination (See for review: Ref. (2). Second, in the absence of pre-BCR function, e.g. in mice with targeted disruption of Ig H chain, Ig-α, Ig-β, SLC components or Syk, Ig κ locus rearrangement is diminished. Third, expression of an activated Ras transgene induces L chain rearrangement in $J_H^{-/-}$ pro-B cells, which lack the ability to assemble Ig H chain variable regions (10). Fourth, the findings of reduced Ig L chain germline transcription and gene rearrangement in SLP-65-deficient pre-B cells (7, 11) and of reduced λ L chain usage in Btk-deficient mice (12-13) points to important roles for these signaling molecules in the initiation of Ig L chain rearrangement. Nevertheless, evidence for a direct involvement of pre-BCR signaling in the induction of L chain V(D)J recombination is lacking. On the contrary, it has also been reported that rearrangement and expression of Ig L chain genes can occur without Ig H chain expression. Ig k transcription and rearrangement is detectable in Ig μ^- pro-B cells (14) and is increased by activation of NF-κB by lipopolysaccharide in transformed pro-B cell lines (15). Removal of IL-7 from cultured pro-B cells from $J_H^{-/-}$ or $\lambda 5^{-/-}$ mice, which are incapable of expressing a proper pre-BCR, resulted in the apparent differentiation into cells that transcribe and rearrange Ig L chain loci (16). Moreover, the observations that SLC- and Ig H chain-deficient pre-B cells rearrange Ig L chain at normal frequencies and with normal kinetics, argue for a model in which the initiation of Ig L chain rearrangement is independent of pre-BCR expression (4, 17).

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

To distinguish between these possibilities, in the current report we investigated the effects of Btk and SLP-65 deficiency on transcriptional activation of Ig L chain loci in pre-B cells. By crossing Btk/SLP-65 double deficient mice onto a Bcl-2 Tg background (18), we determined whether providing an extended time window for Ig L chain rearrangement rescued their severe arrest at the pre-B to immature B transition. Finally, we studied Tg mice that express the constitutively active Btk mutant E41K-Btk under the control of the CD19 promoter region (8, 19-21). The CD19 promoter region

targets expression of transgenes to all stages of B cell development, including pro-B cells (21). Therefore, the E41K-Btk transgenic mice enabled us to investigate whether Btk signaling has the capacity to prematurely induce cell surface marker modulations and Ig L chain rearrangement in pro-B cells, thus independent of the developmental progression of large cycling into small resting pre-B cells.

Materials and Methods

Mice and Genotyping

Mice deficient for Btk (22), RAG-1 (23) or μMT (24) and E_{μ}-*2-22* Bcl-2 Tg mice (18) were on the C57BL/6 background; SLP-65^{-} mice (25) were on the Balb/c background. The VH81X-μ Tg mouse strain (26) was a generous gift from J.F. Kearney. E41K-Btk mice have been described previously (8, 21). The different composite genotypes were on a mixed background and in single experiments littermates were compared. All mice were bred and maintained at the Erasmus MC animal care facility under specific pathogen free conditions. Mice were analyzed at the age of 8-16 weeks. Experimental procedures used in this study were reviewed and approved by the Erasmus University Committee of Animal Experiments. For mouse genotyping, tail DNA was analyzed by Southern blotting of BamHI digests using a partial human Btk cDNA probe (bp 133-1153), as described previously (21-22). Alternatively, the presence of Btk transgenes, endogenous mouse Btk WT or Btk KO alleles were evaluated by PCR (8, 27). PCR assays were also used to determine the genotypes for SLP-65 (8), RAG-1 and μMT mice, using standard primers **(**www.jax.org), and to determine the presence of the E_{μ}-*2-22* Bcl-2 (12) or VH81X-μ transgenes (forward primer specific for framework I: 5'-CGCGCGGCCGCGTGGAGTCTGGGGGAGGCT-3' and reverse primer specific for the transgene CDR3 region: 5'-CCCAGACATCGAAGTACCAGCTACTA-CCATG-3' (26).

Flow cytometric analyses

Preparations of single-cell suspensions, flow cytometry procedures and monoclonal antibodies have been described previously (5, 22). For intracellular staining of cytoplasmic proteins, cells were first stained for cell surface markers and subsequently fixed in 2% paraformaldehyde and permeabilized using 0.5% saponin (21). The anti-SLC hybridoma LM34 ((28) was kindly provided by A. Rolink, University of Basel, Switzerland). Events (1-5 x 10^5) were scored using a FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences, Mountain View, CA).

RNA isolation and analysis

Total bone marrow (BM) cell suspensions were depleted of erythrocytes by standard ammonium chloride lysis. For mice that were on the μMT background, cell suspensions were enriched for B-lineage cells by magnetic sorting using anti-B220-coated microbeads (Miltenyi Biotec). Ig L chain-negative pre-B and Ig L chain-positive immature B cell fractions were obtained from *in vitro* BM cultures. Total BM cultures in the presence of IL-7 (Sigma), and quantification of IL-7 dependent proliferative responses of total BM cells have been described previously (5). Cells were labeled with biotinylated anti-Igκ (187.1) and anti-Igλ (R26-46) antibodies, incubated with streptavidin-coated microbeads (Miltenyi Biotec), and subjected to cell separation by AutoMACS (Miltenyi Biotec).

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

Results

Reduced germline λ L chain transcription in the absence of Btk

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

As shown in Figure 1B, we found that Btk^+ and Btk^- pre-B cells expressed comparable levels of κ^o0.8 and κ^o1.1 germline transcripts, which are initiated in different regions 5' of Jκ and spliced to the C_κ region (29). In contrast, the expression levels of germline λ transcripts were significantly reduced in Btk^- pre-B cells, as compared with Btk^+ pre-B cells. In particular the levels of $\lambda1^o$ and $\lambda2^o$ transcripts, which initiate 5' of the $J_\lambda1$ and $J_\lambda2$ region and are spliced to $C_\lambda1$ and $C_\lambda2$, respectively (29), were reduced

in Btk-deficient pre-B cells (Figure 1B). Interestingly, this effect of Btk appeared to be specific for the germline transcripts of the λ J-C cluster, because transcripts of the Vλ region were present at similar levels in Btk^+ and Btk^- pre-B cells (Figure 1B). Although successful V_L-to-J_L rearrangement results in loss of germline transcription on productive alleles, transcription of unrearranged L chain alleles continues in mature B cells (32). We therefore also analyzed surface Ig^+ B cells and observed a specific reduction in the expression of $\lambda 1^o$ and $\lambda 2^o$ transcripts in Btk-deficient B cells, while Vλ, $\kappa^o 0.8$ and $\kappa^o 1.1$ transcripts were present at normal levels (Figure 1B).

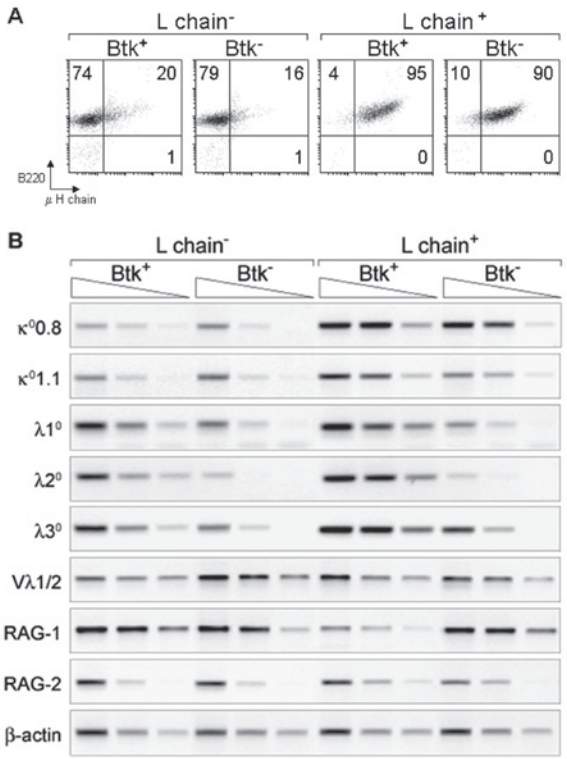

Figure 1. Reduced germline λ L chain transcription in Btk^- pre-B and immature B cells.
(A) Flow cytometric characterization of sorted surface Ig^- pre-B cell and surface Ig^+ immature B cell fractions from IL-7 driven BM cultures. (B) Detection of Ig L chain germline and RAG-1/2 transcripts. Ig κ + λ L chain negative and positive fractions were obtained by magnetic sorting. Total RNA from these cell fractions was reverse transcribed, diluted serially threefold, and used as a template for amplification of the transcripts indicated at the left. Amplification of a β-actin (bottom) was performed as a control. Products were fractionated by gel electrophoresis and detected with ethidium bromide. Data shown are representative for four mice of each genotype. Statistical evaluation of density values showed that expression levels of $\lambda^0 1$ and $\lambda^0 2$ were significantly reduced in Btk-deficient pre-B cells ($p<0.004$ and $p<0.02$, respectively) and immature B cells ($p<0.05$ and <0.009, respectively), when compared with wild type. No significant differences were found for $\kappa^0 0.8$, $\kappa^0 1.1$ or $\lambda^0 3$ expression.

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

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

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

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

Detailed flow cytometric analyses of the four groups of mice (WT, Btk-deficient, SLP-65-deficient and Btk/SLP-65 double deficient mice) on the RAG-$1^{-/-}$$V_H$81X Tg background revealed that Btk- or SLP-65-deficient pre-B cells exhibited elevated expression of CD43 and SLC and reduced expression of CD2, CD25 and MHC Class II, whereby the defects were most pronounced in the Btk/SLP-65 double mutant (Figure 2B). These findings show that the previously reported aberrant phenotypes of Btk and SLP-65 single or double deficient pre-B cells, which reflect their impaired cellular maturation (5, 8), were preserved on the RAG-$1^{-/-}$$V_H$81X Tg background. We additionally found that downregulation of IL-7R and c-kit expression on the surface of pre-B cells was impaired in the absence of Btk and SLP-65 (Figure 2B).

From the four groups of mice, BM cell suspensions were analyzed for the expression of Ig κ and λ L chain germline transcripts. In RT-PCR experiments, we observed a specific reduction in the expression of λ1° and λ2° transcripts in the absence of either Btk or SLP-65. Expression levels of λ3° were not significantly affected by the absence of Btk or SLP-65 (Figure 3). We detected a modest reduction in the expression levels of the κ°0.8 and κ°1.1 germline transcripts in SLP-65 deficient pre-B cells, when compared to wild-type or Btk-deficient mice (Figure 3). In contrast, in the Btk/SLP-65

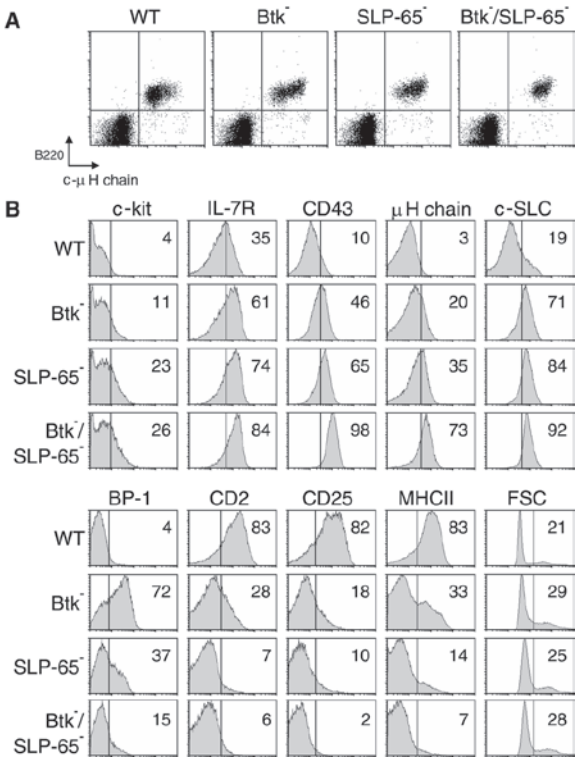

Figure 2. Defective pre-B cell maturation in Btk and SLP-65 deficient pre-B cells.
(A) Flow cytometric characterization of the B cell population from the indicated mice on a RAG-1$^{-/-}$ VH81X Tg background. BM lymphoid cells were gated on the basis of forward and side scatter characteristics. The expression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

double deficient pre-B cells the levels of both κ and λ germline transcripts were severely reduced.

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

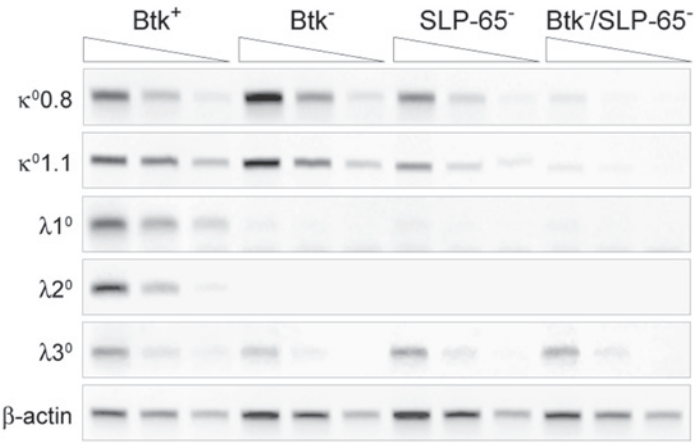

Figure 3. Defective germline transcription of Ig L chain loci in Btk and SLP-65 deficient pre-B cells. Expression of the indicated Ig L chain germline transcripts was analyzed by RT-PCR in the indicated four groups of mice on the RAG-1$^{-/-}$ VH81X Tg background. Total RNA from BM fractions was reverse transcribed, diluted serially threefold, and used as a template for amplification of the transcripts indicated at the left. Amplification of β-actin (bottom) was performed as a control. Products were fractionated by gel electrophoresis and detected with ethidium bromide. Data shown are representative for 3-4 mice examined in each group. Statistical evaluation of density values showed that expression of $\kappa^0$0.8/$\kappa^0$1.1 was significantly reduced in Btk/SLP-65 double deficient pre-B cells (when compared with wild-type; $p<0.03$) and expression of $\lambda^0$1/$\lambda^0$2 was significantly reduced in Btk or SLP-65 single or double deficient pre-B cells (when compared with wild-type; $p<0.05$). No significant differences were found for $\lambda^0$3 expression.

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

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

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

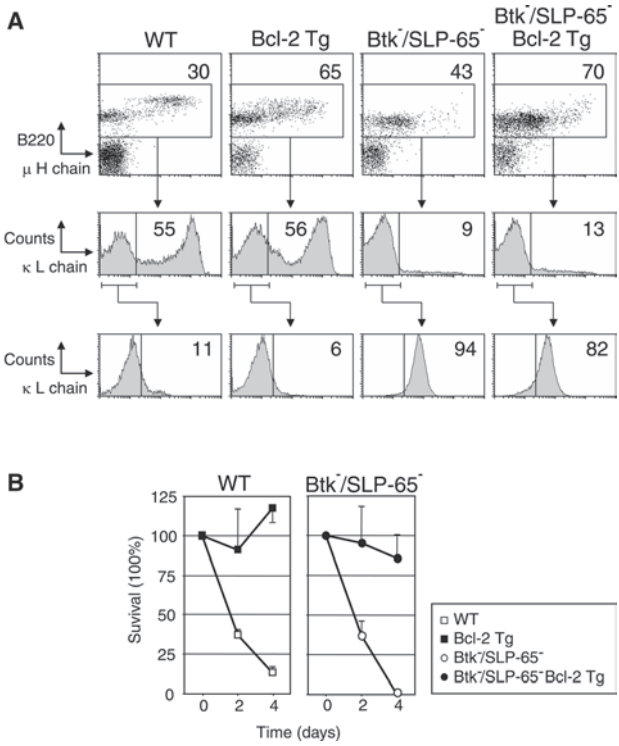

Figure 4. Bcl-2 expression does not rescue the pre-B cell arrest in Btk/SLP-65 double deficient mice.

(A) Flow cytometric analysis of BM lymphoid cells from the indicated mouse groups. Expression profiles of B220 and μ H chain are displayed as dot plots. Total $B220^+$ B-lineage fractions were gated and analyzed for the expression of κ L chain on the cell surface. Next, pro/pre-B cell fractions ($B220^+$, μ^-) were analyzed for cytoplasmic expression of SLC. Data are shown as histograms, whereby numbers indicate the percentage of positive cells. Plots are representative for 3-8 mice of each genotype. (B) In vitro survival after IL-7 withdrawal of BM cultures from the indicated mouse genotypes.

pre-B cell stage (8, 34), and the absence of germline L chain transcripts, would in this model reflect the short life span of Btk/SLP-65 double deficient pre-B cells.

To investigate this issue, we determined whether enforced expression of the anti-apoptotic Bcl-2 gene, which is thought to provide an extended time window per cell for Ig L chain rearrangement (35), is able to rescue the severe block in B cell development in Btk/SLP-65 double deficient mice. We crossed Btk/SLP-65 double deficient mice with Eμ-Bcl-2 Tg mice (18) and investigated the size of the immature B cell population by flow cytometry. We found that in the presence of the Bcl-2 transgene, the size of the immature B cell population was still severely reduced (Figure 4). Moreover, analysis of cytoplasmic μ H chain-positive pre-B cells indicated that increasing survival of Btk/

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

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

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

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

Although expression of transgenic E41K-Btk resulted in a dose-dependent deletion of B cells at the transition of IgM^{low} to IgM^{high} $B220^{low}$ immature B cell stage in the BM (8, 21), it nevertheless completely corrected the impaired modulation of pre-B cell surface markers in Btk-deficient mice (Figure 5A). In vitro BM culture experiments showed that expression of E41K-Btk increased IL-7 responsiveness of pro-B cells and reduced IL-7 responsiveness of pre-B cells (Figure 5B-D). E41K-Btk did not increase IL-7Rα chain transcription or surface expression in pro-B cells (data not shown), and therefore it appears that this effect of E41K-Btk is based on the functional intersection of pre-BCR and IL-7R signaling pathways that induce pre-B cell proliferation (36).

To examine the capacity E41K-Btk to mimic pre-BCR activation and consequently signal for premature cellular maturation of cytoplasmic μ^- pro-B cells *in vivo*, we characterized the $CD19^+B220^{low}$ cytoplasmic μ^- pro-B cell fractions in the BM from Btk^+, Btk^- and E41K-Btk Tg mice (Figure 6A). In Btk^+ and Btk^- mice, almost all $CD19^+B220^+$ cytoplasmic μ^- pro-B cells contained SLC. In contrast, in E41K-Btk Tg mice ~40% of the pro-B cells did not express detectable levels of SLC in the cytoplasm. When compared with Btk^+ or Btk^- mice, the pro-B cell fraction from E41K-Btk Tg mice contained a significantly larger subpopulation of cells expressing CD2, CD25 and MHC class II. In all groups of mice, pro-B cells were largely $CD43^+$. To exclude the possibility that the analyses of pro-B cells are confounded by Ig μ H chain positive pre-B cells that have subsequently lost or downregulated Ig μ H chain expression, we crossed Btk^+, Btk^-, and E41K-Btk Tg mice onto the μMT background (24). In μMT mice, cells are arrested at the pro-B cell stage, because the membrane exon of the Ig H chain μ constant region

is disrupted and therefore Ig μ H chain cannot be expressed on the cell surface. Also on the μMT background, we found that expression of E41K-Btk resulted in downregulation of SLC and the induction of CD2, CD25 and MHC class II in cytoplasmic μ H chain negative pro-B cells (Figure 6B).

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

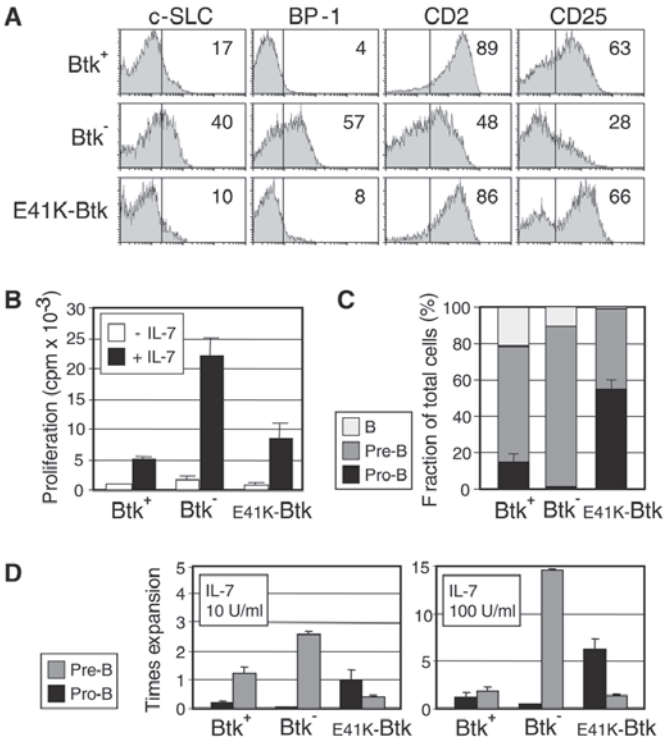

Figure 5. The effect of E41K-Btk expression on pre-B cells.
(A) E41K-Btk expression corrects the impaired modulation of cell surface markers in Btk-deficient pre-B cells. Cytoplasmic SLC and CD2 expression was investigated in pre-B cell fractions ($B220^+IgM^-$, c-μ H $chain^+$) whereas BP-1 and CD25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

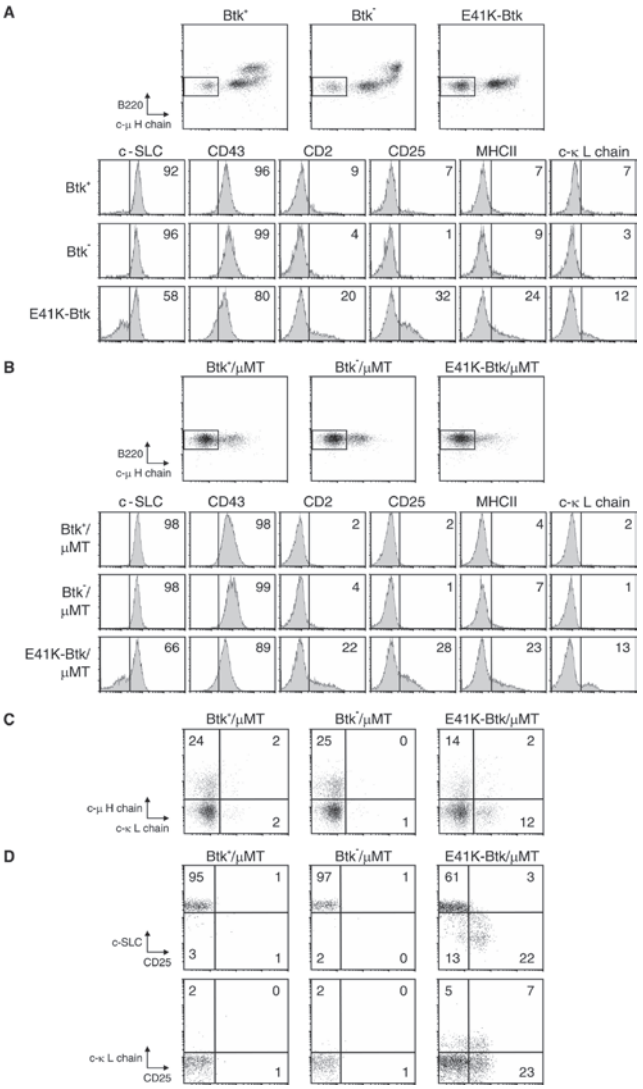

Figure 6. E41K-Btk induces phenotypic changes in cytoplasmic μ⁻ pro-B cells.
Flow cytometric expression profiles of the indicated markers in pro-B cells of Btk⁺, Btk⁻ and E41K-Btk mice on a wild type (A) or a μMT background (B, C, D). Flow cytometric analysis of surface B220 and cytoplasmic μ H chain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

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

Next, we investigated the effect of Tg E41K-Btk expression on Ig L chain rearrangement in pro-B cells, both on the wild-type background and on the μMT background. Whereas in wild-type mice <5% of $CD19^{+}B220^{low}$ cytoplasmic μ H chain negative pro-B cells express detectable levels of Ig κ L chain proteins in their cytoplasm, this proportion was significantly increased to ~10-15% of pro-B cells in E41K-Btk Tg mice (Figure 6AB). In the total population of E41K-Btk μMT $CD19^{+}B220^{+}$ pro-B cells approximately equal proportions of cells exclusively expressed Ig H or Ig L chains, whereas the fraction of H and L chain double positive pro-B cells was low (Figure 6C). This finding indicates that in E41K-Btk μMT pro-B cells the majority of functional κ L chain rearrangements are performed in the absence of a productive H chain rearrangement. As shown in Figure 6D, Ig κ L chain expression was mainly found in those pro-B cells in which expression of pre-B cell specific markers was induced. Moreover, the fractions of pro-B cells in which SLC expression was downregulated and CD2, CD25 or MHC class II were induced were largely overlapping (Figure 6D).

Using similar flow cytometric techniques we were unable to detect Ig λ L chain protein in E41K-Btk pro-B cells. However, when we performed RT-PCR analyses using primers specific for Vλ1 and Cλ1, rearranged λ L chain transcripts were readily detectable in purified E41K-Btk μMT pro-B cell fractions (Figure 7A). The levels of the Vλ1Cλ1 transcripts in E41K-Btk μMT pro-B cells were increased, when compared with control Btk^{+} or Btk^{-} μMT pro-B cell fractions. Consistent with the induction of L chain rearrangement by the E41K-Btk transgene, increased levels of $\kappa^{o}0.8$, $\kappa^{o}1.1$, $\lambda1^{o}$, and $\lambda2^{o}$ germline transcripts were detected in E41K-Btk μMT pro-B cells (Figure 7B).

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

Cooperation of IL-7 withdrawal and pre-BCR signaling

Pre-B cell receptor signaling mediates selective response to IL-7 at the pro-B to pre-B cell transition via an ERK/MAP kinase-dependent pathway (36). It has however been reported that removal of IL-7 induces Ig L chain rearrangement in cultured pro-B cells, even in the absence of functional Ig H chain protein (16). To investigate cooperative effects of pre-BCR signaling and IL-7 withdrawal, we cultured total BM cells from Btk^{+}, Btk^{-} and E41K-Btk mMT mice in the presence of IL-7 for 5 days and subsequently without IL-7 for 2 days. Consistent with reported findings (16), we observed that the Btk^{+} μMT pro-B cell cultures contained cytoplasmic κ L chain-positive cells (~20%, Figure 8). However, the expression of Ig κ L chain was partially dependent on Btk signaling, because in Btk^{-} μMT pro-B cell cultures the proportions of cytoplasmic κ L chain-positive cells were consistently lower (~10%, Figure 8). Likewise, the levels of germline $\lambda^{0}1$ and $\lambda^{0}2$ transcripts were severely decreased in Btk^{-} μMT pro-B cell cultures, when compared

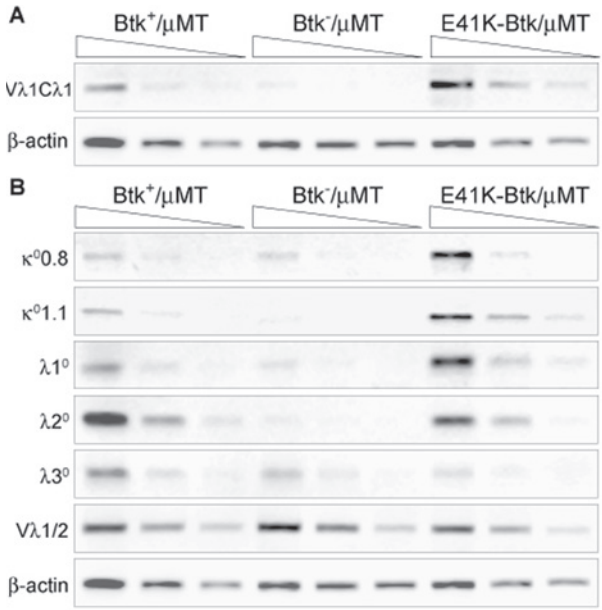

Figure 7. E41K-Btk induces L chain germline transcription and gene rearrangement in pro-B cells. RT-PCR analysis for the detection of λ 1 L chain transcripts from rearranged Vλ1-Cλ1 segments (A) and κ and λ L chain germline transcripts (B) in Btk$^+$, Btk$^-$ and E41K-Btk mice on a µMT background. Total RNA from BM fraction, enriched for B220$^+$ cells, was reverse transcribed, diluted serially threefold, and used as a template for amplification of the indicated transcripts. Amplification of β-actin (bottom) was performed as a control. Products were fractionated by gel electrophoresis and detected with ethidium bromide. Data shown are representative for 3 mice examined in each group. Statistical evaluation showed that expression of $\kappa^0$0.8, $\kappa^0$1.1, $\lambda^0$1 and $\lambda^0$2 were significantly induced in E41K- Btk transgenic pro-B cells ($p<0.05$), when compared with Btk$^-$ pro B cells. No significant differences were found for $\lambda^0$3 expression.

to Btk$^+$ µMT pro-B cell cultures (data not shown). In E41K-Btk µMT pro-B cell cultures the proportions of Ig κ$^+$ cells were similar to those found in Btk$^+$ µMT cultures, and levels of $\lambda^0$1 and $\lambda^0$2 transcripts were slightly increased (Figure 8 and data not shown).

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

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

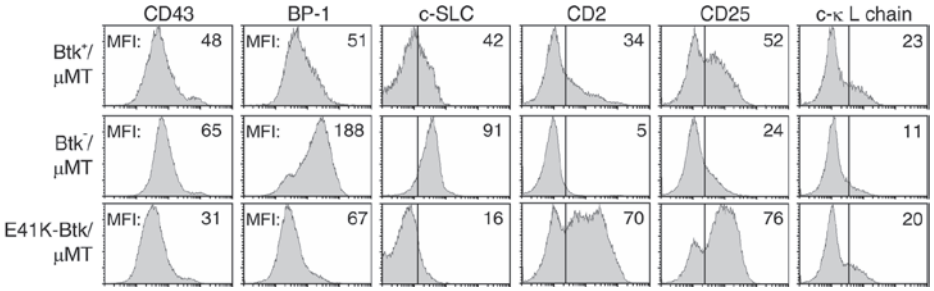

Figure 8. Cooperative effects of pre-BCR signaling and IL-7 withdrawal.
Flow cytometric expression profiles of the indicated markers in pro-B cells of Btk^+, Btk^- and E41K-Btk mice on μMT background that were cultured in the presence of IL-7 for 5 days and for 2 additional days without IL-7. $CD19^+B220^+$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

Discussion

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

In this report, we show (i) that protection of Btk/SLP-65 double deficient pre-B cells from apoptosis did not rescue modulation of pre-B cell surface marker expression or initiation of L chain rearrangement, and (ii) that expression of the E41K-Btk mutant in pro-B cells induced the pre-B cell developmental program, including germline transcription and productive rearrangement and expression of Ig L chain as well as modulation of cell surface markers. Therefore, we conclude that activated Btk does not only act to terminate proliferation or to increase survival of pre-B cells, but has the intrinsic capacity to signal for cellular maturation and the initiation of both k and λ L chain rearrangement and expression.

It is highly unlikely that E41K-Btk signals for cellular maturation and Ig L chain

recombination by inducing cell cycle arrest in pro-B cells, because expression of E41K-Btk increased IL-7 responsiveness in pro-B cells (Figure 5). Although we observed cooperation of IL-7 withdrawal and pre-BCR signaling in vitro (Figure 8), modulation of SLC, CD2 and CD25 expression was not induced by IL-7 withdrawal in the absence of Btk signaling.

The E41K gain-of-function mutation in the pleckstrin homology (PH) domain of Btk induces transformation of 3T3 fibroblasts and this capacity is augmented by mutation of Y223, the main autophosphorylation site of Btk (19-20). The E41K and E41K-Y223F mutants are associated with enhanced Btk membrane localization, activation and calcium signaling in cultured cell lines (19-20, 38-39). Several lines of evidence show that these mutants also represent activated forms of Btk in murine B cells. Tg expression of E41K-Btk (i) enhances blast formation of splenic B cells in culture (40), (ii) drives peripheral B cells efficiently into IgM plasma cell differentiation in vivo (21), and (iii) induces the expression of the early activation antigen CD69 on splenic B cells (R.W.H., unpublished). In addition, Tg E41K-Y223F-Btk manifests increased tyrosine phosphorylation in resting mature splenic B cells and has the ability to prevent tumor formation in SLP-65$^-$ pre-B cells (8). This observation indicates that E41K-Y223F-Btk may not only mimic activation of Btk, but may also transmit signals that substitute for SLP-65 function. Here we demonstrated that E41K-Btk was able to rescue the defects in Btk$^-$ pre-B cells, and to induce premature developmental progression and Ig κ and λ L chain recombination and expression in Ig H chain negative pro-B cells. As Ig λ rearrangements are normally initiated in late small resting pre-B cells when most k rearrangements cease (29, 41), expression of the E41K-Btk transgenic particularly advances Ig λ L recombination. Accordingly, E41K-Btk transgenic mice manifest increased Ig λ usage *in vivo* (~15%, as compared with ~7% in wild-type littermates; see ref. (12). Similar effects were also found for the E41K-Y223F Btk mutant (R.W.H., unpublished results). We did not detect any *in vivo* additional effects of the Y223F mutation on the phenotype of E41K-Btk mice, further supporting our previous conclusion that Y223 autophosphorylation-dependent interactions are not essential for Btk function during B cell development (27).

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

We observed that deficiency of Btk or SLP-65 in pre-B cells specifically reduced λ germline transcription, while κ germline transcription was not dramatically affected. These analyses were performed in VH81X transgenic RAG-1-deficient pre-B cells (Figure 3), which cannot perform receptor editing, a process that is usually

accompanied by increased λ usage (35). Therefore, our findings indicate that Btk/SLP-65 differentially regulate activation of the κ and λ L chain loci, independent of their possible role in receptor editing (12-13). We conclude that the specific reduction of λ germline transcription in Btk- or SLP-65-deficient pre-B cells supports our previous hypothesis that Btk transmits signals that regulate the activation of the λ locus for V(D)J recombination in pre-B cells (12). The finding that the absence of either Btk or SLP-65 affects germline transcription of the λ locus, but not of the κ locus, is consistent with various reports that indicate the importance of pre-BCR signaling specifically in λlocus activation. Firstly, Ig κ and λ rearrangements occur independently, whereby already in pro-B cells Ig κ germline transcription and rearrangements are present (albeit at low levels), while λ rearrangements are only initiated in small resting pre-B cells when most κ rearrangements cease (29, 41). Secondly, Btk-deficient mice have reduced Ig λ L chain usage (12). However, from the observed cooperative effects of Btk and SLP-65 on Ig κ germline transcription, it is clear that - in the absence of SLP-65 - Btk is also required for full activation of the λ locus. This would also be supported by our finding that E41K-Btk induces both Ig κ and λ L chain germline transcription in pro-B cells (Figure 7). Nevertheless, this capacity of the E41K-Btk mutant may alternatively indicate that its expression does not only mimic activation of Btk, but also transmit signals that substitute for SLP-65 function, analogous to our previous findings on the Y223F- E41K-Btk mutant (8).

Our experiments do not discriminate whether signals transmitted by Btk/SLP-65 directly induce opening of the Ig L chain loci for recombination, or whether these signals only act indirectly by inducing the pre-B cell differentiation program. However, Ig L chain locus activation and pre-B cell maturation are probably closely connected, as transcription factors such as E2A, PU.1, Spi-B and IRF-4/8, are implicated both in the expression of e.g. MHC class II or SLC and in opening of Ig L chain loci (42-47). This would be consistent with recent quantitative analyses of κ 3' enhancer chromatin structure and protein association, which indicate that this enhancer is activated progressively through multiple steps as cells mature (48). The transcriptional enhancers within the κ and λ L chain loci contain binding sites for the transcription factors E2A and EBF. Interestingly, transient transfection of E2A and EBF transcription factors into a non-lymphoid cell line was sufficient to activate V(D)J recombination at the Ig κ and λ loci, respectively (43). However, these transcription factors also activate transcription of the SLC genes λ5 and VpreB (42), which are silenced when Ig L chain rearrangement is initiated. Moreover, expression of Tg E41K-Btk in pro-B cells concurrently induced activation of Ig κ and λ L chain transcription and downregulation of SLC expression (Figures 6 and 7). In RT-PCR analyses, expression of E41K-Btk in pro-B cells did not appear to result in a significant increase in the expression levels of EBF or E2A (R.W.H., unpublished results). It remains however possible that Btk or SLP-65 are involved in the induction of EBF or E2A activity by stage-specific regulation of protein stability or expression of

partner molecules that interfere with the action of these transcription factors, such as Id proteins or Ikaros (47). Further experiments are required to investigate if Btk/SLP-65 signaling pathways converge on EBF and E2A.

Recently, the transcription factors IRF-4/8 and Spi-B were shown to be important for downregulation of the λ5 and Vpre-B genes and for the induction of germline Ig κ transcription in pre-B cells (44-45). However, Tg expression of E41K-Btk in pro-B cells did not appear to result in a significant increase in the expression levels of IRF-4 or Spi-B transcripts (R.W.H., unpublished results), indicating that these transcription factors may not function as nuclear effectors of Btk/SLP-65 signaling. This would be supported by recent findings demonstrating that changes in PU.1 and IRF-4 association during κ locus activation are independent of changes in protein expression levels, but rather reflect changes in chromatin accessibility and histone acetylation (48). Already in pro-B cells intermediate levels of the transcriptional activators PU.1 and IRF-4 associate with the κ 3' enhancer, which is moderately accessible at this stage. Therefore, it is likely that pre-BCR signaling results in Ig L chain chromatin structure changes, which are linked to epigenetic marking and affect transcription factor binding site occupancy in the 3' κ enhancer (48). Likewise, it is possible that pre-BCR signaling may downregulate the expression of the SLC chain genes by affecting the epigenetic status of the recently identified regulatory region within the λ5-VpreB locus, which is associated with a tightly localized epigenetic mark of H3 acetylation and histone H3 K4 methylation (47).

Our finding that Btk^- B cells fail to efficiently downregulate RAG-1 expression during the pre-B to immature B cell transition in culture (Figure 1B), implicate Btk signaling in the termination of V(D)J recombination activity upon productive Ig L chain rearrangement in immature B cells. This would be consistent with the finding that peripheral B cells from XLA patients, which have mutations in the Btk gene, show a distinct antibody repertoire consistent with extensive secondary V(D)J recombination (49).

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

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Chapter III

Malignant transformation of Slp65-deficient pre-B cells involves disruption of the Arf-Mdm2-p53 tumor suppressor pathway

Ta VBT, Bruijn MJW, ter Brugge PJ, van Hamburg JP, Diepstraten HJA, van Loo PF, Kersseboom R, Hendriks RW. Blood. 2010 Feb

Abstract

The adapter protein Slp65 is a key component of the precursor-B (pre-B) cell receptor. Slp65-deficient mice spontaneously develop pre-B cell leukemia, but the mechanism by which *Slp65*$^{-/-}$ pre-B cells become malignant is unknown. Loss of Btk, a Tec-family kinase that cooperates with Slp65 as a tumor suppressor, synergizes with deregulation of the c-Myc oncogene during lymphoma formation. Here, we report that the presence of the immunoglobulin heavy chain (IgH) transgene V_H81X prevented tumor development in *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice. This paralleled the reported effect of a human IgH transgene on lymphoma development in Em-*myc* mice, expressing transgenic c-Myc. As activation of c-Myc strongly selects for spontaneous inactivation of the p19^{Arf}–Mdm2–p53 tumor suppressor pathway, we investigated if disruption of this pathway is a common alteration in *Slp65*$^{-/-}$ pre-B cell tumors. We found that combined loss of Slp65 and p53 in mice transformed pre-B cells very efficiently. Aberrations in p19^{Arf}, Mdm2 or p53 expression were found in all Slp65$^{-/-}$ (n=17) and Btk$^{-/-}$Slp65$^{-/-}$ (n=32) pre-B cell leukemias analyzed. Also 9 our of 10 p53$^{-/-}$Slp65$^{-/-}$ pre-B cell leukemias manifested significant Mdm2 protein expression. These data indicate that malignant transformation of *Slp65*$^{-/-}$ pre-B cells involves disruption of the p19^{Arf}–Mdm2–p53 tumor suppressor pathway.

Introduction

B cells are produced in the bone marrow (BM) through a complex process of cellular differentiation, characterized by the ordered rearrangement of immunoglobulin (Ig) heavy (H) and light (L) chain gene segments encoding the B cell antigen receptor (BCR) (reviewed in Ref. (1-2)). In pro-B cells productive V(D)J recombination results in cell surface deposition of the pre-BCR, comprised of Ig μ H chain and the surrogate light chain (SLC) components λ5 and VpreB(2-4). The pre-BCR serves as an important checkpoint to monitor proper expression of a functional IgH chain and triggers clonal expansion, whereby pre-B cells acquire the capacity to respond to low concentrations of the proliferation factor interleukin (IL)-7(2-4). After a limited number of cell divisions, large pre-B cells stop cycling and differentiate into small, resting pre-B cells in which IgL chain rearrangement is initiated.

The adaptor protein Slp65 (also known as Blnk or Bash) is a key component in the signaling pathway downstream of the pre-BCR and the BCR. Slp65, when phosphorylated by Syk, provides docking sites for various molecules, including Bruton's tyrosine kinase (Btk) and phospholipase C (PLCγ). Btk then phosphorylates PLCγ, which leads to its full activation and the generation of second messengers(3, 5).

In human, mutations in *SLP65* or *BTK* result in defective pre-B cell proliferation and an almost complete arrest of B cell development at the pro-B to pre-B cell transition, associated with the immunodeficiency disorder agammaglobulinemia (3). In contrast, mice deficient for Slp65 or Btk show only a partial arrest at the large cycling pre-B cell stage, while a nearly complete block is present in Btk/Slp65 double deficient mice(6-8). Importantly, at the age of ~6 months 5-10% of Slp65-deficient mice develop pre-B cell leukemia expressing high levels of pre-BCR on the cell surface(6, 9). Although Btk-deficient mice do not develop pre-B cell tumors, we found that Slp65 and Btk cooperate as tumor suppressors, whereby Btk exerts its tumor suppressor function independently of its kinase activity(8, 10). It has been reported that in a substantial fraction of human pre-B cell acute lymphoblastic leukemia (ALL), including cases expressing the oncogenic BCR-ABL1 tyrosine kinase fusion protein, SLP65 expression is defective due to aberrant splicing(11-12). As subsequent analyses indicated that SLP65 deficiency may be an infrequent event in human pre-B-lineage ALL (13),(14), the importance of loss of SLP65 expression as one of the primary causes of pre-B ALL in human remains unclear.

The mechanism by which Slp65 exerts its tumor suppression function in mice has not yet been elucidated. During the transition of large cycling into small resting pre-B cells, *Slp65*$^{-/-}$ or *Btk*$^{-/-}$ cells fail to efficiently downregulate SLC and IL-7 receptor (IL-7R) expression, resulting in an increased proliferative response to IL-7 *in vitro*(6, 8). Recent findings show that Slp65 also downregulates IL-7-mediated proliferation and survival

through direct inhibition of Jak3, which is an essential IL-7R signaling component(15). The IL-7R pathway promotes cellular survival, proliferation and maturation, involving induction of the anti-apoptotic protein Bcl-2 and the proto-oncogene c-Myc(16, 17). B cell development can be partially restored in Jak-3-deficient mice when they are bred to mice co-expressing a rearranged IgH chain transgene and a c-Myc transgene(18).

Similar to *Slp65*$^{-/-}$ mice Eμ-*myc* transgenic mice, which express c-Myc under the control of the IgH intronic enhancer, develop rapid-onset pre-B cell malignancies (19). Interestingly, loss of Btk or PLC-γ2 synergizes with deregulation of c-Myc during lymphoma formation in Eμ-*myc* mice(20-21). The presence of the Eμ-*myc* transgene substantially increases the proliferative potential of B cell progenitors in response to IL-7(20-21). Activation of c-Myc is required for progression of quiescent cells into the S phase of the cell cycle, but c-Myc can also induce the p53 protein, which protects against oncogenic transformation of proliferating cells (see Ref. (22 for review)). c-Myc activates the p19^{Arf} tumor suppressor that interferes with the E3 ubiquitin protein ligase Mdm2 and thereby stabilizes and activates p53, resulting in cell-cycle arrest or apoptosis. It has been shown that in Myc-induced lymphomagenesis the p19^{Arf}–Mdm2–p53 circuitry is often disrupted, indicating that c-Myc activation strongly selects for spontaneous inactivation of this pathway(23).

We hypothesized that malignant transformation of Slp65-deficient pre-B cells is not only dependent on constitutive proliferative signals provided by the pre-BCR and IL-7R, but also requires loss of the protective checkpoint function of the p19^{Arf}–Mdm2–p53 pathway. Therefore, we investigated if disruption of the p19^{Arf}–Mdm2–p53 pathway is a common alteration in *Slp65*$^{-/-}$ pre-B cell tumors. We found that loss of p53 enhanced lymphoma formation in Slp65-deficient mice and that Slp65-deficient pre-B cell tumors harbored aberrations in the p19^{Arf}–Mdm2–p53 tumor suppressor pathway.

