Pre-B cell receptor signaling prevents malignant transformation by BCR-ABL1

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1. Introduction

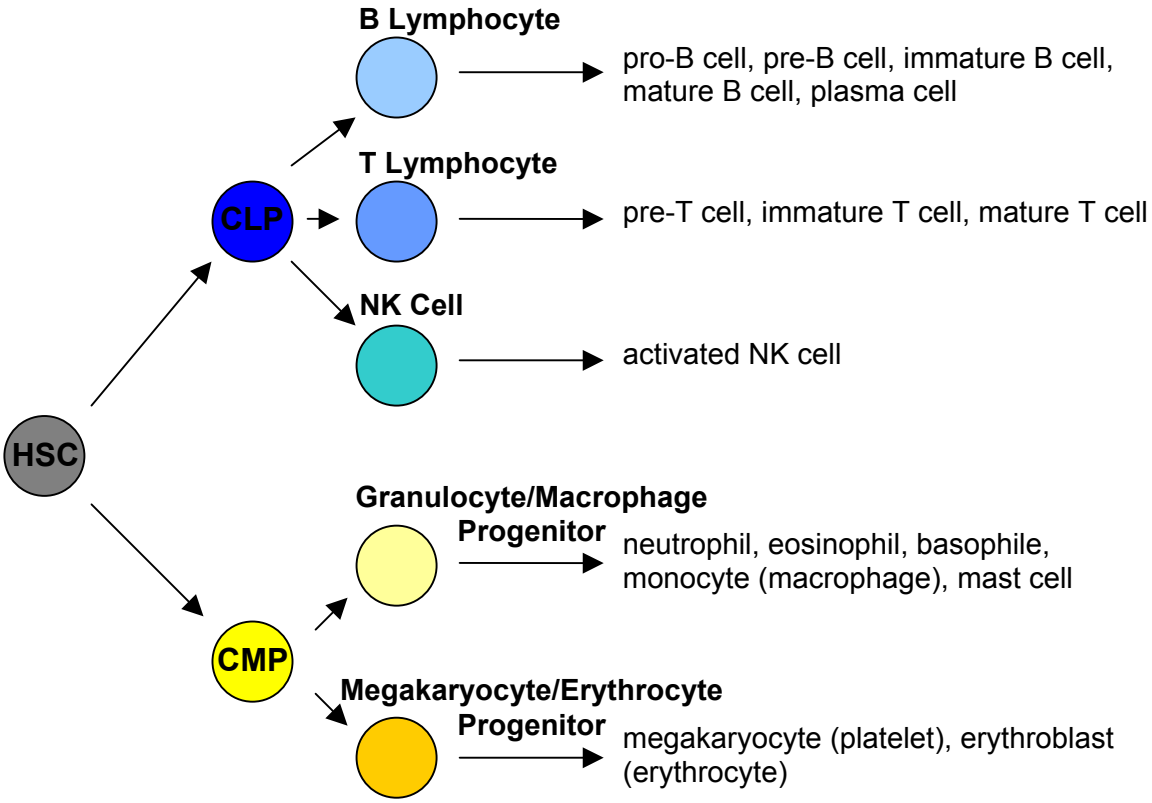
1.1 Hematopoiesis

Hematopoiesis is the developmental process that leads to the production of blood cells throughout life (Ginsburg and Sachs, 1963; Sachs, 1996). This process occurs prenatally in the placenta (embryonic yolk sac), fetal liver, and bone marrow and postnatally primarily in the bone marrow and in other lymphatic tissues (Moore and Metcalf, 1970; Peault, 1996). The origin of all blood cells is a common stem cell population, which is self-renewing and can give rise to all hematopoietic lineages (Osawa et al., 1996). Commitment to a hematopoietic lineage is determined through intermediate progenitors including common lymphoid progenitors (CLPs), from which B, T and natural killer (NK) cells arise and common myeloid progenitors (CMPs), which can differentiate into erythrocytes, granulocytes, monocytes and platelets (Kondo et al., 1997). Progenitors further downstream of the CLPs and CMPs are restricted in the number and type of lineages they can generate (Akashi et al., 2000; Figure 1).

Phenotypically, human hematopoietic stem cells (HSC) and primitive progenitors are suggested to be small quiescent cells expressing the surface glycoprotein CD34 but none of the lineage specific markers (Miller et al., 1999). In the course of hematopoiesis, cells develop other surface markers that characterize their lineage identity.

An interaction between the intrinsic genetic processes of blood cells and their environment, sustains the process of hematopoiesis and determines whether HSCs, progenitors and mature blood cells remain quiescent, self-renew, proliferate, differentiate, or become apoptotic (Domen et al., 2000; Orkin and Zon, 2002). Cytokines and chemokines are among others environmental regulators of hematopoiesis. Cytokines belong to protein families that function by engaging a specific receptor and activating signaling pathways, thus influencing proliferation, differentiation etc. Chemokines are molecules that regulate blood cell trafficking and homing sites. Additionally they mediate processes like inflammation, leukocyte development and tumor cell growth (Wright et al., 2002).

Figure 1: Hematopoiesis



The origin of blood cells lies within a population of hematopoietic stem cells (HSC). Through several differentiation steps the lineage commitment and final cell type is defined.

1.2 B cell development in hematopoiesis

Hematopoietic stem cells (HSCs) found in the bone marrow and fetal liver are the origins of all myeloid and lymphoid cells in the human body. Their extensive self-renewal capacity guarantees the regeneration of all other hematopoetic cells throughout life.

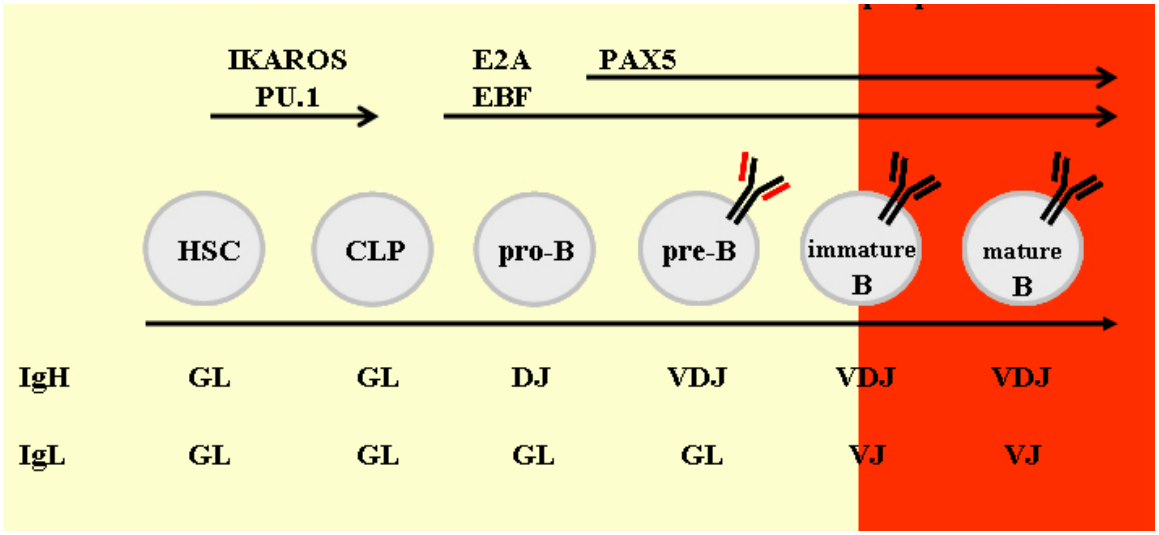
Further development of HSCs is controlled through various transcription factors and in the case of early B cell development can be characterized through the stage of rearrangement of the immunoglobulin heavy chain (*IGH*) locus (Figure 2). This process is mediated by recombinating activation genes 1 and 2 (RAG1/RAG2). HSCs can differentiate either into common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs). For this decision the expression of the transcription factors IKAROS

and PU.1 play a critical role. *IKAROS* is expressed in all hematopoietic progenitors with highest expression levels in thymocytes, T cells, B cells, and NK cells (Georgopoulos et al., 1997). Inactivation of Ikaros in mice lead to a complete loss of lymphoid cells (Georgopoulos et al., 1994). Few week old *Ikaros* knock-out mice display normal T cell development but a complete lack of B cells and an aberrant development in the myeloid lineage (Wang et al., 1996). Mice lacking Pu.1 develop erythrocytes, megakaryocytes and platelets but have a general defect in all other hematopoetic cells (Singh, 1996). Also the *PU.1* expression level plays a critical role in the decision of myeloid or lymphoid lineage as shown by retroviral reconstitution experiments with PU.1 deficient progenitor cells (DeKoter and Singh, 2000). High *PU.1* expression mediates development of the myeloid lineage, whereas low levels of *PU.1* favor the lymphoid lineage.

For entering the B lymphoid pathway, two transcription factors are required:basic helix-loop-helix protein E2A and early B cell factor (EBF). Deficiency of any of these transcription factors results in a B cell differentiation arrest at earliest stages and in the absence of *IGH* V region gene rearrangements (Bain et al., 1994; Lin and Grosschedl, 1995; Urbanek et al., 1994). In agreement with the phenotype of E2A- and EBF-deficient mice, it has been shown that E2A and EBF act in synergy to regulate the expression of *RAG1* and *RAG2* and germline transcription of the *IGH* locus (Romanow et al., 2000), which are both essential for recombination processes within the *IGH* locus.

Not only do E2A and EBF initiate the transcription of several crucial components of the pre-B cell receptor complex, like VpreB and λ5, the components of the surrogate light chain (Kee and Murre, 1998; Sigvardsson et al., 1997), but they are also required for the transition from the pro-B to the pre-B cell stage. However they are not sufficient. Paired box gene 5 (PAX5), another transcription factor that belongs to the family of the paired domain proteins, is required for further development. The absence of Pax5 leads to an arrest in the pro-B cell phenotype and blocks further rearrangement of the *IGH* locus to the V_HDJ_H configuration (Urbanek et al., 1994). Conditional inactivation of Pax5 in mice even reactivates the potential of early B cells to differentiate into cells of the myeloid lineage. Hence, *Pax5* expression is essential for maintaining B cell lineage commitment (Mikkola et al., 2002) and is present in every following stage up to mature B cells (Urbanek et al., 1994).

Figure 2: Schematic diagram of early B cell development



Differentiation of a hematopoietic stem cell (HSC) into a common lymphoid progenitor (CLP) is guided by the transcription factors IKAROS and PU.1. To enter the B lymphoid lineage the transcription factors E2A and EBF are necessary. Further development into pre-B cells and following B cell stages additionally requires *PAX5* expression. Early B cell development is defined by a sequence of somatic rearrangements within the immunoglobulin heavy chain locus (*IGH*): The rearrangement of a D_H to J_H gene segment occurs during the transition from the CLP to the pro-B cell stage followed by a rearrangement of a V_H gene segment to the DJ_H joint during the transition from the pro-B to the pre-B cell stage. Pre-B cells express the μ-heavy chain (black, long) in combination with the surrogate light chain (red) on the surface. Selected pre-B cells downregulate their pre-B cell receptor, initiate the rearrangement of the immunoglobulin light chain loci and further differentiate into immature and mature B cells, which express a conventional k or λ light chain (black, short).

Common lymphoid progenitors

Lymphoid lineages develop from hematopoietic stem cells located within the bone marrow and thymus. Per definition the common lymphoid progenitor (CLP) is a cell that can develop into B, T, natural killer (NK), or lymphoid dendritic cells (DC). Development into cells of the non-lymphoid compartment is not possible (Galy et al., 1995). In the case of murine bone marrow cells CLPs have been characterized through the expression of the interleukin 7 receptor α chain (IL7Rα), the stem cell markers $Sca1^{lo}$ and $c\text{-}Kit^{lo}$ and the absence of Thy1 and any mature blood cell lineage marker (Lin^-; Kondo et al., 1997). Through attachment to stromal cells and their secretion of growth factors in the bone marrow the survival and subsequent differentiation is regulated and characterized by the expression of certain transcription factors (Rolink et al., 2000).

Pro-B cells

Human pro-B cells are characterized through the expression of CD10, CD34 and CD19 on the cell surface (Loken et al., 1987). Another characteristic for pro-B cells is the start of the VDJ recombination process by the joining of D_H and J_H segments (Ravetch et al., 1981). After the initial DJ_H rearrangement step, pro B cells proliferate with the potential to rearrange different V_H gene segments to the already rearranged DJ_H segment (Li et al., 1993). This happens at the transition to the pre-B cell stage, where also CD34 expression stops.

Pre-B cells

If the rearrangement to a V_HDJ_H segment is successful a transcript can be formed that through alternative splicing will be connected to the C_μ constant region. The resulting peptide is the so called μ-heavy chain, a part of the (pre-)B cell receptor complex. If the V_HDJ_H rearrangement on the first allele is non-functional, the recombination step can be performed on the second allele. If the rearrangement on the second allele is also non-productive, the pre-B cell undergoes apoptosis (Lewis, 1994).

The expression of the pre-B cell receptor complex on the cell surface is therefore characterisitic for the late pre-B cell stage. The complex consists out of the μ-heavy chain and the surrogate light chain components λ5 and VpreB, linked with the Igα/Igβ signal transducing heterodimer (Figure 6). Surface expression of the pre-B cell receptor, and thereby initiated signaling induces clonal expansion of $Ig\mu^+$ pre-B cells. On the other hand surface expression also leads to transcriptional silencing of λ5 and VpreB and thereby leading to the limitation of this cell expansion (Grawunder et al., 1995; Parker et al., 2005). To terminate further Ig heavy chain rearrangements, the recombination-activating genes *RAG1* and *RAG2* and *TdT* are temporarily down-regulated assuring allelic exclusion (Grawunder et al., 1995). After a limited number of cell divisions, the pre-B cells stop cycling and the rearrangement machinery is reactivated allowing the joint of V_L and J_L gene segments at the Ig light chain loci (Lu et al., 2003). Functional light chain rearrangement leads to formation of the B cell receptor (BCR) and the transition to immature B cells.

Immature B cells

Differentiation into an immature B cell is dependent on a functional Ig light chain rearrangement at the Ig kappa (*IGK*) or Ig lambda (*IGL*) loci. Rearrangements at the *IGK* loci thereby take place first (Klein et al., 2005). In case of a successful rearrangement, the ability of the light chain to pair with the μ-heavy chain, and finally the surface expression of a non-autoreactive B cell receptor complex, the transition to the immature B cell compartment can take place. If there is no functional light chain expressed after the first rearrangement attempt, the cells might be rescued by a process called receptor editing (Radic and Zouali, 1996). The cells thereby undergo further rearrangement attempts of one or more κ or λ V region genes. In case of a failure in generating a functional light chain by primary and secondary rearrangements apoptosis is induced and the cell dies (Rolink et al., 1999).

Between 10% and 20% of the immature B cells migrate from the bone marrow to the spleen (Rolink et al., 1998). Immature B cells that enter lymphoid follicles mature into naïve B cells and typically express IgM and IgD on the cell surface. Cells that fail to enter lymphoid follicles have only a half-life of about three days and probably die by apoptosis (Rolink et al., 1998).

B1 cells

High expression of surface IgM, low surface IgD and expression of B220 characterize the phenotype of B1 cells in mice (Abrahao et al., 2003). Many B1 cells also express CD5 (Berland and Wortis, 2002). Produced are B1 cells in the fetus in bone marrow, liver and other gut-associated regions. Maintenance of the population is guaranteed through self-renewal capacity (Herzenberg, 2000). The constitutive production of IL10 promoted through CD5 might thereby play a major role (Gary-Gouy et al., 2002). At the same time negative feedback regulation assures the limitation in *de novo* production from progenitors (Herzenberg, 2000). B1 cells carry the B cell receptor complex on their surface and are able to react with foreign antigens like bacteria and parasites as well as with a variety of autoantigens (Hayakawa et al., 1999). In contrast to proliferating B cells in the germinal center, B1 cells are in a so called lowly activated state and therefore do not divide in response to antigen stimulation. In this way they can

escape the short half-life of the immature B cell stage (Potter and Melchers, 2000). Negative signals from CD5, CD22 and CD72 coreceptors regulate the unresponsiveness to B cell receptor mediated signaling (Ochi and Watanabe, 2000).

Mature B cells

Mature naïve B cells that are activated in the secondary lymphoid organs (spleen, lymph nodes, tonsils, Peyer's patches) by antigen-specific binding and that receive appropriate T_H cell help, become a lymphoblast which divides and secrets IgM. Progeny of the lymphoblast undergo class switching and start secretion of IgG, IgE or IgA. As most of these cells become plasma cells, they secret large amounts of immunoglobulins and are short-lived (Smith et al., 1996). A few cells make a transition into long-lived memory B cells with IgG, IgE or IgA as surface receptor (Manz et al., 1997).

Newly formed B lymphocytes enter the spleen via the blood and migrate to the B cell area in the follicle or the marginal zone (MZ) in the white pulp (Brelinska and Pilgrim, 1982). A unique population of resting marginal zone B cells is located in the MZ. The peripheral lymphoid tissues are organized with different T and B lymphocyte compartments. Marginal zone B cells which migrate into follicles become part of the long-lived mature B cell pool (Manz et al., 1997). Migration from the follicles into the lymphatic vessels allows the returning to the circulation. Besides long lived mature B cells, the lymphatic vessels also carry antigens from the site of infection to the lymph nodes. Within the lymph nodes, the B cells are located in the outer cortex while T cells are found separately in the paracortical area. Secondary follicles that are present in the outer cortex can contain areas called germinal centers (Camacho et al., 1998). After secondary antigen challenge, naïve B cells enter the dark zone of the germinal center as centroblasts which rapidly divide in response to strong stimuli from complexes on follicular dendritic cells and from cytokines (IL4) released by T cells. Clonal expansion, isotype switching and somatic hypermutation take place in the dark zone centroblasts (Liu and Arpin, 1997). Thereafter, the centroblasts transform in the nondividing centrocytes, which are vulnerable and die unless rescued by association with antigen on a follicular dendritic cell (MacLennan, 1998). Depending on cytokine expression, cells either expand the memory B cell pool (Manz et al., 1997) or migrate to the sites of

plasma cell activity (lymph node medulla; Smith et al., 1996). Engagement of CD40 by CD40 ligand expressing T cells also allows transition into B cell memory compartment (Arpin et al., 1995). Memory B cells can be found in the MZ, tonsils and Payer's Patches (Laichalk et al., 2002).

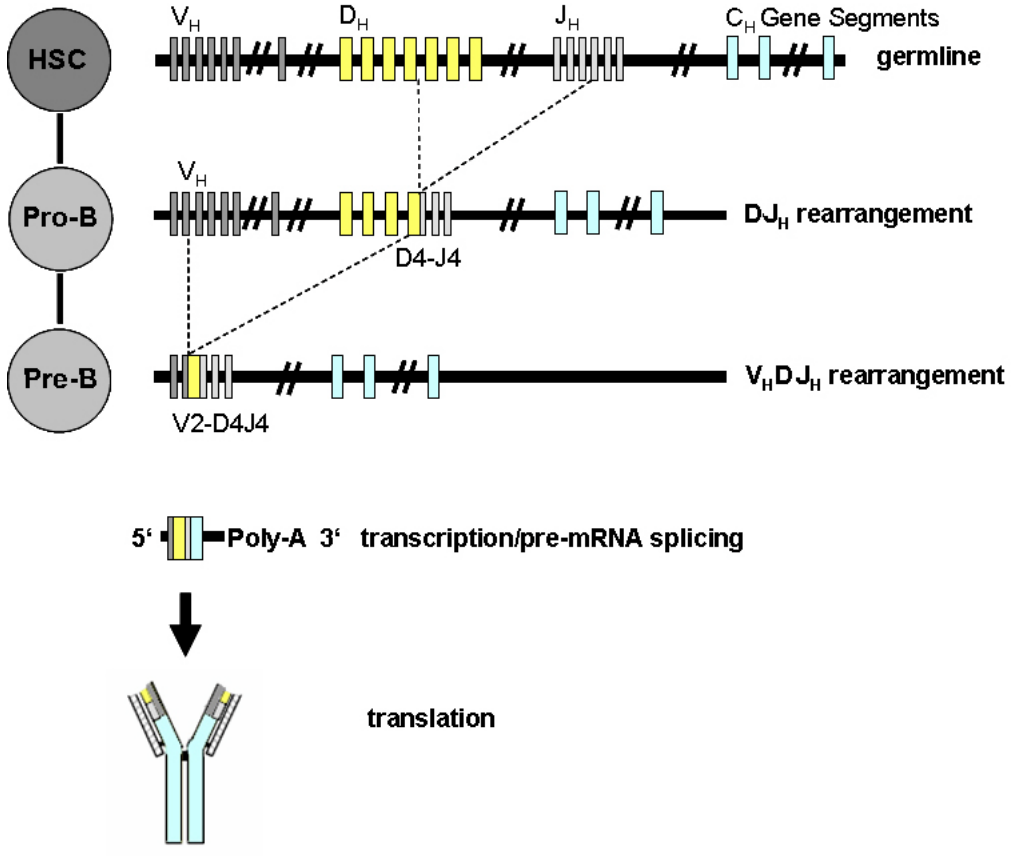
1.3 V(D)J recombination

The success of the immune system is based amongst others on the variable structure of the B cell receptor. Responsible therefore is a sequence of somatic rearrangements within the immunoglobulin heavy chain (*IGH*) and immunoglobulin light chain (*IGL, IGK*) loci. Besides that the recombination steps also characterize the differentiation progress in early B cell development.

There are four different gene clusters located in the *IGH* locus: the V (variable), D (diversity) and J (joining) gene segments, which build the variable region of an immunoglobulin heavy chain, and the C (constant) cluster that encodes the different constant regions (Matsuda et al., 1988; Ravetch et al., 1981). The connection of one V, D and J gene follows an ordered process called V(D)J recombination (Figure 3). The first step of this process is a rearrangement of a D_H to a J_H segment resulting in a DJ_H-joint at the transition to the pro-B cell stage. In the second step, at the transition to the pre-B cell stage, a V_H gene is rearranged to the DJ_H-joint (V_HDJ_H-joint). The constant region is linked afterwards on the transcriptional level through alternative splicing.

A functional μ-heavy chain, building together with the components of the surrogate light chain a functional pre-B cell receptor, enables the cell to proceed with the rearrangement of the light chain, which consists only of a V and a J segment (Grawunder et al., 1995). In case of a non-functional μ-heavy chain, due to out of frame rearrangement or introduced stop codons, the *IGH* locus on the second allele can be rearranged.

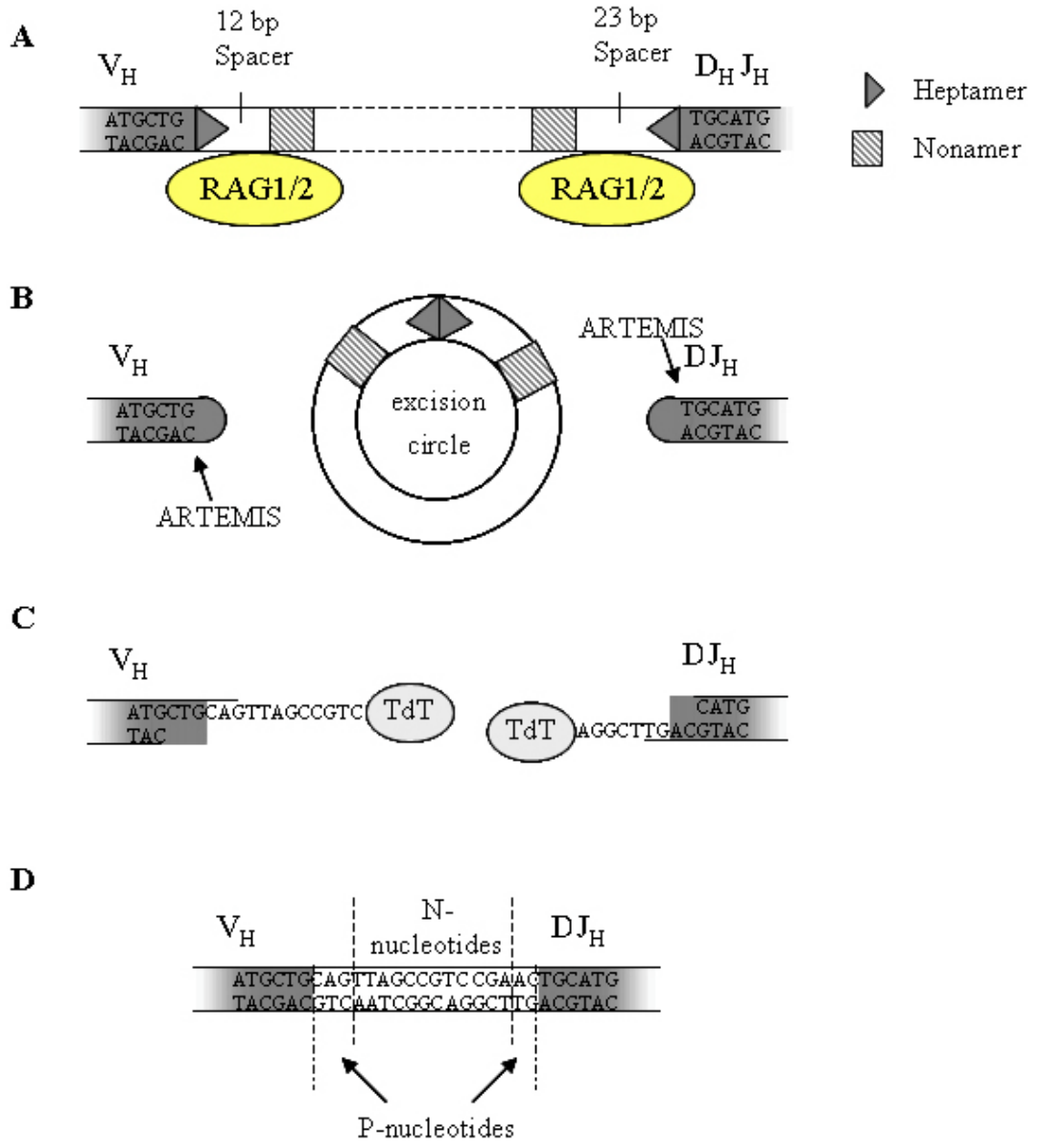
Figure 3: VDJ rearrangement as a prerequisite of the µ-heavy chain



For the expression of the µ-heavy chain two somatic rearrangements are required. In a first step one J_H segment is fused to a D_H gene segment. This DJ_H-joint is in a second step rearranged to a V_H segment. The resulting V_HDJ_H-joint encodes for the variable region and is fused and expressed with the C_μ segment for the constant region through mRNA splicing. The expression of the so generated heavy chain leads together with the expression of the surrogate light chain components to the formation of the pre-B cell receptor.

Responsible for the initiation of the somatic rearrangements is the V(D)J recombinase which is encoded by the recombination-activating genes 1 and 2 (RAG1/2; Oettinger et al., 1990). To ensure the introduction of DNA double-strand breaks (DSBs) at the correct chromosomal positions, RAG1 and RAG2 recognize and bind to recombination signal sequences (RSS), which are flanking V_H segments at their 3’ border, J_H segments at their 5’ border and D_H segments on both sides (Sakano et al., 1981; Figure 4).

Figure 4: Recombination processes during the formation of the V_HDJ_H-joint

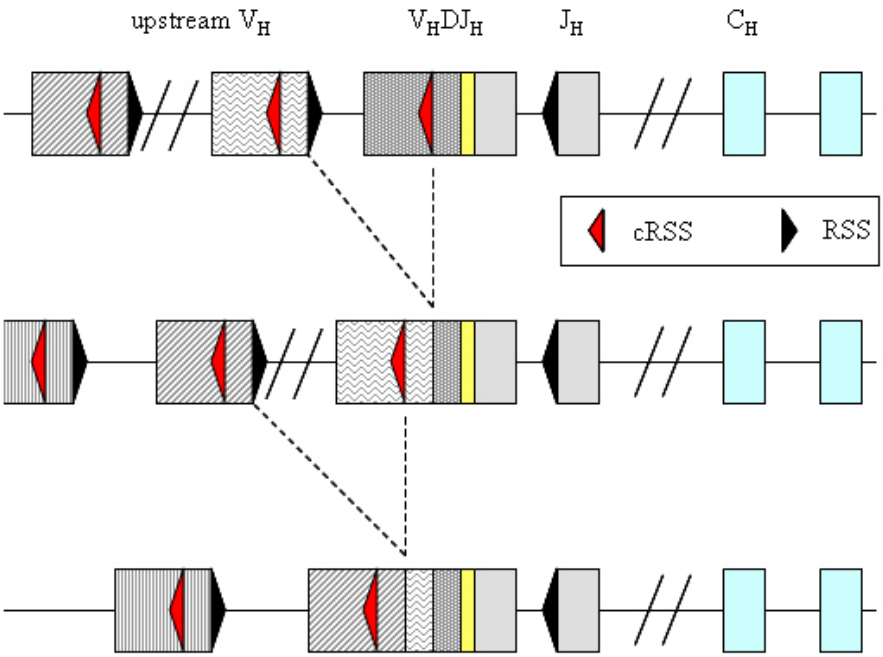


The RAG1 and RAG2 enzymes recognize the recombination signal sequences (RSS), consistin of a heptamer, nonamer and a spacer, and cleave the DNA at the coding ends of these gen segment flanking structures. While intervening DNA is removed through formation of an excisio circle, phosphorylated hairpin structures are generated at the coding joints. ARTEMIS, TdT an components of the NHEJ are responsible for reopening of the hairpin, addition of nucleotides an joining of the coding ends and thereby generating a unique V_HDJ_H junction.

The RSS motif consists of a conserved heptamer and nonamer sequence separated by a spacer of a conserved length of either 12 or 23 bp. Successful recombination can according to the 12/23 rule (Akamatsu et al., 1994) only take place between RSS motifs with different spacer length. As V_H and J_H genes have only RSS with spacers of 23 bp length and D_H genes only RSS with spacers of 12 bp length, this ensures the correct linkage of the three segments. The V(D)J recombinase cleaves between the RSS sites and the flanking coding regions of the two rearranging gene segments. This results in asymmetric DNA ends. While the coding ends are covalently sealed into hairpins, the RSS ends are blunt ended, 5' phosphorylated (Schlissel et al., 1993) and subsequently ligated to build an episomal excision circle. The hairpins are recognized and opened by ARTEMIS through single-strand cleavage at a random position (Ma et al., 2002). This results in a palindromic sequence that can be added to (P-nucleotides) or removed from the coding ends. Furthermore non-templated nucleotides (N-nucleotides) can be added by the activity of the terminal deoxynucleotidyl transferase (TdT) which leads to an increased diversity of the variable receptor region (Alt and Baltimore, 1982). The open ends of the two coding segments are finally joined by components of the non-homologous end joining (NHEJ) pathway (Grawunder et al., 1998).

