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Jelena von Dziembowska

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Abbreviations

AML	Acute myeloid leukemia
ALL	Acute lymphoblastic leukemia
B-ALL	B-cell ALL
BCR	B-cell receptor
BM	Bone marrow
CD	Cluster of differentiation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CRLF2	Cytokine receptor like factor 2
DP	Double positive
DS-ALL	Down syndrome acute lymphoblastic leukemia
ecotropic Env	Ecotropic envelope
FACS	Fluorescence activated cell sorting
fel env	Feline endogenous virus (RD114) envelope
GMP	Granulocyte-macrophage progenitor
HPC	Haematopoietic progenitor cell
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cell
i.v.	intravenous
JAK	Janus kinase
LMPP	lymphoid multipotent progenitor
MEP	Megarkaryocyte-erythrocyte progenitor
MPP	Multipotent progenitor
NK	Natural killer
NSG	NOD/SCID/γ_c^{null}
qRT-PCR	Quantitative real time polymerase chain reaction
PB	Peripheral blood
P/S	Penicillin/streptomycin
rpm	rounds per minute
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STAT	signal transducer and activator of transcription
TSLPR	Thymic stromal lymphopoietin receptor

Zusammenfassung

Der Begriff „Hämatopoese“ beschreibt den Prozess der Bildung der zellulären Blutbestandteile, welcher im Knochenmark stattfindet. Einen wichtigen Teil der Hämatopoese stellt die Bildung von reifen B-Zellen dar, welche sich in den sekundären lymphatischen Organen und dem peripheren Blut befinden und einen wichtigen Bestandteil des adaptiven Immunsystems darstellen. Die Entwicklung der B-Zellen wird über ein komplexes und streng reguliertes Netzwerk von verschiedenen Transkriptionsfaktoren kontrolliert, die zu unterschiedlichen Zeitpunkten exprimiert werden. Die B-Zell-Differenzierung wird außerdem über externe Faktoren wie die Zytokine reguliert. Diese aktivieren Rezeptoren an der Zelloberfläche, um Proliferation und Differenzierung zu induzieren oder Apoptose zu verhindern. Eine Deletion oder eine Mutation dieser Transkriptionsfaktoren oder der Komponenten der Rezeptor vermittelten Signaltransduktion kann zu einer Blockade in der Differenzierung und/oder zu einer veränderten Proliferation und folglich zu der Bildung von neoplastischen Krankheiten wie der B-Zellvorläufer akuten lymphoiden Leukämie (B-ALL) führen.

Die Analyse von Patientenmaterial hat gezeigt, dass der CRLF2 Cytokinrezeptor, ein wichtiger Regulator der frühen B-Zellentwicklung, überexprimiert in B-ALL Fällen mit schlechter Prognose gefunden wurde. Eine hohe CRLF2 Expression tritt oft gemeinsam mit aktivierenden JAK2 Mutationen oder dimerisierenden Mutationen in CRLF2 auf. Bezeichnenderweise zeigten diese B-ALL Fälle auch sehr häufig genetische Veränderungen im *IKZF1*-Gen, welches für den Transkriptionsfaktor IKAROS kodiert, Dieser ist für die Differenzierung in die lymphoide Richtung notwendig und reguliert auch verschiedene, andere Stadien der B-Zellentwicklung. Das Wissen über das Zusammenspiel dieser zwei Arten von Mutationen in Verbindung mit Leukämie ist bisher lückenhaft, andererseits aber essentiell, um effektive Therapien für diese Gruppe von Risikopatienten zu etablieren.

Ziel dieser Diplomarbeit war es daher, Mausmodelle zu entwickeln, um die Rolle dieser Faktoren in der Entstehung der B-ALL zu untersuchen. Die mutierten Gene wurden mittels retroviraler Vektoren in humane bzw. murine Stamm- und Vorläuferzellen (HSC/HPC) eingebracht. Die ersten Mausexperimente zeigten, dass die retrovirale Transduktion der HSC/HPC mit aktiviertem Jak2* sehr ineffizient war. Dies war wahrscheinlich aufgrund der Toxizität und/oder der Vektorgröße von Jak2* und der daraus resultierenden geringen viralen Vektortiter der Fall. Statt der Jak2* Kinase wurde daraufhin die Mutation CRLF2* zusammen mit der IKZFΔ6 Mutation in Primärzellen eingebracht. Hämatpoetische Stamm- und Vorläuferzellen konnten zu hohen Raten infiziert werden, aber der Anteil der Transgen exprimierenden Zellen ging im Blut und Knochenmark der transplantierten Mäuse im Laufe der Zeit stark zurück. Da auch die Mäuse mit doppelt infizierte (CRLF2*+IKZFΔ6) HSC/HPC

nach über drei Monaten noch keine B-ALL entwickelten, wurde eine weitere Studie begonnen, mit der der Einfluß eines zusätzlichen potentiellen Tumorsupressors (LNK) auf die Leukämieentstehung untersucht werden konnte. Es ist bekannt, dass LNK die Aktivität von JAK2 inhibiert und dass *Lnk knock-out* Mäuse eine hohe Anzahl an frühen B-Zellvorläufern besitzen. Folglich wurde der Effekt von einer LNK Überexpression auf CRLF2* vermittelte Signaltransduktion analysiert. Dieser Assay zeigte, dass LNK Überexpression unter Zugabe von IL-3 die Proliferation von einer proB Zelllinie, vermutlich über die Inhibierung von Jak2, deutlich hemmt. Im Gegensatz dazu hatte die LNK Überexpression in Kombination mit CRLF2* und ohne IL-3 keine Auswirkung auf die Proliferation. Dies könnte ein Hinweis darauf sein, dass CRLF2* nicht nur über Jak2 wirkt, sondern auch andere Signalwege nutzt und dass eine Lnk Inaktivierung mit CRLF2* kooperiert, um Leukämie zu induzieren. Bezeichnenderweise haben erst kürzlich veröffentlichte Studien gezeigt, dass inaktivierende LNK Mutationen zusammen mit aktivierenden CRLF2 Mutationen in B-ALL auftreten.

Abstract

„Haematopoiesis“ describes the process of the formation of cellular blood components that takes place in the bone marrow via several differentiation stages. The generation of mature B-cells is one important aspect of this process. They reside in the secondary lymphoid organs and the peripheral blood and represent an important part of the adaptive immune system. B-cell development is regulated via a complex and tightly regulated network of transcription factors that are expressed at different points in time. In addition, B-lymphopoiesis is also tightly regulated by external factors such as cytokines, which activate cell surface receptors to stimulate proliferation, induce differentiation, or prevent apoptosis. Deletion or mutation of these transcription factors or components of the receptor signaling cascade may lead to a block of differentiation and/or aberrant proliferation, and consequently to the formation of neoplastic malignancies, such as B-cell progenitor acute lymphoblastic leukemia (B-ALL). Analysis of patient material has shown that the CRLF2 cytokine receptor, an important regulator of early B-cell development, is overexpressed in high-risk B-ALL cases. High CRLF2 levels are often accompanied by activating mutations in JAK2, or with dimerizing mutations in CRLF2. Significantly, these B-ALL cases also show a high incidence of genetic alterations in the *IKZF1* gene, encoding of the transcription factor IKAROS, which is necessary for the differentiation into the lymphoid lineage, but which also regulates various stages of B-cell development. The interplay of these two types of mutations in leukemia induction is poorly understood, but essential for developing effective therapies in this high-risk group of patients.

Consequently, the aim of this thesis was to develop mouse models to analyze the role of these mutations in connection with B-ALL development. The mutated genes were transduced into human and murine stem and primary cells via retroviral vectors. The first mouse experiments showed that retroviral transduction of activated Jak2* into human CD34+ cells or murine haematopoietic stem or progenitor cells (HSC/HPC) was very inefficient. This was probably due to the toxicity and/or the large vector size of Jak2*, resulting in low viral vector titers. Consequently, a mutated CRLF2* was used instead of the Jak2* kinase to infect primary cells together with a dominant-negative IKAROS (IKZFΔ6). These HSC/HPC were infected at quite high rates, but the percentage of transgene expressing cells in the blood and bone marrow of transplanted mice decreased significantly over time. Due to the fact that mice that carried double-transduced (CRLF2* and IKZFΔ6) HSC/HPC for over three months and did not establish B-ALL, studies were initiated to investigate a potential tumor suppressor that may contribute to leukemia induction. LNK is known to inhibit JAK2 activity, and *Lnk* deficient mice show high levels of early B-cell progenitors. Consequently, the effect of LNK overexpression on CRLF2* mediated signaling was examined. This assay showed

that LNK overexpression in the presence of IL-3 significantly inhibits the proliferation of pro-B-cells, presumably through inhibiting Jak2. In the absence of IL-3 and together with CRLF2* there was no visible effect on the proliferation behavior of the pro-B-cells, suggesting CRLF2* may signal through other pathways. This work suggests that Lnk inactivation may cooperate with CRLF2* to induce aberrant pro-B-cell proliferation. Significantly, recent studies have indeed uncovered LNK inactivation mutations in B-ALL in conjunction with CRLF2 activating mutations.

1 Introduction

1.1 Haematopoiesis

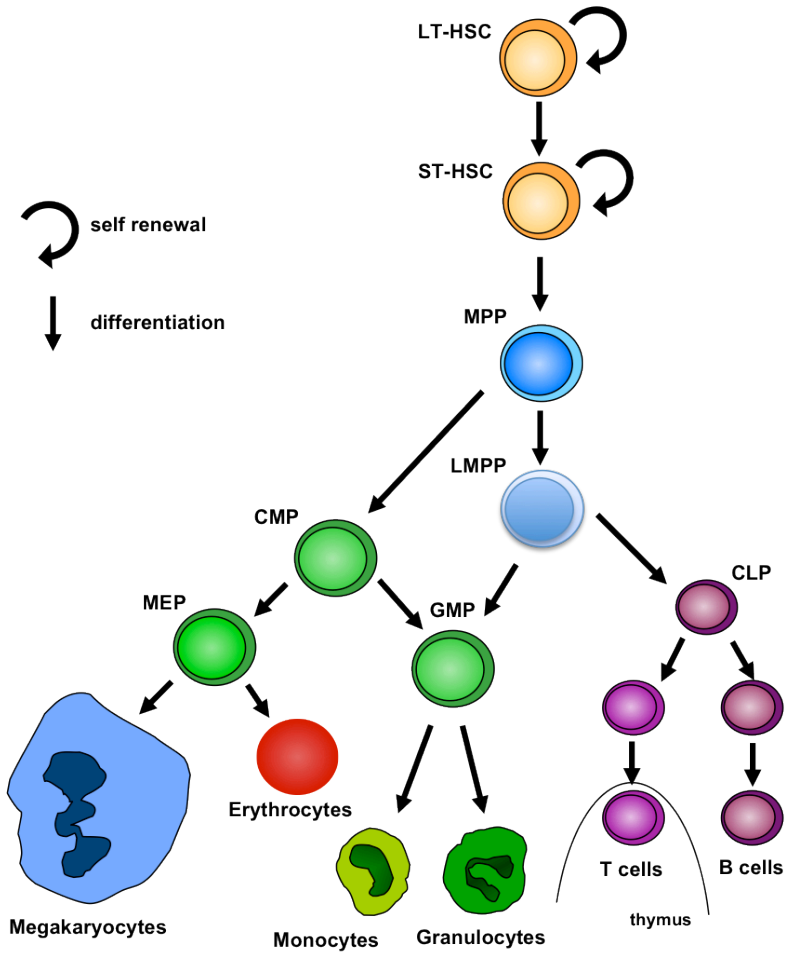

Figure 1 Simplified summary of the process of haematopoiesis.
Haematopoiesis starts with the HSC: haematopoietic stem cell. LT-HSC: long-term haematopoietic stem cell; ST-HSC: short-term haematopoietic stem cell; MPP: multipotent progenitor; LMPP: lymphoid multipotent progenitor CMP: common myeloid progenitor; MEP: megakaryocyte-erythrocyte progenitor; GMP: granulocyte-macrophage progenitor; CLP: common lymphoid progenitor. The figure has been simplified and modified according to Passegue et al., 2003; Cedar et al., 2011.

The term „haematopoiesis“ describes the process of the formation of cellular blood components. During haematopoiesis everything starts with the haematopoietic stem cells (HSCs) in the bone marrow. The HSCs have two functions. First of all they are able to perform self-renewal, which means they produce new HSCs without differentiation (Seita and Weissman, 2010). Due to their pluripotency they also possess the ability to differentiate into the different cell types of the blood. The balance between self-renewal and differentiation is regulated via niches in the bone marrow (Orkin, 2000; Wilson and Trumpp, 2006). There are two different kinds of HSCs: long-term (LT)-HSCs and short-term (ST)-HSCs. LT-HSCs

possess the lifelong ability of self-renewal (Orkin, 2000), whereas the ST-HSC are able to perform self-renewal for about eight weeks (Morrison and Weissmann, 1994).

During the process of haematopoiesis HSCs differentiate into the multipotent progenitors (MPPs), which give rise to the common myeloid progenitors (CMPs) or lymphoid multipotent progenitors (LMPPs) or common lymphoid progenitors (CLPs). The LMPPs as well as the CMPs can give rise to the granulocyte-macrophage progenitors (GMPs), which are able to differentiate into monocytes and granulocytes (Passegue et al., 2003; Cedar and Bergman, 2011), which are part of the innate immune system. Additionally, CMPs are able to form erythrocytes and megakaryocytes via the megarkaryocyte-erythrocyte progenitors (MEPs). The differentiated megakaryocytes are responsible for the release of thrombocytes, whereas the erythrocytes are essential for the transport of oxygen in the blood. The CLPs on the other hand differentiate into the lineage restricted progenitors named pro-DC, pro-B and pro-T, which then form the dendritic, B- and T-cells (Laiosa et al., 2006), representing cells of the adaptive immune system. The decision whether CLPs form B- or T-cells is regulated by the localization of the cells. T-cells are produced in the thymus (Chung et al., 2003), while CLPs that remain in the bone marrow differentiate into the B-cell lineage. The B-cell progenitors leave the bone marrow before they are completely mature and migrate to the secondary lymphoid organs, such as the spleen and the lymph nodes.

1.2 B-cell development

The formation of B-cells takes place in the bone marrow, as described in 1.1, starting from the HSCs. Mature B-cells are formed through a complex process of differentiation stages. These stages are characterized by the expression of different lineage specific proteins on the cell surface (CD molecules). Using antibodies that selectively bind to these surface molecules and are coupled to fluorescence markers, it is possible to distinguish the different stages of B-cell development by their different patterns of surface markers expression via FACS analysis (Hardy et al. 1991; Perez-Vera et al., 2011).

The HSCs, characterized by the expression of Sca and c-kit, differentiate into the LMPP population, which is defined by the occurrence of the cell surface markers Sca1 c-kit and FLT3, as depicted in Figure 2. The HSCs and the LMPPs form the LSK ($Lin^{-}Sca^{+}c\text{-}kit^{+}$) population. The LMPPs, which in contrast to the HSCs also show FLT3 expression, represent the first population that is not able to differentiate into erythrocytes and megakaryocytes anymore (Buza-Vidas et al.,2011). The CLP population is identified by the expression of the IL-7R α-chain (Nutt and Kee, 2007). The CLPs can be divided into early and late CLPs, the latter of which express Ly6D and are specified to the B-cell lineage (Inlay et al., 2009). The CLPs are followed by the pro-B-cells, which are characterized by CD43 expression, which is already present in the HSCs, as well as by CD19 and B220 expression

(Hardy et al., 2007). Subsequent differentiation stages are hallmarked by the accumulation of events leading to the formation of a functional B-cell receptor (BCR).

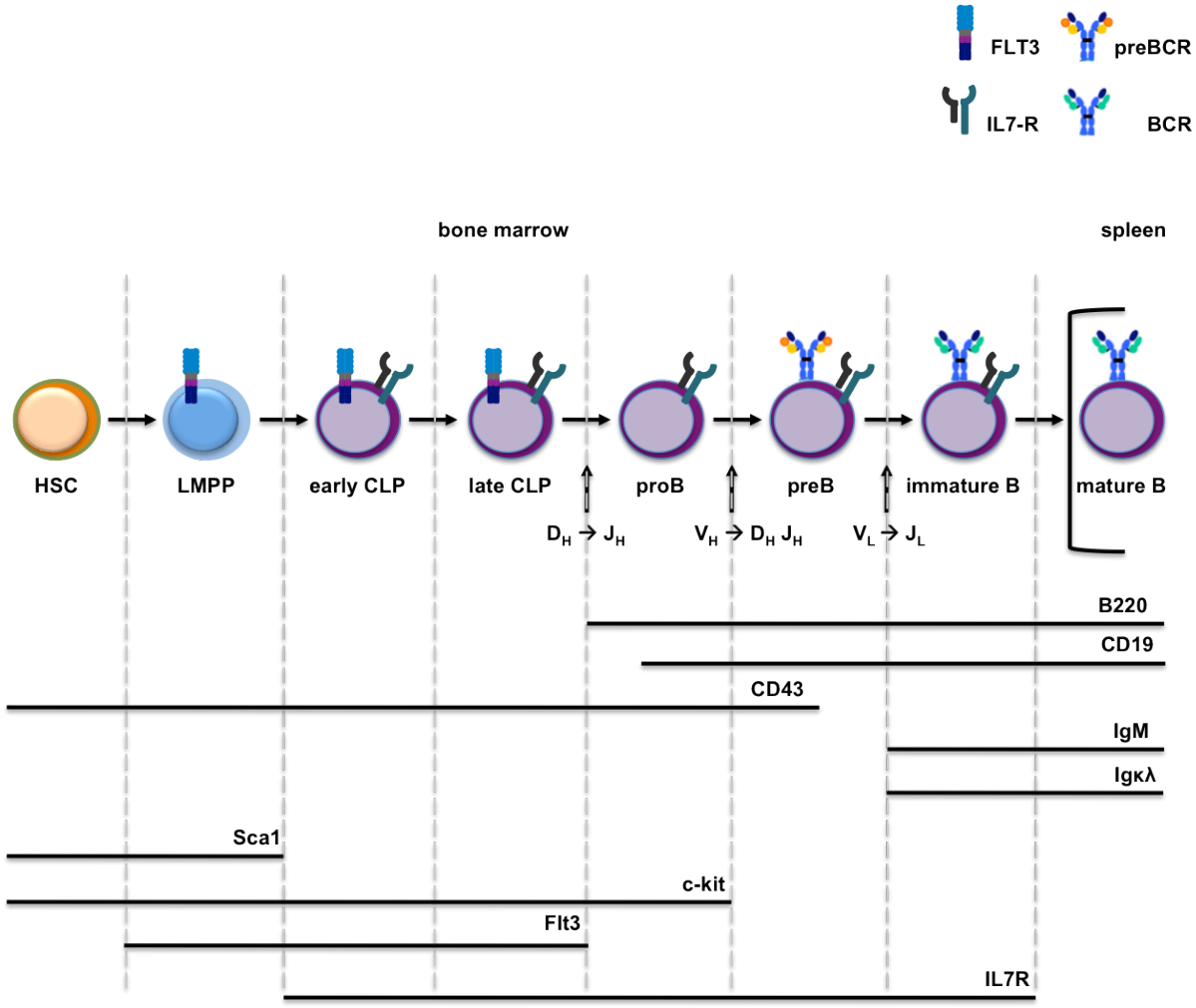

Figure 2 Development and differentiation of B-cells.
The figure shows the variable stages of B-cell differentiation. Additionally, the development of the BCR as well as the expression of different cell surface molecules, which are characteristic for the different stages, are shown. The figure is simplified and modified according to Hardy et al., 2007; Nutt et al., 2007; Welinder et al., 2011.

The formation of the functional BCR is a complex process and characterizes several stages of B-cell development. The process of BCR formation starts in the pre-B-cells, which possess a preBCR. This preBCR possesses a heavy immunoglobulin chain (HC), which is formed by D_HJ_H as well as $V_HD_HJ_H$ rearrangement of the *Igh* locus. The D_HJ_H rearrangement starts already in the pro-B-cell stage and is regulated via a complex containing Rag1 and Rag2 proteins. The expression of the *Rag1* and *Rag2* gene is regulated among others via the important transcription factor IKAROS (Reynaud, 2008). After the rearrangement is terminated, a functional HC protein binds with a so-called surrogate light chain (sLC) to form the preBCR. Surface expression of this receptor represents the transition from the pro-B to the pre-B-cell phase. Surface expression of a functional preBCR stimulates proliferation, leading to the clonal expansion of the cells, but also triggers the stage-specific recombination of the two immunoglobulin LC loci (*Igκ* and *Igλ*). The recombination of the V and J fragments (V_LJ_L rearrangement) leads to the expression of a functional LC and therefore to the

formation of the mature, surface BCR, which marks the crossing to the phase of immature B-cells phase (Melchers et al., 2000). If the BCR on the immature B-cells do not recognize self-antigen after several days in the blood and bone marrow, mature B-cells are formed. This happens via migration of the immature B-cells from the bone marrow to the secondary lymphoid organs, such as the spleen and lymph nodes.
The main function of mature B-cells is the recognition of antigen and pathogens via the BCR and the consequent production of specific antibodies through a complex process. The production of these antibodies contributes to the control of the pathogen. Therefore, B-cells represent an important part of the humoral, adaptive immune response.

1.2.1 Transcription factors

In addition to extracellular cytokines, which bind to their specific receptors and induce signal cascades, there are intracellular transcription factors that play an important role in early B-cell development. The activity of these transcription factors leads to the activation or inhibition of the gene expression of specific target genes, what regulates the process of differentiation.

1.2.1.1 PU.1

The *SFPI1* gene encodes for the purine box factor 1 (PU.1). PU.1 is expressed in all states of B-cell differentiation at different levels (DeKoter et al., 2007). *Sfpi1*$^{-/-}$ mice die during embryogenesis, show severe defects in the lymphoid and myeloid lineage and possess neither fetal B-cells nor T-cells in the thymus (Scott et al., 1994; McKercher et al., 1996). Consequently it is assumed that there is a block before the CLP state in *Sfpi1*$^{-/-}$ mice and PU.1 expression plays an essential role in the MPPs, by regulating the differentiation in the lymphoid direction (DeKoter et al., 2002).

1.2.1.2 E2A

The *TCF3* gene encodes for the E2A protein, which belongs to the *Basic Helix Loop Helix* (bHLH) protein family. *TCF3* encodes for two different proteins: E47 and E12, which are made via alternative splicing. These proteins can form heterodimers with bHLH proteins. But E2A mainly functions as homodimer in the B-cell lineage (Kee et al., 2000).
Tcf3$^{-/-}$ mice show a block at the pro-B-cell stage (Matthias and Rolink, 2005). These mice do not possess CD19 positive B-cells and lack the expression of B-cell specific genes such as *Pax5, Rag1* and *Rag2*, which are necessary for the D_HJ_H *rearrangement* and consequently no functional preBCR can be formed (Bain et al., 1994).

1.2.1.3 EBF1

The *early B-cell factor 1* (EBF1) is mainly expressed in pro, pre and mature B-cells. *Ebf1*$^{-/-}$ mice show a differentiation block at the early pro-B-cell stage as well as a reduced expression of B-cell specific genes such as *Pax5* and of genes, which are important for the formation of the preBCR (Matthias and Rolink, 2005; Nutt and Kee, 2007). E2A and PU.1 activate the expression of EBF1, whereas PAX5 represents a target gene of EBF1 (Lukin et al., 2008). EBF1 expression is activated by the interleukin-7 signaling pathway and is thought to be the key transcription factor that specifies B-cell lineage development (Busslinger et al., 2000; Nutt and Kee, 2007).

1.2.1.4 PAX5

Expression of the *paired homeodomain protein 5* (PAX5) gene starts in the pro-B-cells and its expression is not downregulated until the plasma cell state. *Pax5*$^{-/-}$ mice also show a block at the pro-B-cells stage as well as a loss of B-cell specificity. These cells are able to differentiate into other directions such as into NK cells. This shows that PAX5 is essential for the B-cell specific differentiation. The *PAX5* gene is a target gene of E2A and EBF1, whereas the PAX5 protein itself regulates the expression of genes, which are important for the B-cell specific development such as *CD19* (Nutt and Kee 2007).

1.2.1.5 IKAROS

The *IKZF1* gene, which was of main interest during this thesis, encodes the zinc finger transcription factor IKAROS, which is an important regulator of lymphoid development and differentiation. Ikaros is a member of a family of zinc finger transcription factors that includes Helios, Aiolos, Eos and Pegasus.

The Ikaros protein possesses 6 zinc fingers, four at the N-terminus, which are important for DNA binding, and two at the C-terminus, which are necessary for dimerization. Alternative splicing results in lots of different splicing variants (Molnar and Georgopoulos, 1994). Ikaros expression is found in cells of the myeloid as well as of the lymphoid lineage, but also in the HSCs and multipotent progenitors. Ikaros shows the highest homology to Aiolos and is able to form homodimers or heterodimers with Aiolos (Busslinger, 2004; John and Ward, 2011). Different mouse models with different mutant forms of Ikaros have shown that differentiation into CLPs is not possible without a functional Ikaros protein. Therefore, it has been postulated that Ikaros plays a role in the early multipotent progenitors influencing differentiation in direction of the lymphoid lineage (Busslinger, 2004).

In *Ikzf1*$^{-/-}$ mice all B- and NK-cells as well as B-cell progenitors were lost, but T lymphocytes were still present (Wang et al., 1998).

Ikaros also contributes to the formation of the BCR by regulating the expression of Rag1 and Rag2, which are responsible for the rearrangement of the heavy chain. *In vitro* experiments

have also shown that Ikaros downregulates the expression of the *Igll1* gene, which encodes for λ5, as sLC that is part of the preBCR (Sabbattini et al., 2001).