Material and Methods

Mice and genotyping

Btk$^{-/-}$(24), Eμ-*2-22* Bcl-2 transgenic (Tg)(25), *p53*$^{-/-}$(26), *Aicda*$^{-/-}$(27) and V_H81X Tg(28) mice were on the C57BL/6 background. *Slp65*$^{-/-}$ mice(5) were on the Balb/c background. The different composite genotypes were on a mixed background and in single experiments littermates were compared. For mouse genotyping genomic DNA was analyzed by PCR as previously described (8, 27-28). Animals in tumor panels were sacrificed after indication of tumor formation, or after a maximum period of 26 weeks. Mice were bred and maintained in the Erasmus MC animal care facility under pathogen-free conditions. Experimental procedures were reviewed and approved by the Erasmus MC committee of animal experiments.

Tumors were identified as described[8], and tumor load in lymphoid tissues was determined by flow cytometry on the basis of intracellular expression of SLC components and Ig u heavy chain and surface expression of B220 and CD19 (Suppl. Tables 3ABC).

Quantitative reverse transcriptase (RT)-PCR analysis

Total RNA was extracted using the GenElute Mammalian Total RNA Miniprep system (Sigma-Aldrich). One microgram of total RNA was DNAse digested (Invitrogen, Carlsbad, CA) and used as a template for cDNA synthesis, using Superscript II reverse transcriptase (Invitrogen) and random hexamer primers. For quantitative RT-PCR, primers spanning at least one intron-exon junction were designed manually or using ProbeFinder software (Roche Applied Science, Indianapolis, IN) (Supplementary Table S1). Quantitative RT-PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). C_t-values were obtained using SDS v1.9 software (Applied Biosystems) and normalized to glycereraldehyde-3-phosphate dehydrogenase (GAPDH).

Southern Blotting

Genomic DNA (10 μg) was digested with various restriction endonucleases (New England Biolabs, Beverly, MS) and processed by Southern blotting using nylon membrane (Hybond N+, Amersham or Nytran SPC, Whatman). Fragments were visualized using a PhosphorImager and analyzed with ImageQuant (Molecular Dynamics**,** Sunnyvale, CA). Probes specific for N-myc, a 990 bp PCR fragment from exon 3, for Gata-3, a 800 bp cDNA containing exons 1-3, for p53, a 1123 bp PCR fragment from intron 1 (probe 1) and a 614 bp fragment containing part of intron 10 and exon 11 (probe 2), for Ink4a-Arf, a 815 bp PCR fragment from exon 1α, were labeled by random priming, according to standard procedures.

Flow cytometry, cell culture and in vivo 5-bromo-2'-deoxyuridine (BrdU) labeling

Preparations of single-cell suspensions, flow cytometry procedures, and monoclonal antibodies have been described previously(8, 24, 29). BM cell suspensions were depleted of erythrocytes by standard ammonium chloride lysis and IL-7 driven cultures were performed as described previously(29). For intracellular staining of cytoplasmic proteins, cells were first stained for cell surface markers and subsequently fixed in 2% paraformaldehyde and permeabilized using 0.5% saponin. Procedures for calcium flux measurements have been described previously(10). Events (1–5 x 10^5) were scored using a FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences, San Jose, CA).

BrdU (BD Biosciences) was dissolved in Phosphate-buffered saline 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 Biosciences) in conjunction with cell surface marker expression.

Spectral karyotyping (SKY) and fluorescence in situ hybridization (FISH)

Tumor cells were cultured overnight in the presence of 100 U/ml IL-7 and 10 ng/ml colcemid (KaryoMAX Colcemid solution, Gibco-BRL, Gaithersburg, MD) and subsequently with 30 ng/ml for 4h to arrest proliferating cells at metaphase. Cells were treated with 75 mM KCl and fixed with methanol/acetic acid (3:1). SKY was performed using the Applied Spectral Imaging system (ASI, Migdal Ha'Emek, Israel) following manufacturer's protocols. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) containing DABCO/Vectashield (Vector Laboratories, Burlingame, CA). Chromosomes were analyzed by Zeiss Axioplan 2 microscope equipped with the Spectra Cube system (ASI). At least 10 different metaphases from each sample were analyzed, using Skyview analysis software (ASI). Dual-colored FISH experiments were performed using BAC probes RP23-246B9 for *N-Myc* and CT7-199M11 for IgH chain (Invitrogen, Carlsbad, CA), using standard procedures.

Pre-B cell stimulation and western blotting

Total BM cells were cultured in the presence of 100 U/mL IL-7 (Sigma-Aldrich, St Louis, MO) as described(29). Pre-B cells were stimulated with 20mg/mL F(ab)2 fragment of polyclonal goat anti-mouse IgM (Jackson Immuno Research, Westgrove, PA) at 37°C for 5 minutes. Cultured pre-B cells or pre-B leukemia cells were lysed in lysis buffer (20mM TRIS, 137mM NaCl, 10mM EDTA, 100mM NaF, 1% Nonidet P-40, 10% glycerol, 1mM pefabloc/AEBSF, 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (Roche Applied Science, Indianapolis, IN), 1 mM Na3VO4) on ice for 20 minutes and centrifuged at 16000*g* at 4°C for 20 minutes. Total cell lysates of were immunoprecipitated with antiphosphotyrosine (pTyr-100, Cell Signaling Technology, Beverly, MA). Samples were subjected to Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrotransferred to nitrocellulose membrane (Whatman), using standard procedures. Blots were stained with antibodies to Mdm2 (C18), p53 (DO-1 and FL-393), or Syk (N-19), all purchased from Santa Cruz Biotechnology, Santa Cruz, CA, or to $p19^{ARF}$ (ab80) from Abcam, Cambridge, MA.

Results

Enhancement of cellular survival does not increase pre-B cell leukemia incidence in Slp65-deficient mice

The developmental arrest in Slp65 and Btk single or double deficient mice results in a dominating populating of pre-B cells with increased surface expression of SLC and IL-7R, when compared with wild-type mice(6-7). Although *Slp65*$^{-/-}$ pre-B cells display an increased proliferative response to IL-7 *in vitro*(6, 9), the pre-B cell population in *Slp65*$^{-/-}$ mice contains significantly fewer cycling cells, when compared wild-type littermates(9). In agreement with the absence of increased *in vivo* proliferation of *Slp65*$^{-/-}$ pre-B cells, we found that BM pre-B cell numbers were not increased in *Slp65*$^{-/-}$ mice and even significantly reduced in *Slp65*$^{-/-}$*Btk*$^{-/-}$ mice (Figure 1A).

We previously reported that transgenic expression of the anti-apoptotic protein Bcl-2 enhanced *in vitro* survival upon IL-7 withdrawal, but did not rescue the developmental arrest of *Slp65*$^{-/-}$*Btk*$^{-/-}$ pre-B cells(30). It is therefore conceivable that Bcl-2 overexpression, which is commonly associated with enhanced malignancy of hematological tumors, may increase tumor incidence in *Slp65*$^{-/-}$ mice. To investigate this, we compared pre-B cell leukemia incidence in a panel of *Slp65*$^{-/-}$*Btk*$^{-/-}$ mice and Eμ-*2-22* Bcl-2 Tg *Slp65*$^{-/-}$*Btk*$^{-/-}$ mice. We confirmed that in these crosses Bcl-2 overexpression did not rescue pre-B cell differentiation (Figure 1B) and found that the presence of the Eμ-*2-22* Bcl-2 Tg had no effect on pre-B cell leukemia incidence in *Slp65*$^{-/-}$*Btk*$^{-/-}$ mice (Figure 1C) or *Slp65*$^{-/-}$ mice (not shown).

These findings show that in *Slp65*$^{-/-}$ pre-B cells – in which pre-BCR/IL-7R-mediated proliferation and survival signals are increased – enhancement of cellular survival does not augment tumor formation. Therefore, acquisition of additional genetic changes is required for malignant transformation of *Slp65*$^{-/-}$ pre-B cells.

Expression of the V_H81X IgH chain transgene prevents leukemia in *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice

Because (i) pre-BCR/IL-7R signaling induces c-Myc and its family member N-myc in large pre-B cells(16), and (ii) loss of Btk or PLCγ synergizes with deregulation of the c-Myc oncogene during lymphoma formation(20-21), we hypothesized that transformation of Slp65-deficient pre-B cells may involve enhanced Myc-activation.

We used quantitative RT-PCR to establish that pre-B cell tumors from *Slp65*$^{-/-}$ (n=16) and *Btk*$^{-/-}$*Slp65*$^{-/-}$ (n=27) mice expressed substantial levels of Myc transcripts. When compared with cultured wild-type pre-B cells, c-Myc expression in *Slp65*$^{-/-}$ and *Btk*$^{-/-}$*Slp65*$^{-/-}$ pre-B cell tumors was ~8-fold increased, while n-Myc was moderately increased only in *Slp65*$^{-/-}$ pre-B cell tumors (Figure 2A).

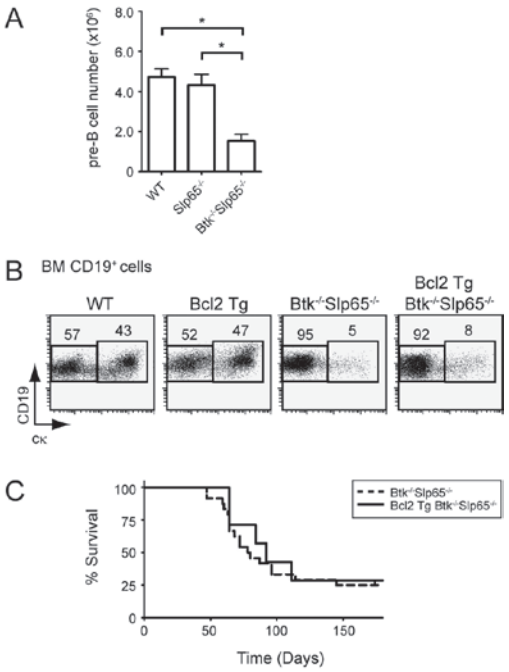

Figure 1. Enhancement of cellular survival does not increase pre-B cell leukemia incidence in Slp65-deficient mice.

(A) Absolute numbers of pre-B cells ($CD19^+Ig\ L^-cm^+$) per hind leg in the indicated mice. Bars represent average values and the standard error of the mean (SEM) of 9-14 animals per genotype. Asterisks indicate significant differences with $p< 0.001$ (t-test). (B) Flow cytometric analysis of BM lymphoid cells from the indicated mouse groups. Total $CD19^+$ B-lineage cells were gated and CD19/cκ profiles are displayed as dot plots. Percentages of cells within the indicated gates are given and data shown are representative of 3-4 animals per genotype. (C) Kaplan-Meier tumor-free survival estimates for *Btk*$^{-/-}$ *Slp65*$^{-/-}$ mice (n=25) and Bcl-2 Tg *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice (n=7).

Interestingly, the incidence of lymphomas in Eμ-*myc* mice is greatly reduced by the introduction of a human IgH transgene(31). Such a transgene is thought to accelerate B cell development, thus reducing the population size of cells most susceptible to oncogenic transformation by c-Myc. To investigate if expression of an IgH chain transgene also reduces pre-B cell tumor incidence in Slp65-deficient mice, we employed mice carrying the IgH chain transgene V_H81X(28). First, we ascertained by flow cytometry that the V_H81X transgene did not significantly affect the generation of large and small pre-B cell compartments in the BM of WT, *Btk*$^{-/-}$, *Slp65*$^{-/-}$ and *Btk*$^{-/-}$ *Slp65*$^{-/-}$ mice (Suppl. Figure S1). Furthermore, the presence of the V_H81X transgene did not affect *in vivo* pre-B cell proliferation: when mice were pulsed with a single dose of the thymidine analogue BrdU, which is selectively incorporated into the DNA of large

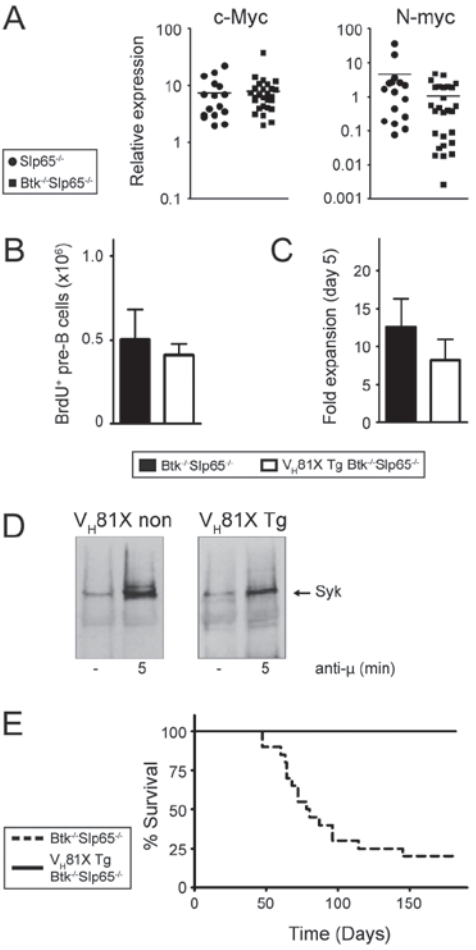

Figure 2. The V_H81X Tg prevents tumor formation in Btk/Slp65 double-deficient mice.
(A) Quantitative RT-PCR analyses of c-Myc and N-Myc expression in *Slp65*$^{-/-}$ (n=16) and *Btk*$^{-/-}$*Slp65*$^{-/-}$ (n=27) pre-B cell tumors, normalized with GAPDH. Values in WT pre-B cells, cultured for 5 days with 100 U/mL IL-7, were set to one. For each of these groups, the horizontal line represents the mean of the relative expression level. (B) Effect of the V_H81X Tg on pre-B cell proliferation *in vivo*. The absolute numbers of BrdU$^+$ CD19$^+$cμ^+cκ^- pre-B cells in the BM, 4 hr after i.p. injection of a single dose of BrdU, as determined by flow cytometry. Average values and SEM of three animals per group are shown. (C) Effect of the V_H81X Tg on pre-B cell proliferation *in vitro*. Results are displayed as fold expansion after 5 days of culture in the presence of IL-7 (100 U/mL), whereby the pre-B cell numbers at the start of the culture where set to one. Bars represent average values and SEM of 7-8 animals per genotype. (D) Syk phosphorylation in pre-B cell cultures from non-V_H81X Tg Slp65$^{-/-}$ and V_H81X Tg Btk$^{-/-}$Slp65$^{-/-}$ mice. Cells were either unstimulated (-) or stimulated for 5 minutes with polyclonal anti-IgM F(ab)2 fragments. The presence of phosphorylated Syk was detected in antiphosphotyrosine immunoprecipitates from total cell lysates, analyzed by Western blotting using Syk-specific antibodies. (E) Kaplan-Meier tumor-free survival estimates for *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice (n=20) and V_H81X transgenic *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice (n=25).

pre-B cells(29), comparable numbers of BrdU$^+$ CD19$^+$IgL$^-$cμ^+ pre-B cells were present in non-Tg and V_H81X Tg *Btk$^{-/-}$Slp65$^{-/-}$* mice (Figure 2B). The high *in vitro* proliferative capacity of *Btk$^{-/-}$Slp65$^{-/-}$* pre-B cells in IL-7 driven cultures(8) was also not significantly affected by the presence of the V_H81X transgene (Figure 2C). We therefore conclude that the phenotypical abnormalities of *Btk$^{-/-}$*, *Slp65$^{-/-}$* and *Btk$^{-/-}$Slp65$^{-/-}$* pre-B cells, which reflect their impaired cellular maturation from large cycling into small resting pre-B cells, were preserved in the presence of the pre-rearranged V_H81X IgH chain transgene. When cultured V_H81X transgenic and non-transgenic *Btk$^{-/-}$Slp65$^{-/-}$* pre-B cells were stimulated with Ig μ H chain-specific antibodies, we found similar Syk tyrosine kinase phosphorylation (Figure 2D), supporting the signaling competence of the V_H81X IgH chain. Consistent with the essential role of Slp65 in calcium mobilization, we found that pre-BCR signaling-associated calcium fluxes in both V_H81X transgenic and non-transgenic *Btk$^{-/-}$Slp65$^{-/-}$* pre-B cells were blunted (Suppl. Figure S2).

To address the effect of the V_H81X transgene on pre-B cell leukemia formation, we followed panels of *Btk$^{-/-}$Slp65$^{-/-}$* mice, which did or did not carry the V_H81X transgene. At 6 months of age, 16 out of 20 *Btk$^{-/-}$Slp65$^{-/-}$* mice developed pre-B cell leukemia. In strong contrast, none of the 23 V_H81X IgH chain transgenic *Btk$^{-/-}$Slp65$^{-/-}$* mice developed pre-B cell leukemia (Figure 2E). Also, when we examined BM and spleen of these mice by flow cytometry at the age of 6 months we did not find evidence for lymphoproliferative disease.

Thus, expression of the pre-rearranged V_H81X H chain transgene in early B cell differentiation prevented oncogenic transformation of *Btk$^{-/-}$Slp65$^{-/-}$* pre-B cells, equivalent to findings in Eμ-*myc* Tg mice(31).

Deficiency of Slp65 and p53 have cooperative effects in tumorigenesis

Next, we investigated the effect of p53-deficiency on pre-B cell tumor formation by crossing *Slp65$^{-/-}$* mice onto a *p53$^{-/-}$* background. In flow cytometric experiments, no significant differences were found between the sizes of the individual B-lineage subpopulations in *Slp65$^{-/-}$* mice and *p53$^{-/-}$Slp65$^{-/-}$* mice: as shown in Figure 3A, *Slp65$^{-/-}$* mice and *p53$^{-/-}$Slp65$^{-/-}$* mice have a similar defect in CD25 upregulation upon pre-BCR signaling(8), and have a similar reduction in the number of mature recirculating IgM^{low}IgD$^+$ B cells. The proliferative capacity of *Slp65$^{-/-}$* pre-B cells in IL-7 driven BM cultures *in vitro* was not affected by the concomitant absence of p53 (data not shown).

Whereas ~17% (6 out of 36) of *Slp65$^{-/-}$* mice developed pre-B cell leukemia at 6 months, all *p53$^{-/-}$Slp65$^{-/-}$* mice (n=24) developed pre-B cell leukemia within 5 months (Figure 3B). All pre-B cell tumors in *p53$^{-/-}$Slp65$^{-/-}$* mice were positive for Ig μ H chain and SLC, and showed variable surface expression of developmentally regulated markers, including CD2 and the metallopeptidase BP-1, similar to those normally found in *Slp65$^{-/-}$* mice (Figure 3C). In contrast, five out of 17 *p53$^{-/-}$* single deficient littermates developed T

cell lymphoma before the age of 5 months. Thus, loss of Slp65 on the *p53*$^{-/-}$ background resulted in rapid onset of pre-B cell tumors before the age at which T cell tumors usually arise in *p53*$^{-/-}$ mice. We conclude that Slp65 and p53 cooperate to limit the oncogenic potential of DNA damage and/or sustained oncogenic signaling.

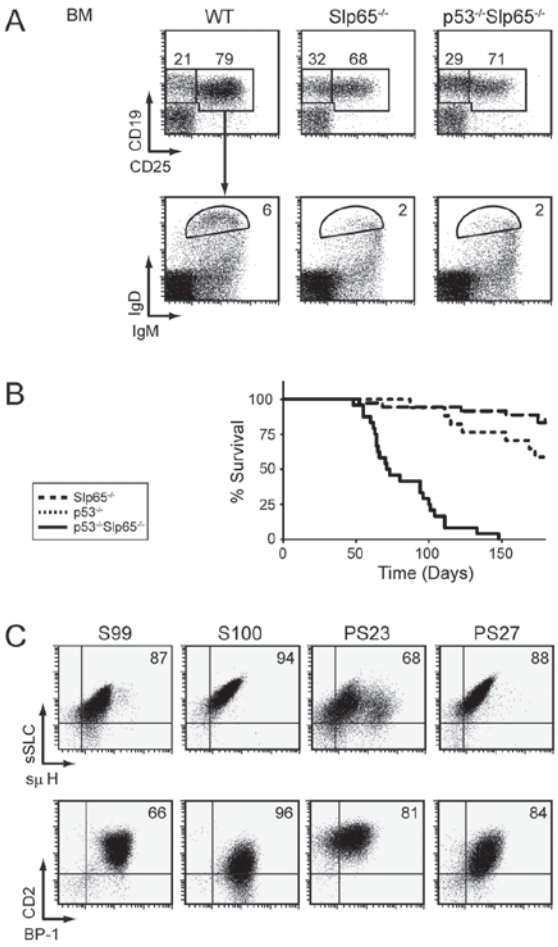

Figure 3. Slp65 and p53 cooperate as tumor suppressors in pre-B cells
(A) Flow cytometric analysis of total lymphoid fractions in BM (top panel). Total CD19^{+} fractions were gated and analyzed for IgM and IgD expression (lower panel). Data are displayed as dot plots, and numbers indicate the percentages of cells within the gates and are representative for four 5-week-old non–tumor bearing mice. (B) Kaplan-Meier tumor-free survival estimates for *Slp65*$^{-/-}$ mice (n=36), *p53*$^{-/-}$ mice (n=17), and *p53*$^{-/-}$*Slp65*$^{-/-}$ mice (n=24). (C) Characterization of pre-B cell tumors by flow cytometry. Dot plots for surface SLC and μ H chain expression (upper panel) and dot plots for CD2 and BP-1 expression (lower panel) in gated CD19^{+} cells from tumor samples of *Slp65*$^{-/-}$ (S99, S100) and *p53*$^{-/-}$ *Slp65*$^{-/-}$ (PS23, PS27) mice.

Activation-induced cytidine deaminase (Aid) is not involved in malignant transformation of *Slp65*$^{-/-}$ pre-B cells

One of the malignancy-associated stress signals that activates p53 is DNA damage. We therefore investigated a possible role of DNA damage caused by the Aid mutator protein or by aberrant V(D)J recombination, leading to chromosomal translocations (32).

Human pre-B ALL that harbor the t(9;22)(q34;q11) Philadelphia translocation encoding the oncogenic BCR-ABL1 tyrosine kinase have defective pre-BCR signaling, express a truncated isoform of SLP65(12, 33) along with AID(12, 33), which acts as a BCR-ABL1-induced mutator. Aid expression is normally restricted to germinal center B cells where it initiates somatic hypermutation (SHM) and class switch recombination. However, when expressed in non-germinal center cells, Aid can induce mutation in various highly transcribed genes and thereby act as a genome-wide mutator(34).

We analyzed Slp65-deficient pre-B cell tumors for Aid expression by quantitative RT-PCR. Remarkably, in most of the samples Aid transcripts were detected, albeit at variable levels. In a small fraction of leukemic samples Aid expression was in the same range as in Peyer's Patches (Suppl. Figure S3A). It was thus conceivable that Aid-induced DNA damage could contribute to tumorigenesis in Slp65-deficient pre-B cells. However, when we sequenced putative Aid target regions, including the 5' regions of the *l5* and *p53* genes, as well as the JH4 intron of the IgH locus, from a panel of 15 *Btk*$^{-/-}$*Slp65*$^{-/-}$ tumors, we did not find evidence for SHM (data not shown). Next, we reasoned that if Aid would play an important role in malignant transformation of *Slp65*$^{-/-}$ pre-B cells, the frequency of pre-B cell leukemias would be significantly reduced in the absence of the *Aicda* gene encoding Aid. But, we found that the pre-B cell tumor incidences in *Slp65*$^{-/-}$ or *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice were similar in the presence (*Aicda*$^{+/-}$) or absence (*Aicda*$^{-/-}$) of Aid (Suppl. Figure S3B).

Therefore, we conclude that Aid-induced DNA damage is not involved in malignant transformation of *Slp65*$^{-/-}$ pre-B cells.

Chromosome 12 abnormalities in *Slp65*$^{-/-}$ pre-B cell tumors

When DNA breaks mediated by the V(D)J recombination machinery are not resolved properly, they can give rise to chromosomal alterations, in particular translocations, and lymphoid tumors(32). We examined metaphase spreads from *Btk*$^{-/-}$*Slp65*$^{-/-}$ and *p53*$^{-/-}$*Slp65*$^{-/-}$ tumor cells by SKY and found evidence for chromosomal instability mainly characterized by chromosome gains, in particular of chromosome 12 and 14 (Supplementary Table S2). Interestingly, BCR-Abl transgenic mouse lymphomas often show gain of chromosome 12 and 14(35).

Two leukemia samples, BS56 (*Btk*$^{-/-}$*Slp65*$^{-/-}$) and PS11 (*p53*$^{-/-}$*Slp65*$^{-/-}$) harbored an aberration involving intrachromosomal gain of chromosome 12 material (Supplementary Figure S4A). Similar enlargements of chromosome 12 have been identified in pro-B cell

leukemia's derived from mice deficient for p53 and Artemis, a component of the non-homologous end-joining DNA repair pathway(36). As the observed chromosome 12 enlargement points to the possibility of *N-myc* amplification, FISH experiments were performed. We found a duplication (BS56) and dramatic amplification (PS11) of the *N-myc* locus (Supplementary Figure S4B). Southern blotting of genomic EcoRI digests with an *N-myc*-specific probe confirmed amplification of *N-myc* in the BS56 and PS11 tumors and identified a third pre-B cell leukemia (PS08) with *N-myc* amplification (3 out of 38 analyzed tumors) (Supplementary Figure S4C). Quantitative RT-PCR experiments demonstrated that the two *p53*$^{-/-}$*Slp65*$^{-/-}$ pre-B cell leukemias (PS08 in red and PS11 in green) that harbored substantial *N-myc* gene amplification expressed extremely high levels of N-myc and severely reduced levels of c-Myc transcripts (Supplementary Figure S4D). Similar cross-regulation of Myc family members has been reported in the context of N-myc-related transformation(36). In contrast to chromosome 12 enlargements involving N-myc in Artemis/p53 double-deficient mice(36-37), we did not detect co-amplification of IgH chain locus sequences in FISH and Southern blotting experiments (data not shown).

In summary, although part of the *Slp65*$^{-/-}$ pre-B cell leukemias exhibit chromosomal instability, we found no evidence for Rag-mediated translocations. Our SKY analyses revealed gain of chromosome 12 or *N-myc* amplification in 6 out of 19 (~32%) pre-B cell tumors.

Only a small subset of Slp65-deficient tumors expresses p53 protein

The p53 response can also be activated by oncogenic signaling. In particular, it has been shown that Myc-induced lymphomagenesis strongly selects for spontaneous inactivation of the p19^{Arf}–Mdm2–p53 tumor suppressor pathway(23). Therefore, we next investigated whether malignant transformation of *Slp65*$^{-/-}$ pre-B cells involves disruption of this pathway.

Loss of p53 function can occur through deletions or point mutations. We did not detect gross deletions in the *p53* gene by Southern blot analyses in a panel of 23 *Slp65*$^{-/-}$ and *Btk*$^{-/-}$*Slp65*$^{-/-}$ tumors (Suppl Figure S5A). Next, we sequenced p53 transcripts and genomic DNA encompassing exons 2-8 (encoding the DNA-binding domain where p53 most frequently undergoes mutation in tumors) from a panel of 29 *Slp65*$^{-/-}$ and *Btk*$^{-/-}$*Slp65*$^{-/-}$ tumors (Suppl. Tables S3A and S3B). This analysis revealed a mutation only in a single *Slp65*$^{-/-}$ tumor (S13): a R270C missense mutation. This mutation has previously been identified in Eμ-*myc* Tg lymphoma and corresponds with a mutation hot-spot of p53 found in human tumors(38). Missense mutations often result in accumulation of mutant p53 protein to supraphysiologic levels, because mutant p53 is often more stable than wild-type(39). Western blot analyses confirmed very high p53 protein expression in the tumor with the R270C p53 mutation (sample S13; Figure 4A).

Table 1. Arf - Mdm2 - p53 protein expression in *Slp65*-deficient leukemias

Tumor	p53	p19ARF	Mdm2
aberrant p53			
S13	high[1)]	high	expressed
S92	expressed	low	expressed
S96	expressed	expressed	expressed
S100	low	high	expressed
S102	low	high	expressed
S116	low	high	expressed
S203	low	high	expressed
S208	low	high	expressed
p53 wild type, Arf expressed/ high			
S14	undetected	high	expressed
S25	undetected	high	expressed
S75	undetected	high	undetected
S99	undetected	high	expressed
S101	undetected	high	expressed
S107	undetected	high	expressed
S109	undetected	high	expressed
S114	undetected	high	expressed
BS27	undetected	high	expressed
BS29	undetected	high	expressed
BS30	undetected	expressed	expressed
BS36	undetected	high	low
BS45	undetected	high	low
BS50	undetected	high	low
BS53	undetected	high	expressed
BS70	undetected	high	expressed
BS91	undetected	high	expressed
BS103	undetected	high	expressed
BS105	undetected	high	expressed
BS108	undetected	high	expressed
BS115	undetected	high	expressed
BS118	undetected	high	low
BS119	undetected	high	expressed
BS123	undetected	high	expressed
BS124	undetected	high	expressed
BS301	undetected	high	expressed
BS303	undetected	high	expressed
BS304	undetected	high	expressed
BS305	undetected	high	expressed
BS306	undetected	high	expressed
BS309	undetected	high	expressed
BS310	undetected	high	expressed

Table 1. Arf - Mdm2 - p53 protein expression in *Slp65*-deficient leukemias (Continued)

Tumor	p53	p19ARF	Mdm2
BS311	undetected	expressed	expressed
BS312	undetected	expressed	expressed
BS316	undetected	high	expressed
BS318	undetected	expressed	expressed
BS326	undetected	high	expressed
BS327	undetected	high	expressed
Arf low/ undetected			
S98	undetected	low	expressed
BS72	undetected	undetected	low
BS93	undetected	undetected	expressed
p53 deficient tumors, Arf expressed			
PS15	undetected	high	expressed
PS16	undetected	high	expressed
PS18	undetected	high	expressed
PS19	undetected	expressed	expressed
PS21	undetected	high	expressed
PS23	undetected	high	expressed
PS24	undetected	high	expressed
p53 deficient tumors, Arf undetected			
PS17	undetected	undetected	expressed
PS22	undetected	undetected	low
PS27	undetected	undetected	expressed

[1)] Immunoblotting experiments were performed twice and a summary of those experiments is shown.

In 7 out of 17 *Slp-65*$^{-/-}$ tumors analyzed we found low to moderate p53 protein levels (Figure 4A; Table 1). Sequencing of three of these tumors (S96, S100 and S102) did not reveal point mutations in exons 2-8 (Suppl. Table S3A). Furthermore, p53 protein was not detected in any of the 32 tumors from *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice (Figure 4C; Table 1). To examine p53 functional activity in these tumors, we compared transcription of the p53 target p21^{Cip1} in *Slp65*$^{-/-}$ tumors without detectable p53 protein expression and tumors with p53 protein (Figure 4B). We did not observe increased p21^{Cip1} transcription in tumors expressing p53, indicating that p53 protein in these tumors has lost part of its normal function.

Taken together, in 8 out of 49 *Slp65*$^{-/-}$ or *Btk*$^{-/-}$*Slp65*$^{-/-}$ tumors we found p53 protein expression, whereby only in a single case we were able to identify a p53 missense mutation.

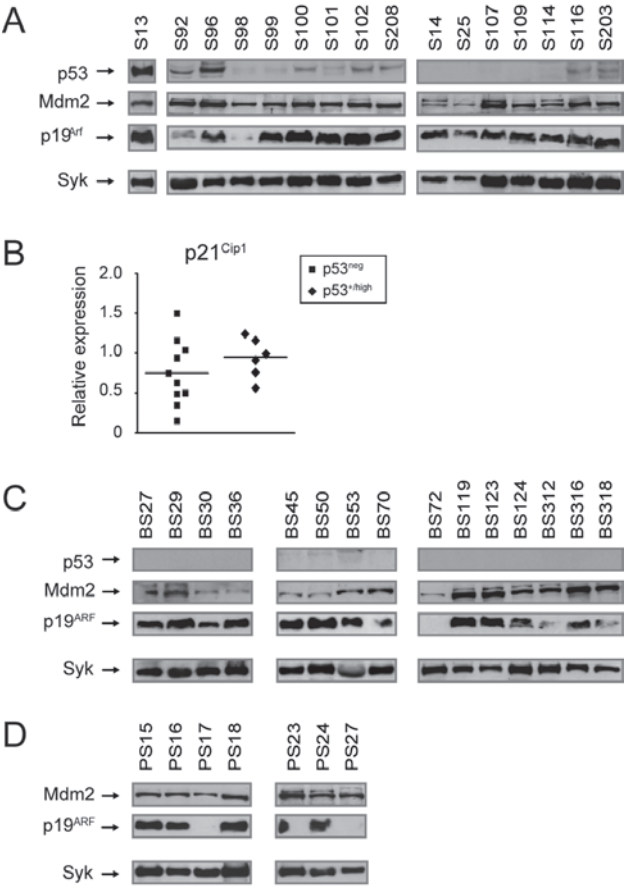

Figure 4. Aberrations of the p19^{Arf}–Mdm2–p53 pathway in Slp65-deficient leukemias.
(A) Western blot analysis of total cell lysates from pre-B cell tumors from *Slp65*$^{-/-}$ mice for the expression of p53, p19^{Arf}, Mdm2 and Syk (loading control). (B) Quantitative RT-PCR analyses of *p21*^{Cip1} expression in *Slp65*$^{-/-}$ pre-B cell tumors without or with detectable p53 protein, normalized to GAPDH. Values in sorted WT BM B220^{+} cells were set to one. Horizontal lines represent mean values of the relative expression levels. (C) Western blot analysis of total cell lysates from pre-B cell tumors from *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice for the expression of p53, p19^{Arf}, Mdm2 and Syk (loading control). (D) Western blot analysis of total cell lysates from pre-B cell tumors from *p53*$^{-/-}$*Slp65*$^{-/-}$ mice for the expression of p19^{Arf}, Mdm2 and Syk (loading control). Protein of p53 was not detected in *p53*$^{-/-}$*Slp65*$^{-/-}$ tumors.

All Slp65-deficient tumors manifest aberrations of the p19^{Arf}–Mdm2–p53 pathway

Consistent with disruption of the p53-p19^{Arf} feedback loop, p19^{Arf} expression was significantly increased in the pre-B cell tumor with the R270C p53 mutation (S13; Figure 4A) and in 6 of 7 additional *Slp65*$^{-/-}$ samples with detectable p53. Remarkably, substantial levels of p19^{ARF} were also present in 38 of 41 *Slp65*$^{-/-}$ or *Btk*$^{-/-}$*Slp65*$^{-/-}$ tumors in which p53

protein was undetectable. The presence of $p19^{Arf}$ protein indicates inactivation of the p53 response, because p53 is a negative regulator of $p19^{ARF}$.

In only two samples $p19^{ARF}$ was undetectable (BS72; BS93; Figure 4C; Table 1). Inactivation of the p53 pathway can also occur through loss of $p19^{ARF}$, which requires deletion of both *Arf* alleles (40). The *Ink4a-Arf* locus encodes two linked tumor-suppressor genes: $p16^{Ink4a}$ and $p19^{ARF}$. PCR amplification of exon 1α (*Ink4a*-specific) and exon 1β (*Arf*-specific*)* in combination with Southern blot analyses for exon 1α, 2 and 3 did not reveal any deletions in the *Ink4a-Arf* locus in these two samples.

Elevated expression of the Mdm2 oncogene augments proliferation, reduces susceptibility to p53-dependent apoptosis and induces chromosomal instability(41). High levels of Mdm2 protein were found in all eight tumors with detectable p53 and in 32 of 38 tumors that expressed $p19^{ARF}$. In six $p19^{ARF}$-expressing tumors Mdm2 levels were low (BS30, BS36, BS45, BS50 BS118) or undetectable (S75) (Figure 4AC, Table 1). From those three tumors that had low or undectable $p19^{ARF}$ two exhibited significant Mdm2 expression.

In summary, we found that all $Slp65^{-/-}$ (n=17) and $Btk^{-/-}Slp65^{-/-}$ (n=32) pre-B cell leukemias analyzed expressed either $p19^{ARF}$ or Mdm2, perhaps with the exception one sample, BS72, with no detectable expression of p53 or $p19^{ARF}$and only low Mdm2 expression. These results provide evidence for the involvement of disruption of the Arf–Mdm2–p53 tumor suppressor pathway in malignant transformation of Slp65-deficient pre-B cells.

Additional aberrations in $p19^{Arf}$ and Mdm2 in p53-deficient pre-B cell tumors

The finding that most Slp65-deficient pre-B cell tumors expressed Mdm2 raised the possibility that Mdm2 might be induced even in the absence of functional p53. To investigate this issue, we analyzed $p19^{ARF}$ and Mdm2 expression in a panel of 10 $p53^{-/-}Slp65^{-/-}$ pre-B cell tumors and found that seven expressed both $p19^{ARF}$ and Mdm2 (Figure 4D; Table 1). In three samples $p19^{ARF}$ was not detected. Because in cells lacking p53 the levels if $p19^{ARF}$ are normally increased, this finding indicates that the p53- $p19^{ARF}$ feedback loop was interrupted. This did not appear to result from genomic *Arf* loss, because no aberrations were found in the *Ink4a-Arf* locus by genomic PCR and Southern blotting analyses (Suppl. Figure S5BD). In these three tumors without detectable $p19^{ARF}$, we found that Mdm2 expression was high in two cases and low in one case (Table 1).

In summary, p53-deficient pre-B cell tumors have additional aberrations in $p19^{ARF}$ and Mdm2, suggesting that Mdm2 promotes transformation of Slp65-deficient pre-B cells by targeting effectors other than p53.

Discussion

In this study we provide evidence for disruption of the $p19^{Arf}$–Mdm2–p53 tumor suppressor pathway in malignant transformation of *Slp65*$^{-/-}$ pre-B cells. We demonstrated that combined loss of Slp65 and p53 in mice transformed pre-B cells very efficiently. Furthermore, we found aberrations in $p19^{Arf}$, Mdm2 or p53 in all 49 Slp65$^{-/-}$ and Btk$^{-/-}$ Slp65$^{-/-}$ pre-B cell leukemias analyzed.

Our findings reveal striking parallels in pre-B cell tumor formation between *Slp65*$^{-/-}$ and Eμ-*myc* Tg mice. First, in both models the expression of a pre-rearranged IgH chain transgene in early B cell differentiation reduces oncogenic transformation. Second, malignant transformation of both *Slp65*$^{-/-}$ and Eμ-*myc* Tg pre-B cells involves disruption of the $p19^{Arf}$–Mdm2–p53 tumor suppressor pathway. Third, Aid-induced DNA damage is not required for tumor development in either of the two models. Also the reported cooperation of Btk as a tumor suppressor in both *Slp65*$^{-/-}$ and Eμ-*myc* Tg pre-B cells point to parallel transformation mechanisms. On top of the failure of Slp65-deficient pre-B cells to efficiently downregulate pre-BCR and IL-7R signaling, it was recently shown that Slp65 has the capacity to inhibit IL-7R signaling by direct inhibition of Jak3 and that *Slp65*$^{-/-}$ tumors acquire autonomous IL-7R signaling by autocrine IL-7 production(15). As c-Myc is induced by IL-7R signaling, it is very well possible that sustained IL-7R signaling results in constitutive high levels of c-Myc expression in Slp65-deficient pre-B cells.

The finding that Slp65-deficient pre-B cell tumors display disruptions of the Arf–Mdm2–p53 pathway implies that oncogenic transformation of *Slp65*$^{-/-}$ pre-B cells does not exclusively result from sustained IL-7R signaling and endocrine IL-7 production(15). Instead, it is conceivable that mutations have accumulated before this stage, which would be supported by the finding that expression of the pre-rearranged V_H81X IgH chain transgene in early B cell differentiation stages prevents malignant transformation. Because early expression of a functional IgH chain is known to considerably shorten or even bypass pro-B cell development(42), acceleration of the passage of B cell precursors through the pro-B cell stage may prevent oncogenic transformation by reducing the size of the cell population most susceptible to transformation by e.g. c-Myc, N-myc or Abl(31). However, there are alternative explanations for the tumor suppressive effect of V_H81X IgH chain. We cannot exclude that V_H81X IgH chain transgene prevents tumor formation by limiting the time window for V(D)J recombination activity. Several reported findings indicate that V(D)J recombination is ongoing in *Slp65*$^{-/-}$ large pre-B cells. First, *Slp65*$^{-/-}$ pre-B cell tumors mostly co-express IgH chain, SLC, IgL chain(8) and the recombination activation genes Rag1 and Rag2 (V.B.T.T., unpublished data, October 2007l). Second, pre-B cell fractions from *CD19*$^{-/-}$*Slp65*$^{-/-}$ mice show increased expression of Rag2 protein(9). Third, In human pre-B cell leukemia SLP65 deficiency correlates with RAG expression and ongoing V_H gene rearrangement activity(43).

However, recently it has been found that Rag1 does not contribute to lymphomagenesis in Eμ-*myc* Tg mice(44), suggesting that malignant transformation of *Slp65*$^{-/-}$ pre-B cells mediated by Myc does not require deregulated Rag activity. Furthermore, it is possible that transformation of *Slp65*$^{-/-}$ pre-B cells is dependent on the expression of IgH chains with specific structural properties that induce strong autonomous pre-BCR signaling. Recently, Kohler et al(45) have described functional similarities between the pre-BCR and autoreactive BCRs, indicating that recognition of self-antigens by the pre-BCR might play a role in the initiation of pre-B cell proliferation. This explanation requires further investigation of the properties of IgH chains in Slp65-deficient pre-B cell tumors. Finally, it is unlikely that the V_H81X IgH chain is signaling incompetent, because it can pair efficiently with IgL chains, it induces strong proliferation and Syk phosphorylation and its presence does not significantly affect pre-B cell differentiation.

We found high levels of p19^{Arf} protein, in the absence of detectable *p53* gene mutations or deletions, indicating that these tumors have inactivated p53 through some other mechanism. In this context, we found by RT-PCR that *Slp65*$^{-/-}$ and *Btk*$^{-/-}$ *Slp65*$^{-/-}$ pre-B cell tumors contain high levels of p53 transcripts and that p53 protein was induced upon 10 gray irradiation in four pre-B cell tumors tested (V.B.T.T., unpublished data, August 2008). Thus, it is possible that p53 protein stability is low in these tumors, e.g. because of the high levels of Mdm2 that was present in the majority of tumors. Nevertheless, six *Slp65*$^{-/-}$ or *Btk*$^{-/-}$*Slp65*$^{-/-}$ pre-B cell leukemias with high p19^{Arf} levels (Table 1) and without detectable p53 did not exhibit Mdm2 overexpression. Importantly, p19^{Arf} has p53-independent functions, e.g it promotes the progression of lymphomas by mediating autophagy, a process of lysosome-mediated self-digestion that occurs during periods of nutrient deprivation(40, 46). Silencing of p19^{Arf} inhibits the progression of Myc-driven lymphoma cells containing mutant or no p53(46). Thus, *Slp65*$^{-/-}$ tumors may retain p19^{Arf} to promote survival under metabolic stress.

Slp65 regulates the activity of the forkhead-box transcription factors Foxo3a and Foxo1, which do not only promote IgL chain recombination, but also suppress Myc-driven lymphomagenesis via direct p19^{Arf} activation(2)·(47). This leads us to propose the following mechanism for malignant transformation of *Slp65*$^{-/-}$ pre-B cells. The absence of Slp65 results in sustained expression of the pre-BCR and the IL-7R in large cycling pre-B cells. Since IL-7R signaling induces c-Myc this also results in constitutively high levels of c-Myc. At this stage, FoxO transcription factors that normally suppress c-Myc-driven lymphomagenesis via direct activation of p19^{Arf} are not properly activated because of the absence of Slp65. Activation of c-Myc in combination with sporadic alterations in the p19^{Arf}–Mdm2–p53 pathway may finally lead to oncogenic transformation of *Slp65*$^{-/-}$ pre-B cells.

Our mouse model links defective pre-BCR signaling to oncogenic c-Myc activation and disruption of the p19^{Arf}–Mdm2–p53 tumor suppressor pathway, which also play an essential role in the pre-B cell transformation process mediated by Abelson

murine leukemia virus(48). Although defective expression of Btk or Slp65 may only be involved in a limited fraction of childhood ALL, and IL-7 signaling does not play a role in human pre-B cell development, our findings have relevance for human ALL. Deletion of CDKN2A (INK4A-ARF) is a significant secondary abnormality in childhood ALL which strongly correlates with phenotype and genotype(49-50). Furthermore, overexpression of the MDM2 gene was found in childhood acute lymphoblastic leukemia cells expressing wild-type p53(51).

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Supplementary Table S1. Primers and probes used in qRT-PCR analyses.