If the resulting V(D)J rearrangement is in-frame and has through the activity of ARTEMIS and TdT no additional stop codon, the transcript leads to a functional μ-heavy chain that might be able to pair with the surrogate light chain and form the pre-B cell receptor. In any other case the second allele can be rearranged and furthermore the already combined V_HDJ_H-joint can undergo an additional recombination step, the so called V_H replacement (Figure 5; Reth et al., 1986; Kleinfield et al., 1986; Covey et al., 1990). Thereby the already rearranged V_H gene is replaced by a different, upstream located V_H gene. As the 5' RSS of the D_H segment no longer exists RAG1/2 recognize a cryptic RSS motif (cRSS) that is highly homolog to the RSS and lies within the extreme 3' region of 40 functional V_H segments. Since the cRSS is located within the original V_H gene, the downstream part is automatically introduced into the new V_HDJ_H-joint and therefore leaves a small footprint of the primary recombination.

Figure 5: V_H replacement



Already formed V_HDJ_H-joints can undergo further rearrangements by V_H replacement. The recombination is thereby taking place between the RSS of an upstream located V_H and the cryptic RSS (cRSS) which can be found in 40 functional V_H gene segments at the 3' end. As the sequence downstream of the cRSS is not affected by the new recombination process a footprint of the original V_H is left in the new V_HDJ_H-joint.

1.4 The pre-B cell receptor

If a V_HDJ_H-rearrangement is successful and a functional μ-heavy chain is expressed, it is further tested for its fitness in association with the surrogate light chain (SL) that is composed of the two polypeptides VpreB and λ5 (Karasuyama et al., 1990). In mice, only half of all newly functional rearranged μ-heavy chains are able to pair with the SL (ten Boekel et al., 1997). If the μ-chain is able to pair with the surrogate light chain, the two can associate with the signaling chains Igα and Igβ on the cell surface, generating together the pre-B cell receptor (pre-BCR) complex (Karasuyama et al., 1990; Lassoued et al., 1996; Tsubata and Reth, 1990). As soon as the pre-B cell receptor is deposited on

the surface signaling is initiated. It is still controversially discussed whether surface expression of the pre-B cell receptor is therefore sufficient or if it has to be triggered by receptor-ligand interaction. As Galectin-1 (GAL1), a stromal cell expressed ligand, can specifically bind to recombinant SL and the pre-B cell receptor in human, it was proposed to be that ligand. The interaction mediates synapse formation between pre-B cells and stromal cells and initiates intracellular tyrosine kinase activity (Gauthier et al., 2002). In mice, the same function of GAL1 could not be shown. Instead heparin sulfate was proposed to act as a pre-B cell receptor ligand (Bradl et al., 2003). Controversially it could be shown that a single pre-B cell suspended in liquid culture is able to proliferate without any ligand stimulation (Rolink et al., 2000). This autonomous signaling can be explained by cross linking of the non-Ig like region of λ5 through a pre-B cell expressed ligand or direct interaction of the SL with each other resulting in aggregation and signaling (Ohnishi and Melchers, 2003).

However, the expression of the pre-B cell receptor leads to limitation in proliferation and induces further differentiation: through activation of the two transcription factors IRF4 and IRF8 a negative feedback loop is initiated resulting in the downregulation of *VpreB* and *λ5* (Lu et al., 2003; Parker et al., 2005) and thereby finally in the termination of the pre-B cell signal. Besides that IRF4 and IRF8 also promote differentiation by initiation of the recombination of the immunoglobulin light chain loci, leading to the expression of a B cell receptor (BCR; Lu et al., 2003).

1.5 Pre-B cell receptor signaling

The transmission of pre-B cell receptor signaling is mediated through three different types of protein tyrosine kinases (PTKs). These are SRC (cellular homolog of *Rous sarcoma virus*) family kinases (Aoki et al., 1994; Saijo et al., 2003), the SYK (spleen tyrosine kinase) family kinases (Schweighoffer et al., 2003) and the TEC family kinases (Ellmeier et al., 2000). The lack of any of these proteins results in aberrant B cell function and development (Campbell, 1999). Another group of proteins, that have no enzymatic activity but possess structural domains that mediate intermolecular protein-

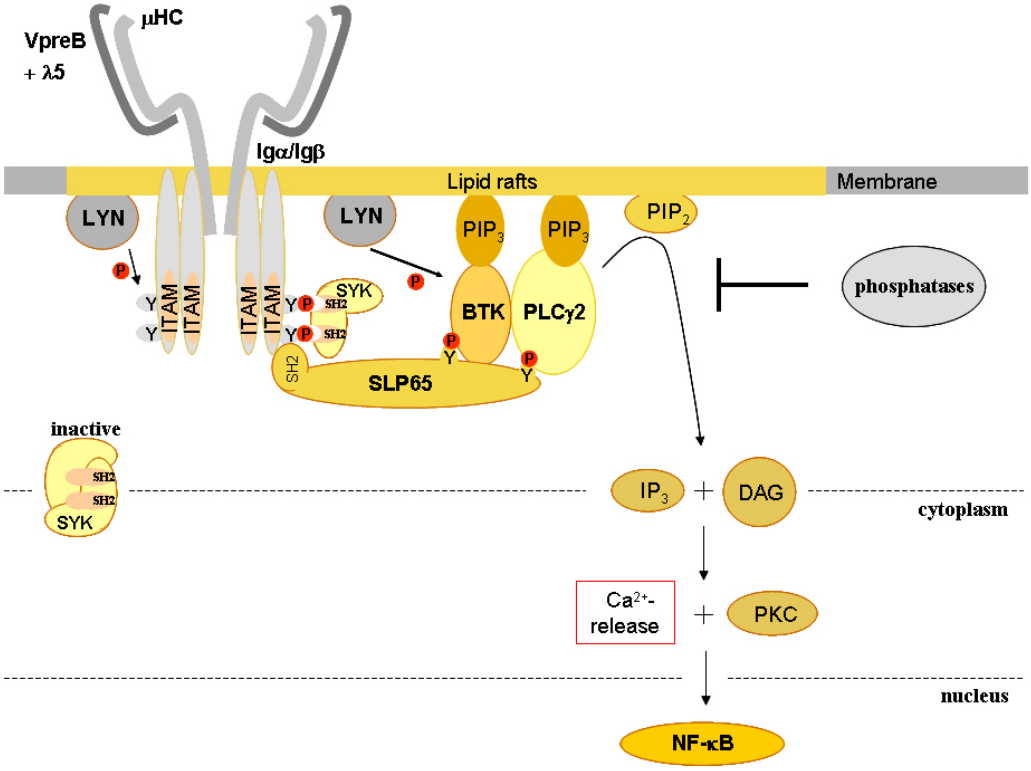
lipid and protein-protein interactions, are the adaptor proteins (Kurosaki, 2002). Through their stabilizing effect they are responsible for the correct assembly of the pre-B cell receptor signalosome (Flemming et al., 2003; Ishiai et al., 1999). As many of the pre-B cell receptor signaling connected proteins have initially been identified as oncogenes (Rodrigues and Park, 1994) or have a tumor suppressive function (Flemming et al., 2003) a strong regulation is necessary. This is guaranteed through autoinhibition, regulation of localization and the activity of inhibitory protein tyrosine phosphatases (PTPs; Shiue et al., 1995; Rolli et al., 2002; Tolar et al., 2005; Bolland et al., 1998; Ravetch and Lanier, 2000).

The crosslinking of the pre-B cell receptor accumulates signaling molecules within cell surface microdomains of high activation, the glycolipid-enriched membrane domains (GEMs) or so-called lipid rafts (Guo et al., 2000). Recruitment of the pre-B cell receptor to lipid rafts is essential for an effective signaling (Saeki et al., 2003) and starts the signaling cascade by phosphorylation of the pre-B cell receptor associated signal chains Igα and Igβ (Figure 6). The SRC kinase LYN is constitutively present in the GEMs and transmits the initial signal by phosphorylation of one tyrosine within the so called immunoreceptor tyrosine-based activation motif (ITAM; Reth, 1989; Cheng et al., 1999) of Igα and Igβ (Flaswinkel and Reth, 1994; Rolli et al., 2002). In the absence of LYN, its function can be substituted by other members of the src-family kinases, like FYN or BLK (Gauld and Cambier, 2004; Johnson et al., 1995). Phosphorylation of ITAM-associated tyrosine residues creates docking sites for SRC homology 2 (SH2)-domain containing proteins and is essential for the recruitment and activation of one of them, namely the spleen tyrosine kinase SYK. Without activated ITAM, SYK is in an autoinihibitory state through binding one of the two N-terminal SH2 domains to the own kinase domain (Wossning and Reth, 2004). The activation of SYK leads to a positive feedback loop by phosphorylation of further ITAMs and as a result to even more phosphorylated SYK molecules (Keshvara et al., 1998; Rolli et al., 2002). Mice that lack expression of SYK family kinases have a complete block at the pro-B cell stage and show thereby the importance of this protein family (Schweighoffer et al., 2003). Besides the positive feedback loop, activated SYK phosphorylates the adapter molecule SLP65 (also known as BLNK or BASH; (Fu et al., 1998; Goitsuka et al., 1998; Wienands et al., 1998)

and induces the recruitment of the TEC family kinase BTK (Bruton's tyrosine kinase). and PLCγ2 (phospholipase C, γ2) by the activation of phosphatidylinositol-3-kinase (PI3K; Beitz et al., 1999). PI3K generates the membrane bound phospho-lipid phosphatidylinositol-3,4,5-triphosphat (PIP_3) that acts as an membrane-anchor for the PH-domains of BTK and PLCγ2 (Saito et al., 2003). The counterpart of PI3K are the phosphatidyl-inositol phosphatases SHIP (Bolland et al., 1998) and PTEN (Satterthwaite et al., 2000) which can degrade PIP_3. SLP65 stabilizes the signalosome through binding to phosphorylated Igα and downstream targets including BTK, PLCγ2, GRB2, VAV and NCK (Fu et al., 1998; Wollscheid et al., 1999). Through the binding to SLP65, BTK is available as a substrate for LYN that in synergy with SYK phosphorylates it (Rawlings et al., 1996; Kurosaki and Kurosaki, 1997). The so activated BTK is now able to phosphorylate PLCγ2, one of the other binding partners of SLP65. Activated PLCγ2 generates the second messengers diaglycerol (DAG) and inositol-3-phosphate (IP_3) through cleavage of the membrane-associated phosphatidylinositol-4,5-biphosphate (PIP_2). By triggering the IP_3 receptors on the endoplasmic reticulum (ER), this reaction causes Ca^{2+} release from intracellular stores. The induction of different pathways, like NF-κB or MAPK, through DAG and elevated Ca^{2+} levels promotes survival, proliferation and differentiation of the cell.

As the whole signaling cascade is primarily depending on the phosphorylation of tyrosine residues and due to the fact that the counteractive PTPs have a 10- to 100-fold higher turnover rate than the PTKs (Honjo, 2004), the pre-B cell receptor signal needs an efficient method for inhibition of PTPs. In the oxidation of a cystein within the catalytic center of PTPs, a mechanism for reversible inactivation of PTPs has been identified (Meng et al., 2002). In response to (pre-)B cell receptor stimulation the membrane-bound NADPH-oxidase complex generates H_2O_2 which is responsible for the cystein-oxidation (Singh et al., 2005).

Figure 6: Scheme of the pre-B cell receptor-induced signaling cascade



Three major tyrosine kinase families mediate the pre-B cell receptor signal transduction: The SRC-family kinases (e.g. LYN), the SYK-family kinases (e.g. SYK) and the TEC-family kinases (e.g. BTK). The presence of adapter molecules like SLP65 is for the accurate assembly of critical importance. Through different phosphorylation-mediated activation steps PLCγ2 generates the second messengers diaglycerol (DAG) and inositol-3-phosphate (IP_3). This finally leads to Ca^{2+} release and activation of transcription factors like NF-κB resulting in survival, proliferation and differentiation.

1.6 B cell precursor leukemia

Leukemia is a malignant transformation of hematopoietic progenitors. According to the lineage commitment it can be divided into myeloid and lymphoid leukemia. In addition both kinds are further split into the acute form that is characterized by the rapid increase of immature blood cells and the chronic form that typically takes month to years to progress. Acute lymphoblastic leukemia (ALL) of B-cell precursors is the most common form of acute leukemia in children and thus the most common malignancy of childhood.

In adulthood, ALL (including T-cell ALL) represent only 20% of all acute leukemia. B-precursor ALL is characterized by aberrant cell growth, differentiation and survival signaling. The most frequent mechanism in leukemic transformation involves chromosomal translocations. In 52% of all childhood and in 68% of all adulthood ALL cases such an aberration was detected (Pui et al., 2004). Chromosomal translocations in cancer typically result in transcriptional deregulation of the target gene or lead to the generation of a fusion gene if the breakpoint involves coding exons on both chromosomes. These chimeras often encode for proteins acting as constitutively active tyrosine kinases or as novel transcription factors. While in almost all instances of mature B cell lymphomas transcriptional deregulation is the case, translocations in leukemia mostly result in the expression of chimeric proteins (Look, 1997).

The most common genetic aberration in childhood ALL (25%) is t(12;21)(p12;q22). It involves the rearrangement of the oligomerisation domain of the *TEL* gene to the entire coding region of *AML1* (Golub et al., 1995). While TEL belongs to the ETS family of transcriptional repressors, AML1 is with its RUNT-DNA binding domain part of the core-binding factor (CBF), a transcription factor with an essential role in hematopoiesis (Hiebert et al., 1996). Normal AML1 recruits other co-activators like histone-acetyl-transferases (HAT) and thereby allows the expression of specific target-genes like TCRδ (Hernandez-Munain and Krangel, 1994), interleukin-3 (Uchida et al., 1997), and PU.1 (Okada et al., 1998). The formation of TEL-AML1 in contrast leads to recruitment of histone-deacetylases (HDACs) that close the chromatin structure by methylation and thereby repress the expression of target-genes (Hiebert et al., 1996). In addition, the normal TEL, expressed on the non-rearranged allele, is also affected either through dimerization with TEL-AML1 or through deletion (Gunji et al., 2004; McLean et al., 1996).

The second most frequent chromosomal translocation in B cell ALL in children (5-6%), t(1;19)(q23;p13), results in the fusion protein E2A-PBX1 (Hunger et al., 1991; Kamps et al., 1991). While the basic helix-loop-helix transcription factor E2A plays an important role in B cell development, the transcriptional repressor PBX1 is usually not expressed in the lymphoid compartment (Monica et al., 1991) but interacts with HOX proteins (Shepherd et al., 1995; van Dijk et al., 1995). E2A-PBX1 activates target-genes

of HOX/PBX1 that are normally suppressed (Lu and Kamps, 1997) and deregulates the expression of E2A target-genes which promotes uncontrolled cell division (Massari et al., 1999; Qiu et al., 1998).

Leukemias bearing translocations involving chromosome 11q23 are found in leukemic blasts from > 70% of leukemias in patients younger than 1 year of age whether the immunophenotype is designated AML or ALL (Biondi et al., 2000). Some infant leukemias express antigens characteristic of both lymphoblasts and monoblasts, and are sometimes designated acute biphenotyic leukemias. Infants diagnosed with ALL harboring an 11q23 rearrangement have a particularly poor prognosis as compared to other children with ALL (Chen et al., 1993). The association of 11q23 rearrangements with either ALL or AML is unique in that most other translocations tend to be associated with leukemias of a particular hematopoietic lineage. These observations prompted the name mixed-lineage leukemia (*MLL*) for the gene on 11q23. One of the most prominent translocations in ALL involving *MLL* is t(4;11)(q21;q23). It encodes the MLL-AF4 fusion protein consisting out of the N-terminal 1439 amino acids from MLL and the C-terminal 865 amino acids from AF4 (Domer et al., 1993; Gu et al., 1992; Tkachuk et al., 1992; Ziemin-van der Poel et al., 1991). Analysis of *Mll* knockout mice suggests that Mll plays an important role in development and hematopoiesis through maintenance of appropriate homeotic (*Hox*) gene expression (Hess et al., 1997; Yu et al., 1995; Ernst et al., 2004a; Ernst et al., 2004b). *AF4* encodes for a putative transcriptional activator (Ma and Staudt, 1996; Li et al., 1998) that functions as a positive regulator of transcriptional elongation by RNA-Polymerase II and acts in combination with AF9, AF10 and Enl as a mediator of histon H3-K79 methylation (Bitoun et al., 2007). The mechanism of MLL-AF4-mediated leukemogenesis is not identified yet. It seems to be likely that transcriptional disregulation of wildtype target genes, like *Hox* genes, play an important role (Slany, 2005).

In adulthood, the most frequent type of lymphoid leukemia (25%) is characterized through the so called Philadelphia (Ph) chromosome. The translocation t(9;22) results in the fusion of the 5' part of the *BCR* gene to almost the whole *ABL1* gene (Nowell and Hungerford, 1960; Pui et al., 2004; Rowley, 1973; Heisterkamp et al., 1983; Heisterkamp et al., 1985). The *BCR-ABL1* oncogene is not only present in B cell progenitor leukemia,

but is also responsible for most if not all malignant transformations that lead to chronic myeloid leukemia (Shepherd et al., 1995). Depending on the breakpoint different sizes of BCR-ABL1 proteins are possible. While in CML the 210 kd (p210) form of BCR-ABL1 is expressed in almost all cases by the leukemic cells, the predominant form expressed in pre-B cell leukemia is 190 kd (p190) in size (Clark et al., 1988). This difference is presented through the lack of the guanine-nucleotide exchange factor homology (GEF) domain and a pleckstrin homology (PH) domain in the p190 oncogene form. The reason why the smaller BCR-ABL1 protein is only capable to transform pre-B cells while the p210 form can give rise to ALL as well as CML remains unclear. Another yet not understood difference between the two isoforms is the clinical outcome of BCR-ABL1 mediated leukemia. While Ph^+-CML patients have a good chance to be cured by treatment with the ABL1-kinase inhibitor STI571 (also known as Imatinib or Gleevec), all patients with *BCR-ABL1*-positive ALL, getting the same treatment, experience a relapse within a median of 4 months (Druker et al., 2001). One might think that the difference of ABL1-kinase activity between the two isoforms that has been demonstrated by comparison and showed that p190 possess a higher kinase activity than p210 (Lugo et al., 1990) might be an explanation. However within the group of pre-B cell leukemia a significant difference in response to treatment between p190 and p210 could not be observed (Gleissner et al., 2002). Functionally, the fusion of *BCR* with *ABL1* leads to the deletion of the autoinhibition domain of ABL1 resulting in a constitutively active BCR-ABL1 tyrosine kinase. The BCR-ABL1 kinase activity leads to the deregulation of signaling pathways and is of critical importance for the survival of the leukemia cells (Huettner et al., 2000). This has also been shown by the use of ABL1 kinase inhibitors against BCR-ABL1 which led to induction of apoptosis in leukemic cells (Druker et al., 1996).

1.7 BCR-ABL1 signaling activity

BCR-ABL1 is capable to induce and/or modulate different signaling pathways promoting cell growth and survival and to reduce sensitivity for apoptosis. Responsible therefore is

the constitutively active ABL1-kinase in the chimeric protein. Due to the translocation process and the formation of the *BCR-ABL1* oncogene the coding sequence for the N-terminal myristoyl group of normal c-ABL1 is deleted. Since this group binds to a deep hydrophobic pocket in the kinase domain, keeping c-ABL1 in a closed inactive conformation, its loss results in an open configuration (Nagar et al., 2003). While c-ABL1 has to be recruited to specific membrane domains, like lipid rafts, where it gains full activation by phosphorylation of tyrosine 412 and others (Hantschel et al., 2003), BCR-ABL1 oligomerizes through its coiled coiled domain within the BCR part (Tauchi et al., 1997). The formation of homotetramers leads to autophosphorylation (Maru et al., 1996), resulting in independence of BCR-ABL1 activity from membrane localization. This allows access to many phosphorylation substrates and formation of multiprotein complexes resulting in induction and affection of different signaling pathways.

BCR-ABL1 prevents cell death through regulation and inactivation of several proteins. STAT5 activation through BCR-ABL1-mediated phosphorylation (Carlesso et al., 1996) leads to an upregulation of the anti-apoptotic protein $BCLX_L$ (Gesbert and Griffin, 2000). Through the activation of the PI3K/AKT pathway another anti-apoptotic protein, BCL2, is activated (Skorski et al., 1997). Besides this, AKT signaling cascades lead to the phosphorylation and thereby inactivation of BAD, a pro-apoptotic protein (Salomoni et al., 2000).

Next to influences on apoptosis, BCR-ABL1 has a strong impact on cell-cycle regulation. Again through PI3K/AKT activation the protooncogene *c-Myc* is upregulated (Skorski et al., 1997), increasing cell proliferation. The same result is achieved through activation of the RAS pathway (Cortez et al., 1997), upregulation of *CYCLIN D2* and downregulation of the cyclin dependent kinase (CDK) inhibitor *p27* (Parada et al., 2001).

BCR-ABL1 also affects the ability of DNA-repair of the cell. While normal human cells primarily repair DNA double-strand breaks (DSBs) through the DNA-PK dependent pathway (Wang et al., 2001), BCR-ABL1 transformed cells downregulate the DNA- protein kinase catalytic subunit (*DNA-PK$_{CS}$*) through deregulation of proteosome inhibitors, resulting in proteosome-dependent degradation (Deutsch et al., 2001). By contrast another protein involved in the repair of (DSBs) and homologous recombination, RAD51, shows increased expression through BCR-ABL1 transformation, which seems to

be connected to enhanced drug resistance (Slupianek et al., 2001). Besides this the protein BRCA1, which is involved in the maintenance of genome integrity, is downregulated through expression of *BCR-ABL1* (Deutsch et al., 2003). These regulations might be part of the mechanism of BCR-ABL1 to promote genomic instability in leukemic cells.

Another way for BCR-ABL1 to cause genomic instability is the induction of the activiation-induced cytidin deaminase (AID). Somatic hypermutation (SHM) and class-switch recombination (CSR) represent physiological processes that modify variable (V) and constant (C) regions of Ig genes in mature germinal center B cells (MacLennan and Gray, 1986). Both SHM and CSR critically depend on expression of *AID*, which introduces single-strand breaks into target DNA (Muramatsu et al., 2000). In fact, AID mediated DNA single-strand breaks leading to SHM and CSR are specifically introduced into V or C regions of Ig genes. However, at much lower frequency AID can also target non-Ig genes in germinal center B cells (Pasqualucci et al., 2001; Shen et al., 2000) and may even act as a genome-wide mutator (Wang et al., 2004). As BCR-ABL1 induces the expression of *AID* (Feldhahn et al., 2007), mutations in Ph^+ ALL are introduced, probably leading to secondary genetic aberrations and/or resistance against ABL1-kinase inhibitors.

For pre-B cells, signaling through the pre-B cell receptor is absolutely critical for proliferation, differentiation and survival. In acute lymphoblastic leukemia, BCR-ABL1 is able to initiate an autonomous oscillatory Ca^{2+} signal (Feldhahn et al., 2005a). Therefore it initiates alternate splicing of *BTK* resulting in a truncated form that is able to act as a linker between BCR-ABL1 and full length BTK. This leads to constitutively phosphorylated BTK and thereby also to phosphorylation of other downstream targets, like STAT5 and PLCγ1. Thus BCR-ABL1 kinase mimics an aberrant pre-B cell receptor signal which leads to proliferation and survival of leukemic cells (Feldhahn et al., 2005a; Figure 26).

1.8 Aim of the thesis

The high frequency of defects in the pre-B cell receptor-related signaling molecules SLP65 (Jumaa et al., 2003; Klein et al., 2004; Sprangers et al., 2006) and BTK (Feldhahn et al., 2005b) in ALL cells suggests that the pre-B cell receptor may have a tumor suppressor function. On the other hand, the pre-B cell receptor delivers critical survival and proliferation signals in early B cell precursors and its expression is required for abnormal lymphoproliferation (Flemming et al., 2003). In addition, previous work demonstrated that the pre-B cell receptor and the pre-B cell receptor-related tyrosine kinase Syk are required for Myc-mediated transformation of pre-B cells (Wossning et al., 2006).

To clarify the function of the pre-B cell receptor as a tumor suppressor versus a requirement for leukemic transformation in ALL, the following questions should be addressed within this thesis:

1. Is there a selection for or against pre-B cell receptor expression in ALL?

2. Which role plays the pre-B cell receptor in *BCR-ABL1*-transgenic ALL during leukemic progressive transformation *in vivo*?

3. Does simultaneous signaling of the pre-B cell receptor and BCR-ABL1 has any effect on the affected cells?

4. Are changes in expression of pre-B cell receptor and related signaling molecules in BCR-ABL1-mediated ALL a result of cell selection and/or transcriptional regulation?

2. Materials and Methods

2.1 Materials

2.1.1 Patient samples and human primary cells for V_HDJ_H analysis

V_HDJ_H gene rearrangements were analyzed from 148 cases of ALL. For 129 cases, we amplified and sequenced clonal V_HDJ_H gene rearrangements from leukemia-derived cDNA, for 9 cases, we reanalyzed previously published sequence data on Ph^+ ALL (Height et al., 1996). Primary data were kindly provided by Dr. Martin JS Dyer, Sutton, UK. For 10 additional cases of Ph^+ ALL, sequence data from EMBL/GenBank (from Dr. Michael J Brisco, Flinders University, Adelaide, Australia; accession numbers L77980.1, L77983.1, L77982., L77984.1, L77985.1, L77986.1, L77987.1, L77989.1, L77971.1, L77974.1) were reanalyzed.

Patient samples were provided from the Department of Hematology and Oncology, University Hospital Benjamin Franklin, Berlin, Germany (WKH) and the Department of Medical Biosciences, Pathology, Umea University, Umea, Sweden (Dr. Aihong Li) in compliance with Institutional Review Board regulations.

Primary human bone marrow samples from four healthy donors were purchased from Cambrex (Verviers, Belgium).

2.1.2 Cell lines

The human ALL cell lines 380, 697, BV173, Kasumi-2, MHH-Call2, MHH-Call3, Nalm1, Nalm6, Nalm19, REH, RS4;11, SD1, SEM, SUP-B15, and TOM1 were purchased from DSMZ, Braunschweig, Germany. BEL1 and HBP-Null were a kind gift from Dr. Ruoping Tang (Paris, France) and Dr. Yoshinobu Matsuo (Okayama, Japan), respectively.

The murine cell lines PLC1 (Mishra et al., 2006), 8093 (Kaur et al., 2007) and T1657 were generated from *BCR-ABL1*-transgenic mice. Therefore a lymph node from a fully leukemic mouse was dissected, the lymphocytes isolated, and afterwards taken into cell culture.

2.1.3 Chemicals

If not specified otherwise, all chemicals have been purchased from Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, USA) and Thermo Fisher Scientific (Waltham, USA).

2.1.4 Oligonucleotides and Primers

The oligo-dTs for the reverse transcription of RNA into cDNA and all primers for PCR, quantitative real-time (qRT) PCR, sequencing, and spectratyping were synthesized by MWG-Biotechnologies (Ebersberg, Germany). All primer sequences are listed in chapter 7.2.1.

2.1.5 Solutions and Buffers

All following solutions were solved in di-destilled water:

Agarose loading buffer	50% (v/v) glycerol, 1mM Na_2EDTA,
	0.1% (v/v) xylencyanol, 0.1% (v/v) orange G
10x BV buffer	166 mM $(NH_4)_2SO_4$, 670 mM Tris·HCl, pH 8.8,
	67 mM MgCl, 100 mM ß-mercaptoethanol
PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4,
	1.4 mM KH_2PO_4, pH 7.4
PBS-EDTA	PBS + 1.3 mM EDTA
TAE	10 mM sodium acetate, 1 mM Na_2EDTA,
	40 mM Tris/HCl, pH 8.0

2.1.6 Media

LB/Amp-Medium	10 g/L tryptic digested peptone, 5 g/L yeast extract, 10 g/L NaCl, 100 µg/L ampicillin
LB-Agar	LB-Medium + 15 g/L agar

2.1.7 Bacterial strain

One Shot® TOP10 chemically competent *E.coli* (F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) have been purchased from Invitrogen (Carlsbad, USA)

2.1.8 Vectors

Following vectors were used for production of viral supernatant and subsequently transfections:

Table 1: List of vectors

Vector	Reporter	Type
pHIT123	-	envelope (ecotropic)
pHIT456	-	envelope (amphotropic)
pHIT60	-	packaging (retrovirus)
p8.2	-	packaging (lentivirus)
pMIG	GFP	retrovirus
pMIG-BCR/ABL^{p210}	GFP	retrovirus
pMICD8	mCD8	retrovirus
pMICD8-µ	mCD8	retrovirus
pMOWS-EcoR-CD8	mCD8	lentivirus

2.1.9 Antibodies

For flow cytometry the following antibodies were used:

Table 2: List of Antibodies

Antigen	Conjugate	Specifity	Isotype	Company
CD10	FITC	human	mouse IgG_1	BD Bioscience
CD19	PE-Cy5	human	mouse IgG_1,κ	BD Pharmingen
CD19	MicroBeads	human	mouse IgG_1	Miltenyi Biotech
CD20	PE	human	mouse IgG_1,κ	BD Quantibrite
CD34	FITC	human	mouse IgG_1	BD Pharmingen
CD179a/VpreB	PE	human	mouse IgG_1	BD-Pharmingen
CD8a(Ly-2)	PE	mouse	rat IgG_{2a},κ	BD Pharmingen
CD19	PerCP-Cy5.5	mouse	rat IgG_{2a},κ	BD-Pharmingen
CD23	PE	mouse	rat IgG_{2a},κ	BD-Pharmingen
CD127/IL7Rα	PE	mouse	rat IgG_{2a},κ	e-Bioscience
AA4.1	FITC	mouse	rat IgG_{2b},κ	BD-Pharmingen
IgD	FITC	mouse	rat IgG_{2a},κ	BD-Pharmingen
IgM	FITC	mouse	rat IgG_{2a},κ	BD-Pharmingen
Ig, κ light chain	PE	mouse	rat IgG_1,κ	BD-Pharmingen

2.1.10 Kits

HiSpeed® Plasmid Maxi Kit	Qiagen
RNeasy® Mini Kit	Qiagen
RNeasy® Micro Kit	Qiagen
RNase-Free DNase Set	Qiagen
TOPO® TA Cloning Kit for Sequencing	Invitrogen

2.2 Methods

2.2.1 *in vitro* culture of cell lines

All human cell lines were cultured using RPMI 1640 GlutaMAX^{TM} medium with addition of 10 % fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, USA). The three ALL mouse cell lines, PLC-I (Mishra et al., 2006), 8093 (Kaur et al., 2007) and T1657 were generated from isolated lymphocytes of *BCR-ABL1*-transgenic mice (Heisterkamp et al., 1990) and grown in complete lymphoblast medium comprising of McCoy's 5A medium supplemented with 15% heat inactivated FBS, 1% penicillin/streptomycin, 1mM sodium pyruvate, 2 mmol/L L-glutamine (Invitrogen, Carlsbad, USA) 10 ng/mL recombinant Il-3 (Peprotech, Rocky Hill, USA) and 50 µmol/L β-mercaptoethanol in the presence of irradiated mouse osteoblasts (MC3T3, from ATCC, Manassas, USA) as feederlayer. All cells were incubated at 37 °C in an atmosphere of 5% CO2. Every two to three days fresh medium was added.