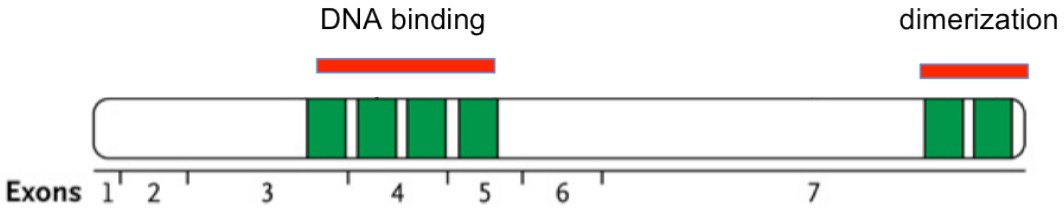

Figure 3 Primary structure of the Ikaros protein.
IKAROS possesses six zinc fingers (Bercovich et al., 2008): four zinc fingers at the N-terminus that are responsible for DNA binding, whereas the two C-terminal zinc fingers mediate dimerization. Modified according to Mullighan et al., 2009.

1.2.2 Cytokine and tyrosine kinase signaling

As mentioned in 1.2, it has been shown that B-cell development is regulated and controlled by a variety of transcription factors and by the expression of different transmembrane receptors, which are involved in different signaling pathways (Busslinger, 2004). Cytokine receptors, such as Flt3 and IL7R, are one important system of regulation. Cytokines bind to these specific receptors, what leads to activation of signal cascades and therefore influences the survival, proliferation and differentiation of cells. These receptors can influence haematopoiesis in a negative and positive way and are expressed at different stages of haematopoiesis.

1.2.2.1 Flt3

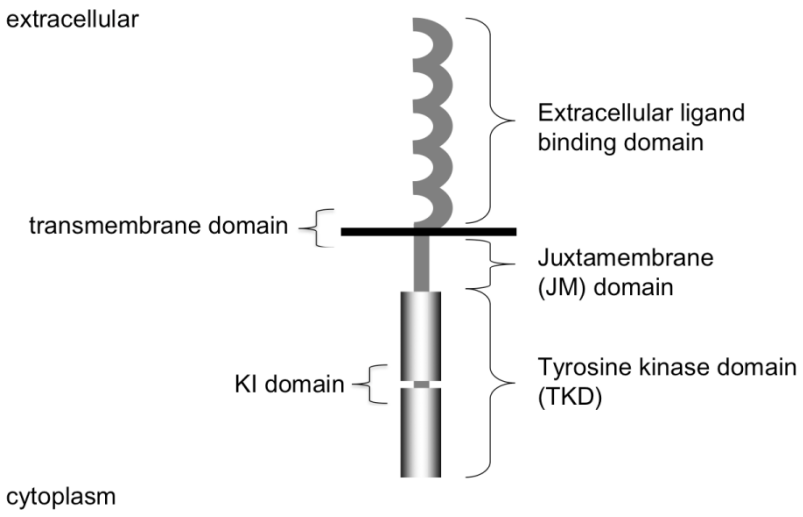

Figure 4 The Flt3 tyrosine kinase.
The Flt3 kinase is composed of an extracellular ligand-binding domain, a transmembrane domain, a juxtamembrane domain and two tyrosine kinase domains, which are connected by a kinase insert domain (KI). The figure is modified according to Takahashi et al., 2011.

The *Flt3* gene encodes for the Flt3 transmembrane receptor, which belongs to the class III receptor tyrosine kinases (Takahashi, 2011). The extracellular N-terminus consists of five Ig-like domains, which mediate the Flt3 ligand binding. The intracellular domain is composed of the juxtamembrane domain and two tyrosine kinase domains. They mediate signal transduction and are connected via a kinase insert domain (Small, 2006).

Flt3 expression can be found in the early myeloid and lymphoid lineage (Maroc et al., 1993). It is not possible to detect Flt3 expression in the LT-HSCs, but it is already possible in the ST-HSCs, which shows that Flt3 expression leads to the loss of self-renewal capacity. Flt3 expression is also detectable in the following LMPPs as well as in the CLPs. Its expression in the LMPPs directs B-cell specific development (Adolfsson et al., 2005; Buza-Vidas et al., 2011). The expression of Pax5 in pro-B-cells terminates the Flt3 expression (Holmes et al., 2006).

The importance of the Flt3 receptor is evidenced by analysis of Flt3 ligand ($Flt3L^{-/-}$) deficient mice. These mice have a significant decrease of pro- and pre-B-cells in the murine bone marrow as well as a decrease of myeloid progenitors, NK cells and dendritic cells (McKenna et al., 2000). Flt3 knockout ($Flt3^{-/-}$) mice also showed a reduced number of early B-cell progenitors (Matsumura et al., 2008). This has shown that the Flt3 receptor and ligand are essential for the formation of the CLPs and consequently the formation of pro- und pre-B-cells.

Binding of the Flt3 ligand to Flt3 leads to homodimerization of two Flt3 receptor tyrosine kinases within the membrane. Consequently, the two cytoplasmic domains gain close proximity and are able to transphosphorylate their juxtamembrane domains. This leads to conformational changes and activation of the receptors. The autophosphorylation of the kinase domains allows the binding of adaptor molecules and leads to the activation of signal cascades via phosphorylation of effector molecules. Flt3 signaling is involved in important pathways such as the PI3K/AKT, Ras/MAPK and JAK/Stat5 pathways. These pathways are involved in apoptosis, proliferation and differentiation, and therefore Flt3 possesses an oncogenic potential (Parcells et al., 2006; Matsumura et al., 2008).

1.2.2.2 Cytokine signaling in early B-cell development

There are two cytokines, interleukin-7 (IL-7) and thymic stromal lymphopoietin (TSLP), which play an essential role at early stages of B-cell differentiation. These two cytokines use two distinct receptors, the IL-7 receptor (IL-7R) and the TSLP receptor (TSLPR), but share a common subunit. Both receptors belong to the Type 1 cytokine receptor family. The IL-7R represents a heterodimeric receptor consisting of two different protein chains: the IL-7R α-chain and the γ_C-chain. The α-chain together with the “cytokine-receptor-like factor 2” (CRLF2) chain form the TSLPR, as depicted in Figure 5 (Park et al., 2000). The binding of IL-7 is mediated by the extracellular part of the IL-7R α-chain, whereas the γ_C-chain increases

the binding affinity. The γ_C-chain is also involved in the formation of interleukin receptors for IL-2, IL-4, IL-9, IL-15 and IL-21 (Milne and Paige, 2006; Palmer et al., 2008). The CRLF2 chain does not show a high affinity for TSLP, but the affinity increases together with the IL-7R α-chain.

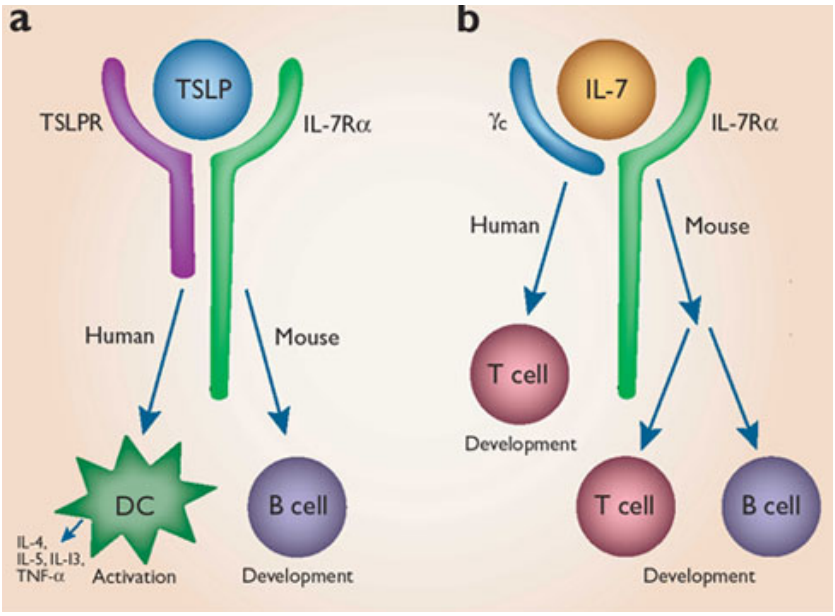

Figure 5 The IL-7R and the TSLPR.
It is visible in (a) that the ligand TSLP binds to a receptor, which consists of the TSLPR chain and the IL-7R α-chain. Binding of TSLP leads to activation of dendritic cells in humans and to activation of the B-cell development in mice. Figure (b) shows that IL7 binds to a receptor consisting of the IL-7R α-chain as well as of a γ_C-chain. Binding of IL7 leads to activation of T cell development in humans and to activation of T and B-cell development in mice Leonard et al., 2002.

The IL-7R is first detected in the CLP compartment and continues to be expressed during T-cell development in T-cell progenitors and mature T-cells. During B-cell development, IL-7R is found in pro-B- and early pre-B-cells, whereas it cannot be found anymore at the transition from pre-B- to IgM^+ B-cells (Sudo et al., 1993). Much evidence supports the hypothesis that its expression induces the B-cell development, and thereby inhibits myeloid differentiation (Kondo et al., 1997). Looking at the *IL*-$7R^{-/-}$ and *IL7*$^{-/-}$ mouse models it is possible to recognize the importance of the IL-7R for the B-cell development. No normal B-cell development is possible and a block at the CLP stage is visible in the *knock-out* mice (Peschon et al., 1994; von Freeden-Jeffry et al., 1995). Compared to wild-type mice, these *knock-out* mice contain just 1/3 of the CLP population, which is not able to differentiate into mature B-cells (Dias et al., 2005). Consequently, it has been postulated that the block probably takes place at the transition from the early ($Ly6.D^-$) to the late ($Ly6.D^+$) CLPs, whereas the down regulation of transcription factors such as EBF1 contributes to the differentiation arrest (Tsapogas et al., 2011).

The IL-7R is involved in the activation of several signaling pathways that are important for survival, proliferation and differentiation of the B-cell progenitors. The α- and γ-chain of the

IL-7R do not possess tyrosine kinase activity, but they are able to activate associated kinases and recruit SH2-domain containing adaptor molecules. The IL-7R is involved in signaling pathways such as the PI3K/AKT and the MAPK/ERK pathway (Corfe and Paige, 2012). It also activates the JAK-STAT pathway. The α- and γ-chains are associated with a JAK3 and JAK1, respectively. Binding of IL7 ligand leads to dimerization of the α- and γ-chain, which increases the proximity of the JAK kinases. This results in transphosphorylation and an increased kinase activity. The STAT proteins are recruited and phosphorylated, what leads to their dimerization. The STAT dimer is then able to translocate into the nucleus and to regulate the expression of target genes (Nosaka et al., 1995; Rodig et al., 1998; Rawlings et al., 2004).

The TSLPR signaling pathways are less understood. These receptors also do not possess any intrinsic catalytic activity but lead to STAT5 activation. The opinions about the mechanism of STAT5 activation are controversial. On the one hand it is postulated that STAT5 activation is independent of JAK proteins, whereas on the other hand it has been shown that TSLP mediated signaling requires JAK1 and JAK2 (Isaksen et al., 1999; Levin et al., 1999; Rochman et al., 2010). The ligand of this heterodimeric receptor complex is TSLP, which is related to IL-7 and is produced by epithelial cells to activate dendritic cells, induce inflammation and allergic responses (Russell et al., 2009). It is also known that TSLP is important for the proliferation, survival and differentiation of B-cell progenitors. CRLF2 is found on many cells of the immune system such as dendritic cells, T- and B-cells as well as on monocytes and mast cells.

Concentrating on B-cells, it is known that TSLP drives the B-cell progression towards IgM^+ B-cells via mechanisms that are different from the IL-7 mediated mechanism. It has been postulated that TSLP is particularly active on pro-B-cells. But $TSLPR^{-/-}$ mice show a normal B-cell development and possess a normal population of IgM^+ cells (He and Geha, 2010).

The opinions concerning the definitive role of CRLF2 for B-cell development are controversial. On the one hand it has been postulated that TSLP overexpression leads to the suppression of the B lymphocytes population and is not able to restore the B-cell differentiation in $IL7^{-/-}$ and $Flt3^{-/-}$ mouse models (Jensen et al., 2007). On the other hand, other groups have shown that TSLP overexpression might restore the B-cell population in IL-7 deficient mice (Chappaz et al., 2007).

1.2.2.3 JAK proteins

As already mentioned it is known that different signaling pathways play an important role during the process of haematopoiesis. These pathways consist of growth factor or cytokine binding to a cytokine receptor on the cell surface, followed by signaling cascades, which finally lead to the regulation of the expression of target genes. These cytokine receptors do not possess an intrinsic catalytic activity and are therefore associated with intracellular

tyrosine kinases such as the JAKs. The occurrence of activating mutations and translocation in these tyrosine kinases lead to constitutive signaling and enhanced proliferation and therefore to the development of haematologic malignancies (Khwaja, 2006; Gery et al., 2009).

There are four different JAKs found in mammalians: JAK1, JAK2, JAK3 and TYK2. Of these four different JAK proteins, JAK2 is most frequently mutated in haematopoietic malignancies. Looking at *Jak2*$^{-/-}$ mice, the specific importance of JAK2 becomes clear. These mice die at an embryonic age of 12 days, due to the failure of definitive erythropoiesis (Neubauer et al., 1998). Although conditional *knock-out* mice have been generated, their role in adult hematopoiesis has only recently been studied (Grebien et al., 2008; Hantschel et al., 2012). Jak2 is known to be pivotal in relaying signals of myeloid-specific cytokines (IL-3, GM-CSF, G-CSF), but may only be important in very early stages of lymphoid differentiation. In contrast, drastic defects in the development of lymphoid cells are visible in *Jak3*$^{-/-}$ mice. They possess a smaller spleen, thymus and lymph nodes and show severe defects in T- and B-cell development (Aringer et al., 1999).

The best understood signaling pathway of JAK proteins is that involving the STAT proteins. In this important pathway, as depicted in Figure 6, cytokine-receptor binding leads to receptor multimerization and juxtaposition of their associated JAK proteins. This is followed by the autophosphorylation and therefore activation of the receptor-associated JAKs on their tyrosine domain. The phosphorylated JAKs are then able to phosphorylate the intracellular part of the cytokine receptor. These phosphorylated sites represent a docking site for signaling proteins such as the STAT proteins, which are located unphosphorylated and monomeric in the cytoplasm upon activation. The STATs are then phosphorylated by the JAKs and consequently are able to dimerize via their SH2-domain. The resulting homo- or heterodimers enter the nucleus via importinα-5 dependent mechanism to regulate transcription of target genes (Ward et al., 2000). To prevent unregulated signaling, the JAK-STAT pathway is controlled via several processes such as receptor internalization and factors such as tyrosine phosphatases, PIAS (protein inhibitor of activated STAT) and SOCS (suppressor of cytokine signaling) proteins (Khwaja, 2006).

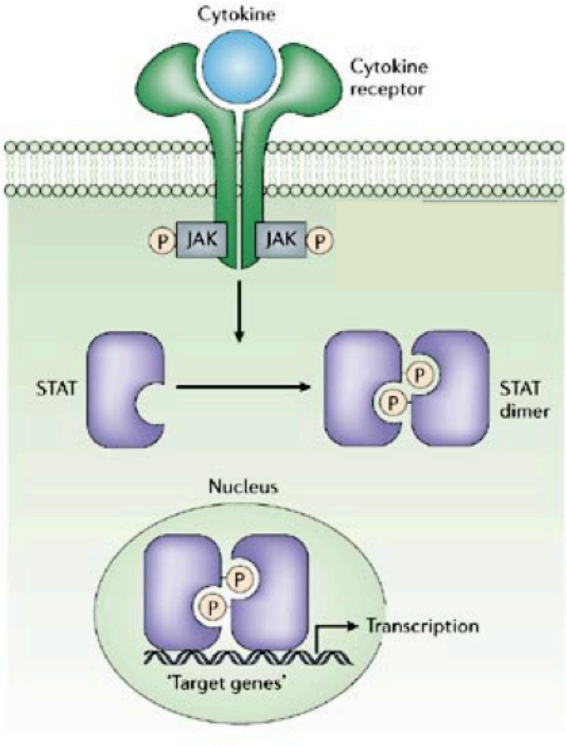

Figure 6 The JAK-STAT pathway.
Binding of a cytokine to the cytokine receptor leads to autophosphorylation of the JAK proteins. They then phosphorylate the receptor, which leads to binding and phosphorylation of the STAT proteins. Consequently, the STATS form dimers and are able to enter the nucleus, where they regulate target gene expression. Modified according to O'Neill et al., 2006.

The fact that the JAK-STAT pathway is used by a wide range of factors, including receptor tyrosine kinases such as Flt3 that are important for haematopoiesis combined with the fact that many key genes contain STAT responsive elements shows its importance and necessity for functional haematopoiesis.

The JAK proteins show a unique structure among tyrosine kinases. The primary structure of JAK2, as depicted in Figure 7, shows that the protein possesses the JH1 domain at the carboxy terminus, which contains the catalytic kinase domain and the JH2 domain, which represents the pseudokinase domain. The pseudokinase domain, which is catalytically inactive, is thought to negatively regulate the catalytic activity of the kinase domain and may mediate protein-protein interactions. Binding to the intracellular part of the cytokine receptors is mediated via the FERM domain.

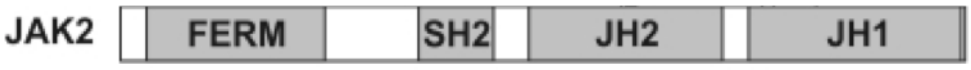

Figure 7 Primary structure of JAK2.
The primary structure of JAK2 shows the N-terminal FERM domain, the SH2 domain as well as the pseudokinase domain (JH2) and the kinase domain (JH1) at the C-terminus. Several known point mutations are indicated. Modified and simplified according to Mullighan et al., 2009.

Next to the main proteins involved in the JAK-STAT pathway, there are other effector proteins that show an impact on some of the events during the JAK-STAT pathway. One

class of these kind of proteins represents the „SH2B/LNK/APS family“ (Rawlings et al., 2004). Due to their possession of a SH2 domain they also present targets of JAK phosphorylation.

1.2.3 LNK

LNK is an adaptor protein, which is mainly expressed in haematopoietic cells such as in the lymphocytes. It represents a 68 kDa protein and its NH_2-terminal domain contains a proline rich region. Furthermore, the protein contains a PH domain and a SH2 domain at the C-terminus. LNK plays an important role in a variety of signaling pathways, starting from growth factor and cytokine receptors, such as c-kit, erythropoietin receptor, and JAK2 associated receptors. With its SH2 domain it is able to bind phosphorylated tyrosines of various signaling molecules and thereby inhibits them. For example, LNK can bind and inhibit mutated tyrosine kinases, as visualized in Figure 8 whereas a mutation in its SH2 domain results in an abolishment of its inhibitory activity (Rudd, 2001).

To access the importance of this protein, the group of Takaki generated *Lnk knock-out* mice (*Lnk*$^{-/-}$) and showed that there is a function for Lnk in limiting B-lymphopoiesis and to regulate the responsiveness to growth factor stimulation (Takaki et al., 2000). They showed that whereas the T-cell development was unaffected, B220^{lo}IgM^{hi} cells, which represent immature, newly generated B-cells accumulated in the spleens of *Lnk*$^{-/-}$ mice. The B220^{lo}IgM^{-} cell population was also increased in the spleens of these mice, which is quite surprising, because normally this kind of progenitor population only occurs in the bone marrow. In the bone marrow the pro- and pre-B-cells were also proportionally increased, which reflects an enhanced proliferation of B-cell progenitors. The responsible mechanism for the B-cell defect has been postulated to be disruption of IL-7R signaling, although molecular and biochemical studies to demonstrate this have not been performed.

It has also been shown that Lnk plays a role in HSC quiescence and self-renewal as well as in the haematopoietic progenitors. The balance between cell cycle quiescence, self-renewal and proliferation plays an important role in HSCs. The process of quiescence and self-renewal is regulated via the binding of TPO to the Mpl receptor, what leads to phosphorylation of JAK2 and consequently the phopshorylation of the receptor. This allows the binding of Lnk. It has been proven that Lnk directly binds to phosphorylated tyrosine residues of JAK2 and represents a negative regulator of JAK2 in the HSCs. Therefore, *Lnk knock-out* leads to an increased activation of JAK2, particularly in the response to TPO, and as consequence to an expanded HSC pool, consisting of an increased number of quiescent cells (Bersenev et al., 2008). A defect in the erythroid compartment has also been attributed to defects in erythropoietin (Epo) receptor signaling (Tong et al., 2005).

Furthermore, it has been postulated that Lnk plays a role in myeloid progenitor cells by binding directly to and interacting with several receptors, such as FLT3, PDGFR and FMS.

Lnk also negatively regulates the SCF/c-kit signaling pathway in haematopoietic progenitors. Their number is increased in *Lnk*$^{-/-}$ mice due to the fact that there is a higher growth signaling through the c-kit receptor in *Lnk*$^{-/-}$ mice. Binding of SCF to the c-kit receptor leads to the recruitment and tyrosine residue phosphorylation of different proteins such as Gab2. Gab2 then leads to the activation of the MAPK signaling pathway, for example. LNK in this correlation leads to inhibition of the c-kit mediated proliferation by inhibiting the phosphorylation of Gab2 and therefore inhibiting the activation of the MAPK pathway, as shown in Figure 8. The haematopoietic progenitor cells compartment increases in the absence of LNK and the cells show an enhanced repopulating ability due to an enhanced c-kit signaling. (Takaki et al., 2002).

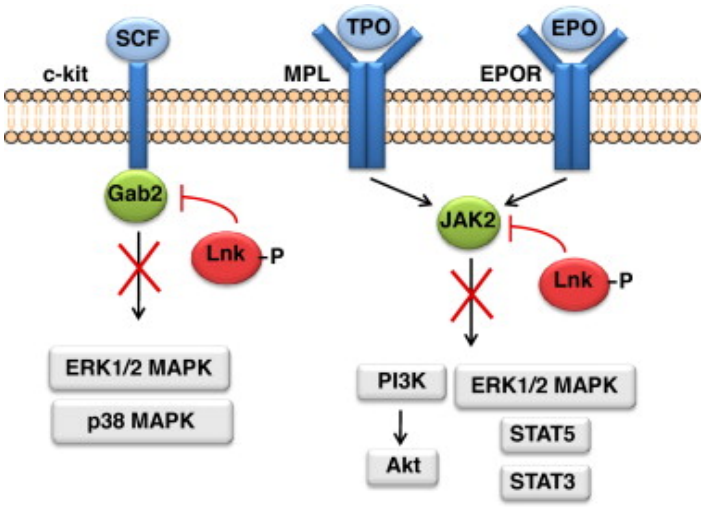

Figure 8 The role of LNK in different signaling pathways.
It has been shown that the adaptor protein LNK inhibits the activity of Gab2 as well as of Jak2, consequently inhibiting the activation of the MAPK/ERK and PI3K/AKT pathway in haematopoietic progenitor cells. An increase of SCF, TPO and EPO mediated signaling leads to enhanced proliferation of the progenitor cells in the absence of LNK (Takaki et al., 2002). Figure adapted according to Devalliere et al., 2011.

1.2.4 Leukemia and B-ALL

1.2.4.1 Leukemia

Cancer of the blood and bone marrow is called leukemia. This kind of cancer is characterized by an increased number of leukocytes in the bone marrow, spleen and peripheral blood, which is the result of a deregulated proliferation and differentiation.

Two kinds of leukemia can be distinguished: the acute and the chronic form. The chronic form is characterized by no or little block in differentiation, which leads to a less drastic or slower progression of the disease. The acute leukemia is defined by a significant proliferation of inoperable progenitors blocked in differentiation. These aberrant progenitors overgrow the functional cells and destroy the function of the immune system as well as several organs. According to the affected cell lineage, acute myeloid leukemia (AML) and acute

lymphoblastic leukemia (ALL) can be distinguished. In contrast to the AML, which is mainly found in adults, ALL mainly occurs in children of 2-4 years (Gilliland and Tallman, 2002). Transformation to a preleukemic or leukemic cell takes place at the level of the HSCs and/or progenitors. These changes include, as Figure 9 shows, increased self-renewal ability, as well as the block of differentiation and apoptosis. Furthermore, these cells do not show controlled proliferation and react differently to growth and anti-growth signals. Additionally, the telomerase activity is changed in leukemic cells, which means that ageing is retarded in these cells (Pui et al., 2004).

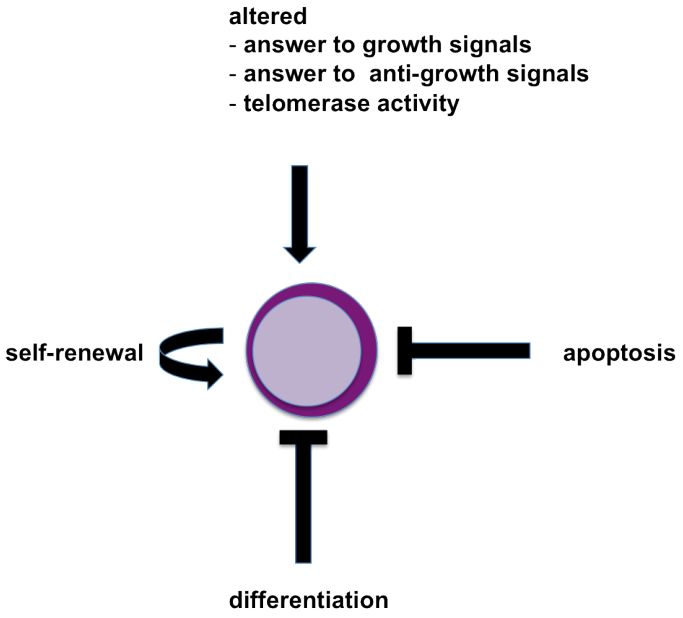

Figure 9 The leukemic cell.
The properties, which differ between a normal HSC or haematopoietic progenitor and a leukemic cell, are shown. Modified according to Pui et al., 2004.