Gene	Forward primer	Reverse primer	Probe
AID	TCCTGCTCACTGGACTTCGG	GTGAACCAGGTGACGCGGTA	CGCTACATCTCAGACTGGGACCTGGACC
c-Myc	CGAGCTGTTTGAAGGCTGGAT	GTCGCAGATGAAATAGGGCTGT	CACGACGATGCCCCTCAACGTGA
Gapdh	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA	TGCATCCTGCACCACCAACTG
N-myc	AGAGGATACCTTGAGCGACTCAGA	ACAGTGATCGTGAAAGTGGTTACC	TGAGGATGACGAGGAGGAAGATGAAGAGGA
$p21^{Cip1}$	TCCACAGCGATATCCAGACA	GGCACACTTTGCTCCTGTG	CAGAGCCA

Supplementary Table S2. Chromosomal Alterations in Slp65-deficient pre-B cell leukemias

	Karyotype	
Tumor	**Numerical**	**Structural**
BS29	+12, +14	der (18;10)
BS30	+12	-
BS36	+14	-
BS47	+14, +19	-
BS48	-	-
BS49	-	der (7;14)
BS50	+14	-
BS51	+19	-
BS52	+12	-
BS53	+12	-
BS56	+14	der (12;12)
BS67	+14	-
BS68	-	-
BS69	-	-
PS2	-Y, +9	der (2;1)
PS5	+11, +17	del (2), del (13)
PS11	-	del (2), der (3;14)
PS14	+3, +11, +16, +17	der (12;12)
PS20	-13	-

[1)] Structural alterations in chromosomes are listed when observed in more than 50% of metaphases (as determined by SKY). BS, *Btk*$^{-/-}$*Slp65*$^{-/-}$; PS, *p53*$^{-/-}$*Slp65*$^{-/-}$

Supplementary Table S3A. Overview of purity of *Slp65*$^{-/-}$ pre-B cell tumor samples used in various assays

	Western Blot		Sequencing of p53			Sequencing of p53			Southern Blot p53	
Tumors	**Tissue**	**Tumor load**	**cDNA**	**Tumor load**	**exons**	**DNA**	**Tumor Load**	**exons**	**Tissue**	**Tumor Load**
S11										
S13	Spl	99%	Spl	58%	2-8	Spl	58%	8		
S14	BM	100%	Tu	99%	2-8				Tu	99%
S19	Spl	90%	Spl	82%	5-8				Spl	82%
S25	Spl	91%	Tu	92%	2-8					
S37			Tu	96%	2-8				Tu	96%
S75	Spl	99%	Tu	95%	5-8				Tu	95%
S92	Tu	92%								
S96	Tu	94%	Tu	94%	2-8	Tu	94%	4,5,6,7,8		
S98	Spl	74%	Spl	74%	2-8	Spl	74%	4,5,6,7,8		
S99	Tu	91%	Tu	91%	5-8	Tu	91%	4,5,6,7,8		
S100	Spl	96%	Spl	69%	2-8	Spl	69%	5,6,7		
S101	Spl	64%	Spl	64%	2-8	Spl	64%	4,5,6,7,8		
S102	Spl	93%	Spl	74%	2-8	Spl	74%	4,5,6,7,8		
S107	Tu	93%								
S109	Tu	94%	Tu	94%	5-8					
S114	Tu	93%	Tu	93%	5-8	Tu	93%	4,5,6,7,8		
S116	Tu	98%								
S203	Spl	82%								
S208	Spl	81%								

[1)] Leukemic samples were cultured for 4 to 12 days with 100 U/mL IL-7 to obtain a higher purity

Supplementary Table S3B. Overview of purity of $Btk^{-/-}Slp65^{-/-}$ pre-B tumor samples used in various assays

	Western Blot		Sequencing of p53			Sequencing of p53			Southern Blot p53	
Tumor	Tissue	Tumor load	cDNA	Tumor load	exons	DNA	Tumor Load	exons	Tissue	Tumor Load
BS15									Tu	92%
BS16									Spl	87%
BS18									Tu	92%
BS22									Tu	88%
BS26									Tu	90%
BS27	Spl	90%	LN	94%	2-8				LN	94%
BS29	Spl	100%	Tu	92%	2-8				Tu	92%
BS30	Tu	97%	Tu	97%	2-8	Spl	72%	5,6,7	Tu	97%
BS36	Spl	93%	Tu	88%	2-8				LN	85%
BS38			Spl	73%	2-8	Spl	73%	7		
BS39			Tu	92%	2-8				Tu	92%
BS40			Tu	96%	2-8				Tu	96%
BS41			Tu	96%	2-8	Tu	96%	8	Tu	96%
BS42			Tu	90%	5-8					
BS43			Spl	94%	2-8	Tu	98%	5,6	Spl	94%
BS44			Tu	88%	2-8				Tu	88%
BS45	Tu	100%	Tu	96%	2-8	Tu	96%	5,6	Tu	96%
BS46									Spl	91%
BS47										
BS48										
BS50	BM	100%	Tu	86%	2-8					
BS52										
BS53	LN	95%	LN	95%	2-8	LN	95%	5,6	LN	95%
BS54										
BS57										
BS59									BM	88%
BS66										
BS70	BM	69%								
BS72	Tu	98%								

Supplementary Table S3B. Overview of purity of Btk$^{-/-}$Slp65$^{-/-}$ pre-B tumor samples used in various assays

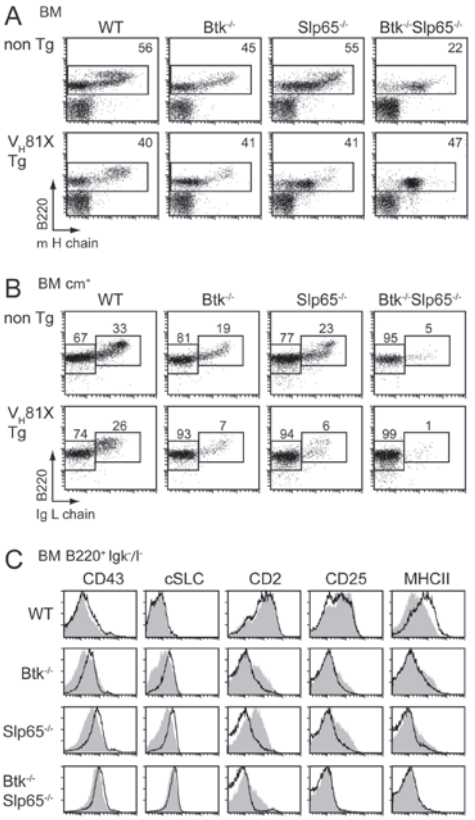
	Western Blot		Sequencing of p53			Sequencing of p53			Southern Blot p53	
Tumor	Tissue	Tumor load	cDNA	Tumor load	exons	DNA	Tumor Load	exons	Tissue	Tumor Load
BS74										
BS77									Tu	96%
BS91	Tu	97%	Tu	98%	2-5					
BS93	Tu	96%								
BS105	Tu	92%								
BS113	Tu	93%								
BS115	Tu	98%								
BS108	Spl	80%								
BS118	Spl	87%								
BS119	Tu	82%								
BS123	Tu	94%								
BS124	Tu	94%								
BS301	Tu	83%								
BS303	Tu	96%								
BS304	Tu	99%								
BS305	Tu	97%								
BS306	Tu	98%								
BS309	Tu	98%								
BS310	Tu	98%								
BS311	Tu	98%								
BS312	Tu	97%								
BS316	Spl	97%								
BS318	Tu	98%								
BS326	Spl	91%								
BS327	Tu	92%								

[1)] Leukemic samples were cultured for 4 to 12 days with 100 U/mL IL-7 to obtain a higher purity

Supplementary Table S3C. Overview of purity of $p53^{-/-}Slp65^{-/-}$ pre-B cell tumor samples used in various assays

	Western Blot		Southern Blot Ink4a/Arf	
Tumors	Tissue	Tumor load	Tissue	Tumor Load
PS15	Tu	87%	Spl	17%
PS16	Tu	95%	Tu	95%
PS17	Tu	88%	Tu	88%
PS18	Spl	88%	LN	68%
PS19	Spl	76%	LN	54%
PS20	Spl	58%	Spl	58%
PS21	Tu	94%	Spl	94%
PS22	Tu	96%	Tu	96%
PS23	Tu	91%	Tu	91%
PS24	Tu	78%		
PS27	Spl	84%	Spl	84%
PS28	Spl	95%	Spl	56%

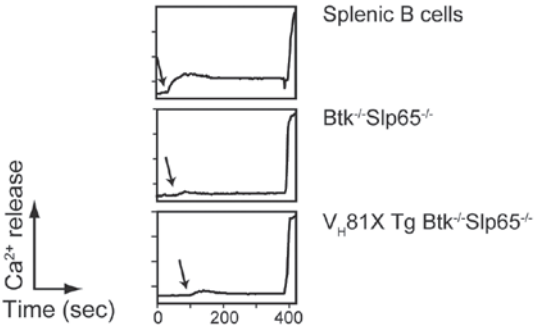
[1)] Leukemic samples were cultured for 4 to 12 days with 100 U/mL IL-7 to obtain a higher purity



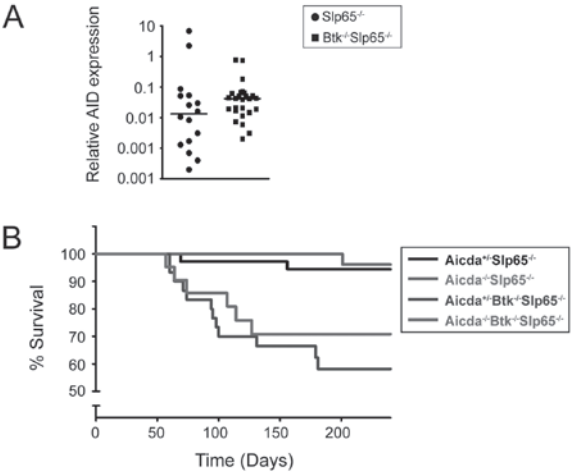
Supplementary Figure S1. Phenotypical abnormalities of Btk and Slp65 single or double mutant pre-B cells are preserved in the presence of the VH81X IgH chain transgene.

We compared eight groups of mice by flow cytometry: WT, *Btk*$^{-/-}$, *Slp65*$^{-/-}$ and *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice, which either did or did not carry the VH81X transgene. (A) B220/μ H chain profiles from BM lymphoid cells are displayed as dot plots. Total B220^{+} B-lineage cells were gated and percentages within the indicated gates are shown. Data are representative for 4 independent experiments. In agreement with reported findings (1,2) in the absence of Btk or Slp65 the proportions of B220^{high}Igμ H chain recirculating B cells in the BM were reduced, while in *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice B cell development was almost completely blocked at the B220^{low}Igμ^{+} pre-B cell stage. (B) Cytoplasmic μ H chain^{+} B-lineage fractions from total BM were analyzed for κλ IgL chain surface expression. Data are shown as dot plots, whereby the numbers indicate the proportions of κλ IgL chain surface expression of B220^{+}cytoplasmic μ^{+} B cells. The proportions of surface IgL chain positive B cells appeared slightly reduced in BM and spleen, when compared with mice of the same genotype that did not carry the transgene. (C) B220^{+}κ^{-}λ^{-} pro-/pre-B cells were gated and analyzed for the expression of CD43, cytoplasmic SLC, CD2, CD25 and MHCII. The results are displayed as histogram overlays of non-VH81X Tg (shaded histograms) and VH81X Tg (black lines) pro-/pre-B cell fractions of the mice indicated on the left. Plots are representative for 4-5 animals of each genotype. In the absence of Btk or Slp65 pre-B cells failed to efficiently downregulate CD43 and SLC, and to efficiently initiate CD2, CD25 and MHC II expression, as reported previously (1,3). For all four genotypes, the presence of the VH81X transgene did not significantly affect the expression of these developmentally regulated markers.

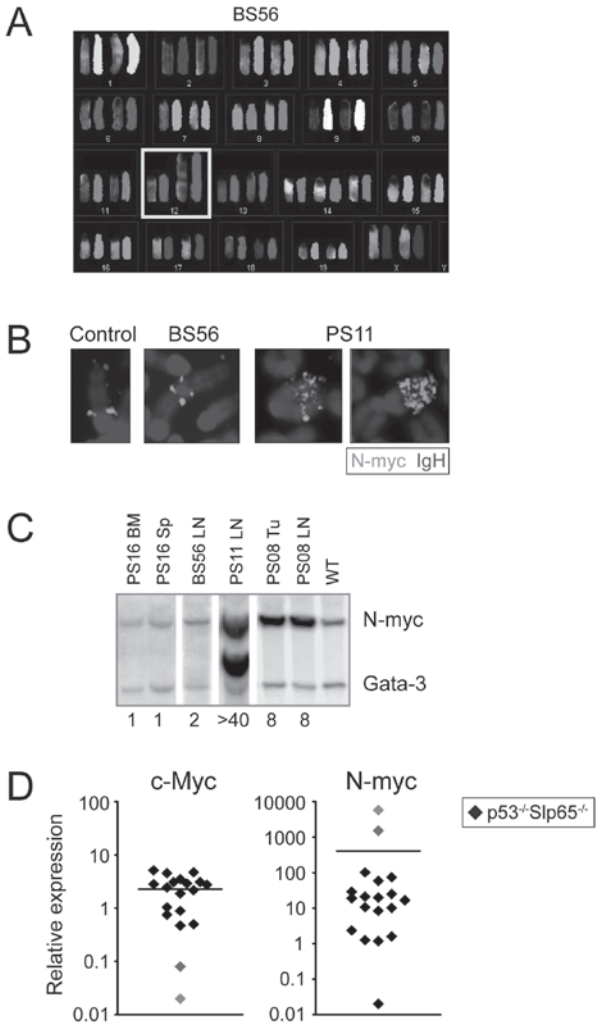
(1) Kersseboom R, Middendorp S, Dingjan GM, et al. Bruton's tyrosine kinase cooperates with the B cell linker protein SLP-65 as a tumor suppressor in pre-B cells. J Exp Med. 2003;198:91-98.
(2) Jumaa H, Mitterer M, Reth M, Nielsen PJ. The absence of SLP65 and Btk blocks B cell development at the preB cell receptor-positive stage. Eur J Immunol. 2001;31:2164-2169.
(3) Middendorp S, Dingjan GM, Hendriks RW. Impaired precursor B cell differentiation in Bruton's tyrosine kinase–deficient mice. J Immunol. 2002;168:2695-2703.



Supplementary Figure S2. Ca2⁺ fluxes are blunted in *Btk*$^{-/-}$*Slp65*$^{-/-}$ pre-B cells and VH81X Tg *Btk*$^{-/-}$ *Slp65*$^{-/-}$ pre-B cells when compared with mature B cells.
$Ca2^+$ fluxes were measured in splenic B cells of WT mice (upper panel), pre-B cells from *Btk*$^{-/-}$*Slp65*$^{-/-}$ (middle panel) and VH81X Tg *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice (lower panel) that were cultured for 4 days with 100 U/mL IL-7. Cells were loaded with Indo-1 AM and stimulated with anti-IgM $F(ab)_2$ fragments (20 µg/ml) and ionomycin as a control. Arrows indicate the addition of anti-IgM $F(ab)_2$ fragments. Histogram plots of gated $B220^+$ cells are representative for 3 to 4 mice of each genotype.

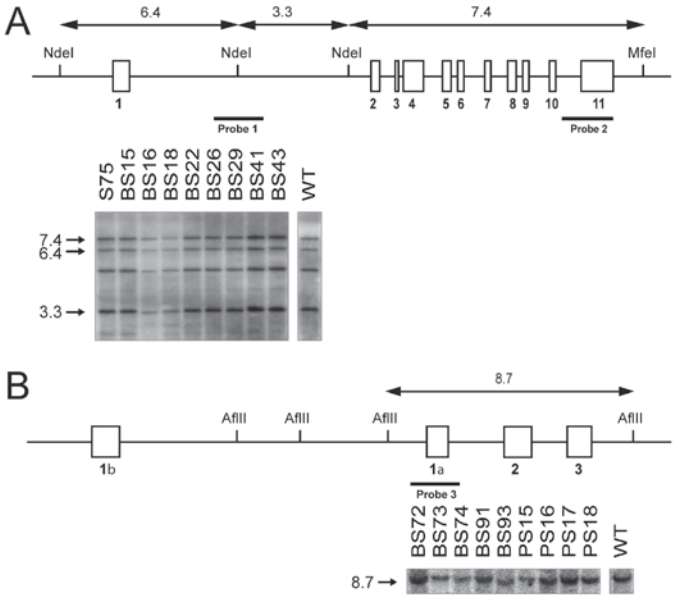


Supplementary Figure S3. AID is not involved in malignant transformation of *Slp65*$^{-/-}$ pre-B cells
(A) Quantitative RT-PCR analysis of AID expression in *Slp65*$^{-/-}$ (n=16) and *Btk*$^{-/-}$*Slp65*$^{-/-}$ (n=27) pre-B cell tumors, normalized with GAPDH. Values in total Peyer's patches were set to one. Horizontal lines represent mean values of the relative expression levels. (B) Kaplan-Meier tumor-free survival estimates for *Aicda*$^{+/-}$ *Slp65*$^{-/-}$ (n=43), *Aicda*$^{-/-}$*Slp65*$^{-/-}$ (n=27), *Aicda*$^{+/-}$*Btk*$^{-/-}$*Slp65*$^{-/-}$ (n=30) and *Aicda*$^{-/-}$*Btk*$^{-/-}$*Slp65*$^{-/-}$ (n=22) mice.



Supplementary Figure S4. Chromosome 12 enlargement and N-myc amplification in *Slp65*$^{-/-}$ pre-B cell tumors

(A) SKY image of a metaphase spread from a *Btk*$^{-/-}$*Slp65*$^{-/-}$ tumor (BS56) with an enlarged copy of chromosome 12. SKY images are on the left and computer-classified colors are on the right. (B) FISH analysis of a control chromosome and an enlarged chromosome from tumor BS56 and tumor PS11 (*p53*$^{-/-}$*Slp65*$^{-/-}$). Panels show N-myc-specific and IgH-specific probes. (C) Analysis of N-myc amplification by Southern blotting of genomic DNA from tumor samples and a BM control digested with EcoRI and hybridized with a N-myc probe and a Gata3 probe as a loading control. Fold amplification relative to the control lane is indicated below each lane. (D) Quantitative RT-PCR analyses of c-Myc and N-Myc expression in *p53*$^{-/-}$*Slp65*$^{-/-}$ (n=19) pre-B cell tumors, normalized to GAPDH. Values in sorted WT BM B220^{+} cells were set to one. Horizontal lines represent mean values of the relative expression levels.



D

Tumors	Southern Blot Ink4a/Arf Tissue	Tumor Load	Genomic status	PCR Ink4a/Arf Tissue	Tumor Load	PCR product
BS72	Spl	68%	intact	LN	94%	+
BS93	Spl	65%	intact	Spl	65%	+
PS17	Tu	88%	intact	Tu	88%	+
PS22	Spl	94%	intact	Tu	94%	+
PS27	Spl	84%	intact	Spl	84%	+

Supplementary Figure S5. *Slp65*$^{-/-}$ pre-B cell tumors do not harbor deletions of the p53 or Ink4a-Arf loci.

(A) Schematic map of the p53 locus including all relevant restriction sites, fragment lengths (in kilobases), and Southern blot probes 1 and 2 (map not drawn to scale).Southern blot analysis of genomic DNA from Slp65$^{-/-}$ tumors (n=4) and *Btk*$^{-/-}$*Slp65*$^{-/-}$ tumors (n=19) to detect deletions at the p53 locus. Genomic DNA was digested with *Nde*I and *Mfe*I and probed with a mixture of probes 1 and 2. (B) Schematic map of the Ink4a-Arf loci including all relevant restriction sites, fragment length (in kb), and Southern blot probe 3 (map was not drawn to scale). Southern blot analysis of genomic DNA from *Slp65*$^{-/-}$ tumors (n=11), *Btk*$^{-/-}$*Slp65*$^{-/-}$ tumors (n=16) and *p53*$^{-/-}$*Slp65*$^{-/-}$ tumors (n=11) to detect deletions of exon 1a, 2 and 3. Genomic DNA was digested with *Afl*II and probed with probe 3. (C) PCR amplification of exon 1a and exon 1b of the Ink4a/Arf locus in *Slp65*$^{-/-}$ tumors (n=15) and *Btk*$^{-/-}$*Slp65*$^{-/-}$ tumors (n=23). Amplification of the Slp65 knock out (KO) allele was performed as a control. (D) Overview of status of the Ink4a-Arf locus and purity of *Btk*$^{-/-}$*Slp65*$^{-/-}$ (BS) and *p53*$^{-/-}$*Slp65*$^{-/-}$ (PS) tumor samples used in Southern blot and genomic PCR assays.

Chapter IV

Highly restricted usage of V_H14 family gene segments in Slp65-deficient pre-B cell leukemia

Ta VBT, Zoller M, Uebelhart R, de Bruijn MJW, Jumaa H, Wardemann H, Langerak AW, Hendriks RW (in preparation)

Abstract

The adapter protein Slp65 is a key component of precursor-B cell receptor (pre-BCR) signaling. Slp65-deficient mice spontaneously develop pre-B cell leukemia, but the mechanisms by which Slp65-deficient pre-B cells become malignant remain to be identified. Because high-level pre-BCR expression on Slp65-deficient leukemia cells is thought to contribute to their strong proliferative capacity, we investigated whether particular pre-BCR specificities may predispose these cells to malignant transformation. DNA sequence analysis of immunoglobulin heavy (IgH) chain V regions of Slp65-deficient leukemias revealed that 55 out of 60 (~92%) used a V_H14 gene segment, despite the small size of this V_H family. In particular, V_H14.1 en V_H14.2 segments were used in 24 (~40%) and 19 (~32%) of cases, respectively. By contrast, the CDR3 regions of these IgH chains were heterogeneous in length and sequence.

When combined with surrogate light chain or various conventional light chains, yielding pre-BCR and BCR, respectively, the V_H14 IgH chains did not exhibit enhanced poly- or autoreactivity. It is therefore not likely that the V_H14 IgH chains provide enhanced proliferative signals. However, Slp65-deficient leukemia cells also exhibited preferential V_H14 segment usage on non-productively rearranged IgH alleles, strongly indicating altered IgH chain V region accessibility. We therefore conclude that malignant transformation of Slp65-deficient pre-B cells occurs almost exclusively in those cells that have aberrant IgH chain accessibility favoring V_H14 segment recombination at the pro-B cell stage.

Introduction

Diversity of the antibody repertoire is generated by recombination of various gene segments at the immunoglobulin heavy (IgH) and light (IgL) chain loci to assemble a B cell antigen receptor (BCR) (1-4). In pro-B cells productive V(D)J recombination results in cell surface deposition of the pre-BCR, comprised of IgH μ chain and the surrogate light chain (SLC) components λ5 and VpreB (3, 5-6). The pre-BCR serves as an important checkpoint to monitor proper expression of a functional IgH chain and triggers clonal expansion, whereby pre-B cells acquire the capacity to respond to low concentrations of the proliferation factor interleukin (IL)-7 (3, 5-6). After a limited number of cell divisions, large pre-B cells stop cycling and differentiate into small, resting pre-B cells in which IgL chain rearrangement is initiated.

It has been hypothesized that the pre-BCR signals in a cell-autonomous fashion (7). Hereby the non-Ig tail of the λ5 SLC component induces ligand-independent pre-BCR cross-linking, signaling for pre-B cell expansion. Recently, Jumaa and colleagues have shown that functional pre-BCR formation and autonomous signaling requires the N-linked glycosylation site in the CH1 domain of μ IgH chain (N46), whereby binding of the SLC component λ5 to N46 mediates autonomous crosslinking (8). However, it remains controversial whether the proliferative expansion of pre-B cells can also be initiated or enhanced by binding of particular self-antigens. This seems possible since the pre-BCR shapes the V_H repertoire at the transition from pro-B to large pre-B cells (9). Furthermore, findings of interaction between the pre-BCR and galectin-1 (10) and binding of the pre-BCR, via the non-Ig tail of λ5, to stromal-cell-associated heparin sulphate (11) would support this notion. Recently, it has been shown that the pre-BCR is polyreactive and capable of recognizing multiple (self-)antigens, including DNA, LPS and insulin, via the non-Ig part of λ5 (Kohler 2008). Thus, pre-BCR autoreactivity may serve to clonally expand those cells that produce a functional μ H chain and ensures that this selection can occur in the absence of foreign antigens. In support of this idea, in SLC-deficient mice mainly autoreactive pre-B cells are selected, resulting in the accumulation of autoreactive antibodies (12). But also in the presence of SLC, more than half of the antibodies expressed in early B cell compartments of healthy individuals are polyreactive (13).

The adaptor protein Slp65 (also known as Blnk or Bash) and the tyrosine kinase Btk are key components in the signaling pathway downstream of the pre-BCR and the BCR. In human, mutations in *SLP65* or *BTK* result in defective pre-B cell proliferation and an almost complete arrest of B cell development at the pro-B to pre-B cell transition, associated with the immunodeficiency disorder agammaglobulinemia (5, 14). In contrast, mice deficient for Slp65 or Btk show only a partial arrest at the large cycling pre-B cell stage, while a nearly complete block is present in Btk/Slp65 double deficient mice (15-17).

Importantly, at the age of ~6 months 5-10% of Slp65-deficient mice develop pre-B cell leukemia, expressing high levels of pre-BCR on the cell surface (15, 18). The lack of Slp65 results in defective pre-BCR internalization (15, 19). Although high-level pre-BCR expression on Slp65-deficient leukemia cells is thought to contribute to their strong proliferative capacity, we showed previously that transgenic overexpression of SLC components is not sufficient to induce leukemia (19). Btk-deficient mice do not develop pre-B cell tumors, but Btk does cooperate with Slp65 as a tumor suppressor, whereby it exerts tumor suppressor function independently of its kinase activity (17, 20). Recent findings show that Slp65 also downregulates IL-7-mediated proliferation and survival through direct inhibition of Jak3, which is an essential IL-7R signaling component (21). The IL-7R pathway promotes cellular survival, proliferation and maturation, involving induction of the anti-apoptotic protein Bcl-2 and the proto-oncogene c-Myc (22-23). Furthermore, loss of p53 enhanced lymphoma formation in Slp65-deficient mice and Slp65-deficient pre-B cell tumors harbored aberrations in the $p19^{Arf}$–Mdm2–p53 tumor suppressor pathway (24).

Because pre-BCR signals in Slp65-deficient leukemia cells may contribute to their strong proliferative capacity, we investigated whether particular pre-BCR specificities may predispose these cells to malignant transformation. We performed DNA sequence analysis of immunoglobulin heavy (IgH) chain V regions of 60 Slp65-deficient leukemias and found highly restricted usage of the V_H14 gene family.

Results and Discussion

Restricted V_H repertoire of expressed IgH μ chains in Slp65-deficient pre-B cell leukemias

To investigate whether particular pre-BCR specificities may predispose Slp65-deficient pre-B cells to malignant transformation, we sequenced the expressed IgH chain V regions in a panel of pre-B cell leukemias from mice that were deficient for Slp65 (n=17) or double deficient for Slp65 and Btk (n=26) or p53 (n=17). Remarkably, we found that 55 out of 60 (~92%) used a V_H14 (SM7) gene segment (Table 1; Supplementary Table S1), despite the small size of this V_H family with only four members. In particular, the members $V_H14.1$ en $V_H14.2$ were used in 24 (~40%) and 19 (~32%) of the cases, respectively. This restricted V_H14 usage is in stark contrast with the diverse repertoire found in large pre-B cells of normal mice: V_H genes belonging to the V_H1 (V_HJ558), V_H2 (V_HQ52), and V_H5 (V_H7183) family are represented in more than 80% of pre-B cells in normal mice (9). However, in Slp65-deficient tumors <8% used a member of either the V_H1, V_H2 or V_H5 family.

Table 1. V_H representation in 60 Slp65-deficient pre-B cell leukemias

V_H family	V_H representation
V_H14.1	24 (40.0%)
V_H14.2	19 (31.7%)
V_H14.3	3 (5.0%)
V_H14.4	9 (15.0%)
V_H1	3 (5.0%)
V_H2	1 (1.7%)
V_H4	1 (1.7%)

Large IgH CDR3 region diversity in Slp65-deficient pre-B cell leukemias

Next, we analyzed the CDR3 region diversity of expressed IgH μ chains. These CDR3 regions were heterogeneous in sequence and in length (Table 2).

The average CDR3 length of the 60 IgH μ chains of Slp65-deficient leukemias was 14.8 ± 2.1 amino acids, which is a substantial increase when compared with the reported average CDR3 length of ~12 amino acids in normal pre-B cells (25). Moreover, the 60 IgH μ chains from Slp65-deficient tumors did not show a normal distribution, with mainly CDR3 lengths of 14-17 amino acids (Figure 1A). The average number of N nucleotide additions in the IgH μ chains from Slp65-deficient tumors was 10.2 ± 4.0 (Figure 1B), which is a substantial increase in comparison with the reported number of ~7 nucleotides in wild-type pre-B cells (26).

Hayashi *et al.* (18) have reported that counter-selection of pro-B cells expressing Dμ protein (Dμ selection) was abolished in Slp65 mutant mice, resulting in similar usage of the three D gene segment reading frames (RF). However, our Slp65-deficient leukemias analyzed still displayed RF2 counter-selection (Figure 1C). The IgH μ chains of Slp65-deficient leukemias showed preferential usage of J_H4 (Figure 1D), in contrast to equal J_H usage normally found (25-26). The four J_H segments differ in length, with J_H4 being the longest one and contributing up to 20 nt to the CDR3 region. Therefore, preferential J_H4 usage and increased N nucleotide addition both contributed to the increased CDR3 length of IgH μ chains of Slp65-deficient leukemias.

Table 2. CDR3 analysis in Slp65$^{-/-}$ pre-B cell leukemias

VH gene	Tumor code	CDR3 sequence	CDR3 length	pI[a]
VH14-1	BS29	CASGGSSYVYYAMDYW[b]	14	13
VH14-1	BS30	CAGGGVYYTFYAMDYW	14	13
VH14-1	BS36	CASGGSMITTWAMDYW	14	13
VH14-1	BS38	CASGGTMVTLYAMDYW	14	13
VH14-1	BS39	CAIGGSTMAFYAMDYW	14	13
VH14-1	BS41	CASGGVVTTRYAMDYW	14	6,44
VH14-1	BS42	CASGGMVTTFYAMDYW	14	13
VH14-1	BS44	CTTTVVGYW	7	13
VH14-1	BS45	CASGGYYRYRYAMDYW	14	8,83
VH14-1	BS47	CASGGTGTVPHAMDYW	14	5,45
VH14-1	BS50	CASGGVVTKVYAMDYW	14	6,43
VH14-1	BS52	CASGPPTMVTTYPFYAMDYW	18	13
VH14-1	BS56	CASGGTMIYYYAMDYW	14	13
VH14-1	BS57	CGIGGMVTTFYAMDYW	14	13
VH14-1	BS73	CASGGPTIGTTRVWYFDVW	17	6,44
VH14-1	BS93	CASGAPTEYYYAMDYW	14	13
VH14-1	PS14	CASGPPITTVVLYAMDYW	16	13
VH14-1	PS15	CASGPPMVTLYYYAMDYW	16	13
VH14-1	PS17	CASGGAMVTTSPWYFDVW	16	13
VH14-1	PS19	CASGGTTRRVYAMDYW	14	9,07
VH14-1	S13	CASGGSMMVTTRWYFDVW	16	6,44
VH14-1	S14	CALGMPSMVFYAMDYW	14	13
VH14-1	S37	CASGAPTMVTTDAMDYW	15	13
VH14-1	S75	CASGGPTMVVYAMDYW	14	13
VH14-2	BS27	CASGGLLWLPLYAMDYW	15	13
VH14-2	BS43	CALGGPLPTVVTYYFDYW	16	13
VH14-2	BS53	CARRAYDYDDAHYYAMDYW	17	4,86
VH14-2	BS54	CGVGGYAPYYYAMDYW	14	13
VH14-2	BS70	CGRSHYYDGSPYAMDYW	15	5,61
VH14-2	BS74	CASGGTVVAYWYFDVW	14	13
VH14-2	PS20	CASGGYYYYGVVTSMDYW	16	13
VH14-2	PS24	CARRSTMITTPYYYAMDYW	17	8,89
VH14-2	PS27	CARRVTTVVAPYYYAMDYW	17	8,89
VH14-2	PS28	CARRSPTTVVDYVPYYYAMDYW	20	6,57
VH14-2	PS29	CGRSSNYRGDYYAMDYW	15	6,58
VH14-2	PS32	CGRNYDGTPYYYAMDYW	15	4,4
VH14-2	S97	CGRGGYYVYAMDYW	12	6,42
VH14-2	S98	CGRSNYYDGSPYAMDYW	15	4,4
VH14-2	S99	CGSGSMMVTTFYAMDYW	15	13
VH14-2	S109	CASGGYYYAPYAMDYW	14	13
VH14-2	S203	CASGGPYYSNPYAMDYW	15	13
VH14-2	S101	CAGGGRDYYAFYAMDYW	15	4,4
VH14-2	S102	CGRGTYYYDGPYAMDYW	15	4,4
VH14-3	BS40	CASGGPYGSSYVWYFDVW	16	13

Table 2. CDR3 analysis in Slp65$^{-/-}$ pre-B cell leukemias (continued)

VH gene	Tumor code	CDR3 sequence	CDR3 length	pI[a]
VH14-3	BS91	CASGGYYGSSYVYAMDYW	16	13
VH14-3	S25	CGEGGPYYGSSPYAMDYW	16	13
VH14-4	BS66	CTTGPPFITTVVVVPPWFAYW	19	13
VH14-4	PS18	CTTGPPITTVVAWYFDVW	16	13
VH14-4	PS21	CTTARNWDVAWFAYW	13	6,44
VH14-4	PS22	CTRGPPRYIFYAMDYW	14	8,96
VH14-4	PS30	CTTGGPIVTTPYYYAMDYW	17	13
VH14-4	S19	CKTGMITTPGFAYW	12	9,07
VH14-4	S96	CTTGPPTVVAPHWYFDVW	16	5,45
VH14-4	S114	CTTGPPITTVVATWYFDVW	17	13
VH14-4	S100	CTTSGWEEWFAYW	11	13
VH1-52	BS72	CARRREGYFFDYW	11	9,07
VH1-64	PS08	CARLRSTMITTGDYW	13	9,24
VH1-75	S92	CARSELPIVTLYYYAMDYW	17	4,4
VH2-2	PS11	CARFYYGSSYYAMDYW	14	6,42
VH4-1	PS16	CASYYRYGAYW	9	8,89

[a] pI indicates isoelectric point

[b] Positively and negatively charged amino acids are underlined. Positively charged underlined amino acids are arginine (R), histidine (H), Lysine (K). Negatively charged underlined amino acids are aspartic acid (D) and glutamate (E).

Finally, the CDR3 regions shared characteristics of IgH chains of autoreactive antibodies, including long CDR3 regions that were enriched in aromatic and positively charged amino acids (Figure 1E). In most cases there was at least one arginine or aspartate acid present (Table 2). While arginines are abundant in CDR3 regions from autoreactive antibodies, in healthy mice only about a quarter of pre-B cells contain arginines in their CDR3 regions (12, 27).

Taken together, these findings indicate that CDR3s of Slp65-deficient tumors are very diverse and have characteristics of polyreactive or autoreactive antibodies, as these are often found to have long CDR3 regions and to contain many charged amino acids, a pattern that is thought to favor self-reactivity especially against DNA binding (13).

IgH m chains from Slp65-deficient pre-B cell leukemias are not highly poly- or autoreactive

We hypothesized that the unique features of the IgH μ chains of Slp65-deficient leukemic cells, *i.e.* restricted V_H14 usage, long CDR3 regions with many aromatic and charged amino acids, reflected a specific selective advantage of these cells at the large cycling pre-B cell stage. Pre-B cell proliferation is thought to be induced by binding of the SLC component λ5 to N-linked sugar groups of the CH1 domain of μ IgH chain (8) and may

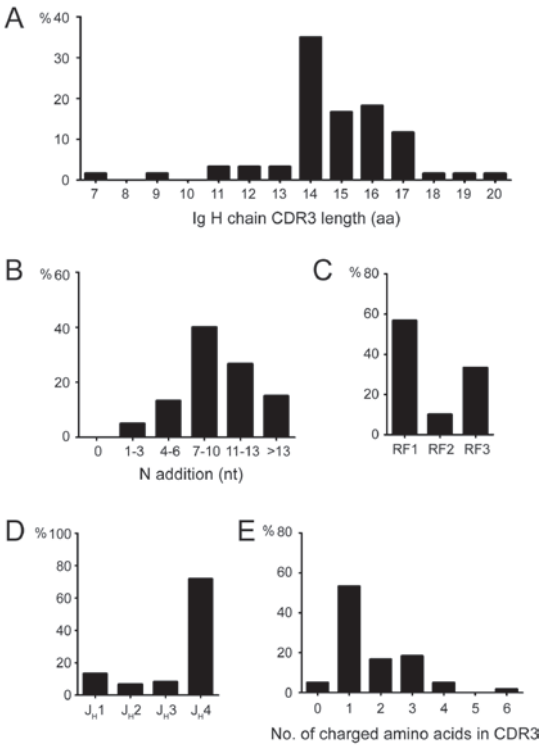


Figure 1. Characteristics of CDR3 regions of IgH m chains of Slp65-deficient leukemias.
DNA sequences from expressed IgH μ chains from Slp65-deficient leukemias (n=60) were analysed. (A) Distribution of V_HDJ_H CDR3 lengths. CDR3 length is in amino acids (aa). The average CDR3 length was 14.8 aa. (B) Distribution of N nucleotide additions. For determination of N nucleotide addition, P nucleotides were not included. The average value was N= 10.2 nucleotises (nt). (C) D_H reading frame (RF) usage (D) Observed relative frequency of J_H usage. (E) Proportions of IgH CDR3s with charged amino acids. Positive (arginine, lysine and histidine) and negatively charged amino acids (aspartic acid and glutamate) were included.

be enhanced by binding self-antigens (10-11). Although to date the V_H14 family has not been associated with autoreactivity as found in rheumatoid arthritis or SLE in human or mice (27-28), it is conceivable that the IgH μ chains of Slp65-deficient leukemic cells have increased pre-BCR polyreactivity or autoreactivity due to their unique CDR3 characterisitics.

Ten different V_H14 Ig H chains (BS40, BS70, BS73, BS93, PS14, PS19, PS27, S13, S25, S99) and five non-V_H14 Ig H chains (S92, BS72, PS08, PS11, PS16) were cloned and expressed together with SLC and various IgL chains, including the non-polyreactive Ig L chains of murine B1-8, the non-polyreactive human mGO53 and the polyreactive human Ig L chain BC62 (29). We used Human epithelial (Hep)-2 enzyme

linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA) techniques (30) to determine polyreactivity, autoreactivity and subcellular staining patterns of the panel of 15 IgH chains of Slp65-deficient leukemias. When combined with SLC to form the pre-BCR, most IgH μ chains showed Hep-2 reactivity (Figure 2A) and efficient binding to all antigens tested by ELISA, including ssDNA, dsDNA, LPS and insulin (Figure 2B; Supplemental Figure 1; Supplemental Table S2), as been previously described for other pre-BCRs (31). Only two (PS14, S13) did not show Hep-2 reactivity, but we could not confirm by Western blotting whether the two pre-BCR were completely assembled. When combined with the two non-polyreactive and the polyreactive Ig L chain, we found that the IgH μ chains of Slp65-deficient leukemic cells behaved quite differently, whereby 8-11 out of the 15 (~53-73%) IgH μ chains tested were not polyreactive or autoreactive. Thus, the IgH μ chains of Slp65-deficient leukemias tested were not intrinsically strongly polyreactive or autoreactive. From these findings, we conlcuded that, similar to regular IgH μ chains, the reactivity of the IgH μ chains of Slp65-deficient leukemic pre-B cells is influenced by the specificity of the paring Ig L chain. Although we cannot rule out that the IgH μ chains of Slp65-deficient leukemic cells have unique structural properties that are associated with strong proliferation induction when combined with surrogate light chain components, this capacity does not appear to be based on enhanced poly- or autoreactivity.

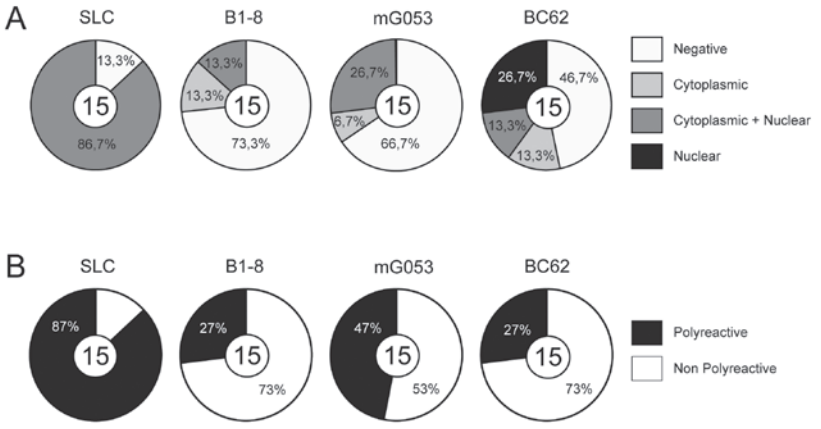


Figure 2. IgH m chains of Slp65-deficient leukemias do not show increased poly – or autoreactivity. Ig H chains of Slp65-deficient tumors were tested for selfreactivity by Hep-2 cell lysate IFA (A) or ELISA (B), in combination with SLC and the indicated IgL chains. Ten different V_H14 and five non-V_H14 Ig H chains were tested. Murine B1-8 and human mGO53 are nonpolyreactive L chains, while BC62 is a human polyreactive Ig L chain. (A) Pie charts summarize the frequency of Hep-2 cells with nuclear (*black*), nuclear plus cytoplasmic (*dark gray*), and cytoplasmic (*light gray*) IFA staining patterns, and the frequency of nonreactive antibodies (*white*). The number of tested antibodies is indicated in each pie chart centre. (B) Data are from ELISAs for reactivity with ssDNA, dsDNA, insulin, and LPS. Percentages represent frequency of polyreactive (*black*) or non-polyreactive (*white*) antibodies.

Restricted V_H segment usage reflect increased accessibility of the V_H14 family gene segments

An alternative explanation for restricted V_H14 family usage and concomitant large CDR3 diversity in the IgH μ chains of Slp65-deficient leukemias would be aberrant V_H gene segment accessibility during the V_H to D_H-J_H recombination process at the pro-B cell stage.

The V_H14 gene family is closely related to the V_HJ558 (V_H1) family, but maps 3' of V_HJ558 (32) (Figure 3). The V_H family usage in Slp65-deficient leukemias could not be explained solely on the basis of a restricted V_H region accessibility, because (i) three Slp65-deficient leukemias utilized distally located V_HJ558 members, and (ii) V_H gene segments belonging to various other families, e.g. V_H11, V_HS107, V_H36-60 or V_HGam3.8, which are interspersed between and adjacent to the V_H14 gene members, were not used in Slp65-deficient tumors (Figure 3).

To investigate whether restricted V_H14 usage could be due to increased accessibility of particular V_H14 gene segments in the IgH μ chain locus of Slp65-deficient leukemias, we determined the V(D)J gene configuration of the non-productively rearranged Ig H chain alleles. As these non-expressed alleles do not confer a selective advantage or disadvantage to pre-B cells, their V(D)J gene configuration should reveal the accessibility of individual V_H gene segments in pro-B cells.

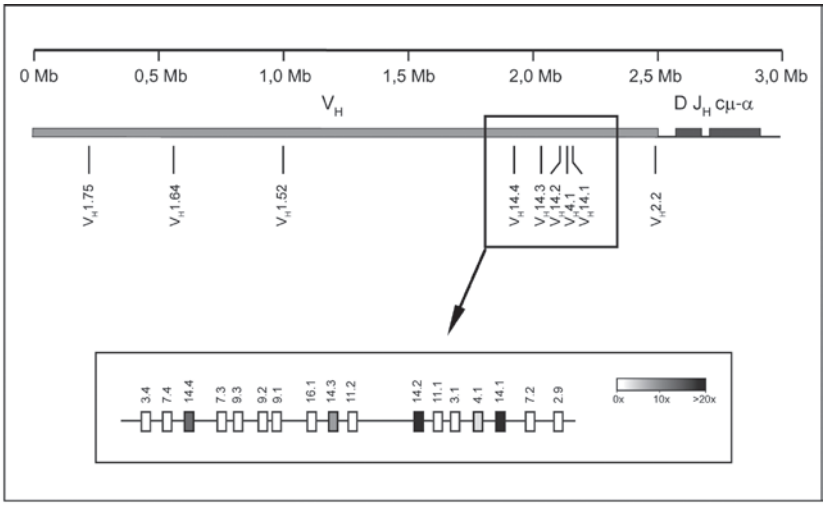


Figure 3. Schematic map of the mouse IgH chain locus and the localization of V gene segments used in Slp65-deficient pre-B cell tumors.

Chromosomal localization of V_H gene segments utilized in IgH μ chains of Slp65-deficient leukemias. The localization of the specific V_H genes that were used is indicated. Below a more detailed view is given of the V_H genes present between and around the V_H14 family members. The black/gray scale shows the usage of individual V_H14 family members, with in white interspersed V gene segments that are not used.

We analysed genomic DNA from our panel of Slp65-deficient pre-B cell leukemias by a set of four PCR reactions. In these experiments, we were able to identify the configuration of the non-productively rearranged allele in 50/60 tumor samples. In ten samples this configuration could not unambiguously be determined, e.g. because of too low tumor load, because some V_H segments may be poorly amplified by degenerate V_H primers or because of chromosome loss.

Non-productively rearranged alleles were first analyzed by performing genomic PCR reactions (i) with primers localized near DQ52 and J_H1 to identify alleles with a complete germline configuration, and (ii) with primers for a D_HJ_H4 PCR, to detect alleles with D_H-J_H rearrangements only, in the absence of V_H gene segment recombination. These analyses showed that 28/50 (~56%) of the non-productive alleles were either in the germline configuration or contained a D_H-J_H rearrangement (Figure 4A; Table 3).