2.2.2 Mouse model

As a model for ALL, p190 *BCR-ABL1* transgenic mice have been used. A construct consisting out of the coding sequence for *BCR* exon 1, *ABL1* exon 2 and *ABL1 exon* 3-11 was generated and linked to a segment (nucleotides -153 to +68) of the mouse metallothionein-1 (MT) promoter (Heisterkamp et al., 1990). As expression of a *BCR-ABL1* construct under control of the *BCR* gene promoter is lethal during embryogenesis (Heisterkamp et al., 1991) this promoter was chosen as alternative. The complete p190/MT construct was than microinjected into one-cell fertilized eggs. The presence of the transgene was confirmed by Southern blot analysis of tail DNA. In the first generation of founders, 6 of the 8 *BCR-ABL1* transgenic mice developed lymphoblastic leukemia/lymphoma. The remaining two animals were diagnosed with myeloid leukemia. As these two animals were found dead a definitive analysis was not possible. The following generated founders and their progeny all developed lymphoblastic

leukemia/lymphoma in a pre-B cell arrested stage. This indicates that either generation of myeloid leukemia seems to be a very rare event in this model or that the above mentioned animals have been misdiagnosed (Heisterkamp et al., 1990; Voncken et al., 1992). Although the MT promoter is active in nearly all tissues (Palmiter et al., 1983), none of the animals developed malignancies other than leukemia/lymphoma. This indicates that the oncogenic potential of BCR-ABL1 is restricted to the hematopoietic system. The reason therefore is the lack of expression of important BCR-ABL1-effector molecules in other cell types than the hematopoeitic lineage.

On a C57Bl/6J background, average age at death for the f10–f15 generation (n = 127) was 100 days (range 38–265 days). To compare the ALL model with the wild type situation C57Bl/6J were purchased from The Jackson Laboratory (Bar Harbor, USA).

For the isolation of primary samples the mice were sacrificed, blood was taken from the heart, the spleen was removed, and the femurs and shanks of the hind legs were separated from the soft tissues. All following steps were performed on ice.

To isolate the lymphocytes out of the peripheral blood, 500 µL of Lysis Buffer (BD Bioscience, Franklin Lakes, USA) was added to 30 µL of the sample. The mixture was incubated for 3 minutes and the reaction stopped by adding 1 mL PBS. After centrifugation for 5 minutes at 300 rcf the supernatant was discarded and the cells were washed with 1 mL PBS.

For the preparation of the bone marrow samples, the isolated femurs and shanks were carefully cut open on both sides and the cells were flushed out of the bone with PBS. After centrifucation for 5 minutes at 300 rcf the supernatant was discarded and the cells resuspended in 2 mL NH_4Cl to lyse remaining erythrocytes. After incubation for 1 minute 10 mL PBS was added and the cells were centrifuged again at 300 rcf for 5 minutes.

To isolate the splenocytes, the spleen was carefully mashed in a dish with 2 mL NH_4Cl. After 5 minutes 5 mL PBS was added and the mashed spleen further homogenized by use of a syringe. The viscous suspension was filtered through a cell strainer (40 µm; BD Falcon, Franklin Lakes, USA) and washed with 10 mL PBS.

For treatment with AMN107 (Nilotinib) 75 mg of AMN107/kg body weight were added to a mixture of 8 parts peanut butter and two parts vegetable oil and fed daily for 7 days to the mice by use of a syringe.

All animal research was performed at the Animal Care Facility of the Research Institute of Childrens Hospital Los Angeles in accordance with institutional guidelines. Animals were maintained in accordance with the *NIH Guide for the care and use of Laboratory Animals*.

2.2.3 Flow cytometry and cell sorting

To analyze and separate cells by flow cytometry the cells were marked with fluorescence labeled antibodies against specific surface markers. An aliquot of cells was therefore centrifuged at 300 rcf for 5 minutes and the obtained cell pellet washed with 1 mL PBS. After another centrifugation step the cells were resuspended in 20 µL of PBS, the appropriate antibodies were added (2 µL of the stock solution for anti-human antibodies, except CD20-PE, or 2 µL of a 1:10 dilution for anti-mouse antibodies and anti-human CD20-PE) and the samples incubated at room temperature for 30 minutes. Afterwards the staining was stopped by addition of 1 mL PBS and the cells were again centrifuged for 5 minutes at 300 rcf. For the flow cytometry measurement the pellet was resuspended in 250 µL of PBS/EDTA containing propidium iodid (PI; 1:10000). EDTA forms complexes with bivalent cations, like Mg^{2+} or Ca^{2+} and therefore inhibits agglutination of cells in the flow cytometer. PI is a fluorescent substance that intercalates into DNA. PI can only enter cells with defects in the cell membrane and is hence an indicator for dead cells. Measurements were done on a BD FACScan Flow Cytometer. Sorting of specific cell populations was done either on a BD FACSVantage SE Cell Sorter or on a BD FACSAria Flow Cytometer.

2.2.4 Measurement of Ca^{2+} release in response to pre-B cell receptor engagement

One way to examine the functionality of the pre-B cell receptor signaling cascade is to measure Ca^{2+} release from cytoplasmic stores in response to (pre-) B cell receptor engagement. Therefore 5 x 10^5 cells/sample were centrifuged for 5 minutes at 300 rcf and resuspended in 500 µL of medium without any supplements. After addition of 2 µL of Fluo-3AM (1mM; Molecular Probes, Carlsbad, USA) the cells were incubated for 15 minutes at room temperature and for another 30 minutes at 37 °C. All incubation steps were performed in the dark. The uncharged Fluo-3AM molecules can thereby permeate the cell membranes. Once inside the cell, the lipophilic blocking groups are cleaved by nonspecific esterases, resulting in a charged form that leaks out of cells far more slowly than its parent compound. After the incubation the sample was directly transferred to the flow cytometer for measurement. After 5 seconds the measurement was paused and 10 µL of an anti-µ chain-specific antibody (human: Jackson ImmunoResearch, West Grove, USA/ murine: Southern Biotech, Birmingham, USA) for engagement of the (pre-) B cell receptor was added. In case of an intact (pre-)B cell signaling cascade the stimulation led to the release of Ca^{2+} that was bound to Fluo-3 and thereby increased its fluorescence intensity more than 100fold resulting in a visible signal change in the flow cytometer.

2.2.5 Isolation of cells by magnetic cell sorting (MACS)

Before the flow cytometry sorting of the human bone marrow samples for single-cell analysis, the samples were pre-purified using anti-CD19 MicroBeads and the MACS® Technology from Miltenyi Biotec (Bergisch Gladbach, Germany). The method was preformed according to the manufacturer protocol. Briefly, the cells are incubated and thereby magnetically labeled with anti-CD19 MicroBeads. After washing, to prevent unspecific binding, the cells are transferred to MACS Columns, which were before placed in a MACS Separator. As this assembly generates a magnetic field in the column matrix, labeled cells stay in the column while unlabeled cells flow through. Afterwards

the column is washed and than removed from the MACS Separator. The release from the magnetic field allows flushing out the cells and collecting them for downstream applications, like cell sorting.

2.2.6 V_H Single Cell PCR on human bone marrow subsets

Normal human pro-B cells ($CD19^+$ $CD34^+$ $VpreB^-$), pre-B cells ($CD19^+$ $VpreB^+$) and immature B cells ($CD19^+$ $CD10^+$ $CD20^+$) were sorted from human bone marrow (from four healthy donors; Cambrex, Verviers, Belgium) by flow cytometry using a FACSVantage SE cell sorter (BD Biosciences, Franklin Lakes, USA). Single cells from each population were sorted into 20 µL PCR buffer in individual PCR reaction tubes. The following procedure is divided into three steps: I. Proteinase K-digestion, II. PEP (primer extension preamplification), III. V_H-PCR (1^{st} and 2^{nd} round).

I. To prepare the DNA of the single cells for amplification, a Proteinase K incubation has been performed. The Proteinase K (PCR grade; Roche, Basel, Switzerland) was diluted 1:2 in 10mM Tris (pH 7.6) and 0.6 µL of the solution added to the 20 µL PCR buffer with the single cell. To avoid evaporation the whole reaction was finally overlaid with a drop mineral oil. After the incubation for 2 h at 50 °C, the enzyme was heat-inactivated for 10 minutes at 95 °C.

II. During the PEP reaction the whole genome of a single cell can theoretically be amplified. It has been estimated that about 95% of the genomic DNA is usually amplified up to 60 times, allowing to perform different gene-specific PCRs with aliquots of the PEP reaction. For the PEP reaction 16 bp long oligonucleotides with random nucleotide composition have been used as primers. Because of the risk of contamination with foreign DNA the PEP reaction was performed in an extra single cell PCR room.

5	µL	dNTPs (2 mM)
4	µL	10 x PCR buffer
5	µL	random-15-mer (400 µM)

6	µL	$MgCl_2$ (25 mM)
18.5	µL	ddH_2O
38.5	µL	
20.5	µL	sample from proteinase K digestion
1	µL	Taq DNA polymerase from Promega for buffer A (5 U/µl)
60	µL	

The reaction was run with the following program:

1	95 °C	10 min
2	80 °C	pause (add Taq DNA polymerase)
3	37 °C	2 min
4	55 °C	special function + 0.1 °C/sec 1 sec.
5	55 °C	4 min
6	95 °C	1 min
7	37 °C	2 min
8	55 °C	special function + 0.1° C/sec 1 sec.
9	55 °C	4 min
10	12 °C	pause

49x

III. After the PEP reaction a first round V_H PCR was performed. Therefore a primer mix was prepared. The same volumes of the 2.5 mM working dilutions of the V_H (1-6) and 3' J_H primers were mixed and afterwards further diluted to a final concentration of 0.125 mM for each primer (1:20).

2.5	µL	dNTP (2 mM)
5	µL	10 x PCR buffer
6	µL	primer mix
4	µL	25 mM $MgCl_2$
28	µL	H_2O

45.5	µL	
0.5	µL	Platinum *Taq* DNA polymerase (Invitrogen)
4	µL	PEP
50	µL	

The following program was used:

1	95 °C	2 min	
2	95 °C	50 sec	
3	60 °C	30 sec	34x
4	72 °C	1 min	
5	72 °C	5 min	
6	4 °C	pause	

The second round was a so called nested PCR. As reverse primer a mix of 5' J_H primers was used. These lie upstream of the 3' J_H primer sequence of the first round and thereby increase the specifity of the reaction. Therefore same volumes of the 5' J_H primers were mixed and used together with a single V_H primer in the following PCR reaction:

5	µL	dNTP (2 mM)
5	µL	10 x PCR buffer
2.5	µL	V_H primer (2.5 µM)
2.5	µL	J_H primer mix
3/5	µL	25 mM $MgCl_2$ (V_H2-6/ V_H1)
1	µL	Platinum *Taq* DNA polymerase (Invitrogen)
30/28	µL	H_2O
49	µL	
1	µL	1st round
50	µL	

The following program was used:

1	95 °C	1 min	
2	95 °C	50 sec	
3	61 °C	30 sec	44x
4	72 °C	1 min	
5	72 °C	5 min	
6	4 °C	pause	

Part of the PCR product was afterwards used for an agarose gel to make possible products visible. Reactions with product were in the following sequenced and the V_HDJ_H rearrangement analyzed.

2.2.7 V_H PCR on primary leukemia samples and cell lines

In order to analyze the functionality of possible V_HDJ_H gene rearrangements in leukemia a V_H PCR on cDNA from patient samples and human leukemia cell lines was performed. Therefore a mix of V_HL (V_H-Leader) and a mix of 3'J_H primers was used in the following reaction:

1	µL	dNTP (10 mM)
5	µL	10 x PCR buffer
6	µL	V_HL primer mix (2.5 µM)
3	µL	J_H primer mix (2.5 µM)
2	µL	50 mM $MgCl_2$
1	µL	Platinum *Taq* DNA polymerase (Invitrogen)
31	µL	H_2O
49	µL	
1	µL	cDNA
50	µL	

The following program was used:

1	95 °C	2 min	
2	95 °C	1 min	
3	63 °C	1 min	44x
4	72 °C	1 min	
5	72 °C	5 min	
6	4 °C	pause	

Part of the PCR product was afterwards used for an agarose gel to make possible products visible. Reactions with product were subsequently used for TOPO cloning, colony-PCR and sequencing.

2.2.8 RNA isolation

To extract the whole RNA of animal cells the RNeasy® Mini Kit from Qiagen was used. In case of cell number below 5 x 10^5 cells/sample the RNeasy® Micro Kit was used instead. The extraction was performed according to the protocol of the manufacturer. To eliminate possible genomic DNA contamination the optional on-column DNase digestion with the RNase-free DNase Set was done. The success of the isolation was afterwards verified by running 1 µL RNA on an agarose gel.

2.2.9 cDNA synthesis

Because RNA is very sensitive to degradation it cannot be stored over longer periods of time. In comparison DNA is much more durable. For this reason mRNA was transcribed into cDNA directly after isolation if mRNA was not needed as substrate in downstream applications. For these purposes the enzyme SuperScript™ III Reverse Transcriptase (RT) from Invitrogen was utilized. It is derived from Moloney Murine Leukemia Virus

and bears the capability to translate RNA into DNA. This RT is highly thermostable and initiates translation starting from a primer bound to the RNA template. For the reaction, an oligo-dT primer that binds to the polyA-tail of mRNA was used. Therefore only mRNA is translated into cDNA. The first step is to add the following components to the isolated RNA:

- 2 µl of oligo-dT, 50 µM manufactured by MWG
- 2 µl of dNTP Mix, 10 mM each: dTP, dCTP, dGTP, dTTP supplied by Peqlab

The whole sample is heated to 65 °C for five minutes to degrade secondary structures of the RNA and allow the primers to bind to the polyA-tails. Afterwards the sample is incubated on ice for at least one minute and a mixture of the following ingredients is added:

- 8 µl of First-strand buffer, supplied with the RT
- 2 µl of DTT, 0,1 M from Invitrogen
- 2 µl of RNaseOUT^{TM} , 40 units/µL from Invitrogen ;
- 2 µl of SuperScript^{TM} III RT, 200 units/µL, supplied by Invitrogen

After carefully mixing the sample by pipetting, it is incubated at 50 °C for 1 hour. To inactivate the RT the reaction is finally heated up to 70 °C for 15 minutes. The obtained cDNA can be stored at -20 °C.

2.2.10 Microarray analysis

For microarray analyses, two sets of experiments were performed: 2 x 10^5 to 2 x 10^6 $CD19^+$ $AA4.1^+$ bone marrow B cell precursors were FACS-sorted from three wildtype mice, three BCR-ABL1-transgenic “pre-leukemic” mice, three mice with full-blown ALL and three leukemic mice after seven days of treatment with the BCR-ABL1 kinase inhibitor AMN107. In a second set of experiments, $Slp65^{-/-}$ pre-B cells were transduced

with *GFP* or with *GFP/BCR-ABL1* with or without subsequent addition of the BCR-ABL1 kinase inhibitor STI571. For each condition, three experiments were performed. Biotinylated cRNA was generated (MicroArray Core, Childrens Hospital Los Angeles, USA), fragmented according to the Affymetrix protocol and hybridized to 430 mouse microarrays (Affymetrix, High Wycombe, UK). After scanning (scanner from Affymetrix), the expression values for the genes were determined using Affymetrix GeneChip software. Therefore the .cel files from the appropriate gene chips were imported to BRB Array Tool (http://linus.nci.nih.gov/BRB-ArrayTools.html) and processed using the RMA algorithm (Robust Multi-array Average) (Irizarry et al., 2003) for normalization and summarization.

To determine relative intensities, the ratio of intensity for each sample in a probeset was calculated by normalizing to the average value. Ratios were exported in Gene Cluster and visualized as a heatmap with Java Treeview.

For the comparison of ALL and B cell subset expression profiles the raw microarray data files of 60 samples from four ALL subgroups (BCR-ABL1, E2A-PBX1, MLL-translocations and TEL-AML1; (Ross et al., 2003) and 14 samples from different stages of B cell development (van Zelm et al., 2005); Figure 10) were obtained from http://www.stjuderesearch.org/data/ALL3/rawFiles.html and http://franklin.et.tudelft.nl/ and analyzed as mentioned above.

2.2.11 Clonality analysis and spectratyping of B cell populations

A normal polyclonal B cell repertoire exhibits a bell-shaped curve of individual size-peaks according to a Gaussian-type distribution of length diversity within the VDJ junction. Conversely, clonal expansions or monoclonal B cell populations have only few or one single size-peak corresponding to few or only one dominating clone(s). To analyze the B cell repertoire on clonality/diversity V_HDJ_H gene rearrangements from cDNAs of mouse peripheral blood, were amplified using PCR primers specific for the J558 V_H region gene together with a primer specific for the Cμ constant region gene.

1	µL	dNTP (10 mM)
5	µL	10 x PCR buffer
2.5	µL	V_HJ558 forward primer(10 µM)
2.5	µL	Cµ reverse primer (10 µM)
1.5	µL	50 mM $MgCl_2$
1	µL	Platinum *Taq* DNA polymerase (Invitrogen)
35.5	µL	H_2O
49	µL	
1	µL	cDNA
50	µL	

The following program was used:

1	95 °C	2 min	
2	95 °C	1 min	
3	60 °C	1 min	34x
4	72 °C	1 min	
5	72 °C	5 min	
6	4 °C	pause	

Using a FAM-labeled Cµ constant region gene-specific primer in a run-off reaction, PCR products were labeled and subsequently analyzed on an ABI3100 capillary sequencer by fragment length analysis. In additional experiments, J_H-specific primers were used instead of the Cµ constant region gene-specific primer. Whereas J558-Cµ fragment analysis generates an overview over the repertoire of B cell clones using this V_H gene segment, J558-J_H1-4 amplifications have a higher level of resolution for individual V_H-J_H combinations.

1	µL	dNTP (10 mM)
5	µL	10 x PCR buffer
2.5	µL	V_HJ558 forward primer(10 µM)
2.5	µL	Cµ-FAM/J_H(1-4)-FAM reverse primer (10 µM)
1.5	µL	50 mM $MgCl_2$
1	µL	Platinum *Taq* DNA polymerase (Invitrogen)
35.5	µL	H_2O
49	µL	
1	µL	1st round
50	µL	

The following program was used:

1	95 °C	2 min	
2	95 °C	1 min	
3	60 °C	1 min	10x
4	72 °C	1 min	
5	72 °C	5 min	
6	4 °C	pause	

2.2.12 Quantitative RT-PCR

Quantitative real-time PCR was performed with the SYBRGreenER mix from Invitrogen (Carlsbad, USA) according to standard PCR conditions. During the PCR amplification, the SYBRGreenER dye in the mix binds to accumulating double-stranded DNA and generates a fluorescence signal proportional to the DNA concentration which can be visualized and measured using an ABI7900HT (Applied Biosystems, Foster City, USA) real-time PCR system. PCR reactions were carried out on 96-well optical plates or 384-

well optical plates (obtained from Applied Biosystems). Results were analyzed with SDS 2.2.2 from Applied Biosystems.

2.2.13 Generation of Retrovirus

5 x 10^6 cells of the adherent fibroblast cell line 293FT (Invitrogen, Carlsbad, USA) were plated on a 10 cm cell culture dish in complete growth medium, namely DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 200 mM L-Glutamine, 10 mM MEM Non-Essential Amino Acids, and 100 mM MEM Sodium Pyruvate (Invitrogen, Carlsbad, USA). About 16 hours later 5 µg of the packaging plasmid pHit60 (gag-pol), 2 µg of the packaging plasmid pHit123 (ecotropic envelope) or pHit456 (amphotropic envelope) and 7 µg of the retroviral vector were diluted in 1.75 mL DMEM without any supplements. In a separate tube 42 µL of Lipofectamine™ 2000 was also diluted in 1.75 mL DMEM without supplements. After 5 minutes of incubation the two solutions were combined and for another 20 minutes incubated. Meanwhile the medium from the 293FT cells was removed and 5 mL DMEM without supplements added. In the following the DNA-Lipofectamine complex was dropwise added to the plate and incubated under normal cell culture conditions. After 3 hours the supplements of the normal growing medium were added and the cells further incubated. 12 hours post-transfection the virus production was further induced through addition of sodium butyrate to a final concentration of 10 mM. Another 8 hours later the medium was substituted with complete growing medium and the first supernatant harvested 48 hours post-transfection. A second batch of virus was collected 72 hours post-transfection. The virus was either directly used or shock frozen in liquid nitrogen and stored at -80 °C.

2.2.14 Retroviral transduction

One well of a non-tissue treated 6-well plate was coated with RetroNectin® (TaKaRa, Shiga, Japan) by incubating it with 2 mL of a 50 µg/mL solution for 2 hours. After

blocking the coated well with 2 mL PBS containing 2% BSA for 30 minutes and washing it twice with 2 mL plain PBS, the well was ready prepared for the transduction procedure. Therefore the coated wells were covered with 2 mL of the viral supernatant and the plate centrifuged at 2000 rcf and 32°C for 2 hours. After repeating this step with fresh supernatant 1 x 10^6 cells in 2 mL of their own growth medium were filled into the virus loaded wells and centrifuged for 30 minutes at 500 rcf and 32°C. Afterwards the cells were further incubated for at least 48 hours under normal cell culture conditions. For following experiments the cells were gently scraped from the RetroNectin® plates and kept under normal culture conditions.

2.2.15 TOPO Cloning

As the amplification step for the cell lines and leukemia samples was performed with a primer mix a set of different products could be present in one reaction tube. To avoid problems with sequence analysis and to identify all V_HDJ_H gene rearrangements additional intermediate steps were performed.

The plasmid vector in the TOPO® PCR cloning kit is provided linearized and conjugated with TOPOisomerase I of Vaccinia virus. PCR products generated with *Taq* polymerases always contain an overhanging adenin at each end. Therefore the vector has to have an overhanging thymidine. The TOPO-isomerase recognizes a PCR product and ligates it into the linearized vector. 4 µL of the PCR product are mixed with 1 µL salt solution provided in the kit and 1 µL of the TOPO vector, in order to perform the cloning reaction. The mixture is then incubated at room temperature for 5 to 30 minutes and can afterwards be stored at -20 °C or directly used for bacterial transformation.

2.2.16 Bacterial transformation

The transformation of chemical competent cells was achieved by heat-shock. To this aim, 2 µL of the TOPO cloning reaction or 500 ng of any other plasmid were added to a vial of

chemical competent TOP10 *E.coli* cells (Invitrogen, Carlsbad, USA). The samples were incubated on ice for 30 minutes and then heat-shocked at 42 °C for 90 seconds. The tubes were immediately transferred on ice to accomplish the temperature shock. Subsequently 200 µL of S.O.C. medium was added to the tubes to attain a suitable growth environment for the cells. Incubation at 37 °C, shaking horizontally at 200rpm for one hour followed. 50 µL of this cell suspension were spread on LB-ampicillin plates and incubated at 37 °C over night. As all used vectors encoded for an ampicillin resistance gene only cells that carried a vector were able to grow and form colonies.

2.2.17 Plasmid preparation

In order to get a sufficient plasmid concentration for virus production a transformed *E.coli* clone was transferred into 5 mL LB-ampicillin medium and incubated for 16 hours at 37 °C in an incubator-shaker. Afterwards the volume was expanded to 200 mL and incubated for another 16 hours under same conditions. For the isolation of the plasmid DNA the HiSpeed® Maxi Kit from Qiagen (Hilden, Germany) was used. All steps were performed according to the manufacturer protocol.

2.2.18 Colony PCR

In order to amplify and isolate the V_HDJ_H segments, 25 µl of the master-mix below were pipetted into a 96 well PCR plate and one *E. coli* clone was transferred into each well. M13 primers bind to vector sequences adjacent to the insert. The colony PCR products were controlled by gel electrophoresis and subsequently purified for sequencing.

2.5	µL	dNTP (10 mM)
2.5	µL	10 x BV buffer
0.0625	µL	M13 forward primer (100 µM)

0.0625	µL	M13 reverse primer (100 µM)
1.25	µL	DMSO
0.2	µL	Platinum *Taq* DNA polymerase (Invitrogen)
18.425	µL	H_2O
25	µL	

The following program was used:

1	95 °C	2 min	
2	95 °C	30 sec	←
3	55 °C	1 min	44x
4	72 °C	1 min	←
5	72 °C	5 min	
6	4 °C	pause	

2.2.19 Purification of PCR products for sequencing

PCR products were purified in 96-well filter plates (Corning Inc., Corning, USA) filled with Sephacryl™ S-400 (Amersham Bioscience, Piscataway, USA). The filter plate was placed on a normal 96 well plate and filled with 300 µL of resuspended Sephacryl in each well. The plates were centrifuged for 2 minutes at 2390 rpm and washed five times with 250 µL LiChrosolph® dH_2O (Merck, Darmstadt, Germany) per well. After the final washing step the filter plate was transferred onto a clean 96-well plate and loaded with PCR products. The plates were once more centrifuged and the PCR products obtained were ready for sequencing.

2.2.20 Agarose gele electrophoresis

RNA and DNA-fragments were analyzed by agarose gel electrophoresis (AGE). The gels consisted of 2% agarose, 1x TAE was used as running buffer. For length determination a 1:10 dilution of a DNA ladder (Invitrogen, Carlsbad, USA) was used.

2.2.21 Software and web interfaces

Table 3: List of software

Software	
BRB Array Tools	http://linus.nci.nih.gov/BRB-ArrayTools.html
Cell Quest	BD Bisoscience
FCS Express	De Novo
Java Tree View	http://jtreeview.sourceforge.net
SDS 2.2.2	Applied Biosystems
Sigma Plot 10	Systat
Office 2003	Microsoft

Table 4: List of web interfaces

Web interfaces	
BLAST	http://www.ncbi.nlm.nih.gov/blast/Blast.cgi
Ensembl Genome Browser	http://www.ensembl.org/index.html
IMGT	http://imgt.cines.fr/
Java Tree View	http://jtreeview.sourceforge.net
Primer3	http://frodo.wi.mit.edu/
Reverse Complement	http://www.bioinformatics.org/sms/rev_comp.html

3. Results

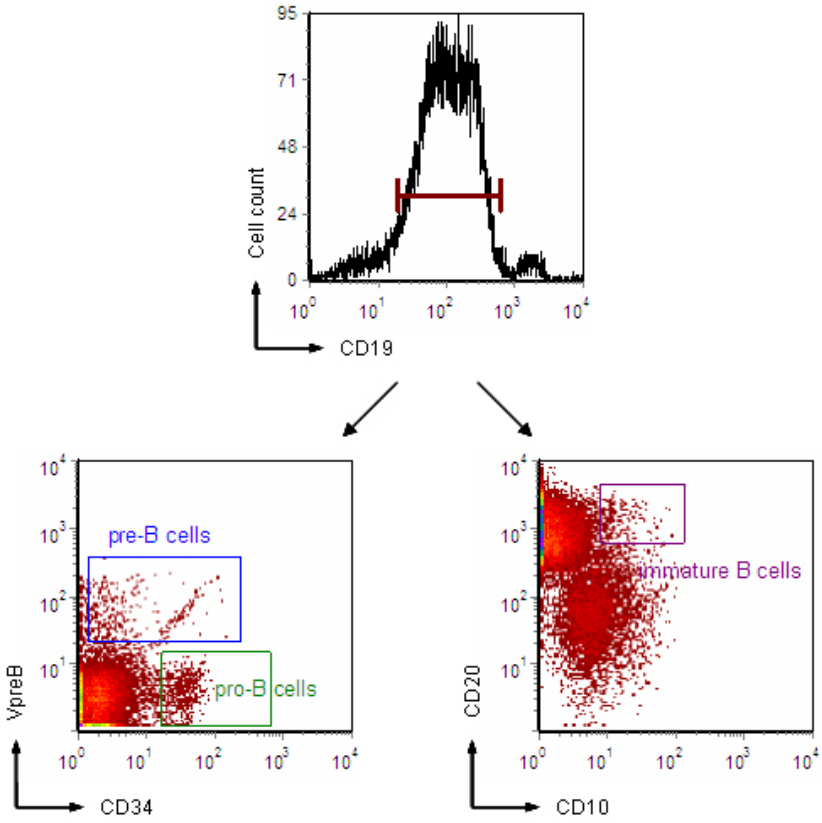
3.1 Lack of pre-B cell receptor function in ALL cells

3.1.1 Functionality of VDJ-rearrangements in ALL

To investigate the role of the pre-B cell receptor in ALL, the configuration of the immunoglobulin heavy chain locus in 148 cases of acute lymphoblastic leukemia and bone marrow B cell precursors from four healthy donors were studied. ALL cases include 57 cases of Ph^+ ALL (*BCR-ABL1*), 13 cases with *TEL-AML1*, 7 cases with *MLL-AF4*, 8 cases with *E2A-PBX1* and 20 cases with sporadic gene rearrangements. In addition, 30 cases with hyperdiploid and 13 cases with normal karyotype were studied (Figure 8, Table 5 and 7). Genomic DNA from these cases was used to amplify VDJ-fragments which subsequently were cloned into TOPO-vector (Invitrogen, Carlsbad, USA), amplified through transformation in *E.coli* and following Colony-PCR, and finally sequenced and analyzed by use of IMGT/V-QUEST web interface.

To be able to compare the results from the ALL cases with the normal situation in the human B-cell development, we did a single-cell sort of different B-cell subsets from four healthy donors (Figure 7). As cell surface markers for the sort we chose CD10, CD19, CD20, CD34 and VpreB. CD10, also known as common acute lymphoblastic leukemia antigen (CALLA), is a transmembrane glycoprotein expressed on a subset of B and T cell progenitors and germinal center B cells (Horejsi et al., 1988; Knapp et al., 1989). During all stages of B-cell differentiation and maturation, the co-receptor CD19 is expressed. Except for plasma cells expression levels are quite high (Knapp et al., 1989; (Bradbury et al., 1993). CD20 is expressed on pre-B cells, resting and activated B cells but not on plasma cells (Knapp et al., 1989; Loken et al., 1987). CD34 is an approximately 75-120kD glycoprotein on the surface of hematopoietic stem cells (McSweeney et al., 1998) and endothelial cells (Bhattacharya et al., 2000). VpreB, as part of the surrogate light chain and thereby part of the pre-B cell receptor (Karasuyama et al., 1990), is a characteristic marker for pre-B cells.

Figure 7: Single cell sort of early B cell subsets



Bone marrow from four healthy donors (one shown exemplarily) were pre-sorted through CD19-MACS and subsequently sorted by flow cytometry. Pro-B cells were sorted as $CD19^{+}CD34^{+}VpreB^{-}$, pre-B cells as $CD19^{+}VpreB^{+}$ and immature B cells as $CD19^{+}D10^{+}CD20^{+}$.

The sorted single cells were proteinase digested, followed by a pre-amplification step of the complete genome (PEP) and a two round nested PCR of the variable region of the µ-chain. The thereby amplified VDJ-segment was purified and directly used for sequencing.

Productive rearrangement of *IGHM* variable region genes is required for the expression of a µ-chain as the central component of the pre-B cell receptor. In the ALL analysis, 191 clonal *IGHM* V region gene rearrangements were analyzed, only 37 of which were potentially functional (19.4%). Among the 148 cases of ALL, only 31 (20.9%) cases had coding capacity for a potentially functional µ-chain (white rows;

Figure 8, Table 5 and 7). Unlike any other ALL subtype, ALL cells carrying an *E2A-PBX1* gene rearrangement had coding capacity for a functional μ-chain in all 8 cases studied. We conclude that ALL cells carrying an *E2A-PBX1* but not other oncogenic gene rearrangement are selected for the expression of a pre-B cell receptor. In non-*E2A-PBX1* ALL cells, a functional *IGHM* allele was amplified in 16.4%.