Genetic changes lead to the transformation of normal haematopoietic progenitor cells to leukemic cells. But at least two mutations are necessary for this kind of transformation. Class 1 mutations, as for example mutations of tyrosine kinase receptors *Flt3* and *c-kit* or of a proto-oncogene such as *Ras,* lead to a survival and proliferation advantage of the mutated cell. Class 2 mutations, in contrast to class 1 mutations, block the differentiation and increase the self-renewal capacity of the cell. This second class of mutations generally affects genes encoding transcription factors. The two classes of mutations cooperate to contribute to leukemia formation (Graf and Beug, 1983; Dash and Gilliland, 2001).

1.2.4.2 B-ALL

1.2.4.2.1 B-ALL and *BCR-ABL1*

Progenitor or precursor B-cell (B-)ALL represents more than 80% of the lymphoid leukemias in children and over 75% in adults. With a cure rate of 80% in children, a big success against this disease has already been reached. This means that ALL is actually curable, but 20% of the B-ALL cases are resistant, representing cases of high-risk B-ALL. Consequently, ALL still represents the second leading cause of pediatric cancer death (Mullighan et al., 2009).

B-ALL is a clonal disease, which arises from a single cell and leads to the accumulation of blast cells resulting in the suppression of normal haematopoiesis and infiltration of various extramedullary sites (Reynaud, 2008; Cobaleda and Sanchez-Garcia, 2009). It is assumed that the development of pediatric B-ALL is initiated by chromosomal changes, such as rearrangements or translocations, or mutations in HSC/HPC that reside in the bone marrow or fetal liver.

Consequently the identification of recurrent chromosomal translocations is of prognostic importance for ALL cases. The most common chromosomal translocation generates TEL-AML1 (*ETV6-RUNX1*), which is present in 25% of pediatric B-ALLs. Another fusion transcript, which occurs in B-ALL, is BCR-ABL, which is present in 33% of adult B-ALL and in 5% of childhood B-lineage ALL. A translocation between chromosome 22 and 9 results in the formation of the *BCR-ABL1* fusion gene. This gene encodes for the oncoprotein BCR-ABL that represents a constitutively active tyrosine kinase, which perturbs several signaling pathways such as the MAPK, JAK/STAT5 and Myc pathways. This leads to an uncontrolled cell proliferation and reduced apoptosis in early leukemogenesis. The outcome of BCR-ABL positive ALL is quite poor (Armstrong and Look, 2005; Piccaluga et al., 2007). High-risk patients without *BCR-ABL1* translocation show comparable gene expression patterns compared to the patients with this chromosomal translocations (Den Boer et al., 2009; Mullighan et al., 2009). These BCR-ABL1 negative cases with a comparable expression pattern represent the majority of high-risk B-ALL cases. Analysis of patients' material of these cases resulted in the identification of among others CRLF2 rearrangements, *JAK2* mutations, *IKZF1* deletions and *LNK* deletions (Hertzberg et al., 2010; Roberts et al., 2012).

1.2.4.2.2 CRLF2* and JAK2* in B-ALL

It has been postulated that genetic alterations at the *CRLF2* locus, such as DNA copy number alterations, *CRLF2* point mutations, as well as deletions or translocations upstream of *CRLF2,* lead to the overexpression of this receptor and may contribute to the formation of malignancies such as B-ALL. The overexpression of CRLF2 seems to occur only in B-ALL cases without BCR-ABL chromosomal translocations. It is known that in 7% of all B-ALL cases grouped together, there is a CRLF2 overexpression, whereas CRLF2 overexpression

is present in 50% of Down syndrome acute lymphoblastic leukemia (DS-ALL). Additionally, a point mutation of *CRLF2* could be found in 10% of the CRLF2 overexpressing patients, with a F232C point mutation being one of the most frequent (Hertzberg et al., 2010).

But it is also important to notice that CRLF2 overexpression alone is not sufficient to transform cells (Hertzberg et al., 2010; Roll and Reuther, 2010). It is postulated that due to the fact that CRLF2 possesses a Jak-binding Box 1 motif, it may be used as a scaffold for activated JAK2 (Mullighan et al., 2009; Yoda et al., 2010; Harvey et al., 2010).

JAK2 also plays a role in acute lymphoblastic leukemia. Activating JAK2 mutations have also been found in high risk ALL (Mullighan et al., 2009). These mutations mainly occur in the highly conserved kinase and pseudokinase domain, as depicted in Figure 10, and result in a constitutive JAK-STAT signaling. It is known that 10% of all high-risk pediatric B-ALL show JAK mutations. Furthermore, it has been shown that there are JAK2 mutations in about half of the cases with CRLF2 overexpression in DS-ALL. 70% of the cases overexpressing CRLF2 in B-ALL also show JAK2 mutations (Mullighan et al., 2009). In contrast to JAK2 mutations identified in myeloproliferative neoplasia (MPN), which is primarily a V617F mutation within the JH2 domain, the most common mutation in B-ALL is the R683G missense mutation (Jak2*), also occurring in the pseudokinase domain, which may result in the loss of the inhibitory activity of the pseudokinase domain, as depicted in Figure 10. This kind of mutation has also been found in patients' samples of BCR-ABL1-negative cases, occurring together with CRLF2 rearrangement and IKZF1 deletion (Roberts et al., 2012).

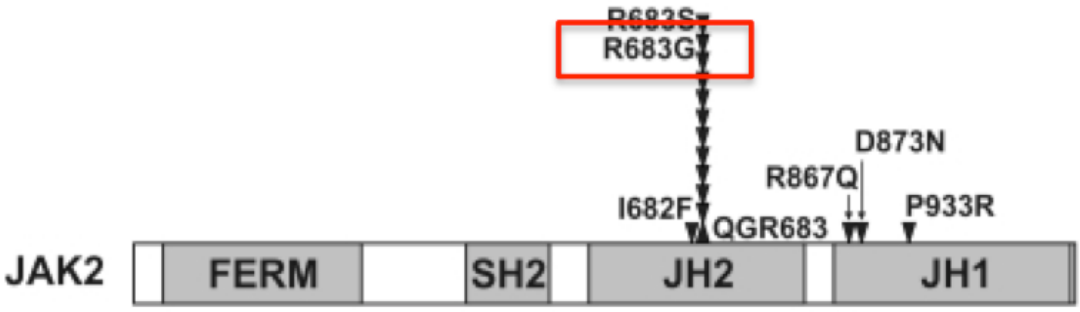

Figure 10 Primary structure of JAK2.
The primary structure of JAK2 shows the N-terminal FERM domain, the SH2 domain as well as the pseudokinase domain (JH2) and the kinase domain (JH1) at the C-terminus. Several known point mutations are indicated. The R683G missense mutation was used for this thesis. Modified and simplified according to Mullighan et al., 2009.

1.2.4.2.3 B-ALL and IKZF1

Genome-wide studies of over 200 children with B-ALL showed that genetic alterations of *IKZF1* are associated with a very poor outcome in B-cell progenitor ALL (Mullighan et al., 2009). This transcription factor plays an important role in high-risk ALL as Mullighan and others have shown that its deletion is frequent in BCR-ABL1-positive ALL: present in 80% of all the cases, but also in BCR-ABL-negative ALL (Figure 12). Overexpression of the dominant negative IKZFΔ6 has been found in 40% of adult pre-B-cell ALL (Rebollo and Schmitt, 2003). This isoform of Ikaros lacks all N-terminal, DNA-binding zinc fingers and is therefore not able to bind DNA. It is only located in the cytoplasm and not in the cell nucleus. This short isoform acts as a dominant negative, which inhibits the binding of the functional isoforms to the DNA and therefore prevents the normal, regulatory function of the Ikaros proteins (Rebollo and Schmitt, 2003).

The similarity of the gene-expression signatures of BCR-ABL-negative ALL with *IKZF1* mutation and BCR-ABL-positive ALL raises the possibility that patients with BCR-ABL negative ALL and IKZF1 mutations might have activating mutations in tyrosine kinases (Martinelli et al., 2009; Mullighan et al., 2009). This hypothesis was born out in several studies showing coincident JAK2 mutations and *IKZF1* deletions within this high-risk group (Mullighan et al., 2009). More recently, using “next-generation sequencing,” activating kinase mutations have been found in all 15 BCR-ABL1-negative ALL cases (Roberts et al., 2012). Whereas the majority has previously described CRLF2 or JAK2 mutations, mutations in the IL-7R or FLT3 were also found, as well as translocations leading to the constitutive activation of ABL, PDGFR, or EPOR. This work provides strong evidence that high-risk B-ALL is driven by activated tyrosine kinase pathways, but nevertheless many questions remain. What is the function of Ikzf1 deletion, a common feature of most (but not all) kinase-activated B-ALL? Can kinase inhibitors be used to effectively treat this patient pool and what are the pivotal downstream effectors?

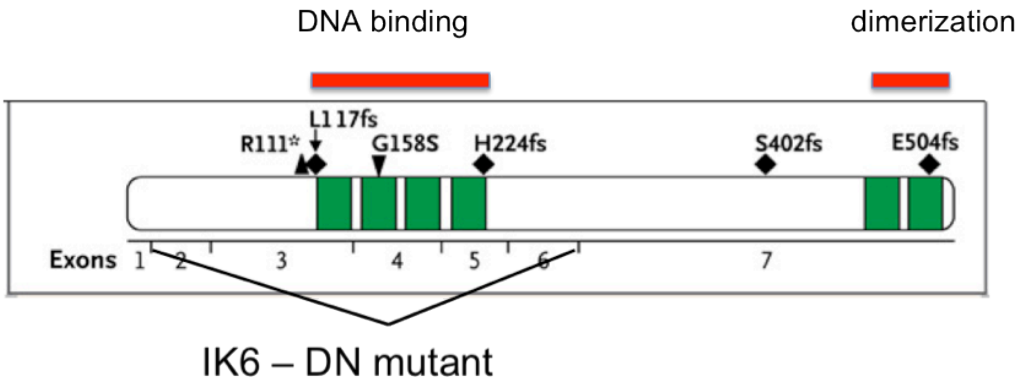

Figure 11 Primary structure IKZFΔ6
IKAROS possesses six zinc fingers (Bercovich et al., 2008): four zinc fingers at the N-terminus that are responsible for DNA binding, whereas the two C-terminal zinc fingers mediate dimerization. The dominant-

negative isoform IKΔ6(IK6) lacks exon three to six and therefore all N-terminal zinc fingers. The black arrows indicate point mutations of IKZF1. Modified according to Mullighan et al., 2009.

In summary, molecular analysis of high-risk B-ALL has identified a common set of genetic mutations that are implicated in disease progression. The first shared feature is high expression of CRLF2, found in 7% of all B-ALL (and 50% of DS-ALL). At least two unique mechanisms are used to promote signaling through CRLF2: activating mutations in CRLF2 itself (e.g F232C, leading to dimerization) or JAK2 mutations (e.g R683G, presumably resulting in inactivation of inhibitory sequences). These two mutation types only account for circa 70 to 80% of these patients, thus other mechanisms are probably at play. In light of the fact that Lnk is a known inhibitor of JAK2 signaling and its disruptions leads to the accumulation of early pro-B-cells, we postulate that Lnk inactivation may also contribute to disease progression. The final shared feature is the disruption of the *IKZF1* gene, in many cases leading to the expression of a dominant negative transcription factor. The importance of this deletion is not known, but it presumably inhibits B-cell development at the proB stage. Why this mutation is so specific for high-risk ALL with tyrosine kinase mutations remains unclear.

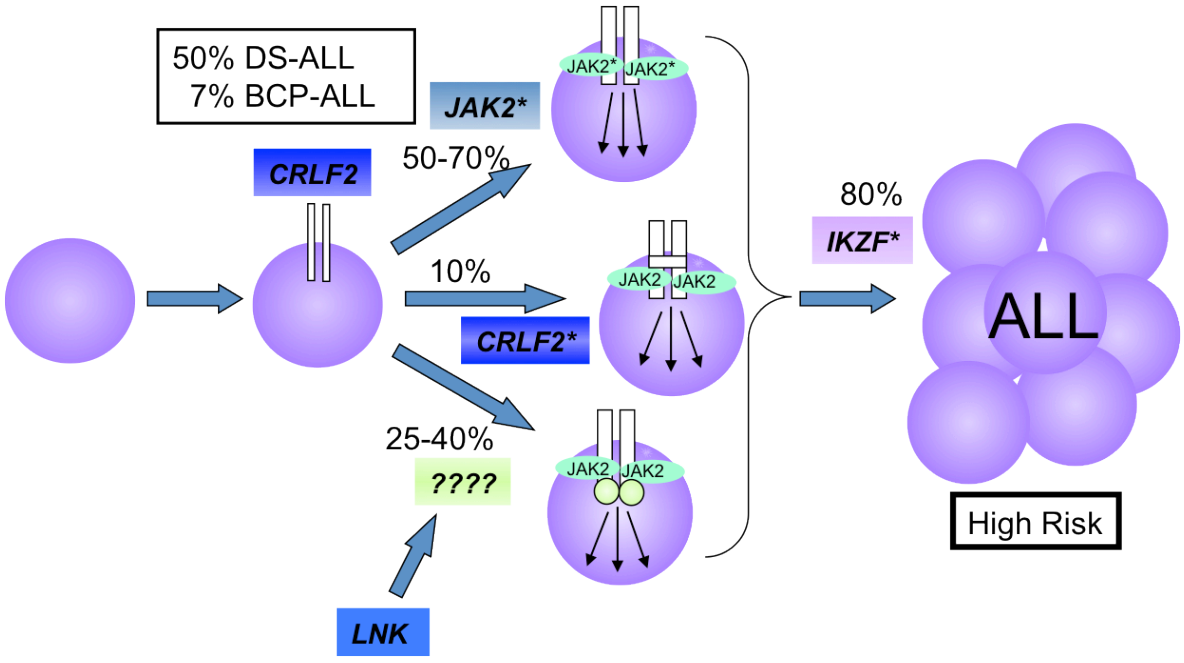

Figure 12 Selected mutations occurring in B-ALL.
Overexpression of *CRLF2* as well as *JAK2*, *CRLF2* and *IKZF* mutations contribute to the formation of high-risk B-ALL. The adaptor protein LNK might also play a role in the disease formation. Modified according to C.Stocking.

2 Aim of this work

It is known that signaling via CRLF2 and the associated JAK2 proteins is important for B-cell proliferation, whereas IKAROS is essential for B-cell differentiation. Deletion or mutation of these transcription factors or components of the receptor signaling cascade may lead to a block of differentiation and/or aberrant proliferation, and consequently to the formation of neoplastic malignancies, such as B-cell progenitor acute lymphoblastic leukemia (B-ALL). Analysis of patient material has shown that the CRLF2 cytokine receptor is overexpressed in high-risk B-ALL cases often accompanied by activating mutations in JAK2. These B-ALL cases also showed genetic alterations of the transcription factor IKAROS. The interplay of these two types of mutations in leukemia induction is poorly understood, but essential for developing effective therapies in this high-risk group of patients.

It was the aim of this work to develop mouse models to analyze the effects of different combinations of these mutated factors in murine and human primary cells and to determine their contribution to the formation of B-ALL. In a first set of experiments, two different approaches were used to infected human CD34+ HSC/HPC with retroviral vectors expressing Jak2* and IKZFΔ6 to determine which approach leads to the highest engraftment and infection of human cells in immune deficient NSG mice. Additionally, murine HSC/HPC isolated from bone marrow (BM) were infected either with Jak2* or CRLF2* together with IKZFΔ6 constructs to determine if one of these combinations contributes to B-ALL formation. LNK is known to inhibit JAK2 activity, and *Lnk* deficient mice show high levels of early B-cell progenitors. Additionally, it was postulated that its inactivation might also contribute to B-ALL formation, possibly by augmenting CRLF2 or JAK2 signaling. Consequently, the effect of LNK overexpression on CRLF2* mediated signaling and following proliferation of pro-B-cells was analyzed *in vitro* as preliminary study for probable following *in vivo* experiments.

3 Material and methods

3.1 Material

All laboratory-ware was obtained, if not noted otherwise, by the companies Merck (Darmstadt), Roth (Karlsruhe), Sigma-Aldrich (Munich) and Serva (Heidelberg).
If not named otherwise, plastic-ware of the companies Becton Dickinson (Heidelberg), Sarstedt (Nümbrecht) and TPP (Trasadingen, Switzerland) was used.

3.1.1 Equipment

Biological safety cabinets *Herasafe*	Thermo Scientific (Karlsruhe)
Centrifuge *Allegra X12R*	Beckmann Coulter (Krefeld)
Desk centrifuge *5415D*	Eppendorf (Hamburg)
Electrophoresis chamber (DNA) *Perfect Blue Mini S*	PeqLab Biotechnologie (Erlangen)
Electrophoresis system (Protein) *Mini-PROTEAN Cell*	Bio-Rad (Munich)
Film processor *AGFA Classic EOS*	Siemens (Erlangen)
Flow Cytometer *FACSCanto*	Becton Dickinson (Heidelberg)
Flow Cytometer *FACSAria*	Becton Dickinson (Heidelberg)
Gel documentation (DNA) *BioDoc Analyze*	Biometra (Göttingen)
Heat block *Thermomixer compact*	Eppendorf (Hamburg)
Hemavet	Drew Scientific (Cumbria, UK)
Incubator *Hera Cell 150*	Heraeus (Hanau)
Lab balance PG503-S DeltaRange	Mettler Toledo (Gießen)
Light Cycler *1.5*	Roche (Mannheim)
Magnetic stirrer *RCT basic*	IKA-Werke (Staufen)
Microscope *Zeiss IM*	Zeiss (Oberkochen)
Power supply *power supply*	
pH meter	Schott (Mainz)
Photometer *Nanodrop Spectrophotometer ND-100*	New Brunswick Scientific (Edison, USA)

Pipettes *(20µl, 200µl, 1000µl)*	Gilson (Middleton, USA)
Protein blotting equipment *Mini Trans-Blot Cell*	Bio-Rad (Munich)
Shaking incubator *innova 4000*	New Brunswick Scientific (Edison, USA)
Sonificator *Bioruptor*	Diagenode (Liège, Belgium)
Vortex mixer V*ortex-2 Genie*	Scientific Industries (Bohemia, USA)
Water bath *Typ 1008*	GFL (Burgwedel)
X-ray film cassette	Rothaar & Schröder (Heidelberg)

3.1.2 Software

FACS*Diva software version 4.2.2*	Becton Dickinson (Heidelberg)
Light Cycler Software 5.32	Roche (Mannheim)
Biodoc 1.8.12.4	Biometra (Göttingen)
Nanodrop ND-1000 V3.7.1	Thermo Scientific (Karlsruhe)

3.1.3 Kits

DNA Gel Extraction Kit	Millipore (Schwalbach)
RNeasy Mini Kit	QIAGEN (Hilden)
Plasmid Midi Kit	QIAGEN (Hilden)
Plasmid Maxi Kit	QIAGEN (Hilden)
QIAprep Miniprep Kit	QIAGEN (Hilden)
ProFection® Mammalian Transfection System - Calcium Phosphate	Promega
Lineage Cell Depletion Kit *mouse*	MACS Milteny Biotech (Bergisch-Gladbach)
Light Cycler® FastStart DNA Master SYBR Green I Kit	Roche (Mannheim)

3.1.4 Media

3.1.4.1 Bacteria culture

LB medium: 10 g/L Bacto-Trypton (BD, Heidelberg); 5 g/L Bacto-yeast extract (DIFCO, Lawrence, USA); 10 g/L NaCl; pH 7,5

LB agar: LB-medium; 15 g/L; agar (DIFCO, Lawrence, USA)

The media have been autoclaved before usage for 20min at 121°C. 100µg/ml of ampicillin were added to the LB medium as well as to the LB agar, after cooling down.

3.1.4.2 Media, solutions and reagents for cell culture and mouse experiments

Solutions and reagents for cell culture

α-MEM *Minimum Essential Medium alpha Modification*	PAA (Pasching, Austria)
BSA (dry chemical) *Bovine Serum Albumin*	Sigma (Taufkirchen)
Chloroquine (stock 25mM)	Sigma (Taufkirchen)
Ciprobay (stock 2 mg/ml)	Bayer (Leverkusen)
DMEM *Dulbecco's Modified eagle Medium*	PAA (Pasching, Austria)
FBS *Fetal Bovine Serum*	Lonza (Basel, Switzerland)
FCS *Fetal* Calf Serum	Biochrom (Berlin)
Glutamine (stock 200mM)	Sigma (Taufkirchen)
HBSS *Hank's Balanced Salt Solution* (1x)	PAA (Pasching, Austria)
Hepes (stock 1M)	PAA (Pasching, Austria)
IMDM	Gibco (Darmstadt)
MEM Alpha Modification (α-MEM)	PAA (Pasching, Austria)
Methylcellulose *M3231*	StemCell Technologies (Grenoble, France)
Penicillin/Streptomycin (stock 10000U/ml)	PAA (Pasching, Austria)
Polybrene (stock 8mg/ml)	Sigma-Aldrich (Munich)
Puromycin (stock 1µg/ml)	Sigma-Aldrich (Munich)
Retronectin (stock 48µg/ml)	Takara Bio inc. (St.Germain en Laye, France)
RPMI 1640 *Cell culture medium*	PAA (Pasching, Austria)
SFEM	StemCell Technologies
Sodium pyruvate (stock 100mM)	PAA (Pasching, Austria)
Trypsin/EDTA (10 x; 0,5%/0,2% (w/v))	Biochrom (Berlin)

IMDM:	IMDM powder solubilized in H_2O to 310 mOS; pH 7,6
10xPBS:	140mM NaCl; 3mM KCl; 10mM Na_2HPO_4; 1,5mM KH_2PO_4; pH 7,4

Cytokines

Solubilized according to manufacturer instruction

Human FLT3 (rhFLT3; stock 100ng/µl)

Human Interleukin-11 (rhIL-11; stock 100ng/µl)

Human stem cell factor (rhSCF; stock 100ng/µl)

Human Tpo (rhTpo; stock 20ng/µl)
Human Interleukin-6 (rhIL-6; stock 20ng/µl)
Murine Interleukin-3 (rmIL-3; stock 10ng/µl)
Murine Interleukin-7 (rmIL-7; stock 20ng/µl)
Murine stem cell factor (rmSCF; stock 100ng/µl)

Cell culture media

α-MEM medium (Bmi-Flt3)	α-MEM; 10%(v/v) FCS (Biochrom); 4mM glutamine; 1mM sodium pyruvate
DMEM medium (HEK293T)	DMEM; 10%(v/v) FCS (Lonza); 4mM glutamine; 1mM sodium pyruvate; 20 mM HEPES
DMEM medium (SC-1)	DMEM; 10%(v/v) FCS (Biochrom); 4mM glutamine; 1 mM sodium pyruvate
Freezing medium	90%(v/v) FCS; 10%(v/v) DMSO
IMDM medium	IMDM; 5%(v/v) FCS; 1%(v/v) Penicillin/Streptomycin; 4mM glutamine
RPMI medium (Baf3)	RPMI 1640, 10%(v/v) FCS, 4mM glutamine, 1mM sodium pyruvate
SFEM medium (BM)	SFEM; 4mM glutamine; 1mM sodium pyruvate; 1%(v/v) Penicillin/streptomycin; cytokines (1:1000)

Solutions and reagents for mouse experiments

Baytril ®	BayerVital GmbH (Leverkusen)
BD Pham Lyse™ (10 x)	Becton Dickison (Heidelberg)
5-Fluoruracil (5-FU) (stock 50µg/µl)	Medac (Hamburg)
CALfix ProTaqs	Biocyc GmBH (Luckenwalde)

3.1.5 Buffer

3.1.5.1 DNA analytical methods

6 x loading buffer	Fermentas (St, Leon-Rot)
TAE (1 x)	40mM Tris; 1 mM EDTA; 20mM acetic acid; pH 8,0

3.1.5.2 Protein biochemical methods

SDS-PAGE and Westernblot

10 x PBS	140mM NaCl; 3mM KCl; 10mM Na_2HPO_4; 1,5mM KH_2PO_4; pH 7,4

4 x loading buffer	500mM Tris; 4% SDS; 20-30% glycerol; 40mM DTT; bromophenol blue; pH 6,8
APS 10 %	1g APS to 10ml ddH_2O
Blocking solution	1 x PBS; 0,1% Tween 20; 5% skimmed milk powder
10% BSA	2g BSA in 20ml 1xPBS
Resolving gel 10%	375mM Tris/HCl (pH 8,8); 5% acrylamid mix (37,5:1); 0,1% SDS; 0,1% APS; 0,01% TEMED
1 x running buffer	25mM Tris; 191mM glycine; 0,35mM SDS
SDS 10 %	1g SDS to 10ml ddH_2O
Stacking gel	125mM Tris/HCl (pH 6,8); 5% acrylamid mix (37,5:1); 0,1% SDS; 0,1% APS; 0,01% TEMED
Stripping buffer	62,5mM Tris; 69,3mM SDS; pH: 6,8
1 x transfer buffer	250mM Tris; 192mM glycine; 20% methanol; pH 8,3
Washing buffer	1 x PBS; 0,1% Tween 20

3.1.6 Enzymes

AMV Reverse Transcriptase	Fermentas (St. Leon-Rot)
RNasin® Plus RNase inhibitor	Promega (Mannheim)
T4 DNA-Ligase	Fermentas (St. Leon-Rot)

3.1.6.1 Restriction enzymes

The used restriction enzymes BamHI, BglII, HindIII, KpnI, NcoI, NotI, SacII, XbaI, derive from the companies Fermentas (St. Leon-Rot).