Table 3. *V_H representation on non-productive alleles of Slp65-deficient pre-B cell leukemias*

	Non-productive allele				
Productive allele	Germline	D_HJ_H	non V_H14	V_H14	Total
$V_H14.1$	3	11	4	2	20
$V_H14.2$	5	4	6	2	17
$V_H14.3$	0	1	0	1	2
$V_H14.4$	2	1	2	3	8
non V_H14	0	1	1	1	3
Total	10	18	13	9	50

Next, using a V_H14-J_H4 PCR to detect recombination of V_H14 gene segments on both alleles but containing a J_H segment, we observed that 8 out of 50 Slp65-deficient tumors contained a rearrangement with V_H14 on both alleles (Figure 4B; Table 3). Remarkably, one pre-B cell leukemia (PS16) expressing a non- V_H14 heavy chain (VH4-1) had a V_H14 segment recombination on the non-productively rearranged allele. Finally, using a PCR with two high degenerate primers located in the framework 1 region (33) together with a primer located in the 5' cμ region (34), we identified 13 pre-B cell leukemias with a rearrangement with a non-V_H14 segment on the non-productive allele (Table 3). To confirm the clonality of the amplified PCR products, we used heteroduplex analysis (35). In these assays, homoduplexes and heteroduplexes resulting from denaturation and renaturation of IgH V region PCR products were separated in nondenaturing polyacrylamide gels based on their conformation (data not shown).

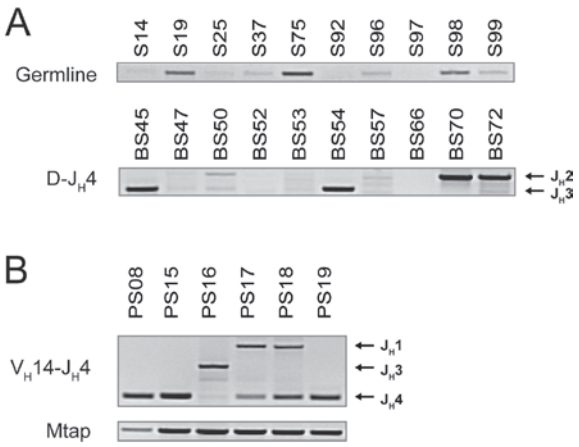


Figure 4. Analyses of non-productive alleles in Slp65-deficient pre-B cell leukemias.
(A) PCR analyses of D_HJ_H rearrangements with a forward primer located in the leader of the D_H segments (upper panel); PCR analyses of Germline configuration of the Ig H chain locus with a primers localized near D_HQ52 and J_H1 (second panel); (B) PCR analyses of V_H14 usage (third panel) with amplification of Mtap performed as a control (lower panel).

Taken together, the finding of V_H14 usage in 9 out of 24 (~38%) alleles with a non-productive V_H-D-J_H-rearrangement indicate altered Ig H chain accessibility at the pro-B cell stage favoring V_H14 segment recombination. At the moment, the reason for increased accessibility of V_H14 family members in Slp65-deficient pro-B cells that are prone to develop into leukemic cells at the large cycling pre-B cell stage is unknown. One explanation might be that only those Slp65-deficient pre-B cells are selectively transformed that carry an additional mutation that affects, next to pre-B cell survival or proliferation, also IgH chain V region accessibility. On obvious candidate would be mutation of the IL-7R signaling pathway: Slp65-deficient pre-B cell tumors carry mutations, e.g. autocrine IL-7 production, leading to constitutive activation of the Jak3/Stat5 pathway. This pathway does not only support proliferative expansion of pre-B cells, but is also thought to be involved in opening of the Ig H chain for recombination in pro-B cells (21, 36-37). In addition, recombination activating gene (Rag)-deficient pro-B cells that are cultured in high levels of IL-7 have active histone marks (H3Kme2 and H3K9ac) specifically at the V_H3609 and V_H14 gene segments (38), indicating high accessibility. Mutations in Slp65-deficient early B-lineage cells, e.g. resulting in constitutive Jak3/Stat5 signaling, may therefore lead to enhanced V_H14 gene segment accessibility at the pro-B cell stage and subsequently contribute to the malignant transformation at the large cycling pre-B cell stage.

Concluding remarks

In this report, we show that Slp65-deficient pre-B cell leukemias had restricted usage of V_H14 gene segments, while their CDR3 regions were heterogeneous in sequence and length. To date the V_H14 gene family has not been associated with autoimmunity in mice or humans and, accordingly, we showed that V_H14 IgH μ chains expressed by Slp65-deficient pre-B cell leukemias do not have enhanced polyreactive or autoreactive capacities. Importantly, also non-productively rearranged IgH alleles exhibited preferential V_H14 gene segment recombination, indicating increased accessibility of particular V_H14 family gene segments during the V to D_H-J_H recombination process in Slp65-deficient pro-B cells. We therefore conclude that malignant transformation of Slp65-deficient preferentially occurs in those pre-B cells that had aberrant IgH chain accessibility favoring V_H14 segment recombination at the pro-B cell stage. We hypothesize that in Slp65-deficient mice preferentially those pre-B cells are transformed that have acquired mutations leading to increased accessibility of V_H14 family gene segments at the pro-B cells stage, e.g. related to constitutive Jak/Stat5 signaling. Nevertheless, in our panel V_H14 usage on productive alleles (~92%) was higher than on non-productive alleles (~38%). Although this can e.g. be explained by the induction of increased V_H14 gene segment accessibility only after a first non-productive non-V_H14 rearrangement has occurred, at the moment we cannot exclude that V_H14 IgH μ chains have a selective advantage at the pre-BCR-checkpoint, contributing to the highly restricted V_H14 usage. The presence of V_H14 IgH μ chains would then result in increased proliferation, independent of autoreactivity. To investigate this issue, we currently aim to measure the signaling capacity of V_H14 and non-V_H14 pre-BCRs, using the reported reconstitution system of Rag2/λ5/Slp65 triple deficent B-lineage cells (TKO cells) to introduce combinations of various Ig H chains with SLC (31, 39).

Preferential usage of particular IgH V_H gene families is not unique to Slp65-deficient pre-B cell leukemias in mice, but has been reported for several leukemias and lymphomas in human and mice, including chronic lymphocytic leukemia (CLL) (40-41). However, on the basis of the presence of stereotypic CDR3 regions and high levels of somatic hypermutation (40), it has been hypothesized that preferential IgH V_H gene usage in CLL reflects antigenic selection rather than preferential V gene segment recombination. To the best of our knowledge, we here for the first time report that a restricted V_H repertoire would originate from altered V_H to D-J_H gene recombination, instead of BCR-mediated antigenic selection. It is conceivable that also in other types of leukemias or lymphomas restricted V_H to D-J_H gene recombination may partially contribute to stereotypic V gene usage.

Materials and methods

PCR analysis

The following primers were used in PCR amplifications: D_HJ_H rearrangements were amplified with the D_H L forward primer (42) and a reverse primer located in J_H4 (TGAGGAGACGGTGACTGAGG); Germline configuration was analyzed by using the D_HQ52 primer (43) and a J_H1 primer (TGAGGAGACGGTGACCGTGG); PCR for V_H14 rearrangements was performed with a V_H14 forward primer (CACAGCTTCTGGCTTCAACA) and the J_H4 reverse primer. Primers to amplify Mtap were located in exon 8 (F: CAGCGCTAAGGAGCCAATAC, R: CGCTCGACATTAACACTGGA).

Heteroduplex PCR and DNA sequencing

Heteroduplex analyses were performed as described (35). For DNA sequence analyses, DNA and cDNA samples were amplified using 2 high degeneracy primers located in the framework 1 region (33) in combination with a primer located in the 5' cμ region (34). PCR products were directly sequenced using the BigDye terminator cycle sequencing kit with AmpliTaq DNApolymerase on an ABI PRISM 377 automated sequencer (Applied Biosystems). All Ig H chain regions were sequenced in 2 directions from at least 2 independent PCR products and analyzed by IMGT/V-Quest (www.IMGT.org, IMGT, the international ImMunoGeneTics information system).

ELISA Studies and Indirect Immunofluorescence Assays

Antibody production and ELISA studies were performed as described (Wardemann et al., 2003). 293A human embryonic kidney fibroblasts were cultured in DMEM supplemented with 10% ultra-low IgG FCS (GIBCO) and cotransfected with 12.5 mg of IgH and IgL chain encoding plasmid DNA by calcium phosphate precipitation. Eight to twelve hours after transfection, cells were washed with serum-free DMEM and thereafter cultured in DMEM supplemented with 1% Nutridoma SP (Roche). Supernatants were collected after 8 days of culture and purified on protein G Sepharose (Amersham Pharmacia Biosciences). For reactivity with specific antigens, microtiterplates (COSTAR Easywash Polystyrene Plates, Corning) were coated with 10 mg/ml of ssDNA, dsDNA, or LPS (Sigma) or 5 mg/ml recombinant human insulin (Fitzgerald). Tissueculture supernatants were used at 1 mg/ml antibody concentrations and three 1:4 dilutions in PBS. All ELISAs were developed with HRP-labeled goat anti-human IgG Fc Ab (Jackson ImmunoReseach) and HRP Substrate
(BIO-RAD). OD405 was measured using a microplate reader (Molecular Devices).
For IFAs Hep-2 cell coated slides (Bion Enterprises, LTD.) were incubated at RT with purified antibodies at 50-100μg/ml for 30 min, washed in PBS and visualized with FITC

anti-human Ig by fluorescence microscopy. Control staining ED38 was included in all experiments.

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Supplemental Table 1. Characterization of Ig H chains of Slp65-deficient tumors

Tumor code	IMGT[a] V_H segment	VH Family	N, P nucleotides	DH Segment	N, P nucleotides	JH Segment	DH RF[b]
BS29	VH14-1	SM7	tggg	DFL16.1	gtc	JH4	3
BS30	VH14-1	SM7	gggggggggt	DST4.2	ccttt	JH4	3
BS36	VH14-1	SM7	cgggggt	DSP2.2	ttgg	JH4	1
BS38	VH14-1	SM7	cggagg	DSP2.1	cct	JH4	1
BS39	VH14-1	SM7	tagggg **ga**[c]	DSP2.4	cgtt	JH4	1
BS41	VH14-1	SM7	tggggg	DFL16.1	acg	JH4	1
BS42	VH14-1	SM7	tgggg	DSP2.8	ctt	JH4	1
BS44	VH14-1	SM7	-	DFL16.1	ggg	JH2	1
BS45	VH14-1	SM7	tggtggg	DSP2.10	cgg	JH4	2
BS47	VH14-1	SM7	cggg	D3-3	ttcctc	JH4	1
BS50	VH14-1	SM7	tgggggag	DSP2.1	aggt	JH4	1
BS52	VH14-1	SM7	cgggccc	DSP2.7	gtacccctt	JH4	1
BS56	VH14-1	SM7	tggagg	DSP2.2	t	JH4	1
BS57	VH14-1	SM7	gcatagggggg	DSP2.1	ctt	JH4	1
BS73	VH14-1	SM7	tgggggac	DSP2.10	agggt	JH1	3
BS93	VH14-1	SM7	cggggcgc	DSP2.10	aat	JH4	3
PS14	VH14-1	SM7	tgggccccc	DFL16.1	cct	JH4	1
PS15	VH14-1	SM7	tggccccc	DSP2.4	ctat	JH4	1
PS17	VH14-1	SM7	tggagggg	DSP2.1	ctcccc	JH1	1
PS19	VH14-1	SM7	tgg	DSP2.10	aggagagt	JH4	3
S13	VH14-1	SM7	cgggg **ga**	D-SP2.9	gagg	JH1	1
S14	VH14-1	SM7	cctaggtat g	DSP2.8	ctt	JH4	1
S37	VH14-1	SM7	tggcgct	DSP2.7	gg	JH4	1
S75	VH14-1	SM7	tgggg **gg**	DSP2.7	gt	JH4	1
S109	VH14-2	SM7	cggggg	DFL16.1	cccc	JH4	3
BS27	VH14-2	SM7	cggagg	DSP2.4	ccct	JH4	2
BS43	VH14-2	SM7	ttaggaggtcccc	DFL16.1	acct	JH2	1
BS53	VH14-2	SM7	gagggc	DSP2.2	gacgccc	JH4	3
BS54	VH14-2	SM7	gagtggg	DST4	gcccc **at**	JH4	2
BS70	VH14-2	SM7	tccc	DFL16.1	cc	JH4	3
BS74	VH14-2	SM7	tggggg	DFL16.1	-	JH1	1
PS20	VH14-2	SM7	tgggggtta	DFL16.1	agtagtcacgt	JH4	3
PS24	VH14-2	SM7	**aga**	DSP2.2	gccc **t**	JH4	1
PS27	VH14-2	SM7	cgag	DFL16.1	ccttt	JH4	1
PS28	VH14-2	SM7	gcgctctcc	DFL16.1	actacgtacctt	JH4	1
PS29	VH14-2	SM7	tc	DST4.2	cgggggg	JH4	3
PS32	VH14-2	SM7	a	DFL16.1	gacgccct	JH4	3
S203	VH14-2	SM7	cggggggc	DST4.2	cc	JH4	3
S101	VH14-2	SM7	gggggcagag	DFL16.2	t	JH4	3
S102	VH14-2	SM7	gggac	DFL16.1	ccct	JH4	3
S97	VH14-2	SM7	gg	D-SP2.9	gt	JH4	3
S98	VH14-2	SM7	tcga	DFL16.1	cc	JH4	3
S99	VH14-2	SM7	ggagtg **ga**	D-SP2.9	-	JH4	1
BS40	VH14-3	SM7	tgggggtcc	DFL16.1	gt	JH1	3

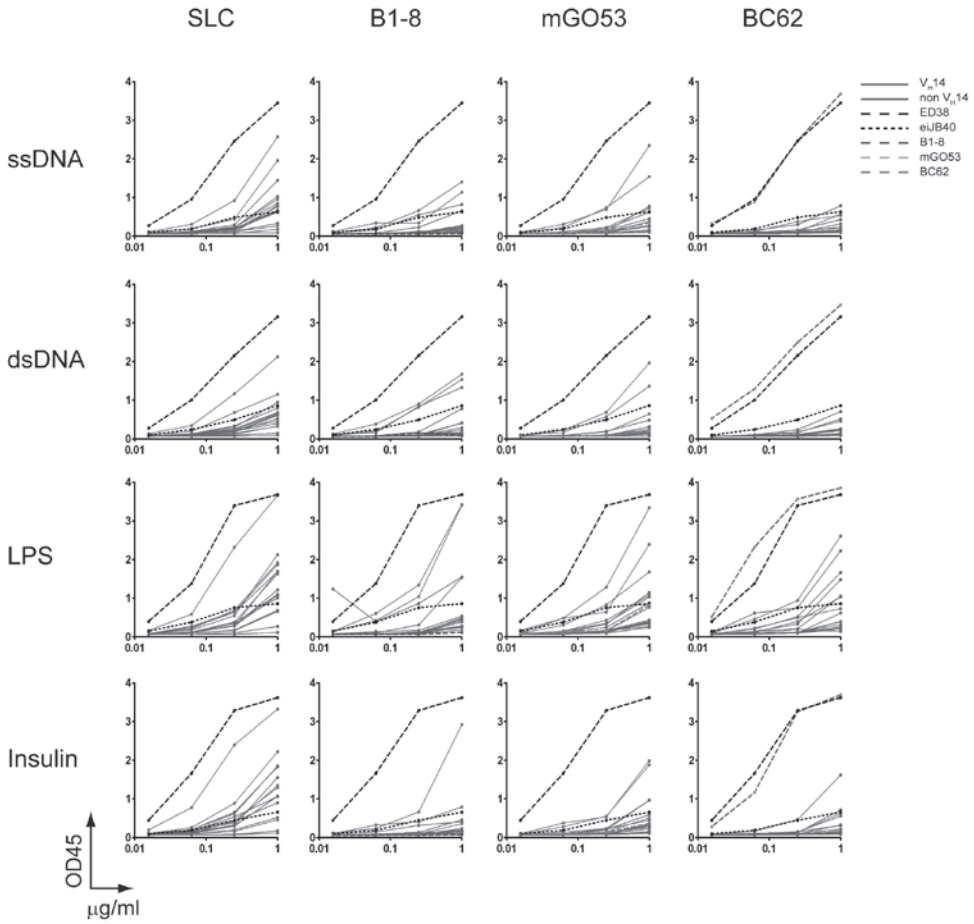
Supplemental Table 1. Characterization of Ig H chains of Slp65-deficient tumors (continued)

Tumor code	IMGT[a] V_H segment	VH Family	N, P nucleotides	DH Segment	N, P nucleotides	JH Segment	DH RF[b]
BS91	VH14-3	SM7	cgggggg	DFL16.1	gt	JH4	3
S25	VH14-3	SM7	gagaggggggacc	DFL16.1	cc	JH4	3
S114	VH14-4	SM7	ggccccccc	DFL16.1	t	JH1	1
BS66	VH14-4	SM7	gggccaccc	DFL16.1	tagtccccccg	JH3	1
PS18	VH14-4	SM7	gggcccccа	DFL16.1	-	JH1	1
PS21	VH14-4	SM7	cgcac **g**	DQ52	gtt **g**	JH3	2
PS22	VH14-4	SM7	gg	D3-3	cgctatat	JH4	1
PS30	VH14-4	SM7	ggggggc	DST4.2	ccc **at**	JH4	1
S100	VH14-4	SM7	-	DST4	gggaggag	JH3	2
S19	VH14-4	SM7	aaacggg	DSP2.2	cccgg	JH3	1
S96	VH14-4	SM7	gggcctc	DFL16.1	ccctc	JH1	1
BS72	VH1-52	J558	-	DST4	aaggtt	JH2	1
PS08	VH1-64	J558	**t** tac **ga**	DSP2.2	ggg	JH2	1
S92 tu	VH1-75	J558	tccgaactcc	DSP2.12	t	JH4	1
PS11	VH2-2	Q52	tt	DFL16.1	-	JH4	3
PS16	VH4-1	X-24	t	DSP2.10	g	JH3	2

[a] IMGT indicates the international ImMunoGeneTics information system (www.imgt.org).
[b] RF indicates the reading frame of the D_H segments
[c] Bold nucleotides refer to P (palindromic) nucleotides

Supplemental Table S2. Polyreactivity as measured by ELISA

Tumor code	Reactivity															
	SLC				B1-8				mG0				BC62			
	ds	ss	LPS	Insulin	ds	ss	LPS	Insulin	ds	ss	LPS	Insulin	ds	ss	LPS	Insulin
non VH14																
S92	+++	+++	+	+++	+++	+	+++	+++	+++	+++	+++	+++				
PS16		+	+	+					+	+	+	+		+	+	+
PS08	+++	+++	+++	+++	+++	+	+++	+	+++	+++	+++	+++	+	+	+++	+
BS72	+	+	+++	+											+	
PS11			+	+						+	+++	+			+	
VH14																
S25	+	+	+	+	+++	+	+	+								
BS93	+	+	+	+											+	
S13													+	+	+++	+
S99			+	+											+	
PS27		+	+	+					+	+	+	+	+	+	+	+
BS40	+	+	+	+							+	+				
PS14																
BS73	+	+	+	+							+	+				
PS19	+	+++	+	+	+	+	+	+			+					
BS70		+	+	+												



Supplemental Figure 1. Ig H chains expressed in Slp65-deficient tumors are not highly polyreactive.

ELISA showing binding to different antigens of ten different VH14 Ig H chains (green) and five non VH14 Ig H chains (red) were cloned and expressed together with SLC and various IgL chains, including the non-polyreactive Ig L chains of murine B1-8, the non-polyreactive human mGO53 and the polyreactive human Ig L chain BC62. The monoclonal polyreactive ED38 (Wardemann, 2003 Science and Meffre, 2004 J Exp Med) and intermediately polyreactive eiJB40 served as control (Wardemann, 2003 Science).

Chapter V

Pre-B cell leukemias in Btk/Slp65-deficient mice arise independently of ongoing V(D)J recombination activity

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Leukemia 2010, in press

Abstract

The adapter protein Slp65 and Bruton's tyrosine kinase (Btk) are key components of the precursor-B (pre-B) cell receptor signaling pathway. Slp65-deficient mice spontaneously develop pre-B cell leukemia, expressing high levels of the pre-B cell receptor (pre-BCR) on their cell surface. Because leukemic Slp65-deficient pre-B cells express the recombination activating genes (Rag)1 and Rag2 and manifest ongoing immunoglobulin light chain rearrangement, it has been hypothesized that deregulated recombinase activity contributes to malignant transformation. In this report, we investigated whether Rag-induced DNA damage is involved in oncogenic transformation of Slp65-deficient B cells. We employed Btk/Slp65 double-deficient mice carrying an autoreactive 3-83μδ BCR transgene. When developing B cells in their bone marrow express this BCR, the V(D)J recombination machinery will be activated, allowing for secondary immunoglobulin light chain gene rearrangements to occur. This phenomenon, called receptor editing, will rescue autoreactive B cells from apoptosis. We observed that 3-83μδ transgenic Btk/Slp65 double-deficient mice developed B cell leukemias expressing both the 3-83μδ BCR and the pre-BCR components λ5/VpreB. Importantly, such leukemias were found at similar frequencies in mice concomitantly deficient for Rag1 or the non-homologous end-joining factor DNA-PKcs. We therefore conclude that malignant transformation of Btk/Slp65 double-deficient pre-B cells is independent of deregulated VD)J recombination activity.

Introduction

Diversity of immunoglobulin (Ig) and T cell receptor specificities is generated through DNA gene rearrangement initiated by lymphocyte-specific proteins encoded by the recombination activating genes Rag1 and Rag2 (see for review: Ref. (1-3). The Rag complex recognizes and cleaves specific DNA elements, called recombination recognition sequences (RSS), which flank rearranging V, D, and J gene segments. The arising double-strand DNA breaks are subsequently resolved by the non-homologous end joining (NHEJ) pathway. Due to this unique feature of lymphoid cells to somatically rearrange antigen receptor genes, these cells are frequent targets for chromosomal translocations and oncogene activation resulting from recombinase targeting mistakes or incorrect repair of the V(D)J recombination intermediates (4-5). It was demonstrated in the mouse that chromosomal reinsertion of broken RSS can target cryptic RSS-like elements via a V(D)J recombination-like mechanism (6). Moreover, cryptic RSS sequences immediately internal to the deletion breakpoints in the *IKZF1* locus, encoding the transcription factor IKAROS, have been identified in human BCR-ABL1$^+$ acute lymphoblastic leukemia (ALL) (7). Cryptic RSS are estimated to have a density of 1 per 600 bp in the genome and have been identified in other loci involved in ALL, including *PAX5* and *CDKN2A/B* in human and *c-Myc* and *Lmo2* in the mouse (8-9). In addition, Rag proteins have the ability to rearrange DNA sequences that do not resemble RSS, as reported in follicular lymphoma (10). Taken together, these findings suggest that gene deletion or translocation arising from aberrant Rag activity contributes to leukemogenesis in human and mouse ALL.

Productive Ig heavy (H) chain V(D)J recombination in pro-B cells results in deposition of IgH μ chain on the cell surface together with the non-rearranging surrogate light chain (SLC) components λ5 and VpreB (11-14). Signaling from this pre-BCR monitors expression of a functional IgH chain and triggers clonal expansion, leading to differentiation into large cycling pre-B cells as pre-B cells acquire the capacity to respond to low concentrations of the proliferation factor interleukin (IL)-7 (11-13, 15). In large cycling pre-B cells, Rag proteins are transiently downregulated to terminate further IgH chain rearrangement, thus ensuring that only one functional IgH chain is assembled, a phenomenon termed allelic exclusion. Moreover, as ongoing recombinase activity during mitosis will interfere with faithful transmission of the genome to daughter cells, Rag2 proteins are degraded during cell division, thus linking V(D)J recombination to the cell cycle (16). Developmental progression from large cycling into small resting pre-B cells is associated with reactivation of the V(D)J recombination machinery for Ig light (L) chain gene rearrangement and with cell surface marker changes (11-14).

The adapter protein Slp65 (also known as Blnk or Bash) and Bruton's tyrosine kinase (Btk) are key components in the signaling pathway downstream of the pre-

BCR and the BCR. Slp65, when phosphorylated by the Syk kinase, provides docking sites for various molecules, including Btk and phospholipase C (PLCγ). Btk then phosphorylates PLCγ, which leads to its full activation and the generation of second messengers (11-14). In human, mutations in *SLP65* or *BTK* result in defective pre-B cell proliferation and an almost complete arrest of early B cell development, associated with the immunodeficiency disorder agammaglobulinemia (11, 17). In mice, deficiency for Slp65 or Btk leads to a partial arrest of early B cell development (11). Importantly, 5-10% of Slp65-deficient mice develop pre-B cell leukemia expressing high levels of pre-BCR on the cell surface (18-19). Btk cooperates with Slp65 as a tumor suppressor, independently of its kinase activity (20-21). In a substantial fraction of human pre-B ALL, including cases expressing the oncogenic BCR-ABL1 tyrosine kinase fusion protein, SLP65 expression is defective due to aberrant splicing (22-23). But it has also been reported that SLP65 deficiency may be an infrequent event in human pre-B-lineage ALL (7, 24), and thus the importance of loss of SLP65 expression as one of the primary causes of pre-B ALL in human remains unclear.

Slp65 function is essential for proper progression of large cycling into small resting pre-B cells. Slp65 collaborates with Btk to down-regulate SLC and IL-7 receptor (IL-7R) expression (18, 20). It also directly inhibits the IL-7R signaling component Jak3 (25), thereby terminating the proliferative response to IL-7 and reducing expression of bcl-2 family anti-apoptotic proteins and c-Myc. Finally it has been shown that Slp65 induces cell cycle arrest through upregulation of Ikaros, which controls gene expression through its association with chromatin remodeling complexes (26). The mechanism by which Slp65 exerts its tumor suppression function in mice has not been completely elucidated to date, but striking parallels exist with pre-B cell tumors initiated by deregulated expression of the c-Myc oncogene. Malignant transformation of both Slp65-deficient and Eμ-*myc* transgenic pre-B cells (i) involves disruption of the $p19^{Arf}$–Mdm2–p53 tumor suppressor pathway (27-28), (ii) is reduced by expression of a pre-rearranged IgH chain transgene in early B cell differentiation (28-29), and is (iii) independent of activation induced deaminase (AID) (28, 30). Moreover, loss of Btk or PLC-γ2 synergizes with deregulation of c-Myc during lymphoma formation in Eμ-*myc* mice (31-32). Several lines of evidence have indicated a role for unregulated Rag activity in Slp65-deficient pre-B cell leukemia. First, Rag proteins are expressed in strongly proliferating Slp65-deficient pre-B cells (18-19). Second, Slp65-deficient pre-B cell leukemias mostly co-express IgH chain, SLC, IgL chain (20). Third, in human pre-B cell leukemia SLP65 deficiency correlates with RAG expression and ongoing V_H gene rearrangement activity (33).

Importantly, it has been recently demonstrated that Rag-mediated DNA breaks are not essential for malignant transformation in Eμ-*Myc* Tg mice (30). We have previously demonstrated the effects of transgenic IgH chain expression and p53-

deficiency on oncogenic transformation of Slp65-deficient pre-B cells (28). However, genetic experiments addressing the requirement of Rag-mediated V(D)J recombination events in leukemia formation have not been performed. Therefore, we investigated whether oncogenic transformation of Slp65 deficient pre-B cells could occur on a Rag1-deficient background. We found that leukemia in Btk/Slp65 double-deficient mice arises independently of V(D)J recombination activity or illegitimate repair by NHEJ.

Material and Methods

Mice and genotyping

Mice deficient for Btk (34), Rag1 (35) and DNA-PKcs (Jackson Laboratories) were on the C57BL/6 background. 3-83μδ mice (36) were on a non-deleting B10.D2 background. Slp65-deficient mice (37) were on the Balb/c background. The different composite genotypes were on a mixed background and in single experiments littermates were compared. For mouse genotyping genomic DNA was analyzed by polymerase chain reaction (PCR), as previously described (20, 28). Animals in tumor panels were sacrificed after indication of tumor formation, or after a maximum period of 40 weeks of age. Mice were bred and maintained in the Erasmus MC animal care facility under pathogen-free conditions. Experimental procedures were reviewed and approved by the Erasmus MC committee of animal experiments.

Southern Blotting

Genomic DNA (10 μg) was digested with *Hin*dIII (New England Biolabs, Beverly, MS) and processed by Southern blotting using nylon membrane (Nytran SPC, Whatman) and standard procedures. Fragments were visualized by phosphor-Imager (Storm, Molecular Dynamics) and analyzed with ImageQuant (Molecular Dynamics, Sunnyvale, CA). Probe specific for J_{κ}, a 1.6kb *Eco*RI fragment, was labeled by random priming.

Flow cytometry and in vivo 5-bromo-2'-deoxyuridine (BrdU) labeling

Preparations of single-cell suspensions, flow cytometry procedures, and mAbs have been described previously (20, 34, 38). The anti-3-83μδ hybridoma 54-1 (39) was kindly provided by D. Nemazee (The Scripps Research Institute, La Jolla, Ca). Bone marrow (BM) cell suspensions were depleted of erythrocytes by standard ammonium chloride lysis and IL-7 driven cultures were performed as described previously (38). For intracellular staining of cytoplasmic proteins, cells were first stained for cell surface markers and subsequently fixed in 2% paraformaldehyde and permeabilized using 0.5% saponin. Events (1–5 x 10^5) were scored using a BD FACSCalibur or LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

BrdU (BD Biosciences) was dissolved in PBS at 2 mg/ml. Mice were injected i.p. with 200 µl. Total BM cell suspensions were analyzed by flow cytometry for BrdU incorporation, using the BrdU flow kit (BD Biosciences) in conjunction with cell surface marker expression, as previously described (38).

Results

Ongoing IgL chain gene recombination in Slp65-deficient pre-B cell leukemias

By flow cytometry we found co-expression of IgL chain and SLC, as previously reported (20), in most Slp65-deficient or Btk/Slp65 double-deficient pre-B cell leukemias. The proportions of Ig κ or λ L chain expressing leukemic cells were 16±2% (n=30) and 1.5±0.3% (n=22), respectively. Normally, large cycling pre-B cells contain low levels of Rag transcripts (3), but when we analyzed the pre-B cell leukemias by quantitative RT-PCR we found substantial expression (Figure 1A). Transcription of Rag1 and Rag2 was ~2-3 fold higher in Btk/Slp65 double-deficient pre-B leukemias than in Slp65-deficient leukemias, most likely reflecting the more severe phenotype of Btk/Slp65 double-mutant mice, as compared with Slp-65 single mutant mice (20, 40). Consistent with the expression of Ig κ L chain protein, various clonal V_κ-J_κ rearrangement patterns were detected next to the germline J_κ restriction fragment in Southern blotting analyses, using a J_κ-specific probe (Figure 1B, 1C). In most of the leukemia samples analyzed, we observed multiple J_κ restriction fragments, indicating oligoclonality. IgL chain recombination is generally initiated in small resting pre-B cells that have terminated SLC expression (1, 11). Therefore, the finding that Slp65-deficient or Btk/Slp65 double-deficient pre-B cell leukemias contained oligoclonal Ig κ^+ B cell fractions co-expressing SLC suggested ongoing IgL chain gene rearrangement after leukemic transformation of pre-B cells.

Slp65/Btk double-deficient pro-B cells are not susceptible to malignant transformation

To investigate the involvement of V(D)J recombination in malignant transformation, we first crossed Btk/Slp65 double-deficient mice with mice deficient for Rag1. Because Btk/Slp65/Rag triple-deficient mice cannot express a functional IgH µ chain, they manifested an arrest of B cell development at the pro-B cell stage, as was found for the Rag1 single-deficient mice (35). Btk/Slp65/Rag triple-deficient mice did not develop leukemia (Table 1), in agreement with the previously reported finding that proliferation is mediated by high pre-BCR expression and not due to the absence of Slp65 per se (18).

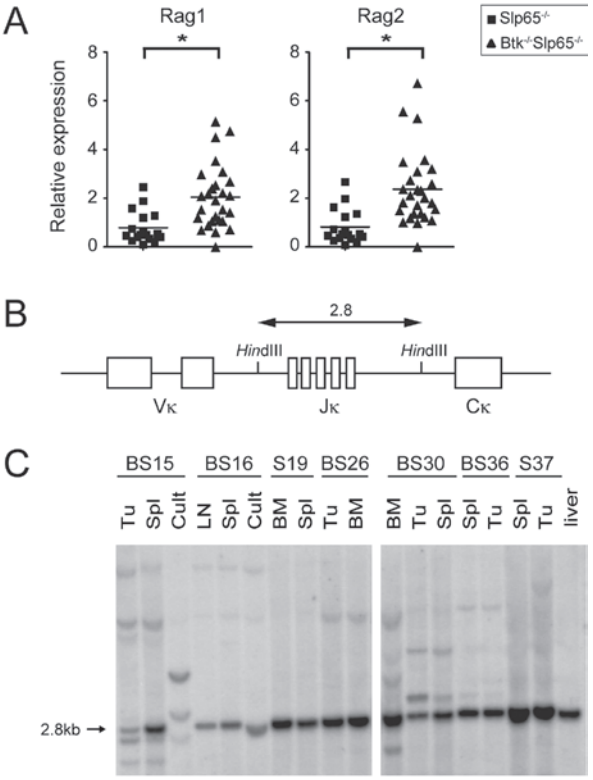

Figure 1. Ongoing V(D)J recombinase activity in Slp65-deficient leukemia
(A) Quantitative RT-PCR analyses of Rag1 and Rag2 expression in Slp65-deficient (n=17) and Btk/Slp65-deficient (n=27) pre-B cell leukemias, normalized with GAPDH. Values in WT B220$^+$ B cells in the BM were set to one. For each of these groups, the horizontal line represents the mean of the relative expression level. *p< 0.001 (t-test). (B) Schematic representation of the germline Ig κ L chain locus. Restriction sites of *Hind*III and the 2.8 kb κ germline fragment are shown. (C) Southern blotting analysis of *Hind*III digests of genomic DNA from the indicated tissues from Slp65-deficient (S) and Btk/Slp65 double-deficient (BS) leukemias. Ig κ L chain gene rearrangements were analyzed using a J_κ probe. The arrow indicates the 2.8 kb Ig J_κ germline band. BM = bone marrow; LN = lymph node; Tu = tumor; Sp = spleen; Cult = cultured leukemic cells.

Table 1. Leukemia development in panels of Btk/Slp65 double-deficient mice

Mouse genotype *Btk$^{-/-}$Slp65$^{-/-}$*	Tumor incidence	Onset range (days)	Follow-up period (days)
Rag-1$^{+/-}$	16/20	47 - 145	180
Rag-1$^{-/-}$	0/25	n.a.	180
DNA-PKcs$^{+/-}$	15/35	60 - 153	180
DNA-PKcs$^{-/-}$	0/23	n.a.	180

n.a. = not applicable

Defective NHEJ does not enhance malignant transformation of Slp65/Btk double-deficient pro-B or pre-B cells

Next, we analyzed whether defective NHEJ, which is associated with increased Rag-mediated double-strand DNA breaks that remain unresolved in B and T lymphocytes, contributed to leukemia formation. We crossed Btk/Slp65 double-deficient mice with mice that harbor a defect in the NHEJ pathway component DNA-PKcs, encoded by the *Prkdc* gene (41). In contrast to Rag1-deficient mice, DNA-PKcs-deficient mice have an incomplete block at the pro-B cell stage and very low but detectable numbers of pre-B cells are present in the BM (42-44). When compared with these DNA-PKcs-deficient mice, the numbers of intracellular IgH μ chain positive pre-B cells in Btk/Slp65/DNA-PKcs triple deficient mice were slightly increased, probably due to enhanced proliferative expansion of pre-B cells (Figure 2A). Accordingly, we found that the membrane IgL chain-negative cell fraction contained more surface SLC^+ pre-B cells in Btk/Slp65/DNA-PKcs triple than in DNA-PKcs single-deficient mice (Figure 1B). Next, we investigated if the increased proliferation capacity of Btk/Slp65-deficient pre-B cells (20) could correct the low generation of pre-B cells in DNA-PKcs-mutant mice. When mice were pulsed with a single dose of the thymidine analogue BrdU, which is selectively incorporated into the DNA of large pre-B cells (38), the absolute numbers of $BrdU^+$ large pre-B cells in the BM of DNA-PKcs-deficient mice were severely reduced, when compared to wild-type mice (Figure 2C). The absolute numbers of $BrdU^+$ large pre-B cells in Btk/Slp65/DNA-PKcs triple mutant mice were variable and only slightly increased, when compared with DNA-PKcs-deficient mice. Within the fractions of pre-B cells the proportions of $BrdU^+$ cells were comparable between wild-type and mice defcient for DNA-PKcs, Btk/Slp65 or Btk/Slp65/DNA-PKcs (Figure 2D). Therefore, proliferation of Btk/Slp65/DNA-PKcs triple mutant pre-B cells was not substantially increased *in vivo*, when compared to Btk/Slp65 double mutant mice, even though the BM in these mice may contain more empty niches or available space because of the block in B cell development.

Whereas in the Btk/Slp65 double deficient control group 15 out of 36 mice developed pre-B cell leukemia (Table 1), none of the 22 Btk/Slp65/DNA-PKcs triple mutant mice that were followed for 6 months showed signs of leukemia formation, even when we examined BM and spleen of these mice in detail by flow cytometry (data not shown). We conclude that pro-B cells and pre-B cells generated in Btk/Slp65/DNA-PKcs triple mutant mice are not particularly susceptible to malignant transformation, in spite of defective NHEJ.

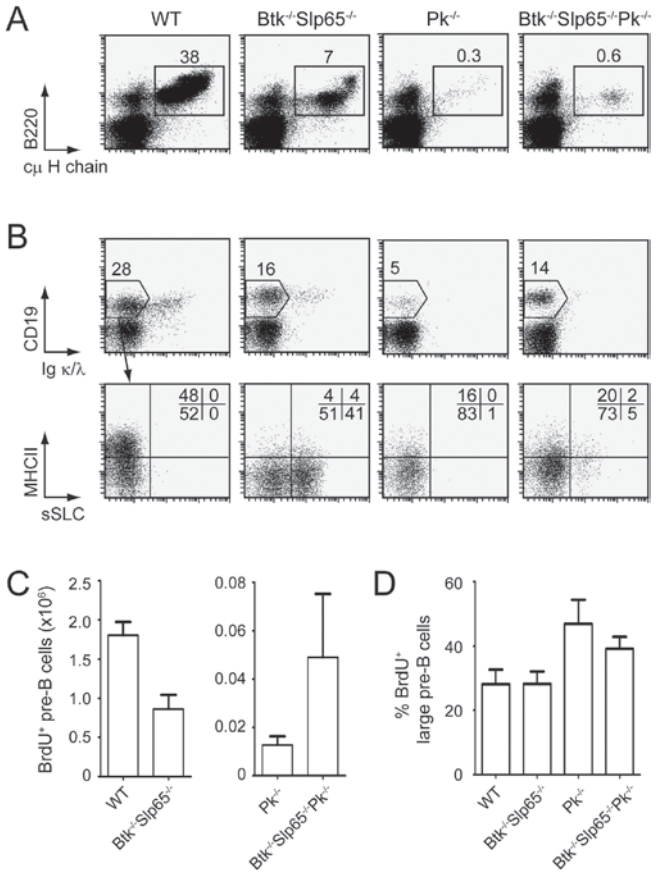

Figure 2. The small pre-B cell population in Btk/Slp65/DNA-PKcs triple-deficient mice is not highly susceptible to malignant transformation.
(A) Phenotypic characterization of BM from the indicated mouse genotypes. B220$^+$cμ H chain$^+$ B cells were gated. Data are displayed as dot plots, and numbers indicate the percentages of cells within the gates. (B) Flow cytometric analyses of surface expression of MHCII and SLC on CD19$^+$ Ig κ$^-$λ$^-$ pro-B and pre-B cells. Data are representative for four to six 8-week-old mice. (C) Absolute numbers of BrdU$^+$ CD19$^+$Ig κ$^-$λ$^-$ pre-B cells, 4 hr after i.p. injection of a single dose of BrdU, as determined by flow cytometry. (D) Percentages of BrdU$^+$ CD19$^+$Ig κ$^-$λ$^-$ pre-B cells in the BM. Average values and SEM of four to six animals per group are shown. PK$^{-/-}$=Prkdc$^{-/-}$.

Autoreactive receptor editing Slp65/Btk double-deficient B cells express high levels of pre-BCR

The complete absence or severe reduction of the size of the pre-B cell compartment in Btk/Slp65 mutant mice with additional mutations in the *Rag1* or *Prkdc* genes, precluded the analysis of leukemia development in these mice. However, when the developmental block at the pro-B cell stage was rescued by transgenic expression of the pre-

rearranged IgH chain transgene VH81X, tumor development was also prevented in Btk/Slp65 mutant mice (28). This could be explained by an acceleration of the passage of B-cell precursors through the pro-B cell stage, which may reduce the size of the cell population most susceptible to transformation.

Therefore, we employed Btk/Slp65 deficient mice carrying the 3-83μδ transgene, which consists of pre-rearranged Ig μ and δ H chain genes and an Ig κ L chain gene encoding an antibody that specifically recognizes MHC class I H-2K^{b,d} (36). In developing B cells in the BM, on a centrally deleting H-2K^{b} C57BL/6 background, the expressed BCR is autoreactive and therefore the V(D)J recombination machinery remains active. As a result, secondary immunoglobulin L gene rearrangements occur and hence autoreactive B cells can be rescued from apoptosis, a phenomenon called receptor editing (45-47). By contrast, the 3-83 μδ Tg on a non-H2-K^{b,d} background reflects an innocuous BCR and the arising B cells are not autoreactive and will not be deleted or edited (45-47).

On a non-deleting background, splenic B cells from 3-83μδ transgenic mice expressed the 3-83μδ BCR, as analyzed by staining with the anti-idiotypic antibody 54-1 (Figure 3A). As previously described splenic B cell numbers were very low in Btk/Slp65 double-deficient mice (20), irrespective of the presence of the 3-83μδ transgene (Figure 3A). When the 3-83μδ Tg was expressed on a WT deleting background, those B cells present had lost 54-1 reactivity and increased usage of Ig λ L chain, indicating that they had edited the 3-83μδ BCR. On a deleting background, 3-83μδ transgenic Btk/Slp65 double-deficient mice had only very few splenic B cells, but those B cells present were 54-1^{low} and had detectable Ig λ usage (Figure 3A), demonstrating that receptor editing is operational in Btk/Slp65 double-deficient mice.

In the BM, the presence of the 3-83μδ transgene on a deleting background resulted in BCR downregulation, as evidenced by low signals for surface μ H chain and 54-1 (Figure 3B). These B cells still expressed high levels of μ H chain in their cytoplasm, but SLC expression was limited precluding abundant surface expression of the pre-BCR. Also 3-83μδ transgenic Btk/Slp65 double-deficient B cells on a deleting background showed downregulation of μ H chain expression, albeit more modest, and had reduced staining with 54-1. As these B cells expressed high levels of SLC, we conclude that these 3-83μδ transgenic Btk/Slp65 double-deficient B cells expressed the 3-83μδ H chain together with SLC as a pre-BCR (Figure 3B).

Taken together, these findings show that on a centrally deleting background autoreactive 3-83μδ transgenic Btk/Slp65 double-deficient B cells undergo receptor editing, whereby the expression of pre-BCR is significantly increased, when compared with WT 3-83μδ transgenic mice.

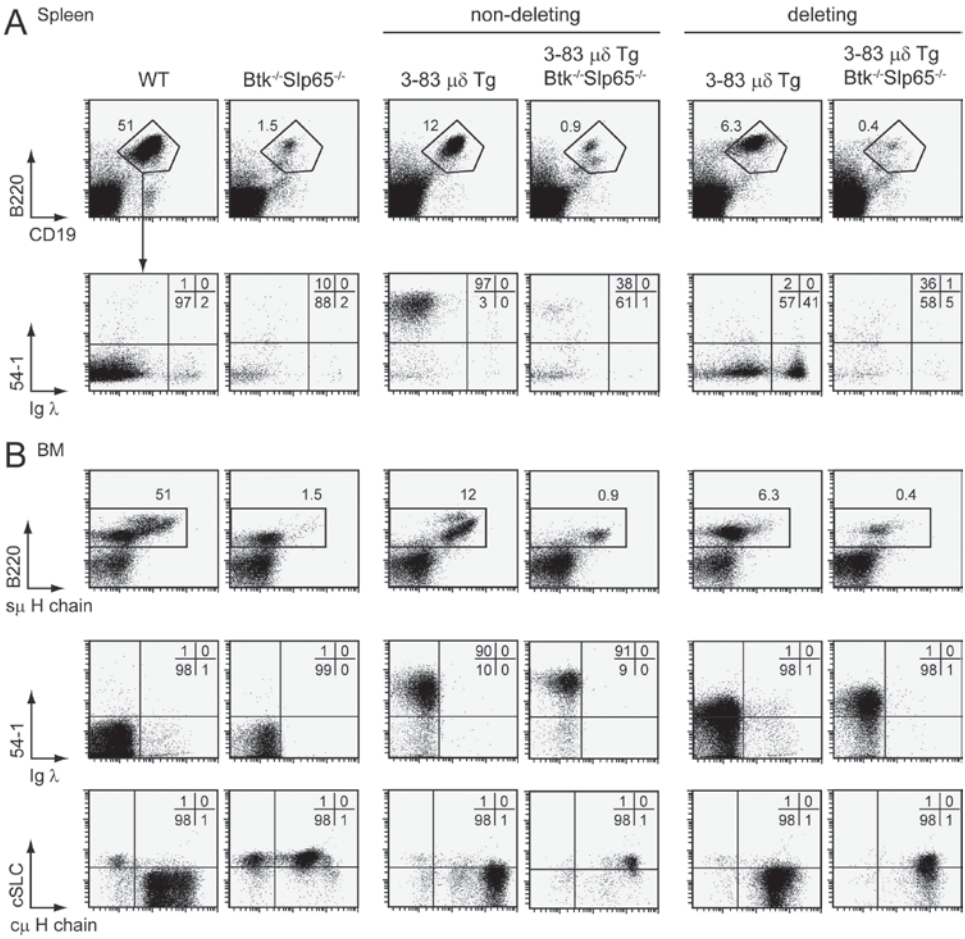

Figure 3. Receptor editing in Btk/Slp65 double-deficient B cells carrying the 3-83μδ BCR.
(A) Flow cytometric analysis of B cells in the spleen from the indicated mice. Total B220$^+$CD19$^+$ fractions were gated (*upper panel*) and analyzed for the 54-1 idiotype and Ig λ expression (*lower panel*). Data are displayed as dot plots, and numbers indicate the percentages of cells within the gates. Data represent four to eight 8-weeks old mice analyzed per genotype. (B) BM expression profiles of B220 and μ H chain are shown as dot plots (*upper panel*). B220$^+$CD19$^+$ B-lineage cells were gated and analyzed for expression profiles of 54-1/Ig λ and cSLC/cμ H chain. Numbers indicate the percentages of cells within the gates.