Table 5: VDJ-junctions of ALL cases*

ALL case	*Amino acid sequence of junction*
TEL-AML1 I	CTTGMVRGV#LYYYYGMD
	CARD#DLDYW
TEL-AML1 II	#VREGTSGAKG
TEL-AML1 III	CAHRPNIRYDAFDIW
TEL-AML1 IV	CARGKWIRHYYYYMDVW
TEL-AML1 V	CAREG**WEPPMLLIYYYG
TEL-AML1 VI	CARDSLRYFDWLG#DYW
TEL-AML1 VII	CARIQRYYYMDVW
TEL-AML1 VIII	CQEKGPA*YYYMD
	PSL*LLGPRNPGH
TEL-AML1 IX	L*QLV*L
TEL-AML1 X	CPVRRLLYYY*GMDV
TEL-AML1 XI	CARKGDYYGMDVW
	CTR*DL*VSAPSSW#DYW
	CARIWGWGGVGGAARRG
TEL-AML1 XII	CP*TYYDILTGYYYYYYGMRRL
TEL-AML1 XIII	CDEFGWW*LLPSLT
MLL-AF4 I	CARGFERVL**YQL#YYYYYYMDVW
	CARDVLL***WLLPGYYYYYYGMDV
	CARDPYSSGWYYYDSSGYYQ#YYYYYYG
MLL-AF4 II	CARD*SRY*WCMLY##WFDPW
MLL-AF4 III	CAHRWDFITMVRGVIIP#YYYYGMDVW
MLL-AF4 IV	CARLL*WW*LPP#YYYYGMDVW
MLL-AF4 V	LC#RTG***GVLLLLRYGTFG
	LCESKTTVTTQVRPLGP#NWFD
MLL-AF4 VI	CARDLL**LLPGDYYGMDVW
MLL-AF4 VII	SELT*NWFDPW
E2A-PBX1 I	SVREAPPFVAETFQDWGQGHW
E2A-PBX1 II	CARGSDDYGDYVAPGGRDQDFD
E2A-PBX1 III	CTTLGGGGFLDQSAAWFD
E2A-PBX1 IV	CARIRPYCSSTSCYNEST
E2A-PBX1 V	CARDPRRVLWFGELT
E2A-PBX1 VI	CAREHPLVRFG*MLL***WLLVN
	CARDFYWGSDYDAFDI

ALL case	*Amino acid sequence of junction*
PH+ ALL I	IPADCGGDC*GGWFDPWGQG
PH+ ALL II	CAGDGYN#YYYYGMDVW
PH+ ALL III	CARGGTCGGDCYH#SSYWYFDLW
PH+ ALL IV	WLQLF#LTTGARE
PH+ ALL V	CARAPDPPSMAVRGKKGLRV*FPYYYGM
PH+ ALL VI	EG*WEPPMLLISGAKG
PH+ ALL VII	CARDSLRYFDWLG#DYW
PH+ ALL VIII	CARRGSGY##FDYW
PH+ ALL IX	CAKDQAWE##YFD#W
PH+ ALL X	CARPL#CFDYW
PH+ ALL XI	CAPL*LERR#DYW
PH+ ALL XII	CA*GVLRYFDWLLYAPA#NWFDPW
PH+ ALL XIII	CARDRGPGRLRLGPGNPGHRLLR*GRIR
PH+ ALL XIV	CARDLGGSGS**P*VNWFDPW
PH+ ALL XV	CARI##FDPW
PH+ ALL XVI	VH*DIVVVPAA
PH+ ALL XVII	CKPSLGDFWSRIDYW
PH+ ALL XVIII	CVRDGDTTSWSFDYW
	CARDPSGWCGGDS*HH#FDYW
PH+ ALL XIX	CASGEKPRSTTVV#FYYYYYGMDVW
PH+ ALL XX	CART#SSTSYDGMDVW
PH+ ALL XXI	CAEVPLIF*LVIIFDYW
PH+ ALL XXII	CARAVSH#YNWFEGPG
PH+ ALL XXIII	CATLA#SGWYRYFDLW
PH+ ALL XXIV	CARDMGSERWIQLWLL*CPF#FDYW
	VYATSPYYYYGMDVW#QG
PH+ ALL XXV	CARGG*GAAAGTR#NWFDPW
PH+ ALL XXVI	CARDTADVL**YP#FDYW
PH+ ALL XXVII	CARALGITMIAVALGE#F
PH+ ALL XXVIII	CARPGRFSSGWYSAFDI#
	ASSGYSYWGQGTTVTVSSV*TCRK
PH+ ALL XXIX	CARHTVRETSPEPV*NPPHRR
	CRMSC*RLTPQGPA
	CARHTVRETSPEPV*NPCRMSC*RLTPQ GPA

ALL case	*Amino acid sequence of junction*
E2A-PBX1 VII	CAREGSIVVVPAATS*YYFDY
	CARGHPRFLEWLLYRYYFDY
E2A-PBX1 VIII	CAREHPLVRYYLLDVW
Hypderdiploid I	CARVPSIVVVPAADGITTTTTVWT##
Hypderdiploid II	H*DIVVVPAAIHYYYGMDV
Hypderdiploid III	CAKDIIDRWE##YFDYW
Hypderdiploid IV	CARGGGFG#YYGMDVW
Hypderdiploid V	CAKDLRE*YQL#YYYYYGMDVW
Hypderdiploid VI	CAHLLPERNS*TYMVRGVIIRKPPYYYYGMDV
Hypderdiploid VII	CARCRSFCIAVA#YFDYW
Hypderdiploid VIII	CARETVGI#PFDYW
Hypderdiploid IX	CARV*V*LRLGELSIE#SDYW
Hypderdiploid X	CAR#FDYW
Hypderdiploid XI	GPWS#
Hypderdiploid XII	CAHRPNSFSSSGGSC#*DYW
Hypderdiploid XIII	CARGHQS*LG*PWADYW
Hypderdiploid XIV	CARDRIAARP*#W
	CARDAVLMVYAIR##NGMDVW
Hypderdiploid XV	CARDPGMVRGVRPTTTTVW
Hypderdiploid XVI	SPSPQ#
Hypderdiploid XVII	GRGVL*WW*LLLGPWGQG
Hypderdiploid XVIII	PPVSGY*RSGLWLLLLRYGRLGPR
Hypderdiploid XIX	CAR#*LLYYFDYW
Hypderdiploid XX	CAKDYVLRFLEQ*#WLLYYYYGMDVW
Hypderdiploid XXI	CASSTIFGVVTSYYY
	CVRDQRRRYSSSSYYYYYMDVW
Hypderdiploid XXII	CARDRGLTESLRYFDWLLWAS#DPW
Hypderdiploid XXIII	CASDGPSGGD*LGI##DYW
Hypderdiploid XXIV	CAKGNPWDDFWSGYYR#NWFDPW
Hypderdiploid XXV	CARGLGATG#YYYYYYMDVW
Hypderdiploid XXVI	CARDSAGCGSSTSCYRG#AFDIW
Hypderdiploid XXVII	CASRGYASHM#YYYYYVDFW
Hypderdiploid XXVIII	YIVVVTAT*TGTSI
	CAGGVAGD*YGMD
Hypderdiploid XXIX	CARDGPGDGR*LGT
Hypderdiploid XXX	CARPHYSNYV#YYYYYGMDVW
	CARGGRTVTTSY*YYYGMDV
Sporadic I	CARPEEAYLITMVRGVIMVPTGARTLG#

ALL case	*Amino acid sequence of junction*
PH+ ALL XXX	CARDPPSITMIVVVIMG#DAFDIW
PH+ ALL XXXI	CARHQQTVVRGPLDPW
PH+ ALL XXXII	CAHSPRDPRG**WLYSGSNSRYYFDYW
	CAHSPRDPRG**WLLLGI
PH+ ALL XXXIII	CTRECITVRETAGYSSWFDF*
PH+ ALL XXXIV	CASQIL*WW*LPI#GAFDIW
PH+ ALL XXXV	CAYQKNLQIYTF*KVVI*RPSLTCTKCRS MRSSQPRSPSPQ
PH+ ALL XXXVI	CARAGSGDYGLYYYYYGMDVWGQGTTVTV SSG
	CARASGWNPTYYYYMDVWGKGTTVTVSS
PH+ ALL XXXVII	CVKPMGPYREAFDIWGQG
PH+ ALL XXXVIII	LVRELPGGGVCY#TGTTTTVWTSGAKG
	CTRVARGWCMLYRYYYYGMDVWGQGTLV TVS
	CARRDCSSTSCYTSDYYYGMDVWGQ#TMV TV#
	CTRVARGWCMLYRYYYYGMDVW
PH+ ALL XXXIX	#CEMG#EQPVYYGMDVWGQG
	CARWAGTTG#YYGMDVW
	CARVRYYDSSGYYHYYYGMDVW
PH+ ALL XL	CAER*KGLRGVL*LLGPG
	DYWGQGTLVTVSSG
	CARELSRRYSDLWGRGTTVTVS##
	CARGVL*LLGPGNPGHRLL
	CAHRSGVL*LLGPGNHGHRLLR#
	ITVLWW*LLLRAVWTSGAKG
	*CEG*KGLRGVL*LLGPG
	CARDKRDYGGYFDYW
	TV#EMGGV#LTTGARE
PH+ ALL XLI	CARVWYQLMPRDSW
PH+ ALL XLII	CVRDGDTTSWSFYYW
	CPEYSSGYGLPGPYYGMVVW
	CARSRRAVTT*#YFDYW
	CAGSTFLDWLLL##AFDVW
PH+ ALL XLIII	CAGG#NWFDPW
	CVRESSYDFWSGY##FDPW
PH+ ALL XLIV	CARDRWV*QQLVRPLRLWGQGTLVT
PH+ ALL XLV	CARPHY#DAFDIW
PH+ ALL XLVI	CSRGRSIAAAGKIPLL##YFDYW
PH+ ALL XLVII	GTTQAGSTP#
PH+ ALL XLVIII	CARV#GDYW

ALL case	*Amino acid sequence of junction*
Sporadic II	CARDDGPYGMDR##
Sporadic III	CARDLG*LGMTTGARAP
Sporadic IV	CARIERFG#FDYW
Sporadic V	CARPQEKQ#YYYYGMDVW
Sporadic VI	YCSSTSCYSLNH*L
Sporadic VII	CPVRRLLLLLRYGR
Sporadic VIII	CARVSHSSGSP#YWYFDLW
	CVPRGAR#E
Sporadic IX	CARCGYHSSGYYYGTAVHYW
Sporadic X	TTWTSGARA
Sporadic XI	CARDRRGEWPPSDYYYYY
Sporadic XII	CARGGY#WFDPW
Sporadic XIII	CARAPPYGYCSSTSCYGK#YYYGMDVW
	CAPPLRYFDW*LFRRRT
Sporadic XIV	CAKDPQQ#FDYW
	CARDKVWIAAA#YYYYYGMDVW
Sporadic XV	C*PGGYYF#YYYYYYMDVW
	CVKGVAVA*RLEHNNLT
Sporadic XVI	CAHSPFAGALFDY*GQG
Sporadic XVII	CARGEGYYDFWSGYYTG
	CAKVRLRPSTTTTVWT
Sporadic XVIII	CVRGRSGW#YW
Sporadic XIX	CARDLG#FDPW
Sporadic XX	CVR*RTREEIFGSAAGS

ALL case	*Amino acid sequence of junction*
PH+ ALL XLIX	CAKDXPRILWW*LLFR
PH+ ALL L	CARHP*GIAAAFCI##
PH+ ALL LI	LLYP#YGMDVW
PH+ ALL LII	CTTRRALVVPAA#YYG
PH+ ALL LIII	CARDRIS*YCXGGSCRERNYY
PH+ ALL LIV	CARDGPGYCSGGSC*WVGITM
PH+ ALL LV	HLLPERNS*TYMVRG
	AVGVH*DIVVVPAAIHYYYGM
PH+ ALL LVI	CAR*PFDPW
PH+ ALL LVII	CARVDRGG*LLRYGRLL
normal Karyo I	CARGPHLDIRCSSTSCYGSGSYYLTTGART LGP
normal Karyo II	CARAGTIKRYFDWLL*SIMITFGGVI#F
normal Karyo III	CAKGLPLKTDF##IDYW
normal Karyo IV	TGAREPLVTVSSG
normal Karyo V	CAKPLLL***WLLK#CFDYW
normal Karyo VI	CARAGGGPSPYYYGMDVW
normal Karyo VII	CARGGDCSGG##CYSGAPHYYYYYMDVW
normal Karyo VIII	CTTDLVVVPAAMPHL*#W
normal Karyo IX	CVREYYDSSGCLA#DYW
normal Karyo X	CARDRVIGGGFFTYYYDSSGYYYVP#YY
normal Karyo XI	CARDPKPAARPDTIF*L
normal Karyo XII	CARGPHLDIRCSSTSCYGSGSYYLTTGART LGP
normal Karyo XIII	CARAGTIKRYFDWLL*SIMITFGGVI#F

*functional rearrangements: white
non-functional rearrangements: grey

Table 6: VDJ-junctions of early B cell subsets*

B cell subset	*Amino acid sequence of junction*
pro-B I.8	ARDRHI*#FDYW
pro-B I.9	CAKDLRRGHFDWSLEYDSW
pro-B II.1	CARSGYSYARRGYYYYYGMDVW
pro-B II.3	CTTVHPSCYSGYGYGYGCGYGYGYTTSM*W
pro-B II.10	CARSGYSYARRGYYYYYGMDV
pro-B II.15	CAKDGAGSGYFDSW
pro-B II.17	CARWRNLEWYPW
pro-B III.3	TRTENYYDSTAYS
pro-B III.9	CFERGYYGLLGPWHLVIRSTSD#HDYYGMDVW
pro-B III.13	CAREKYSGYDLDYYYGMDVW
pro-B III.15	CAKVVMVVMVMVVMVVMVMVMVMVT#DAW
pro-B IV.3	CATPRTRQ*WSKPLFDYW
pro-B IV.7	CAKEGQGVLPYDTGDYW
pro-B IV.9	CARSGYSYARRGYYYYYGMDVW
pro-B IV.11	CVERERFSSGGY*LLQ
pro-B IV.15	CTRDAVVDTIF*RMAS#DYW
pro-B IV.20	CARDSLPSLPMPEGGSFW

B cell subset	*Amino acid sequence of junction*
pre-B I.2	CARRPAAGTGWFDPW
pre-B I.11	CARSNWGTDPDFDYW
pre-B I.14	SARIVYDSRDLYRLGWYFDLWGRG
pre-B II.12	CARERITMIVIMDVW
pre-B II.16	ARGTV*KTTSSE
	CAREGGTIFGVLYYFDYW
pre-B II.20	CARGLEGNSGSFHFDYW
pre-B III.2	CERF*VWIFGVVIIYAP*YYCGMDVW
	CARAHVGASGALDYW
pre-B III.6	CAGGAVFDYW
pre-B III.14	CALRLGVAGTGDDAFDIW
pre-B III.17	CAKDLYGGYGYGGYGCYGYGFYVDAW
pre-B III.20	CARPALPGGQAYGMDVW
pre-B IV.3	CARVTTVR*#YYYYYYYMDVW
pre-B IV.4	CAKDPTPRSSLFDYW
pre-B IV.11	CVRADLSLTAGGHFDSW
pre-B IV.14	CAKVADQQL#YFDSW
pre-B IV.20	CAKDSSCYSGCLYGMDVW

B cell subset	*Amino acid sequence of junction*
IB I.15	CARGVVVAATTWDYW
IB I.17	CARRVEMATKGAFDIW
IB I.18	CARDQPNFGVVRGHYYYGMDVW
IB II.3	CASRYCIYQLLWGPCL#DVW
IB II.5	CAREPNSGSYGWFDPW
IB II.12	CAVGRTYLDYW
IB II.17	CARDRRFDFWGDYYYYMDVW
IB II.20	CARDLQVATTVTTSPYYYYYMDVW
IB III.2	CARTTMIVVVIAGDSTP
IB III.3	CARSGYSYARRGYYYYYGMDVW
IB III.4	CARDPLYGDYDPLEYGMDVW
IB III.5	CARQPDTAGFWFDPW
IB III.6	CASPNYYGSGNPPYYYYGMDVW
IB III.7	CARDRGYGDYGLYFQHWGQGTLSPSPQVSLLSGDSG EPGV
IB III.15	CARGTNAIVPAAISSSWYDVRFDYW
IB IV.5	CARHLL*HGW*WTS*SIYYYYYV#DVW
IB IV.8	CARALGYVVVVPAAMRGWGHYGMDVW

*functional rearrangements: white
non-functional rearrangements: grey

Studying *IGHM* V region gene rearrangements in pre-B cells ($CD19^+$ $VpreB^+$) and immature B cells ($CD19^+$ $CD10^+$ $CD20^+$) from bone marrow of healthy donors by single-cell PCR, we identified potentially functional rearrangements in 14/16 (87.5%) and 15/17

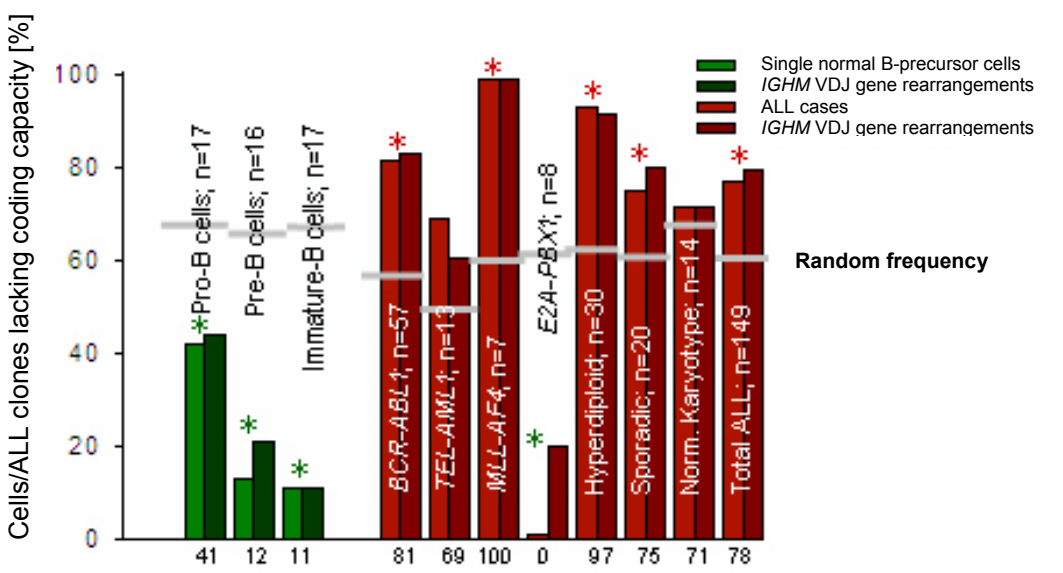
(88.2%) cells (Figure 8, Table 6 and 7). Among normal pro-B cells ($CD19^+$ $CD34^+$ $VpreB^+$), 10/17 cells (58.8%) carried a potentially productive *IGHM* V region gene rearrangement.

Table 7: Summary of µ-chain functionality

ALL subtype	Cases	VDJ rearrangements
BCR-ABL1	10/57 (17.5%)	14/82 (17.1%)
TEL-AML1	4/13 (30.8%)	5/17 (29.4%)
MLL-AF4	0/7 (0%)	0/10 (0%)
E2A-PBX1	8/8 (100%)	8/10 (80%)
Hyperdiploid	1/30 (3.3%)	1/34 (2.9%)
Sporadic	4/20 (20%)	5/25 (20%)
Normal Karyotype	4/13 (30.8%)	4/13 (30.8%)
sum	31/148 (20.9%)	37/191 (19.4%)
sum without *E2A-PBX1*	23/140 (16.4%)	29/181 (16.0%)
B cell subtype	**Cases**	**VDJ rearrangements**
pro-B	10/17 (58.8%)	10/17 (58.8%)
pre-B	14/16 (87.5%)	14/18 (77.8%)
immature B	15/17 (88.2%)	15/17 (88.2%)
sum	39/50 (78%)	39/52 (75%)

Assuming random distribution of functional and non-functional *IGHM* alleles, it can be expect that roughly 69% of cells/ALL clones lack coding capacity for a pre-B cell receptor if one *IGHM* locus is rearranged and 47% of cells/ALL clones if both *IGHM* alleles are rearranged. Two of three possible V_HDJ_H-joints are not in the correct reading frame and 4 in 64 junctional codons can be stop codons if randomly distributed. Based on these assumptions, the expected frequency of cells/ALL clones lacking coding capacity in the absence of positive or negative selection for expression of a functional pre-B cell receptor (grey lines, Figure 8) were calculated. Compared to random distribution of non-functional *IGHM* alleles, we found evidence for positive selection in favor of functional *IGHM* alleles in pre-B cells, immature B cells and *E2A-PBX1*-induced ALL cells ($p<0.05$; green asterisks, Figure 8).

Figure 8: Graphical overview of µ-chain functionality



The configuration of the immunoglobulin heavy chain (*IGHM*) locus was studied in bone marrow B cell precursors from four healthy donors by single-cell PCR and in 148 cases of acute lymphoblastic leukemia (ALL) including main ALL subtypes. In each group the frequency of cells/ALL clones that do not harbor any functional *IGHM* allele (light bars) and the total frequency of non-functional IGHM alleles (dark bars) are given. Based on whether only one or both *IGHM* alleles are rearranged, the random frequencies of cells/ ALL clones lacking coding capacity for a pre-B cell receptor were calculated. Asterisks denote frequencies of cells/ALL clones without coding capacity for a pre-B cell receptor that are significantly higher (red asterisks) or lower (green asterisks) than random. Numbers at the bottom panel denote frequencies of cells/ALL clones that do not harbor any functional *IGHM* allele; *p<0.05

In *BCR-ABL1*- and *MLL-AF4*-induced ALL, and ALL with either sporadic aberrations or a hyperdiploid karyotype, the observed frequency of cases lacking coding capacity was significantly higher than random distribution (red asterisks, Figure 8). These findings suggest that ALL clones in these leukemia subtypes are negatively selected against a functional pre-B cell receptor. In pro-B cells and ALL cells carrying a *TEL-AML1* gene rearrangement or a normal karyotype, the frequency of non-functional *IGHM* alleles was not significantly different from random distribution (Figure 8).

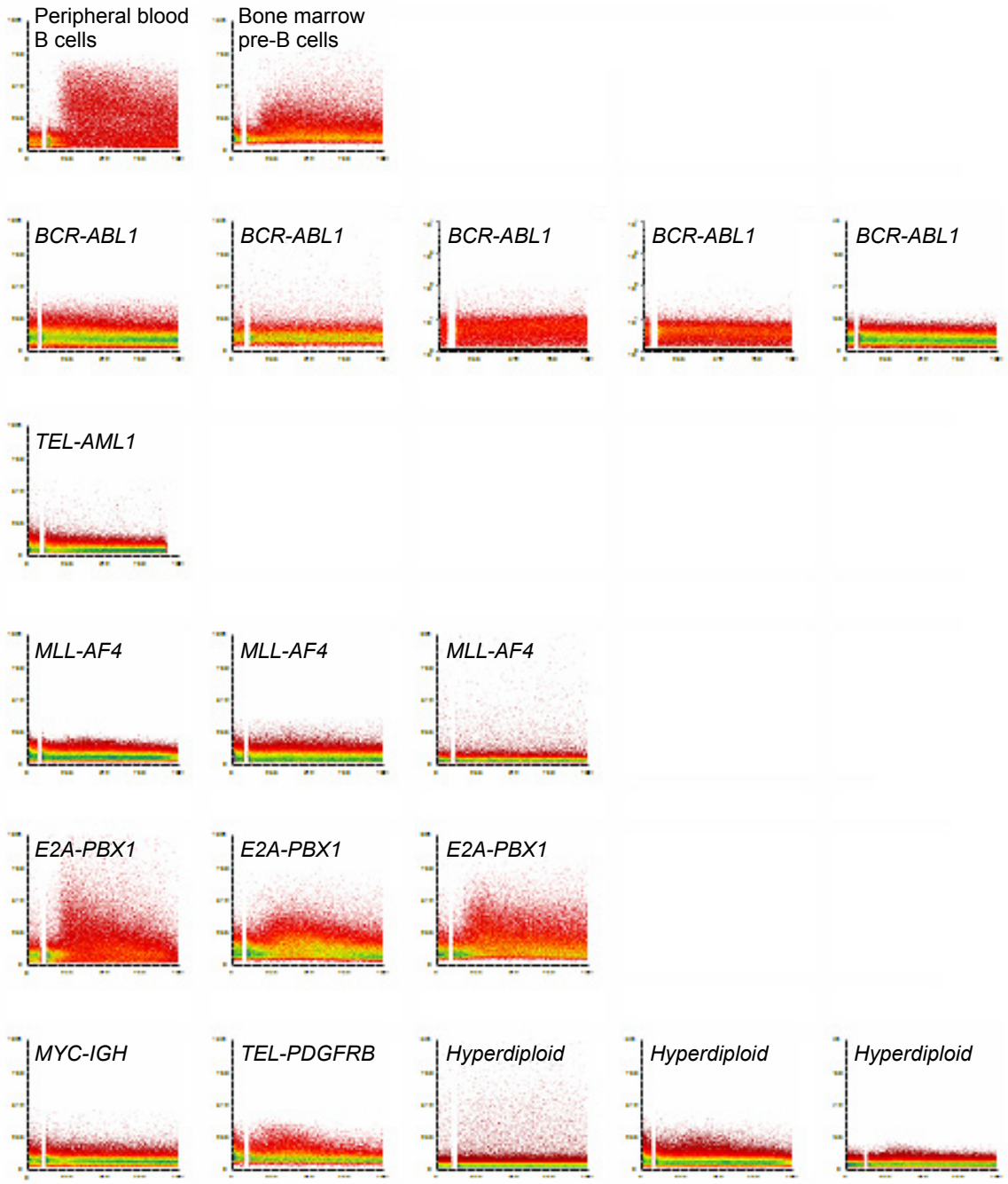
3.1.2 Determination of functional pre-B cell receptor signaling

We next studied the function of the pre-B cell receptor in 17 ALL cell lines:

Table 8: ALL cell lines

Cell line	Karyotype
BV173	BCR-ABL1
Nalm1	BCR-ABL1
SD1	BCR-ABL1
SUP-B15	BCR-ABL1
TOM1	BCR-ABL1
REH	TEL-AML1
BEL1	MLL-AF4
RS4;11	MLL-AF4
SEM	MLL-AF4
697	E2A-PBX1
Kasumi-2	E2A-PBX1
MHH-Call3	E2A-PBX1
380	MYC-IGH
Nalm6	TEL-PDGFRB
HBP-Null	hyperdiploid
MHH-Call2	hyperdiploid
Nalm19	hyperdiploid

Figure 9: Verification of pre-B cell receptor functionality through Ca^{2+} release



17 ALL cell lines were treated with a µ-chain specific antibody for pre-B cell receptor engagement. Ca^{2+} flux in response to pre-B cell receptor ligation was measured by flow cytometry. Normal peripheral blood B cells and bone marrow pre-B cells were used as positive controls. The gap in the density plots indicates the time intervals during which anti-µ antibodies were added for (pre-) B cell receptor crosslinking. Only the three *E2A-PBX1*-induced ALL cell lines showed a strong Ca^{+} signal as stimulation response.

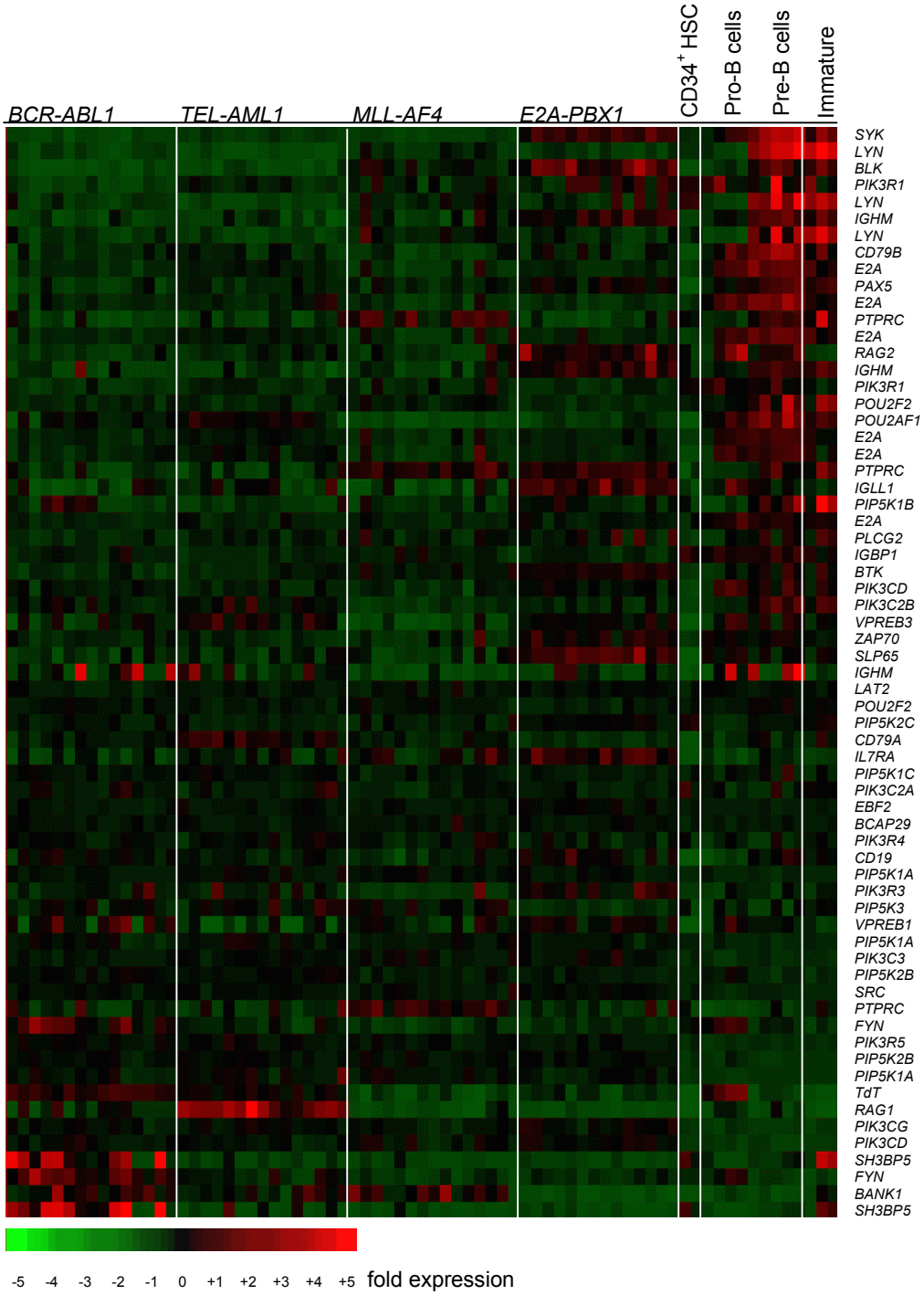
The stimulation of a functional pre-B cell receptor signaling cascade through an anti-μ-chain antibody leads besides others to activation of PLCγ2, resulting in generation of IP_3. Subsequently triggering of the IP_3-receptor causes the release of intra- and extracellular Ca^{2+} sources into the cytoplasm. Through the Ca^{2+}-indicator fluo3 (Invitrogen, Carlsbad, USA) this is visible in form of a change in signal intensity in flow cytometry.