3.1.7 Antibodies

3.1.7.1 Protein biochemical methods

Primary

target prot.	source	clonality	protein size	description	supplier
LNK	goat	polyclonal	68 kDa	sc-19743	Santa Cruz, Biotechnology
GAPDH	mouse	monoclonal	37 kDa	sc-32233	Santa Cruz, Biotechnology

The LNK antibody was used in dilution of 1:100 or 1:200 in 2%(w/v) BSA. The GAPDH antibody was used 1:2000 or 1:4000 in 1 x PBS; 0,1 %(v/v) Tween 20; 5 %(w/v) skimmed milk powder.

Secondary

Anti goat	Donkey IgG	1:5000	sc-2020	Santa Cruz
Anti mouse	Goat IgG	1:5000	sc-2005	Santa Cruz

All secondary antibodies were conjugated with horseradish peroxidase and were obtained from Santa Cruz Biotechnology. They have been solubilized in 1 x PBS; 0,1 %(v/v) Tween 20; 5 %(w/v) skimmed milk powder.

3.1.7.2 Flow cytometry

target protein	clone	manufacturer	fluorochrome
Gr.-1	RB6-8C5	BioLegend	APC
CD11b	M1/70	BioLegend	PE
B220	RA3-6B2	BioLegend	PE
B220	RA3-6B2	BioLegend	APC-Cy7
CD3e	145-2C11	BioLegend	APC
Ter119	Ter-119	BioLegend	PE
ckit	2B8	BioLegend	APC
mCD45	RA3-6B2	BioLegend	PE
hCD45	HI30	BioLegend	APC
Sca1	D7	BioLegend	Pe-Cy7

3.1.8 Oligonucleotides for RT-PCR

gene	description	sequence	qPCR conditions Annealing temperature	fragment length
IgLL1[1]	CS1580 CS1581	TTGGTATGTCTTTGGTGGTGGGAC TAAGGAAGGCAGGAACAGAGTGAC	64°C, 6sec	250bp
mHPRT	MKF35 MKF36	GCTGGTGAAAAGGACCTCT CACAGGACTAGAACACCTGC	62°C, 10sec	250bp
Rag1	CS849 CS850	ATGCTGATGGGAAGTCAAGC AATCCTGGCAATGAGGTCTG	60°C, 10sec	200bp

[1] Lukin et al., PNAS 2010

3.1.9 Plasmids

Helper plasmid for virus production

R690	pSV40-gag/pol; carries the MoMLV gag- and pol-genes under the control of the SV40 promoter (Beyer et al., 2002)
#583	pCMVgagpol/RRE, lentiviral gag/pol/RRE expression vector (Dull et al., 1998)
#584	pRSV-REV, lentiviral REV expression vector (Dull et al., 1998)
#522	pEcoenv-I-puro; carries the ecotropical ecotropic Env-gene under the control of the EF1α promoter (Morita et al., 2000)
R861	pVSV-G_{NJ} (Beyer et al., 2002)
R916	pFeline endogenous virus (RD114) env (Cosset et al., 1995)

γ-Retroviral constructs

R1331	pRMys-iV, γ-retroviral vector with venus as reporter gene (Kitamura et al., 2003)
R1481	pRMys-IKZFv6-ERt2-iV, γ-retroviral vector with venus as reporter gene, containing among others the dominant negative isoform 6 of IKZF as well as the inducible estrogen receptor ERt2
R1496	pRMys-hCRLF2*-iV, γ-retroviral vector with venus as reporter gene, containing among others the mutant form of human CRLF2
R1515	pRMys-strep-ERt2-iV, γ-retroviral vector with venus as reporter gene, containing among others the inducible estrogen receptor Ert2
R1518	pRMys-hCRLF2*-iCer, γ-retroviral vector with cerulean as reporter gene, containing among others the mutant form of human CRLF2
R1558	pRMys-iBFP-2a-puro+, γ-retroviral vector with BFP as reporter gene
R1566	pRMys-Ikzf1Δ6-iBFP-2a-puro+, γ-retroviral vector with BFP as reporter gene, containing among others the dominant negative isoform 6 of IKZF

Lentiviral constructs/ LeGO vectors (Weber et al., 2008)

#834	pLeGO-iG2/Puro+, Lentiviral vector with eGFP as reporter gene, containing among other a puromycin resistance gene
R1346	piCer2, Lentiviral vector with Cerulean as reporter gene
R1505	LeGO-iG2-Puro+CTAP-mJak2.9b, Lentiviral vector with eGFP as reporter gene, containing among others the R683G mutated form of murine Jak2 as well as puromycin as resistance gene

R1507	LeGO-hLNKwt-iCer2, Lentiviral vector with cerulean as reporter gene, containing among others the human LNK (wt)
R1520	LeGO-hCRLF2*-iCer2, Lentiviral vector with cerulean as reporter gene, containing among others the mutated form of human CRLF2
R1541	LeGO-PI3KCA-H1072R-iB2-Puro+, Lentiviral vector with BFP as reporter gene, containing among others a puromycin resistance gene
R1542	LeGO-hLNK-iB2-Puro+, Lentiviral vector with BFP as reporter gene, containing among others the human LNK (wt) as well as puromycin as resistance gene
R1543	LeGO-iB2-Puro+, Lentiviral vector with BFP as reporter gene, containing a puromycin resistance gene

3.1.10 Bacteria strains

The bacteria strain *Escherichia coli* XL10-GOLD® (Stratagene, La Jolla, USA) was used for all experiments of this thesis.

3.1.11 Mouse strains

All mouse strains were maintained under specific pathogen-free conditions at the animal facility of the Heinrich-Pette-Institute.

C57BL/6 Ly5.2 mice were used for isolation of bone marrow cells, which were transplanted into C57BL/6 Ly5.1 mice. These two mice strains are genetically identical, apart from the *Ptprc* gene (= congenic).

$Lnk^{-/-}$ or $Lnk^{+/+}$ 129 mouse strains were used for the isolation of bone marrow cells, which were transplanted into $Lnk^{+/+}$ 129 mice.

NOD/SCID/γ_c^{null} mice (Ito et al., 2002) were used for xenotransplantation of the infected CD34+ cells. This mouse strain represents immunodeficient mice and therefore they were kept in sterile isolator cages and were fed with autoclaved food and water only. Their treatment only took place under sterile conditions.

3.1.12 Cell lines

Ba/F3 cells

Ba/F3 cells are murine bone marrow-derived pro-B-cells, which normally grow IL-3 dependent (Palacios and Steinmetz, 1985). They are suspension cells and were cultured in RPMI medium with 10%(v/v) FCS (Biochrom) and 5%(v/v) BPV-mIL3, produced by a myeloma cell line (Karasuyama and Melchers, 1988).

The cells were passaged every 2-3 days in a ratio of 1:5 or 1:10. Two different Ba/F3 cell lines were used:

1. Ba/F3 →IL-3-dependent

2. Ba/F3 R1496 (containing CRLF2* vector)→ IL-3 independent

Bmi-Flt3 cells

Bmi-Flt3 cells represent murine, bone marrow derived lymphoblasts from FLT3-induced ALL, which possess a constitutive expression of the Flt3 receptor. These cells were cultured in α-MEM medium with 10%(v/v)FCS (Biochrom) .

HEK293T cells

293T cells belong to the HEK cells and represent „Human embryonic kidney cells". This cell lineage (ATCC #CRL-11268) is a transformation product of human embryonic kidney cells with parts of the DNA of the human adenovirus. 293T cells are adherent growing cells, which express the SV40 large antigen and therefore allow the replication of plasmids with SV40 origin of replication. They were used for virus production.
293T cells were cultured at a confluency of 70% with DMEM medium with 10%(v/v)FBS (Lonza). The cells were passaged every 2-3 days in a 1:5 or 1:10 ratio.

SC-1 cells

SC-1 cells present a cell lineage, which is derived from fetal mouse fibroblasts (ATCC #CRL-1404). They were cultured in DMEM with 10% FCS (Biochrom).

CD34 cells

CD34 cells were isolated from human cord blood by Ficoll gradient. These cells represent early progenitor and stem cells. They were cultured in SFEM medium (1%(v/v) P/S, 1%(v/v) glutamine, 1%(v/v) sodium pyruvate) and the cytokines rhFlt3(100ng/ml), rhSCF(100ng/ml), rhTPO(20ng/ml) and rhIL-6(20ng/ml).

3.2 Methods

3.2.1 Cell culture methods

3.2.1.1 Cultivation of different suspension cells over time

Suspension cells were cultivated in non-tissue-culture 6-well plates at 37°C and 5% CO_2. Depending on the cell density, the cells were passaged every 2-3 days at a ratio of 1:2 – 1:10, depending on the cell type.

3.2.1.2 Freezing of cells

The freezing medium consisted of 90%FCS plus 10%DMSO and has to be kept on ice all the time.

Cells were centrifuged for 5min, 1000rpm at 21°C. After centrifugation the supernatant was discarded. The cell pellet was then resuspended in the cold freezing medium (up to $5x10^6$ cells per ml medium). The cells were transferred into a cryotube. The vials were put in a freezing container (4°C) and were cooled down slowly in the -80°C freezer. The cells were stored in -190°C nitrogen after 24 hours.

3.2.1.3 Thawing of cells

The tubes containing the frozen cells were thawed in the 37°C water bath and were then taken up with a pasteur pipette and added drop wise to an adequate amount of corresponding medium to dilute the DMSO. The cells were then centrifuged 5min, 1000rpm at 21°C. The cell pellet was resuspended in 3ml of the corresponding medium and the cells were transferred into the well and cultivated at 37°C with 5%CO_2.

3.2.1.4 Transfection of 293T cells- production of infectious virus particles

On day one 293T cells had to be seeded in the afternoon at a density of $4x10^6$ cells per 9cm tissue culture dish in 10ml of the corresponding cell culture medium. On day two the cells were transfected, via the calcium phosphate method, with the viral constructs and the helper plasmids, encoding for *gag, pol* and *env.* Therefore, the plasmids were diluted in nuclease free water and 62,5µl 2M $CaCl_2$ solution per approach was added like the following:

Lentivirus:

- Vector plasmid 10µg/dish
- Gag/Pol plasmid: 10µg/dish
- Rev plasmid: 5µg/dish
- Envelope plasmid: 2µg/dish phCMV-VSV-G
  4µg/dish ecotropic Env
- $CaCl_2$: 62,5µl/dish
- Nuclease free water: ad 500µl

γ-Retrovirus:

- Vector plasmid 5µg/dish
- Gag/Pol plasmid: 10µg/dish
- Envelope plasmid: 3µg/dish ecotropic Env
- $CaCl_2$: 62,5µl/dish
- Nuclease free water: ad 500µl

After diluting the plasmids, the DNA/$CaCl_2$ mix was added drop wise to 500µl of HEPES buffer (2x), while producing air bubbles with the help of a pasteur pipette in the buffer. This solution was incubated for 20min at room temperature. The old medium was aspirated from

the 293T cells. 10ml of fresh medium were added to the cells as well as 25µM chloroquine per plate. The DNA mix was added drop wise to the cells, the plates were swirled gently and then incubated for 6 -12 hours at 37°C and 5%CO_2.
After the incubation time, the medium was changed to 8ml without chloroquine. Starting 12 hours later, the supernatant containing the virus particles was harvested every 12 hours. The harvest began on day three in the morning by taking up the whole supernatant with a syringe and by filtrating it through a 0,22µm Millex-GP sterile filter into 15ml tubes. Then 6ml of fresh medium were added to the cells and the viral supernatants were frozen at -70°C. This was repeated every 12 hours for 72 hours.

3.2.1.5 Titer determination of produced viruses via FACS analysis

On day one 5x10^4 293T cells or SC-1 cells, depending on the envelope protein of the virus, were seeded in 500µl medium per well in a 24-well plate. Polybrene was added to every well at a final concentration of 8µg/ml. The thawed viral supernatants were added to the cells in duplets, once 20µl and once 100µl. The plate was centrifuged one hour, 2000rpm at 24°C and was the incubated at 37°C overnight. On day two the medium was removed and 1ml of fresh medium was added per well.
On day four the medium was removed, the cells were detached with trypsin/EDTA, centrifuged for 5 min at 2000rpm and resolved in 1xPBS. The cells were examined via FACS analysis and the virus titer was calculated with the following formula:

Titer (gtu/ml) = number of seeded cells x proportion of transduced cells / volume of added supernatant

3.2.1.6 Infection of target cells

Infection via retronectin

To transfect the cells via retronectin, a 6 well plate was coated with 2ml retronectin (48µg/ml) and incubated for 2 hours at RT. Then the retronectin solution was discarded and stopping solution (PBS/2%(w/v) BSA) was added to the well and incubated for 30min at RT. The BSA solution was abolished and HBSS/2,5%(v/v) 1M Hepes was added to the well for neutralization, which was removed shortly before the virus application. Therefore, the HBSS/2,5%(v/v) 1M Hepes solution was discarded from the wells and 1,5ml of the virus supernatant (with ecotropic Env or fel env as envelope protein) was applied per well. Afterwards, the plate was centrifuged for 20min, 2000rpm at 4°C. After centrifugation 1ml of supernatant was discarded of each well and 1ml of fresh virus was added to each well and centrifugation of the plate was performed as before. This whole step was then repeated twice. After the fourth centrifugation the last virus supernatant was removed just before the addition of the cells. Finally, the target cells were transferred to the virus-coated wells and

incubated overnight at 37°C and 5%CO_2. On day two a new 6 well plate was coated with virus as the day before and after virus coating the cells, which were infected the day before, were transferred to the newly coated wells. The detachment of the cells was performed with the help of a spatula. On the following days the transduced cells could either be sorted and cultivated or transplanted into mice.

Infection via spinoculation and following cell sorting

$2x10^5$ cells were transferred to a new well of a 6-well plate. 1ml of the thawed virus supernatant was added directly to the cells as well as medium up to 3ml per well and polybrene at a concentration of 8µg/ml. The plate was centrifuged 1h, 2000rpm at room temperature. The transduction was repeated identically the next day. After the second infection the cells were cultivated for several days. The infected cells were sorted after 4-5 days using the flow cytometer.

To sort a specific cell population out of a mixed cell population the whole content of the well was centrifuged 5min, 1000rpm at 21°C. The supernatant was discarded and the pellet was resuspended in 1xPBS. The sorting was then performed via FACS. The cells were sorted into FACS tubes containing the corresponding medium as well as a broad-spectrum antibiotic, as for example Ciprobay, at a concentration of 1:125. After the sorting process the cells were centrifuged for 5min, 1000rpm at 21°C. The supernatant was discarded and the pellet was resuspended in the corresponding medium. The cell solution was then transferred into a well of a 12-well plate and Ciprobay was added at a concentration of 1:40. The sorted cells were cultured with the addition of Ciprobay for at least one week.

3.2.1.6 Puromycin selection

Several of the vectors, which were used in this study, contained a puromycin resistance gene. If the transduction rates were too low cells, which express this vector, could be enriched by puromycin selection.

The selection was performed by adding 1µg/ml of puromycin (stock 1µg/µl) to the cells, which were infected twice. Fresh puromycin was added to the cells every two days at a concentration of 1µg/ml. The infection rate of these cells was analyzed via flow cytometry four days after the puromycin selection. The preparation for the analysis was accomplished as before.

3.2.1.7 Competitions assay of Ba/F3 cells

To analyze the proliferation behavior of differently infected Ba/F3 cells a competitions assay can be performed *in vitro*. The cells, which have been infected with the target vector and have been puromycin-selected, were counted and the percentage of vector containing cells (=BFP positive cells) was analyzed via flow cytometer. Afterwards these cells were mixed

with uninfected cells of the same cell line. The target was to seed $5x10^5$ cells at the ratio of 70% infected cells to 30% of uninfected cells per well of a 12-well plate. The cells were mixed and then the cell ratio was confirmed on the flow cytometer (day 0). The change of the ratio of infected to uninfected cells was then analyzed every two to three days via flow cytometer using an aliquot of the cell suspension. Furthermore, the cells were passaged 1:5 every two days.

3.2.2 Mouse experiments

3.2.2.1 Enrichment of human and murine primary cells

Primary cells of murine bone marrow and human cord blood were cultured as mentioned above in non-tissue-culture plates.

3.2.2.1.1 Isolation of murine bone marrow

The previously narcotized animals were sacrificed via cervical dislocation. The bone marrow was isolated from the murine tibia and femur. The whole isolation was performed at the sterile bench with a 1ml syringe and cannula. The bones were cut at one end and by perforating the other end the bone marrow was flushed into a falcon tube, containing IMDM medium with 5%FCS, 1%Pen/Strep and 2mM glutamine. The cells were washed once with IMDM. After washing, the cell pellet was resuspended in an adequate volume of lysis buffer, so that the erythrocytes would burst due to the osmotic pressure. After centrifugation of 5min at 1200rpm, the cell pellet was resuspended in murine SFEM medium (4mM glutamine; 1mM sodium pyruvate; 1%(v/v) Penicillin/streptomycin) containing the corresponding cytokines (1:1000) ($5x10^5$-$1x10^6$ cells per ml).

3.2.2.1.2 Lineage depletion of murine bone marrow cells

Stem cells as well as early progenitor cells represent the target cells for infection and subsequent analysis and transplantation. For this purpose they have to be isolated and enriched out of the whole murine bone marrow or human cord blood. There are two different ways, which were performed during this thesis.

<u>Lineage depletion by 5-FU</u>

One way of lineage depletion is 5-FU treatment. 5-FU acts toxically against strongly proliferating cells and therefore leads to the enrichment of early progenitor cells and HSCs in the haematopoietic system. Due to experienced data it is known that the isolation of bone marrow cells three days after 5-FU treatment leads to the enrichment of myeloid progenitors. Enrichment of B-cell progenitors needs more time. Consequently, our data indicates that the isolation of bone marrow five days after 5-FU treatment resulted in a population with lots of

B-cell progenitors. The mice were treated with 150µg 5-FU per g body weight in a volume of 10µl 1xPBS per g body weight via intraperitoneal injection.

Lineage depletion by MACS columns

Another form to isolate the HSCs and early progenitors out of the bone marrow is the depletion of lineage$^+$ cells with MACS column. For this purpose the cells were counted after the bone marrow isolation and then centrifuged for 10min, 1200rpm at room temperature. The pellet was resuspended in 400µl of PBS/0,5%FCS per 1x10^8 cells and mixed. Thereupon 100µl per 1x10^8 cells of Biotin-antibody cocktail was applied to the cells to stain the lineage positive cells. The cells were mixed and incubated on ice for 10min. To dilute unbound antibodies, 300µl per 1x10^8 cells of cold PBS/0,5%(v/v) FCS were added to the cells as well as 200µl per 1x10^8 cells of anti-biotin microbeads (secondary antibody) so that the magnetically labeled cells are held in suspension within the column. The cells were mixed again and then incubated for 15min on ice. After that the cells were washed twice with IMDM/5%FCS medium, were centrifuged for 5min and 1200rpm. The cell pellet was resuspended in IMDM medium/5%FCS (2ml per 1x10^8 cells).

Then the LD column within the magnet had to be equilibrated with IMDM (5%FCS, 1%P/S, 1% glutamine) via the 30µm filter on top of the column. The tubes with this flow-through were abolished and new 15ml tubes were positioned under the columns. 2ml of the labeled cell suspension were applied to the column via the filter. Each column was washed twice by applying 1ml of medium to the column. The flow-through consisted of the lineage negative, unlabeled fraction of the bone marrow cells. The tubes were then centrifuged for 5min and 1000rpm. The pellet was resuspended in 3ml of SFEM medium (1%(v/v) P/S, 2mM glutamine, 1mM sodium pyruvate, rmSCF (100ng/ml), rhFlt3 (100ng/ml), rhIL-11 (100ng/ml) and rmIL-3 (10ng/ml)). The cells were transferred to one 6-well and Ciprobay (1:125) was added.

3.2.2.2 Infection of the murine bone marrow cells

To guarantee an efficient infection with the virus, infection via retronectin was performed as described above. The cells were infected on day one and two after bone marrow isolation. On day four an aliquot of the cells was taken to analyze the infection rate of the cells, whereas the rest of the cells was transplanted, as described in 3.2.2.5.

3.2.2.3 Infection of the CD34+ cells

The cells were thawed 24 hours before the first infection. 1,8x10^6 cells were cultured per well of a 6-well plate in 3ml of SFEM medium (1%(v/v) P/S, 2mM glutamine, 1mM sodium pyruvate) with the corresponding cytokines. The infection with the virus was performed twice via a combination of retronectin-depending (fel env) infection and spinoculation (VSV-G).

3.2.2.4 Colony formation assay of infected bone marrow cells

A methylcellulose assay was used to analyze the proliferation behavior and growth advantage of differently infected and non-infected bone marrow cells. This semisolid medium was used due to the fact that haematopoietic progenitors are able to proliferate and form colonies in this medium as a response to cytokine stimulation. Per approach 3,3ml of mouse methylcellulose (M3231) were thawed. Then $5x10^4$-$2x10^5$ cells/ml were added to the methylcellulose as wells as Pen/Strep (33U), 1:1000 of rmIL7 (20ng/ml) and if necessary IMDM up to a volume of 330µl. An aliquot of the original cells was used for FACS analysis on day zero. After mixing gently to avoid air bubble formation, the methylcellulose approach was performed in triplets on 35mm plates. The colony assays were incubated at 37°C and 5%CO_2. After one week the colonies/cells were analyzed. For this purpose colonies were counted via microscopy and one plate of each approach was analyzed via FACS. The methylcellulose containing the cells was detached by adding 1xPBS to the plates, resuspending, pooling the cells and washing the plates. Then the cells were centrifuged 5min, 1200rpm and the pellet was resuspended in 1xPBS. The cells were then analyzed via flow cytometry directly or they were first stained and then analyzed. The remaining colonies were replated again by seeding the same cell density after detachment, washing, and counting of the cells into new methylcellulose. FACS analysis as well as replating was performed again at day 13. The final analysis of the cells without replating was performed four weeks after the experimental start.

3.2.2.5 Allotransplantation

If the bone marrow cells should be transplanted, they were counted on day three after the bone marrow isolation to estimate the cell number and consequently the number of mice that were going to be transplanted. The recipient mice (129 or C57BL/6J Ly5.1) were irradiated lethally with a dose of 9 Gray (=25min for C57BL/6J Ly5.1 mice) or 7,6 Gray (=21min for 129 mice) of γ-rays. The prepared, infected cells together with fresh spleen support cells were i.v. -injected at the earliest 2 hours after irradiation and not later than 24 hours after irradiation. About $1x10^6$ cells were transplanted per mouse. After transplantation the mice were treated with Baytril ® 2,5% at a concentration of 400µl/100ml drinking water for three weeks. Induction via tamoxifen was initiated seven weeks post transplantation.

Due to lethal irradiation the mice loose the bigger part of haematopoietic cells and therefore need haematopoietic support cells, mainly erythrocytes, to stay alive until the transplanted early progenitor cells engraft and produce new cells. The spleen of a syngeneic mouse was isolated, because this organ possesses percentaged only a few stem cells but many erythrocytes. The spleen cells were transferred into a small tissue culture plate. IMDM (5%(v/v) FCS; 1%(v/v) P/S; 1%(v/v) glutamine) was added to the spleen, which was crushed

with the backside of a syringe. The solution was collected with a 20ml syringe and transferred through a FACS filter into a sterile FACS tube to count the cells. $3x10^4$ splenic support cells were mixed with the prepared, infected cells and the mixture was transplanted.

3.2.2.6 Xenotransplantation

Infected human CD34+ cells, as described in 3.2.2.3, were transplanted into immunodeficient NOD/SCID/γ_c^{null} mice that show a functional incompetence of B-, T- and NK-cells to avoid a rejection of the human cells. The irradiation and transplantation of the NOD/SCID/γ_c^{null} recipient mice was performed as described for the allotransplantation, but just with a dose of 1,6 Gray(=4,5min).

3.2.2.7 Analysis of sick animals

If an animal showed serious disease symptoms according to the guidelines on the care and use of animals for scientific purposes, they were sacrificed and analyzed.

Analysis of the organs

Peripheral blood was taken and analyzed via Hemavet and FACS analysis.
After taking blood, the organs of the sacrificed animal were analyzed. For this purpose the spleen was weighted, parts of the spleen and liver were fixed in formaldehyde. The fixation of the sternum was performed in CALfix. The splenic cells and bone marrow cells were also analyzed via FACS.

Preparation of spleen, peripheral blood and bone marrow for analysis via flow cytometry

The spleen was processed to a single cell suspension by pressing the spleen through a 160µm filter into 1xPBS. The isolation of the bone marrow was performed as it was described for the gain of primary cells and was resuspended in 1xPBS. The spleen and blood were erylysed by adding 1xlyse buffer and after centrifugation the cells were resuspended in 1xPBS. Afterwards the cells could be analyzed directly via flow cytometry or they could be stained for 20min at 4°C with antibodies (amount according to manufacturer instruction) that are coupled to different fluorochromes. After staining, the cells were washed once with 1xPBS and after centrifugation, they were resuspended in 1xPBS and were then analyzed via flow cytometry.

3.2.3 Molecular methods

3.2.3.1 Standard methods

Agarose gel electrophoresis, ethanol precipitation, phenol/chloroform extraction and heat shock transformation were performed according to standard protocols. The isolation and

purification of plasmid DNA out of bacteria culture and agarose gels were performed with the help of the mentioned kits (3.1.3) and according to manufacturer's instructions.

3.2.3.2 Protein biochemical methods

Whole cell lysate and protein extraction

To perform cells lysis and isolate the proteins out of the cells, $1x10^7$ cells were first washed with cold 1xPBS. The cells were centrifuged for 5min, 1000rpm at 4°C and put on ice afterwards. The supernatant was discarded and the cells were resuspended in 200µl of cold lysis buffer II (50mM Tris(pH=7,4), 100mM NaCl, 10% glycerol, 0,1%Triton X-100, 1mM DTT, 1mM EDTA). The cells were then incubated on ice for 0,5 to one hour, while vortexing the cells every 10min. Additionally, a sonificator was used for 30 sec to finally break open the cells. To remove the cell debris the samples were finally centrifuged for 10min, 13000rpm at 4°C and the supernatant was transferred into an Eppendorf tube. The protein concentration was measured via Nanodrop. The protein extracts were stored at -80°C.