Leukemia in Btk/Slp65 double-deficient mice arises independently of V(D)J recombination activity

As editing 3-83μδ transgenic Btk/Slp65 double-deficient B cells expressed high levels of SLC and IL-7R it was conceivable that these mice were also susceptible to leukemia formation. Indeed when we followed panels of 3-83μδ transgenic Btk/Slp65 double-

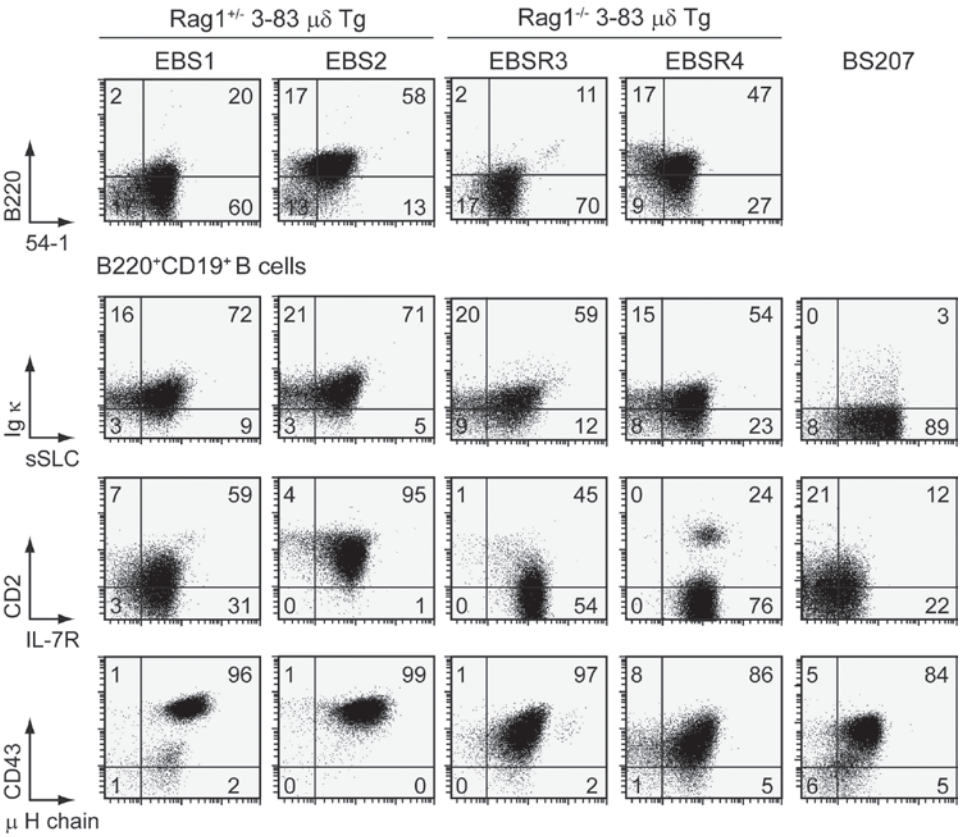

Figure 4. 3-83μδ Tg B cell leukemias have pre-B cell characteristics.
Flow cytometric analysis of two *Rag-1*$^{+/-}$ (EBS1, EBS2) and two *Rag-1*$^{-/-}$ (EBSR3, EBSR4) 3-83μδ Tg Btk/Slp65 double-deficient leukemias. On the right, a non-3-83μδ Tg Btk/Slp65 double-deficient leukemia is shown as a control. Lymphoid cells from lymph nodes (LN) and spleen (Spl) were gated and B220/54-1 profiles are shown as dot plots (*upper panel*). Total B220^{+}CD19^{+} B-lineage cells were gated and analyzed for the expression of surface Ig κ L chain, surface SLC (sSLC), CD2, IL-7R, CD43 and μ H chain (*lower panels*). Data are shown as dot plots, whereby the numbers indicate the percentages of cells within the quadrants.

deficient mice for 40 weeks, about 10% developed leukemia. Therefore, this model was suitable to investigate the involvement of V(D)J recombination in malignant transformation of Slp65-deficient pre-B cells. First, we crossed the mice with mice deficient for Rag1. When we followed panels of 3-83μδ transgenic Btk/Slp65 double-deficient mice that were either *Rag1*$^{+/-}$ or *Rag1*$^{-/-}$, we found that tumor frequencies were similar: 2/23 and 2/20, respectively (Table 2). Irrespective of their Rag1 genotype, all these leukemias were positive for the anti-idiotypic antibody 54-1 (Figure 4). They all

expressed SLC and IL-7R and showed variable expression of the differentiation markers CD2 and CD43 (Figure 4). In this respect, these leukemias were not different from those found in Slp65 single or Btk/Slp65 double mutant mice (20). From these findings, we conclude that leukemias in Btk/Slp65 double-deficient mice can arise in the absence of an intact V(D)J recombination machinery.

Finally, we wanted to test whether an increase of unresolved V(D)J recombinase-mediated double-strand DNA breaks would affect leukemia incidence. To this end, we followed panels of 3-83μδ transgenic Btk/Slp65 double-deficient mice that were either *Prkdc*$^{+/-}$ or *Prkdc*$^{-/-}$ for 40 weeks. Again, we did not find differences in leukemia onset or incidence (Table 2). Consequently, we conclude that defective NHEJ does not enhance B cell leukemia development in Btk/Slp65 double-deficient mice.

Table 2. Leukemia development in panels of 3-83μδ Tg Btk/Slp65 double-deficient mice

Mouse genotype 3-83μδ Tg *Btk*$^{-/-}$*Slp65*$^{-/-}$	Tumor incidence	Onset (days)	Follow-up period (days)
Rag-1$^{+/-}$	2/23	251, 249	280
Rag-1$^{-/-}$	2/20	227, 216	280
DNA-PKcs$^{+/-}$	2/17	147, 226	280
DNA-PKcs$^{-/-}$	3/21	244, 232, 238	280

Discussion

It has been reported that strongly proliferating Slp65-deficient pre-B cells express Rag proteins in mice (18-19) and that SLP65 deficiency in human pre-B cell leukemia correlates with RAG expression and ongoing V_H gene rearrangement activity (33). Furthermore, reconstitution of SLP65-expression in human SLP65-deficient leukemia and lymphoma cells resulted in downregulation of RAG1/2 expression and V(D)J recombination events (33). In agreement with these findings in human SLP65-deficient malignancies, we found that Slp65-deficient pre-B cell leukemias in mice contained oligoclonal Ig κ^+ B cell fractions co-expressing IgH and SLC, suggesting ongoing IgL chain gene rearrangement after leukemic transformation of pre-B cells. Therefore, deregulated V(D)J recombinase activity, involving multiple DNA double-strand DNA break events, in pre-B cells may well introduce genetic aberrations contributing to malignant transformation or progression of Slp65-deficient pre-B cells.

However, in this report we observed that Btk/Slp65 double deficient mice, in which the V(D)J recombination machinery is activated during receptor editing, develop leukemia at similar frequencies when they are concomitantly mutant for Rag1 or the NHEJ factor DNA-PKcs. We therefore conclude that malignant transformation of Btk/Slp65 double-deficient pre-B cells is independent of deregulated V(D)J recombination activity or defective NHEJ. In this respect, Slp65-deficient pre-B cell leukemia does not

differ from leukemia in mice with enforced c-Myc expression (30). It is of note that during normal B cell development, expression of the proto-oncogene c-Myc is induced by the IL-7R pathway (48) and that recently it has been shown that Slp65-deficient leukemias acquire autonomous IL-7R signaling by autocrine IL-7 production (25). As c-Myc is induced by IL-7R signaling, it is very well possible that sustained IL-7R signaling results in elevated levels of c-Myc expression (constitutively or transiently), contributing to transformation of Slp65-deficient pre-B cells. Consistent with this hypothesis, we detected c-Myc expression in Slp65-deficient leukemias by RT-PCR (28). Nevertheless, in contrast to Rag1 deficient c-Myc transgenic mice that may develop IgH μ^- pro-B cell tumors, it has been shown that transformation of Slp65-deficient B-lineage cells is fully dependent on pre-BCR expression and thus on functional IgH chain recombination (18).

Our conclusion that ongoing V(D)J recombination activity is not involved in malignant transformation of Slp65-deficient pre-B cells is in agreement with our previous observation that Slp65-deficient pre-B cell leukemias exhibited chromosomal instability but showed no evidence for Rag-mediated translocations by spectral karyotyping analysis (28). Therefore, ongoing Rag-mediated recombination activity in Slp65-deficient pre-B cell leukemias might well be a consequence of malignant transformation, resulting in correct IgL chain recombination, but not in aberrant V(D)J recombination events that would contribute to tumor progression. Spectral karyotyping analysis of Slp65-deficient pre-B cell leukemias revealed chromosomal abnormalities, characterized by chromosomal gains, losses and gene amplifications that were heterogeneous in nature and did not appear to specifically affect the Ig or T cell receptor gene loci (28). Since we found duplications of the N-myc locus that were associated with N-myc amplification on the p53-deficient background (28), it may be interesting to investigate whether these leukemias contain more regions with deletions or duplications involving tumor suppressors or oncogenes.

The proportions of mice developing leukemia in 3-83μδ Tg Btk/Slp65 double-deficient mice is low (~10%), when compared with Btk/Slp65 double-deficient mice that do not harbor an Ig transgene (~50-80%, dependent on mouse strain background). This is consistent with the previously reported observation that transgenic expression of a functional IgH chain prevents malignant transformation of c-Myc transgenic (29) or Slp65-deficient pre-B cells (28). Such premature transgenic IgH chain expression considerably accelerates the passage of B-cell precursors through the pro-B cell stage, thereby likely reducing the size of the cell population most susceptible to transformation (29, 49). In particular, it was recently shown that an IgH chain induces Stat5 dephosphorylation downstream of IL-7R signaling (50). Because Slp65 is essential for pre-BCR-mediated downregulation of IL-7R-Jak3-Stat5 signaling (25, 50-51) and Slp65-deficient pre-B cell leukemias exhibit autocrine IL-7 production, it is attractive

to speculate that transformation of Slp65-deficient pre-B cells is dependent on early oncogenic events that induce constitutive Jak3-Stat5 signaling at the pro-B cell stage.

It may be argued that dilution of SLC-mediated proliferative signals by the 3-83μδ Tg conventional L chains contributes to the low leukemia incidence in 3-83μδ Tg Btk/Slp65 double-deficient mice. This would be supported by the finding that conventional light chains inhibit the autonomous signaling capacity of the BCR (52). However, the opposite is more likely, because the 3-83μδ is an autoreative B cell receptor that mimics autonomous pre-B cell receptor signaling and induces proliferation of early B cells (53). It is therefore very well possible that Btk/Slp65 double-deficient mice transgenic for only the 3-83μδ IgH chain (which is not autoreactive) would not develop leukemia or at frequencies much lower than 10%. In this context, it is important to note that leukemia formation was completely lacking in Btk/Slp65 double-deficient mice that harbored a VH81X IgH transgene, (in contrast to the frequency of ~10% in the presence of the autoreactive 3-83μδ IgH+L chain transgene). Possibly, transformation of Slp65-deficient pre-B cells is restricted to IgH chains with specific structural properties. Particular IgH chain V regions may be associated with strong autonomous pre-BCR signaling, e.g. because they enhance interaction with self-antigens (53) or with carbohydrates in the μ IgH chain (54). In such a model the 3-83μδ H chain but not the VH81X IgH chain would have these specific structural properties.

Because of the low leukemia frequencies in our mouse panels (~10% for all our genotypes, Table 2), we cannot formally rule out that Rag1 activity may to some extent contribute to the transformation process in a fraction of the leukemias. But, importantly, the finding that two Rag1/Btk/Slp65 triple-deficient mice developed leukemia in our 3-83μδ receptor editing model, demonstrates that Rag1 activity is not absolutely essential for malignant transformation of Btk/Slp65-deficient (pre-)B cells. Conversely, defective NHEJ, which is associated with oncogenic misjoining of Rag-induced breaks (55-56), did not appear to enhance lymphoma formation**.** Taken together, we therefore conclude that pre-B cell leukemias in Btk/Slp65-deficient mice arise independently of ongoing V(D)J recombination activity.

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Chapter VI

Oncogenic activity of the DNA repair protein Rad54 contributes to malignant transformation in Slp65-deficient pre-B cell leukemia

Ta VBT, Essers J, de Bruijn MJW, de Haan AB, Meijerink JPP, Kanaar R, Hendriks RW (in preparation)

Abstract

Mice deficient for the precursor-B cell receptor (pre-BCR) signaling proteins Slp65 and Bruton's tyrosine kinase (Btk) manifest an arrest at the large pre-B cell stage, which is a highly proliferative phase in B cell development. Slp65-deficient mice spontaneously develop pre-B cell leukemia and the incidence is increased by concomitant deficiency for Btk. Although the molecular mechanism of malignant transformation is still unclear, we have recently shown that it is independent of deregulated V(D)J activity and that it involves disruption of the Arf-Mdm2-p53 pathway. Interestingly, Slp65-deficient pre-B cells have high levels of IL-7 receptor and pre-BCR, both of which provide proliferative signals. It is therefore conceivable that cell division in these cells may be accompanied by genomic instability, ultimately leading to oncogenic transformation. To investigate the involvement of genomic instability in proliferating large pre-B cells, we crossed Btk/Slp65 double deficient mice with mice deficient for the DNA repair protein Rad54 involved in homologous recombination (HR). Paradoxically, we found that Rad54-deficiency actually protected, rather than enhanced, Btk/Slp65-double deficient pre-B cells from malignant transformation. At 6 months of age Rad54/Btk/Slp65 triple deficient mice manifested reduced tumor incidence (4 out of 24, ~17%), when compared with Btk/Slp65-double deficient mice (9 out of 21, ~43%). Comparative genomic hybridization analysis of pre-B cell leukemias from Rad54/Btk/Slp65 triple deficient mice showed a similar frequency of genetic aberrations, when compared with those from Btk/Slp65-double deficient mice. However, several gene duplications abundant in Btk/Slp65-double deficient pre-B cell tumors, ranging in size up to 400 kb, were not present in the concomitant absence of Rad54. These results indicate that repair via Rad54 and HR operates on a razors edge: although it has been assumed that repair by HR ensures high fidelity repair, our findings indicate that Rad54 activity can be corrupted at certain locations to promote aberrant HR, resulting in genomic rearrangements. For the first time, we demonstrate that a DNA repair protein can contribute to malignant transformation.

Introduction

B lymphocytes provide humoral immunity and are produced in the bone marrow through a complex process of cellular differentiation. B cell development is characterized by the ordered rearrangement of immunoglobulin (Ig) heavy (H) and light (L) chains and the expression of specific cell surface markers (1-3). In pro-B cells productive in-frame recombination of Ig H chain V, D and J segments results in the expression of Ig μ chain protein, which marks the transition to the precursor-B (pre-B) cell stage. The Ig μ chain is expressed on the cell surface, together with the surrogate light chain (SLC) components λ5 and VpreB and the signaling subunits Igα and Igβ, as the pre-B cell receptor (pre-BCR) complex. The expression of the pre-BCR functions as an important checkpoint in B cell development (2, 4-5). Pre-BCR signaling initiates proliferative expansion, because pre-B cells acquire the capacity to respond to low concentrations of the proliferation factor interleukin (IL)-7 (4-5). It also induces cellular differentiation, thereby redirecting V(D)J recombination activity from the Ig H chain to the Ig κ and λ L chain loci (2, 4-5). Productive Ig L chain recombination leads to surface expression of the BCR.

The adapter protein Slp65 (also known as Blnk or Bash) and the non-receptor kinase Bruton's tyrosine kinase (Btk) are key components in the signaling pathway downstream of the pre-BCR and the BCR. Slp65, when phosphorylated by Syk, provides docking sites for Btk and phospholipase C γ (PLCγ). Btk then phosphorylates PLCγ leading to its full activation and the generation of second messengers (2, 4-6). In human, mutations in *SLP65* or *BTK* result in defective pre-B cell proliferation and an almost complete arrest of early B cell development, associated with the immunodeficiency disorder agammaglobulinemia (6-7). In addition, deficiency of SLP65, due to defective splicing, has been found in ~50% of childhood precursor-B cell acute lymphoblastic leukaemia (ALL) cases (8). Because additional studies with large numbers of patients indicated a low frequency of SLP65 defects (9-10), it is currently not clear if SLP65 loss is a common leukemogenic event.

Slp65-deficient mice spontaneously develop pre-B cell leukemia expressing high levels of pre-BCR on the cell surface (11-12). Although Btk-deficient mice do not develop pre-B cell leukemia, Btk and Slp65 cooperate as tumor suppressors whereby Btk exerts its tumor suppressor function independently of its kinase activity (13-14). Combined deficiency of Slp65 and Btk results in a more complete arrest at the pre-B cell stage (13). Recent findings show that Slp65 limits IL-7-mediated proliferation and survival through downregulation of IL-7R expression (15) and by direct inhibition of Jak3 (16). Conversely, in Slp65-deficient leukemia the Jak/Stat5 signaling pathway is constitutively activated, mostly due to autocrine production of IL-7 (16).

Although the molecular mechanism of malignant transformation is still unclear,

we have recently found that it involves disruption of the Arf-Mdm2-p53 pathway (17). We were able to show that malignant transformation is not dependent on defective repair - by the non-homologous end-joining pathway - of double strand DNA breaks (DSB) generated by V(D)J recombination activity (18). As in Slp65-deficient mice B cell differentiation is arrested at the stage of large cycling pre-B cells, it is conceivable that cell division in these strongly cycling cells may be accompanied by genomic instability, ultimately leading to oncogenic transformation. We therefore argued that erroneous homologous recombination (HR), the pathway which repairs DSBs in cycling cells (19), may be involved in the malignant transformation of Slp65-deficient pre-B cells. In this report, we investigated the effect of Rad54 deficiency, Rad54 is a member of the SWI2/SNF2 subfamily of ATPase (20). Rad54 was shown to interact with Rad51 and this interaction is functionally important in multiple steps of HR (21). Rad51 has a central role in HR, assembling onto single-stranded DNA and catalysing the invasion and exchange of homologous DNA sequences.

Our previous gene targeting experiments demonstrated that HR in Rad54-deficient cells is reduced compared to wild-type cells, but that Rad54-deficient mice exhibit normal V(D)J recombination and Ig H chain class switch (22). In this report, we provide evidence that oncogenic activity of Rad54 contributes to malignant transformation in Btk/Slp65-double deficient pre-B cell leukemia.

Materials and Methods

Mice

Btk-deficient(23) and Rad54-deficient (22) mice were on the C57BL/6 background and *Slp65*$^{-/-}$ mice(24) were on the BALB/c background. For mouse genotyping genomic DNA was analyzed by PCR as previously described (13, 22). Animals in tumor panels were sacrificed after indication of tumor formation, or after a maximum period of 26 weeks. Mice were bred and maintained in the Erasmus MC animal care facility under pathogen-free conditions. Experimental procedures were reviewed and approved by the Erasmus MC committee for animal experiments.

Flowcytometry

Preparations of single-cell suspensions, flow cytometry procedures, and mAbs have been described (13, 25). BM cell suspensions were depleted of erythrocytes by standard ammonium chloride lysis and IL-7 driven cultures were performed as described previously (25). Events (1–5 x 10^5) were scored using a FACSCalibur flow cytometer (BD Bioscience and analyzed using FlowJo software (Tree Star, Ashland, OR).

Pre-B cell cultures and visualization of Rad54$^{GFP/GFP}$ cells

Bone marrow cell suspensions were depleted of erythrocytes by standard ammonium chloride lysis and IL-7 driven cultures were performed as described previously (25). Rad54$^{GFP/GFP}$ cells were visualized using a Zeiss LSM510 fluorescent microscope.

Array based CGH

Analyses were performed by using 180K Genomic mouse DNA Arrays (Agilent Technologies, Palo Alto, CA) according to manufacturer's instructions. Slides were scanned with an Agilent scanner and data was analyzed with Agilent Genomic Workbench (Agilent).

Results and Discussion

Rad54 expression is mainly confined to proliferating cell stages

First, we investigated during which stage of B cell development Rad54 is being expressed. Transcripts of Rad54 are abundantly present in thymus, spleen and testis, but were low in most other tissues (26). This expression pattern of Rad54 would be consistent both with a role for Rad54 in DNA repair of V(D)J recombination-associated DSBs in resting cells and with a role for Rad54 in DNA repair in highly proliferative cells. We used mice carrying a Rad54-GFP knock-in allele to analyze the expression pattern of Rad54 at different stages of B cell development (Suppl. Figure 1). The functionality of the Rad54-GFP fusion protein was tested by performing clonal survival assays in response to DNA damage induction by γ-irradiation (Suppl. Figure 1B).

Rad54-GFP expression was analyzed in pro-B/large pre-B cell ($CD19^+CD2^-IgM^-$), small pre-B cell ($CD19^+CD2^+IgM^-$) and immature/mature B cell ($CD19^+CD2^+IgM^+$) fractions (Figure 1A). Rad54-GFP signals were detected in the pro-B cell/large pre-B cell and in the small pre-B cell fractions, mainly in large cells with high forward scatter values and not in small resting cells with low forward scatter values. Rad54-GFP$^+$ cells present in the $CD19^+CD2^+IgM^-$ small pre-B cell fraction were large pre-B cells that had started the upregulation of surface CD2 expression. In parallel, in the thymus Rad54-GFP expression was almost exclusively detected in large, cycling cells that have past β-selection in the double negative ($CD3^-CD4^-CD8^-$) compartments DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) and in the immature single positive ($CD3^-CD4^-CD8^+FSC^{high}$) fraction (data not shown).

Next, total bone marrow cell suspensions were cultured *in vitro* with IL-7 for 5 days enriching for large, cycling pre-B cells (Rolink, Grawunder et al. 1993). Upon IL-7 removal, culture for 2 additional days strongly induces pre-B cells to exit the cell cycle and to initiate stepwise activation of Ig L chain rearrangements (27-28). Pre-B cells

cultured in the presence of IL-7 displayed nuclear localization of Rad54-GFP (Figure 1B). In contrast, small resting pre-B cells present in the cultures without IL-7 essentially did not express Rad54-GFP. Taken together, these findings indicate that during B cell differentiation Rad54 expression is mainly confined to proliferating cell stages and not in cells that are resting and may undergo V(D)J recombination.

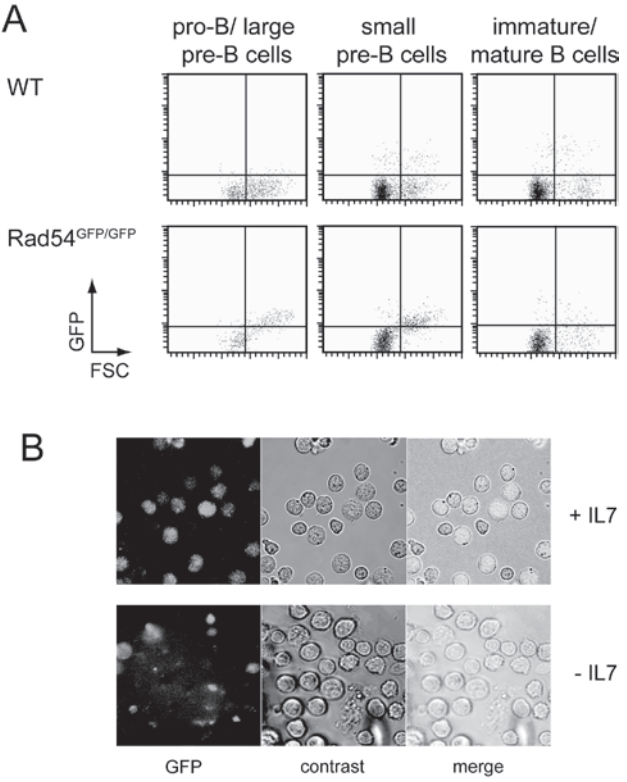

Figure 1. Rad54-GFP expression in proliferating B cells.
(A) Flow cytometry profiles of GFP and forward scatter (FSC) are shown as dot plots for pro-B/large pre-B cells ($CD19^+CD2^-IgM^-$), small pre-B cells ($CD19^+CD2^+IgM^-$), immature and mature B cells ($CD19^+CD2^+IgM^+$) from WT and $Rad54^{GFP/GFP}$ mice. (B) Confocal microscopy images of $Rad54^{GFP/GFP}$ pre-B cells cultured for 5 days with IL-7 (*top*) and for 2 additional days with or without IL-7 (*bottom*). **See Appendix for full-color figure.**

Rad54-deficiency reduced leukemia incidence Btk/Slp65-double deficient mice

We investigated the possible involvement of HR in oncogenic transformation of pre-B cells by crossing Slp65-deficient mice on the Rad54-deficient background. We followed panels of *Rad54*$^{+/+}$*Slp65*$^{-/-}$ and *Rad54*$^{-/-}$*Slp65*$^{-/-}$mice for 6 months. On the Balb/c background, ~10% of Slp65-deficient mice develop pre-B cell leukemia (11-12). We

noticed that only 1/28 Slp65-deficient mice developed pre-B cell leukemia (1/28). In the concomitant absence of Rad54 none of the 25 mice developed a tumor. The low leukemia frequency observed might thus be related to the C57BL/6 genetic background of these mice.

To increase the sensitivity of our analyses, we used Btk/Slp65-double deficient mice in which we previously found tumor frequencies in the range of ~50-80%, dependent on the background strain. We followed panels of *Rad54*$^{+/+}$*Btk*$^{-/-}$*Slp65*$^{-/-}$and *Rad54*$^{-/-}$ *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice. Consistent with previous findings (13-14, 17), we found that at the age of 6 months 9 out of 21 (~43%) Btk/Slp65-double deficient mice developed pre-B cell leukemia (Figure 2). Surprisingly, we found that deficiency of Rad54 significantly reduced leukemia incidence in Btk/Slp65-double deficient mice, because only 4 out of 24 (17%) Rad54/Btk/Slp65 triple deficient mice developed a pre-B cell tumor at 6 months of age ($p<0.03$) (Figure 2). The pre-B cell leukemias found in Rad54/Btk/Slp65 triple deficient mice all expressed high levels of SLC and IL-7R and showed variable expression of the differentiation markers CD2, CD25 and CD43 (data not shown). In this respect, these leukemias were not different from those found in Slp65 single or Btk/Slp65 double mutant mice (13-14).

Collectively, these data show that Rad54-deficiency reduced the incidence of pre-B leukemia formation in Btk/Slp65-double deficient mice, suggesting that Rad54 has oncogenic properties.

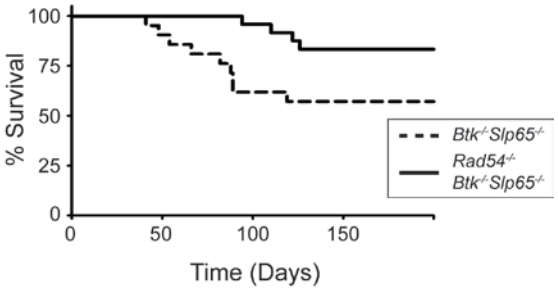

Figure 2. Deficiency of Rad54 protects against malignant transformation.
Kaplan-Meier tumor-free survival estimates for *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice (n=21) and *Rad54*$^{-/-}$*Btk*$^{-/-}$*Slp65*$^{-/-}$ mice (n=24). Tumor-free survival in *Rad54*$^{-/-}$*Btk*$^{-/-}$*Slp65*$^{-/-}$ mice was significantly increased ($P<0.03$) compared with *Btk*$^{-/-}$*Slp65*$^{-/-}$ mice.

Rad54-dependent aberrations in Btk/Slp65-double deficient pre-B cell leukemias

The finding of oncogenic activity of Rad54 is quite remarkable, as deficiency of a component of the HR pathway is expected to increase genomic instability and to enhance malignant transformation. In particular, since point mutations in RAD54 have been found in primary human tumors (29).

Because gene duplications mediated by HR between Alu repeat elements have

been reported in circulating lymphocytes as well as human T-ALL (30), we argued that erroneous Rad54-mediated HR may result in duplications or deletions that contribute to malignant transformation of Slp65-deficient pre-B cells. To investigate this issue, we performed a genome-wide analysis of genetic aberrations present in pre-B leukemia cells from six Btk/Slp65-double deficient and four Rad54/Btk/Slp65-triple deficient mice by comparative genomic hybridization (CGH). We found evidence for genomic duplications and deletions in all samples, with substantial differences in their frequency per chromosome. For example, for chromosome 1, we found that in the absence of Rad54 duplications/deletions were substantially increased, but this was not the case for chromosome 17 (Figure 3). Whereas in Btk/Slp65-deficient leukemias chromosome 17 manifested 11 ± 3.7 deletions and 23 ± 7.7 amplifications, these values in Rad54/Btk/Slp65 triple deficient mice were 11 ± 4.5 and 15 ± 3.5, respectively.

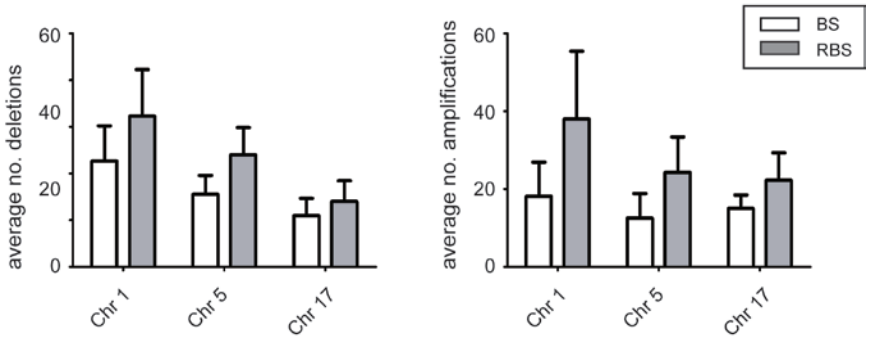

Figure 3. Deficiency of Rad54 does not lead to increased genetic instability.
Array CGH of pre-B cell tumors reveals a similar number of deletions and amplifications present on chromosome 1, 5 and 17 when Btk/Slp65-double deficient tumors (n=6) (BS) are compared with Rad54/Btk/Slp65-triple deficient tumors (n=4) (RBS). Open bars represent BS tumors (n=6) and filled bars represent RBS (n=4) tumors. Deletions are shown on the left and amplifications are shown on the right. Bars represent average normalized values and SEM. Chr, chromosome.

Detailed analyses showed that several genomic duplications (up to 430 kb in size) were consistently present in all six Btk/Slp65-double deficient pre-B cell tumors. Interestingly, many of these were not present in the concomitant absence of Rad54. Two examples of these Rad54-dependent duplications are shown in Figure 4, a 39 kb fragment on chromosome 1 qH3 and a 430 kb fragment on chromosome 17 qA3.3. In general, these amplified fragments contained multiple genes: natural killer cell receptor 2B4 (CD244), BTB (POZ) domain containing 9 protein-protein interactions (Btbd9), glyoxalase 1 and the dynein axomal heavy chain 8 protein (Dnahc8). These findings show that several duplications were specific for Btk/Slp65-double deficient tumors and not present in Rad54/Btk/Slp65-triple deficient tumors. It is therefore possible that particular aberrations contributing to malignant transformation did not occur in the

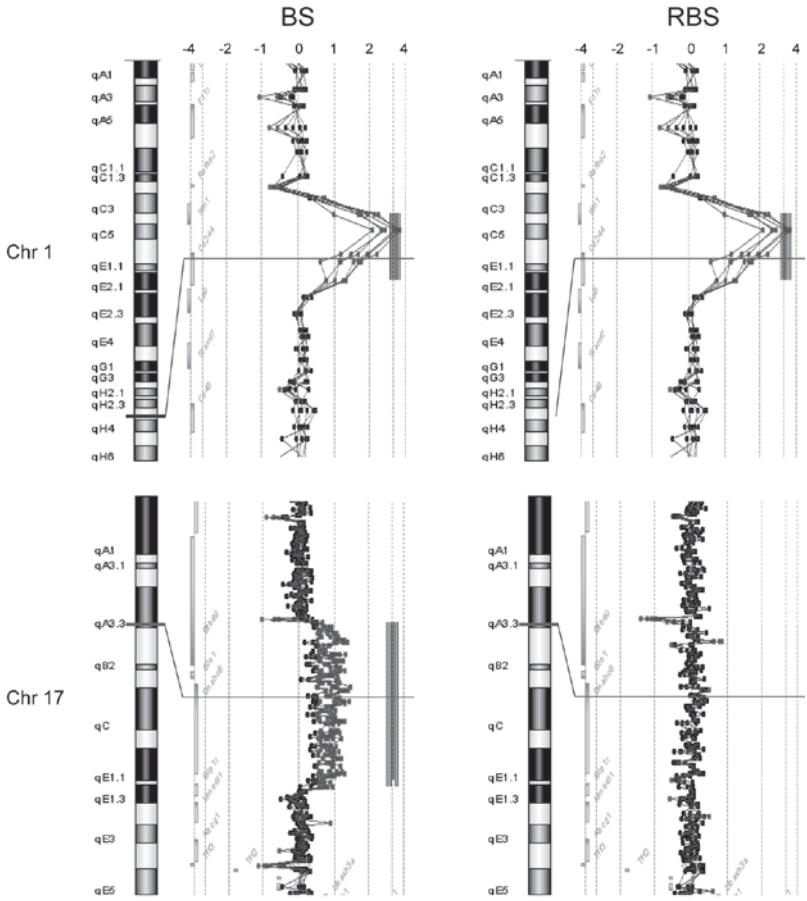

Figure 4. Focal amplifications only present in Btk/Slp65-double deficient tumors.
Overlays of CGH analyses of chromosome 1 (*upper panel*) and 17 (*lower panel*) views of Btk/Slp65-double deficient tumors (BS; n=6) (*left*) and Rad54/Btk/Slp65-triple deficient tumors (RBS; n=4) (*right*), showing different sizes of aberrations exclusively present in BS tumors and not in RBS tumors. Chr, chromosome. **See Appendix for full-color figure.**

absence of Rad54, thus leading to a lower incidence of leukemia in Rad54/Btk/Slp65-triple deficient mice. Although these genes may not be critical for leukemogenesis (as tumors did also arise in Rad54/Btk/Slp65-triple deficient tumors), they may facilitate malignant transformation of Slp65-deficient pre-B cells. In addition, further analyses are required to determine if genes involved in genetic alterations which were found in Slp65-deficient tumors play a role in malignant transformation. In particular, deletions or amplifications common for both Btk/Slp65-double deficient tumors and Rad54/Btk/

Slp65-triple deficient tumors could contain genes which are involved in the malignant transformation of Slp65-deficient pre-B cells. Therefore, our mouse model could be used to screen for new oncogenes or tumor suppressor genes.

Next to these consistently recurrent genomic duplications in Btk/Slp65-double deficient pre-B cell tumors, other genomic alterations (amplifications and deletions) were identified in only a portion of Btk/Slp65-double deficient or Rad54/Btk/Slp65-triple deficient tumors as is shown for chromosome 4 qD1, chromosome 6 qB3 and chromosome 8 qA4 (Figure 5). It was striking that all Btk/Slp65-double deficient pre-B cell tumors contained apparently identical duplications, starting and ending at the same probe. Breakpoint analyses are required to determine the mechanism by which Rad54 mediates amplification. The involvement of repetitive DNA sequences in recurrent chromosomal rearrangements have been found in human tumors (31) and breakpoint regions in Btk/Slp65-double deficient tumors could contain repetitive elements that facilitate amplification, since intrachromosomal rearrangements between Alu elements occur in meiotic cells. Next to repetitive elements, regions of homology could be present and provide a template for disrepair by Rad54-mediated HR. More general properties of eukaryotic chromatin could play a role as common fragile sites are hot spots for chromosomal rearrangements and deletions (32).

The finding of oncogenic activity of Rad54 is quite remarkable, as faithful DNA repair contributes to maintaining chromosomal stability (19, 33-34) and deficiency of proteins involved in DNA repair pathways leads to increased genomic instability and malignant transformation (35-39). BRCA1 and BRCA2 are associated in nuclear foci with Rad51. Individuals with a germline mutation in BRCA1 or BRCA2 are predisposed to breast, ovarian and a few other cancers (40-42). Furthermore, point mutations of RAD54 have been found in primary human tumors (29, 43) resulting in the absence of RAD54 due to aberrant splicing (44). Although no mutations in the HR key protein RAD51 have been found in cancer, overexpression of RAD51 is observed in a wide range of human tumors (45-48). Increased Rad51 expression does not come from amplification but is due to increased transcription (45). High levels of RAD51 lead to increased genomic instability resulting in chromosomal rearrangements (49). Therapies based on inhibition of RAD51 are under investigation (50).

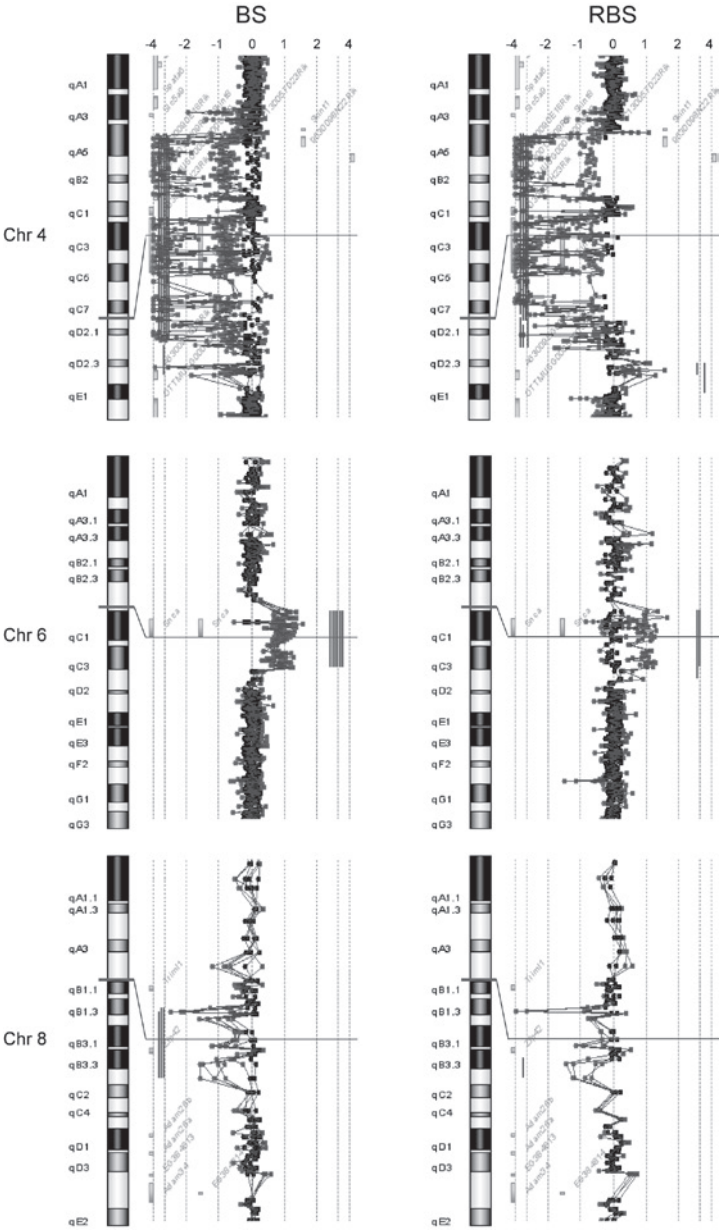

Figure 5. Overview of several genetic lesions in Slp65-deficient tumors.
Overlays of CGH analyses of chromosome 4 (*upper panel),* 6 (*middle panel*) and 8 (*lower panel*) views of Btk/Slp65-double deficient tumors (BS; n=6) (*left*) and Rad54/Btk/Slp65-triple deficient tumors (RBS; n=4) (*right*), showing different sizes of aberrations. Chr, chromosome. **See Appendix for full-color figure.**

Concluding Remarks

In this report, we show that oncogenic Rad54 activity contributes to malignant transformation of Slp65-deficient pre-B cells. Remarkably, deficiency of Rad54 partially protects against malignant transformation. This is in contrast with the current views as HR is thought to promote genome stability through precise repair of DSBs and since mutations in genes encoding HR proteins are associated with tumorigenesis (51). Our results indicate that Rad54-mediated HR operates on a razors edge, as Rad54-mediated HR could result in misrecognition and disrepair. We therefore conclude that Rad54-mediated HR has the capacity to contribute to oncogenic transformation. To investigate the mechanism by which Rad54 mediates malignant transformation, we currently aim to determine the characteristics and specific requirements of the breakpoint areas involved in aberrations found in Slp65-deficient tumors.

Our findings open new perspectives since DNA repair proteins can have the capacity to function as oncogenes. It would be interesting to investigate whether Rad54 also has the capacity to function as an oncogene in other tumor models and whether other proteins involved in HR also have oncogenic properties.

Acknowledgements

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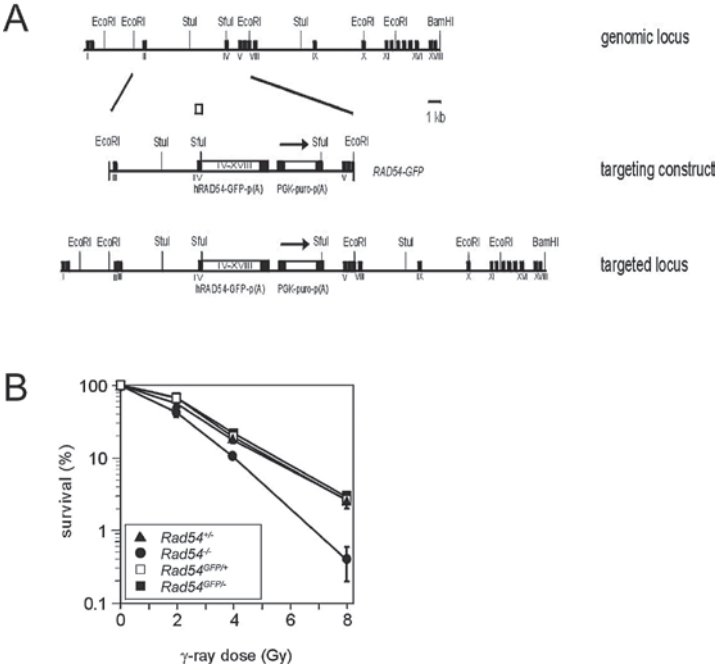

Suppl Figure 1. Generation of the Rad54-GFP mouse model.
(A) Schematic representation of the Rad54 locus and the gene targeting construct. Black boxes indicate exons I-XVIII. The targeting construct contains the human Rad54 cDNA sequence spanning exons IV-XVIII fused to GFP coding sequence. PGK indicates the puromycine resistance gene. The targeting construct replaces the region between exons III and VIII. Integration results in the expression of GFP-tagged Rad54 from the endogenous promoter. (B) Embryonic stem cells of the indicated genotypes were irradiated and followed for survival. Experiments were performed in triplicate and the error bars represent SEM.

Chapter VII

A mouse model for chronic lymphocytic leukemia based on expression of the SV40 large T antigen

Ter Brugge PJ, Ta VBT, de Bruijn MJW, Keijzers G, Maas A, van Gent DC, Hendriks RW. Blood. 2009 Jul

Abstract

The simian virus 40 (SV40) T antigen is a potent oncogene able to transform many cell types and has been implicated in leukemia and lymphoma. In this report, we have achieved sporadic SV40 T antigen expression in mature B cells in mice, by insertion of a SV40 T antigen gene in opposite transcriptional orientation in the immunoglobulin (Ig) heavy (H) chain locus between the D en J_H segments. SV40 T antigen expression appeared to result from retention of the targeted germline allele and concomitant antisense transcription of SV40 large T in mature B cells, leading to chronic lymphocytic leukemia (CLL). Although B cell development was unperturbed in young mice, aging mice showed accumulation of a monoclonal B cell population in which the targeted IgH allele was in germline configuration and the wild-type IgH allele had a productive V(D)J recombination. These leukemic B cells were $IgD^{low}CD5^{+}$ and manifested nonrandom usage of V, D, and J segments. V_H regions were either unmutated, with preferential usage of the VH11 family, or manifested extensive somatic hypermutation. Our findings provide an animal model for B-CLL and show that pathways activated by SV40 T antigen play important roles in the pathogenesis of B-CLL.