While, engagement of the (pre-) B cell receptor using μ-chain-specific antibodies resulted in a strong Ca^{2+} signal in normal B cells and pre-B cells, this was the case for only 3 of 17 ALL cell lines (Figure 9). All three cell lines carry an *E2A-PBX1* gene rearrangement, which is consistent with selection for the expression of a pre-B cell receptor in this subset of ALL. In two cell lines, a minority of the cells exhibit a weak pre-B cell receptor signal (*TEL-PDGFRB* and Hyperdiploid).

Further microarray data of different ALL and B cell precursor subsets (Ross et al., 2003; van Zelm et al., 2005) with regard on pre-B cell signaling components were analyzed. Loss of pre-B cell receptor function in *BCR-ABL1*-, *TEL-AML1*- and *MLL-AF4*-induced but not *E2A-PBX1*-induced ALL is also mirrored at the gene expression level: *IGHM* encoding the μ-chain and central pre-B cell receptor signaling components including *SYK*, *BLK*, *BTK* and *SLP65* are transcriptionally silenced in the cases of *BCR-ABL1*-, *TEL-AML1*- and *MLL* -mediated ALL but not in *E2A-PBX1*-dependent leukemia (Figure 10).

The complete absence of pre-B cell receptor function in most ALL subtypes may indicate that a functional pre-B cell receptor is still needed to deliver survival signals in *E2A-PBX1*-transformed ALL cells but no longer in the presence of *BCR-ABL1*, *TEL-AML1*, *MLL-AF4* or other oncogenic fusion genes. Conversely, one could even hypothesize that the pre-B cell receptor renders a pre-B cell non-permissive to transformation by oncogenic fusion genes other than *E2A-PBX1*.

Figure 10: Expression profiles of ALL subtypes and B-cell subsets



In literature searches, 65 Affymetrix U133A 2.0 probsets were identified that match important components of the pre-B cell receptor signaling pathway. In a meta-analysis of data generated by Ross et al. (Ross et al., 2003) and van Zelm et al. (van Zelm et al., 2005), we compared mRNA levels of pre-B cell receptor signaling molecules in normal hematopoietic stem cells (HSC), normal B cell precursors and *BCR-ABL1*, *TEL-AML1*, *MLL* and *E2A-PBX1* subsets of ALL.

3.2 Expression and function of the pre-B cell receptor in a *BCR-ABL1*-transgenic mouse model

To investigate the expression and function of the pre-B cell receptor during leukemic transformation of B cell precursor cells, we chose a mouse model for either *BCR-ABL1*-, *TEL-AML1*-, or *MLL-AF4*-induced ALL in a proof of principle study. *BCR-ABL1* defines a subset of ALL with a particularly unfavorable prognosis and represents the most frequent gene rearrangement in adult ALL. The currently available mouse models for *TEL-AML1*- and *MLL-AF4*-induced ALL have limitations because *TEL-AML1* only rarely induces ALL in mice (Bernardin et al., 2002) and *MLL-AF4* induces B cell lymphoma rather than ALL after a long latency (Chen et al., 2006). For these reasons, we chose the classical mouse model for *BCR-ABL1*-induced ALL (Heisterkamp et al., 1990). In this model, B cell lineage leukemia driven by p190 BCR-ABL1 develops on average at 100 days of age. We studied B cell populations in the bone marrow, peripheral blood and spleen of i) wildtype littermates, ii) pre-leukemic mice that carry the *BCR-ABL1* transgene in the absence of any indication of leukemia, iii) mice with full-blown leukemia and iv) mice that had full-blown leukemia after seven days of treatment with the BCR-ABL1 kinase inhibitor AMN107 (Nilotinib). Using this model for progressive transformation by BCR-ABL1, we studied the phenotype, clonality, gene expression and pre-B cell receptor function in these four situations (wildtype, pre-leukemic, leukemic, Post-AMN107).

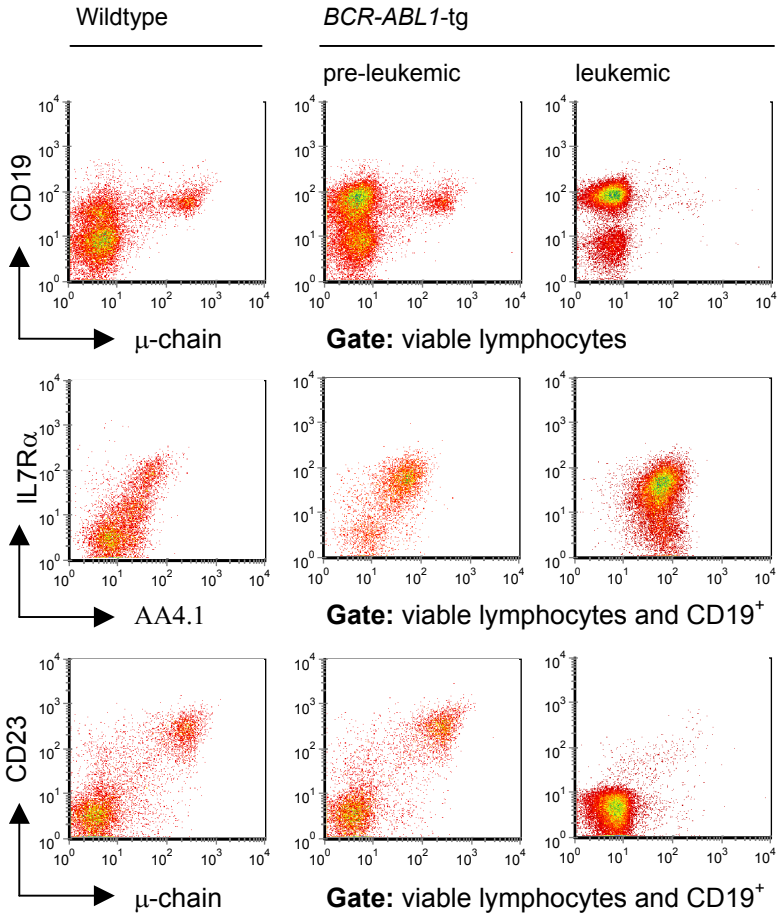
3.2.1 *BCR-ABL1*-transgenic pre-B cells before the onset of leukemia are phenotypically equivalent to wildtype pre-B cells

To examine possible differences in B cell development between the four mouse groups mentioned above, we did flow cytometry measurements on bone marrow, spleen and blood with antibodies against different B cell specific markers. The interleukin 7 receptor α chain (IL7Rα or CD127) as part of the interleukin 7 receptor plays an important role in

the early development of lymphocytes and is therefore expressed on CLPs, early stages of B cell lineage in the bone marrow and most T cells (Akashi et al., 1998; Brugnera et al., 2000; Faust et al., 1993; Henderson et al., 1992; Kouro et al., 2001). The monoclonal antibody AA4.1 reacts with a type I transmembrane protein expressed from early progenitor through the immature B cell stage in murine bone marrow (Li et al., 1996). The μ-chain is part of the pre-B as well as the B cell receptor and therefore expressed from the pre-B cell stage on in bone marrow, spleen and peripheral blood. Besides the μ-heavy chain, mature B cells can express in parallel the δ-heavy chain (Early et al., 1980; Rogers et al., 1980), recognized by an anti-IgD antibody. Other markers for a later stage of B cell development are, as part of the BCR, the κ- and λ-light chain and the low-affinity receptor for IgE CD23 (also known as FcεRII). In addition to mature B cells CD23 is also found on activated macrophages, eosinophils, follicular dendritic cells and platelets (Yokota et al., 1988).

We observed that B cell development in *BCR-ABL1*-transgenic mice with overt leukemia was arrested at a pre-B cell receptor-negative stage of development (Figure 11 and 12). As opposed to wild type B cells, leukemic cells did not express immunoglobulin μ-heavy chains, did downregulate the mature B cell marker CD23 and aberrantly express IL7rα and AA4.1 outside of the bone marrow, reflecting expansion of primitive leukemic B cell lineage clones beyond the site of primary B cell development. In striking contrast to fully developed leukemia, pre-leukemic B cell populations that carry the oncogenic *BCR-ABL1* fusion are phenotypically undistinguishable from wildtype B cell populations (Figure 11 and 12). The presence of the *BCR-ABL1* fusion was confirmed in pre-leukemic B cell populations by Southern Blot. These findings demonstrate that the mere presence of the oncogenic BCR-ABL1 tyrosine kinase is not sufficient to bring about the dramatic phenotypic changes observed after the onset of leukemia. Only after completion of the “transformation” process, the leukemia cells exhibit a characteristic pre-B cell receptor-negative phenotype. From these findings it is not clear, whether “transformation” mainly reflects progressive phenotypic reprogramming of most if not all B cell progenitors or whether it reflects outgrowth of a small, phenotypically primitive, subpopulation, which had a survival advantage over more mature subclones.

Figure 11: Phenotypic changes of B cell populations in bone marrow during progressive leukemic transformation by BCR-ABL1

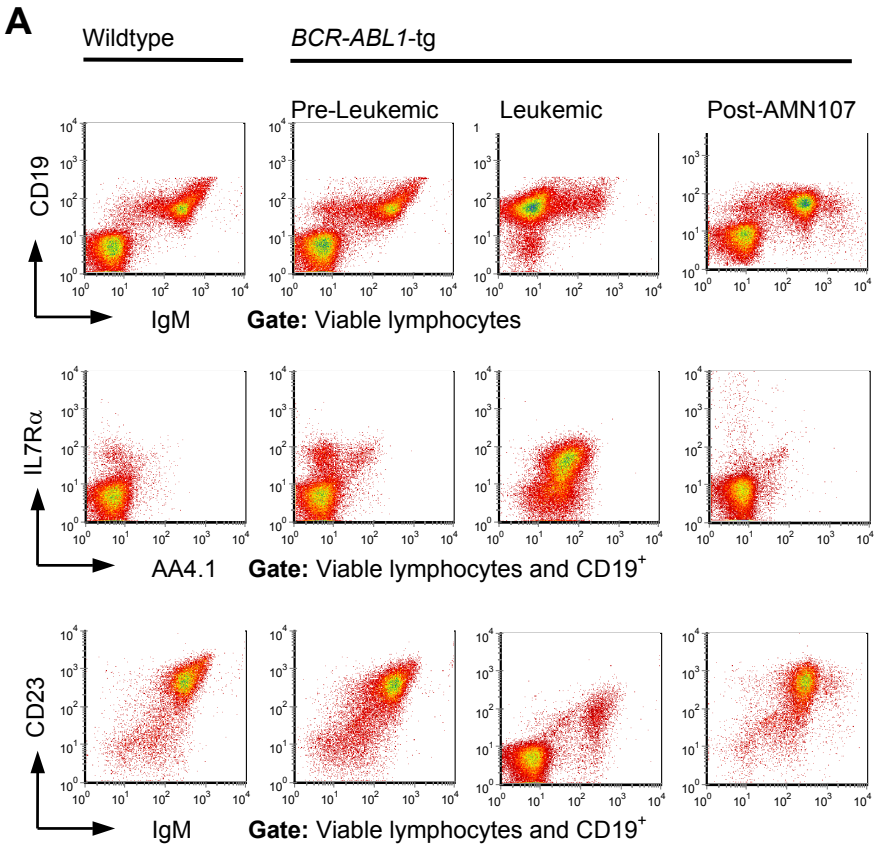


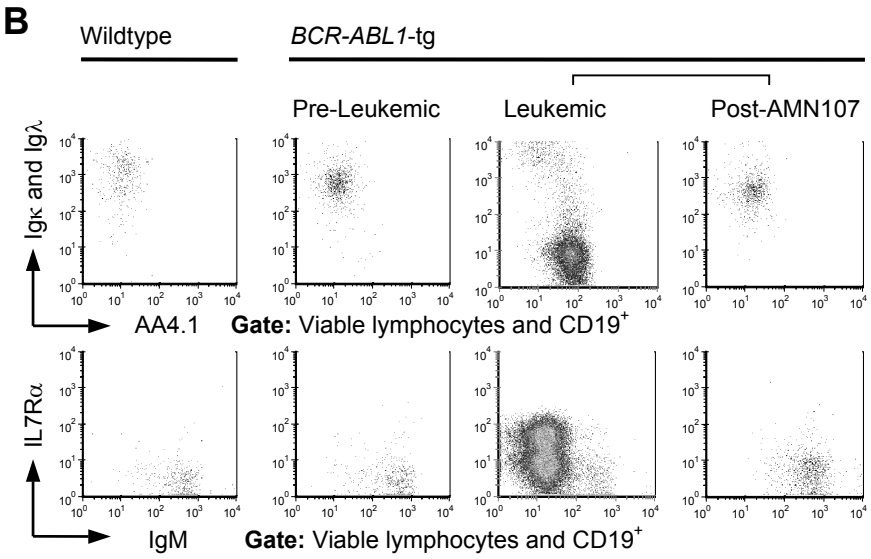
B cell populations from bone marrow of wildtype and *BCR-ABL1*-transgenic mice were analyzed by flow cytometry. Among *BCR-ABL1*-transgenic mice, pre-leukemic and mice with full-blown leukemia were studied. For flow cytometry, antibodies against CD19, the μ-heavy chain or IgM, the interleukin 7 receptor α chain and the AA4.1 (CD93) antigen were used, which are both expressed on early B cell precursor populations. In contrast, CD23 is expressed on more mature B cell populations. The data shown are representative for measurements based on four or more mice in each group.

3.2.2 BCR-ABL1 kinase inhibition by AMN107 reconstitutes normal B cell development within seven days

A subset of *BCR-ABL1*-transgenic mice that developed full-blown leukemia was treated with 75 mg/kg body weight AMN107 for seven days. For some mice with full-blown leukemia, matched peripheral blood sample pairs before and after seven days of treatment with AMN107 were available (Figure 12B and 13). Strikingly, treatment of the leukemic mice with AMN107 almost completely restored phenotypically normal B cell populations in the spleen and peripheral blood (Figure 12 and 13).

Figure 12: Phenotypic changes of B cell populations in spleen and peripheral blood during progressive leukemic transformation by BCR-ABL1

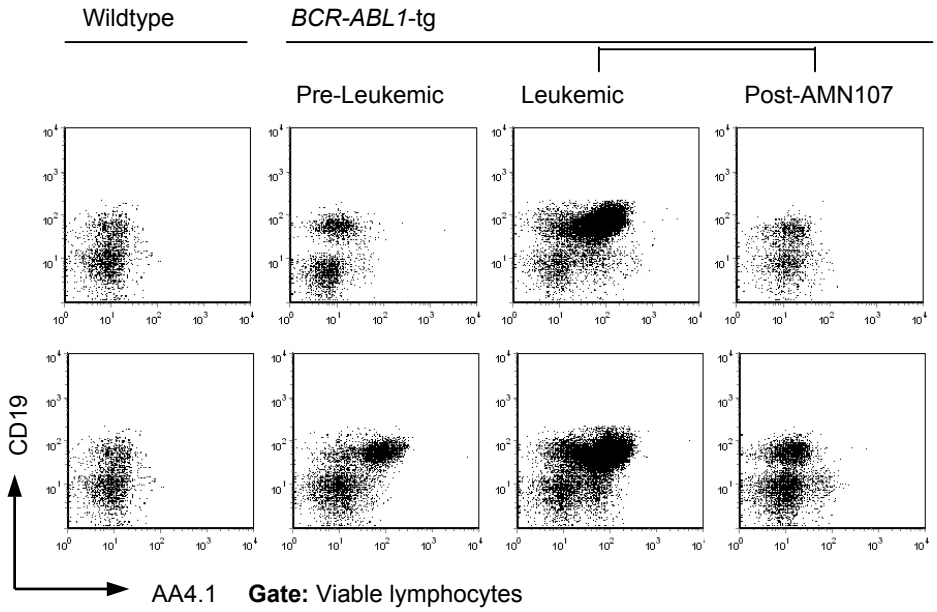




B cell populations from spleen (A) and peripheral blood (B) of wildtype and *BCR-ABL1*-transgenic mice were analyzed by flow cytometry. Among *BCR-ABL1*-transgenic mice, pre-leukemic and mice with full-blown leukemia before and after seven days of treatment with 75 mg/kg AMN107 were studied. For peripheral blood, the same mice were studied before and after treatment. For flow cytometry antibodies against CD19, κ and λ immunoglobulin light chains, the μ-heavy chain or IgM, the interleukin 7 receptor α chain and the AA4.1 (CD93) antigen were used, which are both expressed on early B cell precursor populations. In contrast, CD23 is expressed on more mature B cell populations. The data shown are representative for measurements based on four or more mice in each group.

As $AA4.1^+$ B cells are very rare in the peripheral blood (PB) of normal mice (Hardy et al., 2000) we also analyzed these cells in the four different mouse groups in connection to the complete B cell population ($CD19^+$; Figure 13).

Figure 13: Analysis of AA4.1$^+$ proportion in peripheral blood during progressive transformation by BCR-ABL1



Peripheral blood of two wild type littermates; two overtly leukemic p190 BCR-ABL1 transgenic mice before treatment and the same mice after 7 days of 75 mg/kg AMN107 treatment. Dead cells were excluded based on propidium iodide uptake and the analysis was restricted to viable lymphocytes. Antibodies are as indicated.

Whereas in the normal mice, the percentage of CD19$^+$ cells in PB was low, the PB of the leukemic animals consisted almost entirely of CD19$^+$ cells, of which the majority was AA4.1$^+$ (Figure 13). When these animals were treated for only seven days with AMN107, the numbers of these CD19$^+$AA4.1$^+$ leukemic cells were substantially reduced and other cells re-appeared in the peripheral blood. We also quantitated the proportion of leukemic blasts (CD19$^+$AA4.1$^+$) in the PB of the mice and further characterized the leukemic transformation by white blood cell count (WBC) and splenic weight (Table 9).

Table 9: Characterization of progressive leukemic transformation in *BCR-ABL1*-transgenic mice

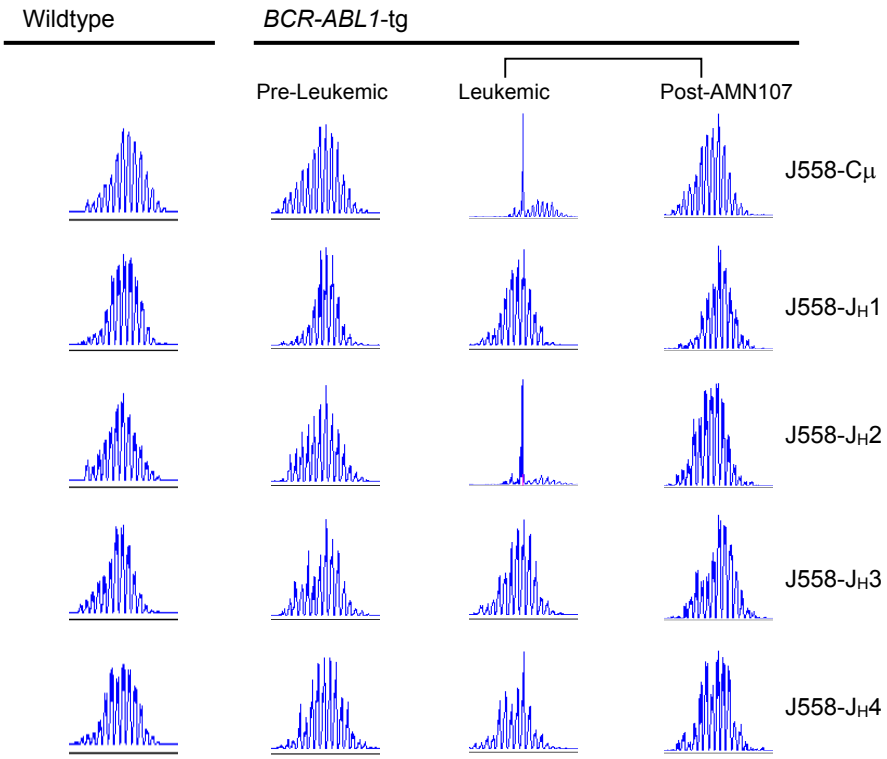
	Wildtype	Pre-Leukemic	Leukemic	Post-AMN107
WBC [x 10^9/l; n=5]	6.0 ± 1.8	15.8 ± 5.2	146.4 ± 56.9	11.4 ± 5.2
Leukemic blasts [%$CD19^+AA4.1^+$; n=5]	0.9 ± 0.7	4.2 ± 7.4	78.4 ± 7.0	1.4 ± 0.7
Splenic weight [mg; n=4]	82.9 ± 15.5	106.3 ± 27.4	289.7 ± 80.8	125.7 ± 19.5

Comparing wildtype mice and pre-leukemic *BCR-ABL1*-transgenic mice, white blood cell counts (WBC) and splenic weights were not significantly increased. Upon full leukemic transformation, however, WBCs were increased ~9-fold, the percentage of leukemic blasts was increased ~20-fold and splenic weights were increased almost 3-fold. These changes were largely reversible upon seven days of treatment with AMN107 (Table 9).

3.2.3 Clonal evolution of *BCR-ABL1*-transgenic pre-B cells during malignant transformation

To study the clonal composition and repertoire of the pre-B cell pools in wildtype and *BCR-ABL1*-transgenic "pre-leukemic" mice and mice with full-blown leukemia before and after treatment with AMN107, we used a modified spectratype length-distribution analysis of VDJ-junctions of rearranged *IGHM* V region genes. At two levels of resolution we analyzed *IGHM* V region gene rearrangements using Cμ- (low resolution) and J_H- (high resolution) specific primers (Figure 14). Interestingly, *BCR-ABL1*-transgenic pre-B cells before the onset of leukemia represent a normal polyclonal population. Pre-B cells from mice with full-blown leukemia however, mainly belong to one single clone, which dominates the repertoire.

Figure 14: Clonal evolution during progressive transformation by BCR-ABL1

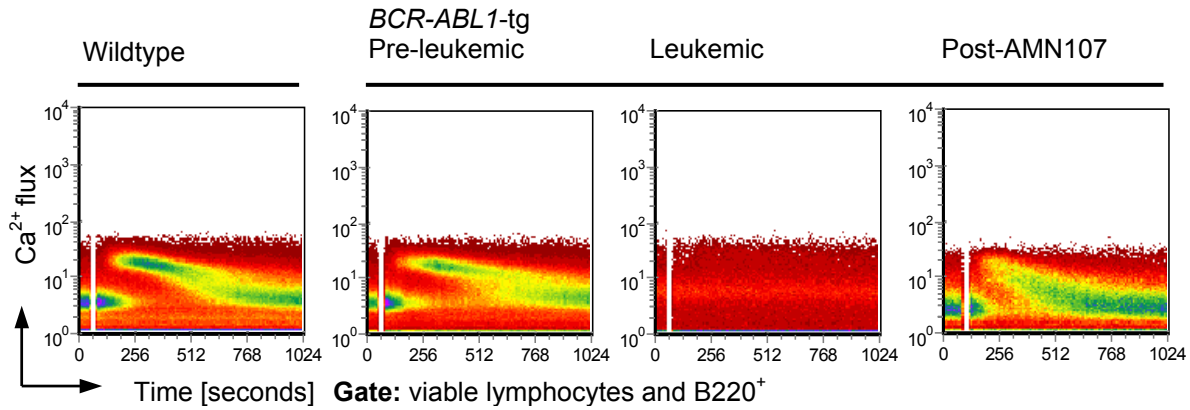


Immunoglobulin heavy chain gene rearrangements were analyzed using the spectratype technique as described in the Materials and Methods section. The chromatograms show the length distribution of VDJ junctions in B lineage cells of wildtype and *BCR-ABL1*-transgenic mice before the onset of leukemia (pre-leukemic), with full-blown leukemia before (Leukemic) and after seven days of treatment with AMN107 (Post-AMN107). Each peak represents one length of VDJ-junctions, which are typically spaced by 3 nucleotides (one amino acid) reflecting the reading frame in functional VDJ gene rearrangements. In the absence of clonal expansions, VDJ junctions typically show a Gaussian-type length distribution as in the wildtype B cells analyzed here (left column). Using a primer specific for the Cμ segment yields low-resolution analysis of the global repertoire of immunoglobulin heavy chain gene rearrangements. J_H segment-specific amplification allows for higher resolution analysis: the leukemic clone in the case shown (3rd column) here uses a J_H2 gene segment.

3.2.4 Pre-B cell receptor function of *BCR-ABL1*-transgenic pre-B cells during progressive transformation

Ca^{2+} release from the endoplasmic reticulum reflects a downstream signaling event upon pre-B cell receptor stimulation (Guo et al., 2000). Therefore, we measured Ca^{2+} release in response to pre-B cell receptor engagement in wild type and *BCR-ABL1*-transgenic mice before the onset of leukemia and full-blown leukemia before and after seven days of treament with AMN107 (Figure 15). While the pre-B cell receptor signal was vigorous both in wildtype and pre-leukemic *BCR-ABL1*-transgenic mice, stimulation of the pre-B cell receptor in mice with full-blown leukemia had no effect (Figure 15). However, treatment of leukemic mice with AMN107 for seven days almost entirely reconstituted pre-B cell receptor responsiveness. These findings indicate that pre-B cell receptor signaling and transformation by BCR-ABL1 are mutually exclusive.

Figure 15: Pre-B cell receptor function during progressive transformation by BCR-ABL1



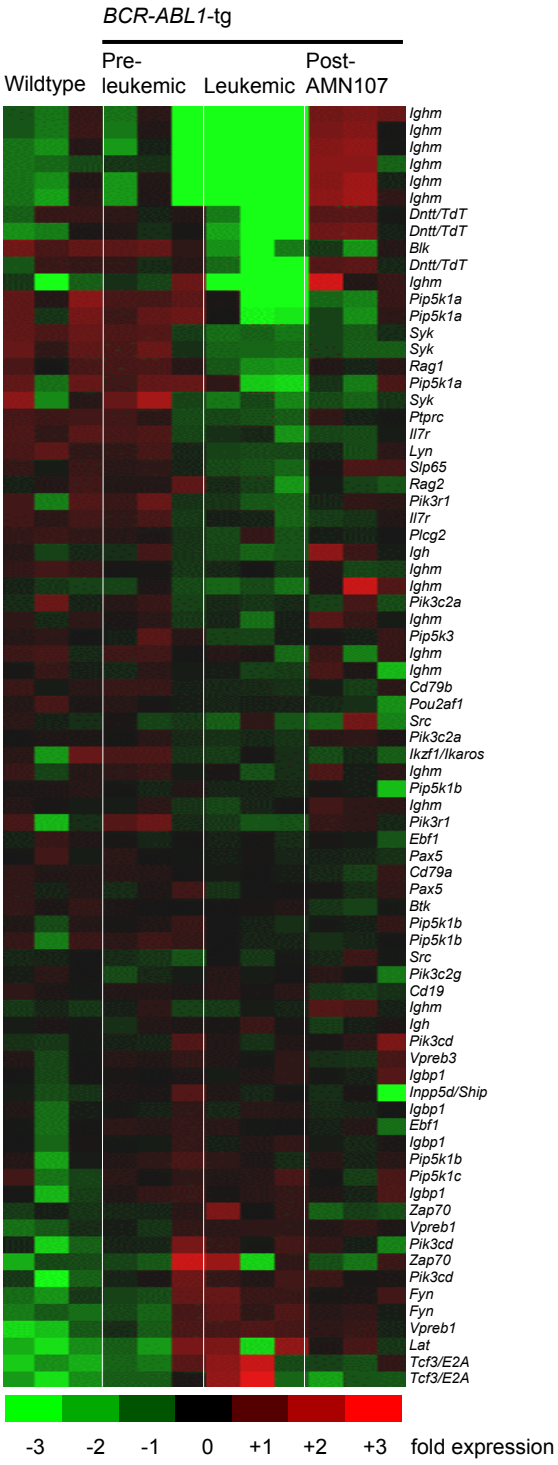
To test pre-B cell receptor responsiveness, we stimulated mouse B cell precursors with an anti-μ chain antibody and measured Ca^{2+} release in response to pre-B cell receptor engagement by flow cytometry. Antibody was added during a short time interval indicated as a gap in the density plots. Normal pre-B cells show a rapid increase of cytoplasmic Ca^{2+} concentrations shortly after stimulation, which return to baseline levels within five to ten minutes.

3.2.5 Gene expression changes during progressive transformation by BCR-ABL1

To elucidate why pre-B cell receptor responsiveness is lost during progressive transformation of pre-B cells by BCR-ABL1, we studied mRNA levels of pre-B cell receptor-related signaling molecules using Affymetrix 430 GeneChips and quantitative RT-PCR (Figure 16 and 17). mRNA from sorted $CD19^+$ $AA4.1^+$ bone marrow pre-B cell populations from wildtype and *BCR-ABL1*-transgenic mice before and after leukemic transformation and after seven days of treatment with AMN107 was isolated, linear amplified through generation of cRNA and hybridized to microarrays. Based on literature searches, we assembled a group of 76 probesets for pre-B cell receptor related signaling molecules. The obtained data for these probesets were sorted based on the ratio of gene expression values in normal bone marrow pre-B cells and bone marrow pre-B cells from mice with full-blown leukemia. As depicted in Figure 16, the μ-heavy chain encoded by the *Ighm* gene is dramatically downregulated upon full transformation by BCR-ABL1. In addition, critical downstream signaling molecules including Syk and Slp65 were downregulated in transformed pre-B cells.

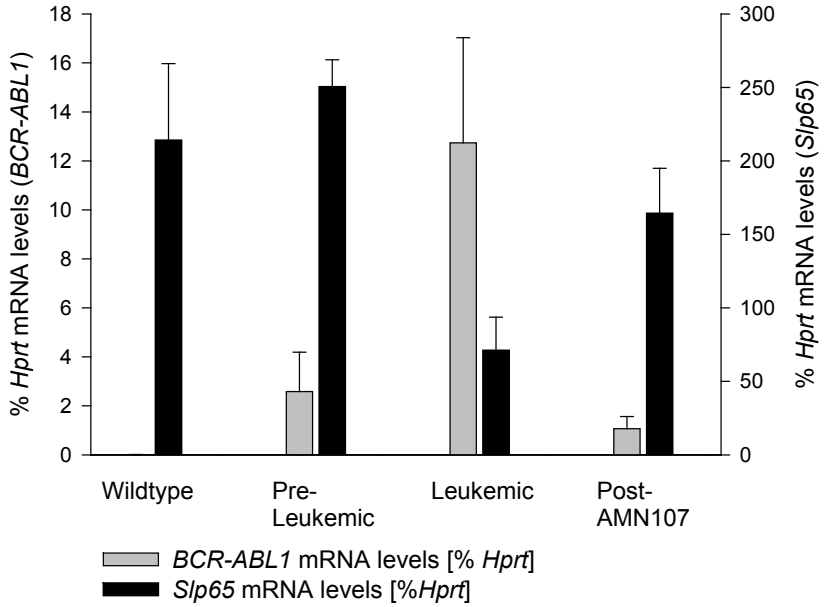
Figure 16: Kinetics of mRNA levels of pre-B cell receptor-related molecules during leukemic transformation by BCR-ABL1

Pre-B cells were sorted for CD19$^+$ AA4.1$^+$ from the bone marrow of wildtype and *BCR-ABL1*-transgenic mice before the onset of leukemia (pre-leukemic), with full-blown leukemia before (Leukemic) and after seven days of treatment with AMN107 (Post-AMN107) and subjected to gene expression analysis using Affymetrix 430 GeneChips. 76 probesets for pre-B cell receptor related (signaling) molecules were identified based on literature searches and sorted based on the ratio of gene expression values in wildtype vs leukemic pre-B cells. For each gene, all available probesets were indicated.