SDS-PAGE and Western blot

To analyze the proteins, which were gained from the whole cell lysates, electrophoretic separation via SDS-PAGE was performed. The proteins were first denaturated by exposing the samples for 5min to 95°C. Afterwards the samples were centrifuged 1min, 12.000rpm at room temperature. 20-80µg of the supernatant of each protein sample were applied to a 10% SDS gel (3.1.5.2) to separate the proteins according to their molecular weight. The stacking gel was run at 80V and the separation gel at 120V. The membrane was activated for 1min in 100% methanol at room temperature. Afterwards it was transferred into dH2O for 2min. The membrane was then incubated for 5min in 1x Towbin transfer buffer (350mM Tris, 192mM glycine, 20% methanol, pH 8,3). To visualize the target proteins with the help of specific antibodies the transfer of the proteins from the gel to the membrane was performed via tankblot at 100V with ice cooling for 1 hour. After blotting, the membrane was incubated in blocking solution (5% skimmed milk powder; 0,1% Tween 20 in PBS) for one hour at room temperature to prevent unspecific binding of the antibodies to the membrane. Then the membrane was incubated with the first antibody at the corresponding concentration (3.1.7.1) overnight at 4°C. On the following day the membrane was washed three times for 10min with washing buffer (1xPBS; 0,1%Tween 20) to remove unbound antibodies. After washing, the membrane was incubated with the peroxidase associated secondary antibody (3.1.7.1) while being shaken. After two hours, washing was performed as before three times to remove unbound secondary antibody.

ECL reaction

The Amershan ECL-Detection™plus kit (GE-Healthcare, Munich) was used according to manufacturer's instructions to visualize the proteins. For this purpose 200µl of the ECL-solution were added to the protein side of the membrane. Then the X-ray film was applied to the membrane and was developed after several seconds up to half an hour, depending on the antibody. The blackening of the film on the side where the secondary antibody has bound happens due to the fact that the HRP catalyzes the reaction H_2O_2 with luminole to an oxide of luminole, which leads to a chemiluminescence reaction.

For further use of the membrane, it could be washed again three times with washing buffer and then be blocked again for one hour with blocking solution and then the primary antibody could be applied again. To completely remove all antibodies, the membrane could be washed three times and then incubated in 50ml of ECL stripping buffer, (350µl β-mercaptoethanol added) for 15min at 50°C. After that the membrane should be washed several times with washing buffer and could then be blocked and incubated with the primary antibody again.

3.2.3.3 Nucleic acid analytical methods

DNA isolation (Maxi-, midi-, miniprep)

The DNA isolation out of bacteria culture was performed by using the Maxi-, Midi- and Miniprep Kit (Qiagen) and were performed according to manufacturer's instructions. The resulting DNA concentration was measured using the Nanodrop. To test whether the isolated DNA correlated with the target DNA a test restriction was performed.

The restriction approach was incubated at 37°C for 1 hour. After that, the restriction was applied to a 1% agarose gel und the resulting bands were compared with the expected ones.

RNA isolation

1-2x10^6 cells were used for RNA isolation and the RNA was isolated according to the protocol of the RNeasy Mini Kit of Qiagen (Hilden). The RNA was diluted in 25µl of RNase-free water. Then the RNA concentration was determined via Nanodrop.

cDNA synthesis

The previously isolated RNA was used for cDNA synthesis. The AMV Reverse Transcriptase (Fermentas, ST. Leon-Rot) was used for reverse transcription. Additionally, random primer (Roche, Mannheim) were used, so that the whole mRNA could be transcribed into cDNA. About 1µg of RNA template was used per reaction and each reaction contained the following: 1µg RNA, 5x AMV buffer (Fermentas, St. Leon-Rot), 1mM dNTP's (Qiagen, Hilden), 1µl random primer, 40U rRnasin Plus® RNase inhibitor (Promega , Mannheim) and 10U AMV reverse transcriptase. If needed, the end volume of 20µl was filled up with H_2O.

The reaction was performed by incubating the samples for 10min at 25°C and then for 60min at 50°C to heat-inactivate the enzymes.

Quantitative Real-time PCR

The Light Cycler® FastStart DNA Master SYBR Green 1 kit of Roche was used for quantitative Real-time PCR. With the help of this method it is possible to quantify the amount of transcripts of different genes in a cDNA sample. Here the fluorescence marker SYBR Green I interacts with double stranded DNA, whereas the fluorescence signal increases with the amount of DNA. 0,5µl of cDNA was used per 20µl approach as well as 5pmol of the *forward* and *reverse* primer. The same PCR program was chosen for all qRT-PCRs, whereas elongation time and annealing temperature varied due to the different primer properties (3.1.8). The PCR cycle consisted of an initial heating step for 10min at 95°C, followed by a cycle of denaturation (10sec, 95°C), the annealing (10sec, x°C) and then the elongation (x sec, 72°C). This cycle was repeated 40 times.

The analysis of the quantitative Real-time PCR was performed using the ΔΔCt-method, which was established by Pfaffl (Pfaffl, 2001). The measurement of the transcription of the *HPRT* gene, as reference gene, was used in all cDNA samples for normalization. The mRNA expression in control cells was set as 1 and consequently the mRNA expression of the target cells was calculated compared to the control cells.

To check the PCR product, a melting point analysis was performed after each PCR reaction. For this purpose the temperature in the reaction capillary was increased slowly so that the double stranded DNA denaturates at a melting point, which is specific for the PCR product. This leads to the release of the whole fluorescence marker and a significant decrease in fluorescence. The resulting melting curve should be comparable in all cDNA samples.

The PCR conditions for the different primer pairs are listed in 3.1.8. Positive controls as well as water control were used for the primer pairs.

After real-time PCR all PCR products were checked on a 1,5% agarose gel to see whether there is only one PCR product with the right size.

Cloning of the LNK vector

The existing vector LeGO-Puro+ was cleaved with NotI. The whole restriction approach was applied to a 0,8% agarose gel. The upper band, which presented the cleaved vector, was cut out of the gel. The gel fragment was applied to a column and by centrifugation for 5min, 5000rpm at room temperature the DNA was eluted from the gel. The DNA in the flow-through was religated. After religation, X_{10}Gold were transformed via heat shock procedure. A miniprep of each of the overnight cultures of ten clones was performed and a test restriction of the isolated DNA was performed as usual with XbaI and BamHI. After the testrestriction,

the approach was zipped to prevent religation of the vector. The restricted and zipped plasmid DNA was applied to a 1% agarose gel. The resulting band of a positive clone was cut out of the gel and a gel elution was performed as before. A phenol-chloroform precipitation of the restricted and zipped vector was performed afterwards.

The existing vector R1507 (human, wt *LNK*) was cleaved with BamHI. The restriction approach was applied to a 1% agarose gel. The lower of the two bands, the *LNK* fragment, was cut out of the gel and the DNA was eluted according to manufacturer's instructions.

The ligation of the *LNK* fragment into the LeGO-iB2 vector was performed according to manufacturer's instructions with 5U T4 DNA ligase, 16µl LeGO-iB2 vector and 10µl *LNK* fragment. After transformation, the existing colonies were checked for the right orientation of the *LNK* fragment by cleaving the gained DNA with convenient restriction enzymes (NotI, BamHI, HindIII).

4 Results

4.1 Modeling B-ALL

4.1.1 Modeling B-ALL in the human system with Jak2* plus IKZFΔ6-ERt2

It is known that the mutation of the tyrosine kinase JAK2, together with aberrantly expressed transcription factors, like IKAROS, is often found in diseases such as high-risk B-ALL. Consequently, we wanted to optimize a mouse model system to analyze the effect of Jak2 mutation occurring together with the dominant negative mutant IKAROS (IKZFΔ6) on normal haematopoiesis *in vivo,* using human CD34+ cells, which after transduction would be transplanted into immune-deficient mice and monitored for clinical symptoms of disease. Retroviral vectors were used to introduce the *JAK2-R682G* mutant and an inducible IKZFΔ6-ERt2 fusion protein, in which IKZFΔ6 is fused to a tamoxifen-inducible estrogen receptor. In the absence of tamoxifen, the IKZFΔ6 mutant remains in the cytoplasm, and only moves to the nucleus to inhibit Ikzf1 function when tamoxifen binds to the ERt2 component. An inducible approach was used because it was not clear, whether lymphoid development would be completely blocked if the dominant-negative form of IKAROS was expressed in HSC.

Previous experiments in our lab had shown that retroviral-transduced human CD34+ cells, which represent pluripotent HSC, do not have or have lost long-term engraftment capacity in immunodeficient NSG mice. This is probably due to the fact the culture conditions used to efficiently infect/transduce human CD34+ cells promoted their differentiation at expense of their self-renewal capacity. Alternatively, long-term repopulating cells may have not been infected, as they make up a small percentage of CD34+ cells. Consequently it was the goal of this experiment to compare different approaches for cell isolation and to test the efficiency of retroviral infection and engraftement in NSG mice. In the first approach, CD34+ cells obtained by magnetic separation were subjected to further purification by sorting a CD38+ and CD38- population. Whereas in the second approach, CD34+ cells were not purified further. The experimental approach is depicted in Figure 13.

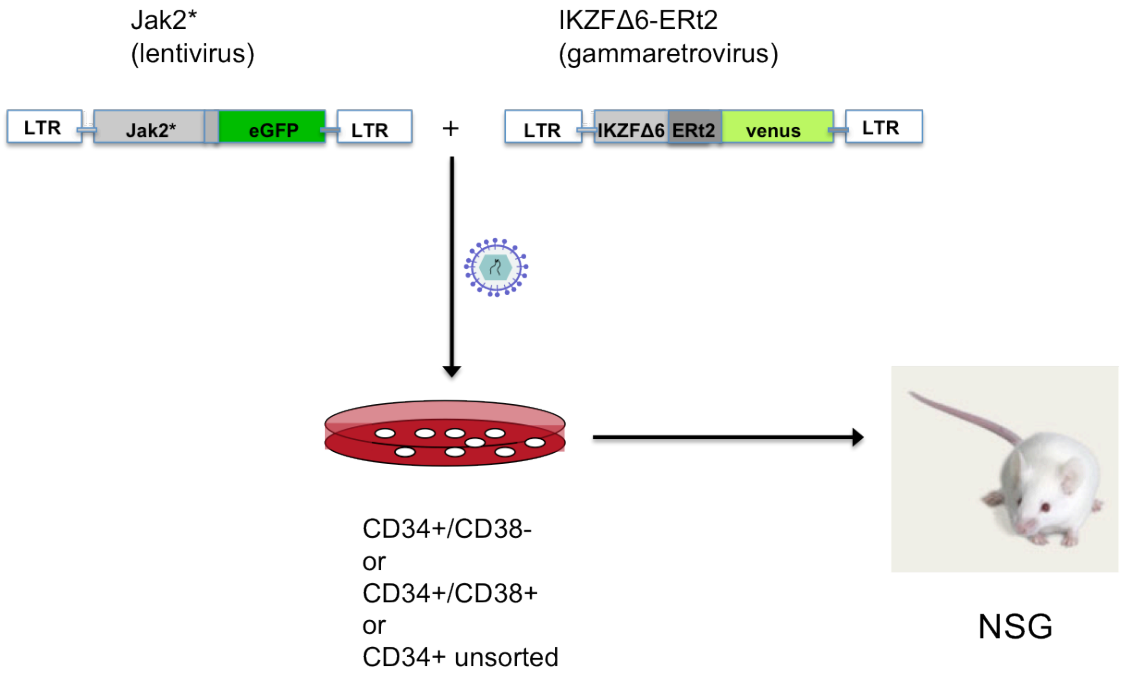

Figure 13 Modeling B-ALL in the human system with Jak2* and IKZFΔ6-ERt2
Three different approaches of human CD34+ were infected with the lentiviral Jak2* and the gammaretroviral IKZFΔ6-ERt2 vectors. The transduced cells were finally transplanted into NSG mice.

In the first approach $3x10^7$ CD34+ cells were thawed and stained with hCD34 and hCD38 for sorting. Consequently, the cells were sorted into two different populations: CD34+/CD38- cells, which represent the earlier progenitor population and CD34+/CD38+ cells, which represent the later progenitor population. Sorting resulted in $5,5x10^5$ CD34+/CD38+ cells and $3,2x10^5$ CD34+/CD38- cells, which were infected on day zero and one with a concentrated Jak2* (lentiviral vector/VSV-G pseudotyped), concentrated eGFP control (lentiviral vector/VSV-G pseudotyped) and IKZFΔ6-ERt2 (gammaretroviral vector/RD114 Env pseudotype) vectors.

In the second approach $1,6x10^7$ CD34+ cells were thawed, not sorted and cultivated overnight to avoid stressing the cells. They were infected on day one and two instead of day zero with the same pseudotyped vectors.

Figure 14 visualizes the FACS analysis of the sorted and infected CD34+/CD38+, which shows that 27% of the cells were infected with the gammaretroviral IKZFΔ6-ERt2, but they did not show lentiviral Jak2* expression at all. The infected CD34+CD38- cells were not analyzed via FACS analysis, because due to the low cell number all cells were transplanted. The unsorted CD34+ cells showed 60% of IKZFΔ6-ERt2 expression and no Jak2* expression again.

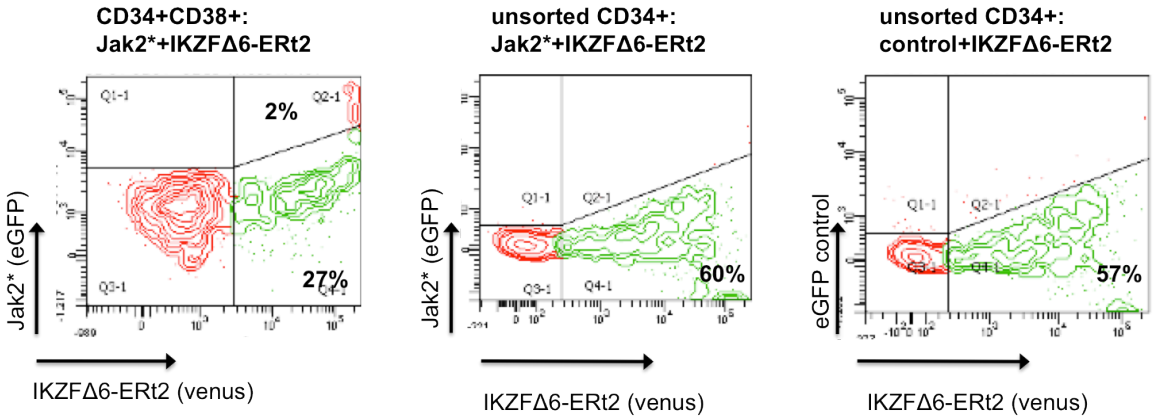

Figure 14 FACS analysis of the CD34+/CD38+ and unsorted CD34+ cells, which were infected with Jak2* plus IKZFΔ6-ERt2 and control vector plus IKZFΔ6-ERt2.
The CD34+CD38+ cells and unsorted CD34+ cells, which were infected twice, were analyzed via flow cytometry using the fluorochromes eGFP for Jak2*/control vector and venus for IKZFΔ6-ERt2. The cells were analyzed right before transplantation into NSG mice.

Looking at the corresponding MOI's and infection rates, as visualized in Chart 1, it can be said that the unsorted cells showed better infection frequencies with IKZFΔ6-ERt2, despite lower initial MOI. Three times higher MOI of IKZFΔ6-ERt2 in the sorted cells resulted in about half the infection frequency compared to the unsorted approach. The high MOI of the lentiviral control vector (30,8) and the following infection frequency of 0% might be the result of technical problems and the fact that the titers were tested on mouse fibroblasts.
In conclusion it can be said that the infection of CD34+ cells with gammaretroviral vectors works more efficiently then with lentiviral vectors especially at lower MOIs.

	CD34+CD38+		**CD34+CD38-**		**unsorted CD34+**	
	MOI	infection	MOI	infection	MOI	infection
Jak2*+IKZFΔ6-ERt2						
DP		2%		N.D.		0%
Jak2*	6,2	0%	10,6	N.D.	2,1	0%
IKZFΔ6-ERt2	19,6	27%	33,8	N.D.	6,4	60%
contr.+IKZFΔ6-ERt2						
DP				N.D.		0%
control	no infection		0,7	N.D.	30,8	0%
IKZFΔ6-ERt2			33,8	N.D.	6,4	57%

Chart 1 MOI during infection of the CD34+ cells.
Three different CD34+ cell populations were infected twice with the IKZFΔ6-ERt2, Jak2* and control vector at different MOI's. This resulted in different infection frequencies. DP stands for "double positive" and indicates the percentage of Jak2*/control+IKZFΔ6-ERt2 infected cells.

The infected cells, which did not proliferate and were low in number, were transplanted into immunodeficient NSG mice that were irradiated for 4,5 min (=1,6 Gray) on day three after thawing, as shown in Chart 2.

Cells	no. of transplanted mice	no. of transplanted cells/mouse
CD34+CD38-: Jak2*+IKZFΔ6-ERt2	2	$4,3x10^4$
CD34+CD38-: control+IKZFΔ6-ERt2	2	$4,3x10^4$
CD34+CD38+: Jak2*+IKZFΔ6-ERt2	8	$6x10^4$
CD34+CD38+: uninfected	4	$3x10^4$
unsorted CD34+: Jak2*+IKZFΔ6-ERt2	2	$1,2x10^6$
unsorted CD34+: control+IKZFΔ6-ERt2	3	$1,3x10^6$

Chart 2 Transplantation of the different CD34+ cell approaches.
The different CD34+ cell populations were transplanted into two to 8 mice, transplanting between $4,3x10^4$ and $1,3x10^6$ cells. The colors indicate the belonging to the respective approach.

Peripheral blood of these mice was taken and analyzed six weeks post transplantation. The blood was stained with hCD45 and mCD45 to determine the percentage of human cells in these mice. Furthermore the blood was analyzed to determine the percentage of human cells transduced with Jak2* and IKZFΔ6-ERt2 vectors.

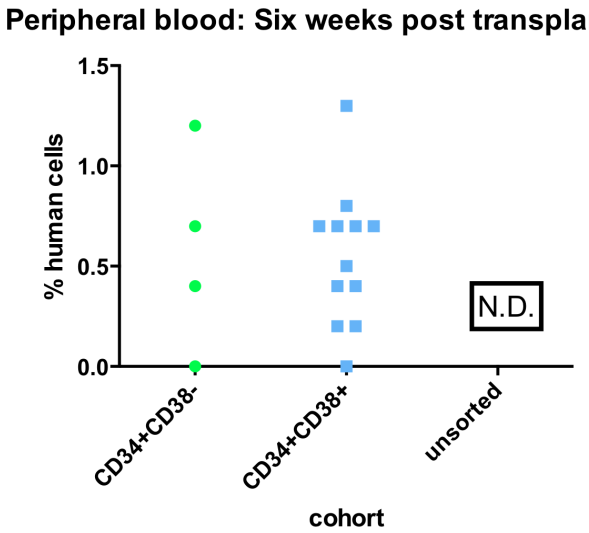

Figure 15 Analysis of the peripheral blood of the NSG mice six weeks post transplantation.
The PB of the NSG mice, which were transplanted with Jak2* and IKZFΔ6-ERt2 infected CD34+ cells (sorted and not sorted) was stained and analyzed via flow cytometry. The cells were stained for human CD45 as well as for mouse CD45 to be able to distinguish between the infected CD34+ cells and the mouse cells. Furthermore the cells were checked for the percentage of transduced cells with Jak2* and IKZFΔ6-ERt2, using eGFP and venus as fluorochromes. The figure shows the percentages of human cells in the peripheral blood of the mice. PB of the mice, which were transplanted with unsorted CD34+ cells, was not analyzed.

Looking at Figure 15, it is visible that all mice showed very low percentages of human cells in the blood of between 0% and 1,3%. It is known that it is uncommon to see high percentages of human cells in the blood after transplantation, but in this experiment the low percentages of human cells additionally did not show any transduction with Jak2* and IKZFΔ6-ERt2. As consequence it was decided not to start tamoxifen induction. Final analysis of ten of these mice was performed five-months post transplantation. The engraftment of the human cells in the spleen and bone marrow of the mice from different cohorts is shown in Figure 16.

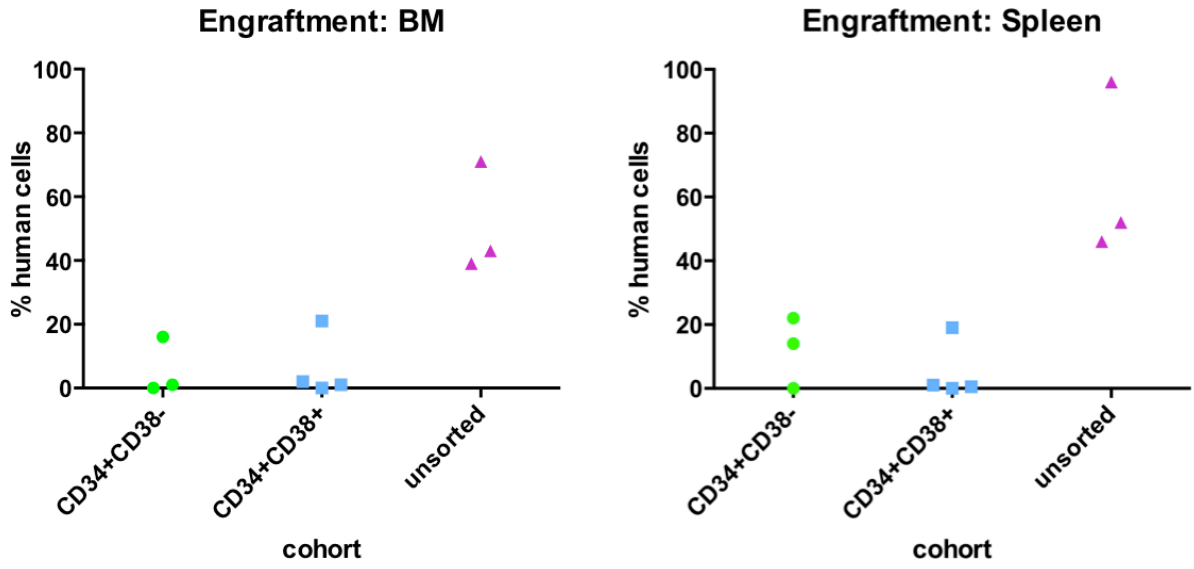

Figure 16 Final analysis of NSG mice.
Ten NSG mice, which were transplanted with Jak2*+IKZFΔ6-ERt2 or Jak2*+control vector infected CD34+/CD38-, CD34+/CD38+ and accordingly unsorted CD34+ cells, were sacrificed for final analysis five month post transplantation. The bone marrow and spleen were isolated and the cells were analyzed via flow cytometry, to analyze the percentage of human and infected human cells in the spleen and bone marrow.

It is clearly visible that all mice showed higher percentages of human cells in the bone marrow and even higher in the spleen compared to the analysis of the peripheral blood six weeks post transplantation. The highest percentage of human cells was found in the mice that were transplanted with transduced, unsorted CD34+ cells. The sorted CD34+CD38- and CD34+CD38+ cohorts showed comparable engraftment.

The percentage of transduced human cells in the different cohorts, visualized in Figure 17, was very low in the sorted CD34+CD38- and CD34+CD38+ cohorts. All mice that were transplanted with unsorted CD34+ cells, in contrast, showed infection of human cells with either Jak2* or IKZFΔ6-ERt2 in the spleen and bone marrow. The percentage of Jak2* vector transduced cells is much higher in the bone marrow then in the spleen. Despite the fact that Jak2* transduction of human cells was not visible in any of the mice before, one mouse even showed 25,5% of Jak2* transduction in the BM, as shown in Figure 18. This indicates that Jak2* might have an advantage for the infected cells. The higher infection frequencies of the

human CD34+ cells with IKZFΔ6-ERt2 was not reflected in the transplanted mice, and most mice analyzed had no or very few transduced cells after 5 months.