Introduction

B cell chronic lymphocytic leukemia (B-CLL) (reviewed in Ref.(1),(2)), the most common leukemia in the Western world, is characterized by the accumulation of a monoclonal population of mature B cells that aberrantly express CD5(3-4). The clinical course of CLL is extremely heterogeneous: whereas some patients survive over a decade with stable disease, others succumb to the disease within months despite aggressive treatment. This heterogeneity is associated with variability in the expression pattern of a number of different proteins, and also with the presence of different chromosomal aberrations(5). Approximately half of the CLL cases have 13q14deletions, apparently involving the linked microRNA molecules miR15 and miR16, which are thought to be negative regulators of the anti-apoptotic gene Bcl2(2). Deletions on chromosome 17p, affecting the p53 protein, are less frequent and are associated with poor prognosis(6-7). CLLs manifest a unique gene-expression signature that differs from other lymphoid cancers, suggesting a common mechanism of transformation or a homogeneous cell population of origin(2).

CLL has been subdivided into two prognostic subsets, based on the presence of somatic mutations of the immunoglobulin (Ig) heavy (H) chain variable (V_H) genes. 50-70% of CLL patients have mutated V_H genes, likely reflecting antigen-driven post-germinal center (GC) selection, and have a more favorable prognosis than those with unmutated B cell receptors. CLL Ig V_H regions also exhibit unique complementarity determining region 3 (CDR3) features that characterize and differentiate aggressive unmutated from more indolent mutated CLLs. In particular, unmutated poor outcome cases frequently contain long CDR3s with amino acid residues that favor polyreactivity. Interestingly, both unmutated and mutated CLLs are thought to derive from self-reactive B cell precursors (8). In this context, it has been found that ~3-4% of healthy individuals over 40 years of age have a population of monoclonal lymphocytes in their blood with immunophenotypic characteristics of CLL cells(2).

The simian virus 40 (SV40) T antigen is a potent oncogene able to transform many cell types(9-10) and has been implicated in the etiology of various cancers(11-12). The SV40 T antigen has transforming activity by inactivating p53 and Rb proteins and inducing genomic instability(13). Several studies point to a causative role of SV40 in the formation of human B-cell malignancies, including non-Hodgkin's lymphoma(14-16). Transgenic expression of the SV40 T gene under the control of the IgH enhancer induced hyperproliferation of multi-lineage hematopoiesis, reminiscent of myelodysplastic syndromes in humans(17).

In this report, we aimed to accomplish sporadic SV40 T gene expression in the B-cell lineage in mice. We introduced the SV40 T gene, without its promoter and in opposite transcriptional orientation between the IgH chain D en J_H segments. As anti-

sense transcription takes place across the D-J_H region in pro-B cells(18), it is possible that in our mouse model SV40 T is expressed as a part of a large anti-sense transcript in early pro-B cells that have not yet performed IgH D to J_H recombination. But, in allmost all pro-B cells the SV40 T gene will be excised during normal IgH D-to-J_H rearrangement. Only in those rare B cells, in which the non-productive targeted allele has a germline configuration and thus still contains the SV40 T gene, it may possibly be expressed in more mature B cell stages. In addition, given the ability of the recombination activating gene (Rag)-1 and -2 proteins to catalyze DNA translocation and transposition reactions both *in vitro*(19-20) and *in vivo*(21-23), it is conceivable that V(D)J-mediated reinsertion of D-J_H circles containing the SV40 T oncogene compromises genomic integrity, leading to tumor formation.

In our model we found that expression of SV40 T antigen is associated with the development of B-cell malignancies with striking similarities to human B-CLL. Tumor formation appears to result from retention of the targeted germline allele and concomitant antisense transcription of SV40 large T in mature B cells.

Materials and methods

Generation of mouse models

To generate the *IgH.T (*and *IgH.TEμ*, with an extra copy of the IgH intronic enhancer) targeting constructs, containing the thymidine kinase gene, neomycin resistance gene, SV40 T antigen, internal ribosome entry site, splice accepter (SA) and flanking homology arms (created by long-range polymerase chain reaction (PCR) from 129 mouse genomic DNA) we performed multiple cloning steps (strategy available upon request). Constructs were linearized and electroporated into E14 embryonic stem (ES) cells, which were subsequently cultured with G418 and gancyclovir. Expanded ES cell clones were screened for homologous recombination by Southern blotting. Chimeric mice were generated by injection of ES cells into blastocysts and bred to C57BL/6 mice. Germline transmission of the targeted allele was verified by Southern blotting and offspring was genotyped by PCR analysis of DNA from tail snips using SV40 T-specific primers. Excision of the neo-cassette was achieved by crosses with transgenic mice expressing Cre-recombinase ubiquitously under the control of the cytomegalovirus immediate early enhancer-chicken beta-actin hybrid promoter.

IgH.T, IgH.TEμ, and *p53*$^{-/-}$ (Jackson Laboratory, Bar Harbor, Maine) mice were bred and maintained in the Erasmus MC animal care facility under specific pathogen-free conditions. Experimental procedures were reviewed and approved by the Erasmus MC committee.

Southern blotting

Genomic DNA samples were digested with SacI or XbaI overnight at 37°C and processed using standard procedures. A 760 bp neomycin probe was generated by PCR. The *DQ52up* probe is an 840 bp BspHI-PciI fragment, located just upstream of DQ52 (Figure 1B). The *CH3* probe is a 1131 bp NspI-XcmI fragment spanning the m CH3 exon. A 1.2 kb XhoI-XbaI fragment (*XX1.2;* Figure 4D) was used as a probe for the H chain DQ52-Eμ region. Probes were labeled using the Prime-it II kit (Stratagene, La Jolla, CA) and hybridized overnight at 65°C. Fragments were visualized using a phospho-Imager and analyzed with ImageQuant (GE Healthcare, Little Chalfont, United Kingdom).

Flow cytometry, cell purification and blood smears

Blood was collected from the tail vein and erythrocytes were lysed using NH_4Cl. Preparation of single-cell suspensions and monoclonal antibody (BD Biosciences, San Jose, CA) incubations for 4-color cytometry have been described(24). Viable cells were counted using a Coulter counter (Beckman Coulter, Fullerton, CA). B cells were purified by magnetic separation using CD19-microbeads and AutoMACS (Miltenyi Biotech, Auburn, CA). To produce blood thin films, 10μl of blood was smeared on a glass slide, dried and fixed in methanol for 3 minutes and stained in 5% Giemsa.

Western blot

Total cell lysates were obtained by addition of lysis buffer (20 mM TRIS, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP-40, 10% glycerol, 1mM pefabloc, 1 mM Na_3VO_4) to cells and incubation on ice for 20 minutes. Lysates were centrifuged (13 000*g*) and supernatants were boiled with loading buffer, separated on sodium dodecyl sulfate-polyacrylamide gels and blotted, using standard procedures. Membranes were stained with biotinylated anti-SV40T antibodies (Pab108, BD Biosciences), using streptavidin-horseradish peroxide as a second step and developed using enhanced chemiluminescence.

RNA isolation and RT-PCR

Total RNA was isolated by GenElute Mammalian RNA purification (Sigma-Alderich, St Louis, MO), DNAse digested and reverse transcribed with Superscript II and random hexamer primers. PCR Primers spanning at least one intron were designed using ProbeFinder software and probes were chosen from the universal probe library (Roche Diagnostics, Indianapolis, IN). For quantitative real-time PCR cDNA was amplified in universal Mastermix containing 200 nM of each primer and 100 nM probe, using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). To confirm specificity of amplification products, samples were analyzed by standard

agarose gel electrophoresis. The obtained C_t values were normalized to those of hypoxanthine-guanine phosphoribosyl-transferase (HPRT) (25).

Heteroduplex PCR and DNA sequencing

Heteroduplex analyses were performed as described(26). For DNA sequence analyses, cDNA samples were amplified using seven primers located in the framework 1 region: two high degeneracy primers MH1-2 and five low degeneracy primers MH3-7(27), in combination with a primer located in the 5' cμ region(28). PCR products were directly sequenced using the BigDye terminator cycle sequencing kit with AmpliTaq DNA polymerase on an ABI PRISM 377 automated sequencer (Applied Biosystems). All H chain regions were sequenced in two directions from at least two independent PCR products and analyzed by IMGT/V-Quest (IMGT®, the international ImMunoGeneTics information system®, http://www.imgt.cines.fr).

Results

Generation of IgH.T and IgH.TEμ mice

To achieve sporadic SV40 T antigen expression, we generated two different mouse models by homologous recombination in ES cells. The SV40 large and small T antigen coding unit(10) was inserted between DQ52 and J_H1 in the IgH locus, either with (IgH.TEμ) or without (IgH.T) an extra copy of the IgH intronic enhancer Eμ (Figure 1). ES cell clones with homologous recombination events were identified by Southern blotting, using the 5'-flanking *DQ52up* probe and the 3'-flanking *CH3* probe (Figures 1BC, left panel and data not shown). ES clones were injected into blastocysts to generate chimeric mice that transmitted the targeted IgH allele through the germline, as identified by SV40 T gene-specific PCR (Figure 1C, right panel). Excision of the neo-cassette was achieved by crossing mice with transgenic mice ubiquitously expressing Cre recombinase under the control of the cytomegalovirus immediate early enhancer-chicken beta-actin hybrid promoter. Successful excision was verified by Southern blotting using neomycin, *DQ52up* and *CH3* probes (Figure 1D; data not shown). Mice harboring a single targeted allele were further bred to C57BL/6 mice. Heterozygous offspring, referred to as IgH.TEμ or IgH.T mice, were born at the expected Mendelian frequencies, did not manifest any developmental defects, appeared normal and were fertile.

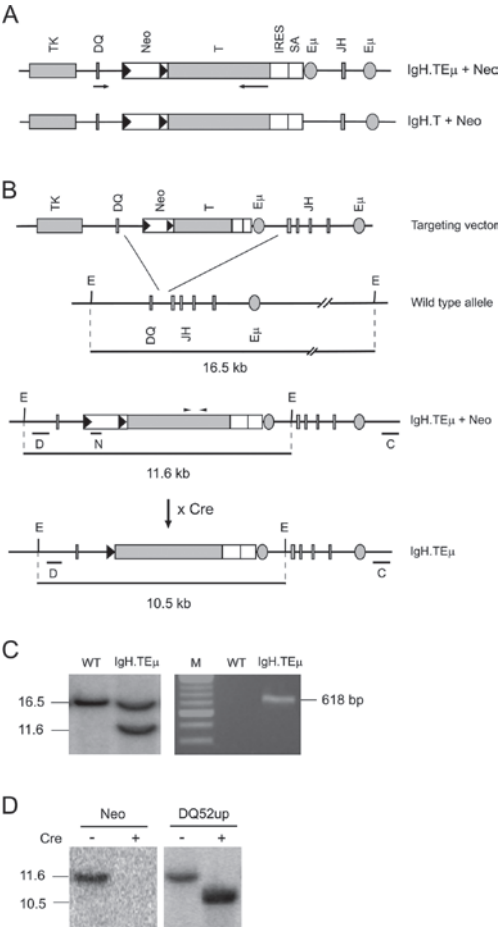

Figure 1. Generation of IgH.TEμ and IgH.T mouse models.
(A) Schematic representation of the targeting constructs with (IgH.TEμ + Neo) and without (IgH.T + Neo) an additional Eμ copy. LoxP sites are represented by black triangles and arrows indicate transcriptional orientation. Neo: neomycin gene; T: T antigen ; IRES: internal ribosome entry site ; SA: splice acceptor. (B) The targeting vectors included the SV40 T antigen gene in opposite transcriptional orientation without its promoter, flanking regions of 5' (DQ52) and 3' (J_H) Ig H chain homology, the herpes simplex thymidine kinase gene for negative selection and the neomycin resistance gene for positive selection. Below the targeting vector: wild-type Ig H chain allele, the locus after homologous recombination, and after Cre-mediated Neo-excision. The position of the PCR primers used to genotype offspring is indicated by arrowheads above the T antigen. Probes used in Southern blots are indicated by horizontal lines. E: EcoRI site, D: *DQ52up*, N: neomycin, C: *CH3*. (C) Southern blot of EcoRV-digested ES cell DNA (left panel) and PCR analysis of tail DNA from the indicated mice. The IgH.TEμ ES cell clone analysis showes a 16.5 kb wild-type fragment and a 11.6 kb fragment recombined fragment containing the Neo insertion. (D) Southern blot of EcoRV-digested tail DNA from IgH.TEμ mice before (-) and after (+) Cre-mediated recombination. Blots were hybridized, either with the Neo (left panel) or the DQ52up probe (right panel).

B cell development in IgH.T and IgH.TEμ mice

First, we investigated whether SV40 T antigen gene insertion in the IgH locus affected B cell development or H chain allele usage. Single cell suspensions from bone marrow (BM) and spleen from ~8-week-old IgH.T and IgH.TEμ heterozygous mice and wild-type littermates were analyzed by flow cytometry. The presence of the targeted IgH alleles did not affect BM or spleen cellularity. Also the proportions of the various stages of B cell development in the BM or the proportions of $CD19^+$ B cells in the spleen were unaffected (Figure 2A). The three groups of mice had similar IgM/IgD profiles or proportions of immature ($CD21^{low}CD23^{low}$), follicular ($CD21^+CD23^+$) and marginal zone ($CD21^{high}CD23^{low}$) B cells in the spleen (Figure 2B).

To determine whether SV40 T gene insertion resulted in an altered frequency of IgH allele usage, we took advantage of IgM allotype differences between the targeted IgH allele (129 strain-derived IgM^a) and the wild-type C57BL/6 allele (IgM^b) in F1 mice. By flow cytometry using allotype-specific antibodies, we found equal usage of the targeted and the wild-type IgH allele in mature splenic $CD19^+$ B cells (Figure 2C).

Quantitative reverse-transcribed (RT) PCR analyses of purified BM $CD19^+$ B-cell lineage fractions from IgH.TEμ/IgH.T mice revealed the presence of SV40 T gene transcripts (Figure 2D). Significantly higher levels of SV40 T transcripts were detected in IgH.TEμ than in IgH.T B cell fractions, consistent with the capacity of the Eμ element to increase transcription. Because the SV40 T gene will be excised during D-to-J_H segment rearrangement, we reasoned that inclusion of SV40 T sequences in IgH transcripts is expected to be largely limited to the early pro-B cells stage in the BM, before the initiation of D-to-J_H gene recombination. Consistent with this, we found that purified $CD19^+$ B-cell lineage fractions from IgH.T and IgH.TEμ on the Rag-$2^{-/-}$ background had increased levels of SV40 T transcription, when compared with IgH.T and IgH.TEμ mice on the wild-type background (Figure 2D). Because B cell development is blocked at the pro-B cell stage in IgH.T;Rag-$2^{-/-}$ and IgH.TEμ;Rag-$2^{-/-}$ mice, this finding indicated that SV40 T antigen gene transcription was indeed particularly present in pro-B cells that have a germline IgH configuration.

Importantly, we detected significant SV40 T antigen transcription in mature splenic $CD19^+$ B cells from IgH.T and IgH.TEμ mice (Figure 2D). Taken together, these findings show that SV40 T gene insertion in the IgH chain locus did not affect B cell development or H chain allele usage and that in a fraction of mature B cells SV40 T is retained and transcribed.

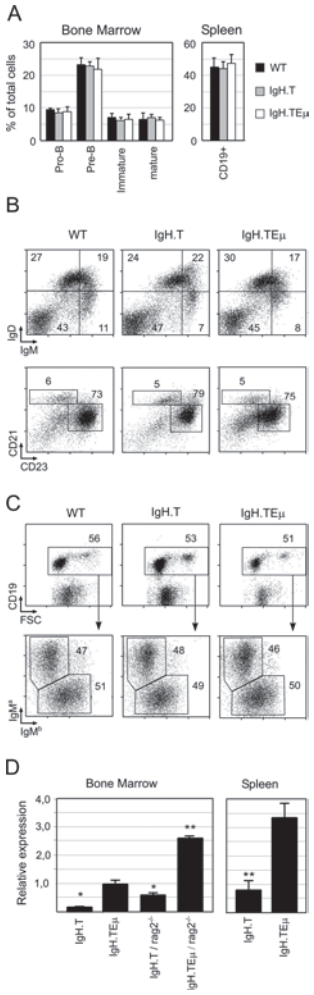

Figure 2. Normal B cell development in young IgH.T and IgH.TEμ **mice.** (A) Proportions of pro-B ($CD19^+/CD43^+/\mu^-$), pre-B ($CD19^+/\mu^+$), immature (IgM^+/IgD^-) and mature (IgM^+/IgD^+) B cells in BM and total $CD19^+$ B cells in the spleen of the indicated mice, as measured by flow cytrometric analysis. Mean values and SD are given for 4 mice per group. (B) Fluorescence-activated cell sorter profiles for IgM and IgD in total spleen cells from the indicated mouse groups (top panel). Total splenic $CD19^+$ B cells were gated and analyzed for CD21 and CD23 expression. The proportions of $CD21^{high}CD23^{high}$ follicular B cells and $CD21^{hi}CD23^{lo}$ marginal zone B cells within the specified gates are given. (C) Flow cytrometric analysis of CD19 expression (top panel) on peripheral white blood cells of WT, IgH.T and IgH.TEμ mice at 2 months of age. The $CD19^+$ fraction was gated and analyzed for the expression of the IgM^a and IgM^b allele (bottom panel). Numbers in the plots indicate the percentage of cells within the specified gates. (D) Quantitative PCR analysis of large T mRNA expression in $CD19^+$ BM cells and spleen cells of the indicated mice. Expression was normalized with HPRT and expression in IgH.TEμ BM was set to 1. Asterisks indicate significant differences, when compared with IgH.TEμ mice (* $p<0.01$, ** $p<0.001$, Mann–Whitney U test). Mean values and SD are given for three 8-10-week-old mice per group.

Unilateral IgH allele usage in aged IgH.T and IgH.TEμ heterozygous mice

To monitor selective outgrowth of monoclonal B cell populations over time, we periodically determined IgM^a/IgM^b profiles in peripheral blood B cells from panels of IgH.T (n=15) and IgH.TEμ (n=31) mice (Figure 3A). Young animals consistently had close to equal proportions of IgM^a and IgM^b-expressing B cells. In contrast, we observed in all aging IgH.TEμ mice and in two out of fifteen IgH.T mice a gradual appearance of a $CD19^+$ population with preferential expression of the non-targeted IgM^b allele. These IgM^b cells were also characterized by large forward scatter values, indicating increased average cell size. In mice with unilateral IgM^b expression (>90% IgM^b, designated UNI mice) significantly higher numbers of peripheral white blood cells were found, when compared with mice with random IgH allele usage (45-55% IgM^b, RAN mice; Figure 3B). Examination of blood smears showed the presence of enlarged cells with clumped chromatin and little cytoplasm (Figure 3C). Nevertheless, animals showed no outward signs of distress in this period.

Collectively, these data show the accumulation over time of atypical, large B cells exclusively expressing the IgM^b allele from the non-targeted IgH locus.

Accumulation of monoclonal B cell populations in aged IgH.T and IgH.TEμ mice

IgH.T and IgH.TEμ mice in the UNI group had normal B-lineage cell numbers in BM, but spleen sizes were significantly increased, when compared with wild-type littermates or IgH.T/IgH.TEμ RAN mice (Figure 4A, Supplementary Table 1). A majority (~60%) of the IgH.T and IgH.TEμ UNI mice had enlarged lymph nodes with cell numbers up to ~15 times those of healthy animals. As shown for three examples of UNI mice in Figure 4B, flow cytometric analyses of the spleen revealed the presence of a population of mature $CD19^+$ B cells with increased cell size, when compared to wild-type control cells (see CD19/FSC profiles in Figure 2C).

Accumulation of B cells expressing the non-targeted IgM^b H chain allele could either reflect a selective polyclonal hyperplasia or a monoclonal expansion of IgM^b-expressing B cells. To distinguish these two possibilities, we did an initial screening for Ig L chain usage in the expanding B cell populations in IgH.T and IgH.TEμ UNI mice and found that the $CD19^+$ B cell population was almost entirely Ig κ positive (with <0.5% of λ^+ B cells) or in two cases largely Ig λ^+ (Figure 4C). Usage of only a single L chain indicated monoclonality of the accumulating B cell populations in IgH.T and IgH.TEμ UNI mice. This was confirmed by detailed analysis of the IgH rearrangement status by Southern blotting of XbaI digests of genomic DNA from purified $CD19^+$ splenic B cell fractions from nine heterozygous IgH.TEμ UNI mice. We used a J_H region probe, which recognizes a 3.5 kb XbaI fragment in a germline wild-type IgH allele and a 2.0/1.7 kb doublet fragment in a germline knock-in IgH.TEμ allele, as present in genomic DNA from non-B cells (Figure 4D and 4E). All nine samples contained prominent ~2.0/1.7

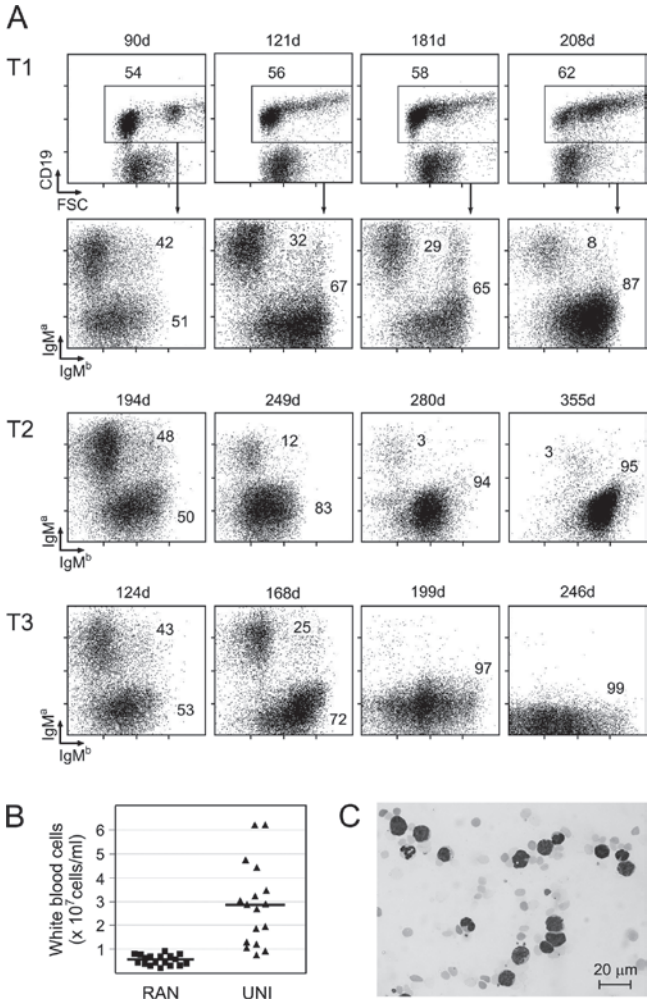

Figure 3. Unilateral Ig H chain usage in aged IgH.TEμ heterozygous mice

(A) Flow cytrometric analysis of surface CD19, IgM^a and IgM^b expression on peripheral blood mononuclear cells from three individual IgH.TEμ mice, T1, T2 and T3, representative for mice analyzed that developed unilateral Ig H chain usage over time. The $CD19^+$ fraction was gated (shown only for mouse T1) and the IgM^a/IgM^b profile was analyzed (shown for all three mice). Data are shown as dot plots; Numbers indicate the percentages of cells within the specified populations. Age of the animal at the time of analysis is indicated by the numbers above the plots. (B) Total white blood cell count in IgH. TEμ mice showing random usage (RAN, n=17) of IgM^a and IgM^b alleles, or unilateral usage of IgM^b (UNI, with <10% IgM^a and >90% IgM^b B cells, n=18; P <.001; Mann–Whitney U test). The RAN mouse group consists of IgH.TEμ (<5 months of age) as well as IgH.T mice (<9 months). The age of onset of UNI group is indicated in Table S1. Each symbol indicates an individual mouse. Bars show average with standard deviation of 17 (RAN) and 18 (UNI) animals. (C) Giemsa staining of blood smear of a UNI mouse, showing the presence of large cells with little chromatin. Size marker = 20 μm.

kb fragments of the IgH.TEμ allele in the germline configuration. In addition, ~3.5 kb fragments of the germline wild-type IgH allele were detected (generally of low density), most likely reflecting non-B cells present in the spleen samples. In addition, uniquely rearranged wild-type IgH alleles were present (in the two cases shown in Figure 4E, these fragments are ~3 and ~4 kb).

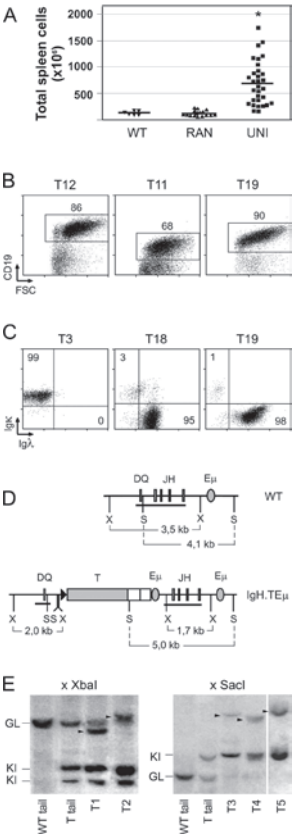

Figure 4: Accumulation of monoclonal B cell populations in aged IgH.T and IgH.TEμ mice. (A) Spleen size of WT (n=6), RAN (n=18) and UNI (n=33) animals. Each symbol indicates an individual mouse; mean values are indicated by a line. *Significant difference with WT (P <.001; Mann–Whitney U test) (Ages of mice are given in Table S1). (B) Flow cytometric analysis of total spleen cells in the three UNI mice indicated. Numbers indicate the percentage of $CD19^+$ B cells. (C) Expression of Igκ or Igλ L chain in splenic IgM^+ B cells of three UNI mice. Numbers indicate the percentage of cells within the specified quadrants. (D) Schematic representation of WT and targeted alleles, and position of restriction sites used for Southern analysis of Ig H chain recombination. Horizontal line indicates the position of the *XX1.2* probe, spanning the DQ-J_H region. S: SacI, X: XbaI. (E) DNA rearrangements in total spleen cells of UNI animals. DNA was digested with XbaI or SacI and hybridized to the *XX1.2* probe. Tail DNA from WT and UNI animals was used as reference for the WT germline fragment (GL) and the knock-in germline fragment (KI). Note that digestion with XbaI results in two germline KI fragments.

Similar results were obtained when we tested DNA from twelve additional heterozygous IgH.TEμ UNI mice using SacI digests. All twelve splenic B cell fractions showed a ~5 kb fragment of the IgH.TEμ allele in the germline configuration, a weak ~4.1 kb germline wild-type IgH allele, as well as uniquely rearranged wild-type alleles in the range of ~7-10 kb (shown for three cases in Figure 4E). The identification of unique, singly rearranged J_H fragments confirmed the expansion of single B cell clones in UNI animals. Furthermore, the presence of germline knock-in IgH.TEμ alleles indicated that the targeted IgH locus with the SV40 T gene was still in germline configuration. This was confirmed by the finding that also a SV40 large T antigen-specific probe hybridized to these germline knock-in IgH.TEμ restriction fragments, without revealing any other unique fragments (in nine out of nine samples tested; data not shown).

In summary, we conclude that aged IgH.T and IgH.TEμ UNI mice show accumulation of monoclonal B cell populations that have retained the targeted IgH allele in the germline configuration. We did not find evidence for the occurrence of Rag-mediated transposition, as no SV40 T sequences were detected on new unique restriction fragments.

Monoclonal B cell populations in IgH.TEμ mice have a CLL-like phenotype

Splenic B cell fractions from aged IgH.T/IgH.TEμ UNI mice were found to express significantly higher levels of SV40 T antigen transcripts than B cells from RAN mice, as determined by quantitative RT-PCR (Figure 5A). SV40 large T protein was identified in all tumor samples by western blotting (Figure 5B). As expected, no SV40 T transcription or protein was detected in wild-type (WT) splenic B cells (not shown).

We characterized the phenotype of the monoclonal B cell populations in IgH.T and IgH.TEμ mice by flow cytometry and quantitative RT-PCR. In these analyses, we did not detect phenotypic differences between clonal B cell populations from IgH.T or IgH.TEμ mice. The clonal B cell populations expressed CD19, B220, CD2 and substantial levels of surface IgM, but surface IgD was generally low (Figure 5C). They were negative for the early differentiation markers surrogate light chain, IL-7R or AA4.1 and the mature B cell markers CD21, CD23 and CD138 (data not shown). Importantly, all B cell leukemia samples expressed significant levels of CD43 and CD5 on the cell surface (Figure 5C), both of which are associated with B-CLL in human. In all UNI mice substantial populations of $CD19^+CD5^+CD43^+$ cells were identified in blood, lymph nodes, spleen and bone marrow (supplementary Table 1).

p53-deficiency increases CLL incidence in IgH.TEμ mice

All mice in the IgH.TEμ panel manifested leukemia formation in blood, spleen and BM before the age of ten months (Figure 6A), but in the IgH.T panel only two out of fifteen (Figure 6B). At one year of age the remaining mice in the IgH.T group were sacrificed and found to have no B cell abnormalities.

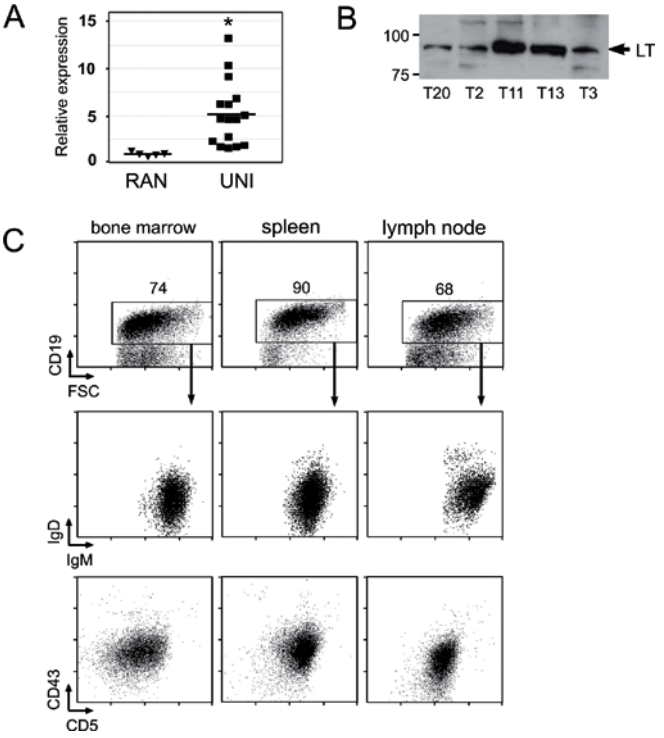

Figure 5. Monoclonal B cell populations in aged IgH.TEμ mice express T antigen and CLL markers. (A) Quantitative RT-PCR analysis of Large T antigen expression in RAN (n=5) and UNI (n=16) purified splenic CD19^{+} B cell fractions. Expression was normalized with HPRT and relative expression in RAN B cell fractions was set to 1. Each symbol indicates an individual mouse; mean values are indicated by a line ($p<0.01$; Mann–Whitney U test). (B) Western blot showing expression of Large T protein (LT, arrow) in purified B cell fractions from IgH.TEμ UNI mice. Numbers on the left indicate size in kD. Examples are representative for 20 CLL samples analysed (C) Expression of CD19, IgM, IgD, CD5 and CD43 on BM, spleen and lymph node cells from a IgH.TEμ UNI mouse. CD19^{+} cells were gated and analysed for their IgM/IgD and CD5/CD43 profiles. Data are representative for 33 animals analyzed (See supplementary Table 1 for age of mice).

In human B-CLL, loss of p53 function is associated with accelerated disease progression and poor prognosis(6-7). To study the effect of p53 loss in our mouse model, IgH.TEμ and IgH.T mice were crossed onto a p53-deficient background. Relative to p53$^{-/-}$ mice, the groups of IgH.TEμ;p53$^{-/-}$ and IgH.T;p53$^{-/-}$ mice did not show a significant increase in tumor formation (Figure 6). However, whereas p53$^{-/-}$ littermate controls all developed T cell tumors, five out of six animals in the IgH.TEμ;p53$^{-/-}$ panel developed B cell tumors, with the remaining animal developing a T cell tumor. Likewise, four out of ten mice in the IgH.T;p53$^{-/-}$ panel had B cell tumors. The age at which malignancies were evident decreased in IgH.TEμ;p53$^{-/-}$ mice, when compared with IgH.TEμ mice

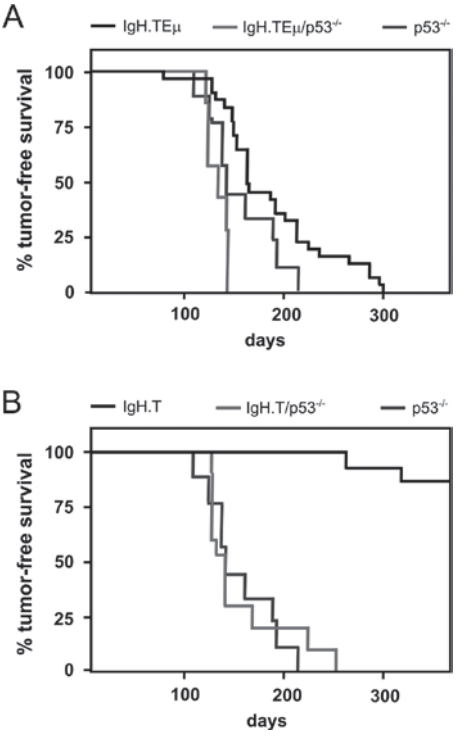

Figure 6. Effect of p53 on CLL development in IgH.TEμ and IgH.T mice. (A) Kaplan-Meier survival curve of IgH.TEμ (n=31), (IgH.TEμ/p53$^{-/-}$, n=6) and p53$^{-/-}$ (n=9) mice. (B) Kaplan-Meier survival curve in IgH.T (n=15); IgH.T/p53$^{-/-}$ (n=10) and p53$^{-/-}$ (n=9) mice. Tumor-free survival is plotted over time.

(median survival of 138 and 161 days, respectively; $p<0.01$, the log-rank test). This was also the case for IgH.T;p53$^{-/-}$ mice, when compared with IgH.T mice (median survival of 143 days and >365 days, respectively; $p<0.001$). The phenotypes of B cell tumors in IgH.TEμ;p53$^{-/-}$ and IgH.T;p53$^{-/-}$ were similar to those present in IgH.TEμ! or IgH.T animals on the wild-type background, as characterized by flow cytometry and southern blotting analysis of IgH alleles (not shown).

Taken together, these results demonstrate that – although SV40 large T antigen acts as an inhibitor of the p53 pathway – complete loss of p53 increases B-CLL incidence in IgH.TEμ and IgH.T mice.

Ig V_H usage in IgH.TEμ leukemias resembles unmutated human CLL

Based on the presence or absence of Ig V_H gene mutations, human CLL patients are categorized into two subgroups with different prognosis. We therefore decided to characterize the V regions from eight IgH.TEμ and one IgH.T mouse. We purified CD19$^+$ splenic B cell fractions and performed Ig V_H family-specific RT-PCR analyses.

First, we confirmed clonality of the leukaemias by heteroduplex analysis, in which homo- and heteroduplexes resulting from denaturation and renaturation of IgH V region RT-PCR products were separated in non-denaturing polyacrylamide gels based on their conformation(29). For most samples we observed clear single homoduplexes, confirming the amplification of clonally rearranged V regions, but one case showed the presence of oligoclonal bands. DNA sequence analysis revealed that four leukemias expressed V_H11.2, another four expressed VH segments from the V_HJ558 family, and one expressed both V_H11.2 and V_HJ558 segments, indicating biclonality.

In the leukemias expressing V_H11.2, which is the only member of the V_H11 family, the sequence was identical to the germ-line gene present in the IMTG database (http://www.imgt.cines.fr)(Table 1). The V_H11.2 gene is preferentially expressed in B-1 lymphocytes and is associated with autoreactivity and oligoclonal expansions of $CD5^+$ B cells in old mice(30). Three of the J558 family genes expressed were (nearly) identical to their most homologous published germ-line genes. By contrast, two V_HJ558$^+$ leukemias manifested extensive somatic hypermutation (SHM). One IgH.TEμ B-CLL expressed V_H1-69, which is often found in human CLL (31), and contained 15 mutations (of which 10 in CDRs) and one IgH.T B-CLL expressed V_H1-74 and contained 13 mutations (5 in CDRs). These two CLL samples likely originated from GC-experienced B cells with high activity of activation-induced cytidine deaminase (AID), which induces SHM.

Non-stochastic usage was not only observed for V_H segments, but also for D and J_H segments: e.g. DST4.2 was found twice (normally DST segment usage is <2%) and all V_H11-2 V_H segments were joined to J_H1 (50%; normally J_H1 usage is <16%). Unmutated poor outcome human B-CLL cases often express IgH chains with long CDR3s, containing multiple neutral tyrosine and serine residues that may confer CDR3 flexibility and favor polyreactivity. The CDR3 length, which is on average ~12 amino acids in murine B cells(32), was 11-12 in six leukemias, whereas two leukemias had a short (6 and 9 amino acids) and two leukemias had a long CDR3 length (15 amino acids). Eight out of ten CDR3s were enriched for tyrosines and serines (3-6 residues), and all CDR3s contained multiple charged amino acids, whereby the pI values were relatively neutral in 7/10 cases. As a result of the biased use of certain V_H, D, and J_H gene segments, the five $V_H11.2^+$ leukemias had identical or strikingly homologous IgH V regions, similar to the sterotypic antigen-binding sites observed in human B-CLL patients.

Similar transcription factor expression in AID^{high} and AID^{low} B-CLL cells

Somatic hypermutation of Ig V_H genes provides prognostic information for CLL patients [1],[2]: whereas hypermutation is predictive of a more favorable prognosis, lack of mutation predicts a poor prognosis. However, both cases identified with somatic hypermutation manifested high splenic cellularity (0.4 and 1.4 x 10^9 cells; see supplementary Table 1).

We investigated AID expression in purified splenic CD19^{+} B cell fractions from leukemic mice by RT-PCR. We found that AID expression was increased in a subset of these CLLs, when compared to B cell populations from wild-type or non-leukemic IgH.T/IgH.TEμ mice (Figure 7A). This AID^{high} subset included leukemic samples expressing unmutated VH11, while AID expression in the two samples with high levels of SHM was low. The AID^{high} B-CLL subset did not show late onset or slow disease progression, when compared to AID^{low} B-CLL.

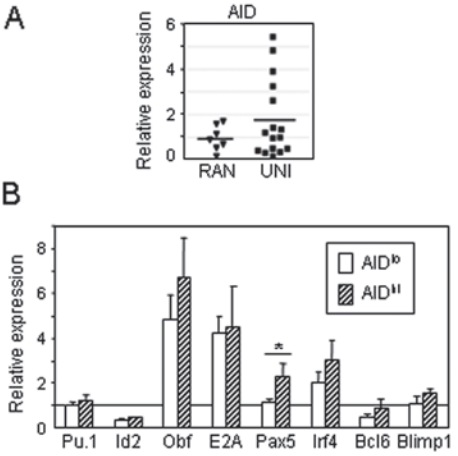

Figure 7. Similar transcription factor expression in AID^{high} and AID^{low} CLL cells
(A) Quantitative RT-PCR analysis of AID expression in RAN (n=7) and UNI (n=16) purified CD19^{+} B cell fractions. Expression was normalized with HPRT and relative expression in wild-type B cell fractions was set to 1. (B) Expression of indicated transcription factors as measured by quantitative RT-PCR in AID^{high} (n= 5; relative expression >2, see panel (A)) and AID^{low} (n=11; relative expression <2). Expression was normalized with HPRT and relative expression in RAN B cell fractions was set to one. Mean values and SEM are given. Asterisk indicates significant differences between AID^{low} and AID^{high} CLL cells ($p<0.001$; t-test).

Finally, we used quantitative RT-PCR to analyze the AID^{low} and AID^{high} subsets for the expression of transcription factors implicated in B cell activation and terminal differentiation. When compared with untransformed B cells, leukemic IgH.T/IgH.TEμ B cells showed markedly increased expression of the transcription factors OBF and E2A (consistent with the reported increased E2A expression in human CLL(33)), while expression of Id2, an inhibitor of E2A, was reduced. Expression of IRF-4, which correlates with clinical outcome in B-CLL patients(34), was ~2-3 times increased. Overall, AID^{low} and AID^{high} leukemia samples did not manifest significant differences in the expression of the transcription factors analyzed. Only expression of Pax-5, a B cell-specific factor that is downregulated upon terminal differentiation, was significantly higher in AID^{high} leukemia samples. This finding does not necessarily indicate that AID^{low}

have differentiated further towards plasma cells, as they did not show higher levels of Blimp-1, which is highly expressed in plasma cells.

Taken together, B-CLL cells with unmutated V regions express substantial levels of AID transcripts, which parallels findings in human B-CLL. Moreover, AID^{low} and AID^{high} B-CLL manifest similar transcription factor expression patterns, supporting a common origin of the two B-CLL subsets.

Discussion

We describe the generation of a B-CLL mouse model based on sporadic SV40 large T antigen expression in mature B cells. Leukemic cells present in these mice displayed many characteristics also found in human B-CLL, in particular in the subgroup of patients with germline IgH V regions associated with poor prognosis. These features include (i) a mature $CD19^{+}CD5^{+}$ phenotype, (ii) V_H regions with predominantly germline-encoded sequences, (iii) non-stochastic V_H-family usage, and (iv) CDR3 regions with high serine/tyrosine content.

The predominant use of V_H11 in our IgH.TEm leukemic cells cannot result from random transformation of $CD5^{+}$ B-1 cells, because only ~10% of this B cell population use V_H11(35). It rather suggests the involvement of (auto)antigen-driven selection. Increased V_H11 usage was also found in clonal expansions of splenic $CD5^{+}$ B lymphocytes in aging mice(30). Moreover, the V_H11- DFL16.1- J_H1 H chain present in leukemias T4 and T20 was identical to the published CLL clone TCL1-005 from a Tcl1 transgenic mouse(36). Thus, the B-CLL cells express a restricted B-cell receptor (BCR) repertoire with several cases of (nearly) identical CDR3s. In this respect, our model replicates the human B-CLL BCR sequences that closely resemble those of known autoreactive or polyreactive antibodies. We found that V_H regions were either unmutated, with preferential usage of the VH11 family, or manifested extensive somatic hypermutation and used VHJ558. The finding of high AID expression in unmutated VH11-expressing CLL - together with the similar transcription factor expression patterns in AID^{low} and AID^{high} leukemia samples - would support a common post-GC origin of both CLL subsets.

In our IgH.T/IgH.TEμ models expression of SV40 large T antigen was achieved by insertion of a promoterless SV40 T gene in opposite transcriptional orientation between the D en J_H segments. We detected SV40 T transcripts in mature B cells of IgH.T/IgH.TEμ mice, most likely from those rare targeted IgH alleles that have retained their germ-line configuration. The SV40 large T antigen is probably expressed as part of an anti-sense transcript within the D-J_H region of the IgH locus, similar to the anti-sense transcripts described in pro-B cells(18). The apparent absence of pro-B cell leukemias in IgH.T/IgH.TEμ mice, indicates that transient expression of the SV40 T gene (until it

is lost as a result of D-to-J_H recombination) did not result in malignant transformation of pro-B cells. Likewise, we did not detect early T-cell lineage leukemias, despite the presence of IgH chain anti-sense transcription in early thymocytes(18). From the finding of equal usage of the targeted and the wild-type IgH allele, we conclude that insertion of the T gene or the additional Eμ element did not affect V(D)J recombination. However, as IgH.TEμ mice manifested a substantially higher tumor incidence than IgH.T mice, the presence of an extra copy of Eμ within the D-J_H region may increase the level of anti-sense transcription. Thus, efficient transformation might be dependent on synergistic effects of Eμ and the IgH 3' enhancers(37) that lead to upregulation of SV40 T expression in more mature B cell stages. Conversely, the absence of transformation of pre-B cells or immature B cells that have retained the SV40 T gene could be explained by a level of T expression that is below the threshold for transformation(38-39). It is also possible that transformation requires secondary events that only take place in mature B cells.

The phenotypes of the leukemias in our mouse models resemble those found in Eμ-TCL1 mice, which express the T-cell leukaemia/lymphoma-1 proto-oncogene under the control of the Eμ element and an Ig V_H promoter(40). TCL1 has been functionally linked to enhanced PKB/Akt-mediated signaling pathways involved in cell proliferation and survival. In contrast, SV40 large T protein interacts with numerous cellular proteins and pathways, most notably the Rb and p53 pathways(10, 13, 41-42), but may also induce cell survival via Akt activation(43). In our mouse model, animals that lack p53 show accelerated tumor formation, even though SV40 large T inactivation of p53 is thought to be necessary to generate lymphoid tumors(44). This implies that in the presence of p53, some of the potential transforming properties of large T are inhibited or diminished, as was shown in a mouse model of pancreatic islet carcinogenesis(38)·(45). Together with the existing communication and coordination between the p53 and Akt pathways, it is very well possible that the mechanisms of malignant transformation by SV40 T antigen and TCL1 are related. When compared to the Eμ-TCL1 mice, in which V_H regions of leukemias consistently differed only marginally (<2.0%) from the germ-line, our IgH.TEμ/IgH.T mouse models are unique in that – next to leukemias with unmutated BCR - we also observed leukemias with extensive SHM (Table 1). It is presently not clear whether this difference between the Eμ-TCL1 and IgH.TEμ mice would point to differences in the mechanism of malignant transformation or would be related to secondary events in leukemia formation.