We next determined mRNA levels of *BCR-ABL1* in parallel with *Slp65* in sorted pre-B cells on a per cell basis. Therefore cDNA from mRNA of the same cells as used for the generation of microarray data (Figure 16) was generated and quantitative RT-PCR, using a SybrGreen® Mastermix (Invitrogen, Carlsbad, USA) was performed. The resulting expression levels of the target genes were normalized to the expression of the house keeping gene *Hprt* (*hypoxanthine guanine phosphoribosyltransferase*). Levels of *BCR-ABL1* and *Slp65* in wildtype, pre-leukemic, leukemic and AMN107-treated mouse samples showed inverse kinetics in these four situations: BCR-ABL1 transcripts were not detected in wildtype animals, expressed at low levels in pre-leukemic pre-B cells, with a 6-fold increase upon transformation (Figure 17). Treatment with AMN107 *in vivo* resulted in a 10-fold decrease of *BCR-ABL1* mRNA levels. Conversely, mRNA levels for *Slp65* were generally high, but 4-fold downregulated in full-blown leukemia (Figure 17).

Figure 17: Kinetics of mRNA levels of *BCR-ABL1* and *SLP65* during leukemic transformation by BCR-ABL1



From the same samples used for GeneChip analysis (Figure 16), cDNAs were synthesized and used for quantitative RT-PCR analysis of *BCR-ABL1* (gray bars, left vertical axis) and *Slp65* (black bars, right vertical axis) mRNA levels. mRNA levels are given in percent *Hprt* mRNA and as means of measurements based on pre-B cells from three individual mice (± S.E.M.).

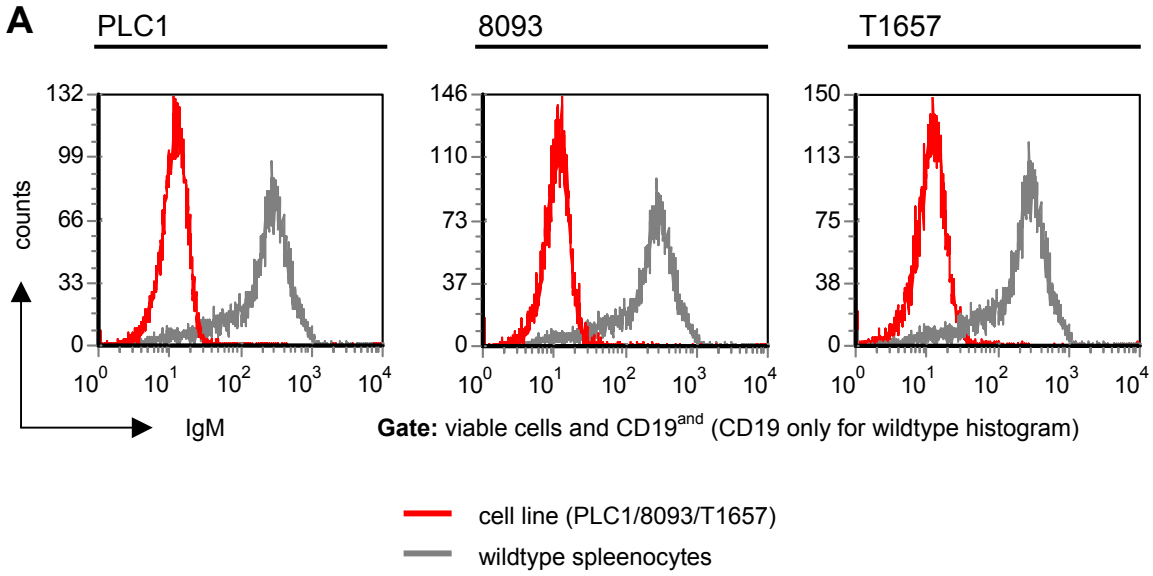
Together these results corroborate that active signaling from a functional pre-B cell receptor and the BCR-ABL1 kinase are mutually exclusive.

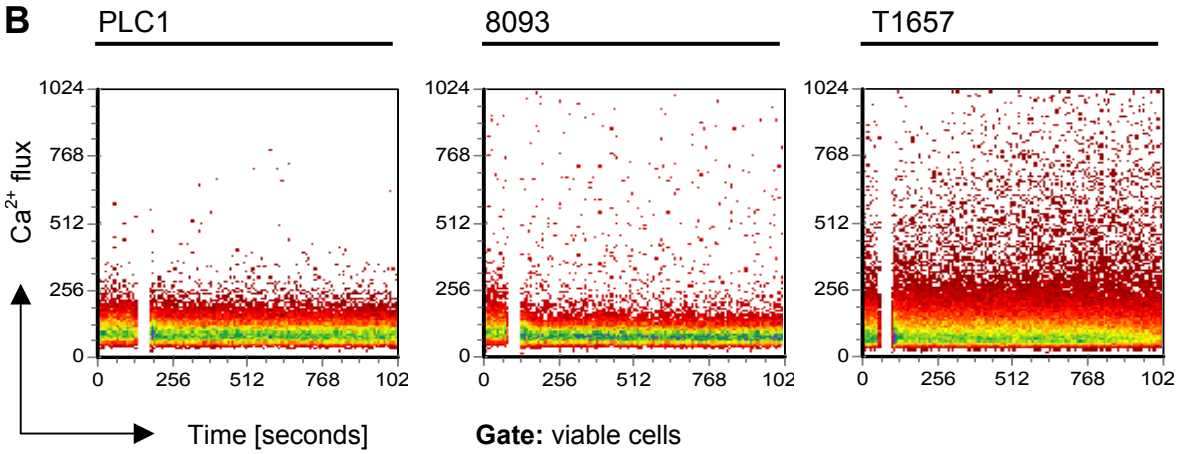
3.3 Mutual exclusion of pre-B cell receptor signaling and *BCR-ABL1* expression in cell culture experiments

3.3.1 Reconstitution of pre-B cell receptor expression suppresses leukemic growth in BCR-ABL1-transformed mouse pre-B ALL cells

To test this hypothesis in a formal experiment, we reconstituted expression of the μ-heavy chain (*Ighm*) and *Slp65* in BCR-ABL1-transformed pre-B ALL cells lacking expression of *Ighm* and *Slp65*, respectively. The two already established pre-B ALL cell lines PLC1 (Mishra et al., 2006) and 8093 (Kaur et al., 2007) and the new generated cell line T1657, each from a mouse with full-blown BCR-ABL1-driven leukemia, were tested for μ-chain expression and pre-B cell receptor signaling capacity and were all negative for both (Figure 18).

Figure 18: μ-chain expression and pre-B cell receptor signaling in murine *BCR-ABL1*-transgenic cell lines

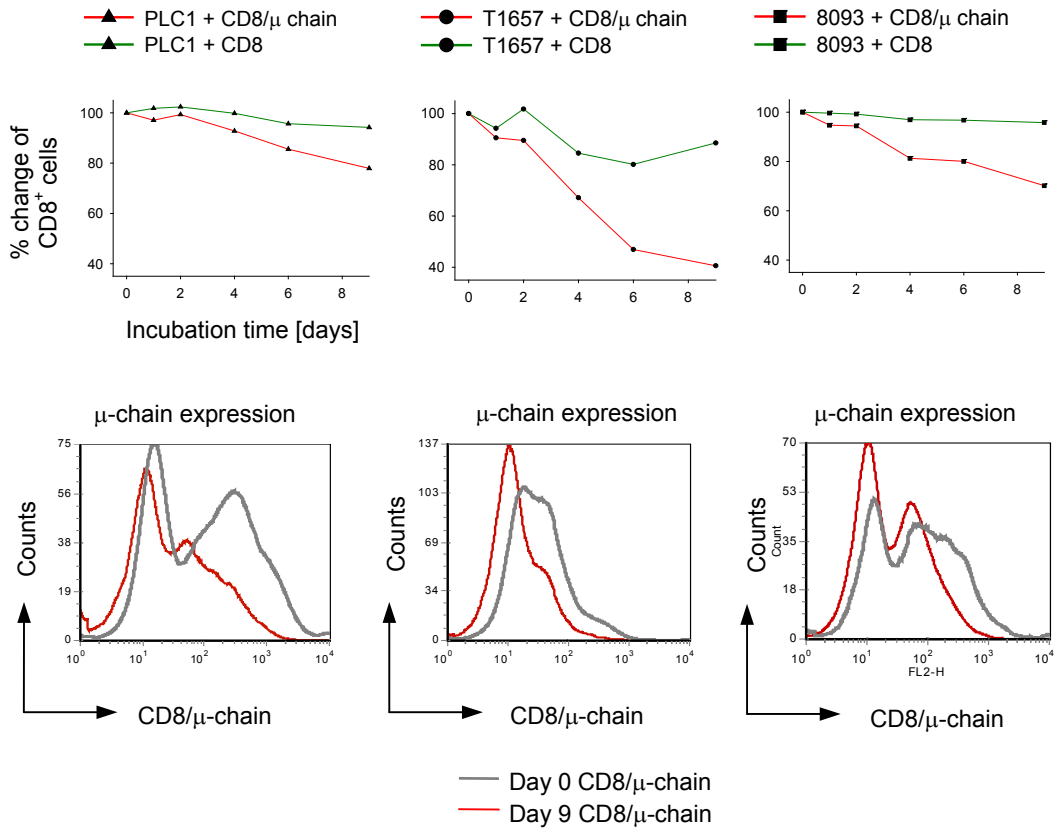




From three leukemic BCR-ABL1-transgenic mice, pre-B ALL cell lines were established (PLC1, T1657, 8093). The lack of pre-B cell receptor expression was verified in two ways: I, the cell lines were stained with an anti-IgM antibody that recoginzes the µ-heavy chain. Missing cell surface expression was determined by flow cytometry measurements as shown (A, red line). As positive, control measurements on splenocytes from wildtype mice are shown (A, grey line). II, the cell lines were stimulated with an anti-µ-chain antibody and Ca^{2+} release in response to pre-B cell receptor engagement was measured. Antibody was added during a short time interval indicated as a gap in the density plots. None of the cell lines showed an increase of cytoplasmic Ca^{2+} concentrations and Ca^{2+} release in response to pre-B cell receptor engagement (B).

We transduced the three cell lines with a retroviral vector encoding a functional µ-chain (*Ighm*) together with mouse CD8 or CD8 alone (control). Enrichment or depletion of µ/$CD8^+$ cells vs $CD8^+$ cells was monitored for 9 days by flow cytometry. In all three cell lines, we observed progressive loss of µ/$CD8^+$ cells as compared to $CD8^+$ cells (Figure 19, upper panel). Also mean fluorescence intensities for µ/CD8 expression were downregulated comparing cells two (set as day 0) and eleven days after transduction (Figure 19, lower panels).

Figure 19: Reconstitution of the µ-heavy chain suppresses leukemic growth in BCR-ABL1-transformed pre-B cells

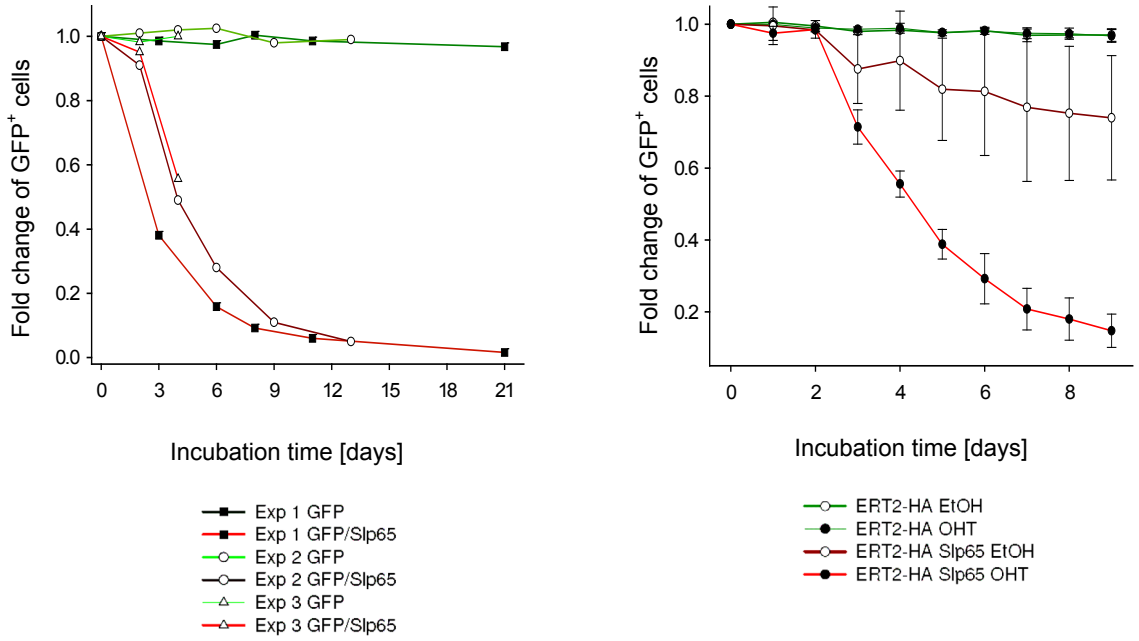


The three cell lines PLC1, T1657 and 8093 were transduced with retroviral expression vectors encoding either CD8 alone or CD8 together with a functional µ-heavy chain. Two days after transduction (set as day 0), measurements of CD8 were started by flow cytometry (set as 100%). Changes of the percentages of $CD8^+$ and µ /$CD8^+$ cells were monitored over nine days (eleven days after transduction; upper panel). The fluorescence intensities for µ /CD8 were compared between day 0 (grey line) and day 9 (red line) in the histogram overlays depicted (lower panel).

Because Slp65 was previously identified as a critical transducer of pre-B cell receptor signals (Flemming et al., 2003; Jumaa et al., 1999) and downregulated by BCR-ABL1 in our analysis (Figure 16 and 17), we studied the effect of *Slp65*-reconstitution in BCR-ABL1-transformed $Slp65^{-/-}$ pre-B ALL cells. To this end, bone marrow pre-B cells from $Slp65^{-/-}$ mice (Jumaa et al., 1999) were purified and transformed with a retrovirus encoding BCR-ABL1 and neomycin-resistance in the presence of mouse Il7. Unlike wildtype pre-B cells, $Slp65^{-/-}$ pre-B cells can be transformed by BCR-ABL1 at a high

efficiency. We established an Il7-independent pre-B ALL cell line and reconstituted *Slp65* expression in the BCR-ABL1-transformed pre-B ALL cells either constitutively (Figure 20, left panel) or inducibly (Figure 20, right panel). For the inducible system Slp65 was fused to the mutated estrogen receptor ligand binding domain (ERT2). Either ERT2 fused to the N-terminus of Slp65 (ERT2-*Slp65*/GFP) or ERT2 alone (ERT2/GFP) were expressed in the Slp65$^{-/-}$ BCR-ABL1-transformed pre-B ALL cells. Addition of 1 µmol/L 4-hydroxy-tamoxifen (OHT) dissolved in ethanol resulted in full activation of ERT2-Slp65 fusion molecules. Ethanol was used as a vehicle control. Enrichment or depletion of GFP$^+$ cells was monitored during 9 days of cell culture after addition of OHT.

Figure 20: Reconstitution of SLP65 in BCR-ABL1-transformed Slp65$^{-/-}$ pre-B cells suppresses leukemic growth*



Slp65$^{-/-}$ pre-B cells were transformed with a retroviral vector encoding BCR-ABL1 and neomycin resistance and kept under cell culture conditions. These cells were then reconstituted with a *Slp65*/GFP expression vector or transduced with a GFP vector as a control. The percentage of viable GFP$^+$ cells two days after transduction was set as 100% and then subsequently monitored for up to three weeks. Three experiments are shown (left panel). To test the effect of Slp65-reconstitution in Slp65$^{-/-}$ BCR-ABL1-transformed pre-B ALL cells using an inducible model, the cells were transduced with an ERT2-*Slp65*/GFP vector or an ERT2/GFP control vector. Addition of 1 µmol/L 4-hydroxy-tamoxifen (OHT) solved in ethanol resulted in full activation of ERT2-Slp65 fusion molecules. Ethanol was used as a vehicle control. Enrichment or depletion of GFP$^+$ cells (measured as percentage of GFP$^+$ cells) was monitored during 9 days of cell culture after addition of OHT. Data represent means of three experiments ± S.E.M (right panel).

*experiments done by Sebastian Herzog, group of Hassan Jumaa, MPI of Immunobiology, Freiburg, Germany

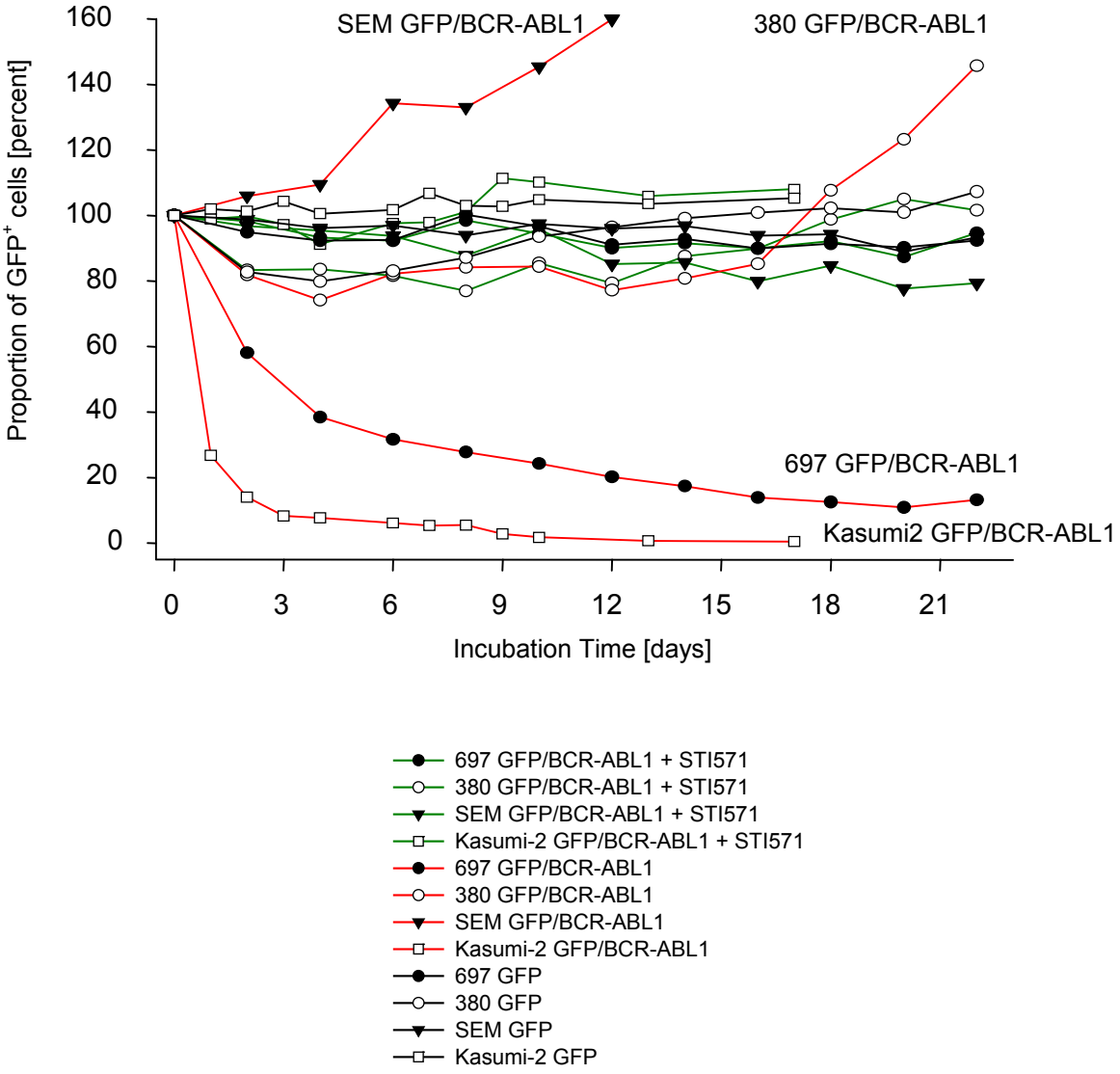
In both systems, we observed selective depletion of *Slp65*-reconstituted pre-B ALL cells within nine days after transduction (constitutive system) or after induction (inducible system) of Slp65. We conclude that reconstitution of pre-B cell receptor signaling (μ-chain or Slp65) effectively prevents leukemic growth of BCR-ABL1-transformed mouse pre-B ALL cells.

3.3.2 Pre-B cell receptor signaling prevents BCR-ABL1-transformation of human pre-B ALL cells

To test whether pre-B cell receptor signaling also renders human pre-B cells non-permissive to transformation by *BCR-ABL1*, we super-transformed pre-B ALL cell lines with BCR-ABL1. To this end, we identified two *E2A-PBX1*-induced pre-B cell receptor-positive (697, Kasumi-2) and two pre-B cell receptor-negative (380, SEM) ALL cell lines based on their ability to respond to pre-B cell receptor engagement (Figure 9). The four human ALL cell lines were then first transduced with a lentiviral vector encoding the ecotropic retrovirus receptor (to allow for secondary transduction with the ecotropic MSCV virus) and CD8. $CD8^+$ cells were then sorted by flow cytometry and transduced with the ecotropic MSCV retrovirus encoding either *BCR-ABL1* and GFP or GFP alone. Two days after transduction, the percentage of GFP^+ cells was measured by flow cytometry and set as reference value for subsequent measurements. For each ALL cell line, three different conditions were compared: i) ALL cells transduced with GFP, ii) ALL cells transduced with *BCR-ABL1*/GFP and iii) ALL cells transduced with *BCR-ABL1*/GFP in the presence of 10 μmoL/L of the BCR-ABL1 kinase inhibitor STI571 (Imatinib; Figure 21). ALL cells transduced with GFP alone or with *BCR-ABL1*/GFP in the presence of STI571 did not show a significant change in the fraction of GFP^+ cells regardless of whether or not they express a functional pre-B cell receptor. ALL cells transduced with *BCR-ABL1*/GFP in the absence of STI571, however, showed either rapid depletion (697 and Kasumi-2 cells) or selective outgrowth (380 and SEM cells) of *BCR-ABL1*/GFP^+ cells, depending on whether the ALL cells carry a functional pre-B cell receptor or not. These findings indicate that super-transformation of ALL cells with

BCR-ABL1 confers a survival advantage in the absence but not in the presence of active pre-B cell receptor signaling (Figure 21). We propose that only "crippled" pre-B cells lacking a functional pre-B cell receptor are permissive to transformation by *BCR-ABL1*.

Figure 21: Pre-B cell receptor signaling renders ALL cells non-permissive to BCR-ABL1 transformation.

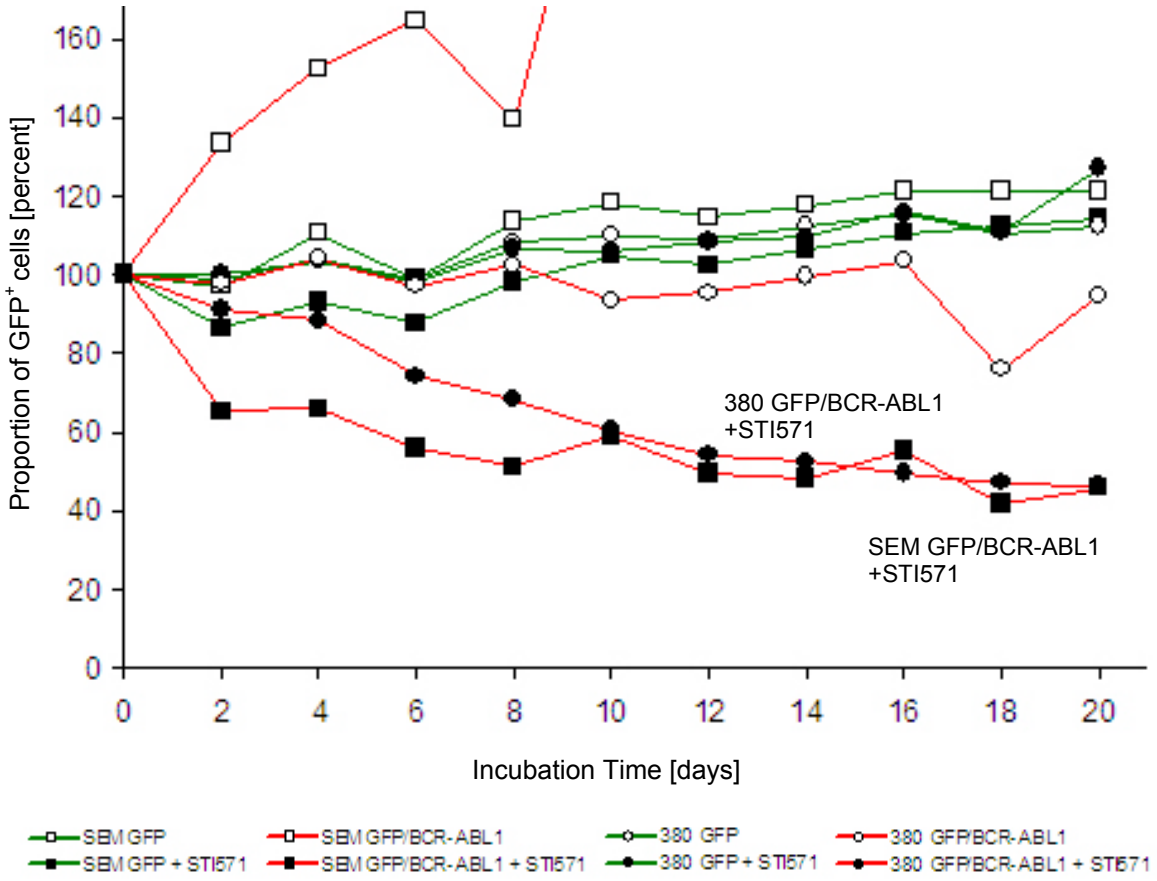


Pre-B cell receptor responsiveness in four B cell lineage ALL cell lines (697, Kasumi-2, 380 and SEM) was measured by flow cytometry as shown in Figure 8. The four B cell lineage ALL cell lines were then cultured under three different conditions: they were either transduced with GFP alone (GFP), transduced with GFP/BCR-ABL1 (GFP/BCR-ABL1) or transduced with GFP/BCR-ABL1 and cultured in the continuous presence of 10 µmol/L STI571 (Imatinib) (GFP/BCR-ABL1 and STI571). The fraction of GFP^+ cells two days after transduction was set as 100% and depletion or enrichment of GFP^+ cells was monitored for up to 22 days.

3.4 Oncogene-Addiction through BCR-ABL1

Philadelphia-chromosome negative ALL cells that do not carry a functional pre-B cell receptor (SEM, 380) showed selective outgrowth after super-transformation with *BCR-ABL1*/GFP (Figure 21). This finding indicates that *BCR-ABL1*-expression confers even in these cells an additional survival advantage. The same cells did not show any reaction in the presence of STI571 as BCR-ABL1 was inhibited directly after transduction. Still we wanted to test whether the cells became sensitive to STI571-treatment after adaption to BCR-ABL1-kinase activity. To this end we let the super-transformed cells grow for further 6 weeks, than added 10 µL/mL of STI571 to the cell culture medium and measured together with the corresponding controls the proportion of transduced cells.

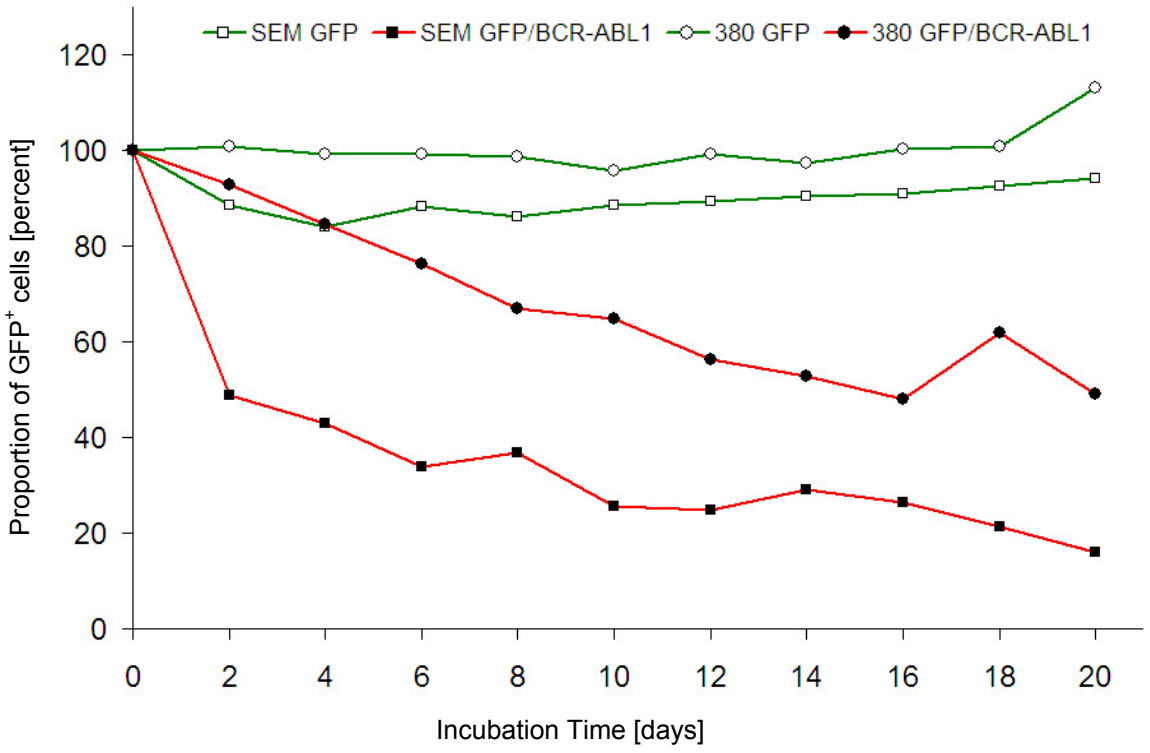
Figure 22: Addiction of ALL cells to BCR-ABL1-kinase



After adaption to BCR-ABL1-kinase activity treatment with STI571 leads to a decrease in the proportion of super-transformed cells. Untreated cells show either an ongoing outgrowth (SEM) or steady-state (380). Experiment was done twice.