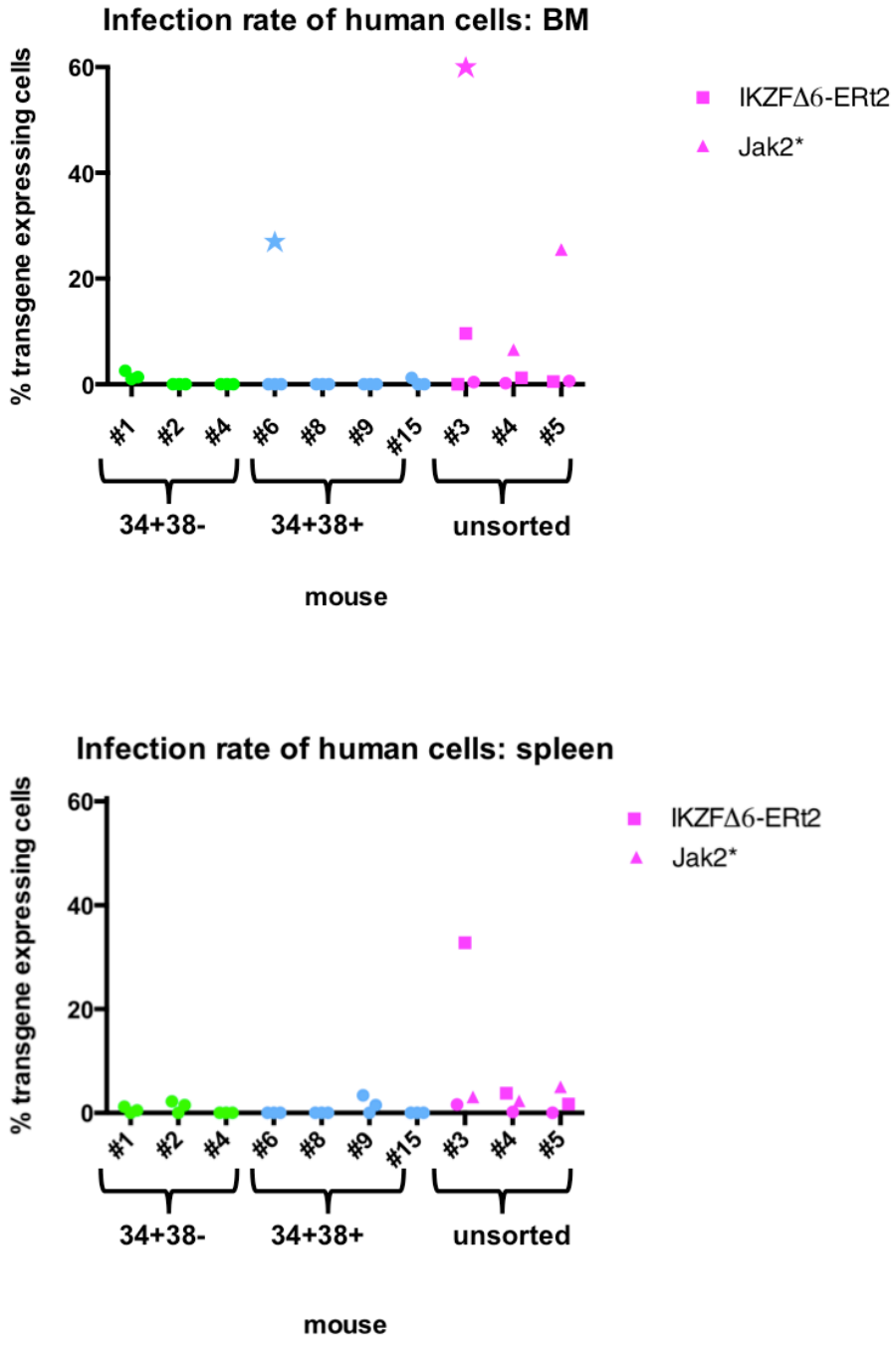

Figure 17 Transduced human cells in BM and spleen.
The human cells in spleen and bone marrow were analyzed for Jak2* and IKZFΔ6-ERt2 expression. Each column represents the human cells of one mouse. Each dot indicates the percentage of Jak2*, IKZFΔ6-ERt2 or Jak2*+IKZF-ERt2 expression of these cells. Same color indicates the same cohort. The triangle indicates Jak2* expression, whereas the square indicates IKZFΔ6-ERt2 expression. The stars indicate the original infection frequency of the bone marrow

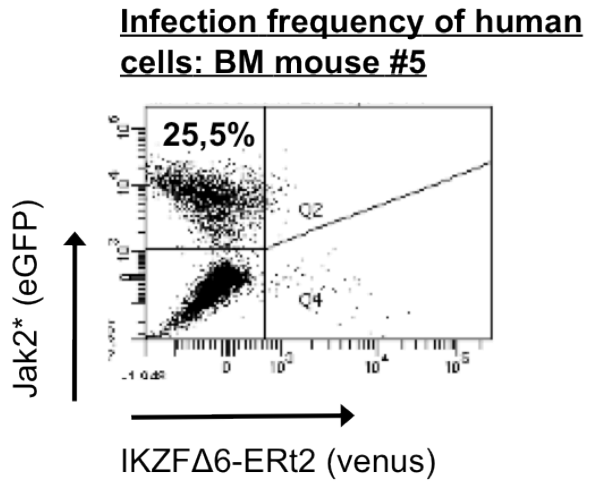

Figure 18 Percentage of JAK2*-transduced human cells in the bone marrow of mouse #5.
FACS analysis of the BM cells of mouse #5 that was transplanted with infected, unsorted CD34+ cells shows 25,5% Jak2* transduced human cells. eGFP stands for Jak2* and venus for IKZFΔ6-ERt2.

4.1.2 Modeling B-ALL in the mouse system with Jak2* plus IKZFΔ6-ERt2

Due to the fact that the human CD34+ could not be infected with Jak2* efficiently (due to low titers/MOIs and/or an initial negative effect of Jak2* on the cells) and IKZFΔ6-ERt2-infected cells were lost in the mice (no long-term engraftment), it was decided to examine the effects of mutant Jak2 together with the dominant negative IKZFΔ6-ERt2 in mouse cells as depicted in Figure 19.

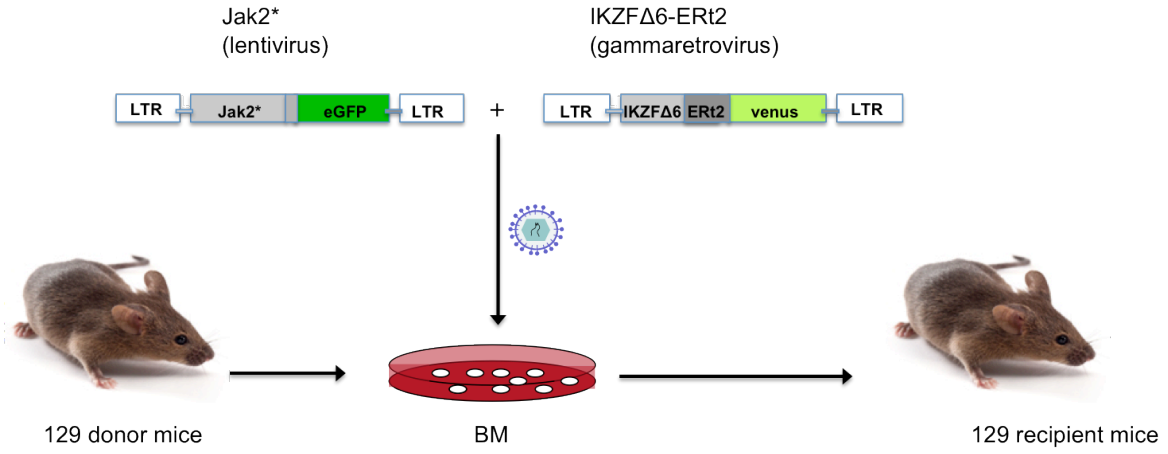

Figure 19 Modeling B-ALL in the mouse system with Jak2* and IKZFΔ6-ERt2
BM cell were isolated from 129 mice and transduced with the lentiviral Jak2* and the gammaretroviral IKZFΔ6-ERt2 vector. The transduced cells were finally transplanted into 129 mice.

Consequently, the bone marrow of five 129 mice was isolated and the lineage negative progenitor cells, representing stem cells and early progenitor cells, were enriched using *MACS columns*. The resulting $9x10^5$ cells were infected on day three after BM isolation.

The FACS analysis, shown in Figure 20, clearly shows that the BM cells of the 129 mice, which were infected with Jak2* and IKZFΔ6-ERt2, only showed IKZFΔ6-ERt2 infection and no Jak2* infection at all. The 129 BM cells only infected with the control vector showed infection. The BM cells infected with IKZFΔ6-ERt2 and control vector showed double positive cells, control vector infected and IKZFΔ6-ERt2 infected cells.

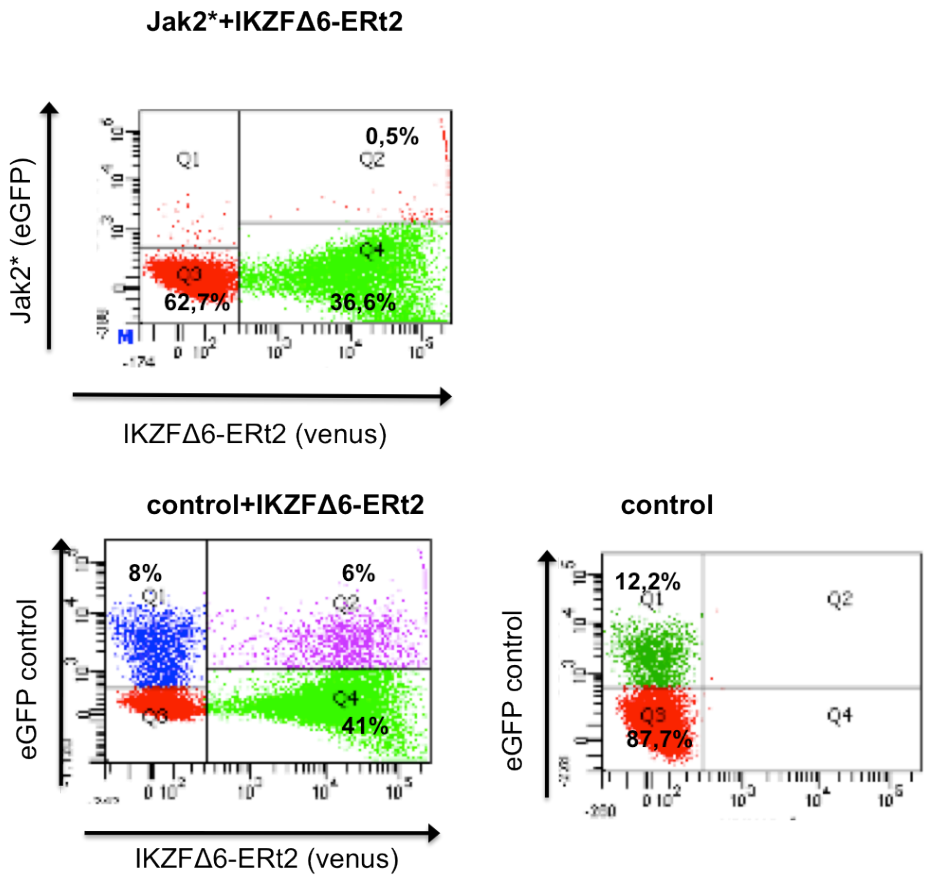

Figure 20 Analysis of the infected bone marrow on the day of transplantation.
The bone marrow, which was isolated from 129 mice, was infected twice with the Jak2*/control vector and IKZFΔ6-ERt2 vector. Analysis of the infected cells was performed on the day of transplantation using flow cytometry. The fluorochrome eGFP stands for the Jak2* and control vector, whereas the venus fluorochrome stands for the IKZFΔ6-ERt2 vector.

The corresponding MOI's and infection frequencies of the cells are presented in Chart 3. The MOIs of the lentiviral Jak2* vector and the gammaretroviral IKZFΔ6-ERt2 vector were comparable, but the infection frequency with the IKZFΔ6-ERt2 vector was significantly higher (36,6%) in comparison to the Jak2* infection frequency (0,5%). Double infection with Jak2*+IKZFΔ6-ERt2 vectors was not possible. The lentiviral control vector showed a five times higher MOI in comparison to the gammaretroviral IKZFΔ6-ERt2 vector, but its infection frequency (8%) was lower by a factor of five compared to the IKZFΔ6-ERt2 infection frequency (41%). Only 6% of the cells were double-infected with the control+IKZFΔ6-ERt2 vector. In conclusion it can be said that the percentage of IKZFΔ6-ERt2 transduced mouse

cells was comparable to the percentage of transduced CD34+ cells, but the problem of the infection with the lentiviral Jak2* vector was still present.

vector	MOI	infection frequency
Jak2*+IKZFΔ6-ERt2		
DP		0%
Jak2*	0,3	0,5%
IKZFΔ6-ERt2	0,42	36,6%
control+IKZFΔ6-ERt2		
DP		6%
control	2,26	8%
IKZFΔ6-ERt2	0,42	41%
control		
control	2,26	12,2%

Chart 3 MOI and infection frequency of the 129 murine bone marrow cells.
The bone marrow cells of the 129 mice were infected twice with the IKZFΔ6-ERt2 plus Jak2* and accordingly control vector. The infection frequency of the cells was determined via FACS analysis on the day of transplantation. DP stands for "double positive" and indicates the percentage of Jak2*/control+IKZFΔ6-ERt2 infected cells.

The infected cells were transplanted into 129 mice, which were lethally irradiated for 21 min (=7,6 Gray) on day five after bone marrow isolation, as shown in Chart 4.

vector	no. of transplanted mice	no. of transplanted cells/mouse
Jak2*+IKZFΔ6-ERt2	9 (129 mice)	$1{,}36 \times 10^6$

Chart 4 Transplantation of the infected murine bone marrow.
The infected bone marrow cells were transplanted into nine mice, transplanting $1{,}36 \times 10^6$ cells per mouse.

Peripheral blood was taken six and 15 weeks post transplantation.

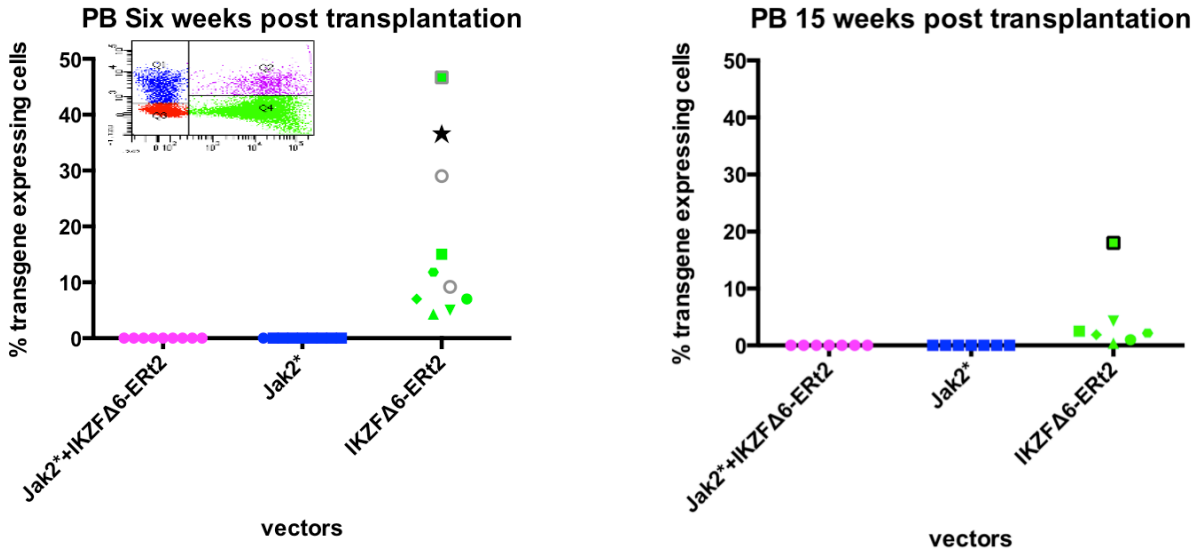

Figure 21 Analysis of the peripheral blood six and 15 weeks post transplantation.

The percentage of Jak2*+IKZFΔ6-ERt2, Jak2* only and accordingly IKZFΔ6-ERt2 only expressing cells in the peripheral blood of 129 mice is shown six and 15 weeks post transplantation. Every data point presents the venus, BFP and accordingly venus+BFP expression value of the peripheral blood of one mouse, which was determined via flow cytometry. Every different point shape stands for one mouse six and 15 weeks post transplantation. The grey circles stand for mice, which died between the sixth and 15th week post transplantation. The black star indicates the original infection frequency with IKZFΔ6-ERt2.

Figure 21 clearly shows that there were no Jak2* transduced cells in the blood of all mice six weeks and 15 weeks post transplantation. The percentage of IKZFΔ6-ERt2 transduced cells were again lost over time. Only one mouse still showed an adequate percentage of IKZFΔ6-ERt2 transduced cells of 18%. The other mice showed IKZFΔ6-ERt2 transduction frequencies of between 0,4% and 4,3%.

4.2 Modeling B-ALL with CRLF2* plus IKZFΔ6

4.2.1 CRLF2* and IKZFΔ6 in cell culture: colony formation assay

Due to the fact that Jak2* infection of primary HSC/HPC cells was very inefficient in the two previous experiments and the percentage of IKZFΔ6-ERt2 transduced cells was lost over time, it was decided to start an *in vitro* experiment with some changes. First of all, the progenitor population was enriched by treating mice with 5-FU for 5 days. Five days was chosen, as previous work in the laboratory also showed the enrichment of early B-cell at this time point (B. Niebuhr, unpublished results). An IKZFΔ6 vector without fusion to ERt2 was used for infection, because if pro-B-cell are infected, an inducible system would not be needed. Additionally, CRLF2* was used instead of Jak2* because it is known, as mentioned in 1.2.4, that CRLF2 overexpression is found in 7% of BCP-ALL and 10% of these cases show CRLF2 mutations. Furthermore, it is known that CRLF2* cooperates with other mutated factors to contribute to the formation of haematologic diseases. Additionally, all vectors used were based on gammaretroviruses, and ecotropic Env was used as envelope protein, instead of lentiviruses with VSV-G proteins. Based on previous experience in the lab, it has been determined that the percentage of transduced murine cells are much higher with ecotropic Env as envelope then with VSV-G.

The CRLF2*+IKZFΔ6 double infected cells were analyzed *in vitro* in a colony formation assay, as preliminary study for following mouse experiments. The proliferation behavior and growth advantage of CRLF2* plus IKZFΔ6 infected cells in comparison to control vector and non-infected HSC/HPC cells was analyzed via a methylcellulose assay. This semisolid medium was used due to the fact that B-cell progenitors are able to proliferate and form colonies in this medium as a response to cytokine stimulation (mIL-7). Two C57BL/6 mice were treated with 5-FU and BM cells of these mice were isolated five days after the start of 5-FU treatment, as shown in Figure 22.

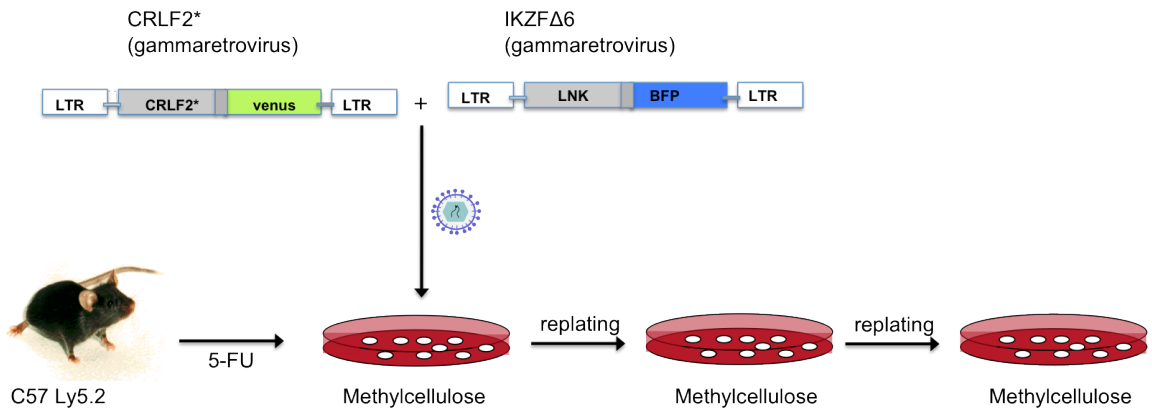

Figure 22 Modeling B-ALL with CRLF2* and IKZFΔ6 *in vitro.*
BM was isolated from C57 Ly5.2 mice that were treated with 5-FU for 5 days. The cells were transduced with the gammaretroviral CRLF2* + IKZFΔ6 vectors. The transduced cells were then seeded in methylcellulose and replated every week for two weeks.

The cells were cultivated overnight and then infected via retronectin on day one and two after isolation with different vectors as shown in Chart 5.

Approaches	**MOI**	**infection frequency**
CRLF2*+IKZFΔ6		
DP		48%
CRLF2*	15,2	5,2%
IKZFΔ6	14,8	14,4%:
CRLF2*+BFP control		
DP		61,9%
CRLF2*	15,2	6,3 %
BFP control	14,8	9,6%:
IKZFΔ6+venus control		
DP		59,1%
venus control	14,8	9,7%
IKZFΔ6	14,8	8%

Chart 5 Used vectors, corresponding MOI and infection frequencies for the colony assay.
The cells for the colony assay were infected in three different approaches. The used vectors, the corresponding MOI's and infection frequencies are shown in the chart. DP stands for "double positive".

On day three after BM isolation $1x10^5$ infected cells per approach were seeded as triplet in methylcellulose. The cells were stained for myeloid and accordingly lymphoid lineage, analyzed via flow cytometry and replated in the same cell density on day seven and 14 post first seeding in methylcellulose. Lots of colonies were already visible for all approaches after seven days and after 14 days.

Figure 23 clearly shows, that the double infected cells overgrow the single infected and uninfected cells in the methylcellulose. The high levels of double infected cells

(CRLF2*+IKZFΔ6 or CRLF2*+BFP control or IKZFΔ6+venus control) at the beginning resulted in a significantly increased probability that an double infected subclone would overtake the cultures. CRLF2* single infection as well as CRLF2*+IKZFΔ6 double infection of the cells did not result in a proliferation advantage of the cells. Staining of the cells showed that nearly all double positive cells were B220/CD11b positive and therefore do not represent B-cells but cells of the myeloid lineage.

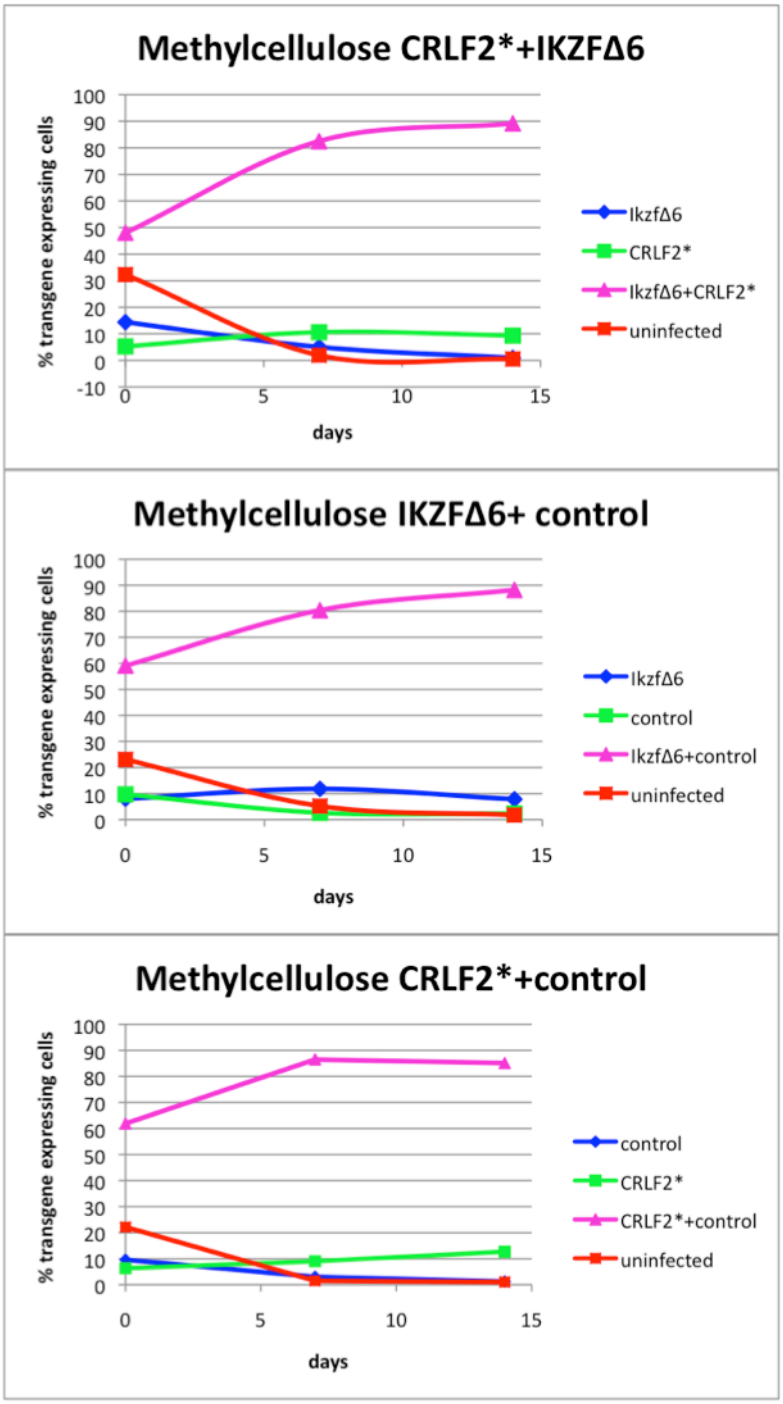

Figure 23 Colony assay: The infected cells, grown in methylcellulose, were analyzed via flow cytometry on day one, seven and 14 after the second infection.
The bone marrow cells of two C57BL/6, which were infected twice with CRLF2* and IIKZFΔ6, respectively with CRLF2* and BFP control vector, and accordingly with IKZFΔ6 and venus control vector were grown in methylcellulose with IL-7(20ng/ml). They were replated every week. The cells were additionally analyzed the day after the second infection as well as seven days and 14 days after the second infection.

The assumption that the double positive cells did not represent B-cells but cells of the myeloid lineage was confirmed using cytospin technology. Looking at the cytospin, shown in Figure 24, it became clear that the colonies mainly consisted of monocytes and macrophages.