Table 1. Characterization of IgH chains of B-CLL.

Tumor #	IMGT[1] VH Segment	VH Family	% difference from GL[2]	N, P	DH Segment	N, P	JH Segment	DH RF	CDR3	CDR3 Length	pI[2]
T1	VH11-2	VH11	0	-	DST4.2	-	JH1	3	MRYSNYWYFDV	11	6.4
T5	VH11-2	VH11	0	-	DST4.2	-	JH1	3	MRYSNYWYFDV	11	6.4
T20	VH11-2	VH11	0	-	DFL16.1	-	JH1	3	MRYGSSYWYFDV	12	6.4
T23	VH11-2	VH11	0	-	DSP2.1	-	JH1	3	MRYGNYWYFDV	11	6.4
T4	VH11-2	VH11	0	-	DFL16.1	-	JH1	3	MRYGSSYWYFDV	12	6.4
T9	VH1-18	VHJ558	0	aggg	DFL16.1	gtagg	JH3	3	ARRDYGSSYVGWFAY	15	9.0
T16	VH1-55	VHJ558	1	ggg	-	-	JH2		ARGFDY	6	6.4
T3	VH1-58	VHJ558	0	gagagagtg	DSP2.2	ctgg	JH3	3	ARERVYDYDLAWFAY	15	4.7
T23	VH1-69	VHJ558	6,8	t	DFL16.2	t	JH2	3	ARYDYYYYYC	9	6.3
T32	VH1-74	VHJ558	5,8	gggat	D3-3	-	JH4	3	ARDDPLGYAMDY	12	4.1

IMGT indicates the international ImMunoGeneTics information system (www.imgt.org); GL, germline; pI, isoelectric point; —, not applicable; RF, reading frame; VH, Ig H chain V segment; N, P, N,P nucleotides; JH, Ig H chain J segment; and DH, Ig H chain D segment.

Although evidence accumulated that the V(D)J recombinase system is capable of mediating transposition of cleaved signal ends into non-specific sites in the genome, both *in vitro* and *in vivo*(19, 21), we did not find evidence for transposition events: all CLLs analyzed had retained the T gene in the IgH locus, which still had the germline configuration. Obviously, we cannot exclude that in some B cells transposition did take place, but with tranposition into transcriptionally inactive regions and therefore not resulting in leukemia. Even though transposition has been shown to occur *in vivo* (21, 23, 46) its frequency is very low. Therefore, our mouse model would argue for the existence of mechanisms that very effectively protect against transposition *in vivo*(47).

To be able to investigate the molecular mechanisms involved in CLL pathogenesis, next to the New Zealand Black mouse strain which is a naturally occurring model of late-onset CLL, several transgenic mouse models have been generated (Reviewed in Ref. (48)). Because in human B-CLL is a heterogeneous disease, each of the current models will provide invaluable knowledge about the molecular mechanisms of transformation in B-CLL. As a pre-clinical model to test novel treatment strategies for CLL, our leukemia model has the advantage that tumor progression can be monitored by an IgM allotype-specific flow cytometry assay. Finally, gene profiling studies have shown that the SV40 T antigen genetic signature, is (i) composed primarily of genes regulating cell replication, proliferation, DNA repair, and apoptosis, (ii) does not reflect a general cancer signature, (iii) is uniquely activated primarily in tumors with aberrant expression of p53, Rb, or BRCA1 and not in tumors initiated through Myc or ras overexpression(49). As human breast, lung, and prostate tumors expressing this set of genes represent subsets of tumors with the most aggressive phenotype(49), our SV40 large T-dependent CLL model should allow the identification of genes that are associated with treatment-resistance and poor prognosis.

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Chapter VIII

General discussion

The focus of this thesis is on the role of the pre-BCR signaling molecules Btk and Slp65 in early B cell development and the mechanisms by which Btk/Slp65-deficient pre-B cells become malignant. By using genetically modified mice, signal transduction in pre-B cells is put into an *in vivo* perspective regarding the regulation of V(D)J recombination activity, developmental progression and cellular proliferation. Furthermore, by understanding the mechanisms which are responsible for malignant transformation during B cell development insight is given into the forces that control genomic stability in general.

In this thesis we describe several surprising findings. First, the restricted usage of V_H14 gene segments in Slp65-deficient tumors which could not be explained by a selective proliferative advantage but was determined by aberrant Ig H chain accessibility (Chapter III). Second, our findings revealed striking parallels in pre-B cell tumor formation between Slp65-deficient and Eμ-myc transgenic mice (**Chapter III).** Third, although several lines of evidence point to the possible involvement of V(D)J recombination, we show that V(D)J recombination activity is not necessary for malignant transformation of Slp65-deficient pre-B cells (Chapter V). Last and remarkably, we found that deficiency of the DNA repair protein Rad54 protects Slp65/Btk-double deficient pre-B cells against malignant transformation and reduces the frequency of genetic aberrations **(Chapter VII)**.

Btk and Slp65 and the induction of cellular maturation and Ig L chain recombination
By studying the possible involvement of Btk and Slp65 in cellular maturation and in the initiation of Ig L chain recombination, we found that Btk or Slp65 single deficient pre-B cells have a specific defect in Ig λ L chain germline transcription (**Chapter II**). In addition, Btk/Slp65-double deficient pre-B cells have an additional defect in Ig κ L chain germline transcription. This defect is not due to reduced cell survival, as it cannot be corrected by enforced expression of a Bcl2 transgene. Our experiments did not discriminate between a function for signals transmitted by Btk and/or Slp65 directly in the opening of the Ig L chain loci for recombination or an indirect role for Btk/Slp65-mediated signals in the induction of the pre-B cell differentiation program. Recent findings of Jumaa and colleagues suggest that Foxo transcription factors connect pre-BCR signaling to the activation of the recombination machinery (1). They found that signaling through PI(3)K and Akt, which induces proliferation, negatively regulates Foxo3a and Foxo1 activation and consequently impairs Ig κ chain rearrangement in developing B cells (**Figure 1**). In addition, Foxo1 directly activates the transcription of the *Rag1/Rag2* locus throughout early B cell development (2). The main targets of the Akt kinases are the Foxo proteins, which have crucial roles in a wide spectrum of biological processes, including lymphocyte development. Interestingly, loss or gain of Foxo function can alter cell fate and lead to tumorigenesis (3-4). Thus, signaling via the pre-BCR recruites and phosphorylates Slp65, which inhibits activation of PI(3)K and Akt and thereby promotes

the accumulation of Foxo proteins to activate Rag1/Rag2 transcription (5) (**Figure 1)**.

Activation of Ig L chain recombination is associated with germline transcription (6-8) and it is not clear how the Foxo proteins induce germline transcription. Germline transcripts originate from promoters upstream of recombining segments in a developmental and tissue-specific manner (6, 9). Blocking transcription from a germline promoter in the TCRα locus prevented rearrangements of downstream Jα segments (10). Therefore, it is thought that germline transcription does not simply reflect open chromatine structure and accessibility of the locus for recombination, but rather is required for recombination to occur. Since pre-BCR signaling results in the rapid onset of germline transcription on both Ig k alleles (11), it is conceivable that Foxo proteins directly bind to regulatory elements within the Ig L chain locus. Alternatively, activation of Ig L chain gene recombination events by Foxo proteins could be an indirect effect.

An interesting candidate involved in the activation of the Ig L chain locus for recombination is Irf-4. This transcription factor binds the Ig 3'Eκ and Eλ enhancers and also regulates expression of the chemokine receptor Cxcr4, which induces migration of pre-B cells away from stromal cells expressing IL-7 (12). Another interesting candidate is the transcription factor E2A, since several lines of evidence implicate the E2A factors E47 or E12 in Ig L chain recombination. First, expression of E47 or E12 is sufficient to activate germline Ig κ transcription and recombinase accessibility in non-lymphoid cells (13-14). Second, E47 expression induces Ig κ VJ gene rearrangements (15). Third, The E2A proteins initiate Ig κ VJ gene rearrangements by directly binding to the E-box sites located within the iEκ and 3'Eκ (16). Fourth, E12 and E47 play critical roles in promoting Ig λ germline transcription, as well as Ig λ V-to-J gene rearrangement. Finally, attenuation of IL-7R signaling elevates E12 and E47 expression (17). More studies are necessary to determine how these different transcription factors interact with each other, in particular as Irf-4 has been shown to bind cooperatively with the E proteins to sites present in the Ig κ enhancers (12).

Not only interactions between transcription factors regulate targeting of V(D)J recombination activity, but also alterations in chromatin structure. The Rag1/Rag2 complex interacts with histone H3 trimethylated at the lysine position 4 (H3K4me3) and gene segments poised to undergo V(D)J recombination are enriched for H3K4me3 (18-20). The functional importance of the Rag2-H3K4me3 interaction for V(D)J recombination provides a new insight into the potential function of germline transcription. Trimethylating H3K4 requires transcription (21). Therefore, the main function of germline transcription might be to mark Ig loci with H3K4me3 to allow efficient V(D)J recombination. Furthermore, H3K4me3 plays an important role in recruiting recombination centers (22). Rag2 binds at thousands of sites in the genome containing H3K4me3, while Rag1 binding is only detected at regions containing RSS. Thus, Rag protein binding occurs in a highly focal manner to a small region of active chromatin.

More studies are required to determine how Slp65 and Btk activate germline transcription and Ig L chain recombination, taking into account that Slp65 and Btk both act together (23), but also have independent functions (24).

Mice deficient for important pre-BCR signaling molecules, such as Syk, Slp65, Btk or Plcγ2 show only a partial block at the pre-B cell stage. Analyses of compound knock-out mice have indicated that homologous signaling proteins, which were previously known to be important in other cell lineages, also contribute to pre-BCR signaling. The partial block in early B cell development in Slp-65-deficient mice (25-28) can be complemented by Slp76 and Lat (linker for activation of T cells), both of which are well-known adaptor proteins essential for signaling from the T cell receptor (28-31). In addition, Zap-70, the only other known Syk family kinase, can compensate for Syk (32). This requirement was unexpected as Zap-70 was previously reported to be expressed exclusively in T cells and natural killer cells but not in B cells. Likewise, the Btk homologue Tec has the ability to compensate for defective Btk function in pre-B cells in vivo (33) and Plcγ1 can compensate for loss of Plcγ2 (34). Therefore, activation of Plcγ1 and Plcγ2 in pre-B cells can occur via two parallel pathways: one containing Syk, Slp65 and Btk and the other containing Zap-70, Slp76, Lat and Tec.

Tec has a role during B cell development in the generation and/or function of the peripheral B cell pool and Tec and Btk have been shown to have redundant functions in B cell development and activation (33). The defects in early B cell development in Btk/Tec double-deficient mice have not been studied in detail. Hence, it would be interesting to study the possible role of Tec in the induction of germline transcripts of the Ig κ and λ L chain loci, in the termination of pre-B cell proliferation and as a tumor suppressor. We have recently crossed mice deficient for Btk, Tec and Slp65. Our first results indicate that Btk and Tec cooperate in the regulation of Ig light chain accessibility, because we observed that Btk/Tec double-deficient pre-B cells had severely reduced Ig κ and λ germline transcripts (V.T., unpublished results). In contrast to Btk, loss of Tec did not lead to an increase in the proliferative response to IL-7 *in vitro*. Furthermore, we observed that Tec did not function as a tumor suppressor and did not collaborate in this context with Slp65 (V.T., unpublished results).

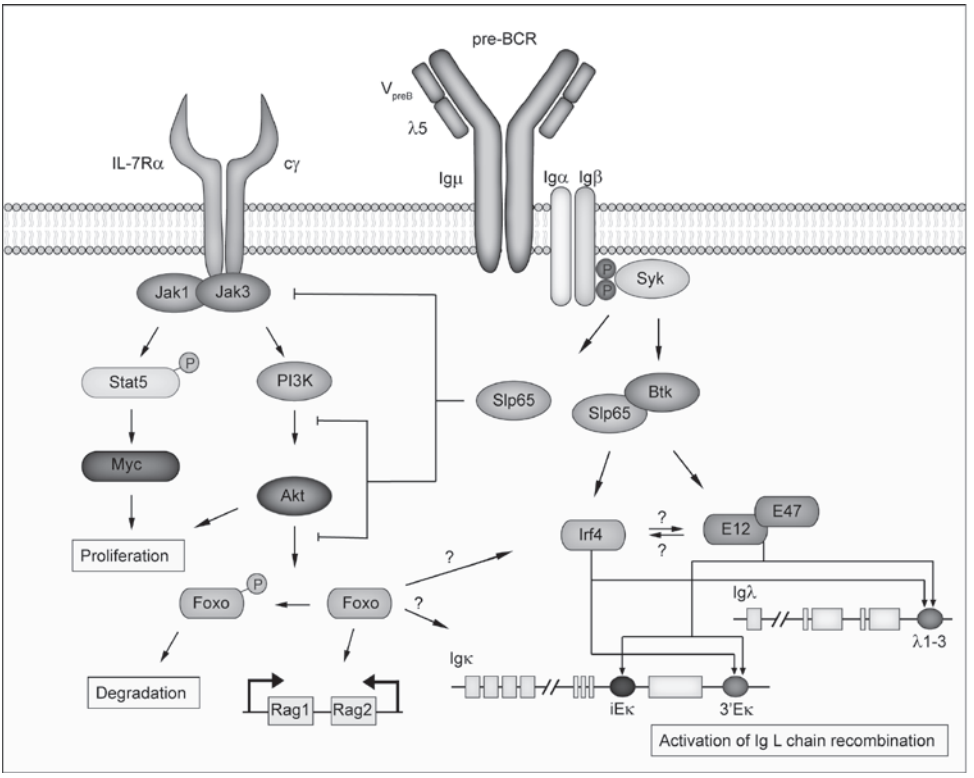

Figure 1. Regulation of Ig L chain recombination
Interaction between pre-BCR signaling and IL-7R signaling in pre-B cells. The figure depicts signaling pathways and transcriptional regulators that are required for proliferation and Ig L chain recombination at the pre-B cell stage. Arrows represent positive regulation and barred lines represent repression. Slp65 plays a central role in inducing light chain recombination, by counteracting Akt activation and thereby promoting activation of Foxo proteins, and inhibition of proliferation by inhibition of Jak3 and PI(3)K-Akt signaling. **See Appendix for full-color figure.**

Btk and Slp65 and pre-BCR-mediated selection

Pre-BCR signaling is different from BCR signaling in that BCR signaling is induced by antigen binding. The non-Ig-like tail of the SLC component λ5 induces ligand-independent pre-BCR cross-linking and as a result cell-autonomous signaling, which is essential for pre-B cell expansion (35). Recently, Jumaa and colleagues show that functional pre-BCR formation and autonomous signaling requires the N-linked glycosylation site in the C_H1 domain of Ig μ H chain (N46) and that crosslinking is mediated by binding of the λ5 SLC to N46 autonomous (36). This implies an unexpected mode of pre-BCR function in which binding of the SLC to N46 mediates autonomous crosslinking and receptor formation.

The importance of a ligand for the pre-BCR is not clear; it is possible that a ligand exists on stromal cells, on other pre-B cells or on the membrane of the same cell. This seems likely since the pre-BCR shapes the V_H repertoire at the transition from pro-B to large pre-B cells (37). Furthermore, several groups have identified interactions between the non-Ig tail of λ5 and galectin-1 (38) or stromal-cell-associated heparin sulphate (39). In addition, the pre-BCR is a polyreactive receptor and capable of recognizing multiple (self-)antigens, including DNA, LPS and insulin, via the non-Ig part of λ5 (Kohler 2008). It is unlikely that foreign antigens play a major role in initiation of pre-BCR signaling. First, pre-B cells are selected for the expression of a functional Ig μ H chain which can pair with the SLC (40-42). This is a selection different from BCR selection, when B cells are selected for appropriate antigen specificity. It would be a highly inefficient mechanism when selection relies on the availability of foreign antigens in the BM that can interact with V_H regions of pre-BCRs. Secondly, the antigen specificity of the Ig μ H chain when paired with the SLC is different from the antigen specificity when paired with a conventional Ig L chain (43-44).

Because high-level pre-BCR expression on Slp65-deficient leukemic cells is thought to contribute to their strong proliferative capacity, we investigated whether particular pre-BCR specificities may predispose these cells to malignant transformation (**Chapter III**). We found that Slp65-deficient leukemias preferentially use a member of the V_H14 family (55 out of 60). By contrast, the CDR3 regions of these Ig H chains were heterogeneous in length and sequence. This restricted usage seems to be mainly based on aberrant Ig H chain accessibility favouring V_H14 segment recombination at the pro-B cell stage. Nevertheless, we currently cannot exclude the possibility that these particular V_H14 Ig H chains have unique structural properties, which could confer an advantage in proliferative expansion. This is, however, not supported by the findings of Van Loo et al., showing that constitutive expression of pre-BCR components throughout B cell development did not alter tumor frequencies in Slp65-deficient mice. These results imply that enhanced pre-BCR may not be important in the oncogenic transformation of Slp65-deficient cells, in constrast to consititutive IL-7R signaling.

Preferential usage of particular Ig H V_H gene families is not unique to Slp65-deficient pre-B cell leukemias in mice, but has been reported for several leukemias and lymphomas in human and mice, including chronic lymphocytic leukemia (CLL) (45-46). However, on the basis of the presence of stereotypic CDR3 regions and high levels of somatic hypermutation it has been hypothesized that preferential Ig H V_H gene usage in CLL reflects antigenic selection rather than preferential V_H gene segment recombination (45).

Interestingly, recent findings show that upregulation of Bcl6 by small pre-B cells is critical for the development of a diverse primary B cell repertoire (47). Phosphorylated Stat5 activates Myc and thereby repress Bcl6 (48), which functions as a transcriptional

repressor in normal and malignant GC B cells (49). It is conceivable that when Slp65-deficient leukemias acquire constitutive activation of JAK3/STAT5 signaling, mostly due to autocrine production of IL-7 (50), this would lead to repression of Bcl6 and the prevention of a diverse primary B cell repertoire. The mechanism by which Bcl6 is regulating B cell repertoire diversification is not known and requires additional research. Nevertheless, it would be interesting to investigate whether specific V_H gene segments have selective advantage at the pre-B cell checkpoint and what the molecular basis of the positive or negative selection process would be. To this end, creating and comparing pre-B cell differentiation in panels of knock-in mice carrying various autoreactive and innocuous Ig H chain V regions would be informative.

Disruption of the p19^{Arf}–Mdm2–p53 tumor suppressor pathway in Slp65 deficient pre-B cell leukemia

Recent findings show that Slp65 downregulates IL-7 mediated proliferation and survival through direct inhibition of Jak3 (50). The IL-7R pathway promotes cellular survival, proliferation and maturation, involving induction of the proto-oncogene c-Myc (51). B cell development can be partially restored in Jak-3-deficient mice when they are bred to mice co-expressing a rearranged Ig H chain transgene and a c-Myc transgene (52). Loss of Btk synergizes with deregulation of the c-Myc oncogene during lymphoma formation (53).

In Chapter IV, we found that disruption of the p19^{Arf}–Mdm2–p53 tumor suppressor pathway plays an important role in malignant transformation of Slp65-deficient pre-B cells. Our findings reveal striking parallels in pre-B cell tumor formation between Slp65-deficient and Eμ-*myc* Tg mice. First, in both models the expression of a pre-rearranged Ig H chain transgene in early B cell differentiation reduces oncogenic transformation (Chapter IV and (54)). Second, malignant transformation of both Slp65-deficient and Eμ-*myc* Tg pre-B cells involves disruption of the p19^{Arf}–Mdm2–p53 tumor suppressor pathway (Chapter IV and (55)). Third, somatic hypermutation-associated, Aid-induced DNA damage is not required for tumor development in either of the two models (Chapter IV and (56)). The finding that Slp65-deficient pre-B cell tumors display disruptions of the p19^{Arf}–Mdm2–p53 pathway implies that oncogenic transformation of Slp65-deficient pre-B cells does not exclusively result from sustained IL-7R signaling and endocrine IL-7 production. Slp65 regulates the activity of the forkhead-box transcription factors Foxo3a and Foxo1, which do not only promote Ig L chain recombination, but also suppress Myc-driven lymphomagenesis via direct p19^{Arf} activation (5, 57). These parallels suggest that oncogenic Myc activation contributes to malignant transformation of Slp65-deficient pre-B cells. In addition, disruption of the p19^{Arf}–Mdm2–p53 tumor suppressor pathway also plays an essential role in the pre-B cell transformation process mediated by Abelson murine leukemia virus (58).

There are also differences between the Slp65-deficient and Eμ-*myc* Tg tumor models. Many Eμ-*myc* lymphomas sustained either p53 or $p19^{Arf}$ loss of function and elevation of Mdm2 levels. Almost all Slp65-deficient or Btk/Slp65-double deficient leukemias expressed substantial levels of $p19^{Arf}$, suggesting inactivation of the p53 response, as p53 is a negative regulator of $p19^{Arf}$. Arf is highly expressed in many human tumors that contain p53 and in up to 40% of Burkitts lymphomas (59). $P19^{Arf}$ has also p53-independent functions, e.g it promotes the progression of lymphomas by mediating autophagy, a process of lysosome-mediated self-digestion that occurs during periods of nutrient deprivation (60-61). Autophagy plays a complex role in the initiation and progression of tumors. It appears that autophagy suppresses tumor initiation, but promotes the survival of established tumors (62). Silencing of $p19^{Arf}$ inhibits the progression of Myc-driven lymphoma cells containing mutant or no p53 (61). Thus, Slp65-deficient leukemias may retain $p19^{Arf}$ to promote survival under metabolic stress. Autophagy inhibitors chloroquine and 3-methyladenine are effective anti-tumor drugs for Burkitts lymphoma and chronic myeloid leukemia (63-65). Chloroquine-induced cell death was dependent on p53 but not on the modulators Atm or Arf (63). Therefore, it would be interesting to test the effect of small molecule inhibitors of autophagy on the survival of Slp65-deficient tumors and to administer Chloroquine to Slp65/Btk-double deficient mice and follow the mice for tumor free suvival.

It is tempting to speculate that transformation of Slp65-deficient pre-B cells is dependent on early oncogenic events that induce constitutive Jak3-Stat5 signaling at the pro-B cell stage. Furthermore, signaling via the IL-7R represses Bcl6 and its negative regulation of $p19^{Arf}$ (47). Therefore Slp65-deficient pre-B cells which have acquired constitutive IL-7R signaling will be cleared via the $p19^{Arf}$–Mdm2–p53 pathway which protects against malignant transformation. The dramatic increase in tumor incidence when both Slp65 and p53 are lacking supports this notion (Chapter IV). However, after initiation of malignant transformation, expression of $p19^{ARF}$ protects the cells against metabolic stress. Thus, p53 mediated apoptosis is an important mechanism in preventing transformation of Slp65 deficient pre-B cells.

This leads us to propose the following mechanism for malignant transformation of Slp65-deficient pre-B cells (Figure 2). The absence of Slp65 results in sustained expression of the pre-BCR and the IL-7R in large cycling pre-B cells. Since IL-7R signaling induces c-Myc this results in constitutively high levels of c-Myc. At this stage, Foxo transcription factors that normally suppress c-Myc-driven lymphomagenesis via direct activation of $p19^{Arf}$ are not properly activated because of the absence of Slp65. Furthermore, IL-7R signaling and aberrant pre-BCR signaling represses Bcl6 and thereby induces $p19^{Arf}$ expression. Subsequently, Slp65-deficient pre-B cells acquiring sporadic alterations in the $p19^{Arf}$–Mdm2–p53 are selected to become malignant. Furthermore, expression of $p19^{Arf}$ in Slp65-deficient leukemias possibly promotes

survival under metabolic stress. Malignant transformation of Slp65-deficient pre-B cells thus is not a one hit model, but a complex multistep process that requires different acquired mutations.

The role for Btk in suppression of malignant transformation remains unclear. It is conceivable that the increased frequency of malignant transformation in Slp65/Btk-double deficient mice reflects the increased pool size of proliferating pre-B cells. Mice which are deficient for Btk/Plc-γ2 and Irf-4/Irf-8, however, are arrested at the large pre-B cell stage. Most of these mutant pre-B cells express pre-BCR on their cell surfaces, but so far the development of pre-B cell leukemia has not been reported (66-67). Therefore, a complete block at the large pre-B cell stage is not sufficient to initiate malignant transformation. The absence of Btk might alter the proliferative capacity of Slp65-deficient pre-B cells. However, Btk does not cooperate with Slp65 in the suppression of the Jak3-Stat5 signaling pathway. Instead, Btk seems to play a role in the accumulation of $p27^{Kip1}$ and to induce cell cycle arrest (50). A third possibility would be that Btk influences the malignant transformation of pre-B cells by c-Abl. C-Abl is a Src family non-receptor protein tyrosine kinase (68). Alteration of the c-Abl structure and function as a consequence of chromosomal translocation (Bcr-Abl) results in fusion protein. This translocation accounts for the majority of cases of human chronic myeloid leukemia and a part of the human ALL cases (69-70). In human pre-B ALL Btk contributes to multiple aspects of the BCR-ABL1-driven survival signaling (71). Currently, the idea is that Slp65 and Btk may act on different levels and thereby synergistically increase the incidence of leukemias.

Deregulated V(D)J recombination is not essential for malignant transformation of Slp65-deficient pre-B cells

One of the fundamental features of lymphoid neoplasms is the presence of chromosomal translocations (72-74). Involvement of the V(D)J recombinase in lymphomagenesis was found when translocational breakpoints in certain tumors consisted of the antigen receptor loci fused with an oncogene (75-76). Because leukemic Slp65-deficient pre-B cells express Rag1/Rag2 and manifest ongoing immunoglobulin light chain rearrangement (23, 77-78), we hypothesized that deregulated recombinase activity contributes to malignant transformation. In Chapter V and VII we investigated the possible involvement of V(D)J recombination in two different models. First, by inactivation of Rag we found that the V(D)J recombinase is not essential for malignant transformation of Slp65-deficient pre-B cells (Chapter V). However, we can not exclude that V(D)J recombination does contribute to malignant transformation as the incidence of leukemias in our model of Btk/Slp65-double deficient mice with the autoreactive 3-83μδ transgene is much lower (~11%) than in Btk/Slp65-double deficient mice without this transgene (40-80%) (23, 79). Thus, V(D)J recombination may contribute to malignant

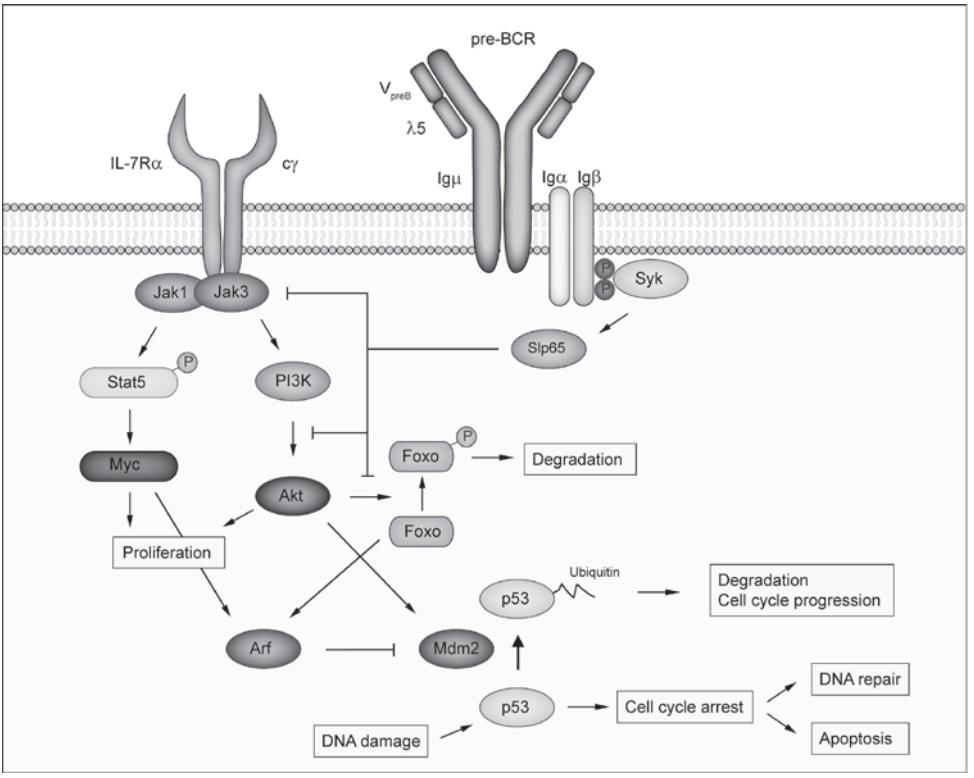

Figure 2. Model for tumor suppression in pre-B cells.
Signaling via Slp65 inhibits IL-7R signaling and induces activation of Foxo. Acativation of Arf occurs via Foxo proteins as well as through sustained and increased signaling induced by overexpressed Myc. Once expressed, the Arf protein interferes with the activity of Mdm2, leading to p53 stabilization and the triggering of a complex p53-dependent transcriptional program. The activation of p53 classically occurs in response to many other cellular stresses that produce DNA damage. Target genes induced by p53 can generate different biological outcomes depending on the tissue type and convergence of different activating signals. Arf induction primarily triggers cell-cycle arrest. **See Appendix for full-color figure.**

transformation but we have shown that it is not essential for leukemogenesis.

In addition, the ability of the Rag1/2 proteins to catalyze DNA translocation and transposition *in vitro* and *in vivo* (74, 80-83) prompted us to investigate in a mouse model the possibility of *in vivo* transposition of D-J_H circles containing the SV40 T oncogene and thereby leading to lymphomagenesis. In Chapter VII we did not find evidence for transposition events because all leukemias analyzed had retained the SV40 T oncogene in the Ig H chain locus in germline configuration. We obviously cannot exclude that transposition did not take place at all, because transposition into transcriptionally inactive regions probably does not lead to malignant transformation. In

our IgH.TEμ model all mice showed accumulation of a monoclonal population of $CD5^+$ leukemic B cells at 6-9 months of age. This model represents a new mouse model to study CLL, next to the mouse models for CLL that have been described in the literature. One of these is the NZB strain, which is a model for late-onset CLL. Another model is represented by, double transgenic mice overexpressing Bcl2 and dominant-negative TNFR-associated factor 2, which develop a clonal $CD5^+$ B-CLL resembling human CLL. Malignant $CD5^+$ B-cells also accumulate in transgenic mice expressing the T-cell leukemia gene TCL-1, which is a proto-oncogene functionally linked to enhancement of Akt-mediated signaling. Analysis of IGHV genes in TCL-1 transgenic CLL suggested that this model represents the more aggressive U-CLL subset (46).

Our model is unique as it gives rise to both molecular subsets, with either unmutated (aggressive CLL) or extensively mutated (indolent CLL) Ig H V regions. As CLL is a heterogeneous leukemia, the IgH.TEμ mouse model makes it possible to study which mechanism of transformation leads to either the aggressive or the more indolent form of CLL. In addition, this mouse model would be valuable to address the issue of how antigenic selection contributes to malignant transformation as leukemic cells present in these mice displayed many characteristics which were also found in human B-CLL (84-85).

In the IgH.Tμ model, expression of the SV40 large T antigen is probably expressed as part of an antisense transcript within the D-JH region of the Ig H chain locus (86). The absence of pro-B cell leukemias in IgH.TEμ mice indicates that transient expression of the SV40 gene does not result in malignant transformation of pro-B cells. Efficient transformation might depend on synergistic effect of the Eμ and the Ig H 3' enhancers that lead to upregulation of SV40 T expression in more mature B cell stages. It is possible that pre-B cells or immature B cells have a level of large T antigen expression which is below the threshold for transformation or that transformation requires additional events that only take place in mature B cells after antigenic stimulation.

Rad54-mediated genomic duplications contribute to malignant transformation in Slp65-deficient leukemia

Faithful DNA repair by diverse repair pathways contribute to maintaining chromosomal stability (87-88). Deficiency of proteins involved in DNA repair pathways lead to increased genomic instability and malignant transformation (89) (90-92). However, in **Chapter VI** we show that repair by Rad54 and homologous recombination operates on a razors edge. Homologous recombination ensures high fidelity repair, but our findings show that Rad54-mediated repair can lead to misrecognition and thus genomic instability. Therefore, in our model Rad54 does not function as a tumor suppressor but as an oncogene. It would be interesting to investigate whether Rad54 also functions as an oncogene in other tumor models next to our Slp65-deficient leukemia model.

Interestingly, comparative genomic hybridization analysis of Slp65-deficient leukemias showed genetic alterations which were found in all samples investigated. Investigation of these lesions could lead to the identification of new oncogenes or tumor suppressors which are involved in the malignant transformation of Slp65-deficient pre-B cells. In addition, analyses of genetic aberrations which differ between Slp65/Btk-double deficient leukemias and Slp65/Btk/Rad54-triple deficient leukemias will give insight into the mechanism by which Rad54 mediates duplications. In particular, cloning of breakpoints of the duplications may reveal specific requirements and characteristics of these areas.

The role of SLP65 and BTK in human ALL

The importance of SLP65 as a tumor suppressor in human ALL has still not been elucidated. Deficiency of SLP65 has been found in ~50% of childhood precursor-B cell ALL cases (93). The loss of SLP65 protein was found to be due to defective splicing, leading to premature stop codons. The SLP65 gene contains alternative exons (exon 3a and 3b) located in intron 3. When these alternative exons are included into the SLP65 mRNA they interrupt the open reading frame of SLP65 and prevent protein expression. Expression profiling studies with larger numbers of patients reported a lower frequency of SLP65 alterations (94-95). In the latter studies, protein expression of SLP65 was not analyzed in detail. Because in these cases detectable SLP65 mRNA expression could still be associated with defective splicing and reduced protein expression, this analysis does not exclude the possibility that loss of SLP65 could be a common leukemogenic event in pre-B ALL.

Cancer is caused by alterations in oncogenes, tumor suppressor genes and microRNA genes. MicroRNA genes can possibly regulate every cellular process. Aberrant microRNA expression is associated with the development and progression of tumors (96-98). Examples of the role of microRNA include miR-15a and miR-16-1, which are deleted or down-regulated in cases of CLL (99). Regions of the genome that are consistently involved in chromosomal rearrangements in cancer but that lack oncogenes or tumor suppressor genes appear to harbor microRNA genes (98). Therefore, it would be interesting to investigate microRNA which may suppress Slp65 and their expression in human ALL.

Concluding remarks

Although our understanding of the role of Slp65 and Btk as critical regulators of pre-BCR induced proliferation, differentiation and Ig L chain recombination has greatly increased, the nuclear targets of Slp65 and Btk mediated signaling are still largely unknown. To identify downstream target genes of Slp65 and Btk signaling, DNA microchip array comparisons should be performed of pre-B cells lacking Btk, Slp65

or both. As signaling networks in pre-B cells consist of many interactions, constructing global networks consisting of transcriptional regulators, signaling and survival factors are becoming more and more important. The first studies which perform genome wide analysis for occupancy of the transcription factors E2A, EBF1 and Foxo1 have recently been published (100). They described the pro-B cell stage in terms of a global network of transcription factors with confirmed previous connections and they identified previously unknown links and participants. This network is a starting point for further analyses that should include other key regulators (Pax5, Pu.1). This approach should also be applied to the large pre-B cell stage and the events unfolding after pre-BCR activation.

CLL is a heterogeneous leukemia and the IgH.TEμ mouse model makes it possible to study which mechanism of transformation leads to either the aggressive or the more indolent form of CLL. CLL cells must interact with stroma in BM or peripheral lymphoid tissues to survive. It would be interesting to explore these interactions as target for therapy, for instance to inhibit BCR signaling by anti-CD20 therapy. Furthermore, comparative genomic hybridization analyses should be performed to identify genomic alterations which differ between the aggressive (unmutated) and indolent (mutated) variant.

This thesis has revealed an unexpected role for the DNA repair protein Rad54, which next to its role in homologous recombination also seems to function as an oncogene, thereby contributing to oncogenic transformation. This new characteristic of Rad54 opens new perspectives and possibilities for research. It would be interesting to test the function of Rad54 in other tumor models.

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Chapter IX

Summary

B lymphocytes are necessary for humoral immunity against a wide variety of antigens. A prerequisite for the generation of an effective humoral response is the diversity and specificity of the antibody repertoire. Diversity of the antibody repertoire is generated by recombination of various gene segments at the immunoglobulin (Ig) heavy (H) and light (L) chain loci which occurs in an ordered manner. Ig in mice have a four chain structure and are composed of two identical H chains and two identical L chains. Signalling by membrane Ig governs a number of distinct checkpoints in B cell differentiation that shape the antibody repertoire by a combination of cellular and molecular selection. A central checkpoint is the pre-B cell receptor (pre-BCR), which monitors successful expression of the Ig H chain and signals for proliferation, differentiation and Ig L chain recombination. The adapter proteins Slp65 and the cytoplasmic tyrosine kinase Btk are key components of the signaling pathways downstream of the pre-BCR. In humans, mutations in *SLP65* or *BTK* result in defective pre-B cell proliferation and an almost complete arrest of early B cell development, associated with the immunodeficiency disorder agammaglobulinemia. Furthermore, in a substantial fraction of human pre-B acute lymphoblastic leukemia (ALL) SLP65 expression is defective due to aberrant splicing. In mice, deficiency for Slp65 or Btk results in a partial arrest of early B cell development. Importantly, 5-10% of Slp65-deficient mice develop pre-B cell tumours expressing high levels of the pre-BCR. Btk has been shown to cooperate with Slp65 as a tumour suppressor.

This thesis describes studies aimed at understanding the pre-BCR checkpoint and the role of the signalling proteins Btk and Slp65 in pre-B cell differentiation, proliferation and the suppression of malignant transformation.

By studying the possible involvement of Btk and Slp65 in cellular maturation and in the initiation of Ig L chain recombination, we found that Btk or Slp65 single deficient pre-B cells have a specific defect in Ig λ L chain germline transcription (**Chapter II**). In addition, Btk/Slp65-double deficient pre-B cells have an additional defect in Ig κ L chain germline transcription. This defect is not due to reduced cell survival, as it cannot be corrected for by enforced expression of a Bcl2 transgene. Therefore, we analyzed transgenic mice expressing the constitutively active Btk mutant E41K. The expression of E41K-Btk in Ig H chain-negative pro-B cells induced 1) surface marker changes that signify cellular differentiation, including down-regulation of the surrogate L chain pre-BCR component and up-regulation of CD2, CD25, and MHC class II; and 2) premature rearrangement and expression of κ and λ light chains. These findings demonstrate that Btk and Slp65 transmit signals that induce cellular maturation and Ig L chain rearrangement independently of their role in termination of pre-B cell expansion.

Similar to Slp65-deficient mice Eμ-*myc* transgenic (Tg) mice develop rapid-onset pre-B cell malignancies. Myc-induced lymphomagenesis often disrupts the $p19^{Arf}$–Mdm2–p53 circuitry, indicating that c-Myc activation strongly selects for spontaneous

inactivation of this pathway. In **Chapter III** we investigated whether malignant transformation of Slp65-deficient pre-B cells also requires loss of the protective checkpoint function of the $p19^{Arf}$–Mdm2–p53 pathway. We found that disruption of the $p19^{Arf}$–Mdm2–p53 tumour suppressor pathway plays an important role in malignant transformation of Slp65-deficient pre-B cells. Our findings reveal striking parallels in pre-B cell tumour formation between Slp65-deficient and Eμ-*myc* Tg mice. First, in both models the expression of a pre-rearranged Ig H chain transgene in early B cell differentiation reduces oncogenic transformation. Second, malignant transformation of both Slp65-deficient and Eμ-*myc* Tg pre-B cells involves disruption of the $p19^{Arf}$–Mdm2–p53 tumor suppressor pathway. Third, somatic hypermutation-associated, Aid-induced DNA damage is not required for tumour development in either of the two models. These data indicate that malignant transformation of *Slp65*$^{-/-}$ pre-B cells involves disruption of the $p19^{Arf}$–Mdm2–p53 tumour suppressor pathway.

Because high-level pre-BCR expression on Slp65-deficient leukemic cells is thought to contribute to their strong proliferative capacity, we investigated whether particular pre-BCR specificities may predispose these cells to malignant transformation (**Chapter IV**). DNA sequence analysis of Ig H chain V regions of Slp65-deficient leukemias revealed that Slp65-deficient leukemias preferentially use a member of the V_H14 family (in 55 out of 60 leukemias analysed), despite the small size of this V_H family. The CDR3 regions of these Ig H chains were heterogeneous in length and sequence. When combined with surrogate light chain or various conventional light chains, yielding pre-BCR and BCR, respectively, the V_H14 IgH chains did not exhibit enhanced poly- or autoreactivity. It is therefore not likely that the V_H14 IgH chains provide enhanced proliferative signals. Slp65-deficient leukemia cells also exhibited preferential V_H14 segment usage on non-productively rearranged IgH alleles, strongly indicating altered IgH chain V region accessibility. We therefore conclude that malignant transformation of Slp65-deficient pre-B cells occurs almost exclusively in cells with aberrant IgH chain accessibility favouring V_H14 segment recombination at the pro-B cell stage.

Because leukemic Slp65-deficient pre-B cells express Rag1/Rag2 and manifest ongoing immunoglobulin L chain rearrangement we hypothesized that deregulated V(D)J recombinase activity contributes to malignant transformation. In **Chapter V** we investigated the possible involvement of V(D)J recombination in the malignant transformation of Slp65-deficient pre-B cells. We employed Btk/Slp65 double-deficient mice carrying an autoreactive 3-83μδ BCR transgene. When developing B cells in their bone marrow express this BCR, the V(D)J recombination machinery will be activated, allowing for secondary Ig L chain gene rearrangements to occur. This phenomenon, called receptor editing, will rescue autoreactive B cells from apoptosis. We observed that 3-83μδ transgenic Btk/Slp65 double-deficient mice developed B cell leukemias expressing both the 3-83 μδ BCR and the pre-BCR components λ5/VpreB. Such

leukemias were found at similar frequencies in mice concomitantly deficient for Rag1 or the non-homologous end-joining factor DNA-PKcs. We therefore conclude that malignant transformation of Btk/Slp65 double-deficient pre-B cells is independent of deregulated VD)J recombination activity.

In Slp65-deficient mice, pre-B cell tumors arise from large, cycling pre-B cells which are highly proliferative. It is therefore conceivable that genomic instability in these cells may accompany cell division, ultimately leading to oncogenic transformation. Therefore, we investigated in **Chapter VI** whether homologous recombination (HR), the pathway which repairs DNA double strand breaks (DSB) in cycling cells, plays a role in the malignant transformation of Slp65-deficient pre-B cells. Remarkably, we found that deficiency of Rad54 protected Slp65/Btk-double deficient pre-B cells from malignant transformation, since Slp65/Btk/Rad54-triple deficient mice manifested reduced tumour incidence when compared with Slp65/Btk-double deficient mice. Comparative genomic hybridization analysis of pre-B cell leukemias from Slp65/Btk/Rad54 triple deficient mice showed a reduced frequency of genetic aberrations, when compared with those from Slp65/Btk-double deficient mice. In particular, many gene duplications abundant in Slp65/Btk-double deficient pre-B cell tumours, ranging in size up to 600 kb, were not present in the concomitant absence of Rad54. These results indicate that repair via Rad54 and HR operates on a razors edge: although it has been assumed that repair by HR ensures high fidelity repair, our findings indicate that Rad54-mediated HR results in misrecognition and disrepair. We therefore conclude that Rad54-mediated HR in the absence of Slp65 and Btk has the capacity to contribute to oncogenic transformation.

In **Chapter VII** we investigated in a mouse model the possibility of *in vivo* transposition of D-J_H circles containing the SV40 T oncogene and thereby leading to lymphomagenesis. Although B-cell development was unperturbed in young mice, aging mice showed accumulation of a monoclonal B-cell population in which the targeted Ig H allele was in germline configuration and the wild-type Ig H allele had a productive V(D)J recombination. These leukemic B cells were $IgD^{low}CD5^{+}$ and manifested nonrandom usage of V, D, and J segments. V_H regions were either unmutated, with preferential usage of the V_H11 family, or manifested extensive somatic hypermutation. Our findings provide an animal model for B-CLL and show that pathways activated by SV40 T antigen play an important role in the development of a monoclonal population of $CD5^{+}$ leukemic B cells. We did not find evidence for transposition events because all leukemias analyzed had retained the SV40 T oncogene in the Ig H chain locus in germline configuration. We cannot exclude that transposition did not take place at all, because transposition into transcriptionally inactive regions would probably not lead to malignant transformation.

In conclusion, we obtained important information about the role of Slp65 and Btk at the pre-BCR checkpoint involved in differentiation, proliferation and suppression of malignant transformation. This information will be helpful for the identification of new

markers for early diagnosis and prognosis for XLA, ALL and CLL. Clinical research has proved that markers for early and detailed diagnosis, stratification and prognosis are of high value for the development of tailormade and more effective treatments for these diseases. Moreover, our studies revealed a remarkable new characteristic for the DNA repair protein Rad54 as it contributes to malignant transformation. This finding may change the general idea about the tumor suppressive function of repair proteins and it opens new perspectives for future research.