The untreated super-transformed cells showed either an ongoing outgrowth (SEM) or stayed on the same level (380). In contrast the proportion of BCR-ABL1$^+$ cells under treatment with STI571 decreased during 20 days of measurement by 50% (Figure 22). During the same time frame the cells transformed with a control vector showed no response on STI571. Considering that the same cells that show a decrease in cell number during treatment with STI571 show an outgrowth without any treatment makes the effect even more striking. In order to make this visible we calculated the proportion of super-transformed cells during STI571 treatment in subject to changes in the non-treated population (Figure 23).

Figure 23: Oncogene addiction of ALL cells depicted as calculation in subject to control cells

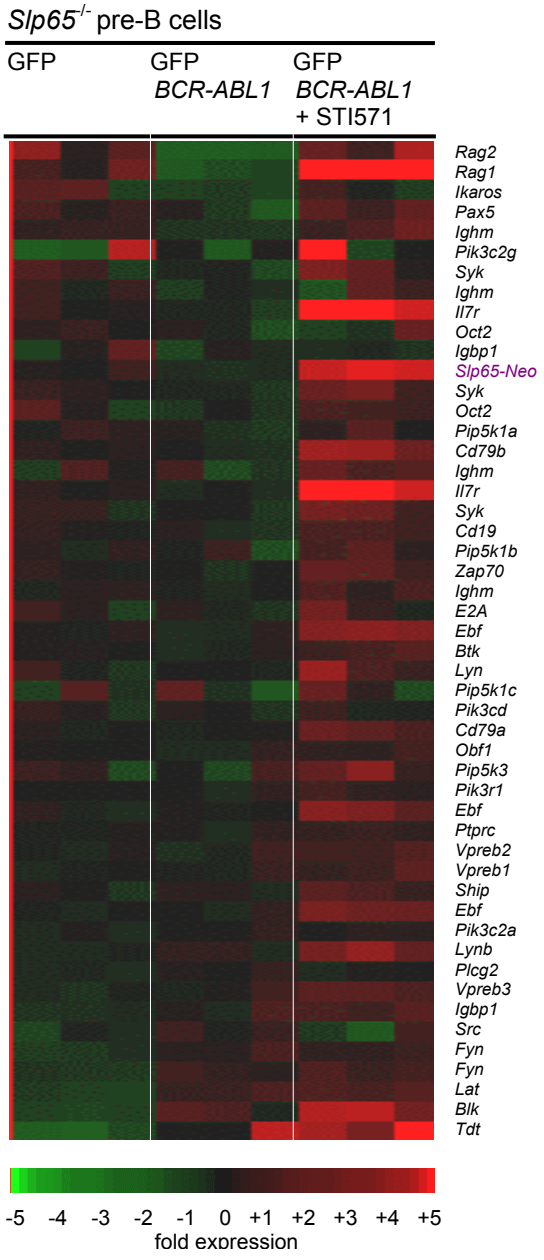


Decrease of BCR-ABL1 super-transformed ALL cells through treatment with STI571 calculated in subject to the non-treated population of the same cells. The decrease of BCR-ABL1-positive cells in the case of SEM is in this depiction with less than 20% after 20 days even more tremendous.

3.5 Transcriptional repression of pre-B cell receptor-related signaling molecules by BCR-ABL1

The findings above suggest that BCR-ABL1 kinase activity negatively selects pre-B cell receptor expression in developing B cell precursors. As a consequence, pre-B cell receptor related molecules would be expressed at lower levels in *BCR-ABL1*-transformed pre-B cells (Figure 16 and 17).

Figure 24: Transcriptional repression of pre-B cell receptor-related signaling molecules by BCR-ABL1 in Affymetrix gene chip analysis*



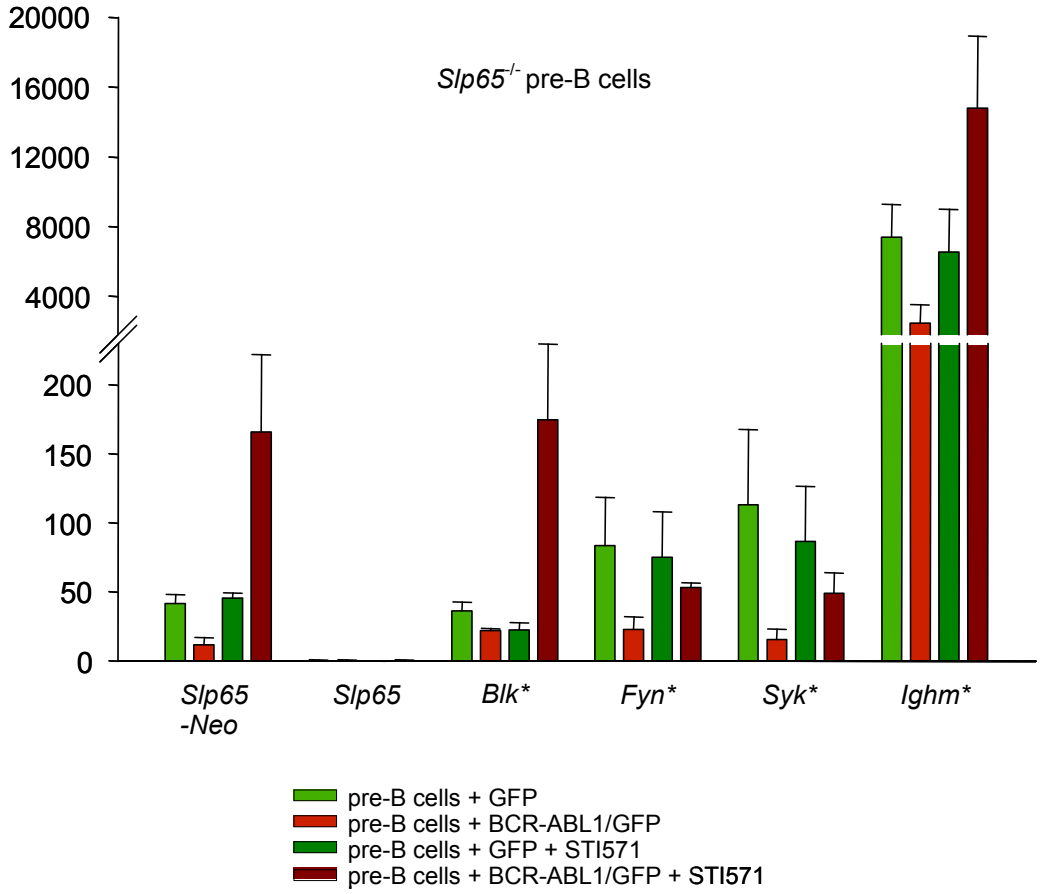
To distinguish between selection effects and transcriptional regulation as two divergent causes of gene expression changes induced by *BCR-ABL1*, we chose a model, in which negative selection effects by *BCR-ABL1* can be ruled out. Unlike wildtype pre-B cells, *Slp65*-deficient pre-B cells do not undergo apoptosis upon transduction with *BCR-ABL1* because pre-B cell receptor signal transduction is disrupted in *Slp65*-deficient cells. Slp65$^{-/-}$ pre-B cells were cultured in the continuous presence of Il7 and under three different conditions: they were either transduced with a retroviral expression vector encoding GFP alone (GFP) or *BCR*-ABL1/GFP in the presence (*BCR*-ABL1/GFP and STI571) or absence (*BCR*-ABL1/GFP) of 10 μmol/L STI571. Continuous addition of 10 ng/mL Il7 inhibited apoptosis that usually occurs if BCR-ABL1-transformed pre-B cells are treated with STI571. After 16 hours of STI571-treatment, RNA was isolated and subjected to gene expression analysis using the Affymetrix 430 GeneChip platform.

*cell culture setup and mRNA isolation done by Cihangir Duy, group of Prof. Müschen, Children's Hospital Los Angeles, USA

Conversely, BCR-ABL1 may also have a direct effect on transcriptional regulation of pre-B cell receptor-related molecules. To test whether reduced expression levels of pre-B cell receptor-related signaling molecules reflects negative selection or transcriptional silencing by BCR-ABL1 or both, the effect of BCR-ABL1 kinase activity in pre-B cells in the absence of any selection effects was studied.

To this end, *Slp65*-deficient pre-B cells were chosen as a model. Unlike wildtype pre-B cells, *Slp65*-deficient pre-B cells do not undergo apoptosis upon transduction with *BCR-ABL1* because pre-B cell receptor signal transduction is disrupted in *Slp65*-deficient cells. We transduced *Slp65*-deficient mouse pre-B cells with a retrovirus encoding either GFP or GFP and *BCR-ABL1*. Transduced pre-B cells were treated with mouse Il7. Inhibition of BCR-ABL1 kinase activity by STI571 usually induces apoptosis in *BCR-ABL1*-transformed pre-B cells, unless the cells are simultaneously incubated in the presence of Il7. *Slp65*$^{-/-}$ pre-B cells transduced with GFP or *BCR-ABL1*/GFP were cultured in the presence of Il7 and in the presence or absence of STI571 and subjected to Affymetrix GeneChip analysis to measure the effects of BCR-ABL1 kinase activity on gene expression in the absence of selection (Figure 24). Pre-B cell receptor-related molecules including *Ighm*, *Syk* and *Slp65* were downregulated by BCR-ABL1 and upregulated by STI571. As a consequence of targeting the *Slp65* locus in the *Slp65*$^{-/-}$ mice, pre-B cells express a non-functional *Slp65*/*neomycin*-resistance gene fusion transcript. *Slp65-Neo* fusion mRNAs are recognized by the Slp65-probeset on the 430 GeneChips used in our analysis. Validation experiments using quantitative RT-PCR confirmed that *Ighm*, *Syk* and *Slp65-Neo* fusion transcripts were downregulated by BCR-ABL1 and upregulated by STI571, whereas wildtype *Slp65* was undetectable (Figure 25). Given that *Slp65-Neo* fusion transcripts do not encode functional Slp65 protein and, hence, cannot be subject to selection, these findings indicate that BCR-ABL1 interferes with transcriptional regulation in the absence of selection effects. Contrary to GeneChip results (Figure 24), the SRC family kinases *Blk* and *Fyn* were also found downregulated by BCR-ABL1 and upregulated by STI571 in the quantitative RT-PCR analysis (Figure 25).

Figure 25: Transcriptional repression of pre-B cell receptor-related signaling molecules by BCR-ABL1 in quantitative RT-PCR



From the same samples used for GeneChip analysis (Figure 24), cDNAs were synthesized and used for validation experiments by quantitative RT-PCR analysis. It should be noted that targeting of the *Slp65* locus in *Slp65*$^{-/-}$ mice involved replacement of the Slp65 exon 4 by a neomycin restance cassette in opposite transcriptional orientation. Therefore, from the targeted locus Slp65-Neo fusion mRNAs are transcribed that were recognized by the GeneChip probeset for Slp65 (Figure 24) and were detected in our RT-PCR validation.

*quantitative RT-PCR for *Blk, Fyn, Syk* and *Ighm* done by Tanja Gruber, MD, group of Prof. Müschen, Childrens Hospital Los Angeles, USA

4. Discussion

4.1 Human ALLs can be divided into two major groups through the expression status of the pre-B cell receptor and related signaling molecules

Through its ability to initiate signaling that results besides others in Ca^{2+} release and the activation of transcription factors like NF-κB, the pre-B cell receptor represents a central signaling unit in the regulation of survival, proliferation and differentiation of early B cell precursor cells (Dolmetsch et al., 1997; Hashimoto et al., 1999; Ishiai et al., 1999; Melchers et al., 2000; Melchers, 2005). Previous work demonstrated that pre-B cell-derived acute lymphoblastic leukemia cells frequently carry defects in the BTK and SLP65 molecules, which are part of the pre-B cell receptor signaling cascade (Feldhahn et al., 2005b; Jumaa et al., 2003; Klein et al., 2004; Sprangers et al., 2006). While these findings argue for a tumor suppressor function of the pre-B cell receptor in ALL, other studies showed that pre-B cell receptor expression is required for malignant lymphoproliferation (Flemming et al., 2003). Likewise, transformation of mouse pre-B cells by the *Myc* oncogene requires pre-B cell receptor expression (Wossning et al., 2006).

To clarify the function of the pre-B cell receptor as a tumor suppressor vs a requirement for leukemic transformation, we first studied 148 cases of human ALL covering all main subgroups of the disease in regard to the VDJ-rearrangement at the heavy chain locus. The result of this analysis was remarkable: only 20.1% of the cases expressed a possibly functional μ-chain (Figure 8, Table 7). A closer look on the single ALL subgroups showed that *MLL-AF4*-induced ALL (0% functional VDJ rearrangement), *BCR-ABL1*-induced (17.5%) ALL and ALL with either sporadic aberrations (20.0%) or a hyperdiploid karyotype (3.3%) showed statistical significant selction against a functional pre-B cell receptor. By contrast 100% of all analyzed *E2A-PBX1*-transformed ALL cases have a functional VDJ-rearrangement. This subgroup of ALL seems to be highly selected for the expression of a pre-B cell receptor and thereby different from all other ALL subgroups.

Additional tests on pre-B cell receptor signaling capacity in human ALL cell lines corresponded with these results. Again, all ALL cell lines with the *E2A-PBX1*

translocation showed a strong Ca^{2+} signal upon receptor engagement comparable with the signal of primary pre-B cells from human bone marrow (Figure 9). In addition only two other cell lines (hyperdiploid and *TEL-PDGFRB*-induced ALL) showed a very weak increase in Ca^{2+} levels after receptor stimulation. The remaining 14 cell lines did not show any signaling capacity at all.

Based on these results we conclude that ALL cells carrying an *E2A-PBX1* gene rearrangement are subject to selection for a functional pre-B cell receptor like normal pre-B cells. Conversely, in other ALL subsets, the leukemia cells carry mostly non-functional *IGHM* alleles that lack coding capacity for a pre-B cell receptor. Even in the few remaining cases in which a μ-chain can be expressed, the ALL cells lack pre-B cell receptor function in most, if not all, instances.

If expression of a functional μ-chain does not lead to a proper signal of the pre-B cell receptor, one or more of the related signaling molecules and/or components of the pre-B cell receptor complex must be negatively affected, e.g. through transcriptional repression. The analysis of Affymetrix GeneChip data (Figure 10) from Ross et al. (Ross et al., 2003) and van Zelm et al. (van Zelm et al., 2005) shows that important pre-B cell receptor-related molecules, like SYK and SLP65, and the μ-chain (*IGHM*) itself are downregulated in *BCR-ABL1-, TEL-AML1-* and *MLL*-translocation-induced ALL. In cases of very weak signaling capacity (Figure 9; hyperdiploid and *TEL-PDGFRB*-induced ALL), expression levels of certain signaling components could be sufficient to generate a small increase in cytoplasmic Ca^{2+} level but are not high enough to reach full strength capacity. In the case of *E2A-PBX1*-mediated ALL, the picture is quite different. Although many of the shown molecules in the microarray data (Figure 10) are also downregulated in these cases, there is a considerable number of important signaling components that show comparable or even higher expression levels to pre-B and immature B cells.

In summary, this analysis revealed that *E2A-PBX1*-transformed ALL cells differ from all other ALL subgroups in that they are highly selected for the expression of a pre-B cell receptor, express a functional pre-B cell receptor on the surface and express pre-B cell receptor-related signaling molecules at similar levels as normal pre-B cells. Conversely, ALL clones carrying a *BCR-ABL1*, *MLL-AF4* gene rearrangement, a

sporadic genetic aberration or a hyperdiploid karyotype appear to be negatively selected against expression of a pre-B cell receptor. These findings indicate that human ALL can be subdivided into two groups, in which the pre-B cell receptor has divergent functions. This situation resembles human B cell lymphoma, in which subgroups can be distinguished based on the presence (e.g. follicular lymphoma, Burkitt's lymphoma, diffuse large B cell lymphoma) and absence (Hodgkin's lymphoma, angioimmunoblastic lymphoma; Brauninger et al., 2001) of a functional B cell receptor.

4.2 Pre-B cell receptor signaling renders pre-leukemic BCR-ABL1-transgenic pre-B cells non-permissive to leukemic transformation

4.2.1 Transformation through *BCR-ABL1* is a rare clonal event that leads to high sensitivity against ABL1-kinase inhibitors

To study the function, if any, of the pre-B cell receptor during the transformation process of ALL subgroups in which the pre-B cell receptor appears to be counterselected (*BCR-ABL1*, *MLL-AF4*, sporadic aberrations, hyperdiploid karyotype), we chose a *BCR-ABL1*-transgenic mouse model. Surprisingly *BCR-ABL1*-transgenic mice before the onset of leukemia (day 30 – 120) showed exactly the same surface marker phenotype in B cell development as wildtype mice (Figure 11 - 12). One could have expected that B cell development in this transgenic model, where all cells express the *BCR-ABL1* oncogene, would show aberrations much earlier. However no influences of the *BCR-ABL1* transgene were detectable. After complete leukemic transformation through *BCR-ABL1*, the cells showed striking differences. All signs of maturation, like the μ-heavy chain or CD23, disappeared almost completely even in the spleen and peripheral blood. Therefore, immature markers like AA4.1 and IL7Rα were expressed by almost 100% of the cells in the examined tissues. This reflects the lock in early B cell development through the transformation of BCR-ABL1. The very small remaining population of mature cells thereby represented the non-transformed cells. Treatment of fully leukemic mice with the

ABL1-kinase inhibitor AMN107 (Nilotinib) not only resulted in rapid resolution of bulky leukemic tumors within seven days (Kaur et al., 2007) but also almost completely restored the phenotype of wildtype mice (Figure 12 and 13). Further characterization by white blood cell counts (WBC), quantitation of leukemic blasts ($CD19^+$ $AA4.1^+$ cells) and splenic weights (Table 9) confirmed these findings. While wildtype and pre-leukemic *BCR-ABL1*-transgenic mice showed no significant differences, fully leukemic mice had increased numbers in WBC (~9-fold), leukemic blasts (~20-fold) and splenic weight (~3-fold). Treatment with AMN107 also largely reversed these changes (Table 9). Corresponding with these findings were the tests on pre-B cell receptor signaling capacity through stimulation induced Ca^{2+} release (Figure 15). Wildtype and pre-leukemic mice showed an increase in Ca^{2+} levels after receptor engagement, thereby reflecting normal signaling capacity. As hardly any μ-chain expressing cells were left after complete transformation through *BCR-ABL1* (Figure 12), stimulation of splenocytes from fully leukemic mice showed no receptor signaling. In agreement with the reset of the B cell surface marker phenotype after AMN107 treatment (Figure 12 and 13), receptor stimulation in splenocytes from these mice led to an increase of Ca^{2+} levels in the cytoplasm (Figure 15). The signaling capacity was almost completely regained.

The study of the VDJ-fragment length-distribution in peripheral blood of wildtype, pre-leukemic and leukemic mice before and after the treatment with AMN107 confirmed once more the effect of BCR-ABL1 inhibition in restoring a phenotype comparable to the wildtype situation (Figure 14). While wildtype and pre-leukemic mice had a broad spectrum of fragment sizes following the pattern of a Gaussian distribution and thereby reflecting the diversity of B cell receptors in the immune system, fully leukemic mice showed clonal expansion. AMN107-treatment was again successful in restoring the wildtype pattern in the form of a Gaussian distribution.

Given that all pre-B cells carry the oncogenic *BCR-ABL1* transgene it is surprising that only one leukemic clone emerges after transformation. These findings are consistent with a similar study that involved bone marrow transduced with *BCR-ABL1*-encoding retroviruses: Transplantation of *BCR-ABL1*-transduced bone marrow led to the outgrowth of clonal leukemia in the mouse recipients (Li et al., 1999). These findings suggest that the *BCR-ABL1*-transgene, although expressed in all pre-B cells, is not sufficient for

transformation. Rare additional events appear to be required for the outgrowth of one emerging leukemia clone. Interestingly, treatment with AMN107 completely eradicates the dominant leukemia clone but seems to spare other polyclonal pre-B cells, even though they also carry the *BCR-ABL1* transgene. As a consequence, treatment with AMN107 restores a largely polyclonal repertoire of the pre-B cell pool within seven days of treatment. These findings suggest that the rapidly proliferating leukemia clone is more sensitive to AMN107 than other *BCR-ABL1*-carrying pre-B cells that did not (yet) undergo leukemic transformation.

4.2.2 BCR-ABL1-transformation leads to changes in expression profiles of pre-B cell receptor signaling molecules

Analysis of Affymetrix GeneChip data, generated from $CD19^{+}AA4.1^{+}$ sorted bone marrow cells of wildtype, pre-leukemic and fully blown leukemic mice before and after the onset of leukemia, showed clear differences in the expression levels of important pre-B cell receptor signaling molecules as a consequence of *BCR-ABL1*-mediated transformation (Figure 16). The verification of the results for SLP65, an important linker molecule in the pre-B cell receptor signaling cascade (Jumaa et al., 1999), in parallel to expression level analysis of the *BCR-ABL1*-oncogene showed inverse kinetics (Figure 17). While *BCR-ABL1* levels increased during the transformation process and almost vanished through AMN107-treatment, *SLP65* levels are considerably decreased in full blown leukemia and restored after inhibition of BCR-ABL1. The low level of *BCR-ABL1* in pre-leukemic mice explains the phenotypical analogy to the wildtype situation in FACS measurements (Figure 11 – 13), VDJ-fragment length-distribution (Figure 14), and Ca^{2+} measurements (Figure 15). The finding of reduced expression of *BCR-ABL1* mRNA in leukemic pre-B cells from AMN107-treated mice was unexpected because this inhibitor primarily blocks BCR-ABL1 kinase activity at the protein level. This may suggest that AMN107 downregulates *BCR-ABL1* mRNA expression in the leukemia cells. Conversely, treatment with AMN107 may select for leukemia subclones that are less dependent on high levels of BCR-ABL1 kinase activity. It is conceivable that

leukemia subclones with lower sensitivity to AMN107 express lower mRNA levels of *BCR-ABL1*. In fact, a recent study demonstrated that leukemia subclones with high *BCR-ABL1* expression levels are particularly sensitive to STI571 (Imatinib; Modi et al., 2007), another ABL1-kinase inhibitor.

4.2.3 Reconstitution of pre-B cell receptor signaling in BCR-ABL1-transformed cells suppresses leukemic growth in murine cell lines

So far the results have shown that *BCR-ABL1*-transformed cells appeared to be negatively selected for the expression of a functional pre-B cell receptor. However, it was not clear (yet) which effect the parallel expression of a receptor and the *BCR-ABL1*-oncogene will have on already fully transformed cells.

As a result of transduction of cell lines generated from *BCR-ABL1*-transgenic mice with a μ-chain encoding retrovirus the proportion of the so receptor-reconstituted cells decreases within a measured time period of 9 days (Figure 19). Although the effect in two (PLC1, 8093) of the three analyzed cell lines is not impressive (decrease of about 20%), the trend is clearly visible. As shown in the analysis of expression profiles of the pre-B cell receptor-related molecules (Figure 16), the downregulation of these signaling components through *BCR-ABL1* should and likely do prevent a stronger effect.

In contrast to *BCR-ABL1*-transgenic mice in which the leukemic transformation seems to be a rare and time consuming process (100 days in average), pre-B cells from $Slp65^{-/-}$ mice that express a μ-heavy chain (Flemming et al., 2003) can be easily transduced by *BCR-ABL1*-encoding retrovirus and become thereby independent from Il7-stimulation. As a consequence of the *SLP65*-deficiency, the μ-chain expression had no influence on leukemic proliferation (Figure 20, empty vector controls). The reconstitution of SLP65 and thereby the reconstitution of pre-B cell receptor signaling confirmed the results from the μ-chain reconstitution (Figure 19) in an impressive manner. The $SLP65^{+}$ fraction was reduced by more than 50% within 4 – 5 days after transduction (Figure 20, left panel) or after induction, in the case of an inducible system (Figure 20, right panel).

These results clearly indicate that the expression of *BCR-ABL1* and the presence of a functional pre-B cell receptor signaling cascade are mutually exclusive in mouse cells and thus show that the expression of the *BCR-ABL1*-oncogene is not sufficient to lead to an outgrowth of a leukemic clone. Additional events appear to be required. This also delivers an explanation for the long latency for transformation of the transgenic mice and might also be the explanation for the rare appearance of *BCR-ABL1*-mediated ALL in human childhood.

4.2.4 Pre-B cell receptor signaling prevents leukemic growth in human ALL cell lines super-transformed by BCR-ABL1

To test whether the findings in murine cell culture also hold true in the human system, two *E2A-PBX1*-expressing ALL cell lines (697, Kasumi-2) that showed a functional pre-B cell receptor signaling (Figure 9) were super-transformed by transduction with a *BCR-ABL1*-encoding retrovirus. To be able to connect possible effects with pre-B cell receptor signaling two cell lines (SEM, 380) that did not show any reaction on receptor stimulation were treated the same way. As already seen in the mouse cell line experiments, human ALL cells that are forced to express a functional pre-B cell receptor signaling cascade as well as the *BCR-ABL1*-oncogene vanish with time. Within 4 days after the start of the measurements, the proportion of these cells diminished to fewer than 40% (697) and fewer than 10% (Kasumi-2) respectively (Figure 21). At the same time the two receptor-signaling negative cell lines did not show any growth disadvantages or apoptotic signs. On the contrary, after 4 days (SEM) and 16 days (380) respectively, both cell lines showed an outgrowth of BCR-ABL1-super-transformed cells. Both described effects were suppressed by the inhibition of BCR-ABL1-kinase activity through STI571.

These results clearly show that the presence of BCR-ABL1-kinase activity together with pre-B cell receptor signaling has a suppressive effect on leukemic growth and thereby confirm the findings of the mouse cell line experiments in a human system. As already immortalized cells (through transformation by E2A-PBX1) are negatively

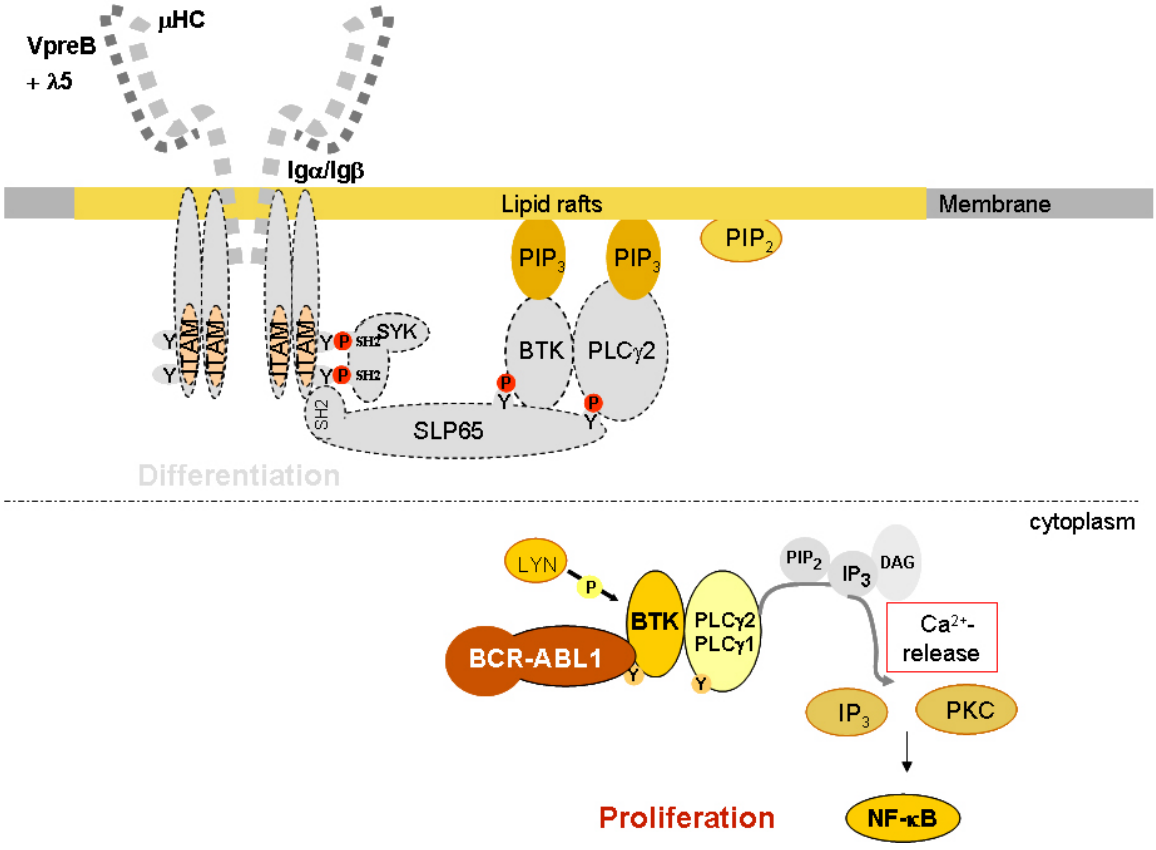
affected by *BCR-ABL1*-expression, this result verifies the mutual exclusivness of pre-B cell receptor signaling and BCR-ABL1 activity in an impressive manor.

4.3 Posssible mechanisms of apoptosis induction

4.3.1 Simultaneous signaling might lead to Ca^{2+}-overload and apoptosis

A possible explanation for the mutual exclusion might be the overlap of pre-B cell receptor (Figure 6) and BCR-ABL1-mediated signaling. Through interaction and activation of BTK and downstream located signaling molecules, BCR-ABL1 mimics pre-B cell receptor signaling in transformed cells (Figure26; Feldhahn et al., 2005a). This also results in a calcium signal which is autonomous and oscillatory and can be visualized by confocal laser microscopy on single cells (Klein et al., 2004). Since every single cell has its own autonomous signal that is not triggered by any stimulant, synchronization of signals in a pool of cells is missing. This is probably the reason for the absence of oscillatory signals in the flow cytometry measurements of BCR-$ABL1^+$ ALL cell lines (Figure 9).