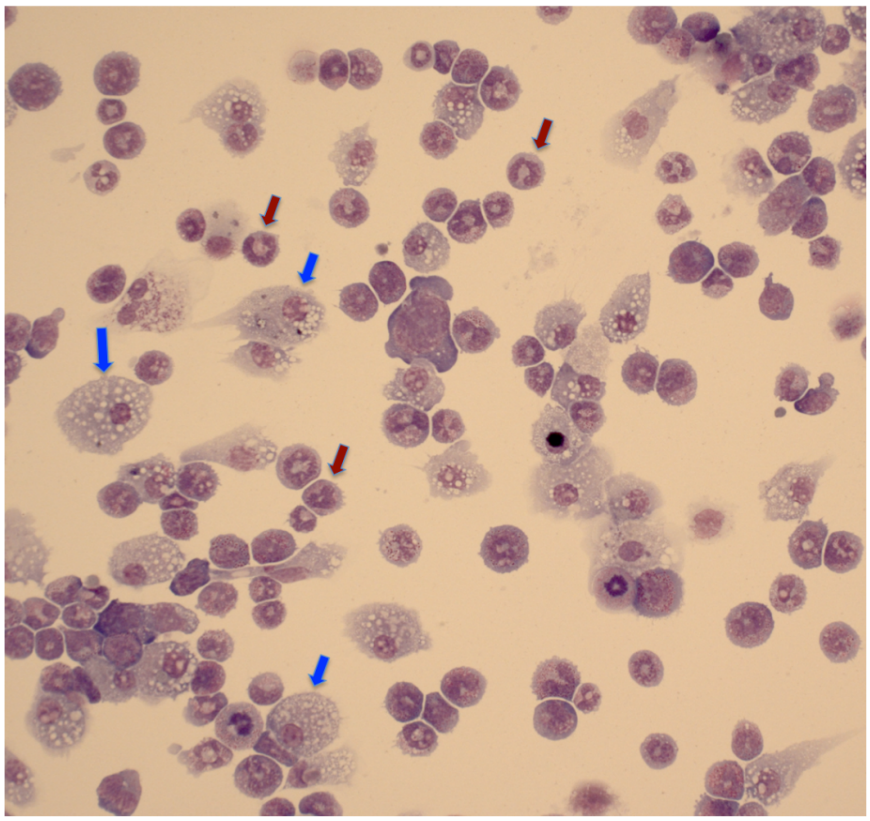

Figure 24 Cytospin of the cells of the colony formation assay.
A cytospin of the cells (after seven days; $1x10^5$ cells/ml), which were infected with CRLF2* and IKZFΔ6 and cultured in methylcellulose for one week is shown. The blue arrows indicate macrophages, whereas the red arrows indicate monocytes.

4.2.2 Modeling B-ALL in the mouse system with CRLF2* plus IKZFΔ6

The chance that the 5-FU enriched stem and progenitors cells differentiate in B-cell direction is higher *in vivo* then *in vitro*. It was the goal to analyze the contribution of CRLF2*+IKZFΔ6 to B-ALL formation. Consequently, the previous experiment (4.2.1) was repeated as an *in vivo* experiment, as shown in Figure 25.

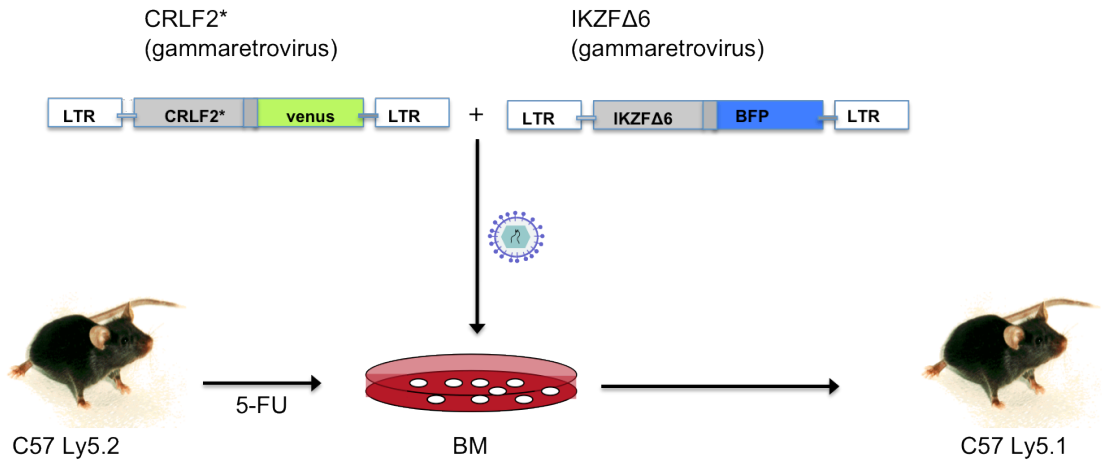

Figure 25 Modeling B-ALL In the mouse system with CRLF2* and IKZFΔ6
BM was isolated from C57 Ly5.2 mice that were treated with 5-FU for 5 days. The cells were transduced with the gammaretroviral CRLF2* + IKZFΔ6 vectors. The transduced cells were finally transplanted into C57 Ly5.1 mice.

The BM cells were isolated from 14 C57BL/6 CD45.2 mice, which were treated with 5-FU for five days. The resulting $9x10^7$ bone marrow cells were cultivated overnight. The cells were then infected on day one and two after bone marrow isolation. FACS analysis of the infected bone marrow cells on the day of transplantation is shown in Figure 26.

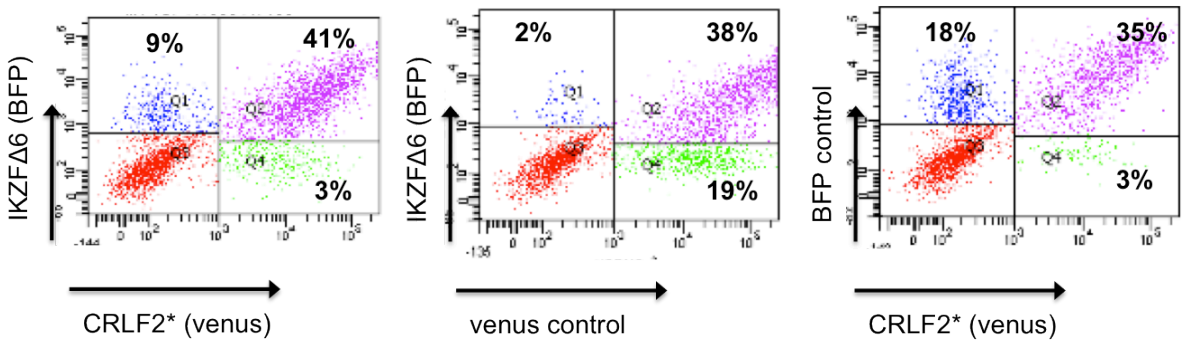

Figure 26 FACS analysis of the infected C57BL/6 bone marrow.
The bone marrow cells of the C57BL/6, which were infected twice, were analyzed via flow cytometry using the fluorochromes venus standing for CRLF2*/control and BFP standing for IKZFΔ6/control. There were three experimental approaches: CRLF2*+IKZFΔ6, IKZFΔ6+control and CRLF2*+control. The cells were analyzed right before transplantation into C57BL/6 CD45.1 mice.

The corresponding MOI's and infection frequencies are shown in Chart 6.

vector	MOI	infection of the BM
CRLF2*+IKZFΔ6		
DP		41%
CRLF2*	0,56	3%
IKZFΔ6	0,46	9%
IKZFΔ6+venus control		
DP		38%
venus control	0,6	19%
IKZFΔ6	0,46	2%
CRLF2*+BFP control		
DP		35%
CRLF2*	0,56	3%
BFP control	0,7	18%

Chart 6 MOI and infection frequency of the infected murine bone marrow cells.
The bone marrow cells of the C57BL/6 mice were infected twice with the IKZFΔ6 plus CRLF2* and accordingly control vectors. The infection frequency of the cells was determined via FACS analysis on the day of transplantation. DP stands for "double positive"

The infected cells were transplanted into lethally irradiated C57BL/6 Ly5.1 mice on day three after bone marrow isolation, as shown in Chart 7.

vector	no. of transplanted mice	no. of transplanted cells/mouse
CRLF2*+IKZFΔ6	15	$1,5x10^6$
IKZFΔ6+venus control	6	$1,6x10^6$
CRLF2*+BFP control	5	$1,7x10^6$

Chart 7 Transplantation of the infected murine bone marrow cells.
The infected bone marrow cells were transplanted in three different approaches, transplanting between 1,5 and $1,7x10^6$ cells per mouse.

Peripheral blood of these mice was taken and analyzed five and 11 weeks post transplantation. The corresponding percentages of transduced cells are shown in Figure 27.

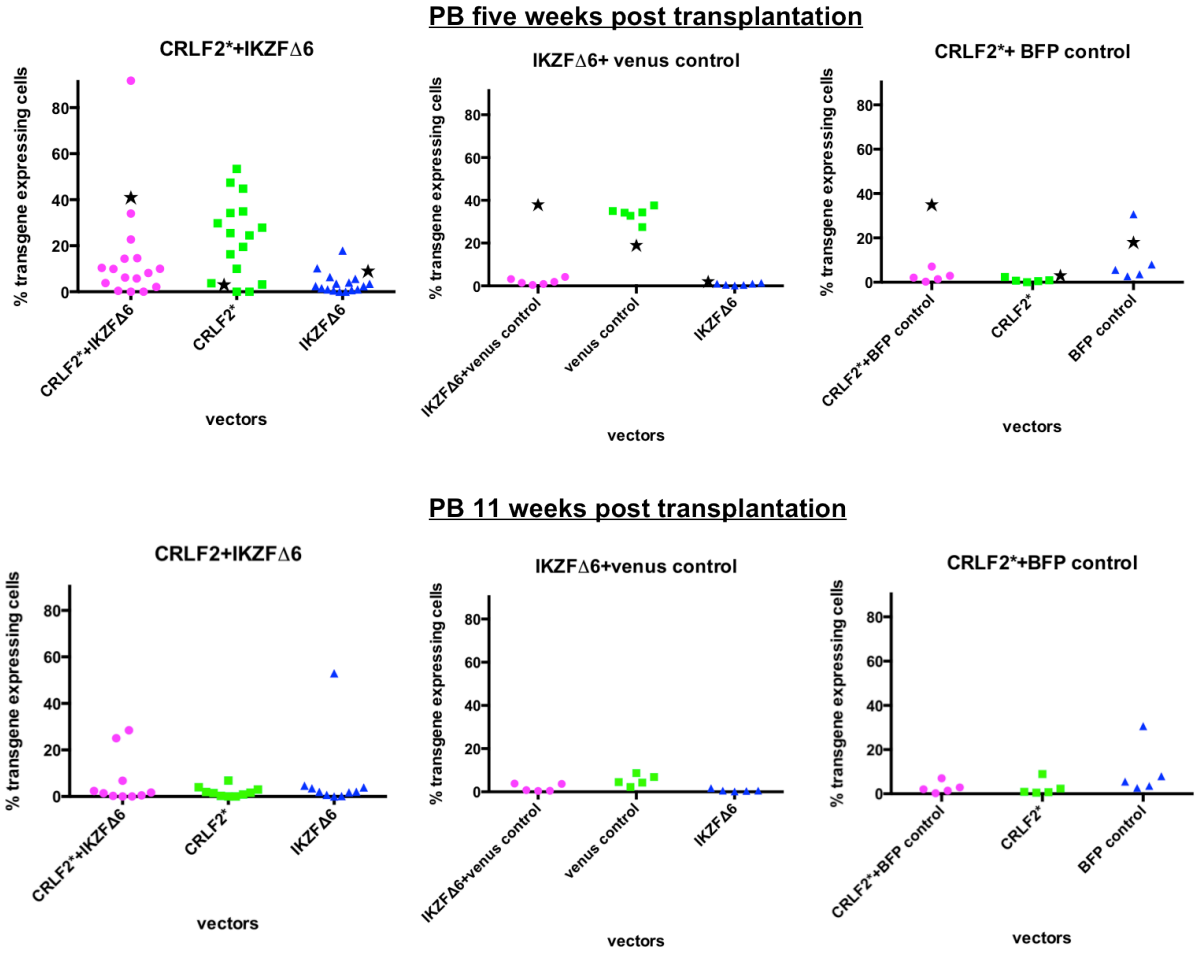

Figure 27 Analysis of the peripheral blood five and 11 weeks post transplantation.
The percentage of double and single positive cells in the peripheral blood of three different cohorts of C57BL/6 mice is shown five (1st row) and 11 weeks (2nd row) post transplantation. Starting from left to right, the first cohort consists of mice, which were infected with CRLF2* and IKZFΔ6. The second cohort consists of mice, which were infected with CRLF2* and a BFP control vector. The third cohort consists of mice, which were infected with IKZFΔ6 and a venus control vector. Every data point presents the venus, respectively BFP and accordingly venus+BFP expression value of the peripheral blood of one mouse, which was determined via flow cytometry. The black stars indicate the corresponding original infection frequency of the BM on the day of transplantation.

Analysis of the peripheral blood after five weeks showed that the highest percentage of transduced cells could be found in the CRLF2*+IKZFΔ6 infected cohort. CRLF2* single infected cells showed the highest expression frequency in this cohort with up to 71% positive cells. There were also high percentages of double infected cells (up to 33%) visible in this cohort. Only IKZFΔ6 expression remained at lower levels. Looking at the CRLF2*+BFP control and IKZFΔ6+venus control cohort it became clear that all animals show percentages of transduced cells that are below 10%. The only exception was the venus expression in the IKZFΔ6+venus control cohort, which had much higher transduction rates at the start.

Strikingly, the blood analysis six weeks later showed completely different results. The percentage of transduced cells dropped in nearly all animals. Only two animals still showed a high percentage of CRLF2*+IKZFΔ6 expression, whereas the percentage of CRLF2* expression decreased in all animals significantly. Only the percentage of IKZFΔ6 expression

remained constant in most animals. This significant decrease of the percentage of transduced cells might signify that transduced HSC are being lost.

Nine animals of the CRLF2*+IKZFΔ6 cohort became sick between day 56 and 103 post transplantation and were sacrificed, as depicted in Figure 28. The peripheral blood, bone marrow and spleen cells of these mice were analyzed via flow cytometry, as shown in Figure 29.

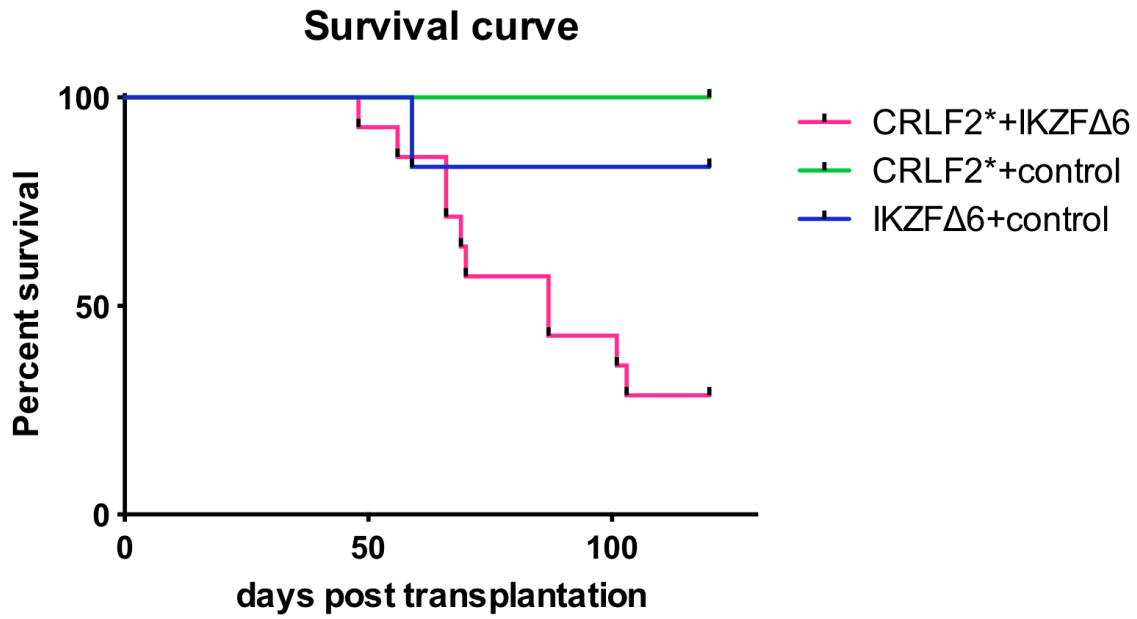

Figure 28 Survival curve of the transplanted C57BL/6 mice.
The survival of the mice, which were transplanted with CRLF2*+IKZFΔ6; CRLF2*+control and IKZFΔ6+control infected cells, is plotted against the time, presenting the days after transplantation.

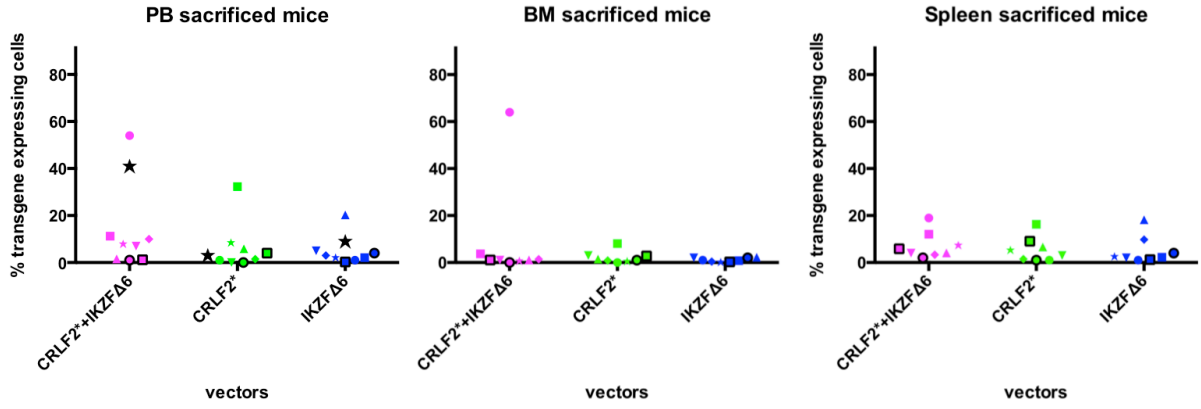

Figure 29 Infection frequency of the peripheral blood, bone marrow and spleen of the sacrificed mice.
Peripheral blood, bone marrow and spleen of the sick mice were analyzed. The infection rates were determined using flow cytometry. The black stars indicate the original infection frequency of the bone marrow at the day of transplantation. Each point shape stands for one mouse.

It is visible, that all percentages of transduced cells are quite low with the exception of three different mice with showed transgene expression frequencies of over 20% in the peripheral blood and BM. These mice were analyzed and the results, shown in Chart 8, indicate that the

mice did not die because of ALL, but because of diseases such as leukocytosis, thrombocytopenia, anemia and unknown reasons. None of the mice possessed an extremely large spleen and none of them showed any other signs of ALL. This disease phenotype is similar to that observed after induced HSC exhaustion, e.g. after repeated treatments with cytotoxins such as 5-FU.

mouse number	time of death	spleen weight (mg)	leukocyte count (K/µl)	hematocrit (%)	abnormalities
#11	56	274	4,54	41,9	Thrombocytopenia
#13	67	168	7,72	36	none
#2	67	146	N.D.	N.D.	none
#7	70	319	2,74	31,5	Anemia, thrombocytopenia
#6	71	230	14,86	43,6	Leukocytosis, thrombocytopenia
#3	87	132	2,24	52,9	Thrombocytopenia, enlarged thymus
#15	87	311	2,38	34,9	Thrombocytopenia
#12	101	137	4,1	51,1	Thrombocytopenia, polycythemia
#8	103	249	3,6	40,2	small liver, enlarged thymus, thrombocytopenia

Chart 8 Analysis of the sick C57BL/6 mice.
Sick animals were sacrificed, their peripheral blood was taken, and bone marrow and spleen were isolated. Time of death is specified as days post transplantation. The hematocrit is defined as the ratio of red blood cells to the total volume of blood.

4.3 LNK- an additional hit?

4.3.1 LNK *in vitro:* overexpression in Ba/F3 cells

Due to the fact that CRLF2* together with IKZFΔ6 did not show an effect on B-lymphopoiesis in the mouse models during the previous experiments, it was decided to look for an additional factor that might play a role in the formation of high-risk B-ALL.

It has been published that there is a function for LNK in limiting B-lymphopoiesis (Takaki et al., 2000), presumably by inhibiting JAK2 function. Consequently, we first wanted to analyze whether LNK alone and LNK together with CRLF2* has an effect on B-cell progenitor proliferation by inhibiting Jak2. The effect of *LNK* overexpression was first analyzed in cell culture.

Ba/F3 cells, which represent an early pro-B-cell line, were infected with a *LNK* expressing vector twice to gain cells with *LNK* overexpression. For these study, IL3-dependent Ba/F3 and IL3-independent Ba/F3 cells (transduced with CRLF2*-vector) were used. After two rounds of infection, infected cells were then selected with puromycin. After infection and selection it was tested whether the cells, which were infected with the LNK vector really showed a *LNK* overexpression in comparison to the endogenous *Lnk* expression in the uninfected and control vector infected cells. Western blot analysis was performed to analyze the *Lnk* expression at the protein level. Whole cell lysis, protein extraction and measurement of protein concentration of the differently infected and non-infected cells were performed as described in 3.2.3.2.

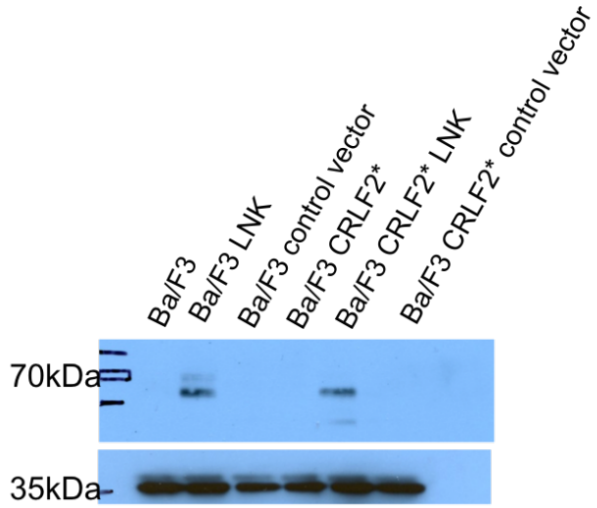

Figure 30 Western blot analysis of Lnk expression in Ba/F3 cells.
80µg of cell lysates of Ba/F3 cells and accordingly Ba/F3 CRLF2* cells, which were infected with LNK, respectively with a control vector, were applied to a 10% SDS gel, separated and plotted onto a PVDF membrane. Uninfected Ba/F3 and accordingly uninfected Ba/F3 CRLF2* cells were used as a control as well as cells, which were infected with the control vector. The detection of Lnk expression as well as of GAPDH as internal control was determined using specific primary antibodies (3.1.7.1) as well as corresponding secondary antibodies.

A polyclonal goat IgG-anti-Lnk antibody was used as primary antibody. LNK could be identified as a band at about 65-68 kDa. To be able to compare the signal strength of the bands, the blot was also incubated with the primary antibody against the housekeeping gene GAPDH. It is clearly visible in Figure 30 that a LNK band was only present for the Ba/F3 cells alone and the Ba/F3 cells with CRLF2*, which were infected with the LNK vector and no bands were visible for the uninfected and control vector infected cells. The GAPDH bands were consistent in all cells. This showed that the LNK vector works and *LNK* overexpression was present in the LNK vector infected cells.

4.3.2 Competition assay

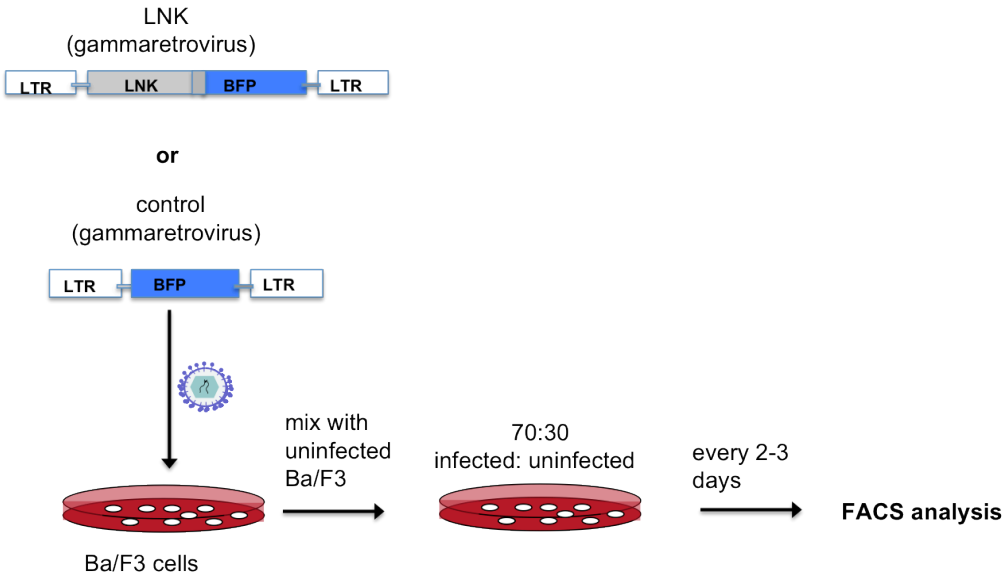

Figure 31 Competition assay
Ba/F3 cells were infected with the LNK vector and accordingly BFP control vector. They were mixed with uninfected Ba/F3 cells and analyzed via FACS every 2-3 days.

To see the effect of the confirmed *LNK* overexpression in cell culture a competition assay between the *LNK* overexpressing, control vector expressing and uninfected cells was performed. Two different kinds of Ba/F3 cell lines were used again to study the role of *LNK* overexpression in these cells: Ba/F3 alone and Ba/F3 cells with CRLF2*. The experiment was performed twice, as shown in Figure 31. The Ba/F3 cells, which present murine bone marrow derived pro-B-cells, were infected with the LNK vector and accordingly the control vector via spinoculation on two days one after another during both experiments. The corresponding MOI's and infection frequencies after puromycin selection are shown in Chart 9. A puromycin selection of the cells was performed directly after the second infection, by adding puromycin at a concentration of 1µg/ml to the cells because the infection rates were too low. After the second infection only 2,2% of the cells were LNK vector positive and 7,7% of the cells were control vector positive, which increased to close to 100% after puromycin selection.

vector	**Infection frequency after puromycin selection 1st experiment**	**Infection frequency after puromycin selection 2nd experiment**
LNK	98,2% (Ba/F3) 98,4% (Ba/F3+CRLF2*)	99% (Ba/F3) 97% (Ba/F3+CRLF2*)
control	97,8%(Ba/F3) 94,1%(Ba/F3+CRLF2*)	96% (Ba/F3) 94% (Ba/F3+CRLF2*)

Chart 9 Competition assay: infection frequencies of the Ba/F3 cells.