Nederlandse samenvatting

Leukemie wordt in Nederland bij ongeveer 1600 mensen per jaar vastgesteld. Leukemie is de verzamelnaam voor verschillende soorten beenmergkanker waarbij er sprake is van ongecontroleerde deling van witte bloedcellen. Er ontstaan grote hoeveelheden witte bloedcellen die vaak niet rijp zijn en hierdoor komt de productie van normale bloedcellen in het beenmerg in het gedrang. Er kan onderscheid worden gemaakt tussen twee vormen:

- acute leukemieën waarbij de kwaadaardige cellen onrijp zijn;
- chronische leukemieën met een opeenhoping van redelijk uitgerijpte cellen.

Leukemie ontstaat via celdelingen uit één voorlopercel en wordt als een klonale ziekte beschouwd. Zo kunnen alle leukemiecellen dezelfde genetische afwijkingen hebben. Veel voorkomende afwijkingen zijn translocaties waarbij stukken erfelijk materiaal (DNA) zijn verplaatst van het ene chromosoom naar een ander chromosoom. Hoe leukemie ontstaat, is nog grotendeels onbekend. Kwaadaardige ontsporing staat in verband met veranderingen in de functie van verschillende genen, zogenoemde pro-tumorgenen en tumorsuppressorgenen die zijn betrokken bij celdeling en celrijping. Enkelvoudige veranderingen in het genetische materiaal leiden niet tot de ontwikkeling van leukemie, maar een reeks van opeenvolgende veranderingen kan zorgen voor kwaadaardige ontsporing.

In dit proefschrift hebben we precursor-B (pre-B) cel leukemie in een muizenmodel bestudeerd en onderzocht wat het mechanisme achter kwaadaardige ontsporing is. Muizen die de tumorsuppressoreiwitten Slp65 en Btk missen ontwikkelen vanaf 2 maanden pre-B cel leukemieën. Ook zijn er aanwijzingen dat in ~50% van de pre-B cel leukemieën bij kinderen het tumorsuppressoreiwit Slp65 defect is. Hoewel er verscheidene genetische afwijkingen zijn gevonden in bepaalde leukemieën, is er over het algemeen nog weinig bekend over de oorzaak van dergelijke tumoren. Leukemieën zouden bijvoorbeeld kunnen ontstaat door storingen in de regulatie van de celdelingsactiviteit of door defecten in het DNA schade herstel mechanisme.

De eiwitten Slp65 en Btk zijn betrokken bij signalering op het pre-B cel stadium en zorgen voor het stoppen van een fase van sterke celdeling. B cellen zijn witte bloedcellen die onderdeel uitmaken van het immuunsysteem en uiteindelijk uitrijpen tot plasmacellen, die antistoffen kunnen maken die bescherming kunnen bieden tegen een infectie. B cellen ontwikkelen zich in het beenmerg en tijdens dit proces zijn er diverse fasen te onderscheiden waarin de voorlopers van de B cellen een groot aantal celdelingen doormaken. Tijdens andere fasen delen deze cellen zich juist niet of maken ze specifieke veranderingen door. Om uiteindelijk de productie van antistoffen ofwel immuunglobulinen mogelijk te maken, zijn herschikkingen in het DNA nodig, waarbij op een nauwkeurig gereguleerde manier breuken in de genen die coderen voor de immuunglobuline eiwitketens worden aangebracht. Dit gebeurd door een uniek zogenoemde V(D)J-recombinatie systeem en – nadat tussenliggende stukjes DNA

zijn weggenomen - worden de gevormde breuken vervolgens weer hersteld door DNA schadeherstel eiwitten.

In de verschillende hoofdstukken van dit proefschrift hebben wij onderzocht wat er fout gaat in cellen die het eiwit Slp65 niet hebben. Naast het signaleringseiwit Slp65 zijn wij ook geïnteresseerd in het sigaleringseiwit Btk, omdat beide eiwitten betrokken zijn op hetzelfde stadium in B cel ontwikkeling en muizen die beide eiwitten niet hebben sneller en eerder tumoren ontwikkelen.

In hoofdstuk II hebben wij de rol van Btk en Slp65 in celrijping en het starten van DNA herschikkingen van genen die coderen voor de immuunglobuline eiwitketens beschreven. Immuunglobulines bestaan uit twee zware en twee lichte eiwitketens die samen een antigeen kunnen binden. Eerst worden de genen voor de zware ketens herschikt en vervolgende de lichte keten genen. Wij zijn tot de ontdekking gekomen dat Btk en Slp65-deficiënte B cellen een defect hebben in de activatie van de genen die coderen voor het immuunglobuline lichte keten eiwit en dat dit effect onafhankelijk is van celdeling en overleving. Hiermee tonen wij aan dat Btk en Slp65 betrokken zijn bij het toegankelijk maken van de immuunglobuline lichte keten genen en dat dit proces onafhankelijk gebeurt van celdeling en overleving.

Het Slp65-deficiënte tumormodel is heel vergelijkbaar met een ander tumormodel: het tumormodel waarin het pro-tumor eiwit c-Myc tot expressie wordt gebracht. Het c-Myc protumor eiwit komt in veel cellen tot expressie. Verhoogde of niet goed gereguleerde c-Myc expressie is aanwezig in een grote verscheidenheid aan humane tumoren (borstkanker, darmkanker, baarmoederhalskanker, enz.). Muizen met hoge expressie van het c-Myc eiwit ontwikkelen pre-B cel tumoren die vaak inactivatie hebben van een belangrijk tumor onderdrukkend mechanisme. In hoofdstuk III hebben wij onderzocht of in Slp65-deficiënte muizen tumoren ontstaan via activatie van het protumor eiwit c-Myc en of er ook inactivatie plaatsvindt van dit tumor onderdrukkend mechanisme. We vonden in Slp65-deficiënte tumoren duidelijke parallellen met het c-Myc muizenmodel. Ten eerste, in beide tumormodellen onderdrukt de expressie van een kant-en-klare immuunglobuline zware keten de tumorvorming. Ten tweede, tumoren in beide modellen hebben het tumor onderdrukkend mechanisme geïnactiveerd. Ten derde, DNA schade geassocieerd met vele mutaties in de immuunglobuline ketens spelen geen rol bij tumorvorming in beide modellen. Deze resultaten geven aan dat tumorvorming in Slp65-deficiënte pre-B cellen een vergelijkbaar tumor onderdrukkend mechanisme inactiveerde als tumoren die het protumor eiwit c-Myc tot expressie brachten.

Slp65-deficiënte leukemie cellen brengen de pre-B cel receptor hoog tot expressie op hun celoppervlak en wij zijn in de veronderstelling dat deze hoge expressie bijdraagt aan sterke celdeling. In hoofdstuk IV hebben wij onderzocht of bepaalde zware ketens vaker geassocieerd worden met tumoren en een bepaald selectievoordeel met

zich meebrengen. Door middel van specifieke DNA amplificatietechnieken hebben we de herschikkingsstatus van Slp65-deficiënte en Btk/Slp65 dubbel deficiënte tumoren onderzocht. Deze analyse toonde aan dat de immuunglobuline zware ketens die deze tumoren op hun celoppervlak dragen een heel beperkt gebruik hebben van het variabele deel van de immuunglobuline, het zogenoemde V_H gebied. Ze gebruiken voornamelijk genen uit de V_H14 familie. Dit is uitzonderlijk omdat V_H14 een relatief kleine familie van V_H genen is met maar 4 segmenten. Normaal gesproken wordt deze familie niet zo veel gebruikt en worden er andere segmenten van de in totaal 150 verschillende genen gebruikt. We hebben onderzocht waarom deze V_H14 ketens zo veel gebruikt worden. We hebben gekeken of deze V_H14 ketens sterker zouden signaleren omdat ze reageren op lichaamseigen moleculen, maar dat bleek niet het geval. Bij de analyse van de niet-productieve immuunglobuline zware keten vonden we ook regelmatig gebruik van V_H14. Ons onderzoek laat zien dat het specifieke V_H gebruik van Slp65-deficiënte tumoren het best verklaard kan worden doordat in de getransformeerde cellen vooral dit gebied van het DNA toegankelijk was voor herschikkingen.

Gedurende V(D)J recombinatie ontstaan DNA breuken en als deze breuken niet goed worden gerepareerd kan dat leiden tot chromosomale afwijkingen en leukemie. Slp65-deficiënte tumorcellen brengen eiwitten tot expressie die DNA breuken maken en lijken hun DNA herschikkingactiviteit niet stop te zetten. Daarom wilden we de hypothese testen dat tumorvorming kan worden veroorzaakt door niet goed gereguleerde V(D)J recombinatie activiteit. In hoofdstuk V hebben wij in verschillende modellen onderzocht of V(D)J recombinatie activiteit noodzakelijk is en of een foutief herstel van de DNA breuken leidt tot tumorvorming. Er was geen verschil in het ontstaan van tumoren tussen een muizengroep die wel DNA kon herschikken en een muizengroep die niet kon herschikken. Ook bij muizen die wel of niet in staat waren DNA breuken te repareren vonden we geen verschil in de mate van tumorontwikkeling. Daarom hebben wij geconcludeerd dat tumorvorming in Btk/Slp65-deficiënte muizen onafhankelijk is van de V(D)J recombinatie activiteit en foutief DNA herstel.

In hoofdstuk VI hebben wij onderzocht of verminderde DNA stabiliteit een rol speelt in de tumorvorming van Slp65-deficiënte voorloper-B cellen. Het DNA herstel eiwit Rad54 is betrokken bij de DNA schadeherstelroute dat via homologie (overeenkomst in structuur) repareert. Wij hebben onderzocht of DNA herstel via het eiwit Rad54 invloed heeft op het ontstaan van pre-B cel tumoren. Verrassenderwijs vonden wij dat het gemis van Rad54 muizen beschermt tegen tumorvorming, omdat minder muizen leukemie ontwikkelden als ze - naast Btk en Slp65 - ook deficiënt waren voor Rad54. Analyses van het DNA van de tumoren liet zien dat Btk/Slp65 dubbel deficiënte tumoren specifieke genetische afwijkingen bevatten die niet terug te vinden zijn in Rad54/Btk/Slp65 drievoudig deficiënte tumoren. Onze resultaten laten zien dat DNA herstel via Rad54 en homologe recombinatie op het scherpst van de snede opereert. Aan de ene

kant garandeert herstel op basis van homologie een hoge nauwkeurigheid, maar aan de andere kant kan foutieve herkenning leiden tot verkeerd herstel van DNA breuken en daardoor genomische instabiliteit. Dit is voor het eerst dat er wordt aangetoond dat een DNA hersteleiwit kan bijdragen aan tumorvorming.

In hoofdstuk VII onderzochten wij in een muismodel de mogelijkheid van het verspringen van DNA stukken door V(D)J recombinatie en of dit kan leiden tot tumorvorming. In jonge muizen was B cel ontwikkeling niet aangedaan, maar oude muizen ontwikkelden chronische lymfatische leukemie (CLL). In deze tumoren waren geen tekenen te vinden van verspringende DNA stukken. Deze muizen hadden tumoren ontwikkeld omdat ze een protumor eiwit oncogen tot expressie brachten. Ons onderzoek heeft geleid tot het ontstaan van een nieuw muismodel voor CLL onderzoek.

Samenvattend hebben wij belangrijke informatie verkregen over de rol van Btk en Slp65 op het pre-B cel stadium en hun betrokkenheid in celrijping, celdeling en tumor onderdrukking. Deze informatie kan behulpzaam zijn voor verdere studies betreffende de identificatie van nieuwe kenmerken van tumoren die gebruikt kunnen worden voor diagnose, prognosebepaling en de behandeling van acute lymfatische leukemie en chronische lymfatische leukemie. Verder hebben we een nieuwe eigenschap gevonden voor het DNA hersteleiwit Rad54 en deze bevinding biedt nieuwe interessante invalshoeken voor toekomstig onderzoek.

List of abbreviations

Aid	activation-induced cytidine deaminase
ALL	acute lymphocytic leukemia
BCR	B cell receptor
BM	bone marrow
BrdU	bromodeoxyuridine
BSAP	B cell specific activator protein
Btk	Bruton's tyrosine kinase
C	immunoglobulin constant region
CDR	complementarity determining region
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CSR	class switch recombination
D	immunoglobulin diversity region
DAG	diacylglycerol
DSB	double strand break
Ebf1	early B cell factor 1
ELP	early lymphoid progenitor
Erk	extracellular signal-regulated kinase
Foxo	forkhead box
GC	germinal centre
HR	homologous recombination
HSA	heat stable antigen
HSC	hematopoietic stem cell
Ig	immunoglobulin
Ig H	immunoglobulin heavy
Ig κ	immunoglobulin kappa light chain
Ig λ	immunoglobulin lambda light chain
Ig L	immunoglobulin light
IL-7	interleukin-7
IL-7R	interleukin-7 receptor
IP3	inositol triphosphate
Irf	interferon regulating factor
ITAM	immunoreceptor tyrosine-based activation motif
J	immunoglobulin joining region
LMPP	lymphoid primed multipotential progenitor
Mapk	mitogen-activated protein kinase

MHC	major histocompatibility complex
MPP	multipotential progenitor
MZ	marginal zone
NHEJ	non-homologous end-joining
PI3K	phosphatidylinositol 3 kinase
PIP	phosphatidylinositol phosphate
PIP2	phosphatidylinositol 4,5-biphosphate
PIP3	phosphatidylinositol 1,4,5-triphosphate
PKB	protein kinase B
PKC	protein kinase C
Plcγ2	phospholipase cγ2
Pre-BCR	precursor B cell receptor
PTEN	phosphatase and tensin homolog
Rag	recombination activating gene
RSS	recombination signal sequence
SCID	severe combined immune deficiency
SH2	src homology 2
SH3	src homology 3
SLC	surrogate light chain
Slp65	SH2-domain leukocyte protein of 65 kD
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TI-II	thymus-independent type II response
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia
V	immunoglobulin variable region

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Dan zijn er mijn ouders: jullie hebben een belangrijke bijdrage geleverd aan mijn promotie. Altijd hebben jullie achter mijn keuzes gestaan en zijn jullie betrokken bij elke fase van mijn leven. Ook kon ik altijd een hapje komen eten als ik het weer eens druk had!
Tot slot, Geerd. Ik wil jou bedanken voor je onvoorwaardelijke steun, liefde en vertrouwen. Het is niet altijd makkelijk met een partner die heel veel werkt, maar jij vond het nooit een probleem dat wij niet zo'n regelmatig leven hadden zoals andere mensen. Je was er ook altijd om mij op te halen als het weer zo laat op het lab was geworden. De afgelopen maanden zijn voor jou niet gemakkelijk geweest om naast jouw drukke baan ook een groot deel van het huishouden te doen. Ik was de hele tijd maar druk bezig; overdag bezig met mijn coschappen en in de avonden en het weekend voltijd aan het schrijven. Het is voor mij heel bijzonder dat jij zonder biologische achtergrond altijd veel van mijn onderzoek hebt begrepen en betrokken bent geweest. Jij bent degene die mij af en toe met beide benen weer op de grond zet en de goede kant op wijst. Jouw ondernemendheid is bijzonder en jouw harde werken heeft ons Spanjewijn opgeleverd! Het is nu de hoogste tijd om een mooie fles wijn open te trekken.

Van

About the author

The author of this thesis was born in Spijkenisse, The Netherlands, on September 26th 1981. She attended secondary school (VWO) at the Penta College CSG Blaise Pascal in Spijkenisse and graduated in 2000. After graduation she started with her Medicine study at the Erasmus University Rotterdam. In 2001 she got the opportunity to combine her Medince study with the Master of Sciene Molecular Medicine program. In 2004 she received her doctoral degree in Medicine and in 2005 she conducted her MSc research project 'The effects of an immunoglobuline heavy chain transgene on the development of precursor-B cell leukemia in mice' at the department of Immunology at Erasmus MC in Rotterdam under supervision of dr. R.W. Hendriks. After receiving her MSc degree in 2005 she obtained the NWO Moziëk grant for 'The role of the V(D)J recombinase system in the development of precursor-B cell leukemia'. With this grant she started her PhD research at the departments of Immunology and Pulmonary Medicine, which resulted in this thesis. Next to research she also was a member of the PhD committee of the Postgraduate Molecular Medicine research school. During this period she was involved in organizing various scientific meetings and courses. Currently, she has started with clinical internships in order to finish her medical school.

List of publications

Kersseboom R, **Ta VBT**, Zijlstra AJE, Middendorp S, Jumaa H, van Loo PF, Hendriks RW. Bruton's tyrosine kinase and SLP-65 regulate pre-B cell differentiation and the induction of immunoglobulin light chain rearrangements. J Immunol. 2006 Apr 15;176(8):4543-52.

Ta VBT, de Bruijn MJW, ter Brugge PJ, van Hamburg JP, Diepstraten HJA, van Loo PF, Kersseboom R, Hendriks RW. Malignant transformation of Slp65-deficient pre-B cells involves disruption of the Arf-Mdm2-p53 tumor suppressor pathway. Blood, Feb 2010; 115 1385-1393.

Ta VBT, de Haan AB, de Bruijn MJW, Dingjan GM, Hendriks RW. Pre-B cell leukemias in Btk/Slp65-deficient mice arise independently of ongoing V(D)J recombination activity. Leukemia, Oct 2010 (epub ahead of print).

Ter Brugge PJ, **Ta VBT**, de Bruijn MJW, Keijzers G, Maas A, van Gent DC, Hendriks RW. A mouse model for chronic lymphocytic leukemia based on expression of the SV40 large T antigen. Blood. 2009 Jul 2;114(1):119-27.

Pothof J, Verkaik NS, van IJcken W, Wiemer EA, **Ta VT**, van der Horst GT, Jaspers NG, van Gent DC, Hoeijmakers JH, Persengiev SP. MicroRNA-mediated gene silencing modulates the UV-induced DNA-damage response. EMBO J. 2009 Jul 22;28(14):2090-9.

PhD Portfolio
Summary of PhD training and teaching

T.B.V. (Van) Ta
Pulmonary Medicine and Immunology

Research School:	Postgraduate Molecular Medicine
PhD period:	Sept 2005 - Mei 2010
Promotors:	Prof.dr. H.C. Hoogsteden
	Prof.dr. R. Benner
Supervisor:	Dr. R.W. Hendriks

General courses

2009 Biomedical English Writing and Communication
2007 Didactic skills
2006 Basic Radiation Protection (art.5B)
2004 Laboratory animal science (art.9)

In-depth courses

2008 Workshop on basic data analysis on gene expression arrays II
2008 Workshop on bioinformatic analysis, tools and services (BATS)
2007 Workshop Browsing genes and genomes with ensemble
2007 Course basic and translational oncology
2006 Course Molecular Medicine
2006 Course transgenesis and gene targeting
2005 Course Molecular Immunology
2004 Course Biomedical Research Techniques II

(Inter)national conferences

2010 '8th B cell forum', Dresden, Germany, April 29 – May 01.
2009 'Emerging Themes in Tumor Suppressors: Function and Clinical Implications in the Post-Genomic Era', Keystone Symposium, Taos, New Mexico, United States of America, January 25-30.
2009 '3rd Dutch Hematology Congress' Dutch Society for Hematology (NVvH), Congresscenter 'Papendal', Arnhem, Januari 21.
2007 'Biology of B cells in health and disease', Keystone Symposium, Banff, Alberta, Canada, February 6-12.

2006 'Gene expression and signaling in the immune system', Cold Spring Harbor Laboratory Conference, Cold Spring Harbor, New York, United States of America, April 26-30.
2006 'Annual Meeting Dutch Society for Immunology (NVVI)', Congresscenter 'De Leeuwenhorst', Noordwijkerhout, December 7-8.
2005 Annual Meeting Dutch Society for Immunology (NVVI), Congresscenter 'De Leeuwenhorst', Noordwijkerhout, December 8-9.

Seminars and workshops

2010 14th Molecular Medicine Day.
2009 Mozaiëk workshop meeting 2009, NWO, Den Haag, November 18, 2009. Workshop: 'Career Planning'.
2009 13th Molecular Medicine Day.
2008 Mozaiëk meeting in Madurodam, NWO, Den Haag, November 13, 2008.
2008 12th Molecular Medicine Day.
2007 Mozaiëk workshop meeting 2007, NWO, Den Haag, November 8, 2007. Workshop: 'Onderhandelen'.
2006 10th Molecular Medicine Day.
2006 'Talent Days', NWO, Het Vechthuis, Utrecht, March 14, 2006. Workshops: 'Netwerken' and 'Career Development'.
2006 Symposium on Frontiers of Mucosal Immunology, Erasmus MC Postgraduate School Molecular Medicine, WTC, Rotterdam, January 26, 2006.
2006 Mozaiëk workshop meeting 2006, NWO, Den Haag, November 9, 2006. Workshop: 'Beter promoveren'.
2005 9th Molecular Medicine Day.
2004 8th Molecular Medicine Day.

Presentations

2009 Highly restricted IgHV usage in Slp-65-defiicent pre-B cell leukemia. Minisymposium "Chronic Antigenic Stimulation in Leukemia-Lymphoma Development" department of Immunology, Erasmus MC Postgraduate School Molecular Medicine, June 16, 2009
2009 Mechanism of malignant transformation in Slp-65$^{-/-}$ pre-B cell tumors.'3rd Dutch Hematology Congress' Dutch Society for Hematology (NVvH), Congresscenter Papendal, Arnhem, Januari 21, 2009.
2008 Generation of antibody diversity and Slp-65$^{-/-}$ pre-B cell leukemia. Minisymposium "Biology of B cell differentiation", department of Immunology, Erasmus MC Postgraduate School Molecular Medicine, March 4, 2008.
2007 Role of V(D)J recombination in Slp-65 deficient pre-B cell leukemia formation.

"Biology of B cells in health and disease", Keystone Symposium, Banff, Alberta, Canada, February 10, 2007.

2006 Role of V(D)J recombination in leukemic transformation of Slp-65-deficient pre-B cells. Minisymposium "Childhood leukemia", department of Immunology, Erasmus MC Postgraduate School Molecular Medicine, June 13, 2006.

2005 Precursor B cell differentiation and the role of the V(D)J recombinase system in the development of pre-B cell leukaemia. Experimental Hematology, Erasmus MC Postgraduate School Molecular Medicine, June 1, 2005.

Other

2006 Travelling Fellowship Erasmus University Trust Fund.

2005 NWO Mozaiëk grant. The role of the V(D)J recombinase system in the development of precursor-B cell leukaemia

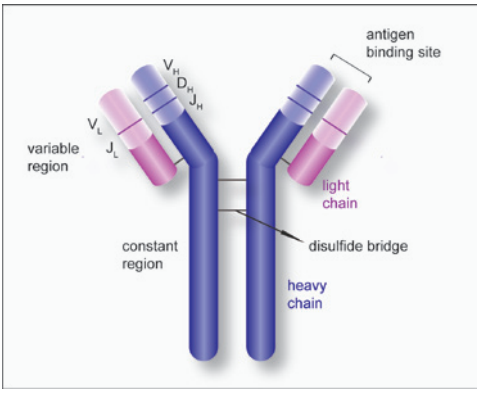
2007-2010 Member of the PhD committee of the Postgraduate School Molecular Medicine. Involved in organizing the 12th (2008) and 13th (2009) Molecular Medicine Day, Get out of your lab days 2009 and the Course Biomedical Research Techniques VII.

Teaching

2007 Supervising practicals Immunology 2nd year medical students

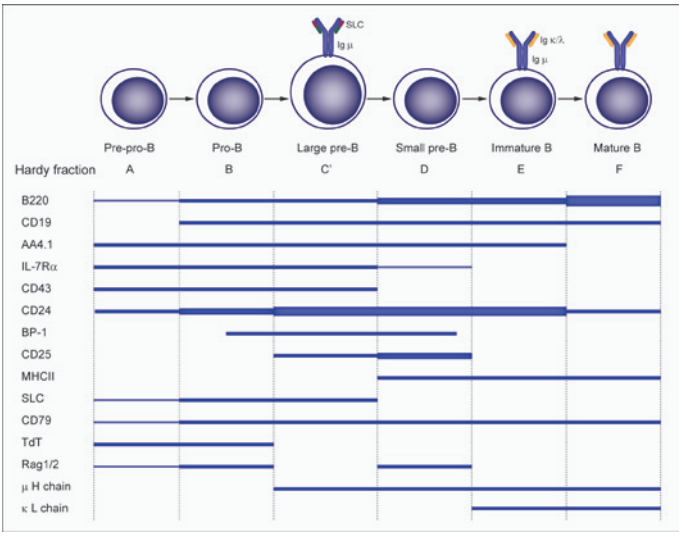
2006 Supervising practicals Immunology 2nd year medical students

2007 Supervising Master's thesis 2007 and 2008: Master of Science Molecular Medicine student Annemieke de Haan.



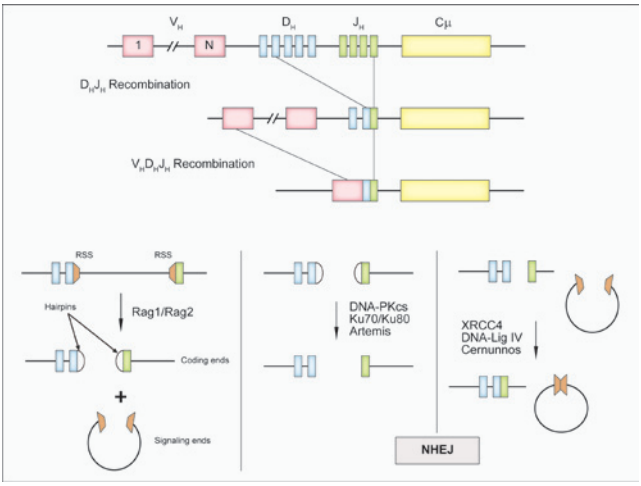
Chapter I: Figure 2

Figure 2. Schematic drawing of an immunoglobulin molecule.
An immunoglobulin molecule is composed of two identical heavy chains and two identical light chains which are held together by disulfide bonds. Each chain consists of a constant region (darker part of an H or L chain) and a variable region (lighter part of an H or L chain). The antigen binding sites are formed by a complex of the variable regions of both light and heavy chains.



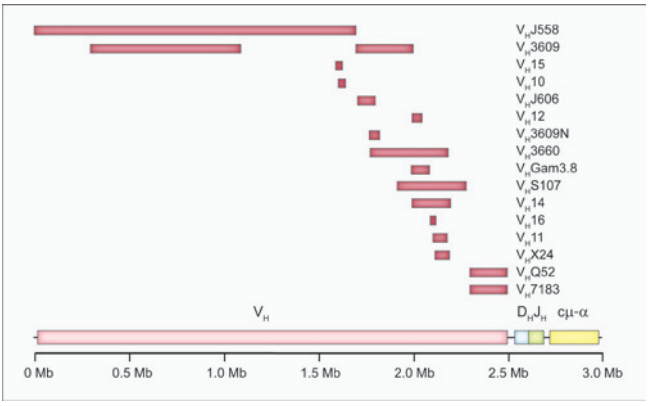
Chapter I: Figure 3

Figure 3. Framework of B cell lineage development based on ordered changes in cell surface molecules.
Relative expression levels are indicated by line thickness. AA4.1 is also known as CD93 and PB493; CD24 as HSA (heat stable antigen); CD25 as IL-2R; MHCII, major histocompatibility complex type II; CD79, Igα and Igβ; SLC, surrogate light chain (λ5 and VpreB). TdT and Rag1/2 (recombination activating gene) expression assessed by analysis of mRNA. Figure adapted from ref. (11).



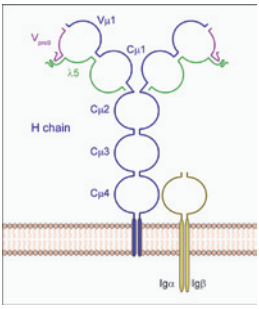
Chapter I: Figure 4

Figure 4. VDJ recombination.
Schematic representation of the Ig H chain locus and V(D)J recombination process. The V(D)J reaction can be divided into three steps. First, the Rag1/2 complex introduces a DNA DSB at the border between D_H and J_H segments and their respective recombination signal sequences (RSS), creating hairpin-sealed coding ends and blunt signaling ends. Artemis, which is recruited and phosphorylated by the DNA-PKcs/Ku70/Ku80 complex, opens the hairpins through its endonuclease activity. The XRCC4/ Cernunnos/DNA-Ligase IV complex finally seals coding and signal joints. NHEJ, non-homologous end-joining. Figure adapted from ref. (31).



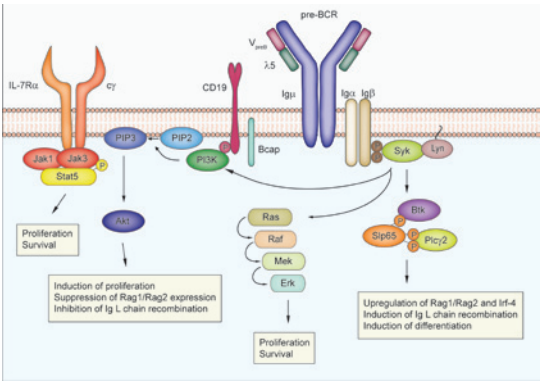
Chapter I: Figure 5

Figure 5. Organization of Ig H chain locus on chromosome 12.
Schematic map of V_H gene family distribution within the mouse Ig H chain V region according to IMGT (www.imgt.org). The length of boxes indicates the distance spanned by members of the gene families and is not related to the number of genes.



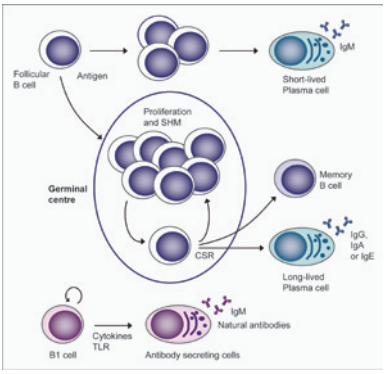
Chapter I: Figure 6

Figure 6. Structure of the pre-BCR.
The pre-BCR complex consists of the heterodimeric Igα/Igβ signal transducer and two covalently associated Ig H chains which are paired with the surrogate light chain, consisting of the invariant proteins λ5 and VpreB. The SLC contains two oppositely charged non-Ig-like tails located at the C-terminus of VpreB and the N-terminus of λ5. The unique tails protrude from the pre-BCR at the position where the CDR3 (complementarity-determining region) of a conventional L chain is located in the BCR.



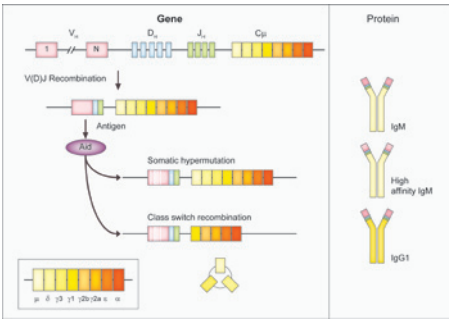
Chapter I: Figure 7

Figure 7. Signaling pathways in pre-B cells.
Pre-BCR activation results in activation of Syk which together with Src family protein kinases, such as Lyn, phosphorylate downstream signaling proteins resulting in pathways involved in proliferation, differentiation and induction of Ig L chain recombination. Activation of Slp65 recruits and activates Btk and Plcγ2 resulting in upregulation of Rag1/Rag2 and Irf-4, induction of Ig L chain recombination and induction of differentiation. An important pathway for proliferation and survival is the activation of the PI3K pathway. Phosphorylation of the co-receptor CD19 and the adaptor protein BCAP (B cell PI3K adaptor) recruit and activate PI3K resulting in the generation of the second messenger PIP3 (Phosphatidylinositol (3,4,5)-trisphosphate) from PIP2 (Phosphatidylinositol 4,5-bisphosphate). PIP3 recruits Akt which is the dominant mediator for controlling cellular proliferation. Ongoing signaling through the PI3K-Akt pathway induces proliferation, suppresses the Rag1/Rag2 genes and blocks Ig κ gene recombination. Proliferation in pre-B cells is also induced via the pathway involving Erk MAP kinase. In addition to the role of the pre-BCR, signaling via the interleuking-7 receptor (IL-7R) plays a central role in controlling the survival and proliferation of pre-B cells.



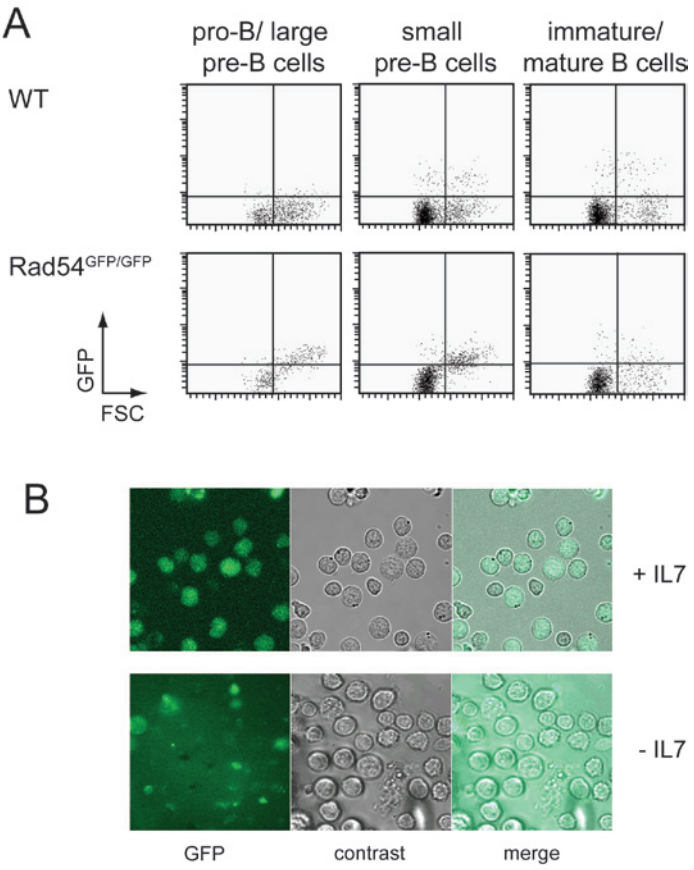
Chapter I: Figure 8

Figure 8. Activation of B cells.
Productive interaction of follicular B cells with antigen results in proliferation and differentiation. The primary response generates pre-germinal centre plasma cells that are short-lived and usually secrete IgM. Some activated follicular B cells form a germinal centre. Antigen-activated lymphoblasts that enter a germinal centre are subjected to multiple rounds of SHM and antigen selection. Cells that express high-affinity antigen receptors are selected for survival with subsequent differentiation to memory B cells or post-germinal centre plasma cells. Post-germinal centre plasma cells that undergo Ig H chain CSR typically home to the bone marrow where they reside and become long-lived (122).
Upon activation of a distinct population of B cells, B-1 cells, by cytokine and/or TLR activation antibody secreting cells are formed which secrete natural IgM antibodies, which often recognize bacterial antigens. SHM, somatic hypermutation; CSR, class switch recombination.



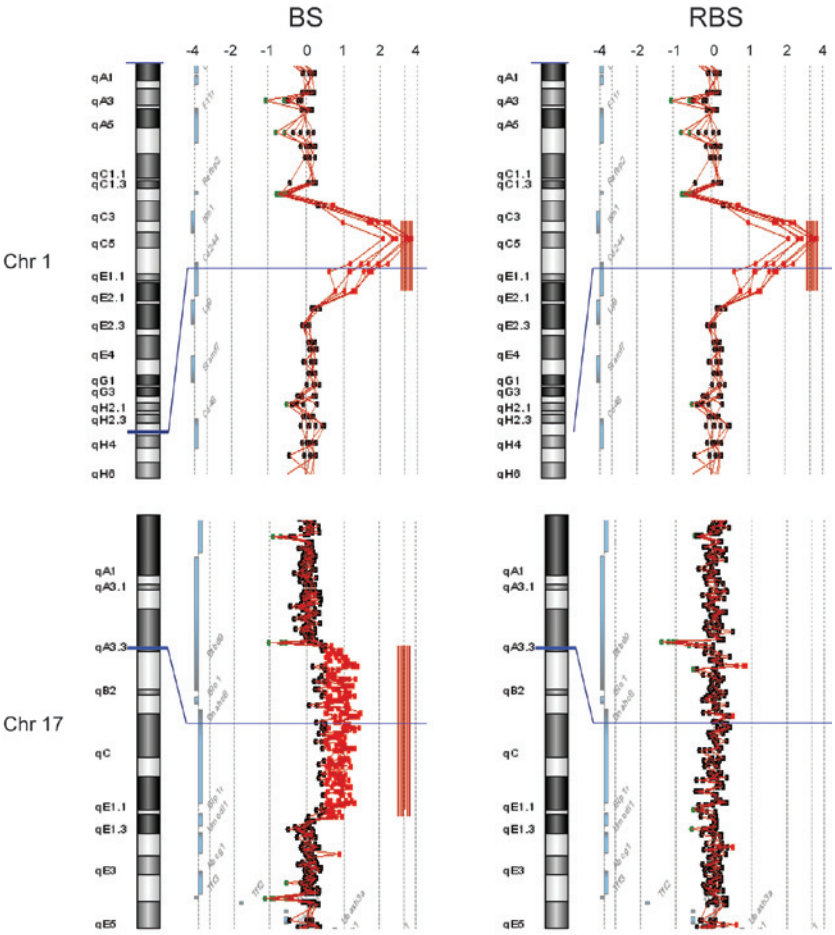
Chapter I: Figure 9

Figure 9. Generating antibody diversity.
Antibodies are encoded by immunoglobulin genes; these include V, D and J regions and C regions. In pro-B cells an immunoglobulin gene includes the full range of V,D, J and C regions. V(D)J recombination generates an antibody (IgM) with a variable region that recognizes a particular antigen. When the B cell encounters antigen, two other processes — both catalysed by the Aid protein — are triggered. Somatic hypermutation generates mutations (thin white lines) in the variable regions, potentially generating an IgM with higher affinity for its antigen. Class switch recombination results in the excision of some of the constant regions, generating antibodies with distinct effector functions (IgG1 is shown here). Figure adapted from ref. (125).



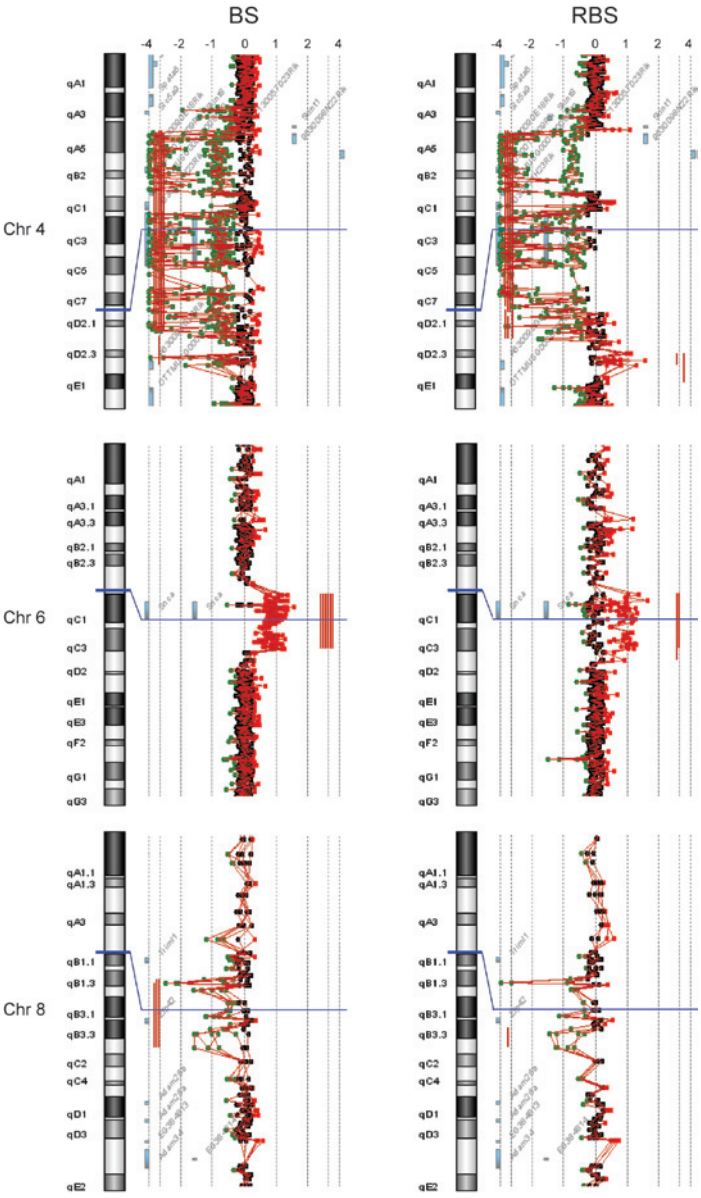
Chapter VI: Figure 1

Figure 1. Rad54-GFP expression in proliferating B cells.
(A) Flow cytometry profiles of GFP and forward scatter (FSC) are shown as dot plots for pro-B/large pre-B cells ($CD19^+CD2^-IgM^-$), small pre-B cells ($CD19^+CD2^+IgM^-$), immature and mature B cells ($CD19^+CD2^+IgM^+$) from WT and $Rad54^{GFP/GFP}$ mice. (B) Confocal microscopy images of $Rad54^{GFP/GFP}$ pre-B cells cultured for 5 days with IL-7 (*top*) and for 2 additional days with or without IL-7 (*bottom*).



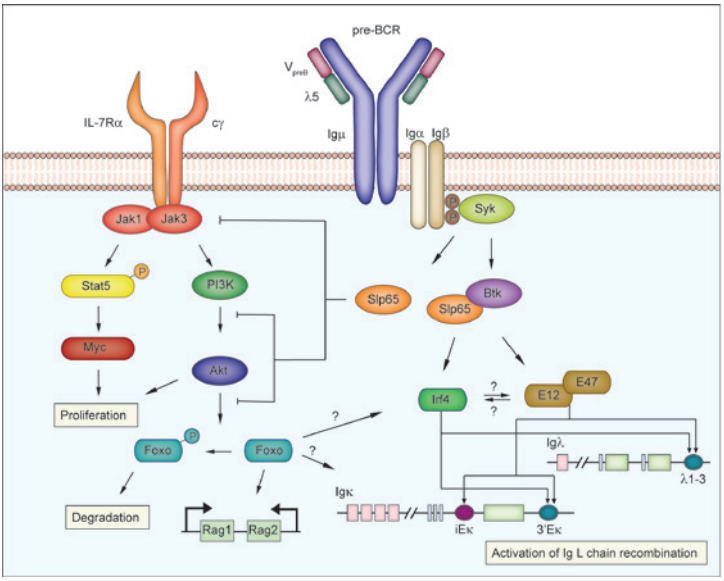
Chapter VI: Figure 4

Figure 4. Focal amplifications only present in Btk/Slp65-double deficient tumors.
Overlays of CGH analyses of chromosome 1 (*upper panel*) and 17 (*lower panel*) views of Btk/Slp65-double deficient tumors (BS; n=6) (*left*) and Rad54/Btk/Slp65-triple deficient tumors (RBS; n=4) (*right*), showing different sizes of aberrations exclusively present in BS tumors and not in RBS tumors. Chr, chromosome.



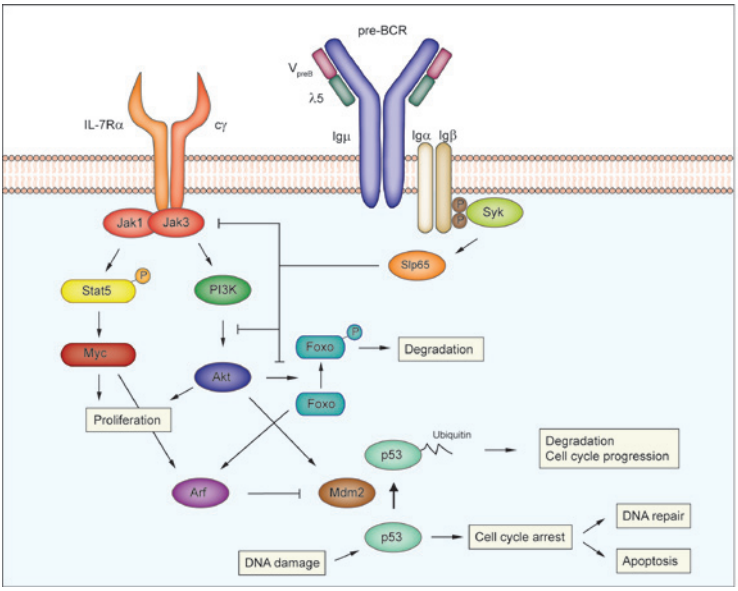
Chapter VI: Figure 5

Figure 5. Overview of several genetic lesions in Slp65-deficient tumors.
Overlays of CGH analyses of chromosome 4 (*upper panel),* 6 (*middle panel*) and 8 (*lower panel*) views of Btk/Slp65-double deficient tumors (BS; n=6) (*left*) and Rad54/Btk/Slp65-triple deficient tumors (RBS; n=4) (*right*), showing different sizes of aberrations. Chr, chromosome.



Chapter VIII: Figure 1

Figure 1. Regulation of Ig L chain recombination



Chapter VIII: Figure 2

Figure 2. Model for tumor suppression in pre-B cells.