Figure 26: Mimicry of pre-B cell signaling through BCR-ABL1

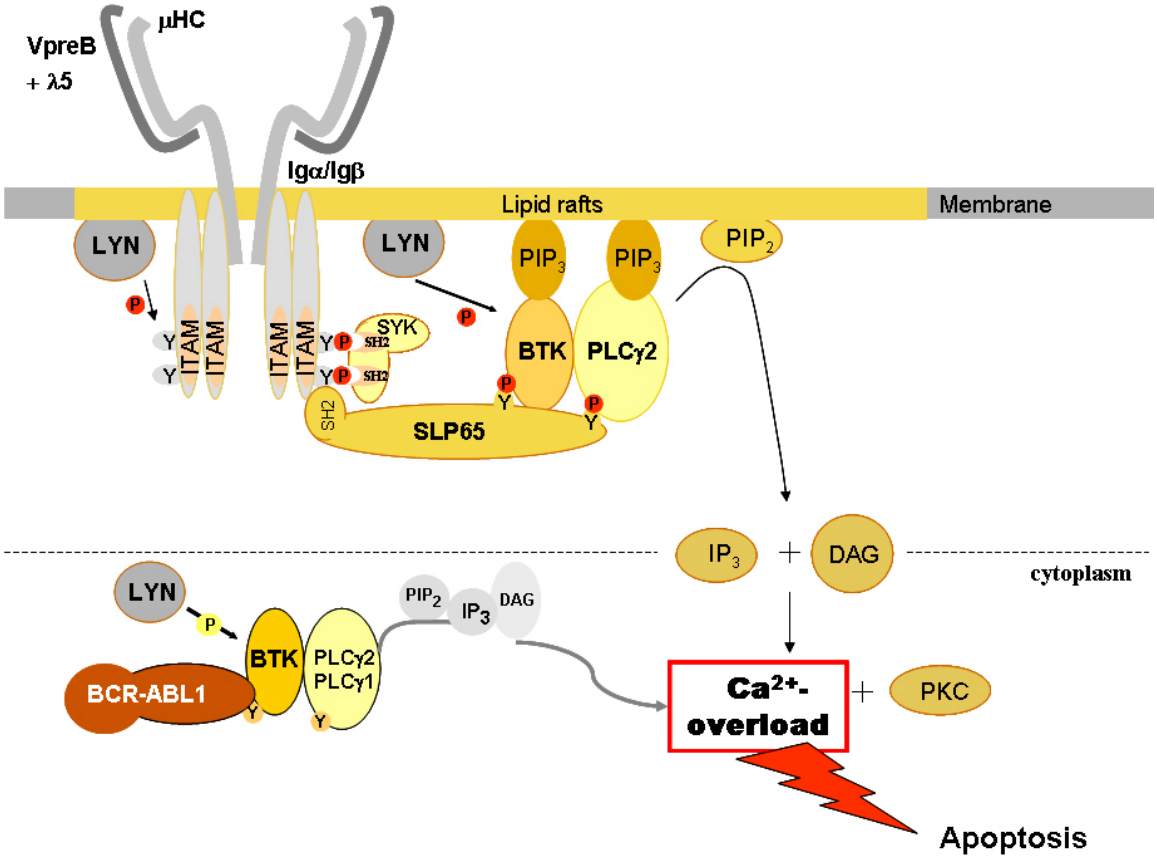


BCR-ABL1 interacts with and constitutively activates BTK and thereby induces a signaling cascade highly similar to that of the pre-B cell receptor, resulting in Ca^{2+} –release and activation of transcription factors like NF-κB (Feldhahn et al., 2005a).

In addition to proliferative effects, Ca^{2+} is known as an apoptosis-trigger in pre-B cells (Zornig et al., 1995; Kato et al., 2002). Studies on the B cell line WEHI-231 indicate that death signals from the B cell receptor may first cause mitochondrial alterations followed by activation of both necrotic and apoptotic cascades (Doi et al., 1999). Although mitochondria are known to participate actively in intrcellular Ca^{2+} compartmentalization (Carafoli, 2002), their uptake ability under physiological conditions is low (Thor et al., 1984). However, high intracellular Ca^{2+} concentrations (Thor et al., 1984) and proximity of mitochondria to the endoplasmic reticulum (ER) can overcome the low affinity (Hajnoczky et al., 1995). High mitochondrial Ca^{2+}-levels cause permeabilization of the mitochondria and lead to induction of apoptosis (Weis et al., 1994; Halestrap et al., 1998;

Crompton et al., 1999). A simultaneous signaling through the pre-B cell receptor and BCR-ABL1 could lead to a cytoplasmic Ca^{2+}-overload resulting in cell death as described above (Figure 27). This could hence be the reason for the tumor suppressive effects of the pre-B cell receptor and any other essential signaling component, like SLP65 (Flemming et al., 2003).

Figure 27: Simultaneous pre-B cell receptor and BCR-ABL1-mediated signaling



Ca^{2+}-overload as consequence of simultaneously active pre-B cell receptor and BCR-ABL1-mediated signaling could lead to cell death and therefore be the reason for the tumor suppressive effect of the pre-B cell receptor.

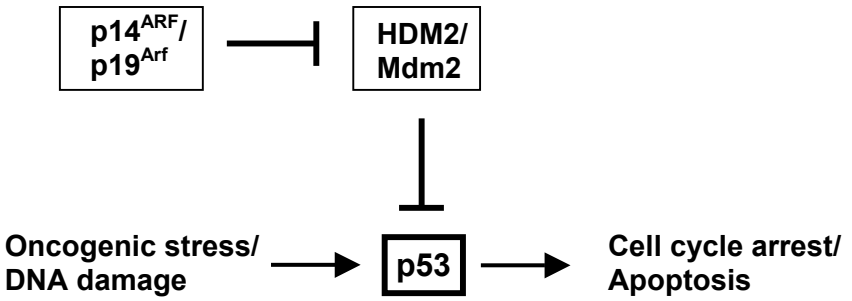
4.3.2 Induction of apoptosis through the ARF-p53-pathway

INK4A-ARF encodes two distinct tumor suppressor genes. The first, $p16^{INK4A}$, acts to inhibit cyclin D-dependent kinases, thereby regulating transcriptional programs that depend upon the retinoblastoma (RB) protein and its related family members, p130 and

p107, for their proper execution. Mice lacking $p16^{Ink4a}$ are tumor prone and develop a wide spectrum of cancers, particularly after exposure to chemical carcinogens or X-rays (Serrano et al., 1996; Krimpenfort et al., 2001; Sharpless et al., 2001).

The second protein, $p14^{ARF}$ ($p19^{Arf}$ in the mouse), blocks the E3 ubiquitin ligase activity of HDM2 (Mdm2 in the mouse) to stabilize p53 and so induce a p53-dependent transcriptional program that triggers either cell cycle arrest or apoptosis in response to oncogenic stress (Figure 28; Sherr and McCormick, 2002).

Figure 28: Influence of ARF on p53-mediated apoptosis



$p14^{ARF}$/$p19^{Arf}$ blocks the E3 ubiquitin ligase activity of HDM2/Mdm2 thereby stabilizing p53. This allows induction of a transcriptional program that triggers either cell cycle arrest or apoptosis in response to cell stress.

At least 30% of Ph^+ ALL patients have sustained deletions at chromosome 9p21 that compromise the *INK4A-ARF* locus (Heerema et al., 2004; Primo et al., 2005). This speaks for one possible pathway to induce apoptosis in cells that are non-permissive for transformation through BCR-ABL1.

4.4 Addiction to BCR-ABL1-kinase activity

"Oncogene addicition" is a term that is used to describe the apparent acquisition of dependency by tumor cells on a single oncogenic activity (Weinstein et al., 1997; Weinstein, 2000; Weinstein, 2002). After a certain time of oncogene expression, the cell

gets reprogrammed and adapted to the oncogene-mediated signaling. Inhibition of the oncogene leads to initiation of apoptosis.

Super-transformation of pre-B cell receptor negative cells through BCR-ABL1 led to their outgrowth (Figure 21). Direct treatment with the ABL1-kinase inhibitor STI571 prevented reprogramming of the same cells through BCR-ABL1 and kept their proportion in cell culture on a constant level. However, super-transformed cells that were grown for 6 weeks without any treatment constantly disappeared over time after start with STI571-traetment (Figure 22 and 23). Since these cells were already transformed before (through MLL-AF4 or MYC-IGH) and therefore independent of any additional survival signals from BCR-ABL1, the only explanation for this result can be the "oncogene addiction" to BCR-ABL1-kinase activity.

Sharma et al. reported that differential attenuation of prosurvival and proapoptotic signals in oncogene-dependent cells contribute to cell death following oncogene inactivation (Sharma et al., 2006). They showed for SRC-, BCR-ABL1- and EGF-receptor-dependent cells a rapid diminution of phospho-ERK, -Akt and -STAT3/5 as prosurvival signaling components following oncogene inactivation. A delayed accumulation of the proapoptotic effector p38-MAPK was also visible. They propose that this reflects a signaling imbalance resulting from the fact that survival signals are relatively short-lived following acute inactivation of the oncogene, while proapoptotic signals persist sufficiently long to produce an apoptotic outcome. However, the cell culture models used were not optimal to prove this hypothesis. For the BCR-ABL1- and EGFR-model, variants of the IL3-dependent cell line BAF3 were used. Through either doxycycline-mediated induction of BCR-ABL1 (Klucher et al., 1998) or expression of a mutationally activated form of the EGF receptor (Jiang et al., 2005), the cells become IL3-independent. Since in this setup no IL3 was added after oncogene inactivation the results could reflect the behavior of "normal" BAF3 cells after IL3 withdrawal and hence be independent from oncogene-addiction. The presented Src-model demonstrates a similar discrepency. The BalbA1 cells used express a temperature sensitive activated c-Src mutant (Maroney et al., 1992). For the experimental setup the temperature was lowered to 35 °C, allowing c-Src expression, and the cells were starved to 0.2% serum. Samples were taken at various time points after increasing the temperature to 39.5 °C and

thereby inactivating c-Src. Whether Src-transduced cells in which c-Src was never activated do not undergo apoptosis at 39.5 °C and 0.2% serum was not shown.

Because the ALL cell lines used for super-transformation grew independently before BCR-ABL1-expression, the aforementioned problems do not occur in the here presented model (Figure 22 and 23). Instead, the signaling background mediated through the already present chromosomal aberrations (MLL-AF4, MYC-IGH) and possible interactions with BCR-ABL1 that are independent from oncogene-addiction might cause problems in confirming the results from Sharma et al. or in discovering other mechanisms that are involved in this phenomenon.

Besides the proposed model of Sharma et al. that differential attenuation of prosurvival and proapoptotic signals in oncogene-dependent cells contribute to cell death following oncogene inactivation another scenario would be possible. In order to disrupt BCR-ABL1-mediated prosurvival signals, inhibition, inactivation or silencing of one or at least a few of the components of the signaling cascade would be enough and should therefore be achieved in a short time. To rebuild their own survival signals, for example through the pre-B cell receptor, all the involved components must be expressed, activated and located at the right positions. As this might take longer than disrupting oncogene-mediated survival signals, a time gap in maintenance with prosurvival signals in the cell could occur, leading to induction of apoptosis and cell death.

This hypothesis is supported by the fact that pre-B cells rescued from apoptosis through Il7 (Figure 24) upregulate important signaling components after BCR-ABL1-inactivation, thereby trying to rebuild their own survival signals.

4.5 Pre-B cell receptor-related signaling molecules are transcriptionally downregulated by BCR-ABL1

The reconstitution and super-transformation experiments clearly indicate that BCR-ABL1-transformation selects for aberrant pre-B cell receptor signaling. As a consequence, involved signaling molecules showed reduced expression in analyzed

microarray and quantitative RT-PCR data. However, additional transcriptional regulation of these molecules through BCR-ABL1 was not yet excluded.

In order to clarify this question further microarray data on *Slp65*-deficient mouse pre-B cells were generated (Figure 24). As these cells proliferate either through stimulation by Il7 or transformation by BCR-ABL1 the probability of selective effects was reduced. To show the reversibility of BCR-ABL1-mediated effects an additional set of data, showing the results of BCR-ABL1-kinase inhibition by STI571, was generated. The results of this experiment exhibit the transcriptional regulation of molecules involved in pre-B cell receptor signaling through BCR-ABL1-activity as well as reversal effects through BCR-ABL1-inhibition. Through an additional setup in the form of quantitative RT-PCR (Figure 25), the regulatory effects were verified, although the results partially showed inverse regulation as seen in the microarray data (*Fyn* and *Blk*). To exclude all possibility of doubt, additional quantitative RT-PCR data was generated. The *Slp65*-deficiency in these cells was achieved through inverse insertion of a neomycin cassette in exon 4 of *Slp65* (Jumaa et al., 1999). Consequently, a *Slp65-neomycin* fusion transcript occurred. While this transcript does not lead to a functional protein, the visible changes in expression levels (Figure 24 and 25) must be the consequence of transcriptional regulation through BCR-ABL1 instead of selective effects.

Therefore, we conclude that reduced expression of pre-B cell receptor-related signaling molecules (e.g. *Ighm, Syk, Slp65*) in BCR-ABL1-transformed pre-B cells reflects both negative selection of pre-B cell receptor signaling and transcriptional downregulation of these molecules by BCR-ABL1. The latter guarantees the permanent absence of a functional pre-B cell receptor signaling cascade even if cells subsequently succeed in productively rearranging an *IGHM* V region gene. In fact, secondary VDJ gene rearrangements at the *IGHM* locus frequently occur in *BCR-ABL1*-transformed ALL cells (Klein et al., 2004) and are occasionally productive. However, by transcriptional silencing of key components of the pre-B cell receptor signaling chain (including *Ighm, Syk, Slp65*), BCR-ABL1 locks the transformed pre-B cells in a pre-B cell receptor-unresponsive state.

4.6 Conclusions and perspectives

Aim 1) The studied ALL subtypes show no consistent pattern in selection for or against a pre-B cell receptor. *Tel-AML1*-mediated ALL and ALL with normal karyotype show no statistical significance for any selection. However, *BCR-ABL1*-induced ALL, *MLL-AF4*-induced ALL, and ALL with either sporadic aberrations or a hyperdiploid karyotype select against a functional pre-B cell receptor. In contrast, *E2A-PBX1*-induced ALL cells are compatible with the expression and show a positive selection in favor of a functional pre-B cell receptor. In conclusion human ALL can be subdivided into groups, in which the pre-B cell receptor has divergent functions.

Aim 2) In the *BCR-ABL1*-transgenic mouse model, the pre-B cell receptor is normally expressed before the onset of leukemia. The B cell precursors show normal development and receptor responsiveness, probably caused by the low expression level of *BCR-ABL1* in pre-leukemic cells. After complete transformation, nearly all pre-B cell receptor positive cells vanish. The μ-heavy chain and pre-B cell receptor-related molecules in the proliferating leukemia clone are downregulated, whereas *BCR-ABL1* is upregulated. Hence *BCR-ABL1*-transformed cells are *in vivo* negatively selected for the expression of a functional pre-B cell receptor. Through treatment with the ABL1-kinase inhibitor AMN107 a phenotype comparable with the wildtype situation is regained. Since all cells have the *BCR-ABL1*-transgene, leukemic clones with high *BCR-ABL1* expression seem to be more sensitive to Abl1-kinase inhibitors than pre-leukemic cells with low expression levels.

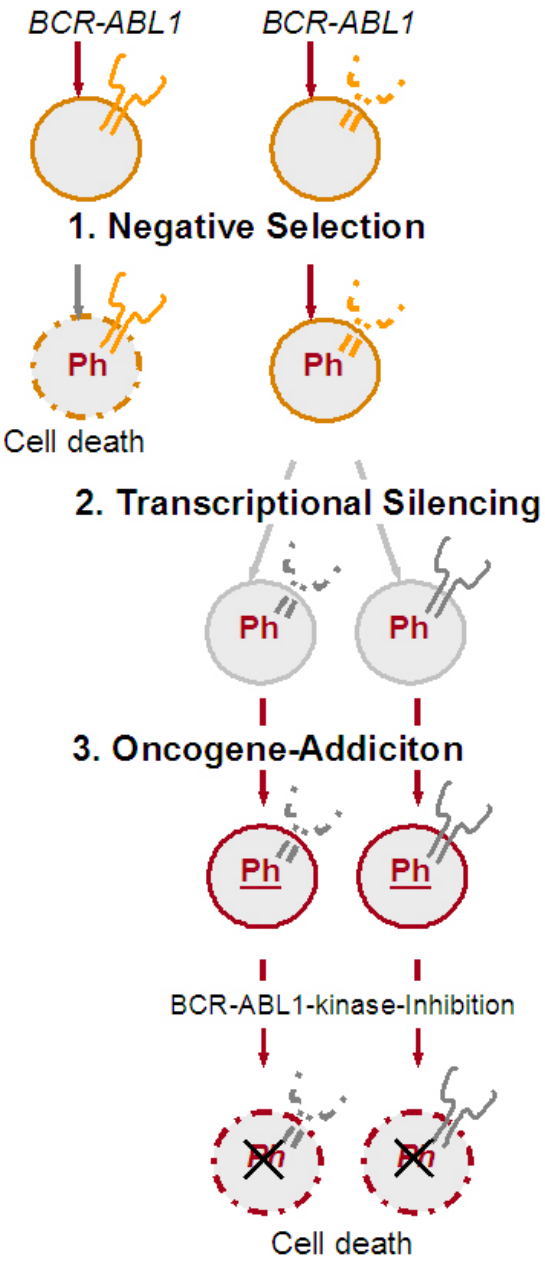
Aim 3) *BCR-ABL1*-expression in pre-B cell receptor negative cells causes proliferation. In contrast, transduction of pre-B cell receptor positive cells with *BCR-ABL1* induces apoptosis. BCR-ABL1-transformed cells display a similar effect when the pre-B cell receptor signaling is reconstituted through transduction of missing components. Pre-B cell receptor and *BCR-ABL1* expression are mutually

exclusive. This indicates that pre-B cell receptor signaling prevents malignant transformation of pre-B cells through BCR-ABL1. In conclusion, only cells that fail to express a functional receptor are permissive to transformation.

Aim 4) Transformation by BCR-ABL1 causes selection against a functional pre-B cell receptor as indicated by analysis of VDJ segments, the characterization of the BCR-ABL1 mouse model and the mutual exclusiveness of signaling. However, microarray analysis on mouse pre-B cells also shows transcriptional regulation of pre-B cell receptor components and related signaling molecules. BCR-ABL1-mediated downregulation of a non-productive *Slp65-neomycin* fusion transcript in Slp65$^{-/-}$ pre-B cells is therefore strong indication. Hence, changes in the expression profile of BCR-ABL1-transformed cells are the result of both, selection and transcriptional regulation. Selection, and thereby the outgrowth of single leukemia clones, is the consequence of the inability of BCR-ABL1 to transform pre-B cell receptor positive cells. Transcriptional regulation, however, guarantees the permanent absence of a functional pre-B cell receptor signaling cascade even if cells subsequently succeed in productively rearranging an *IGHM* V region gene.

The pre-B cell receptor mediated tumor suppression and BCR-ABL1-mediated transformation can be summarized as follows: Because pre-B cell receptor signaling prevents malignant transformation, only “crippled” pre-B cells that do not carry a productively rearranged *IGHM* V region gene or otherwise fail to express a functional pre-B cell receptor are permissive to transformation by *BCR-ABL1*. Thus, BCR-ABL1 has a strong effect on selective outgrowth of B cell progenitors with defective pre-B cell receptor expression. In addition, BCR-ABL1 also interferes with expression of a number of pre-B cell receptor-related molecules at the transcriptional level thereby preventing functional signaling after possible successful rearrangements. Complete transformation finally leads to addiction to BCR-ABL1 (Figure 29).

Figure 29: Scheme for pre-B cell receptor mediated tumor suppression and BCR-ABL1-mediated transformation



BCR-ABL1 selects for pre-B cell receptor negative cells. Through transcriptional silencing of signaling components BCR-ABL1 ensures survival in case of functional secondary VDJ-rearrangement. As the cell becomes addicted to the oncogene, inactivation of BCR-ABL1 would lead to cell death.

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The presented findings clarify the role of the pre-B cell receptor during leukemic progressive transformation by BCR-ABL1. But they also raise a lot of interesting questions to follow up with:

1. The identification of the exact mechanism to induce apoptosis in pre-B cell receptor and BCR-ABL1 positive cells would be very interesting. Is Ca^{2+} overload in the cytoplasm the cause for apoptosis? Is the in Ph^{+} ALL often deleted *INK4A-ARF* locus involved? Has this mechanism therapeutical relevance?

2. Further it would be interesting to have a closer look on other ALL subgroups that showed negative selection for a functional pre-B cell receptor in VDJ-sequence analysis. Can the negative selection be confirmed *in vitro* and *in vivo*? Has the pre-B cell receptor also a tumor suppressive function in these ALL subgroups? Is there a common pattern in these ALL subtypes?

3. Than there is also the question for the positive selection in *E2A-PBX1*-mediated ALL. Can the positive selection be confirmed *in vitro* and *in vivo*? In which way is E2A-PBX1 dependent on the pre-B cell receptor? Is pre-B cell receptor signaling involved in the transformation process?

Hopefully some of these questions can be answered during the next month and years.

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6. Abstract

Abstract

Pre-B cell receptor signaling prevents malignant transformation by *BCR-ABL1*

The pre-B cell receptor plays a critical role in the early B cell development. As a failure of expression results in the lack of survival signal it represents an important checkpoint for further proliferation and differentiation. It has been shown that cells in acute lymphoblastic leukemia frequently carry defects in important pre-B cell receptor related signaling molecules like BTK or SLP65, indicating a tumor suppressive function. In contrast pre-B cell receptor expression is required in malignant lymphoproliferation and at least in mouse pre-B cells for transformation by the *Myc* oncogene. Therefore we investigated the role of the pre-B cell receptor as tumor suppressor vs. a requirement for leukemic transformation.

We found that only *E2A-PBX1*-mediated ALL carry a functional pre-B cell receptor and therefore seems to be essential for this type of leukemic transformation. The remaining investigated ALL subtypes are receptor independent or even show strong negative selection against a functional pre-B cell receptor.

To clarify the role of the pre-B cell receptor during leukemogenesis, we studied the progressive transformation of pre-B cells in a transgenic mouse model for *BCR-ABL1*-induced ALL. Interestingly, before the onset of leukemia *BCR-ABL1*-transgenic pre-B cells respond normally to pre-B cell receptor engagement and express *BCR-ABL1* at low levels. In full-blown leukemia, ALL cells do not respond to pre-B cell receptor engagement and express *BCR-ABL1* at high levels.

Signaling from the pre-B cell receptor and BCR-ABL1 are mutually exclusive: i) Treatment of leukemic mice with the BCR-ABL1 kinase inhibitor, AMN107, reinstates normal pre-B cell receptor signaling within seven days. ii) Reconstitution of pre-B cell receptor signaling in *BCR-ABL1*-transformed pre-B cells induces rapid cell death. iii) Transduction of human ALL cells with the *BCR-ABL1* oncogene induces apoptosis in the presence of pre-B cell receptor signaling and accelerates proliferation in its absence.

We conclude that pre-B cell receptor signaling -while compatible with expression of *E2A-PBX1*- renders B cell precursors non-permissive to transformation by *BCR-ABL1* and likely other leukemogenic fusion genes.

Zusammenfassung

Prä-B Zell Rezeptor Signaling verhindert maligne Transformation durch *BCR-ABL1*

Der Prä-B-Zell-Rezeptor spielt eine wichtige Rolle in der frühen B-Zell-Entwicklung. Die fehlende Expression des Rezeptors die auch gleichzeitig das Fehlen von Überlebenssignalen bedeutet verdeutlicht die aussergewöhnliche Stellung des Prä-B-Zell-Rezeptors als Kontrollpunkt für die weitere Proliferation und Entwicklung. Wie gezeigt wurde, weisen Zellen in ALL häufig Defekte in wichtigen downstream gelegenen Signalmolekülen, wie BTK und SLP65, auf und deuten somit auf eine tumorsuppressive Funktion hin. Im Gegensatz dazu wird die Expression des Prä-B-Zell-Rezeptors für maligne Lymphoproliferation und zumindest in Maus-Prä-B-Zellen zur Transformation durch das *Myc*-Oncogen benötigt. Aus diesem Grund haben wir die Rolle des Prä-B-Zell-Rezeptors als Tumorsuppressor gegenüber seiner Beteiligung an leukämischer Transformation näher untersucht.

Wir haben dabei herausgefunden, dass nur *E2A-PBX1*-vermittelte ALL einen funktionsfähigen Prä-B-Zell-Rezeptor besitzen, der somit für diesen Leukämie-Typ unerlässlich zu sein scheint. Die übrigen untersuchten ALL Formen sind dagegen rezeptorunabhängig oder zeigen sogar eine aktive Selektion gegen diesen.

Um die Rolle des Prä-B-Zell-Rezeptors während der Leukemogenese zu klären haben wir die progressive Transformation von Prä-B-Zellen in einem transgenen Mausmodel für *BCR-ABL1*-induzierte ALL untersucht.

Interessanter Weise reagieren *BCR-ABL1*-transgene Prä-B-Zellen vor dem Beginn der Leukämie völlig normal auf Rezeptorstimulation und exprimieren *BCR-ABL1* nur in geringen Mengen. Nach vollständigem Ausbruch der Leukämie reagieren ALL Zellen nicht mehr auf die Stimulation des Prä-B-Zell-Rezeptors und exprimieren ein hohes Level an *BCR-ABL1*.

Signaltransduktion von Prä-B-Zell-Rezeptor und BCR-ABL1 schließen sich gegenseitig aus: i) die Behandlung leukämischer Mäuse mit dem BCR-ABL1-Inhibitor AMN107 führt innerhalb von 7 Tagen zur Wiederherstellung des normalen Rezeptorsignals, ii) die Wiederherstellung des Prä-B-Zell-Rezeptorsignals in *BCR-ABL1*-transformierten Zellen führt zu schnellem Zelltod, iii) die Transduktion humaner ALL

Zellen mit *BCR-ABL1* führt in Anwesenheit eines funktionellen Prä-B-Zell-Rezeptors zur Induktion von Apoptose und in dessen Abwesenheit zu verstärkter Proliferation.

Damit ergibt sich als Schlussfolgerung, dass ein funktioneller Prä-B-Zell-Rezeptor -obwohl kompatibel mit der Expression von *E2A-PBX1*- Vorläufer-B-Zellen unfähig zur Transformation durch *BCR-ABL1* und wahrscheinlich auch anderer leukemogener Fusionsgene macht.

7. Appendix

7.1 Abbreviations

ALL	acute lymphoblastic leukaemia
AID	activation-induced cytidine deaminase
BCR	B cell receptor
BTK	Bruton's tyrosine kinase
C	constant
CLP	common lymphoid progenitor
CML	chronic myeloid leukemia
cRSS	cryptic RSS
CSR	class switch recombination
D	diversity
DAG	diacylglycerol
ER	endoplasmic reticulum
GL	germline
HSC	hematopoietic stem cell
HPRT	hypoxanthine guanine phosphoribosyltransferase
IGH	immunoglobulin heavy-chain
IGK	immunoglobulin κ light chain
IGL	immunoglobulin λ light chain
ITAM	immunoreceptor tyrosine-based activatory motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
J	joining
MT	metallothionein
NHEJ	non-homologous end joining
Ph	Philadelphia chromosome
PH	pleckstrin homology domain
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
PTK	protein tyrosine-kinase
PTP	protein tyrosine-phosphatase

RB	retino blastoma
RSS	recombination signal sequence
SH2	SRC homology domain 2
SHM	somatic hypermutation
SRC	cellular homolog of *Rous sarcoma virus*
TdT	terminal deoxynucleotidyl transferase
V	variable
WBC	white blood cell count

7.2 Supplementary information

7.2.1 List of Primers

Clonality and spectratyping analysis (mouse)

V_HJ558_F	5'-AAG GCC ACA CTG ACT GTA GAC-3'
Cμ_R	5'-TGG CCA CCA GAT TCT TAT CAG-3'
Cμ -FAM_R	5'-AGA CGA GGG GGA AGA CAT TTG-3'
J_H1-FAM_R	5'-GAC GGT GAC CGT GGT CCC TGT-3'
J_H2-FAM_R	5'-GAC TGT GAG AGT GGT GCC TTG-3'
J_H3-FAM_R	5'-GAC AGT GAC CAG AGT CCC TTG-3'
J_H4-FAM_R	5'-GAC GGT GAC TGA GGT TTC TTG-3'

Quantitative RT-PCR (mouse; *BCR-ABL1* human)

Slp65_F	5'-GAA GGG ACT ACG CAT TAG ACA G-3'
Slp65_R	5'-GCA TCA CAT ACA TCT CGG AGT-3'
Slp65/Neo_F	5'-TGA AGG TGG AAT AAT GGA CAA-3'
Slp65/Neo_R	5'-CTT CCT CGT GCT TTA CGG TAT-3'
*BCR-ABL1*_F	5'-ATC GTG GGC GTC CGC AAG AC-3'
*BCR-ABL1*_R	5'-GCT CAA AGT CAG ATG CTA CTG-3'
Hprt_F	5'-GGG GGC TAT AAG TTC TTT GC-3'
Hprt_R	5'-TCC AAC ACT TCG AGA GGT CC-3'
Fyn_F	5'-AGA GCT CTG GGT ACC GCT AT-3'
Fyn_R	5'-AGT CAT AAA GCG CCA CAA AC-3'
Blk_F	5'-GTT GTC TTC AAC CAC CTT GC-3'
Blk_R	5'-CTT CCT GTG ACG AGT GAC CT-3'
Syk_F	5'-ATG AGA AGA TGC CCT GGT TC-3'
Syk_R	5'-ACT TCT TCC CCT CAG GAA TG-3'
Ighm_F	5'-CAT CTC AAA ACC CAA TGA GG-3'

Ighm_R	5’-GGG CAC TGG TCA CAT ACT TC-3’

Single Cell PCR for IGHM gene rearrangements (human)

V_H1	5'-CAG TCT GGG GCT GAG GTG AAG A-3'
V_H2	5'-GTC CTR CGC TGG TGA AAC CCA CAC A-3'
V_H3	5'-GGG GTC CCT GAG ACT CTC CTG TGC AG-3'
V_H4	5'-GAC CCT GTC CCT CAC CTG CRC TGT C-3'
V_H5	5'-AAA AAG CCC GGG GAG TCT CTG ARG A-3'
V_H6	5'-ACC TGT GCC ATC TCC GGG GAC AGT G-3'
$3'J_H1.2.4.5$	5'-ACC TGA GGA GAC GGT GAC CAG GGT-3'
$3'J_H3$	5'-ACC TGA AGA GAC GGT GAC CAT TGT-3'
$3'J_H6$	5'-ACC TGA GGA GAC GGT GAC CGT GGT-3'
$5'J_H1.4.5$	5'-GAC GGT GAC CAG GGT KCC CTG GCC-3'
$5'J_H2$	5'-GAC AGT GAC CAG GGT GCC ACG GCC-3'
$5'J_H3$	5'-GAC GGT GAC CAT TGT CCC TTG GCC-3'
$5'J_H6$	5'-GAC GGT GAC CGT GGT CCC TTK GCC-3'

PCR for *IGHM* gene rearrangements (human)

V_H1L	5'-CTG ACC ATG GAC TGG ACC TGG AG-3'
V_H2L	5'-ATG GAC ATA CTT TGT TCC ACG CTC-3'
V_H3L	5'-CCA TGG AGT TTG GGC TGA GCT GG-3'
V_H4L	5'-ACA TGA AAC AYC TGT GGT TCT TCC-3'
V_H5L	5'-ATG GGG TCA ACC GCC ATC CTC CG-3'
V_H6L	5'-ATG TCT GTC TCC TTC CTC ATC TTC-3'
$3'J_H1.2.4.5$	5'-ACC TGA GGA GAC GGT GAC CAG GGT-3'
$3'J_H3$	5'-ACC TGA AGA GAC GGT GAC CAT TGT-3'
$3'J_H6$	5'-ACC TGA GGA GAC GGT GAC CGT GGT-3'

7.2.2 List of Figures

7.2.3 List of Tables

7.3 Curriculum vitae

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Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 14.05.2008

(Daniel Trageser)