The Ba/F3 cells were infected twice with a Lnk vector and accordingly a control vector at MOI's of between 0,4 and 2,3. Puromycin was added twice over period of four days at a concentration of 1µg/ml. The puromycin selection resulted in infection frequencies of between 94% and 99%.

To analyze the effect of LNK on the proliferation behavior of pro-B-cells in comparison to the proliferation of control vector-transduced cells or uninfected cultures, a competition assay with the two different cells lines was performed. The BFP positive cells, representing the cells containing the LNK vector or the control vector, were mixed with the corresponding uninfected cells at a ratio of about 70:30 infected to uninfected cells. The proliferation of the cells was analyzed via flow cytometry every two to three days for 12 days.

Concerning the Ba/F3 cell line, cultured with IL-3, it is visible in both experiments that the cells with *LNK* overexpression show a constant decrease in proliferation in comparison to the cells containing no vector over the whole 12 days of measurement, as shown in Figure 32. In contrast the cells containing the control vector show a slight increase in proliferation at the beginning of measurement followed by a mainly constant percentage distribution in comparison to the cells without any vector.

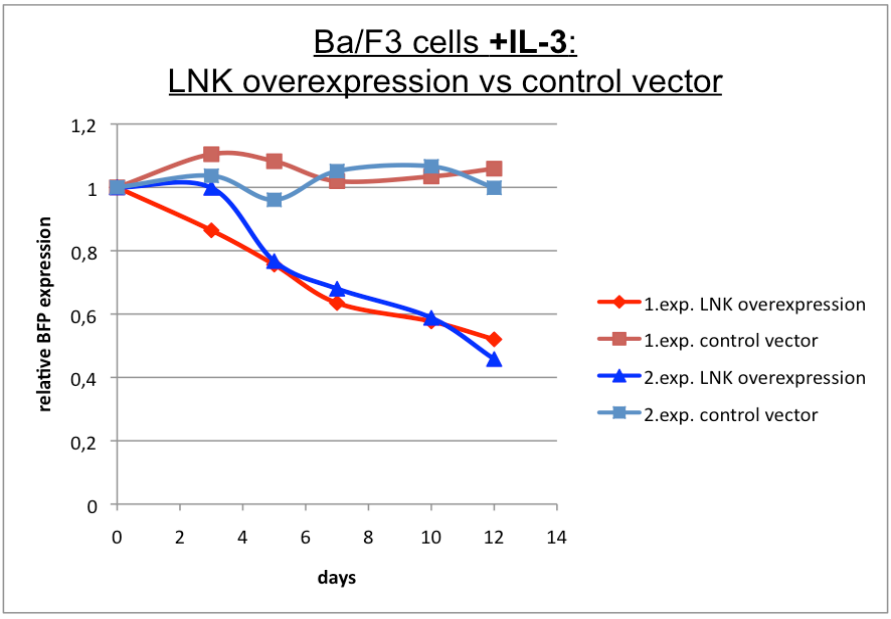

Figure 32 Competition assay of Ba/F3 cells, which overexpress LNK and were cultured with IL-3.
Determination of relative BFP expression of the cells, which were infected with either the LNK vector or the control vector. FACS analysis was performed every 48-72 hours.

Looking at the Ba/F3 cells with CRLF2*, which were grown without IL-3, it is visible that the cells show a completely different proliferation behavior. It is shown in Figure 33 that cells with *LNK* overexpression as well as the control vector infected cells show no significant difference in their proliferation behavior. The cell number of the differently infected cells nearly stayed constant overtime as well as the cell number of the uninfected cells. These results show that whereas LNK inhibits IL3-mediated proliferation, it does not inhibit proliferation stimulated by CRLF2*.

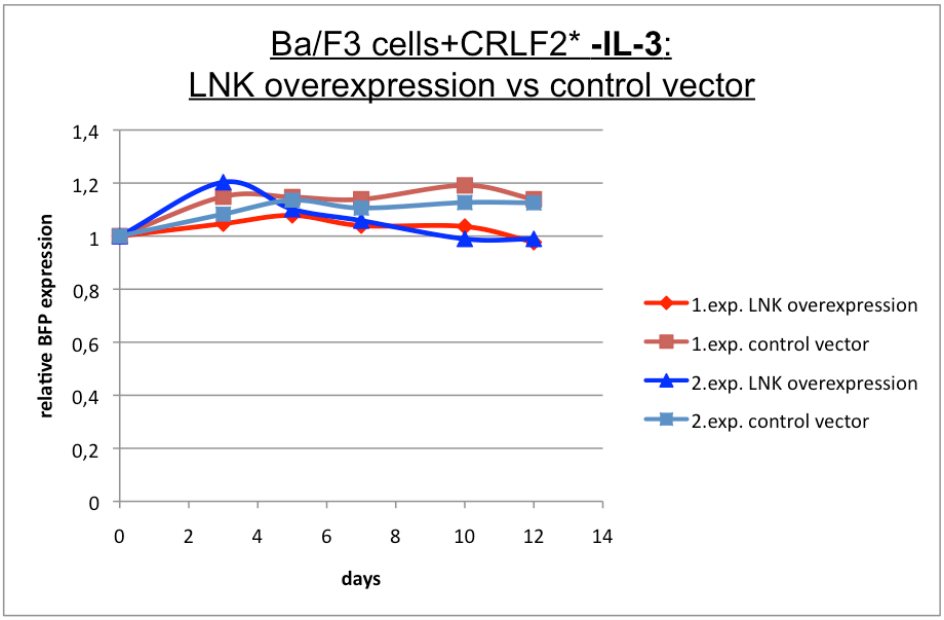

Figure 33 Competition assay: LNK overexpression in Ba/F3 cells, already containing CRLF2*.
Determination of the percentage of BFP positive cells, which were infected with either the LNK vector or the control vector. FACS analysis was performed every 48-72 hours.

5 Discussion

The aim of this thesis was to optimize a mouse model system to analyze the effects of mutated kinases and transcription factors, such as Jak2* and IKZFΔ6, on B-cell development *in vivo*. The reasons for developing such a model system are two-fold: 1) It allows the testing of the hypothesis that mutations that provide a proliferative stimulus coupled with mutations that inhibit differentiation are required (but not necessarily sufficient) to induce a leukemia; and 2) it provides an *in vivo* system to test the efficacy of small kinase inhibitors that target the proliferative stimulus of the mutated kinase.

To establish this model, a retroviral transduction, stem cell transplantation approach was taken, first using human primary HSC/HPC and then mouse primary HSC/HPC. Although the work presented here was not able to establish such a model system, insights into the problematic of this approach were obtained and will be discussed. In addition, suggestions for improving this approach, for instance by targeting other signaling pathways, are presented.

5.1 Retroviral transduction of Jak2* in primary cells is problematic for modeling B-ALL in mice

The double infection of human CD34+ cells with the concentrated lentiviral Jak2* vector particles packaged with VSV-G and with the gammaretroviral IKZFΔ6-ERt2 vector packaged with ecotropic Env, resulted in up to 60% of venus expressing (IKZFΔ6-ERt2) target cells, but at most 2% of Jak2* transduced cells. Similarly, analysis of the peripheral blood six weeks post transplantation showed rarely any single or double-transduced human CD34+ cells either. Final analysis of the sacrificed mice 5 month post transplantation showed that there were single IKZFΔ6-ERt2 or Jak2* transduced human cells within the spleen and bone marrow, but never as double positive cells. The problem that we could not get any double infection of the human CD34+ cells as well as of the murine BM cells started with the transduction protocol.

Previous experiments in our lab had shown that Jak2* vectors based on gammaretroviral vectors had very poor titers when tested on murine SC-1 fibroblasts, whereas much better vital titers were obtained when Jak2* was expressed via a lentiviral vector pseudotyped with VSV-G as envelope protein. Furthermore, it has been shown that the titers of the vector containing the murine *Jak2** were significantly higher then the titers of vectors, containing the human *Jak2**. Additionally, it is possible to concentrate lentiviral particles packaged with VSV-G via ultracentrifugation, whereas gammaretroviruses packaged in the less stable

ecotropic Env, loose infectivity during this process. Consequently, Jak2* viral supernatants were always produced as lentiviruses, exposing VSV-G, and were concentrated.

The titer determination on SC-1 cells during this work showed that the lentiviral mJak2* titers (max. $9x10^5$ gtu/ml) were at best only half as high as the gammaretroviral IKZFΔ6-ERt2 titers (max. $2x10^6$ gtu/ml). The low titers of the lentiviral Jak2* vector in comparison to the gammaretroviral IKZFΔ6-ERt2 vector resulted in lower MOIs of the lentiviral Jak2* vector then needed, despite the fact that Jak2* was concentrated.

In experiments in which human CD34+ cells were infected, the MOIs of the lentiviral Jak2* vector packaged in VSV-G were about one third lower then the MOIs of the gammaretroviral IKZFΔ6-ERt2 vector packaged with ecotropic Env, as shown in Chart 1. We used comparable MOIs of the lentiviral Jak2* and the gammaretroviral IKZFΔ6-ERt2 vector for the infection of murine bone marrow cells (Chart 3). Nevertheless this infection resulted in only 0,5% Jak2* positive cells and 36,6% IKZFΔ6-ERt2 positive cells. Additionally, the MOI of the IKZFΔ6-ERt2 vector during the infection of the murine BM cells was one-fifth that of the MOI of the lentiviral control vector, but 60% of the cells expressed venus (IKZFΔ6-ERt2) and only 8% eGFP (control vector). This indicates that regardless whether human or murine primary cells are infected, the lentiviral constructs need much higher MOIs for high percentages of transduced target cells in comparison to the gammaretroviral constructs. Consequently, the MOI of Jak2* was too low for the transduction of target cells with a lentiviral construct in both experiments.

It is important to note that for the gammaretroviral constructs, ectotropic Env was used to mediate cell entry. This entry system appears to be much more efficient in haematopoietic cells than VSV-G mediated cell entry, whereas in SC1 fibroblasts, the system used to titer the viral vectors, no noticeable difference is observed. Whether this reflects differences in expression levels of cellular receptors used by the viruses in the two cell types, or higher sensitivity to the known cytoxicity of VSV-G glycoproteins is not clear. Although several MuLV Env proteins (including ecotropic Env) can be used to pseudotype lentiviral vectors, the titers are generally much lower. It would be of interest to try other viral glycoproteins for packaging of the lentiviral vectors (e.g. LCMV; Cocal) (Bhella et al., 1998; Beyer et al., 2002; Trobridge et al., 2010).

Additionally, the Jak2* vector presents a really large construct. The Jak2* viral vector is ca 9.5 kb in size. In contrast, the gammaretroviral vectors, such as CRLF2* (ca 4.6 kb), IKZFΔ6-ERT2 (5.3 kb) and LNK (7.3 kb) were smaller in size and led to higher percentages of transduced target cells. The large size of the Jak2* vector might contribute to the fact that the titers were always low due to poor packaging efficiencies..

Another important fact to mention is that the Jak2* virus was concentrated before infecting the human and murine primary cells, because of the relatively low titers. The proteins of the

serum were also concentrated during this process and, consequently, might be toxic for the primary cells, but not for the more robust SC-1 cells during titer determination. Supernatants from the virus producers may also produce large quantities of the VSV-G protein, which are known to be cytotoxic.

This toxicity, the large size of the vector and the relatively low MOI could be reasons for the problems of infecting primary cells. Another possibility that we cannot rule out is that early HSC/HPC may be sensitive to high expression levels of activated Jak2*, leading to differentiation or death of the cells.

5.2 Optimization of the conditions for the xenograft model

The aim of the *in vivo* experiment using CD34+ cells (4.1.1) was to compare different experimental settings to optimize a xenograft model for B-ALL. Three different cell populations were compared for this experiment: sorted CD34+CD38- (most primitive cells), sorted CD34+CD38+ (later progenitor population) and unsorted CD34+ (used in previous experiments) cells.

Nearly twice the number of CD34+ cells was thawed for the sorted approach ($3x10^7$ cells) in comparison to the unsorted approach ($1,6x10^7$ cells). But as a result of sorting only one seventh (CD34+CD38+) and accordingly one twelfth (CD34+CD38-) of sorted cells were infected in comparison to the unsorted CD34+ cells. Additionally, only $4,3x10^4$ (CD34+CD38-) and $6x10^4$ (CD34+CD38+) cells/mouse were transplanted for the sorted approach in comparison to $1,2x10^6$ cells/mouse, which were transplanted for the unsorted approach.

Looking at the engraftment (Figure 16) of the three approaches, it is clearly visible that the engraftment of human cells was much better in the unsorted CD34+ cell approach. The mice, which were transplanted with the infected and unsorted CD34+ cells, showed up to 71% of human cells in the bone marrow compared to a maximum of 16% (CD34+CD38-) and 21% (CD34+CD38+) of human cells in the bone marrow of the mice, transplanted with the sorted populations. On the one hand, the worse engraftment of the sorted populations is probably the result of stress of the sorted cells due to sorting and to the infection on day zero and one. The unsorted population did not experience that stress, because it was not sorted and was infected on day one and two. This stress resulted in less proliferation and the following low cell density was not the ideal condition for the sorted CD34+CD38- and CD34+CD38+ populations. On the other hand, the i.v. injection might not be the ideal form of injection for the sorted cells. During i.v injection the place of injection is quite far away from the target niche of the stem cells in the bone marrow. As a result, the low number of the sorted cell population might not be able to find their niche. Consequently, intra-femoral injection, what leads to injection of the cells near the niche, might be a better alternative for the injection of the sorted populations (McKenzie et al., 2005).

The transgene transduction frequencies (Figure 17) of the human cells were also much higher in the unsorted CD34+ population, with up to 33% of venus (gammaretroviral IKZFΔ6-ERt2, RD114 env) expression and 25,5% eGFP (concentrated lentiviral Jak2*, VSV-G) expression compared to a maximum of 2,6% eGFP expression (CD34+CD38-) and 1,2% of venus (gammaretroviral IKZFΔ6-ERt2, RD114 env) expressing CD34+CD38+ cells. But by looking at the IKZFΔ6-ERt2 expression frequencies before transplantation and after five month it is visible that also the unsorted, infected human cells were lost selectively over time. The worse infection frequencies of the sorted populations were probably the result of the lack of proliferation of these cells. Despite the fact that lentiviruses are known to also infect non-proliferating cells, no infection of the sorted cells with Jak2* (lentivirus) was visible. Double positive cells were present in none of the cohorts probably due to the reasons mentioned in 5.1.

Consequently the xenograft model needs to be optimized by infecting and transplanting a higher number of the sorted, early progenitors CD34+CD38-. The problem was that these cells did not proliferate. Consequently, a much higher cell number of CD34+ should be thawed. The infected cells should then be injected intra-femoral to improve the homing and engraftment of the sorted populations

Alternatively, the next approach could be the infection of unsorted CD34+ cells on day one and two after thawing with gammaretroviral vectors that are pseudotyped with RD114 Env such as CRLF2*+IKZFΔ6, instead of the lentiviral Jak2* vector.

5.3 Optimization of the *in vitro* assay

Murine bone marrow cells were double infected with two gammaretroviral vectors, expressing CRLF2* and IKZFΔ6, and seeded in methylcellulose with rmIL-7 to analyze the effect of CRLF2* and IKZFΔ6 on B-cell proliferation and colony forming ability *in vitro*.

A cytospin showed that the CRLF2*+IKZFΔ6 infected bone marrow cells differentiated into the myeloid direction and mainly formed colonies of macrophages and monocytes, as shown in 4.2.1. FACS analysis of the cells confirmed this finding. This kind of differentiation happened although the bone marrow cells were cultured with rmIL-7 in methylcellulose, what normally drives differentiation into the B lineage direction (Kikuchi et al., 2008). But the problem was that rmIL-7 was not used from the beginning. The bone marrow cells were isolated from the mice five days after 5-FU treatment, cultured in SFEM with the whole cytokine cocktail (rhFlt3, rhIL11, rmSCF, rmIL3) for two days during infection and were then seeded in methylcellulose with rmIL-7 only. These two days in culture with the complete cytokine cocktail probably drove the differentiation of the progenitor cells in direction of the myeloid lineage. To analyze the effects of CRLF2*+IKZFΔ6 on B-cell proliferation and colony

forming ability *in vitro* rmIL-7 should be used from the beginning, right after bone marrow isolation to repress differentiation in the myeloid direction.

Another problem of the colony formation assay was that all double infected cells, independent of the vector combination, showed a proliferation advantage in comparison to the single infected and uninfected cells. Figure 23 clearly shows that the double infected cells overgrow the single infected and uninfected cells in the methylcellulose. The high levels of double infected cells (CRLF2*+IKZFΔ6 or CRLF2*+BFP control or IKZFΔ6+venus control) at the beginning resulted in a significantly increased probability, that a double infected subclone would overtake the cultures. CRLF2* single infection as well as CRLF2*+IKZFΔ6 double infection of the cells did not result in a proliferation advantage of the cells. To prevent too high infection frequencies the cells should be infected just once and should then directly be seeded into methylcellulose.

Due to the fact that the CRLF2* single infected cells did not establish themselves and did not lead to B-lineage differentiation it became clear that CRLF2* alone is not able to promote the expansion of the cells and is not strong enough to have an significant influence on B-cell development and differentiation in this assay, as (Roll and Reuther, 2010) had already postulated.

Nevertheless, with some changes, as the cultivation with rIL-7 from the beginning and the prevention of too high infection frequencies, the colony assay seems to be a good preliminary study for possible following mouse experiments. But these assays also demonstrated the difficulties of assaying primary cells in culture, due to inherent fluctuations in cell proliferation of single clones and maintenance of B-cell differentiation, even under optimal B-cell conditions

5.4 Murine model using CRLF2* and IKZFΔ6

5.4.1 CRLF2* transduction is more efficient

In the experiment 4.2.2, the lentiviral Jak2* vector was replaced by a gammaretroviral CRLF2* vector to circumvent the problems mentioned in 5.1. This CRLF2* vector showed higher titers (CRLF2*: 1,5-2,9x10^6 gtu/ml; Jak2*: 9x10^5 gtu/ml) on SC-1 cells during titration and was smaller in size in comparison to the Jak2* vector.

The MOIs of the used vectors IKZFΔ6 (MOI:0,46) and CRLF2* (MOI:0,56) were comparable and the resulting percentage of double positive bone marrow cells was much higher then in the previous experiments. 41% of the bone marrow cells were double positive, 9% expressed IKZFΔ6 only and 3% showed CRLF2* expression. This showed that using CRLF2* instead of Jak2* and gammaretroviruses instead of lentiviruses improved the percentage of double positive bone marrow cells significantly.

5.4.2 5-FU progenitor enrichment results in fewer long-term HSC

Looking at the analysis of the peripheral blood five and 11 weeks post transplantation, depicted in Figure 21, it becomes clear that the percentage of transduced cells decreased significantly in nearly all animals over time. Five weeks post transplantation between 0,4% and 23% (one case 92%) of the blood cells were double positive, 3%-53% showed CRLF2* expression and 0,6%-18% IKZFΔ6 expression. Eleven weeks post transplantation only two animals still showed a high number of CRLF2*+IKZFΔ6 positive cells, whereas CRLF2* positive cells decreased in all animals significantly. The transduced cells (single and double positive) did not benefit from the transgene expression and did not show an enhanced proliferation. This rather negative selection leads to the loss of the infected cells over time unless another mutation occurs, which leads to an enrichment of the cells.

Additionally, the treatment of the mice with 5-FU for five days resulted in a heterogeneous population of cells in the bone marrow (Ogawa et al., 1994). The isolated bone marrow cells probably still contained lots of ST-HSCs and progenitor cells and not only LT-HSCs. These ST-HSCs and progenitor cells are probably easier to infect, which might result in the high infection frequencies of the bone marrow right before transplantation. These cells then differentiate in the mice and are lost.

In summary we found out that using the combination of 1) CRLF2* as transgene in comparison to Jak2*, 2) only gammaretroviral vectors with ecotropic Env instead of lentiviruses with VSV-G, and 3) comparable MOIs worked much better for double-infecting murine primary cells. We speculate that the infection of the ST-HSCs and progenitor cells instead of LT-HSCs, followed by the take over of the niche by these cells and the loss of stem cells due to expression of either CRLF2* or IKZFΔ6 or both, resulted in “stem cell exhaustion” and death of the mice.

5.4.3 CRLF2* and IKZFΔ6 do not synergize to induce ALL

The fact that none of the mice died of acute lymphoblastic leukemia indicates that the double negative IKZFΔ6 was not strong enough as second hit to cause ALL together with CRLF2*. But by looking at Figure 27 and Figure 28 it is visible that the CRLF2* together with IKZFΔ6 seem to have an effect on the cells. The percentage of transduced bone marrow cells was comparable in all cohorts before transplantation. But analysis of the peripheral blood after five weeks showed that the control cohorts (control+CRLF2*; control+IKZFΔ6) showed much less double positive as well as CRLF2* single positive cells in comparison to the CRLF2*+IKZFΔ6 cohort. Additionally, nine out of ten mice that became sick were of the CRLF2*+IKZFΔ6 cohort. Consequently, CRLF2* and IKZFΔ6 seem to synergize, but they are not strong enough to induce B-ALL formation. Probably an additional mutation is necessary for B-ALL induction.

To further improve the B-ALL model another mouse model should be used, in which leukemia can be induced. Consequently, a *knock-out* mouse, in which a tumor suppressor gene is deleted, would be an alternative to the C57BL/6 BM cells. Furthermore, the search for an additional hit next to CRLF2* and IKZFΔ6 would increase the chances of modeling B-ALL in mice.

5.5 LNK is able to inhibit IL-3 mediated pro-B-cell proliferation *in vitro*

A minimum of two mutations are generally thought necessary for the transformation of a stem cell or progenitor cell to a leukemic cell (Dash and Gilliland, 2001). We have chosen CRLF2* and IKZFΔ6 for our experiments, as they occur together at a high incidence of high-risk B-ALL. This work provided evidence that CRLF2* and IKZFΔ6 synergize but do not induce B-ALL. The mice, which were transplanted with CRLF2*+IKZFΔ6 infected cells, did not establish B-ALL although nine of them died but due to other diseases and reason. This demonstrates that CRLF2* together with IKZFΔ6 does not contribute to B-ALL formation in our mouse model. Consequently, we chose *Lnk* as additional hit, due to the fact that *Lnk* plays a role in haematopoeisis and B-cell differentiation (Takaki et al., 2000; Takaki et al., 2002)

It has been shown that IL-3 mediated signaling happens via the Jak2 kinase (Hara and Miyajima, 1996) and my experimental data (4.3.2) showed that LNK overexpression in the presence of IL-3 significantly inhibits the proliferation of pro-B-cell line Ba/F3, presumably through inhibiting Jak2. In the absence of IL-3 and together with CRLF2* there was no effect visible on the proliferation behavior of the pro-B-cells, suggesting that TSLP may signal through other pathways and kinases. Thus the high levels of pro-B- and pre-B-cells in *Lnk*$^{-/-}$ mice may not be due to inhibition of the TSLP or IL7 pathways, but perhaps via the preBCR and BCR pathway and associated kinases. This is supported by the fact that Lnk is present during all stages of B-cell differentiation, whereas CRLF2 and IL7Ra are not. But the opinions about the TSLP mediated signaling are still controversial. On the one hand, it has been postulated that TSLP mediated phosphorylation of the STATs happens independently of JAK proteins (Isaksen et al., 1999; Levin et al., 1999). On the other hand, it has been postulated that in primary T-cells TSLP uses a combination of Jak1 and Jak2 to induce STAT5 activation (Rochman et al., 2010). They showed that Jak1 is associated with the IL7Rα chain and Jak2 with the TSLPR and that Jak1 and Jak2 are essential for TSLP induced signaling and STAT5 phosphorylation.

Because LNK overexpression showed an effect on IL-3 mediated pro-B-cell proliferation *in vitro* in my experiment, the next aim would be to investigate the effects of LNK *in vivo.* Consequently it would be interesting to analyze the effects CRLF2* and IKZFΔ6 together in *Lnk knock-out* mice. In support of this approach, very recent studies have indeed uncovered

LNK inactivation mutations in B-ALL in conjunction with CRLF2 activating mutations (Roberts et al., 2012).

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8 Curriculum vitae

Jelena von Dziembowska
Emilienstr.76
20259 Hamburg
Deutschland

Mobile Germany: +49 151 18435415
Email: dziembowska@gmail.com

Date of Birth: 02.02.1986
Born in Munich, Germany

Citizenship: Germany

Education

September 1996 – June 2005	Gymnasium Landschulheim Kempfenhausen Starnberg, Germany 5th to 13th grade Degree: Abitur (grade: 1,7)
October 2005 – November 2005	Berlitz Language school Munich, Germany Berlitz proficiency level: 7
November 2005 –December 2005	St. Clare's Language school Oxford, Great Britain General english language ESU level: 6/7
October 2006 – now	Universität Wien/ Department of Microbiology, Immunbiology & Genetics Vienna, Austria Program of study: Mikrobiologie Genetik

Work experience

Year 2000 (2 weeks)	Internship Städtischer Kindergarten, Söcking
February 2003 – March 2003	Internship Prinzregententheater, Munich Assistant producer
January 2006 – April 2006	Internship Bagan Language centre, Burma English teacher
May 2006 – August 2006	Fair Hostess Profi-Tess, Munich Different fairs
October 2011 – July 2012	Diploma thesis Group "Molekulare Patholgie", HPI, Hamburg Graduand